Investigating the role of the stress-related transcription factor, HSF1, upon breast cancer tumourigenesis and progression

by

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GENERAL DECLARATION

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General Declaration

In accordance with Monash University Doctorate Regulation 17 Doctor of Philosophy and Research Master's regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis contains **one** unpublished publications. The core theme of the thesis is **investigating the role of the heat shock transcription factor HSF1 in breast cancer**. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the **Department of Biochemistry and Molecular Biology** under the supervision of **Dr John Price**.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of Chapter 4, my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status	Nature and extent of candidate's contribution
Chapter 4	Heat Shock Factor 1 Impacts Both Positively and Negatively Upon Mammary Epithelial and Cancer Cell Clonogenicity Depending Upon p53 Status	Published at Biochemistry Journal in June 2013	Participated in development of project hypothesis, designed and performed all experimental procedure except experiments in Figure 2, analysed data, prepared and wrote the manuscript.

I have renumbered section of the submitted paper in order to generate a consistent presentation within the thesis.

Signed: ……

Date: ……24/01/2013……

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SUMMARY

Cancer cells are exposed to numerous forms of extrinsic and intrinsic cellular stresses such as hypoxia, acidosis, nutrient deprivation, as well as genotoxic and oxidative stress, and as a result are dependent upon stress support pathways for their survival. One such pathway is the heat shock response (HSR), which results in the enhanced expression of heat shock proteins (HSPs) that function as molecular chaperones that restore cellular protein homeostasis and prevent stress-induced cell death. However, cellular stress has also been shown to be an important contributor to cancer cell growth, progression and metastasis. The elevated expression of HSPs has been identified in many tumour types and correlates with poor patient outcomes. As such, HSPs have emerged as significant therapeutic targets within many cancer types. Consistent with this, the master transcription factor regulating the HSR, Heat Shock Factor 1 (HSF1), has also been shown to function as a powerful modulator of malignancy. Interestingly, HSF1 is found to not only support the malignant phenotype by regulating the expression of the HSPs but also regulates the expression of a complex network of genes that are involved in many cellular processes essential for tumourigenesis and cancer progression. However, while there have been many studies that document the important roles of HSF1 in cancer; the precise mechanisms by which HSF1 achieves this are still relatively unknown.

Despite significant improvements over the years in cancer treatment, breast cancer remains a major cause of death among women worldwide. In particular, individuals diagnosed with triple negative forms of breast cancer that are more refractory to current therapies and have a higher likelihood to undergo metastasis, have particularily poor outcomes. Thus, the identification of novel and more effective therapeutic drug targets to improve patient survival is required. Previous studies have revealed that elevated levels and increased activity of HSF1 are strongly correlated with breast cancer aggressiveness and outcome. Analysis of breast cancer cell lines has also demonstrated that HSF1 levels and activity are increased in highly aggressive and metastatic triple negative cancer cell lines in comparison to the lower migratory and less invasive luminal breast cancer cell line subtypes. As HSF1 has emerged as a potential anticancer

therapeutic target, this study aims to validate and determine the mechanisms by which HSF1 may act in breast cancer tumourigenesis and progression.

In this study, to investigate the role of HSF1 in breast cancer tumourigenesis and progression, wild-type HSF1 and a constitutively active form of HSF1, HSF1ΔRDT, were ectopically expressed in the normal human mammary epithelial cell line, MCF10A, and in MCF10A H-Ras^{V12} transformed cells. This study demonstrates that while ectopic expression of HSF1 has little impact upon the cell biology of the normal MCF10A cells, HSF1 uniquely enhances the malignant phenotype of cells that have been transformed with oncogenic Ras, especially in regard to the cells' migratory and invasion abilities. Similar effects were observed when HSF1 was ectopically expressed in the luminal breast cancer cell line, SkBr3, which exhibits a constitutive activation of Ras. Further analysis reveals that while HSF1 exerts little effects on signal transduction pathways downstream of Ras, the factor co-operates with oncogenic Ras to alter the expression of genes and pathways that promote cancer progression. This study thus confirms that HSF1 is a positive modulator of cancer progression and shows that the cancer promoting effects of HSF1 are mediated via the modulation and/or co-operation of the factor with other oncogenic proteins within the tumour cells.

In addition to its co-operative actions with activated oncogenic Ras, this study also demonstrates that HSF1 can regulate breast cancer cell clonogenicity and this activity is dependent upon the tumour suppressor p53. Wild-type p53 functions as a "guardian of the genome" that regulates the expression of genes involved in DNA damage repair, cell-cycle arrest and apoptosis. Mutations in the TP53 gene lead to the production of mutant p53 proteins that not only exhibit a loss in their tumour suppressor activity but can also exert 'gain-of-function' properties that have been shown to be important at key stages of metastatic progression. This study demonstrates that HSF1 can enhance both wild-type and mutant p53 transcriptional activities, mediating disparate outcomes in clonogenic cancer cell survival and growth in a p53 status dependent manner. Knockdown of mutant p53 abrogates HSF1's ability to enhance clonogenic survival and growth in cancer cells, while knockdown of wild-type p53 rescues the reduced clonogenicity that is mediated by HSF1 ectopic expression. Moreover, in the cellular context of endogenous wild-type p53 and the exogenous expression of mutant p53^{R273H}, activation of HSF1 reduces cell clonogenicity; however, when wild-type p53 is knocked down leaving a cellular context of mutant p53^{R273H}, activation of HSF1 can support

$p53^{R273H}$ activities, thereby greatly increasing clonogenic survival and growth. Therefore, these findings demonstrate that HSF1 actions can be cell context dependent with respect to p53 status.

In addition, this study has also generated HSF1 shRNAmir constructs and examined the effects of HSF1 knockdown within differing cellular contexts. While previous studies have demonstrated that inhibition of HSF1 can abrogate the malignant phenotype of many high-grade cancer cells, this study demonstrates that inhibition of HSF1 exerts little impact upon the cell biology of normal and $H\text{-}Ras^{V12}$ transformed MCF10A cells. However, HSF1 knockdown reduces the clonogenicity of these cells, not only by the reduction of HSP expression, but also potentially through increasing the steady state levels and activity of wild-type p53. Together with previous studies, this work indicates that the inhibition of HSF1 would uniquely abrogate the growth of high-grade tumours while exerting minimal toxicity to normal cells. Moreover, HSF1 inhibition could potentially be used to enhance the efficacy of cancer therapies that activate wild-type p53.

Finally, while there is currently a lack of specific and/or potent HSF1 inhibitors, this study has also successfully developed a novel cell-based reporter system that could be used for large-scale HSF1 inhibitor screening. The development of this model could lead to the identification of new therapeutic compounds for anticancer treatment.

In summary, these studies support the notion that HSF1 is not an oncogene *per se* but rather functions as an enhancer of cancer progression by supporting the maintenance of malignant phenotypes induced by other genetic and epigenetic alterations within tumour cells. In particular, this study shows that HSF1 exerts disparate effects upon cancer tumourigenesis and progression with respect to differing cellular oncogenic contexts. Therefore this work adds to our understanding of the role of HSF1 in cancer cell survival and progression and has important implications for its therapeutic targeting in cancer treatments.

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LIST OF ABBREVIATIONS

17AAG	17-allylamino-17-demethoxygeldanamycin
17-DMAG	17-dimethylaminoethylamino-17-demethoxygeldanamycin
5'UTR	5’ untranslated region
ABD	N-terminal ATP binding domain or ATPase domain
ACCa	Acetyl CoA carboxylase a
ACM	Astrocyte conditioned media (ACM)
AMPK	AMP-activated protein kinase
Apaf-1	Apoptotic protease activating factor 1
APC/C	Anaphase promoting complex C
ARF-BP1/mule	ADP ribosylation factor-binding protein 1
ATCC	American Type Culture Collection
ATM	Ataxia-telangectasia mutated protein
ATR	ATM- and RAD3-related protein
BAD	Bcl-2-associated death promoter
BAG3	Bcl2-associated athanogene 3
BAX	Bcl-2 associated X
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
BDT	Big Dye Terminator (BDT)
BRG1	Brahma-related gene 1
BSA	Bovine serum albumin
c-Abl	Abelson kinase
CaMKII	Calcium/calmodulin-dependent protein kinase II
CBP/p300	CREB binding protein
CDKN1A	Cyclin-dependent kinase inhibitor 1A
CHIP	C-terminal Hsc70-interacting protein
Chk	Checkpoint kinase
Cop1	Constitutive photomorphogenic 1 protein
CoREST	Transcriptional co-repressor
COX2	Cyclooxygenase 2
Ct	Threshold cycle value
DAPI	4’6-diamidino-2-phenylindole
DAXX	Death domain associated protein
DBD	DNA binding domain
DCs	Dendritic cells
DMEM	Dulbecco’s modified Eagle Medium

DMEM/F12	Dulbecco's modified Eagle Medium / Ham's nutrient mixture F12
DMSO	Dimethyl sulfoxide
DNA-PK	DNA- protein kinase
DSIF	DRB sensitivity-inducing factor
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
eEF1A	Elongation factor 1A
EGF	Epidermal growth factor (EGF)
EGFP	Enhanced green fluorescence protein
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal (EMT)
ER	Estrogen receptor
Erk	Extra cellular-regulated kinase
Ets-1	v-ets erythroblastosis virus E26 oncogene homolog 1
FACS	Fluorescence-activated cell sorting
FasL	Fas ligand
FASN	Fatty acid synthase
FBS	Fetal Bovine Serum
Fbx4	F-box only protein 4
FGF	Fibroblast growth factor
FKBP52	FK506-binding Protein
GAPs	GTPase activating proteins
G-CSF	Granulocyte-colony stimulating factor
GEFs	Guanine exchange factors
Grb	Growth factor receptor bound
GRP94	Glucose regulated protein 94
HAUSP	Deubiquitinase complex Herpesvirus-Associated Ubiquitin-Specific Protease
HCC	Hepatocellular carcinoma
HDAC6	Histone deacetylase
HIF-1α	Hypoxia inducible factor 1α
HMEC	Human mammary epithelial cells (HMEC),
HMGB1/TLR	High mobility group box 1/Toll-like receptor
HNE	4-Hydroxynonenal
HR-A/B	Heptad repeat regions
HSBP1	HSF1-binding protein
HSE	Heat shock element
HSF1	Heat shock factor 1
HSF1-DN	Hominant negative form of HSF1
HSPs	Heat shock proteins

HSR	Heat shock response
HSR-1	Heat-sensing RNA molecule
HuR	Human antigen R
IARC	International Agency for Research on Cancer
IL-1β	Interleukin 1β
IR	Ionizing radiation
IRES	Internal ribosome entry site
JNK	c-jun N-terminal kinase
KNK437	N-Formyl-3,4-methylenedioxy-benzylidene-gamma-butyrolaetam
KRIBB11	N(2)-(1H-indazole-5-yl)-N(6)-methyl-3-nitropyridine-2,6-diamine)
LDH	Lactate dehydrogenase
MAP	Mitogen-activated protein
MAPK	MAP kinase
MARs	Matrix attachment region DNA elements
Mcl-1	Myeloid cell leukemia 1
Mdm2	Human murine double minute 2 protein
MDR-1	Multi-drug resistance protein
MEFs	Mouse embryonic fibroblasts
MEK	MAPk-activated protein kinase
MHC	Major histocompatibility complex
MK2	MAPK-activated protein kinase 2
MMP	Matrix metalloproteinase
MOI	Multiple of infection
MRE11	Meiotic recombination 11
MTA1	Metastasis associated protein 1
mTOR	Mammalian target of rapamycin
NELF	Negative elongation factor
NF-Y	Nuclear factor Y
NSAIDs	Non-steroid anti-inflammatory drugs
nSBs	Nuclear stress bodies
p53I3	p53 inducible 3
PARP1	Poly(ADP-ribose) polymerase-1
PBD	Peptide binding domain
PBS	Phosphate bufferred saline
PDGF-B	Platelet-derived growth factor subunit B
PDK1	3-phosphoinositide-dependent protein kinase-1
PGE2	Prostaglandin E2
PGE2	Prostaglandin E2
P-gp	P-glycoprotein
PI3K	Phosphoinositide 3-Kinase

Pin1	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1
Pirh2	p53-induced ring H2 protein
PKC	Protein kinase C
PLCε	Phospholipase C gamma
PLK1	Polo-like kinase 1
PML1	Promyelocytic leukemia 1
PR	Progesterone receptor
pRb	Retinoblastoma protein
Psmb5	Proteasome (prosome, macropain) subunit beta type 5
P-TEFb	Positive transcription elongation factor b
RAGE	Receptor for advanced glycation end products
RALGDS	Ral guanine dissociation stimulator
RD	Regulatory domain
RNAP II	RNA Polymerase II
ROS	Reactive oxygen species
SD	Standard deviation
sHSP	Small HSP
SOS	Son of Sevenless
Sp1	Specificity protein 1
SREBP	Sterol regulatory element binding protein
STK33	Serine/threonine protein kinase 33
SUMO	Small ubiquitin related modifier
SWI/SNF	SWItch/Sucrose NonFermentable
TAD	Transactivation domain
TAFs	TBP associated factors
TBP	TATA binding protein
TF	Transcription factor
TFIIH	Transcription factor II H
TNBC	Triple negative breast cancers
TNF-α	Tumour necrosis factor α
TopBP1	DNA topoisomerase II-beta-binding protein 1
TRAP1	Tumour necrosis factor receptor associated protein
uPA	Urokinase-type plasminogen activator
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor
XAF1	XIAP-associated factor 1
XIAP	X-linked inhibitor of apoptosis protein

CHAPTER 1

INTRODUCTION

1.1. BREAST CANCER

1.1.1. Overview

Breast cancer is a malignant tumour arising from cells of the breast tissue. The disease is the most common type of cancer in women and is the leading cause of cancer related death among women worldwide. In Australia, the risk of breast cancer for a woman before the age of 75 is 1 in 11 (Siegel et al., 2012). The causes of breast cancer are not yet fully understood although several risk factors have been identified such as gender, age, genetics, race and ethnicity. Despite advances in early detection and treatments available, breast cancer remains one of the biggest public health concerns due to its high incidence, complexity and economic costs. Breast cancer treatment requires multidisciplinary management which may include local treatments such as surgery and radiotherapy and systemic treatments such as chemotherapy and hormonal therapy. Improvement of treatment efficacy over the years and the development of new neoadjuvant and adjuvant therapies, such as Herceptin and Tamoxifen, have led to a significant improvement in survival rate for early stage breast cancer, with patients diagnosed with a stage I breast cancer having a more than 95% 5-year survival rate. However, once the cancer cells have metastasised to multiple distant organs (Stage IV), the survival rate is significantly reduced to only 15-20% (DeSantis et al., 2011). In addition, about 40% of all patients with breast cancer will suffer a recurrence and most of these patients will ultimately die from metastatic breast cancer. Effective local treatments are very limited in advanced breast cancer and the currently available systemic treatments are mainly to palliate symptoms and prolong survival rather than being curative (Gerber et al., 2010). Moreover, the patients on such therapies can face severe side effects due to the toxicity of these agents and often drug resistance to these treatments can develop. Therefore, the development of better therapeutic compounds with less toxicity, higher efficacy and a reduced ability to induce resistance are required for advanced and metastatic breast cancer.

1.1.2. Breast cancer subtypes and prognosis

Breast cancer is a highly heterogeneous and phenotypically diverse disease which has been classified into five different molecular subtypes based on gene expression patterns identified through gene expression profiling. These subtypes are referred to as (1) Luminal A, (2) Luminal B, (3) HER2/neu over-expressing, (4) triple negative and (5) normal breast like tumours (Fig. 1.1) (Perou et al., 2000; Sotiriou et al., 2003). Each breast cancer subtype displays distinct histopathological and clinical behaviours with differences in prognosis, patient outcome, response to therapy and likelihood of metastasis. Luminal A and luminal B tumours display an expression profile similar to that of luminal epithelial cells found in normal breast tissue. HER2/neu overexpressing tumours are characterised by an amplified expression of the HER2 oncogene and other genes located in the chromosome 17q11 amplicon. Triple negative breast cancers (TNBC) are tumours lacking the expression of estrogen receptor (ER), progesterone receptor (PR) and HER2/neu. The majority of these tumours display a basal-like transcriptome and are referred to as basal-like breast cancer (Luck et al., 2008)

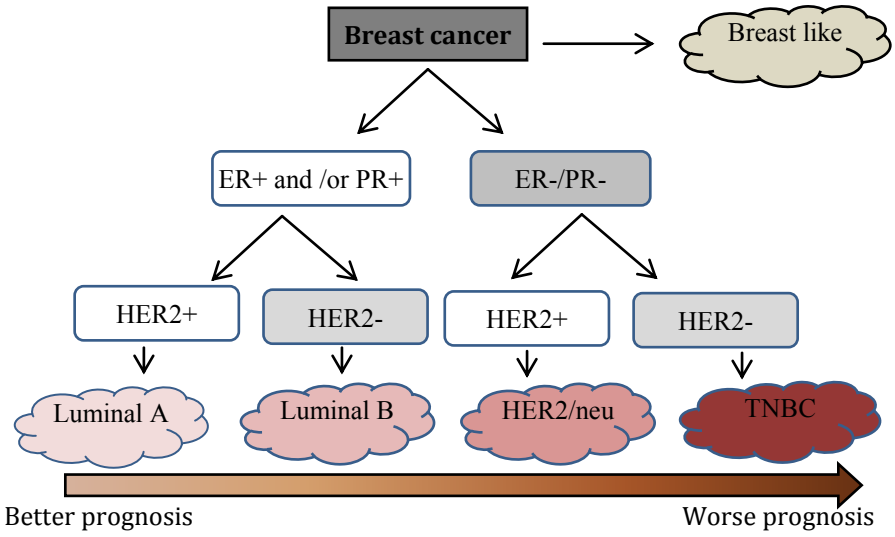


Figure 1.1. Breast cancer molecular subtypes
Breast tumours are classified into five molecular subtypes according to gene expression patterns, which are Luminal A (ER+/PR+/HER2+), Luminal B (ER+/PR+/HER2-), HER2/neu overexpressing (ER-/PR-/HER2+), triple-negative (ER-/PR-/HER2-) and breast like tumours. The aggressiveness of each subtype increases from Luminal A to Luminal B, followed by HER2/neu overexpressing and triple negative tumours, with triple negative tumour displaying the worst clinical outcome.

Among the breast cancer subtypes, luminal A tumours have the best overall survival rate and responsiveness to hormonal therapies (Sorlie, 2004). Luminal B tumours have poorer prognosis due to their enhanced proliferation rate caused by aberrant expressions of cell cycle promoters. HER2/neu overexpressing tumours do not respond to endocrine therapies and are also associated with poor clinical outcome (Kaptain et al., 2001). However, this subtype is highly responsive to HER2 targeted drugs and neoadjuvant therapies used in combination with those drugs (Goldstein et al., 2007). Triple negative breast cancers, especially the basal-like tumours, are the most aggressive among the subtypes and are highly refractory to most current therapies. These tumours are highly metastatic and have been shown to preferentially metastasise to the lungs and brain and less frequently to the liver, lymph nodes and bones. Since current therapies for this subtype lack efficacy with drug resistance often developing, there is a requirement for the identification of better therapeutic targets in highly metastatic breast cancers to improve treatments and ultimately patient survival.

1.2. HALLMARKS OF CANCER

Cancer is characterized by the uncontrolled, abnormal growth of malignant cells, leading to the formation of a cell mass or tumour that has the potential to spread throughout the body. In healthy tissues, normal cells grow, divide and die under a tightly controlled process. Cells become malignant through a multi-step process referred to as tumourigenesis. This process involves multiple sequential genetic and epigenetic alternations, which result in activation of oncogenes and inactivation of tumour suppressors. These alterations enable the acquisition of several abnormal capabilities, allowing the cell to escape from the tight constraints that control normal cells. Common traits of cancer cells have been observed and described by Hanahan and Weinberg (2004) as the six hallmarks of cancer. These are (1) self-sufficiency in growth signals, (2) insensitivity to growth suppressors, (3) evasion of apoptosis, (4) limitless replicative potential, (5) sustained angiogenesis, and (6) tissue invasion and metastasis (Hanahan and Weinberg, 2000; Mansur, 1997). This list has recently been updated by the authors with the addition of another four hallmarks, namely: (1) abnormal metabolic pathways, (2) evading the immune system, (3) genomic instability, and (4) inflammation (Hanahan and Weinberg, 2011).

1.2.1. Self-sufficiency in growth signals

In normal cells, proliferation is activated by mitogenic signals, which include cytokines, hormones and growth factors present in the extracellular matrix and cell-to-cell adhesion/interaction molecules on the surface of adjacent cells. These molecules bind and activate cell-surface receptors, typically containing intracellular tyrosine kinase domains, leading to activation of downstream signalling pathways regulating cell growth and division. Cancer cells acquire the ability to sustain cell proliferation by exploiting a number of alternative ways. For example, they may generate growth factors themselves which results in autocrine proliferation stimulation. They may also alter gene expression and/or mutate key signalling molecules and/or activate oncogenes, leading to the constitutive activation of mitogenic signalling pathways independent of external stimuli (Hanahan and Weinberg, 2011). Some of the pathways that play a central role in the autonomous growth of cancer are the SOS/Ras/Raf/MAPK and PI3K/Akt pathways, which are downstream of ligand activated growth factor tyrosine kinase receptors (TKRs). Constitutive activation of these pathways is frequently observed in cancer cells.

1.2.2. Insensitivity to growth suppressors

The proliferation of normal cells is also regulated by growth inhibitory signals, which force cells into a quiescent or post-mitotic state whereby they can no longer proliferate and can thereby maintain tissue structure and homeostasis. This is primarily controlled by the activity of two tumour suppressors, the retinoblastoma protein (pRb) and p53. Cancer cells exhibit insensitivity to many anti-proliferative signals, mainly via inactivation and mutation of pRb and p53, with approximately 30% containing a mutation in pRb and 50% containing a mutation in p53 (Weinberg, 1995). This capability enables the continuous growth of tumours.

1.2.3. Evasion of apoptosis

Apoptosis is the programmed cell death essential for normal development and tissue homeostasis. This process involves the activation of a family of cysteine proteases, named caspases, which act as proteolytic enzymes to dismantle and remove dying cells (Fulda and Debatin, 2006). Apoptosis is triggered by two major pathways: intrinsic and extrinsic. The extrinsic pathway is initiated through the stimulation of transmembrane

cell death receptors by signals released from other cells, such as cells involved in the immune response. In contrast, the intrinsic pathway is initiated by the release of signal factors from the mitochondria in response to various cellular stresses. Virtually all cancer cells harbour mutations that enable them to evade apoptosis. Common mutations are the loss of the tumour suppressor p53, or mutations that lead to reduced pro-apoptotic and/or increased anti-apoptotic proteins. Alternatively, cancer cells may activate signalling pathways responsible for cell survival which enable them to resist the apoptotic pressure.

1.2.4. Limitless replicative potential

Normal cells do not proliferate indefinitely either *in vitro* or *in vivo*. After a period of rapid proliferation, cells enter a permanent dormant state where they become unresponsive to mitogenic stimuli and cell growth is arrested at the G0-G1 phase of the cell cycle. This process is termed cellular senescence and has been shown to play critical role in regulating cellular lifespan. Cellular senescence is normally induced by the shortening of telomeres, which are DNA structures located at the end of every human chromosome that could not completely replicate during each cell division (de Magalhaes, 2004; Stanulis-Praeger, 1987). Tumour cells can overcome this mitotic 'clock' and escape cellular senescence by reactivating the expression of the enzyme telomerase, which can preserve the telomeres and thereby enable unlimited cell division.

1.2.5. Sustained angiogenesis

Like normal tissues, to remain healthy, tumours require a continuous supply of oxygen and nutrients and a method to remove metabolic waste products. To address these needs, tumour cells stimulate the formation of a network of tumour-associated neovasculature from existing blood vessels – a process called tumour angiogenesis. Low oxygen levels trigger the release of angiogenic signals from the tumour cells, such as members of the fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) families, which stimulate the quiescent vascular endothelium to enter the cell cycle and proliferate. Tumour cells can also promote angiogenesis by secreting extracellular proteases such as urokinase-type plasminogen activator (uPA) and the matrix metalloproteinases MMP2, MMP7, MMP9 and MMP12 that play important roles in the bioavailability of angiogenic activators and inhibitors. Sustained

angiogenesis is a key step in the development and growth of solid tumours (Eckhardt and Pluda, 1997; Ribatti and Djonov, 2011).

1.2.6. Tissue invasion and metastasis

Cancer cells can break away from their site of origin and form new tumours at distant sites. This process is referred to as invasion and metastasis. To escape from the primary site, tumour cells first acquire an invasive phenotype, allowing them to invade the surrounding tissues and intravasate into the microvasculature of the lymph and blood vessels. The cancer cells, which are able to survive, travel through the circulation to micro-vessels at distant sites where they exit the bloodstream and invade the foreign tissue. At this secondary site, the tumor cells survive and adapt to the new microenviroment in ways that facilitate cell proliferation and the formation of secondary tumours. Cancer cells that acquire these properties are considered to be aggressive and are often resistant to cancer therapies (Duffy et al., 2008; Price and Thompson, 2002).

1.2.7. Abnormal metabolic pathways

In order to support rapid proliferation and expansion at different sites within the body, cancer cells alter their metabolism, exhibiting preferential use of the glycolytic pathway and lactic acid production within the cytosol, which is termed the "Warburg effect". This is in contrast to normal cells that exhibit low levels of glycolysis and carry out oxidation of pyruvate within the mitochondria (Annibaldi and Widmann, 2010; Chen et al., 2007; DeBerardinis et al., 2008; Warburg, 1956). The acquisition of this glycolytic phenotype confers significant growth and survival advantages to cancer cells over their normal counterparts by favourably adapting them to the hypoxic tumour microenvironment (Hsu and Sabatini, 2008; Huber et al., 2011). The Warburg effect is explained by the fact that when a tumour expands beyond the diffusion limits of its local blood supply, the hypoxic tumour microenviroment triggers the stabilization of the hypoxic-inducible transcription factor (HIF). HIF stimulates the transcription of target genes involved in angiogenesis and glucose metabolism. Alternatively, oncogene activation or tumour suppressor gene loss can also drive this alteration in glucose metabolism in the cancer cell (Annibaldi and Widmann, 2010).

In addition to increased glycolytic activity, alterations of lipid metabolism are also often observed in cancer. Tumour cells are able to perform *de novo* synthesis of fatty acids,

which are used for membrane synthesis and to modify membrane-targeted proteins that support the rapid cell division. They exhibit an increased expression and activity of a number of lipogenic enzymes such as fatty acid synthase (FASN), ATP citrate lyase, acetyl CoA carboxylase a (ACCa) and Spot14. High levels of these enzymes in cancer are also associated with invasive phenotypes and poor prognosis (Santos and Schulze, 2012; Yecies and Manning, 2010).

1.2.8. Evasion of the immune system

In cancer, the accumulation of mutations in oncogenes and tumour suppressor genes can lead to the production of mutant proteins that may be recognized as tumour-specific antigens. These antigens can initiate immune responses that lead to the detection and elimination of those tumour cells. Tumour formation therefore involves the ability of cancer cells to escape the recognition and attack by the immune system. Cancer cells may express self-antigens that recruit suppressor T cells to the site of the tumour and dampen the immune response, thereby maintaining tolerance to self-antigens. Cancer cells may also suppress the cytotoxic T cell response by the down-regulation or loss of the major histocompatibility complex (MHC) class antigen. Cancer cells may also become resistant to FAS or TRAIL induced apoptosis by T cells or may inhibit T cell activity via the expression of natural killer (NK) cell inhibitory receptors. In addition, the tumour microenvironment can also become resistant to T cell infiltration (de la Cruz-Merino et al., 2011; Seliger, 2005). All of these strategies enable cancer cells to evade the host immuno-surveillance, allowing the formation of tumours.

1.2.9. Genomic instability

One common characteristic of cancer is the genomic instability which is referred to as an increased tendency of alterations in the genome during the cell cycle. Cancer cells typically possess numerous genomic mutations and chromosome aberrations such as point mutations, gene amplifications and deletions, as well as aneuploidy. Genomic instability is the result of the breakdown in one or several components of the genomic maintenance machinery. These involve mutations in proteins responsible for DNA replication, DNA repair, cell cycle progression and other proteins that function to maintain cellular homeostasis. Among these, the tumour suppressor gene TP53 is known to have an important role in preventing genome mutation and is frequently observed to be mutated or deleted in tumour cells. Genomic instability endows tumour

cells with numerous genetic alterations that drive tumour formation and progression (Coleman and Tsongalis, 1995; Negrini et al., 2010).

1.2.10. Tumour-promoting inflammation

Cancer is known to be supported by inflammation. Analyses from epidemiological, preclinical and clinical studies indicate that about 25% of all cancer cases are related to a chronic infection and other types of sustained inflammation (Hussain and Harris, 2007). The relationship is further evidenced by the fact that prolonged use of non-steroid anti-inflammatory drugs (NSAIDs) protects against many types of cancers. Inflammation is part of the innate immune response generated by the body in response to injury, infection or irritation. This response involves activation of the innate immune cells such as macrophages, mast cells, dendritic cells (DCs) and NK cells, leading to the release of pro-inflammatory mediators facilitating the elimination of pathogens and the repair of damaged tissues. Acute inflammation is vital to the healing process; however, failure in the precise control of the immune response, which results in chronic inflammation, can generate a pathological microenvironment conducive to cancer initiation and progression. The sustained inflammation present in the tumour microenvironment can provide a constant supply of pro-inflammatory mediators that can promote several aspects of cancer (Lu et al., 2006). Within the tumour cells, alterations in oncogenes and tumour suppressors can activate the inflammatory signalling pathway, leading to the release of pro-inflammatory molecules and the promotion of inflammation within the tumour microenvironment. The interplay between extrinsic and intrinsic inflammatory pathways is one of the crucial components that drive tumourigenesis (Balkwill and Mantovani, 2001).

1.3. THE RAS ONCOGENE

1.3.1. Ras GTPase

Advances in molecular oncology have led to the discovery of many oncogenes and tumour suppressors implicated in the tumourigenesis process. Among oncogenes, members of the Ras family are the most frequently mutated in cancer and activation of Ras is found to play a central role in cancer development.

Human cells contain three Ras genes which encode four highly homologous proteins: H-Ras, N-Ras, K-Ras4A and K-Ras4B. Ras is a membrane bound GTPase protein, which functions as a binary switch, alternating between the active GTP-bound and inactive GDP-bound states. It functions as a secondary messenger molecule, transferring signals from cell surface growth factor receptor tyrosine kinases to multiple intracellular signalling pathways.

Activity of Ras is regulated by two classes of proteins: guanine exchange factors (GEFs) and GTPase activating proteins (GAPs). Stimulation upstream of Ras promotes GEFs to catalyse the exchange of GDP for GTP, thereby activating Ras. Although Ras has an intrinsic ability to hydrolyse GTP, the rate of this hydrolysis is very slow. Inactivation of Ras is accelerated by the GTP hydrolysis activity of GAPs (Fig.1.2; Sprang, 1997).

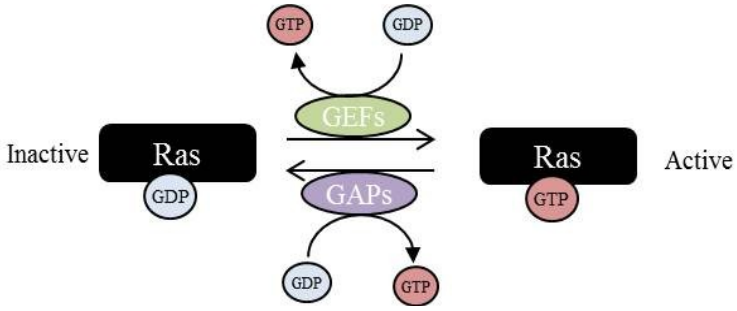


Figure 1.2. Activation / inactivation of Ras

Ras is a GTPase protein which functions as a binary switch, alternating between the active GTP-bound and inactive GDP-bound states. Ras is activated by guanine exchange factors (GEFs), which facilitate the exchange of GDP for GTP. Although Ras can inactivate itself by its intrinsic ability to hydrolyse GFP, the rate of this hydrolysis is slow and the inactivation of Ras is accelerated by the GTP hydrolysis of GTPase activating proteins (GAPs).

Receptor tyrosine kinases (RTKs) stimulate Ras activation via a highly conserved pathway involving an adaptor protein, growth factor receptor bound (Grb) 2 and a Ras GEF, Son of Sevenless (Sos). Grb2 binds specifically to phosphorylated tyrosine residues on activated TRKs via its src homology (SH)-2 domain. The recruitment of Grb2 to the plasma membrane results in the assembly of multi-molecular complexes that interact with Ras and promote the exchange of GDP for GTP. The GTP-bound Ras subsequently interacts with a variety of proteins via its effector domain and activates a cascade of signalling pathways. Several Ras activating proteins have been identified including the serine/threonine kinase Raf, the p110 catalytic subunit of PI3-kinase and

the Ral GDP-GTP exchange factor, RalGDS, Tiam1 and PKCε (Downward, 2003; Graham and Olson, 2007; Pylayeva-Gupta et al., 2011).

1.3.2. Ras signalling pathways

1.3.2.1. Raf/MEK/ERK pathway

The best characterised signalling pathway downstream of Ras is the one initiated by activation of the serine/threonine kinase Raf. Raf phosphorylates and activates the mitogen-activated protein (MAP) kinase kinases MEK1 and MEK2. MEKs in turn phosphorylate the extracellular signal regulated (ERK) family of MAP kinases, ERK1 and ERK2. Upon activation, ERKs phosphorylate and thereby regulate a variety of cytoplasmic and nuclear substrates, such as the transcription factors Elk1, cMyc and estrogen receptors. This signalling pathway controls multiple crucial cellular processes such as cell cycle progression, survival, angiogenesis and invasion (Davies et al., 2002; Peyssonnaux and Eychene, 2001).

1.3.2.2. PI3K/Akt/mTOR pathway

Ras can also interact directly with the catalytic subunit of type I PI3K, leading to the membrane translocation and conformational changes that activate the lipid kinase. PI3K controls the activity of several downstream targets, one of which is Akt that can be phosphorylated directly by PI3K or indirectly via 3-phosphoinositide-dependent protein kinase-1 (PDK1). Akt exerts multiple biological effects by activating substrates that regulate expression of genes involved in apoptosis, cell cycle progression, gene expression, and metabolism. One important and well-studied Akt target is mTOR (mammalian target of rapamycin), which regulates components of the translation machinery for protein synthesis (Osaki et al., 2004).

1.3.2.3. RalGDS

Another well studied effector of Ras is the Ral guanine dissociation stimulator RALGDS which promotes the GDP/GTP exchange of Ral GTPases. As members of the Ras related GTPase subfamily, Ral proteins (RalA and RalB) also alternate between active GTP bound and inactive GDP-bound states. Active Ral binds to Ral-BP1 which is a GAP for cdc42 and Rac. These two proteins are involved in the regulation of actin

cytoskeleton. Ral GTPases are thus implicated in the regulation of vesicle trafficking, cell morphology and transcription (Ramocki et al., 1998).

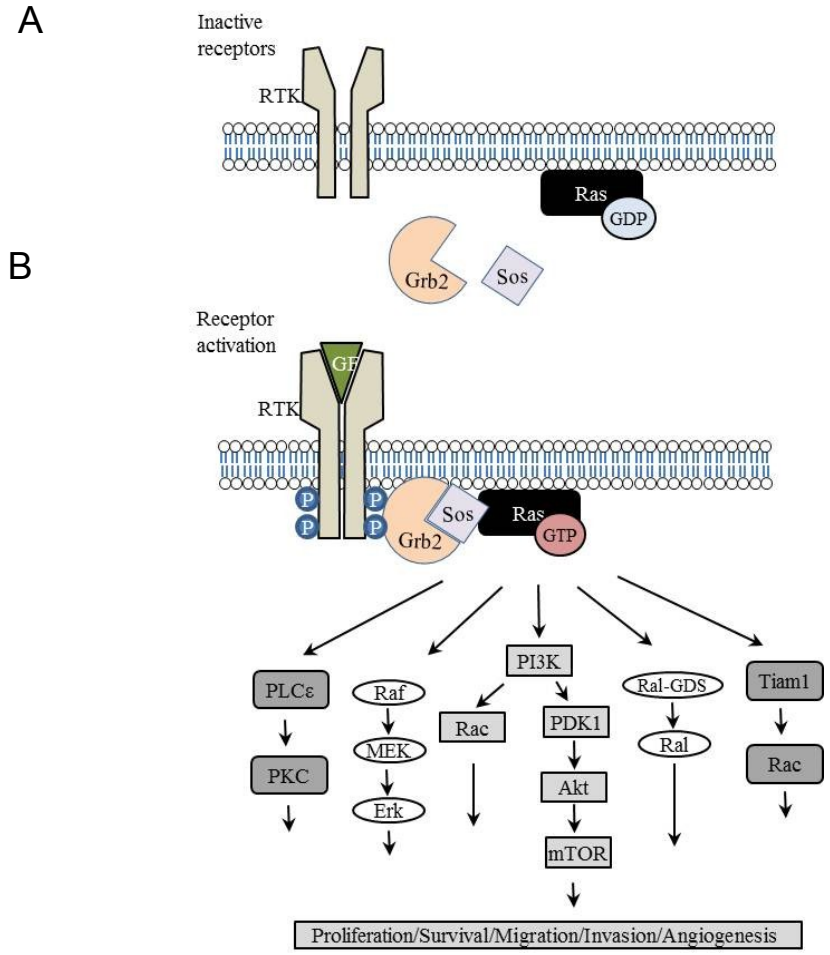


Figure 1.3. Cellular functions of Ras GTPase

(A) Ras at resting state. **(B)** Activation of Ras upon receptor tyrosine kinase (RTK) stimulation. At resting state, Ras is maintained in an inactive GDP-bound state. Stimulation of receptor tyrosine kinases (RTK) by extracellular ligands such as growth factors (GF) results in the assembly of multi-molecular complexes (e.g., Grb2, SOS) that interact with Ras at the plasma membrane, thereby promoting the exchange of GDP for GTP. Activated Ras subsequently activates a cascade of signalling events leading to alterations in several biological processes such as cell proliferation, survival, migration, invasion and angiogenesis.

1.3.2.4. Other Ras downstream targets:

Ras also activates Tiam1 which is a GEF that activates Rac, leading to changes in the actin cytoskeleton critical for a cancer cells' potential to invade and metastasise (Malliri et al., 2002; Strumane et al., 2006). In addition, Ras has been shown to activate phospholipase C (PLC), which links Ras activation to activation of protein kinase C

(PKC). PKC has numerous effects in cells including stimulation of proliferation and calcium mobilisation that is involved in cellular motility and thus migration and metastasis (Wing et al., 2003).

1.3.3. Ras mutation in cancer

In cancer, aberrant Ras signalling pathways are commonly observed, with ~ 90% pancreatic cancer, ~50% of colon cancer and ~30% of all cancers exhibiting constitutive activation of Ras (Bos, 1989). Activation of Ras occurs as the result of several different types of mutations in tumour cells within the Ras family of genes. Approximately 20% of human tumours contain an activated missense mutation in Ras, most frequently in K-Ras, then N-Ras and H-Ras. Most missense mutations are at codons 12 or 61, and more rarely at 13. These mutations prevent the hydrolysis of GTP to GDP on Ras, therefore causing an accumulation of the active GTP-bound Ras form. Ras can also be activated in cancer by the loss of GAPs or overexpression of growth factor receptors. In addition, mutation and/or amplification of Ras effectors has also been shown to up-regulate Ras signalling pathways in tumours (Fernandez-Medarde and Santos, 2011).

As Ras controls a complex network of interconnecting signalling pathways, activation of Ras affects multiple processes that drive tumourigenesis and cancer progression (Pylayeva-Gupta et al., 2011). For example, expression of activated Ras is sufficient to stimulate cell proliferation in the absence of a growth stimulus. Ras activation can also suppress apoptosis and drive metabolic alterations toward glycolysis to support the high energy demands of cancer cells. In addition, oncogenic Ras has also been shown to promote angiogenesis and evasion of the host-mediated immune response. Transformation by Ras has been shown to promote changes in cell motility and adhesion, thereby facilitating acquisition of an invasive and metastatic phenotype in cancer cells (Drosten et al., 2010).

With the compelling clinical and experimental evidences relating to elevated Ras signalling to tumour growth and progression, targeting Ras signalling pathways has become a popular target for the development of novel cancer therapeutics (Downward, 2003; Graham and Olson, 2007).

1.3.4. Targeting Ras in cancer therapy

To date, a wide variety of agents targeting Ras and its downstream signalling pathways have entered clinical trials (Table 1.1). The first group of drugs are the Farnesyl transferase inhibitors, which target the post-translational modification of Ras and are well tolerated by patients. However, the exact molecular mechanism of this class of drugs is still unclear and their efficacy in solid tumours is not sufficient to be utilised as a mono-therapy. A more specific approach to target Ras is the use of antisense oligonucleotides to down-regulate Ras or Raf expression, however the efficacy of these agents, again appears to be inadequate for single agent use (Graham and Olson, 2007).

Table 1.1. *Anti-Ras signalling agents which has been in clinical development (Graham and Olson, 2007)*

Farnesyl transferase inhibitor	R115777 (tipafarnib) SCH66336 (lonafarnib) BMS-214662
Ras antisense inhibitors	ISIS 2503
Raf antisense inhibitors	ISIS 5132 LErafAON
Raf kinase inhibitors	BAY-439006 (sorafenib)
MEK kinase inhibitors	CI-1040 PD-325901 ARRY-142886
Alkylphospho cholines	Miltefosine Perifosine
mTOR inhibitors	CCI-779 AP23573
HSP90 inhibitors	17-AAG 17-DMAG

While identifying a small molecule that can bind and inhibit mutant Ras proteins remains challenging, targeting downstream pathways of Ras is now the major focus of clinical research. Inhibition of the Raf/MAPK signalling pathways has been achieved by the development of Raf kinase inhibitors and MEK kinase inhibitors. The PI3K/Akt pathway can be targeted by Akt inhibitors and mTOR inhibitors such as alkylphosphocholines and the antibiotic rapamycin respectively. In addition, inhibitors of the heat shock protein 90 have been found to target multiple signalling molecules within the Raf/MAPK and PI3K/Akt pathways. Although recently been proven to be ineffective (Neckers and Workman, 2012), these molecules have also been evaluated in clinical trials (Graham and Olson, 2007).

In parallel, current studies also focus on identifying novel proteins that Ras depends on for malignant transformation. Using loss-of-function RNAi high-throughput screens, several proteins, including STK33 and PLK1, have been identified that are non-oncogenic but are required for mutant Ras-mediated transformation (Barbie et al., 2009;

Luo et al., 2009; Scholl et al., 2009; Vicent et al., 2010; Wang et al., 2010b). Inhibition of these proteins induces cell death in cancer cells that harbour mutant Ras proteins while having no effect on normal cells that contain wild-type Ras – a phenomenon known as synthetic lethality. These molecules may represent the next generation of novel cancer therapeutic targets that have been previously underestimated.

1.4. THE TUMOUR SUPPRESSOR p53

1.4.1. p53 structure and function

Among tumour suppressors, p53 stands out as the master regulator of various signalling pathways implicated in tumourigenesis. In normal cells, wild-type p53 acts as a transcription factor that regulates genes involved in a diverse group of biological activities that include DNA metabolism (Helton and Chen, 2007), apoptosis (Vousden, 2006), senescence (Garbe et al., 2007), cellular cycle regulation (Kastan et al., 1992), cell differentiation (Molchadsky et al., 2008; Tedeschi and Di Giovanni, 2009), metabolic processes (Green and Chipuk, 2006; Won et al., 2012), angiogenesis (Gaiser et al., 2009; Nagayama et al., 2000; Teodoro et al., 2007; Teodoro et al., 2006), immune response (Taura et al., 2008), motility and migration (Qin et al., 2009; Roger et al., 2006; Singh et al., 2007), and transcription and translation (Table 1.2). In addition, wild-type p53 possesses transcriptionally independent functions such as the direct binding and activation of proteins involved in the apoptotic pathways within the cytosol and mitochondria, and the direct association with proteins involved in genomic stability and chromatin modification. The activation of p53 upon stress triggers multiple cellular responses to prevent the multiplication of DNA damaged cells that could lead to tumour formation. The protein therefore has been regarded as “the guardian of the genome” (Lane, 1992).

Human p53 is a 393-amino-acid protein containing several functional domains: an N-terminal transactivation domain, a central core DNA-binding region, followed by a tetramerization domain and a C-terminal regulatory domain (Fig.1.4). Wild-type p53 protein exists in solution as a tetramer, which recognizes and binds to p53 responsive elements (p53REs) present on the promoters of the target genes by its central core DNA-binding region. p53REs typically contain four repeats of a consensus DNA sequence 5’-PuPuPuC(A/T)-3’ repeated in two pairs, each arranged as inverted repeats.

The DNA-binding activity of p53 is regulated by the C-terminal domain, which can bind to different forms of DNA such as DNA breaks or internal mismatches. Deletion or phosphorylation of this domain activates the sequence specific DNA binding activity of p53 (Bourdon et al., 1997; el-Deiry et al., 1992; Funk et al., 1992). Once activated, the N-terminal transactivation domain of p53 interacts with other general transcription factors such as transcription factor II D (TFIID) and transcription factor II H (TFIIH). These proteins form part of a large basal transcriptional complex that interacts with RNA polymerases and initiates RNA transcription.

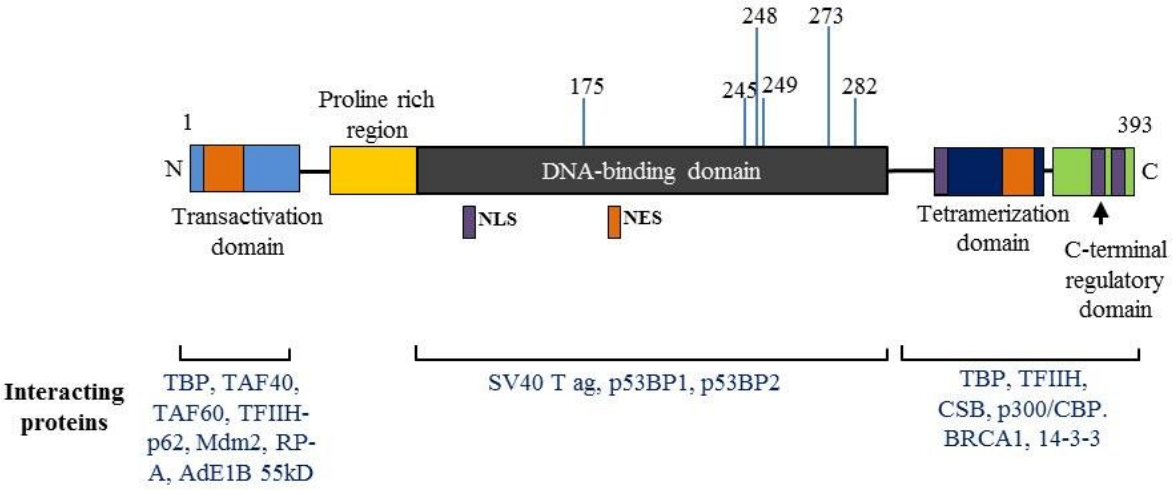


Figure 1.4. Schematic domain structure of human p53 protein with hot-spots for mutation and interacting proteins

Human p53 is a 393 amino-acid protein containing various domains. The N-terminus transactivation domain interacts with the other proteins such as transcription factor II D (TFIID or TBP, TAFs) and transcription factor II H (TFIIH), which form part of the basal transcriptional machinery regulating gene expression. The central core domain of p53 contains the sequence-specific DNA-binding region where most missense mutations of p53 are found. The native p53 protein is a tetramer in solution and the process of tetramerization requires the region of p53 from amino acid 324-355. The C-terminal domain regulates the ability of p53 to bind to specific DNA sequences. This domain binds to different forms of DNA such as DNA breaks or internal mismatches and is required for p53 specific DNA binding activation. NLS: Nuclear localization signal, NES: nuclear export signal (Ryan et al., 2001; Somasundaram, 2000).

***Table 1.2**. Known transcriptional targets of wild-type p53 (Gomez-Lazaro et al., 2004; Menendez et al., 2009; Riley et al., 2008)*

DNA repair	
Positively regulated	
DDB2	Damage-specific DNA binding protein 2
p53R2	p53 ribonucleotide reductase
PCNA	Proliferating cell nuclear antigen
RAP80	Receptor associated protein 80
BRCA1	Breast cancer 1
LIG1	DNA ligase I
ERCC5	DNA excision repair-related gene
Negatively regulated	
DNMT1	DNA (cytosine-5-)-methyltransferase 1
Cell cycle inhibition	
Positively regulated	
14-3-3σ	n/a
CDK2	Cyclin-dependent kinase 2
CDK4	Cyclin-dependent kinase 4
CDKN1	CDK interacting protein 1 (p21)
Cyclin A/B1/B2/D1/D2/E	
GADD45α	Growth arrest and DNA-damage-inducible, alpha
TGF-α	Transforming growth factor α
PTEN	Phosphatase and tensin homolog
IGFBP3	IGF binding protein 3
MIC-1	Colon cancer -associated protein
Negatively regulated	
PLK2	Polo-like kinase 2
CDC25C	Cell division cycle 25 homolog C
Senescence	
Positively regulated	
Ras	n/a
Raf	n/a
p14/ARF	CDKN2 alternative reading frame
MAPK	Mitogen-activated protein kinase 2
E2F1	Retinoblastoma-associated protein 1
P16	n/a
PML	Promyelocytic leukemia
Cytokine production and inflammation	
Positively regulated	
IRF5	Interferon regulatory factor 5
TLR3	Toll like recptor 3
Transcription and translation	
Positively regulated	
EEF1A1	Eukaryotic translation elongation factor 1 α1
ATF3	activating transcription factor 3
Apoptosis	
Positively regulated	
Bax	BCL2-associated X protein
APAF-1	Apoptotic peptidase activating factor 1
p53AIP1	p53-regulated apoptosis-inducing protein 1
PUMA	n/a
FAS/APO1	Fas (TNF receptor superfamily, member 6)
NOXA	n/a
PERP	p53 apoptosis effector related to PMP-22
BID	BH3 interacting domain death agonist
CASP1/2/3/6/9/10	Caspase 1/2/3/6/9/10
PIDD	p53-induced death domain protein
Killer/RD5	DNA damage-inducible *p53*-regulated death receptor 5
Negatively regulated	
BIRC5	Baculoviral IAP repeat-containing 5 (survivin)
STMN1	Stathmin 1
Bcl-2	B-cell lymphoma 2
Bcl-XL	B-cell lymphoma-extra large
HSP90AB1	Heat shock protein 90kDa alpha B 1
HSPA8	Heat shock 70kDa protein 8
Angiogenesis, cell adhesion and migration	
Positively regulated	
MMP2	Matrix metalloproteinase-2
FLT1	Fms-related tyrosine kinase 1
P4HA2	Prolyl 4-hydroxylase subunit alpha-2
COL18A1	Collagen type XVIII, α1
CAV1	Caveolin 1, caveolae protein, 22kDa
Maspin	Protease inhibitor 5
KAI1	Kangai 1 (Metastasis suppressor homolog)
Negatively regulated	
VEGFA	Vascular endothelial growth factor A
PTK2(FAK)	PTK2 protein tyrosine kinase 2
ANLN	Anillin, actin binding protein
Metabolism	
Positively regulated	
HK2	Hexokinase-2
SCO2	Synthesis of cytochrome c oxidase
TIGAR	*TP53*-induced glycolysis and apoptosis regulator
Negatively regulated	
SCD	Stearoyl-CoA desaturase

Human p53 protein is encoded by the gene TP53, which belongs to a family of highly conserved genes containing two other members: TP63 and TP73, encoding p63 and p73 proteins respectively. p63 and p73 are structurally related to p53 and also function as transcription factors. In addition to their own specific transcriptional targets, they are capable of trans-activating some p53-responsive genes. Although not functionally redundant to p53, p63 and p73 are also considered as tumour suppressors. Together with p53, they form a distinct family of transcription factors which can co-operate or act independently in regulation of transcriptional targets involved in cell cycle arrest, apoptosis and other biological processes critical for normal cellular development and differentiation (Flores et al., 2005; Kaghad et al., 1997; Lin et al., 2009; Yang et al., 1998).

1.4.2. p53 regulation

Activity and the level of p53 protein within the cell are tightly regulated. Under normal conditions, p53 is subject to rapid degradation mediated largely by the human murine double minute 2 protein (Mdm2), which acts as an E3 ubiquitin ligase that continuously ubiquitinates and targets p53 for degradation via the proteasome. Mdm2 is also a transcriptional target of p53, and both proteins form an auto-regulatory feedback loop by which they mutually control their cellular levels (Haupt et al., 1997; Kubbutat et al., 1997). In addition to Mdm2, wild-type p53 is also targeted for degradation by other E3 ligases such as constitutive photomorphogenic 1 protein (Cop1), p53-induced ring H2 protein (Pirh2), ADP ribosylation factor-binding protein 1 (ARF-BP1/mule) and C-terminal Hsc70-interacting protein (CHIP) (Newton and Vucic, 2007). These regulations ensure a low level of p53 protein in cells under normal physiological conditions.

Upon exposure to stress, such as DNA damage, hypoxia, and reactive oxygen species (ROS) production, p53 is stabilized and activated through numerous mechanisms that lead to the disruption of the Mdm2-p53 interaction. Many phosphorylation sites have been identified on p53 that significantly reduce Mdm2 binding. For example, phosphorylation of threonine 18, serine 15 and serine 20 in the transactivation domain of p53 by stress induced kinases, such as ataxia-telangectasia mutated protein (ATM), ATM- and RAD3-related protein (ATR), checkpoint kinase 1 (Chk1), checkpoint kinase 2 (Chk2), and DNA- protein kinase (DNA-PK), have been shown to stabilize p53 by

inhibiting Mdm2-p53 interaction (Minamoto et al., 2001). Mdm2-p53 interaction can also be inhibited by the stress-induced phosphorylation of Ser395 and Tyr394 on Mdm2 by the kinases ATM and Abelson kinase (c-Abl), respectively (Meek and Knippschild, 2003). In addition, acetylation of Mdm2 by CREB binding protein (CBP/p300) and acetylation of p53 at eight lysine residues in its C-terminal domain can also activate the transcription factor (Eichenbaum et al., 2010; Tang et al., 2008). Alternatively, Mdm2-p53 interaction can be inhibited by Mdm2 sequestration mediated by the stress-induced tumour suppressor protein ARF (Alternative Reading Frame) or by inducing Mdm2 degradation through the disruption of its interaction with the deubiquitinase complex herpesvirus-associated ubiquitin-specific protease (HAUSP) (Brooks et al., 2007; Li et al., 2004; Sheng et al., 2006). Through these multiple mechanisms of regulation, activity of p53 is tightly control to ensure normal cell growth and cell cycle progression.

1.4.3. p53 mutation in cancer

1.4.3.1. TP53 mutation

Mutation of the p53 pathway is one of the most common events in all types of cancers, with more than 50% of human cancers exhibiting a TP53 gene mutation (Hollstein et al., 1991). According to the latest release of the International Agency for Research on Cancer (IARC) p53 database, there are more than 27,000 somatic and up to 600 germ line p53 mutations found in humans (Petitjean et al., 2007). Among these, more than 70% are missense mutations and most of these mutations cluster within the core DNA-binding domain. Of the mutations in this domain, about 30% fall in 6 ‘hotspot’ residues, which are R175, G245, R248, R249, R273, and R282.

Missense mutations in TP53 often lead to the production of mutated proteins with a partial or complete loss of wild-type p53 tumour suppressor activities. While wild-type p53 under normal conditions is a very short-lived protein, mutant p53 proteins have significantly prolonged half-lives (Strano et al., 2007a). In fact, mutant p53 proteins are often found at very high levels in tumours and cancer cells. This is explained by the fact that mutant p53 proteins are incapable of transactivating Mdm2, and other events occurring during tumourigenesis also abrogate the Mdm2-mediated degradation of p53. For example, mutant p53 proteins have been found to be stabilized by the heat shock

protein (HSP) 90 chaperone complex, which prevents them from Mdm2-mediated ubiquitination and degradation (Li et al., 2011b).

Mutation in one allele of the TP53 gene often results in the loss of activity of the remaining wild-type protein, a phenomenon known as a dominant-negative effect. This is because the more abundant mutant p53 can form co-tetramer with wild-type p53, p63 or p73, leading to the cytoplasmic sequestration or inhibition of DNA binding activity of the wild-type proteins (Roemer, 1999; Willis et al., 2004). Moreover, the mutant p53 proteins can also interact and sequester proteins and co-factors necessary for wild-type p53-dependent transcription (Donzelli et al., 2008).

1.4.3.2. Mutant p53 gain-of-function

Interestingly, a mutation in the TP53 gene is often not equivalent to the loss of wild-type p53 activity. The proportion of missense mutations in p53 is much higher than that

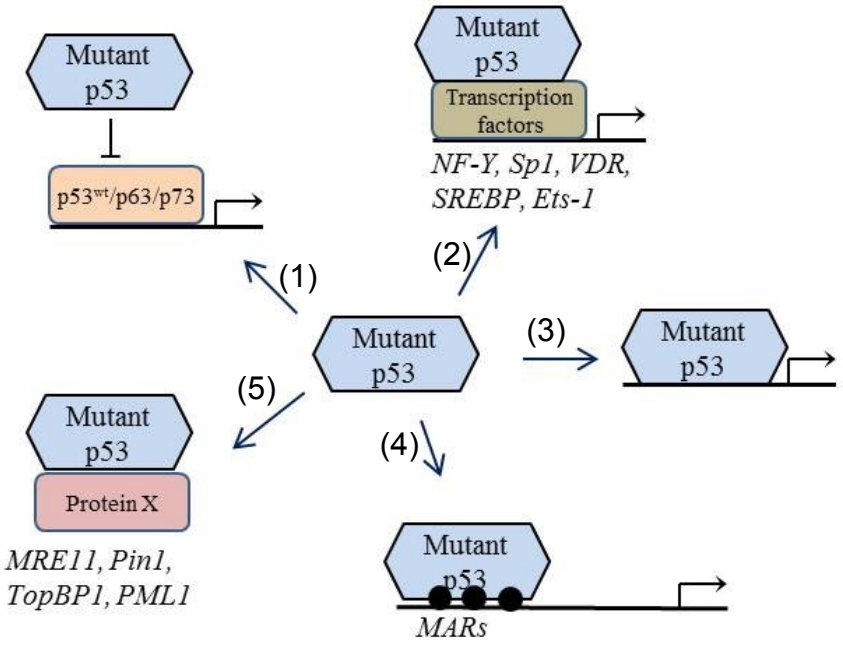


Figure 1.5. Mechanisms of mutant p53 gain-of-function
Several mechanisms have been proposed to explain the dominant negative and gain-of-function properties of mutant p53 proteins. (1) Mutant p53 can inhibit the activity of the remaining wild-type p53, p63 and p73 by directly associating with them or by sequestering cofactors that are required for their activity. (2) Mutant p53 can function as a transcription factor regulating the expression of novel target genes. (3) Mutant p53 can interact and modulate the activity of other transcription factors such as NF-Y, Sp1 and VDR. (4) Mutant p53 can bind to specific DNA sequences on the chromosome such as matrix attachment region DNA elements (MARs) and regulate activity of the relevant promoters. (5) Mutant p53 can associate with other proteins in the cells and modulate their cellular functions. Other mechanisms are also likely to exist (Adapted from Freed-Pastor and Prives, 2012).

of other tumour suppressor genes, suggesting that the expression of p53 mutants may confer selective advantage distinct from that of the loss of wild-type p53 function (Hussain and Harris, 2000; Strano et al., 2007a; Strano et al., 2007b). In fact, many p53 mutants have been shown to promote cell proliferation, survival, migration, invasion and chemo-resistance (Dittmer et al., 1993; Gualberto et al., 1998; Hsiao et al., 1994). This effect is described as "gain-of-function" or dominant-positive effects.

Several mechanisms have been proposed to explain mutant p53 gain-of-function (Fig.1.5; Freed-Pastor and Prives, 2012). Yeast and mammalian cell-based assays have revealed that mutant p53 proteins can lose certain tumour suppressor functions of wild-type p53 yet still retain and/or exaggerate some of the transactivation activities upon a number of wild-type p53 transcriptional target genes (Di Como and Prives, 1998; Kato et al., 2003; Resnick and Inga, 2003). In addition, mutant p53 proteins may also function as transcription factors directly regulating distinct transcriptional targets involved in many different cancer promoting processes. Alternatively, p53 mutant can indirectly regulate gene expression by interacting with several transcription factors such as nuclear factor Y (NF-Y), specificity protein 1 (Sp1), vitamin D receptor (VDR), sterol regulatory element binding protein (SREBP), v-ets erythroblastosis virus E26 oncogene homolog 1 (Ets-1), p63 and p73, and altering their transcriptional activity. Other mutant p53 interacting partners that are not transcription factors have also been found to contribute to the gain-of-function properties of the mutant proteins such as meiotic recombination 11 (MRE11), DNA topoisomerase II-beta-binding protein 1 (TopBP1), Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (Pin1) and promyelocytic leukaemia 1 (PML1) (Albor et al., 1998; Girardini et al., 2011; Haupt et al., 2009; Hu and Wulf, 2011; Liu et al., 2009; Liu et al., 2011a; Song et al., 2007). Furthermore, mutant p53 proteins can bind directly to structure specific DNA regions on the chromosome, such as matrix attachment region DNA elements (MARs), and regulate the activity of the relevant promoters (Will et al., 1998). Other mechanisms leading to the 'gain-of-function' properties of mutant p53 proteins are also likely to exist. However, not all mutant p53 proteins are equal. Each mutation can be translated into different phenotypic affects; thus the 'gain-of-function' properties of mutant p53 proteins are mutant specific.

Taken together, mutation of the TP53 gene leads not only to the inactivation of the tumour suppressor functions of the wild-type protein but also leads to the expression of mutant p53 proteins with novel cancer promoting properties. In fact, many cancer cells are reliant upon the hyper-stable mutant p53 proteins for proliferation and survival, making these molecules potential targets for cancer treatment (Lim et al., 2009).

1.4.4. Targeting p53 in cancer therapy

Since wild-type and mutant p53 exert opposing effects in cancer, current cancer therapies targeting p53 are aimed at activating or restoring wild-type p53 function while eliminating gain-of-function p53 mutants (Chen et al., 2010). Approaches that activate wild-type p53 activity include the use of conventional anticancer therapies such as chemotherapy and ionizing radiation (IR) which cause substantial DNA damage triggering wild-type p53 activation and stabilization (El-Deiry, 2003). In addition, p53 function can be restored by reintroducing wild-type p53 in to tumour cells. This has been achieved by the use of viruses to deliver p53 (Fujiwara et al., 1993). Alternatively, cell-based screening has identified several compounds that reactivate wild-type p53 functions by various mechanisms, such as activating p53 family members, modulating p53 post-translational modifiers, inhibiting Mdm2-p53 interaction and aiding in mutant p53 refolding (Wang and Sun, 2010). These compounds are currently under intensive investigation.

Expression of mutant p53 proteins have been shown to render cancer cells susceptible to synthetic lethality whereby cancer cells containing mutant p53 are more prone to death via inhibition of other genes that would otherwise be nonlethal. Many recent studies are focusing on this phenomenon to develop specific cancer therapy against mutant p53 tumours. Through synthetic lethal screening, some novel small molecules that are cytotoxic to mutant p53 cells have been identified. However, the mechanisms of those compounds remain to be elucidated (Robinson et al., 2003; Wang and Sun, 2010). Approaches that target mutant p53 proteins also include the discovery of molecules that reduces mutant p53 levels. Recent studies have suggested that inhibition of heat shock protein 90 (HSP90) or heat shock factor 1 (HSF1) would lead to an enhanced ubiquitination and degradation of mutant p53 proteins as the proteins are stabilized by the HSP90 chaperone complex (Li et al., 2011b).

Taken together, cancer therapies targeting p53 requires a thorough understanding of the genetic and epigenetic alterations of each individual cancer, followed by the rational design of combinational therapies. Although p53 has long been known to be an important regulator of tumourigenesis, p53 targeting therapies are still in their infancy.

1.5. THE HEAT SHOCK RESPONSE IN CANCER

Cells respond to elevated temperature and various chemical and physical stresses by synthesizing a cohort of highly conserved and homologous proteins called heat shock proteins (HSPs). This process is called the heat shock response (HSR) (Lindquist, 1986). HSPs function primarily as molecular chaperones that assist protein folding, assembly, translocation and degradation. During stress, they facilitate the refolding of misfolded and denatured proteins, targeting denatured proteins for degradation, preventing protein aggregation and blocking apoptotic and cellular senescence pathways. Activities of HSPs upon stress ensure the maintenance of intracellular protein homeostasis and protect cells against stress-induced cell death (Lindquist and Craig, 1988). This response is universal and is one of the most ancient and evolutionarily conserved cytoprotective mechanisms found in nature (Neckers and Workman, 2012).

In several types of solid tumours, increased expression of HSPs is frequently observed (Ciocca and Calderwood, 2005). The pathophysiological features of the tumour microenvironment such as low oxygen, low glucose, and acidosis leads to HSP induction (Calderwood, 2010; Witkin, 2001), as does the emergence of mutated and conformationally altered oncoproteins and tumour suppressors that require permanent chaperoning. The demand for a high level of HSP expression increases as a cancer progresses and consistent with this, high grade tumours have highly elevated HSP expression. Moreover, the levels of HSPs also correlate with poorer patient prognosis in terms of overall survival and poor response to therapy (Calderwood et al., 2006). Although HSPs are not oncogenes, the phrase ‘non-oncogene addiction’ has been used to describe how cancer cells rely on these proteins for survival, proliferation and regulation of essential cellular functions (Solimini et al., 2007). As human cancer is a highly complex and heterogeneous disease at both the molecular and cellular levels with a remarkable diversity in genetic and epigenetic alterations, the HSR is one of the essential key pathways that is universally utilized by cancer cells for survival but is not

critical for host cells under normal physiological conditions. Therefore, there is a great deal of interest in the development of targeted cancer therapies towards HSPs and their upstream modulators (Ciocca and Calderwood, 2005).

1.5.1. Heat shock proteins

Heat shock proteins are a class of functionally related proteins that are further divided in to families according to their molecular weight. At least six families of HSPs have been identified, which are HSP110, HSP90, HSP70, HSP60, HSP40 and small HSPs. Among these, the small HSP, HSP70 and HSP90 families are the most widely studied.

1.5.1.1. Small heat shock proteins

The human small HSP (sHSP) family contains members that range from 12 to 43 kDa in size with the best characterised being HSP27/HSPB1, αA-crystallin/HSPB4, αB-crystallin/HSPB5 and HSP22/HSPB8 (Fig.1.6). Structurally, all sHSPs share a C-terminal domain, referred to as the α-crystallin domain. The domain typically composes the bulk of the protein, consisting of eight beta strands, which form an intermolecular β-sheet interaction site responsible for protein oligomerization (Hayes et al., 2009; Theriault et al., 2004). The N-terminal domain of sHSPs is less conserved, but often contains an WDPF motif followed by a short variable region (Fig.1.6; Gusev et al., 2002).

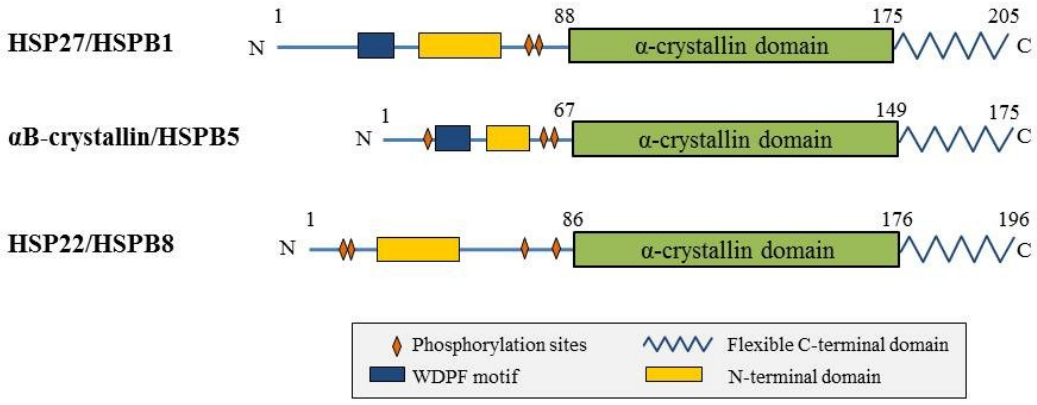


Figure 1.6. Schematic domain structures of small heat shock proteins
All small heat shock proteins share a C-terminal α-crystallin domain that forms an intermolecular β-sheet interaction site for oligomerization. A WDPF motif is often present at the N-terminus. Phosphorylation of serine residues is also a common feature of small heat shock proteins (Acunzo et al., 2012).

The most striking feature of sHSPs is their ability to oligomerize to form large protein complexes from monomeric or dimeric building blocks. They can form either homo- or hetero-oligomers containing up to 50 subunits. This oligomerization is a dynamic process and the rapid subunit exchange can be further accelerated by stress. sHSPs exhibit an ATP-independent holdase activity that traps protein targets as they unfold to prevent amorphous protein aggregation and precipitation (Carver et al., 2003; Jakob et al., 1993). The sHSP oligomers act in a coordinated way with the ATP-dependent chaperones of the HSP70/HSPA, allowing the proper assembly and disassembly of protein complexes and regulation of the ubiquitin-proteasome pathway (Arrigo et al., 2007).

1.5.1.2. HSP70

The HSP70 family is comprised of several members of around 70kD in size with each differing in their cellular localization and response to stresses (Tavaria et al., 1996). HSP70 proteins contain two distinct functional regions: a C-terminal peptide binding domain (PBD), which includes a carboxyl-terminal EEVD motif responsible for substrate binding and refolding; and an N-terminal ATP binding domain or ATPase domain (ABD) which facilitates the release of the client protein (Fig.1.7; Chernorizov and Svedas, 2010; Feige and Polla, 1994; Osipiuk et al., 1999). Activity of HSP70 is regulated by co-chaperones that can be classified into three groups: The J-domain co-chaperones (such as HSP40) which bind to the HSP70 ABD and stimulate the ATPase activity; the nucleotide factor co-chaperones (such as Bag-1 and HSP110) which

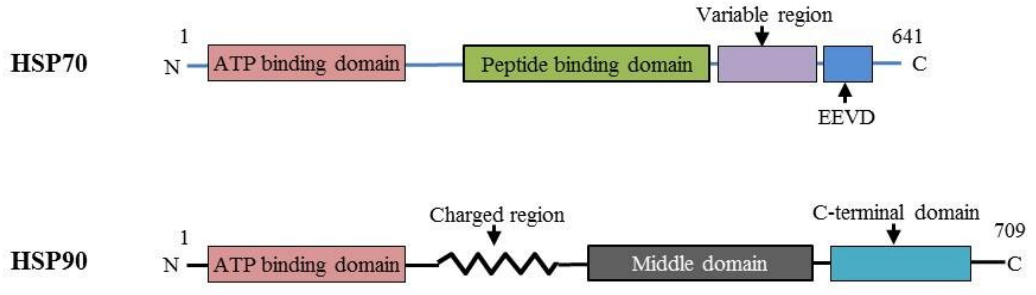


Figure 1.7. Schematic domain structures of HSP70 and HSP90
HSP70 and HSP90 are large heat shock proteins functioning as ATP-dependent chaperones with an ATP binding domain at the N terminus. The C-terminus of HSP70 contains a peptide binding domain, which includes an EEVD motif and a variable region. In addition to its C-terminal domain, HSP90 contains a middle domain with a charged linker region.

catalyse the release of ADP to complete the HSP70 ATPase cycle; and the TPR domain co-chaperones (such as Hop and CHIP) that bind to the EEVD motif and is responsible for the combinational assembly of HSP70 and HSP90 and the ubiquitination of some client proteins (Mayer and Bukau, 2005).

HSP70 proteins normally function in the cytoplasm as ATP-dependent molecular chaperones, assisting the folding of newly synthesized proteins, the assembly of multi-protein complexes and the transport of proteins across cellular compartments (da Silva and Borges, 2011). During stress, HSP70 family members exhibit cytoprotective effects where they are responsible for degrading unstable proteins and the inhibition of proteins involved in apoptosis and cellular senescence (Gabai et al., 1998; Gabai et al., 2009; Saleh et al., 2000; Yaglom et al., 2007). HSP70 proteins have also been shown to be located on the cell surface or secreted into the extracellular space, where they bind to specific receptors on natural killer (NK) cells, macrophages, monocytes and B-cells, thereby promoting the activation of the immune system (Sondermann et al., 2000; Theriault et al., 2005).

1.5.1.3. HSP90

HSP90 is a highly conserved and abundant chaperone protein that comprises 1-2% of total cellular proteins. In humans, the most prominent members of the HSP90 family are the cytoplasmic inducible Hsp90α and the constitutively expressed Hsp90β isoforms, which are expressed by two distinct genes. Hsp90 homologues are also found in the endoplasmic reticulum (glucose regulated protein GRP94), in the mitochondria (tumour necrosis factor receptor associated protein TRAP1) and on the cell surface with its active domain facing the extracellular space (Altieri et al., 2012; Krukenberg et al., 2009).

HSP90 exists as a homodimer with each monomer containing three relevant domains (Fig. 1.7). The N-terminal domain consists of an ATP binding pocket responsible for the protein's ATPase activity (Panaretou et al., 1998; Prodromou et al., 1997). The middle domain with a charge linker region regulates the ATPase activity and contains binding sites for co-chaperones and client proteins. The C-terminal dimerization domain contains a conserved EEVD motif responsible for recruiting co-chaperones with tetratricopeptide repeats (Pearl and Prodromou, 2000).

Hsp90 functions as an ATP-dependent molecular chaperone whose activity involves the formation of a large multiprotein complex, comprised of several co-chaperones and adaptor molecules. This multichaperone complex promotes protein folding, assembly and transport and maintains client proteins in an active conformation that allows them to express their cellular functions. Client proteins initially bind to a HSP70/HSP40/HIP complex. This complex then recruits ADP-bound-HSP90 via the HSP70/HSP90 adaptor protein, Hop. This leads to the binding of co-factor, Aha1, which stimulates the exchange of ADP for ATP by HSP90, and results in a transient dimerization of its N-terminal domain. The conformational change in the HSP90 structure triggers the release of the HSP70/HSP40/Hop complex (Hernandez et al., 2002a; Hernandez et al., 2002b) and the mature HSP90 chaperone complex is formed by the subsequent association of another set of co-chaperones including p23, cdc37 and immunophilins (Pearl and Prodromou, 2001). This active form of the complex is known to regulate more than 200 client proteins, such that HSP90 is considered to be critical in the regulation of multiple cellular processes and signalling pathways (Pearl and Prodromou, 2000; Prodromou, 2012).

1.5.2. Roles of HSPs in cancer

1.5.2.1. HSPs promotes tumourigenesis and cancer progression

Studies have established that HSPs contribute to almost every aspect of cancer and these proteins can be used as indicators of cancer aggressiveness, poor prognosis and resistance to therapies. HSPs have been shown to promote cancer stimulus-independent growth. The chaperone activity of HSPs, especially HSP90, is required for the maintenance and stabilization of client proteins which are components of mitogenic pathways driving cellular proliferation. In addition, during tumourigenesis, activity of HSPs allow cells to tolerate altered expression and mutated conformation of several key mitogenic molecules, thereby supporting activation of cell proliferation independent of external stimuli (Nielsen et al., 2004; Sawai et al., 2008; Smith et al., 2002; Xu et al., 2001; Calderwood and Gong, 2011).

Apart from the chaperoning activity of HSPs that protects cancer cells from apoptosis induced by disruption of cellular protein homeostasis; HSPs can also directly interact and inhibit key effectors of the apoptotic machinery (Garrido et al., 2006). For example,

HSP27 has been found to inhibit apoptosis by direct interactions with cytosolic cytochrome c, after its release from mitochondria, thereby sequestering it from the apoptotic protease activating factor 1 (Apaf-1) and preventing the formation of the caspase activating complex apoptosome (Paul et al., 2002). Similarly, HSP70 has been shown to bind and block several mediators of the caspase activation cascade such as Apaf-1, c-jun N-terminal kinase (JNK1), caspase 8, procaspase 3 and procaspase 7 (Komarova et al., 2004; Park et al., 2001a; Stankiewicz et al., 2005). HSP90 has also been shown to inhibit cytochrome-c induced oligomerization of Apaf-1 and blocks the activation of procaspase 9 (Pandey et al., 2000). Thus, expression of HSPs is an important mechanism enabling tumour cells to evade apoptosis.

Expression of HSPs can also promote unlimited growth of tumour cells by inhibiting cellular senescence. HSP70 has been shown to interact with the catalytic unit of telomerase (hTERT), which is the key enzyme protecting cells from senescence, and down-regulation of HSP70 reducing its activity. In addition, HSP90 chaperone activity is important for the assembly of the active telomerase complex. Studies by Sherman et al. have revealed that HSP70 is required for both p53-dependent and -independent suppression of cellular senescence induced by oncogenes such PI3K, cMyc and HER2 (Meng et al., 2011; Sherman, 2010; Yaglom et al., 2007), and HSP27 has been shown to support the suppression of senescence by inhibiting p53-mediated induction of p21, the major regulator of the senescence program (O'Callaghan-Sunol et al., 2007).

HSP70 and HSP90 chaperone complex has been shown to participate in the invasion and migration steps of metastasis. The complex can bind and assist the activation of MMP2, which is a cell surface localised enzyme essential for cell invasion (Sims et al., 2011; Tsutsumi and Neckers, 2007). Extracellular HSP90 can increase cancer cell motility by activating other client proteins such as HER2 and plasmin in the extracellular matrix (Eustace et al., 2004; McCready et al., 2010; Sidera et al., 2008). In addition, tumour angiogenesis also requires activity of HSPs as blocking HSP90 leads to a diminished secretion of tumor cell-derived pro-angiogenic growth factors and cytokines (Schmitt et al., 2007). HSP90 is also required for activity of hypoxia inducible factor 1α (HIF-1α), which is a nuclear factor that activates numerous hypoxia inducible genes including VEGF genes. In addition, HSP90, HSP27 and HSP70 can enhance cancer cell invasion and metastasis by supporting cancer cell survival in the

circulatory system and at secondary sites during metastasis through their apoptosis inhibiting effects (Calderwood et al., 2006).

Altogether, as tumour cells up-regulate expression of HSPs to cope with alterations in protein homeostasis, this selecting pressure also fosters cancer progression and metastasis through the cancer promoting properties of HSPs (Calderwood, 2010; Witkin, 2001).

1.5.2.2. HSPs promotes drug resistance

Expressions of HSPs, such as HSP70 and HSP27, have been shown to correlate with resistance to therapies in many cancer types (Mazurov et al., 2003; Morii et al., 2010; Oesterreich et al., 1993). HSP27 was found to correlate with a shorter disease-free survival in advanced cancer patients treated with neoadjuvant therapy in breast, ovarian, and head and neck cancers, as well as oesophageal squamous cell carcinoma, and leukaemia (Arts et al., 1999). Similarly, HSP70 is an emerging predictor of a reduced response to radiation, hyperthermia and chemotherapy treatments of many cancers such as breast cancer, lung cancer and multiple myeloma (Tutar, 2010).

Multiple molecular mechanisms have been proposed to explain the mechanisms involving HSPs in resistance to cancer therapies. For example, HSPs can confer therapeutic resistance by their cytoprotective chaperoning effects, which efficiently prepare damaged proteins resulting from cytotoxic drug administration. HSP27 and HSP70 can also protect cells from apoptosis caused by these drugs (Ciocca et al., 2003; Nadin et al., 2003). In addition, HSP27 is found in endothelial cells and protects the microvasculature within tumours (Ciocca et al., 2003). HSPs can also enhance DNA-damage repair by stimulating endonucleases (Ciocca and Calderwood, 2005; Mendez et al., 2003). As HSPs promote drug resistance, inhibition of HSPs can sensitize cancer cells to several anti-cancer therapies (Matsumoto et al., 2005; Mesa et al., 2005).

1.5.2.3. The dual role of HSPs in cancer immune response

In cancer, the accumulation of mutations in oncogenes and tumour suppressors lead to the production of mutant proteins that can be recognized as tumour-specific antigens. These antigens can elicit an anti-tumour immune response that mediates tumour regression. Intracellular HSPs exhibit immunomodulatory activity, whereby HSP70 and

HSP90 have been shown to be associated with antigenic peptides in the cytosol and serve as intracellular antigen transporters mediating the ATP-dependent translocation of the peptides to the endoplasmic reticulum. The increased expression of HSPs in tumour cells can also act as 'danger signals' enabling the generation of an amplified immune response (Todryk et al., 2000). In addition, HSPs have been shown to be released from cells undergoing necrosis into the extracellular space and then enter the bloodstream under inflammatory conditions. Such extracellular HSPs exert profound pro-inflammatory effects, enhancing both innate and adaptive immune responses that mediate tumour regression (Asea et al., 2000; Calderwood et al., 2005; Chen et al., 2009b). Extracellular HSPs can stimulate professional antigen presenting cells, leading to the release of cytokines and expression of cell surface molecules. Extracellular HSPs can also promote the cross presentation of HSP-bound peptide antigens to MHC class I molecules in dendritic cells, leading to efficient induction of antigen-specific cytotoxic T-lymphocytes.

Nevertheless, the immunno-modulatory activities of HSPs are dependent upon cellular conditions and can exert either negative or positive effects on tumour progression. Under resting conditions, HSP70 can be actively secreted by tumour cells and act as a component of the tumour defences against immuno-surveillance (Mambula and Calderwood, 2006). Membrane surface HSP70 from tumour-derived exosomes can facilitate the escape from immuno-surveillance of tumour cells by inducing the release of immunosuppressive cytokines and activation of T regulatory cells that suppress cytotoxic lymphocytes (Chalmin et al., 2010).

The dual role of HSPs in modulating tumour immunity leads to opposing HSPs targeting mechanisms in cancer therapy. Hyperthermia or heat therapy, which activates the expression of HSPs, has been used as an adjunct to standard cancer immunotherapy. In fact, hyperthermia is reportedly an effective way to sensitize tumour cells and potentiate the efficacy of the cancer treatments (Torigoe et al., 2009). HSPs are also currently employed as vaccines in cancer immunotherapy to assist in the presentation of tumour antigens to the immune system (Murshid et al., 2011). On the other hand, several HSP inhibitor compounds have been developed and tested in clinical trials for use as anticancer therapeutic drugs and some of these exhibit promising anticancer properties. It has been suggested that the best anticancer therapy targeting HSPs would

be inhibiting intracellular HSPs that are required for cancer progression and increasing extracellular or membrane-bound HSPs that bolster the immune response against tumour development.

1.5.3. Targeting HSPs in cancer treatments

1.5.3.1. Targeting HSP90

Among the HSPs, HSP90 has emerged as one of the most attractive targets for cancer treatment. A number of compounds have been identified or rationally designed to target HSP90 and its activity (Table 1.3), several of which are currently in clinical trials. Most HSP90 inhibitors block the ATPase activity of HSP90 by binding to the N-terminal ATP-binding pocket. However, a number of other compounds inhibit HSP90 activity function by binding the C-terminal domain and disrupting the cochaperone-HSP90 or client-HSP90 interactions. These inhibitors induce a rapid degradation of client proteins, leading to simultaneous disruption of multiple oncogenic signal transduction pathways essential for tumour formation and progression (Jhaveri et al., 2012; Neckers and Workman, 2012).

The best-characterised HSP90 inhibitor compounds belong to structural classes that are similar to that of geldanamycin or radicicol, which target the N-terminal ATP binding domain. Although these compounds themselves have proved to be highly toxic and unstable, their core structures have led to the development of a range of more clinically suitable drugs such as 17-allylamino-17-demethoxygeldanamycin (17AAG), 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) and NVP-AUY922 (Jhaveri et al., 2012; Rodrigues et al., 2012; Zaidi et al., 2012).

A number of studies have established that HSP90 inhibitors exhibit potent anti-tumour properties against several types of cancers such as glioblastoma, breast, prostate and ovarian cancers (Travers et al., 2012). Interestingly, HSP90 inhibition appears to specifically target cancer cells. HSP90 complexes isolated from tumour cells were found to have a much higher affinity towards HSP90 inhibitors than did the complexes isolated from normal tissues (Kamal et al., 2003). In addition, *in vivo* studies have shown that 17AAG selectively concentrates in tumour tissues while being rapidly cleared from normal ones (Chiosis et al., 2003; Eiseman et al., 2005). However, the basis for the anticancer selectivity of HSP90 inhibitors remains controversial. Further

studies are required to investigate the existence of higher affinity complexes in cancer cells and the basis for retention of certain inhibitors in tumors.

Table 1.3. *Classes of HSP90 inhibitors with different sites of action*

Site of action	Class	Examples	Selected references
N-terminal ATPase	Benzoquinone ansamycin	Geldanamycin 17AAG 17DMAG KOS-953 IPI504	(Gorska et al., 2012)
	Macrolide	Radicicol Zearalenol Pochonins KF55823 KF58333	(Moulin et al., 2005; Wang et al., 2008a; Whitesell and Lindquist, 2005)
	Purine scaffold	PU24FC1 BIIB021/CNF2024 SNX-5422	(Chiosis, 2006; Chiosis et al., 2003; Taldone and Chiosis, 2009)
	Pyrazole	CCT018159 CCT01293297/VER-49009 VER-50589 NVP-AUY922	(Barril et al., 2006; Cheung et al., 2005; McDonald et al., 2006)
	Hybrid	Radamycin GA dimer	(Hadden et al., 2009)
	Peptide mimetics	Shepherdin AICAR	(Gyurkocza et al., 2006; Plescia et al., 2005)
Middle region	Human recombinant antibody	Mycograb®	(Louie et al., 2011; Matthews et al., 2003)
C-terminus	Noviosylcoumarin crosslinker	Novobiocin A4 Coumermycin Cisplatin	(Donnelly and Blagg, 2008)
	Polyphenol extract	EGCG	(Yin et al., 2009)
Others	Histone deacetylase inhibitor	Depsipeptide SAHA FK228	(Dokmanovic et al., 2007; Li et al., 2011a)
	Tetranotriterpenoid	Gedunin	(Brandt et al., 2008; Kamath et al., 2009)
	Quinone methide triterpine	Celastrol	(Chadli et al., 2010)

Although several compounds have entered clinical trials, HSP90 inhibitors are yet to show significant therapeutic benefits in cancer patients (Neckers and Workman, 2012). It has also been demonstrated that HSP90 inhibition is likely to cause clinical side effects, as 17AAG has been shown to induce bone lesions by enhancing osteoclast formation in breast and prostate cancer models of metastasis (Price et al., 2005; Yano et al., 2008). In addition, HSP90 inhibitors that target the N-terminal ATP binding site can disrupt the HSF1-HSP90 association, which leads to activation of the heat shock factor and its cytoprotective effect (Gabai et al., 2005). This potentially reduces the efficacy of HSP90 inhibition and can cause drug resistant tumour cells. In agreement with this, studies have demonstrated that HSP90 inhibitors are more effective in cells in which the heat shock response has been compromised (Zaarur et al., 2006). Therefore, combination of HSP90 inhibitors with other therapeutic compounds such as chemotherapeutic agents are currently being examined (Zhao et al., 2011).

1.5.3.2. Targeting other HSPs

Studies have also shown that HSP70 can also be a target for cancer treatment. There are a number of compounds that have been identified as HSP70 inhibitors, however, none of these compounds provide specific HSP70 inhibition and are clinically available (Wang, 2011). HSP27 has also been considered as a potential anticancer therapeutic target. However, the structural complexity of this molecule is still a challenge for the design of viable therapeutic inhibitors (Jego et al., 2010).

1.6. HEAT SHOCK TRANSCRIPTION FACTOR 1

1.6.1. Heat shock transcription factors

HSPs are transcriptionally regulated by members of a family of transcription factors called heat shock factors (HSFs). Four HSFs (1-4) are found in vertebrate cells. Among them, HSF1 plays a central role in inducing and regulating the HSR. HSF2 plays a supportive role in the HSR and is only activated under specific conditions. HSF3 has only been found in avian species and in mice while HSF4 regulates a number of genes during development and is expressed predominantly in the lens and the brain (Abane and Mezger, 2010; Akerfelt et al., 2010; Akerfelt et al., 2007; Pirkkala et al., 2001; Zhang et al., 2011b; Fujimoto et al., 2010). Consistent with the role of the HSR in

cancer, HSF1 is found to be elevated and activated in several types of cancers and its elevated activity is positivel correlated with cancer aggressiveness (Calderwood, 2012a; Cen et al., 2004; Santagata et al., 2011; Whitesell and Lindquist, 2005). Interestingly, many recent studies have reported that the role of HSF1 in cancer goes far beyond that of regulating the expression of HSPs. The factor was found to regulate several other non-HSP genes involved in a variety of cellular functions. In addition, gene array analyses of HSF1 transcriptional targets has revealed that HSF1 regulates a large number of genes which regulate a broad spectrum of biological processes that are critical for tumourigenesis and metastasis (Mendillo et al., 2012; Page et al., 2006). HSF1 has therefore emerged as an attractive target for the development of anticancer treatment.

1.6.2. HSF1 structure

The human HSF1 protein is composed of several functional domains which have been thoroughly characterized and are schematically represented in Fig.1.8A. Structurally, HSF1 contains an N-terminal DNA binding domain (DBD), followed by two hydrophobic heptad repeat regions (HR-A/B) and a loosely defined regulatory domain (RD). Adjacent to the regulatory domain is an additional heptad repeat (HR-C) and a C-terminal transactivation domain (TAD) (Morimoto, 1998; Wu, 1995).

1.6.2.1. DNA binding domain

The HSF1 DBD is the most conserved region within the heat shock factor family and is the only functional domain with available structural data (Harrison et al., 1994). The domain belongs to the family of winged helix-turn-helix DBDs characterized by a three α-helical bundle and a four stranded anti-parallel β-sheet. These secondary structures form a compact globular tertiary structure with a flexible wing or loop located between the last two β strands. The DBD is capable of binding to the heat shock element (HSE) DNA sequences present in the promoter regions of its transcriptional targets. (Cicero et al., 2001; Littlefield and Nelson, 1999).

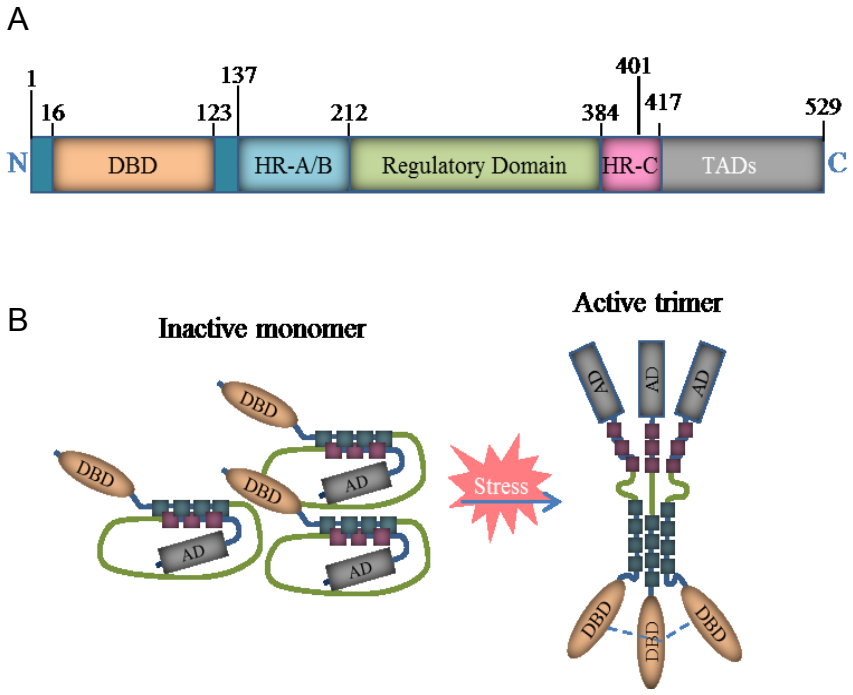


Figure 1.8. Schematic representations of human HSF1 structure
HSF1 structure contains a DNA binding domain (DBD), three heptad repeat domains (HR-A/B and HR-C), a regulatory domain and a C-terminus transcription activation domain (TAD). **(A)** Schematic organization of HSF1 structural domains indicated by amino acid residues. **(B)** Relative positions of HSF1 functional domains in the inactive monomer and in the active trimer structure formed upon stress (Tonkiss and Calderwood, 2005).

1.6.2.2. Heptad repeat A/B and C

HSF1 contains two middle hydrophobic heptad repeat regions, HR-A/B and HR-C, which are separated by the regulatory domain. These heptad repeats form characteristic coiled-coil structures (i.e. leucine zippers), which provide hydrophobic surfaces for intramolecular and intermolecular interactions (Peteranderl and Nelson, 1992; Wu, 1995). Upon stress, HSF1 trimerization occurs through intermolecular interactions among the HR-A/B regions of the three HSF1 subunits. At steady state, the HR-C domain is thought to fold back and interact with the HR-A/B domain to keep the factor in an inactive monomeric structure which prevents spontaneous trimerization of the factor (Fig.1.8B; Peteranderl et al., 1999; Rabindran et al., 1993).

1.6.2.3. Regulatory domain

The stress responsive ability of HSF1 is regulated by the regulatory domain (RD) located between the HR-A/B and HR-C domains (Newton et al., 1996). This domain carries an intrinsic capacity of sensing stress and contains sites for various forms of post-translational modifications regulating the trans-activation activity of HSF1 such as phosphorylation, sumoylation and acetylation. The RD also contains a nuclear localization signal (NLS) responsible for the nuclear translocation of the factor upon stress (Green et al., 1995).

1.6.2.4. Trans-activation domain

The C-terminal region of HSF1 contains two TADs, TAD1 and TAD2, which are located between amino acids 401-420 and between amino acids 431 and 529 respectively (Brown et al., 1998). The structure of the C-terminal region of HSF1 is not yet fully characterised. TAD1 is rich in hydrophobic residues and is predicted to form a helical structure capable of interaction with the basal transcription component TAF-9 (Choi et al., 2000). TAD2 contains both hydrophobic and acidic residues. This domain is proline rich and is predicted to be non-helical (Newton et al., 1996). Both TAD1 and TAD2 are each sufficient to stimulate transcriptional initiation and elongation of HSF1 transcriptional targets (Sorger, 1990; Sullivan et al., 2001).

1.6.3. Molecular mechanism of the heat shock response

In a typical HSR, expression of HSPs increases shortly after exposure to stress; continues for a period of time and then decreases gradually to a low rate approximately corresponding to the expression rate before stress (DiDomenico et al., 1982; Morimoto et al., 1997). This response is mediated through a co-ordinated regulation program controlling the activity of HSF1 (Fig.1.9). In the normal resting state, HSF1 is maintained as an inactive monomer through direct interactions with proteins within the HSP90 complex. Following stress, HSF1 is activated. Several mechanisms have been proposed to explain this activation. Firstly, the increase in denatured proteins is thought to liberate HSF1 from the chaperone complex and subsequently facilitate HSF1 trimerization (Ananthan et al., 1986; Zou et al., 1998). Secondly, HSF1 activation is suggested to be coupled with protein translation by the association between HSF1 and a protein complex containing the heat-sensing RNA molecule HSR-1 and the elongation

factor eEF1A. During stress, the change in conformation of this complex triggers HSF1 activation (Kugel and Goodrich, 2006; Shamovsky et al., 2006). Alternatively, HSF1 has been shown to have a built-in ability to sense stress as purified HSF1 is able to trimerize by itself *in vitro* upon heat shock and other stresses without any other

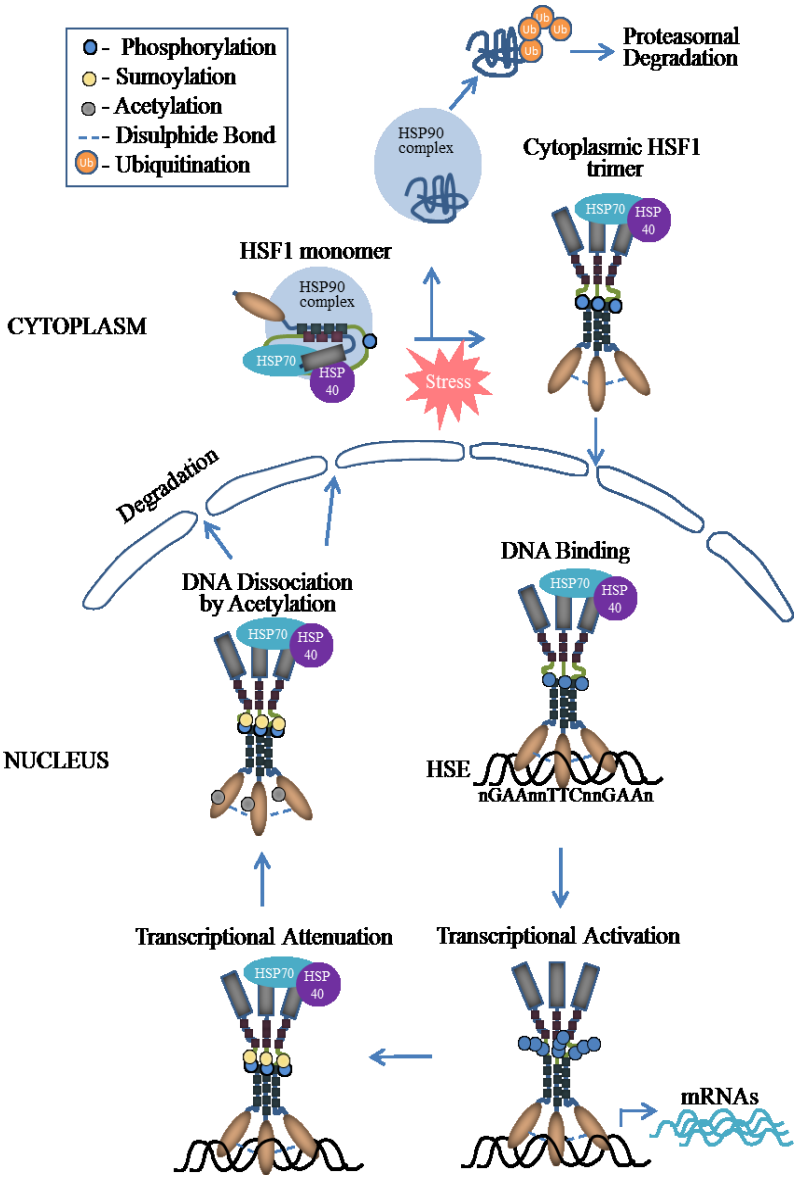


Figure 1.9. HSF1 activation and attenuation cycle in the heat shock response. Under normal resting conditions, HSF1 exists as an inactive monomer stabilized by the HSP90 chaperone complex present predominantly in the cytoplasm. In response to stress, HSF1 is released from the chaperone complex, oligomerizes and translocates into the nucleus, becomes hyperphosphorylated, binds to HSEs and mediates the expression of stress responsive genes. When cells recover from stress, activity of HSF1 is attenuated by post-translational modifications comprising of phosphorylation, sumoylation and acetylation. HSP70 and HSP40 rebinds to HSF1 and act as a feedback mechanism to attenuate HSF1 activity. The subsequent recruitment of an HSP90 complex facilitates the dissociation of HSF1 trimers. Inactive monomeric HSF1 can be targeted for degradation or exported back to the cytoplasm (Adapted from Neef et al., 2011).

stimulating factor (Goodson and Sarge, 1995; Mosser et al., 1990; Zhong et al., 1998).

Upon stress, to become transcriptionally activated, HSF1 proceeds through a multi-step activation process involving trimerization, acquisition of DNA binding activity, nuclear accumulation and post-translational modifications (Baler et al., 1993). Once in the nucleus, activated HSF1 binds to HSEs present within the promoters of the gene targets and facilitates transcription. The factor also concentrates into nuclear stress bodies (nSBs) on specific chromosome loci and induces the transcription of non-coding RNA molecules (Biamonti, 2004; Pirkkala et al., 2001; Sarge et al., 1993).

When cells recover from stress, the available HSPs interact and attenuate HSF1 transactivation activity. HSF1 attenuation involves several repressive post-translational modifications such as phosphorylation, sumoylation and acetylation. The subsequent assembly of a multichaperone complex at the HSF1 transcription activation region then leads to the return of the factor into its HSP90-complex associated resting state (Fig.1.9; Anckar and Sistonen, 2011; Tonkiss and Calderwood, 2005).

1.6.4. HSF1 activation regulations

1.6.4.1. HSF1 DNA binding

In response to stress, HSF1 acquires DNA binding ability upon trimerization and the HSF1 trimer binds to HSEs present on the promoter regions of target genes to induce gene transcription (Cotto et al., 1996). A HSE is composed of multiple adjacent inverted repeats of the pentameric nucleotide motif nGAAn (where n is any nucleotide). Since each individual DBD of a trimeric HSF1 binds to one nGAAn sequence, a typical HSE contains at least three repeating units. Perfect-type HSEs are those containing consecutive inverted repeats of the nGAAn units (i.e. nGAAnnTTCnnGAAn). These HSEs are commonly found in the promoter regions of HSP genes. HSF1 preferentially binds to the continuous perfect HSEs (Enoki and Sakurai, 2011), however, the factor can also tolerate and bind with lower affinity to HSEs with five base-pair insertions between two repeating units called gap-type (one insertion; nGAAnnTTCn(5bp)nGAAn) or step-type (two insertions; nGAAn(5bp)nTTCn(5bp)nGAAn) HSEs. At these HSE variants, HSF1 trimer dissociates from two nGAAn units and quickly rebinds to another two units, thereby facilitating the stabilization of the HSF1-DNA complex (Fig.1.10; Sakurai and

Takemori, 2007). Gap-type HSEs mediate moderate stress induced gene transcription whereas step-type HSEs are found to be involved in basal constitutive transcription and in low-level stress activation. The divergence of HSE architecture is believed to provide specific response to various types of stimuli (Sakurai and Takemori, 2007; Santoro et al., 1998).

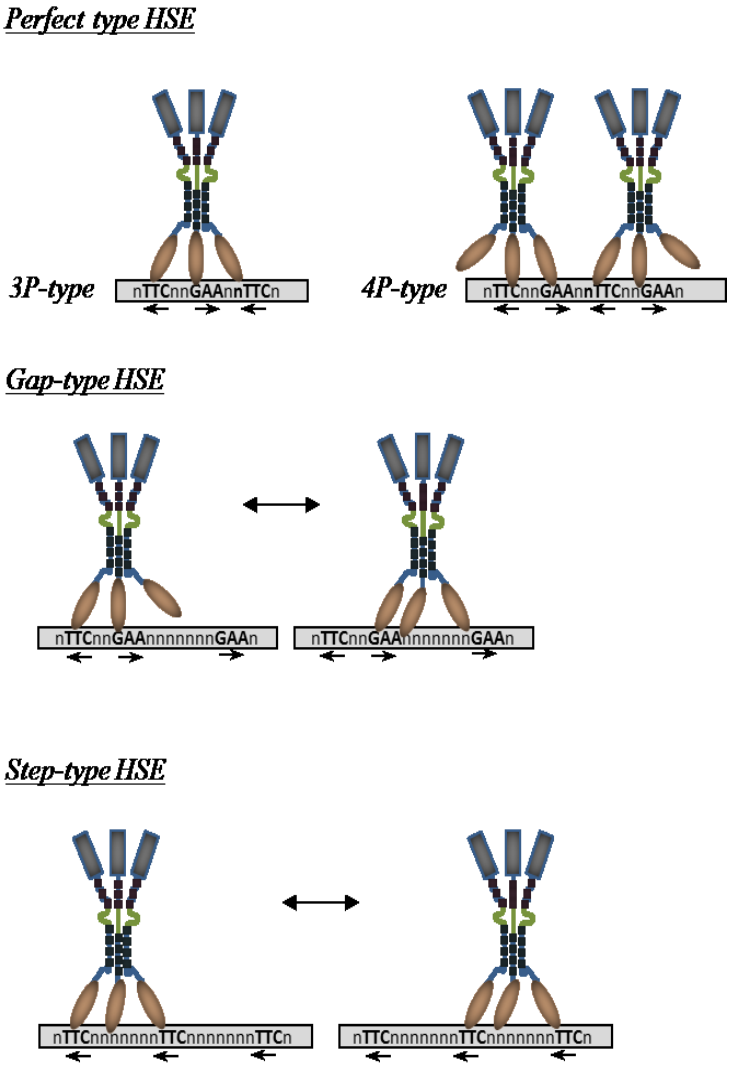


Figure 1.10. HSE types and HSF1-HSE interactions

HSF1 preferentially binds to perfect-type HSE, which contains at least three consecutive inverted repeats of the pentameric nucleotide motif nGAAn (3P-type). HSE with four consecutive inverted repeating units (4P-type) is capable of binding to two HSF1 trimers with two HSF1 subunits not making DNA contact. HSF1 can also tolerate and bind with lower affinity to HSEs with one (gap-type) or two (step-type) five base pair insertions between two repeating units. At these HSEs, HSF1 trimer dissociates from two repeating units and quickly rebinds to another two repeating units, thereby stabilizing the protein-DNA association.

The promoter regions of many HSF1 target genes are found to contain HSEs with extended arrays of nGAAn units or multiple HSEs, which can simultaneously bind to multiple HSF1 trimers. An electrophoretic mobility shift assay has revealed that a perfect-type HSE containing four continuous nGAAn units (4P-type) can bind to two HSF1 trimers with two HSF1 sub-units not making DNA contact. Multiple HSF1 molecules associate with long arrays of the nGAAn sequence in a co-operative manner, whereby the binding of one HSF1 trimer to the HSE facilitates the binding of another one to the adjacent HSE (Kroeger and Morimoto, 1994; Wang and Morgan, 1994; Xiao et al., 1991). The number of HSF1 trimers bound to an HSE affects the subsequent acquisition of the transactivation activity. For example, a trimer-trimer interaction facilitates conformational changes that allow HSF1 to be transcriptionally activated independently to hyperphosphorylation (Hashikawa et al., 2006). It is thus suggested that the composition of HSEs including the number of nGAAn repeating units, fidelity to consensus, orientation and spacing of the repeating units governs HSF1 affinity and ultimately controls the inducibility of the gene targets.

1.6.4.2. HSF1 activating phosphorylation

HSF1 is phosphorylated at multiple serine and threonine sites and these post-translational modifications are essential for the regulation of HSF1 transactivation activity. Analysis of exogenously expressed HSF1 in Hela cells by mass spectrometry reveals that it is phosphorylated on at least 12 serine residues (i.e. Ser121, Ser230, Ser292, Ser303, Ser307, Ser363, Ser329, Ser326, Ser344, Ser363, Ser419 and Ser444) and most of these residues reside in the regulatory domain (Guettouche et al., 2005). Additional phosphorylation sites identified include Ser320, T142, S216, T323, T367, S368, and T369 (Lee et al., 2008a; Olsen et al., 2006; Soncin et al., 2003). The phosphorylation of HSF1 can either activate or inactivate the transcriptional activity of the factor depending upon the sites of phosphorylation. Although the role of each phosphorylation site and the exact signalling pathways mediating each phosphorylation are still poorly defined, it is suggested that the ratio between the activating and repressing phosphorylation sites determines the magnitude of HSF1 transcription activity (Holmberg et al., 2002).

To date, four phosphorylation sites have been confirmed to have stimulatory effects on HSF1 transcriptional activity, which are Ser230, Ser320, Ser326 and Ser419 (Fig.1.11; Boellmann et al., 2004; Holmberg et al., 2001; Kim et al., 2005; Lee et al., 2008a; Murshid et al., 2010). Ser230 is phosphorylated by the calcium/calmodulin-dependent protein kinase II (CaMKII). Overexpression of CaMKII enhances both Ser230 phosphorylation and HSF1 transactivation activity (Holmberg et al., 2001). Phosphorylation of HSF1 at Ser320 is mediated by protein kinase A. This phosphorylation is found to be required for HSF1 nuclear localization, DNA binding and transcription activation activity (Murshid et al., 2010). Ser419 is phosphorylated by the direct interaction between HSF1 and polo-like kinase 1 (PLK1) upon stress. This phosphorylation is required for HSF1 nuclear translocation (Kim et al., 2005). It is still unknown how HSF1 Ser326 is phosphorylated. However, this phosphorylation has been shown to play an important role in HSF1 transactivation activity by facilitating the association of HSF1 with the transcription co-activator death domain associated protein DAXX (Boellmann et al., 2004).

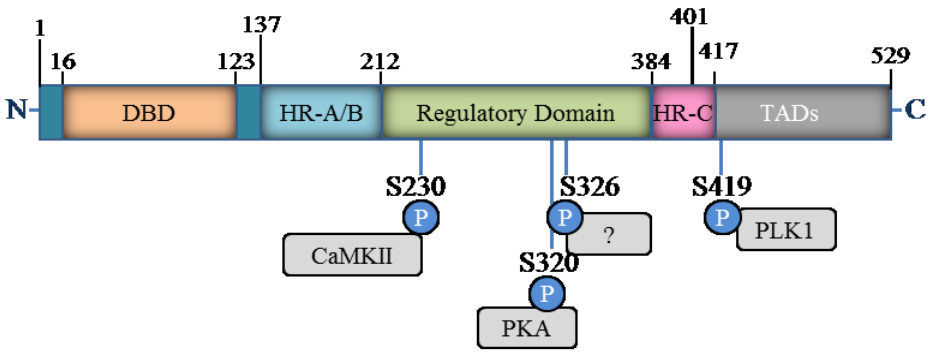


Figure 1.11. HSF1 transactivation stimulatory phosphorylation
Four phosphorylation sites have been identified to date to have stimulatory effect on HSF1 activity, which are serine 230, serine 320, serine 326 and serine 419. Serine 230 is phosphorylated by the calcium/calmodulin-dependent protein kinase II (CaMKII) while serine 320 and serine 419 are phosphorylated by protein kinase A and polo-like kinase 1 respectively. Phosphorylation of serine 326 remains to be characterised.

1.6.4.3. HSF1 transcriptional activation

Most of the understanding of the transactivation activity of HSF1 arises from studies on the expression regulation of the drosophila HSP70 promoter, which resembles the transcription regulation of the mouse and human HSP70.1 gene (Fig.1.12; Anckar and Sistonen, 2011). In the absence of stress, RNA Polymerase II (RNAP II) binds to the

promoter and initiates transcription of the HSP70 gene. However, this polymerase is stably arrested at approximately 20-40 nucleotides downstream of the transcription start site, in a stable complex with the DRB sensitivity-inducing factor, DSIF, and the negative elongation factor, NELF, which bind to the nascent HSP70 mRNA (Wu et al., 2003; Yamaguchi et al., 1999).

Upon stress, HSF1 binds to the heat shock promoter and recruits the positive transcription elongation factor b (P-TEFb), which then phosphorylates the C-terminal domain of RNAP II and facilitates the transition of RNAP II into a mature transcription elongation mode. P-TEFb also phosphorylates DSIF and NELF, causing the release of these proteins from RNAP II. Although the localization of P-TEFb to heat shock promoters is HSF1 dependent, the factor does not directly bind to P-TEFb (Bres et al., 2008; Lis et al., 2000; Ni et al., 2008; Ni et al., 2004). The mechanism by which P-TEFb is recruited to heat shock promoters during stress by HSF1 is yet to be characterised.

Under normal conditions, the wrapping of DNA around nucleosomes in the compact chromatin structure in front of RNAPII also prevents the polymerase from mediating RNA elongation. Upon binding to the promoter, HSF1 also facilitates transcription by inducing a rapid nucleosome displacement across the entire HSP70 gene. In addition to recruiting P-TEFb, HSF1 also recruits the chromatin remodelling complex SWI/SNF (SWItch/Sucrose NonFermentable) by binding to its ATPase subunit BRG1 (Brahma-related gene 1). Once recruited to the promoter, SWI/SNF complex uses energy from ATP hydrolysis to disrupt DNA-histone interactions, thereby facilitating the displacement of nucleosomes in front of RNAP II (Sullivan et al., 2001). Additionally, HSF1 is found to facilitate nucleosome displacement through the activity of the Poly(ADP-ribose) polymerase-1 (PARP-1) (Fossati et al., 2006). However, it remains to be determined how the activities of HSF1 and PARP-1 are coordinated.

HSF1 transactivation activity is also regulated by interactions of the factor with other transcription co-factors. Among these are the Drosophila transcription co-activator dTRAP80 (Park et al., 2003; Park et al., 2001b) and the transcriptional co-activator activating signal co-integrator ASC-2 (Hong et al., 2004). Although the exact mechanism is still unknown, these co-activators are thought to facilitate the maturation of RNAP II into the elongation complex. Alternatively, HSF1 has been shown to

interact with CHIP and DAXX. These proteins prolong HSF1 transactivation activity by opposing the HSF1 repressing effect of HSP multichaperone complexes (Boellmann et al., 2004). In addition, HSF1 is found to be involved in co-transcriptional mRNA

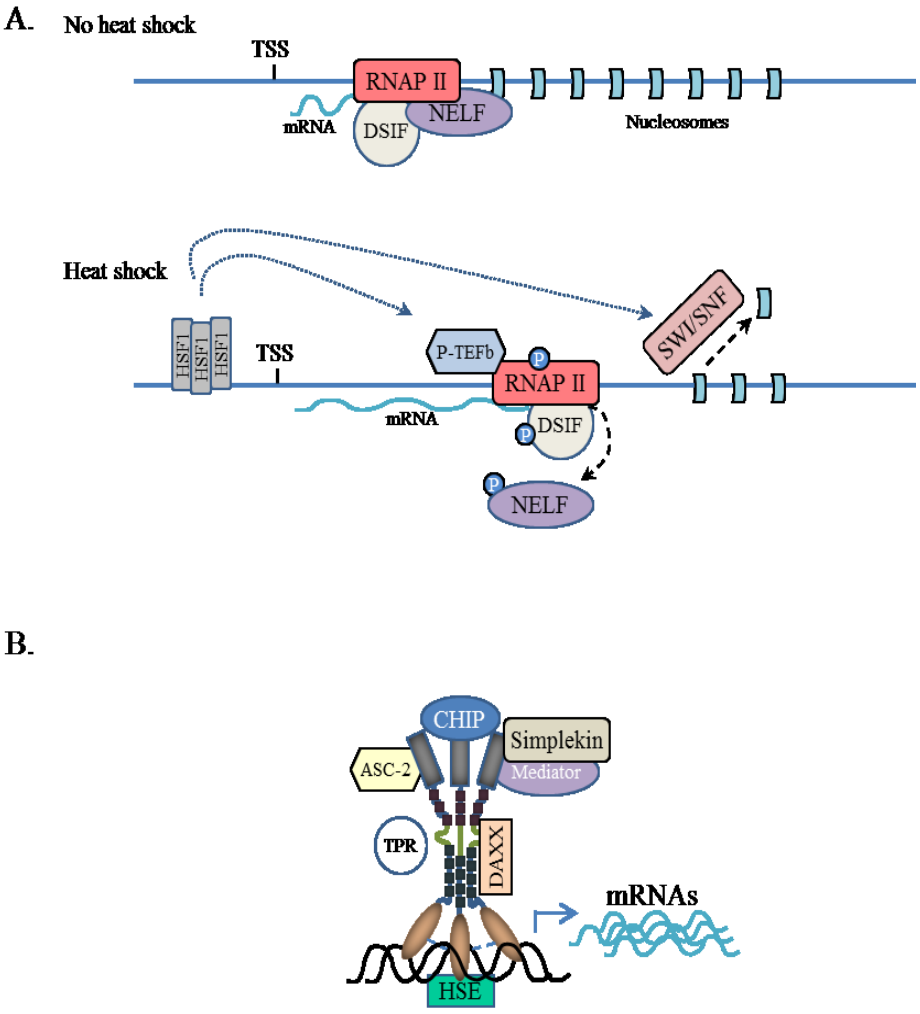


Figure 1.12. HSF1 Transcriptional Activation.

(A) Activation of gene transcription by HSF1. In the absence of stress, RNAP II is kept in a paused state in association with the DSIF-NELF protein complex, approximately 20-30 nucleotides downstream of the transcription start site. Transcription elongation is also prevented by the compact nucleosome structures of the gene downstream of RNAP II. Upon stress, HSF1 binds to the promoter upstream of RNAP II and recruits P-TEFb, which phosphorylates RNAP II and DSIF thereby facilitating the release of DSIF and NELF from RNAP II. HSF1 also recruits the chromatin remodelling complex SWI/SNF, which mediates the displacement of the nucleosomes structures and enables transcription elongation across the gene. **(B)** Hypothetical model of HSF1 interacting proteins during transcription activation.Other proteins have been found to interact with HSF1 and enhance its transactivation activity and include the transcriptional co-activator activating signal co-integrator ASC-2, simplekin, the nuclear pore associating translocated promoter region protein (TPR), HSP70-interacting protein (CHIP) and the nuclear FAS death domain associated protein DAXX (Adapted from Anckar and Sistonen, 2011).

processing and the nuclear export of mRNAs transcribed from the heat shock promoters by direct interactions with the nuclear pore associating translocated promoter region protein TPR and symplekin, which is a scaffold for polyadenylation factors (Fig.1.12B; Skaggs et al., 2007; Xing et al., 2004).

In summary, HSF1 enables transcription of its gene targets primarily by facilitating RNAP II maturation and nucleosome displacement. This transactivation activity of HSF1 is subject to the regulations of other HSF1 binding partners, which ensure a well-coordinated transcription activation of proteins upon stress.

1.6.5. HSF1 attenuation regulation

1.6.5.1. HSF1 repressive post-translational modifications

Stress induces activation of HSF1 but also concurrently induces several repressive post-translational modifications to negatively control activities of the factor. Many phosphorylation events have been shown to repress HSF1 transactivation and addition, HSF1 can also be repressed by sumoylation and acetylation reactions (Fig.1.13).

Phosphorylation: Four phosphorylation sites are known to have repressive effects on HSF1 activity and are serine 121, serine 303, serine 307 and serine 363. Serine 307 is phosphorylated by the extra cellular-regulated kinase 1 (Erk1). This phosphorylation subsequently facilitates the phosphorylation of serine 303 by the glycogen synthase kinase 3β (GSK3β). These two phosphorylation sites promote the association of HSF1 with the regulatory protein 14-3-3ε, leading to the cytoplasmic sequestration of the factor (Chu et al., 1996; Chu et al., 1998; Seo et al., 2006; Wang et al., 2003; Wang et al., 2004a). Serine 121 is phosphorylated by MAPK-activated protein kinase 2 (MK2). In addition to inhibiting HSF1 transactivation activity, phosphorylation at serine 121 promotes HSP90-HSF1 binding, which renders HSF1 in an inactive conformation (Wang et al., 2006b). Serine 363 can be phosphorylated by either Protein kinase C (PKC) or Jun-N terminal kinase (JNK). This phosphorylation rapidly inactivates HSF1 transactivation activity. During severe heat stress, this phosphorylation has been shown to promote cell death by inhibiting HSP production (Dai et al., 2000).

Sumoylation: HSF1 undergoes stress-induced sumoylation at lysine 298 by small ubiquitin related modifier 1, 2 and 3 (SUMO-1, -2 and -3), which represses the

transactivation activity of the factor (Hietakangas et al., 2003; Hong et al., 2001). HSF1 sumoylation is facilitated by phosphorylation at serine 303. In addition, this sumoylation is also facilitated by the association of HSF1 with HSP27 oligomers when cells recover from stress (Simioni et al., 2009).

Acetylation: A study by Westerheide et al. (2009) demonstrated that HSF1 activity can be attenuated by the acetylation of at least nine lysine residues by the acetylase p300/CBP (Westerheide et al., 2009). Among these, acetylation of lysine 80 in the DNA-binding domain is the most important since acetylation at this site abrogates HSF1 DNA-binding to the HSEs. This acetylation, however, can be reverted by the deacetylation activity of the sirtuin deacelylase SIRT1. Activation of SIRT1 has been shown to prolong the DNA-binding activity of the factor (Westerheide et al., 2009).

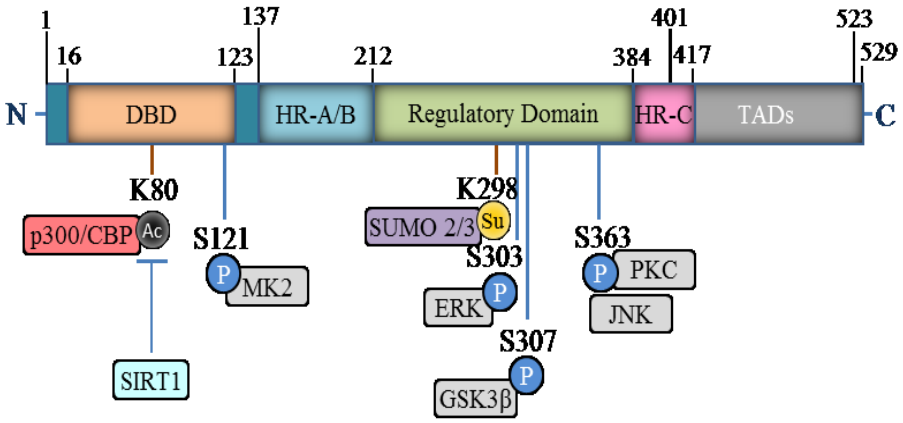


Figure 1.13. HSF1 repressive post-translational modifications

HSF1 undergoes repressive post-translational modifications including phosphorylation, sumoylation and acetylation. Four phosphorylation sites known to have inhibitory effect on HSF1 activity are serine 121, serine 303, serine 307 and serine 363, which are calalysed by MK2, ERK, GSK3β, PKC and JNK respectively. Inhibition of HSF1 activity by sumoylation is mediated by SUMO1/2/3 at lysine 298. HSF1 is also inhibited by acetylation at lysine 80 by p300/CBP, which can be reverted by the deacetylase activity of SIRT1

1.6.5.2. HSF1 transcription attenuation by protein interactions

Once protein homeostasis within cells has been restored, activity of HSF1 is also attenutated through protein interactions. During the recovery phase, HSP70 and HSP40 are known to interact with HSF1 and inhibit its transactivation activity. The HSP90 complex is then recruited to the HSP70/HSP40 bound HSF1 trimers and form a mature

HSP90 complex that facilitates the dissociation and cytoplasmic translocation of HSF1 as well as targeting the factor for proteasomeal degradation (Neef et al., 2010). Within the HSP90 complex, monomeric HSF1 has been shown to be stabilised through the interaction with the histone deacetylase HDAC6, which dissociates from HSF1/HSP90 complex upon high levels of protein aggregates, thereby linking the activity of HSF1 to proteasomal stress (Boyault et al., 2007).

Aside from the feedback regulation of HSPs, activity of HSF1 is also attenuated by the binding of other HSF1 binding partners. During the recovery phase, the protein phosphatase PP5, and the small HSF1-binding protein HSBP1, have also been shown to physically interact with HSF1 and inhibit its transactivation (Conde et al., 2005; Satyal et al., 1998). In addition, HSF1 transactivation is also inhibited by the transcriptional co-repressor CoREST, which is recruited to heat shock promoters through interaction with HSP70 (Gomez et al., 2008). Altogether, these repressive regulations ensure a co-ordinated response of HSF1 to the expression of its target genes and the state of the protein folding environment.

1.6.6. HSF1 and HSF2

In addition to forming homotrimers, HSF1 also forms transcriptionally active heterotrimers with HSF2. Studies have shown that HSF2 can potentiate HSF1-mediated transactivation and this transcription factor also contributes to the constitutive and stress-inducible expression of HSP genes (He et al., 2003; Mathew et al., 2001; Ostling et al., 2007; Wilkerson et al., 2007). However, HSF2 activity is only activated under certain specific conditions such as down-regulation of the ubiquitin-proteasome pathway (Mathew et al., 1998), during differentiation (Pirkkala et al., 1999; Pirkkala et al., 2001; Sarge et al., 1994) and in early development (Eriksson et al., 2000; Mezger et al., 1994). Unlike HSF1, HSF2 has a high affinity for discontinuous gap-type and step-type HSEs (Kroeger and Morimoto, 1994). Additionally, although being a less active transcription regulator, HSF2 can retain its DNA binding activity for extended periods. The differences in HSF1 and HSF2 activation and transactivation activities are suggested to provide a mechanism for more precise regulation of gene expression in response to distinct stresses and developmental stimuli. In addition, with increasing evidence of a role of HSF1 beyond regulating HSP expressions in cancer, HSF1 and

HSF2 heterotrimers are suggested to regulate a novel sub-set of genes or signalling pathways that promote cancer progression (Sandqvist et al., 2009).

1.6.7. Nuclear stress bodies (nSBs)

In human cells, upon stress, activated HSF1 and HSF2 accumulate in large amounts to a particular sub-nuclear structure called nuclear stress bodies (nSBs) on the pericentromeric region of chromosome 9 (9q12). The two factors directly bind and transcribe satellite III repeated sequences present in numerous copies at this locus. The products of this transcription are non-coding RNA molecules called satellite III transcripts, which remain associated with the 9q12 locus several hours after synthesis. These transcripts are proposed to play roles in regulating RNA splicing activities during and after stress by providing scaffolds for splicing factors and other RNA-processing proteins to attach (Biamonti, 2004; Biamonti and Vourc'h, 2010; Denegri et al., 2001).

While normal cells have two nSBs, tumour cells are often found to have several. In tumour cells, HSF1 has been found to also bind to satellite II and satellite III repeated sequences present on the pericentromeric region of chromosome 14, 12 and 15 (Denegri et al., 2002; Eymery et al., 2010). The roles of these nSBs are yet to characterise.

1.7. ROLES OF HSF1 IN CANCER

Aside from regulating HSP expression, HSF1 is capable of regulating multiple non-HSP targets that contain an appropriate HSE sequence. This was evident in a study showing that HSF1 can regulate up to 3% of the yeast genome (Hahn et al., 2004). Similar results have also been reported in Drosophila and mammalian cells (Birch-Machin et al., 2005; Trinklein et al., 2004; Westwood et al., 1991). In addition, HSF1 can also play a direct role in modulating many biological processes by its direct interactions with other binding partners. Extensive investigations on the role of HSF1 in cancer in recent years have revealed several novel roles of HSF1 in supporting cancer progression, leading to the potential use of HSF1 as an anti-cancer therapeutic target (Fig.1.14; De Thonel et al., 2011).

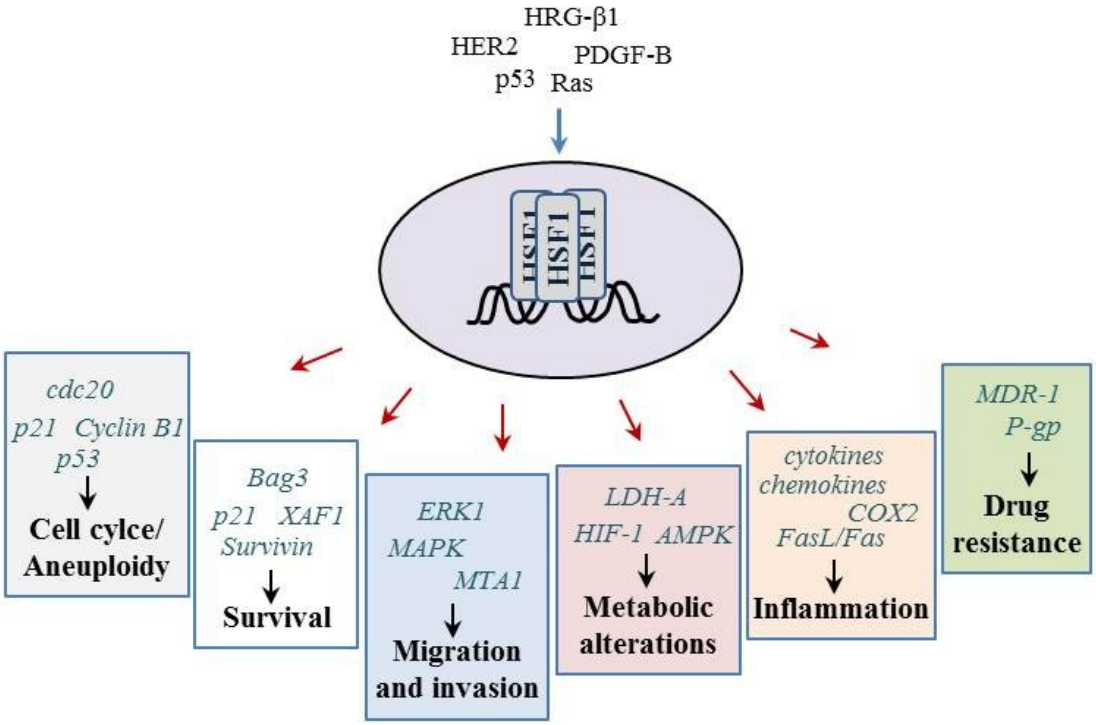


Figure 1.14. Identified non-HSP effects of HSF1 activation in cancer
In cancer, HSF1 is required for tumourigenesis induced by oncogenes and mutated tumour suppressor such as Heregulin β1, HER2, platelet-derived growth factor subunit B (PDGF-B), Ras and p53. HSF1 can directly or indirectly regulate levels and activities of several proteins promoting multiple aspects of cancer including aneuploidy, cell survival, migration and invasion, metabolic alterations, inflammation and drug resistance.

1.7.2. HSF1 regulates cell cycle

Studies have shown that HSF1 can play a direct role in regulating cell cycle and mitotic exit. During mitosis, HSF1 is activated similarly to heat stress but this activation does not lead to elevated level of HSPs (Bruce et al., 1999). Instead, during the cell cycle, HSF1 is phosphorylated by Plk1 at serine 216 and this phosphorylation triggers localization of the factor to the centrosomes during mitosis, especially to the spindle poles in metaphase (Kim et al., 2005). In normal cells, expression of HSF1 is essential for cell cycle arrest in G2 phase, assisting with the cell cycle checkpoint (Chang et al., 2012b) such that a null mutant or knockdown of HSF1 can cause defective mitotic progression (Lee et al., 2008a).

Active HSF1 in malignant cells, however, was found to contribute to the production of aneuploidy, which is the condition of having less than or more than the normal diploid

number of chromosomes. Aneuploidy is the result of incorrect segregation of whole chromosomes or part of the chromosomes during cell division. It has been demonstrated that prolonged expression of a dominant negative form of HSF1 (HSF1-DN), which lacks the transactivation domain, in p53-null PC-3 prostate carcinoma cells prevents the formation of aneuploidy cell populations. Expression of HSF1-DN also protects cells from chemical agents that disrupt the mitotic spindle and prevent cell cycle progression through metaphase (Wang et al., 2004b).

The role of HSF1 in supporting genomic instability and aneuploidy in cancer is explained by the fact that HSF1 blocks cyclin B1 degradation, which is a key step in mitotic exit and its degradation is mediated by the E3 ubiquitin ligase anaphase promoting complex C (APC/C) that targets cell cycle proteins for proteasomeal degradation (Peters, 2006). HSF1 can directly interact with Cdc20, which in turn inhibits the interaction between Cdc20 and Cdc27, the phosphorylation of Cdc27 and the ubiquitination activity of APC (Lee et al., 2008b). As HSF1 mediates aneuploidy and genomic instability in cancer, consistent with this, the double knockdown of Plk1 and HSF1 have been reported to decrease cell proliferation and increase apoptotic cell death in a synergistic fashion in human oral carcinoma cells (Kim et al., 2010).

HSF1 supported mediated aneuploidy in cancer has been shown to require a defective function of the tumour suppressor protein p53 (Kim et al., 2009c). Increased phosphorylation of HSF1 at Ser216, which leads to increased stability of securin and cyclin B1 in mitosis, was only observed in p53 defective cells but not in p53 wild-type cells. This indicates a novel role of p53 in HSF1-mediated mitotic regulation and genomic instability although the association between p53 and this activity of HSF1 requires further investigation.

1.7.3. HSF1 promotes cellular survival

Studies by Khaleque et al. (2005) demonstrated that activation of HSF1 by heregulin β1 in breast cancer cells leads to protection of the cells from apoptosis and enhances clonogenic survival and growth (Khaleque et al., 2005). To elucidate the mechanism of how HSF1 elicits its cytoprotective effects, Page et al. (2006) performed a genome-wide analysis of human HSF1 signalling networks under both stress and unstressed conditions and revealed that HSF1 regulates an extended transcriptional program linked

to cellular adaptation and survival. Analysis of these HSF1 inducibly regulated genes shows enrichment in a variety of categories including amongst others, protein refolding, anti-apoptosis, RNA splicing and ubiquitination (Page et al., 2006).

Of the genes identified by Page et al, HSF1 was reported to directly regulate the expression of BAG3 (Bcl2-associated athanogene 3), which is a member of the BAG family of co-chaperones. BAG3 is known to interact with the ATPase domain of HSP70 and the HSP70-BAG3 chaperone complex reportedly sustains cell survival and enhances therapeutic resistance in many cancer cells by stabilizing several anti-apoptotic members of the B-cell lymphoma 2 (Bcl-2) family proteins such as Bcl-xL, myeloid cell leukemia 1 (Mcl-1), and Bcl-2 (Du et al., 2009; Franceschelli et al., 2008; Jacobs and Marnett, 2009; Rosati et al., 2011; Song et al., 2010). Consistent with the direct role of HSF1 in regulating BAG3 expression, BAG3 has been shown to be induced by many HSF1 activating agents such as MG132 (Du et al., 2009), 4-Hydroxynonenal (HNE) (Jacobs and Marnett, 2009) and pyrrolidine dithiocarbamate (Song et al., 2010). Additionally, HSF1 has been shown to directly bind to the promoter and down-regulate the expression of XAF1 (XIAP-associated factor 1), which functions as an inhibitor of the cytoprotective protein XIAP (inhibitor of apoptosis-interacting protein) (Wang et al., 2006a).

In further support of a role of HSF1 in cellular survival, Meng et al. (2010) demonstrated that HSF1 knockdown in HER2 transformed cells leads to an increase in p21 and decrease of survivin, subsequently causing cell cycle arrest and growth inhibition. Suppression of HSF1 was also seen in fibroblast cells undergoing senescence in response to DNA damaging treatments (Kim et al., 2012). It is therefore suggested that HSF1 is required for cancer cells to escape cellular senescence and maintain indefinite proliferation.

1.7.4. HSF1 promotes migration and invasion

Activation of HSF1 in cancer can promote metastasis by enhancing cell migration and invasion. O'Callaghan-Sunol et al. (2006) demonstrated that immortalized MEF cells derived from *hsf1(-/-)* animals were deficient in both basal and EGF-induced migration as HSF1 knockout causes a defect in MAP kinase signalling, which leads to the reduction in activation of Erk and JNK pathways following EGF stimulation

(O'Callaghan-Sunol and Sherman, 2006). In further support of a role of HSF1 in metastasis, Khaleque et al. (2008) demonstrated that HSF1 when induced by the transforming ligand heregulin β1 via its downstream signalling pathways such as the HER2/neu and PI3K/Akt cascades, can associate and control the activity of MTA1 (metastasis associated protein 1), which is a co-repressor co-factor that promotes metastasis in cancer (Khaleque et al., 2008; Khaleque et al., 2005). MTA1 is a component of the NuRD co-repressor complex, which contains multiple proteins such as the histone deacetylases HDAC1 and 2 and chromatin remodelling protein Mi2α that repress expression of anti-metastatic genes in cancer (Lai and Wade, 2011). Upon activation, the HSF1-MTA1 containing NuRD complexes assemble on the chromatin, associate with the promoters and repress the expression of estrogen-responsive genes. The reduction of some anti-metastatic estrogen-responsive genes such as pS2 and c-Myc is suggested to result in an enhanced metastasis in cancer cells (Khaleque et al., 2008).

Consistent with the notion that HSF1 may promote metastasis, Kouspou (2009) demonstrated that expression of HSF1-DN in TNBC cell lines decreases cell migration and invasion both *in vitro* and *in vivo*. This study also demonstrated that HSF1 regulates several molecules that play key roles in cell migration such as Rac1, cortactin and cofilin1 (Kouspou, 2009). In addition to this, recently, Fang el al. (2011) demonstrated that HSF1 enhances invasion and metastasis of hepatocellular carcinoma (HCC) (Fang et al., 2011). Analysis of clinical samples with HCC reveals the association of the expression levels of HSF1 with multiple nodules, venous invasion, absence of capsular formation, and poor overall survival and disease-free survival. In addition, HSF1 overexpression and knockdown in HCC cell lines increased and decreased cell migration and invasion respectively both *in vitro* and *in vivo*. The authors suggested that this role of HSF1 in HCC is the result of HSF1 regulation of HSP27, such that knockdown of HSP27 in the cells abolished HSF1 effects on cell migration and invasion. However, taken together with results from previous studies on HSF1 activity, regulation of HSP27 expression is likely to be only one of the many mechanisms by which HSF1 is involved in migration, invasion and metastasis.

1.7.5. HSF1 is involved in metabolic alterations in cancer

1.7.5.1. Glucose metabolism

Studies have demonstrated that HSF1 contributes to the increased glycolytic activity in cancer. Dai et al., (2007) demonstrated that *hsf1-/-* MEFs and C2 cells with HSF1 knockdown have reduced sensitivity to glucose deprivation. In glucose-replete conditions, these cells also produce less lactate, which is a glycolysis product, due to having lower lactate dehydrogenase (LDH) activities than wild-type cells. Consistent with this, Zhao et al. (2009) reported that overexpression of the oncogene HER2/ErbB2 in breast cancer cells leads to the up-regulation of LDH-A levels through HSF1 activation. Activated HSF1 was found to directly bind to the LDH-A promoter. Down-regulation of the factor reduces LDH-A expression and subsequently leads to decreased cancer glycolysis and growth (Zhao et al., 2009). Consequently, inhibition of HER2 in cancer, which has been shown to reduce tumour growth by inhibiting glycolysis, was found to be less effective in cells expressing high level of HSF1. The use of ErbB2 inhibitor in combination with HSF1 inhibitor or glycolysis inhibitor, therefore, has been shown to synergistically inhibit tumour growth (Zhao et al., 2011).

Recently, HSF1 was found to regulate HIF-1 translation by regulating the expression of the RNA-binding protein HuR (Human antigen R). Down-regulation of HSF1 was shown to suppress angiogenesis, which is associated with suppression of the HIF-1 pathway (Gabai et al., 2012). Although the study has not linked HSF1 directly to the altered glucose metabolism in cancer, it is possible that HSF1 promotes the glucose metabolic change under hypoxic conditions by enhancing HIF-1 translation.

1.7.5.2. Lipid metabolism

It was found in the genome-wide analysis of HSF1 regulated genes that HSF1 controls several aspects of lipid metabolism at the basal level; and these functions are preserved following heat shock (Page et al., 2006). In further support of this finding, recently, Jin et al. (2011) demonstrated that inactivation of HSF1 inhibits N-diethylnitrosamine (DEN)-induced HCC formation by impairing the deposition and accumulation of lipid in hepatocytes. HSF1 deficient mice exhibit enhanced insulin sensitivity and higher basal and insulin induced activation of AMP-activated protein kinase (AMPK), which is

an inhibitor of lipid synthesis (Jin et al., 2011). HSF1 thus appears to control metabolic alterations enabling oncogenesis and cancer progression.

1.7.6. HSF1 contributes to the link between inflammation and cancer

Studies have indicated that HSF1 regulates the expression of many inflammatory mediators by binding to their promoters either directly or indirectly. These include interleukin 1β (IL-1β) (Cahill et al., 1996), tumour necrosis factor α (TNF-α) (Singh et al., 2002), c-fos (Chen et al., 1997; He et al., 2000; Xie et al., 2003), c-fms (Xie et al., 2003) and granulocyte-colony stimulating factor (G-CSF) (Ma et al., 2012; Zhang et al., 2011a). It was also found that 28 out of 29 human and mouse CXC chemokine genes have multiple putative HSEs present in their promoters and hyperthermia increased expression of CXC chemokines in mice (Nagarsekar et al., 2005). Chromatin analysis revealed that HSF1 was recruited to several CXC chemokine genes. However, the effects of HSF1 on the expression of these genes are variable and gene specific. For example, HSF1 was found to repress CXCL-5 expression whereas having no effect on the expression of CXCL-1 and CXCL-2 despite being recruited to their promoters. In contrast, HSF1 up-regulates expression of IL-8/CXCL-8 upon stimulation by TNF-α (Singh et al., 2008).

It was speculated that the effect of HSF1 on CXC chemokine expression is largely dependent on the physical proximity and interactions of other transcription factors and co-regulators (Singh et al., 2008). In agreement with this, HSF1 was reported to inhibit expression of IL-6 by inducing the expression of the activating transcription factor ATF3 in mouse embryonic fibroblasts and macrophages (Takii et al., 2010). In contrast, the factor was found to augment IL-6 production in human intestinal epithelial cells by activating the transcription factor c/EBP-β (Hungness et al., 2002). Recently, in breast cancer, HSF1 was found to trigger demethylation of the IL-6 promoter, thereby facilitating the constitutive expression of IL-6. A high IL-6 level in turn activates the phosphorylation signalling cascade leading to increased HSF1 activation. Inactivation of this inflammatory circuit was shown to abrogate oncogenic transformation and the maintenance of the transformed state (Rokavec et al., 2012). HSF1 is thus a transcriptional regulator of inflammatory cytokines, although, this effect is rather complex and largely dependent on cellular context.

Aside from regulating expressions of inflammatory cytokines and chemokines, HSF1 is also involved in the expression of other key inflammatory modulators. In human endothelial cells, heat activated HSF1 was found to be recruited to the promoter and activate the expression of Cyclooxygenase 2 (COX-2), which is an inducible enzyme catalysing the inflammatory formation of the second messenger protein prostaglandin E2 (PGE2) (Rossi et al., 2012). Degregulation of the COX-2/PGE2 pathway has been shown to promote tumour initiation, maintenance and progression and stimulate metastatic spread (Greenhough et al., 2009). In addition, HSF1 was also found to regulate the expression of the tumour necrosis factor Fas ligand (FasL) and its receptor, Fas (Bouchier-Hayes et al., 2010; Shunmei et al., 2010). Activation of FasL/Fas pathway induces caspase-dependent apoptosis and is the main mechanism by which T-cells stimulate cell death. However, studies have also demonstrated that Fas can activate multiple non-apoptotic signalling pathways and that activation of these pathways leads to enhanced tumourigenicity and metastasis (O'Brien et al., 2005). Altogether, current findings suggest that HSF1 is a key molecule linking inflammation to cancer, although, further comprehensive experiments are required to investigate the complexity of this effect and on how it may be used to benefit cancer treatments.

1.7.7. HSF1 promotes drug resistance

The acquisition of drug resistance is the major cause of treatment failure in cancer patients. In cancer cells *in vivo* and clinically, the use of many therapeutic compounds such as HSP90 and proteasome inhibitors leads to HSF1 activation, which can subsequently activate the cytoprotective responses in tumour cells and promote drug resistance. Targeting the HSF1 pathway has been shown to enhance the efficacy of a number of anticancer drugs (de Billy et al., 2009; Whitesell and Lindquist, 2009; Zaarur et al., 2006).

One important mechanism for the development of drug resistance in cancer cells is the overexpression of the multi-drug resistance protein MDR-1 and its product P-glycoprotein (P-gp), which is an energy-dependent drug efflux pump. HSF1 has been implicated in promoting the drug resistance phenotype in cancer by transactivating the MDR-1 gene. MDR-1 was found to contain two HSEs upstream of its promoter (Kioka et al., 1992). Endogenous expression of P-gp could be transiently induced by heat-shock while ectopic expression of a constitutively active mutant HSF1 induces MDR-1

expression in Hela cervical carcinoma cells (Chin et al., 1990; Miyazaki et al., 1992; Vilaboa et al., 2000). In addition, cells with a multidrug resistance phenotype, FM3A/M and P388/M, exhibit constitutively activated HSF1 (Kim et al., 1997). Inhibition of HSF1 by quercetin in these cells leads to a down-regulation of MDR-1 expression and subsequently sensitizes the cells to anticancer drugs (Kim et al., 1998). Alternatively, the induction of MDR-1 expression and multidrug resistance phenotype by HSF1 can also occur at the posttranslational level and is independent of the induction of the heat shock response (Tchenio et al., 2006).

However, the regulation of HSF1 on MDR-1 expression appears to be cell-type dependent. Recently, Krishnamurthy et al. (2012) reported that HSF1 knockout induces MDR-1b expression and enhances P-gp based drug extrusion in the heart, which alleviates doxorubicin-induced heart failure and reduced mortality in mice (Krishnamurthy et al., 2012). The repression of HSF1 on MDR-1b in cardiocytes is explained by the fact that MDR-1b expression is regulated by NF-κB. The binding of HSF1 to the MDR-1b promoter hinders the binding of NF-κB to this promoter, thereby preventing its transcription. These findings suggest that systemic inhibition of HSF1 would provide cardioprotection while effectively sensitizing tumour cells to conventional chemotherapeutics and drugs.

1.8. HSF1 ACTIVATING COMPOUNDS

Although HSF1 activation promotes cancer progression, the activation of HSF1 can be beneficial for the treatment of diseases that are associated with the disruption of protein homeostasis and accumulation of misfolded proteins, such as neurodegenerative and cardiovascular diseases. As such, several HSF1 activating compounds have been identified and are currently being examined for their therapeutic efficacy in such diseases (Neef et al., 2010). However, these compounds may also provide a benefit due to the sustained stressed phenotype of cancer cells, in that these stress activating compounds may further disrupt cellular protein homeostasis to levels that exceed the buffering capacity of tumour cells but not of normal host cells. This may provide a unique therapeutic opportunity by which tumour cells are more susceptible to cell death by these stress-inducing compounds. Consistent with this, compounds known to increase stress within tumour cells, such as HSP90 inhibitors, proteasomal inhibitors, or

those that generate reactive oxygen species within tumour cells, have already been shown to exhibit potent anticancer properties (Santagata et al., 2012; Whitesell and Lindquist, 2005).

Multiple HSF1 activating compounds have been identified that activate HSF1 by a variety of mechanisms (Westerheide and Morimoto, 2005 and Table 1.4). HSF1 can be activated by compounds that lead to the appearance of misfolded proteins such as

Table 1.4. *List of HSF1 activating compounds (Westerheide et al., 2005).*

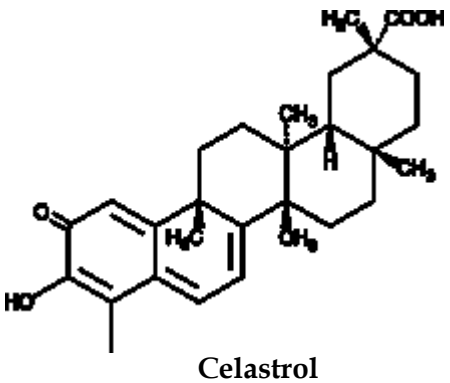
Compounds	References
Protein synthesis inhibitors:	
Puromycin	(Hightower, 1980; Lee et al., 1987)
Azetidine	(Hightower, 1980)
Proteasome inhibitors:	
MG132	(Holmberg et al., 2000)
Lactacystin	(Holmberg et al., 2000)
Bortezomib	
Serine protease inhibitors:	
DCIC (3,4 dichloroisocoumarin)	(Rossi et al., 1998)
TPCK (Tosyl phenylalanyl chloromethyl ketone)	(Rossi et al., 1998)
TLCK (Tosyl-L-lysinyl-chloromethylketone)	(Rossi et al., 1998)
Hsp90 inhibitors:	
Radicicol	(Bagatell et al., 2000)
Geldanamycin	(Bagatell et al., 2000; Kim et al., 1999)
17-AAG	(Bagatell et al., 2000)
Inflammatory mediators:	
Cyclopentenone prostaglandins	(Amici et al., 1992; Ohno et al., 1988)
Arachidonate	(Jurivich et al., 1994)
Phospholipase A_2	(Jurivich et al., 1994)
Glutamate inhibitor:	
Riluzole	(Yang et al., 2008)
ROS:	
Ethanol	(Mandrekar et al., 2008)
H_2O_2	(Bruce et al., 1993; Nishizawa et al., 1999)
Menadione	(Bruce et al., 1993)
Steroidal Lactone:	
Withaferin A	(Xu et al., 2009)
Triterpenoids:	
Celastrol	
Co-inducers	
NSAIDS:	(Jurivich et al., 1992; Seo et al., 2005)
Sodium salicylate	(Lee et al., 1995)
Indomethacin	
Hydroxylamine derivatives:	(Hargitai et al., 2003; Vigh et al., 1997)
Bimoclomol	(Calderwood et al., 2008; Calderwood et al., 2006; Kieran et al., 2004)
Arimoclomol	

protein synthesis inhibitors (puromycin and azetidine) (Hightower, 1980; Lee and Dewey, 1987), proteasome inhibitors (MG132, lactacystin and bortezomib) (Holmberg et al., 2000) and serine protease inhibitors (Rossi et al., 1998). In addition, HSF1 oligomerization and its DNA binding activity can be stimulated by reactive oxygen species (ROS) or agents that are able to generate ROS within cells, such as ethanol. Moreover, HSP90 inhibitors, which bind to the ATP binding domain of HSP90, can de-repress HSF1 from its inactive monomeric structure, leading to its activation (Bagatell et al., 2000; Kim et al., 1999). Another class of HSF1 inducers are the inflammatory mediators (cyclopentenone prostaglandins, arachidonate and phospholipase A2), which cause alteration in protein homeostasis within cells (Amici et al., 1992; Jurivich et al., 1994; Ohno et al., 1988). Alternatively, the glutamate inhibitor rizulole, has also been shown to promote HSF1 activation by the suppression of HSF1 degradation (Yang et al., 2008). Other compounds are known to be co-inducers of the HSR by HSF1 activation such as NSAIDS and hydroxylamine derivatives (Hargitai et al., 2003; Jurivich et al., 1992; Kieran et al., 2004; Lee et al., 1995; Seo et al., 2005; Vigh et al., 1997).

Despite evidence demonstrating the beneficial effects of HSF1 activators in neurodegenerative and cardiovascular diseases, the use of HSF1 activators in cancer still has limited success (Neckers and Workman, 2012). Recently, two HSF1 activators have emerged as potential anticancer therapeutic compounds and are under intensive investigation, namely celastrol and withaferin A. These two compounds may represent new leads in the development of new, broadly effective anticancer drugs which disrupt cellular protein homeostasis.

1.8.1. Celastrol

Celastrol is a naturally occuring quinone methide triterpene compound derived from a Chinese medicinal herb traditionally used as a remedy for inflammatory and autoimmune diseases (Kim et al., 2009a; Kim et al., 2009b). *In vitro* studies have revealed that celastrol can inhibit LPS-induced inflammatory response and platelet activation. The compound also exhibits potent



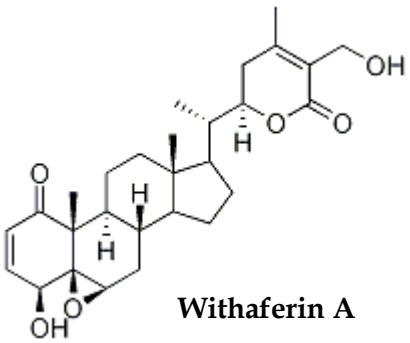
Celastrol

anticancer activity against a variety of tumours by inhibiting cancer cell proliferation, prevention of cancer cell invasion, inhibition of angiogenesis and inducing apoptosis (Chen et al., 2009a; Dai et al., 2009; Davenport et al., 2010a; Ge et al., 2010; Mou et al., 2011; Wang et al., 2010a; Yadav et al., 2010; Yang et al., 2006; Zhou and Huang, 2009). Celastrol can also sensitize resistant cancer cells and potentiate radiotherapy when used in the combination therapeutic setting.

Celastrol has been found to activate HSF1 with similar kinetics similar to that of heat stress (Westerheide et al., 2004; Zhang and Sarge, 2007). Molecular structure analysis revealed that celastrol contains an electrophilic site within its quinone methide ring which can react with the nucleophilic thiol groups of cysteine residues present within proteins (Sreeramulu et al., 2009; Trott et al., 2008). Although the exact molecular mechanism of HSF1 activation is not fully understood, celastrol induces HSF1 DNA binding and its hyperphosphorylation leading to increased HSP expression. However, due to the chemical nature of this mechanism, celastrol affects a number of other molecular targets (Kannaiyan et al., 2011). For instance, celastrol can directly inhibit IKKα and β kinases, thereby inhibiting NF-κB signalling pathway. Celastrol can also inactivate Cdc37 and p23 proteins that are co-chaperones of HSP90, as well as inhibiting the function of the proteasome (Salminen et al., 2010). Although largely non-specific, its broad proteotoxic stress effects coupled with the many recent studies demonstrating its therapeutic potential in preclinical cancer models has led to it being pursued as a potential cancer treatment.

1.8.2. Withaferin A

Withaferin A is a natural compound isolated from the medicinal plant *Withania somnifera*. This compound belongs to the category withanolides, which are a group of naturally occurring C28-steroidal lactone triterpenoids. Withaferin A and celastrol share the same chemical motif, which is an α,β-unsaturated carbonyl functionality that exhibits strong thiol-reactivity. Withaferin A has been identified as a potent activator of the HSR, most recently via an unbiased screen of compounds that targeted protein homeostasis via HSF1-dependent HSR (Xu et al.,



Withaferin A

2009). The compound has been validated as a potent anticancer compound which can inhibit cell growth and induce apoptosis in a variety of cancer types (Hahm et al., 2011; Liu et al., 2011b; Munagala et al., 2011; Zhang et al., 2012b). Combined treatment of withaferin A can potentiate conventional chemo- and radio-therapies (Yang et al., 2011a; Yang et al., 2011b), however, similar to celastrol and other thiol-reactive compounds, the molecular targets of withaferin A are diverse and are dependent upon the conditions maintained in specific intracellular compartments. It is also unclear whether the compound depends on HSF1 for its anticancer effects. Due to the complex biology and chemical reactivity of the compound its use in cancer treatment is still under investigation.

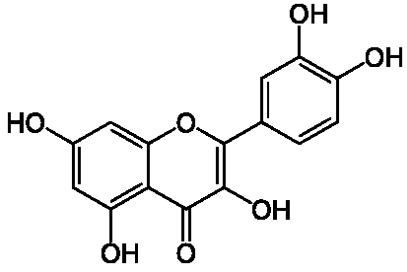
1.9. HSF1 INHIBITORS

As HSF1 emerges as a potential therapeutic target, identification of HSF1 inhibitors has been of great interest in recent years, with an array of drug-like compounds identified and shown to display promising anticancer properties (Table 1.5).

Table 1.5. *List of HSF1 inhibitors*

Compounds	References
Quercetin	(Hansen et al., 1997; Nagai et al., 1995)
KNK437	(Yokota et al., 2000)
Triptolide	(Westerheide et al., 2006)
Dehydroemetine (NZ28 and emunin)	(Zaarur et al., 2006)
Quinacrine and 9-aminoacridine (9AA)	(Gurova, 2009; Neznanov et al., 2009)
KRIBB11	(Yoon et al., 2011)
Trizole nucleoside analog	(Xia et al., 2012)
PI103	(Yih et al., 2012)
Linear polyamidea	(Wang et al., 2012c)

1.9.1. Quercetin



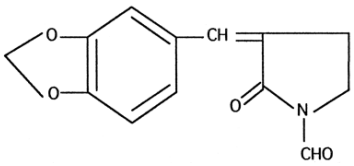
Quercetin

Quercetin is a natural bioflavonoid compound present in various vegetables, fruits, leaves and grains. The compound was found to inhibit HSP induction by reducing HSF1 DNA binding ability in breast cancer cells and by reducing HSF1 levels in various cell

types (Hansen et al., 1997; Nagai et al., 1995). Quercetin does not directly bind to HSF1 but inhibits HSF1 phosphorylation by blocking the activity of a range of protein kinases (Matter et al., 1992). However, since several kinases are inhibited, the activity of quercetin is seen as non-specific. Quercetin has also been identified as an anti-oxidant. Several recent studies have indicated that quercetin is a multi-target inhibitor which can suppress cancer by a variety of mechanisms, such as inhibiting cell proliferation and inducing apoptosis (Duo et al., 2012; Liu et al., 2012b), blocking EMT (Chang et al., 2012a), inhibiting angiogenesis and sensitising cancer cells to hyperthermia and chemotherapy (Li et al., 2012b; Wang et al., 2012b). Phase I clinical trials of quercetin and its water soluble derivative, QC12, confirmed that doses of quercetin sufficient to modulate the HSR in patients can be achieved with no significant adverse effect (Hirpara et al., 2009). However, due to its low potency and lack of specificity, quercetin has not been proven to be an effective anticancer compound in either monotherapy or in combination with other chemotherapeutic drugs in the clinical setting (Dajas, 2012).

1.9.2. KNK437

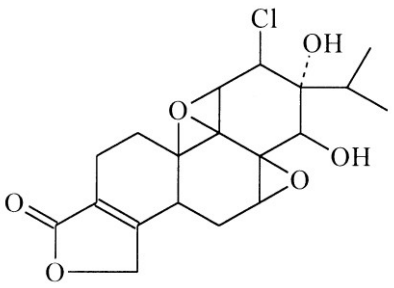
KNK437 (N-Formyl-3,4-methylenedioxy-benzylidene-gamma-butyrolaetam) is a synthetic benzylidene lactam compound which can inhibit the heat induced expression of HSPs without affecting the basal expression of their constitutive forms (Yokota et al., 2000). Studies have shown that KNK437 can sensitise tumour cells to irradiation (Ohnishi et al., 2006), hyperthermia (Sahin et al., 2011) and therapeutic agents such as arsenic trioxide (Wu et al., 2009), gemcitabine (Taba et al., 2011) and HSP90 inhibitors (Davenport et al., 2010b). The compound can inhibit cancer cells acquiring thermotolerance (Sakurai et al., 2005) and can abrogate hypoxia induced radio-resistance by targeting the Akt and HIF-1α survival pathways (Oommen and Prise, 2012). In addition, KNK437 can also induce apoptosis by caspase 3 activation (Inoue et al., 2010). However, the precise molecular mechanism of KNK437 remains unknown. Unlike quercetin, the compound does not appear to inhibit HSF1 phosphorylation. Although being relatively non-toxic, KNK437 has poor potency and as such, the compound has not gained much interest in recent years as a lead compound in the development new anticancer drugs.



KNK437

1.9.3. Triptolide

Triptolide is a diterpene tripoxide found in the Chinese medicinal herb *Tripterygium wilfordii.* The compound is the most potent HSF1 inhibitor described to date, working at low nanomolar concentration range. Triptolide has been shown to interfere with HSF1 transcriptional activity without affecting its trimer formation, hyperphosphorylation or DNA binding ability (Westerheide et al., 2006). However, Titov et al. (2011) has demonstrated that Triptolide acts as a potent, highly selective inhibitor of RNA Polymerase II via direct binding to XPB, a subunit of TFIIH. Other studies have also demonstrated that the activity of Triptolide is not HSF1 specific. The compound can impair the transactivation activities of other transcription factors such as necrosis factor κB (NF-κB) and activator protein 1 (AP-1) (Geng et al., 2012) and interact with other binding partners such as polycystin-2 (PC-2), a disintegrin and metalloproteinase domain 19 (ADAM19) and dCTP pyrophosphatase 1 (DCTPP1). Studies have shown that triptolide has a variety of biological effects, including immunosuppressive, anti-inflammatory and anti-tumour functions (Wang et al., 2012a; Yan et al., 2012). Triptolide can inhibit cell proliferation and invasion (Liu et al., 2012a; Liu et al., 2012c; Wen et al., 2012; Zhang et al., 2012a), induce apoptosis (Wang et al., 2012d) and sensitise cells to chemotherapeutic drugs in a number of cancer cell lines *in vitro* (Huang et al., 2012; Zhu et al., 2012) and suppress tumour development *in vivo* (Ding et al., 2012). Due to its poor water solubility, efforts have been made to modify the compound. Some derivatives, for example, LLDT-8, show promising therapeutic properties with reduced toxicity. As its mechanism is unspecific, it is still unknown whether the anticancer properties of triptolide stem from its ability to disrupt HSF1 function.

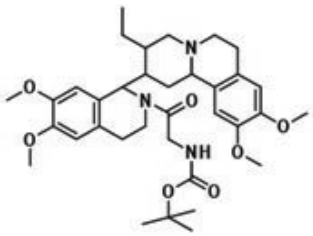


Triptolide

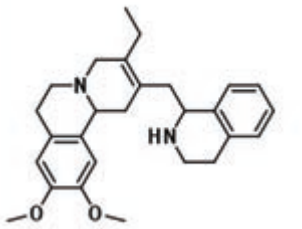
1.9.4. Dehydroemetine

In a screen of 20,000 compounds for structures that block HSP induction, Zaarur et al. (2009) identified two analogs of the general translational inhibitor dehydroemetine, NZ28 and emunin (Zaarur et al., 2006). These compounds were found to sensitize

cancer cells to proteasome and HSP90 inhibitors. NZ28 and emunin were found to work at low micromolar concentrations and exhibit low toxicity. The precise mechanism of these compounds remains unanswered but it has been proposed that they act downstream of HSF1 at the posttranslational level, leading to concerns over their specificity.



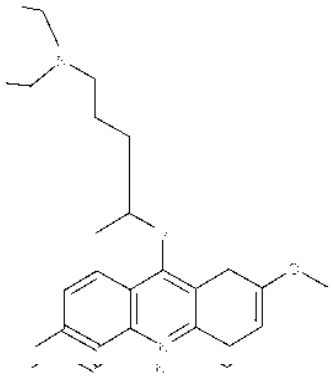
Emunin



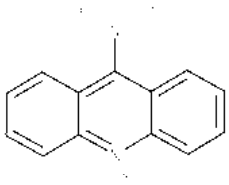
NZ28

1.9.5. Quinacrine

Emetine and its derivatives have been approved for use as anti-malarial drugs. Similar to that of the cancer cell, the malarial parasite has to overcome proteotoxic stresses to survive, and inhibition of this response is the mechanism by which anti-malaria drugs take their effect. In an analysis of a range of anti-malarial drugs for their ability to suppress the HSR in cancer cells, quinacrine and its related compound, 9-aminoacridine (9AA), were identified as inhibitors of HSP expression (Neznanov et al., 2009). Unlike emetine, these compounds do not affect general protein synthesis but rather suppress the HSF1-inducible expression of HSPs in a relatively selected manner. Quinacine and 9AA have been shown to not interfere with HSF1 cytoplasmic activation, translocation or DNA-binding, but localize in the nucleus and affect the transactivation activity of HSF1. As Quinacrine is a general DNA intercalating agent that can interfere with the transcription of many active genes in open areas of chromatin, it is possible that the observation that the compound interfered with the production of HSPs in the cells with stress is not HSF1 specific. Although the precise mechanism of their action remains to be characterised, quinacrine and 9AA exhibit potent anticancer properties. Combined treatment of the compounds with HSP90 inhibitors, such as 17-DMAG, synergistically suppresses tumour growth *in vivo* (Gurova, 2009).



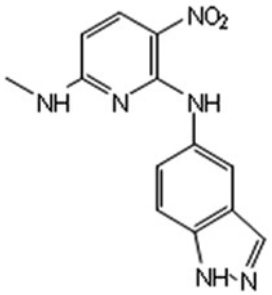
Quinacrine



9-aminoacridine

1.9.6. KRIBB11

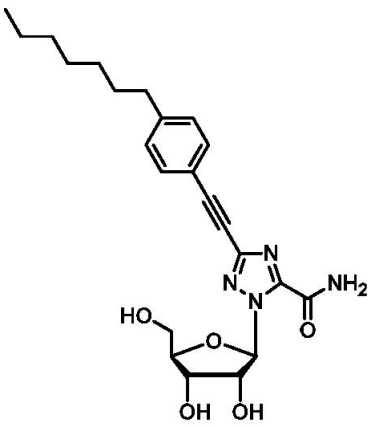
KRIBB11 (N2-(1H-Indazol-5-yl)-N 6-methyl-3-nitropyridine-2,6-diamine) is a compound identified in a screen for HSF1 inhibitors from a synthetic chemical library using a luciferase reporter under the control of a HSE containing promoter (Yoon et al., 2011). The compound has been shown to specifically reduce the heat-induced expression of HSPs by directly interacting with HSF1, preventing the factor from recruiting the transcription co-factor pTEFb. The association of KRIBB11 and HSF1 does not affect HSF1 activation, hyperphosphorylation or DNA-binding abilities. By reducing HSP expression, KRIBB11 has been demonstrated to inhibit cancer cell proliferation *in vitro* and suppress tumour growth *in vivo*. The compound causes cell cycle arrest at G2/M in cancer cells *in vitro* at concentrations up to 10μM, andinduces apoptosis at higher concentrations. This is the first compound known to have specific activity against HSF1 and will be subject to further validation in a clinical setting (Yoon et al., 2011).



KRIBB11

1.9.7. Triazole nucleoside analog

In a screen for HSF1 inhibitors, Xia et al. (2012) reported that expression of HSF1 can be inhibited by a triazole nucleoside analog modified from the 5-arylethynyltriazole ribonucleoside (Xia et al., 2012), which was previously shown to inhibit HSP27 expression and exhibit anticancer properties (Xia et al., 2009). The triazole nucleoside analog was shown to reduce HSF1 expression at the mRNA level, which subsequently lead to the simultaneous reduction of several HSPs including HSP27, HSP70 and HSP90. This compound displays anticancer properties by inducing caspase-dependent apoptosis and treatment of drug-resistant pancreatic tumour xenografts in mice with the compound effectively suppresses tumour growth. Further study revealed that the compound does not inhibit general DNA or RNA synthesis and as such, it is still unknown how this compound specifically inhibits

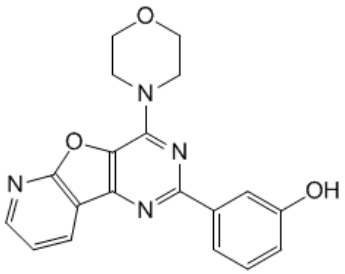


Triazole nucleoside analog

HSF1 mRNA expression (Xia et al., 2012). The use of this compound for cancer treatment requires further study.

1.9.8. PI-103

PI-103 is an ATP-competitive inhibitor of members of the PI3K family. The compound has recently been shown to enhance the cytotoxicity of arsenic trioxide in cancer cells by inhibiting the activating phosphorylation of HSF1 at serine 326. Treatment with the compound leads to a reduction in HSF1 transactivation activity, abrogating the arsenic trioxide-induced expression of HSPs and sensitising cancer cells to arsenic trioxide (Yih et al., 2012). However, as PI-103 inhibits PI3K, the mechanism of function of PI-103 is not HSF1 specific and thus the compound would not represent a new lead in the development of HSF1 targeted inhibitors as anticancer therapeutics.



PI-103

1.9.9. Linear polyamides

Recently, Wang et al. (2012) demonstrated that HSF1 transactivation activity can be inhibited by synthetic linear polyamides that bind to HSE in an 1:1 ratio (Wang et al., 2012c). These linear polyamides have been shown to compete with HSF1 for binding to HSE and prevent the formation of HSF1 trimer on the HSP70 promoter. However, the biological effect and anti-cancer property of these compounds awaits further study.

1.9.10. Summary of current HSF1 inhibitors

The ability of HSF1 inhibitors to inhibit the HSR and thereby sensitise cancer cells to stress-induced death make these compounds promising therapeutic candidates for the treatment of cancer. However, all compounds identified so far suffer from problems of low potency and/or poor specificity. In addition, as there is accumulating evidence demonstrating that HSF1 regulates multiple pathways and cellular processes, it will be challenging to determine whether the effects seen by HSF1 inhibition are specific or are due to off-target effects. Compounds that specifically and directly interact with HSF1 and inhibit its activity would be of the most interest in the development of HSF1 inhibitors. With recent advances in understanding the structure of HSF1 and its

activities in cancer, as well as the development of cell-based screening strategies, it is expected that a number of HSF1 inhibitors will be identified.

1.10. PROJECT RATIONALES AND OBJECTIVES

Recent studies have established that high levels and activation of HSF1 in breast cancer is associated with cancer aggressiveness, poor prognosis and resistance to therapies. A study by Santagata et al. (2011) investigating 1841 clinical samples from breast cancer patients demonstrated that nuclear HSF1 levels are increased in up to 80% of *in situ* and invasive breast cancer carcinomas and these levels are strongly associated with high mortality. High levels of HSF1 correlate with more advanced clinical stages and more malignant phenotypes. At the molecular level, tumours with high HSF1 levels are more likely to be ER-, HER2/neu positive or triple-negative (Calderwood, 2012a; Santagata et al., 2011). These findings support the notion that HSF1 up-regulates an epigenetic program that promotes cancer progression. However, as HSF1 is not a '*bona-fide*' oncogene, mechanisms by which HSF1 achieves this remains to be elucidated. Recent studies suggest co-operations between HSF1 and oncogenes and/or tumour suppressors (Dai et al., 2007; Khaleque et al., 2005; Meng et al., 2010), including Ras and p53.

1.10.1. Stimulation of Ras signalling pathways in breast cancer

Although mutations in Ras genes are infrequent in breast cancer, activated Ras contributes significantly to the tumourigenic and invasive potential of breast cancer cells (Lundy et al., 1986; Stamatakos et al., 2010). Permanent activation of Ras and its downstream signalling pathways are in fact commonly observed in breast tumours (Eckert et al., 2004; Guerra et al., 2003). Ras can be activated in these cells through the activities of the ErbB receptor family, whose members are commonly overexpressed in breast cancer (Sircoulomb et al., 2010). In particular, overexpression of ErbB2/HER2 is detected in 20-30% of breast tumours (Sircoulomb et al., 2010), which leads to the activation of receptor tyrosine phosphorylation, the recruitment of Grb2/Sos complex and Ras activation (Janes et al., 1994; von Lintig et al., 2000). Increased expression of other tyrosine kinases in breast cancer such as the insulin receptor (IR), IGF-R, and c-Src, also lead to the activation of the Ras signaling pathway (Biscardi et al., 2000; Hynes, 2000; Zhang and Yee, 2000). In addition, studies have shown that approximately 50% of breast cancers contain greater than a two-fold increase in Grb2

mRNA level compared to that of normal breast epithelial cells, leading to the amplification of Ras signaling pathways in these breast cancer cells (Daly et al., 1994; Kairouz and Daly, 2000). Moreover, amplifications of Ras expression and the Ras-like GTPase TC21 in breast cancers have also been reported (Barker and Crompton, 1998; Janes et al., 1994; Rokavec et al., 2008). Proteins involved in Ras signalling pathways such as Erk, Akt, PI3K have also been shown to be elevated and activated in breast cancer specimens compared to benign breast lesions, leading to an increase Ras downstream signalling in these cells (Li and Sparano, 2003; Rochlitz et al., 1989; von Lintig et al., 2000).

1.10.2. Roles of HSF1 in oncogenic Ras activity

Previous studies have reported that HSF1 modulates Ras signalling pathways in cancer. Dai et al. (2007) demonstrated that mouse embryonic fibroblasts (MEFs) isolated from HSF1 knockout mice (*hsf1-/-*) cells developed fewer numbers of foci in adhesion-independent growth assays when incubated with retroviruses expressing the activated H-Ras^{V12D} oncogene, when compared to wild-type MEFs. In addition, *hsf1* knockout mice develop fewer tumours induced by activated H-Ras^{V12D} compared to their wild-type counterparts in a skin carcinogenesis model. It has also been demonstrated that the Ras downstream signalling pathway is blunted in *hsf1-/-* MEF cells following serum stimulation (Dai et al., 2007). In agreement with this, a previous study by O'Callaghan-Sunol et al. (2006) demonstrated that *hsf1-/-* MEFs are defective in their MAPK signalling pathways, leading to a significant reduction in EGF stimulated migration compared to wild-type cells (O'Callaghan-Sunol and Sherman, 2006). These findings indicate roles of HSF1 in initiating signalling pathways downstream of Ras and in the maintenance of malignant phenotypes induced by Ras activation.

Conversely, HSF1 has been shown to be a downstream target, either positively or negatively regulated, by a number of signalling pathways downstream of Ras. For example, activation of the MAPK pathway leads to the activation of MAPKAP kinase 2 (MK2), which phosphorylates HSF1 at serine 121 and inactivates HSF1 (Wang et al., 2006b). A member of the MAPK family, ERK1, has been shown to cause activation of ribosomal s6 kinase 2 (RSK2), which also represses HSF1 (Wang et al., 2000). In contrast, HSF1 is activated by the PI3K/Akt signalling pathway due to the ability of Akt to phosphorylate and inhibit GSK3β, which is a repressor of HSF1 activity (He et al.,

1998; Xavier et al., 2000). Similarly, HSF1 can be activated via the phosphorylation activity of PKA, which is a downstream effector of Ras (Murshid et al., 2010). In addition, PKA also phosphorylates and inactivates GSK3β, leading to further HSF1 activation (Fang et al., 2000; Tsujio et al., 2000). Altogether, these findings suggest that HSF1 does not only function as a regulator of many Ras downstream signalling pathways but is also a downstream effector utilised by these pathways to exert their biological effects. This leads to a hypothesis that HSF1 activity co-operates with Ras signalling pathways to regulate the promotion of breast tumourigenesis and progression.

1.10.3. p53 in breast cancer

In breast cancer, the overall frequency of TP53 gene mutation is approximately 20% to 40%. Although this frequency is lower than that of other solid tumours, TP53 mutation is a strong predictor of breast cancer aggressiveness (Coutant et al., 2011). Breast tumours expressing high levels of p53 are more likely to be ER negative and PR negative (Guerra et al., 2003). A high level of p53 is frequently observed in HER2 overexpressing breast cancers and the co-existence of high HER2 with mutation of the TP53 gene is associated with poorer prognosis (Yamashita et al., 2004). In addition, among TNBC, TP53 is the most frequently mutated gene, with up to 44% of the tumours expressing a mutant p53 protein (Chae et al., 2009; Jiang et al., 2011; Nakagawa et al., 2011). High levels of p53, which are indicative of TP53 missense mutations, is associated with a high proliferation rate, high histological and nuclear grade, aneuploidy, poor prognosis and chemo-resistance (Borresen-Dale, 2003; Langerod et al., 2007; Rahko et al., 2003). As a result, many breast cancer cells are found to rely on mutant p53 activity for survival and proliferation (Lim et al., 2009). Mutant p53 therefore may represent a more effective therapeutic target for treatment of high-grade breast cancers which are resistant to most current therapies.

1.10.4. Role of HSF1 in p53 activity

Accumulating evidence demonstrating an association of HSF1 and the activity of p53 is emerging. Studies have shown that HSF1 enhances wild-type p53 degradation with cells that are deficient in HSF1 expressing higher levels of wild-type p53 protein, due to the role of HSF1 regulating the expression of genes involved in the ubiquitin-proteasome degradation pathway (Jin et al., 2009; Lecomte et al., 2010). Small heat shock proteins regulated by HSF1, HSP27 and αB-Crystallin, have also been shown to be responsible

for associating and targeting proteins for ubiquitin-dependent degradation. Wild-type p53 interacts with αB-crystallin and this interaction subsequently targets the tumour suppressor protein for degradation mediated by the ubiquitin ligase Fbx4 (Jin et al., 2009). In addition, HSF1 and HSF2 complex regulates the expression of proteasome subunits such as Psmb5 and Gankyrin (Lecomte et al., 2010).

By showing that HSF1 is required for p53 degradation, studies by Jin et al. (2009) also demonstrated that the accumulation of p53 in HSF1 deficient cells leads to an enhanced cell sensitivity to DNA damaging agents such as etoposide and doxorubicin (Jin et al., 2009). However, in contrast to this, other studies have reported that HSF1 knockdown leads to a reduction of p53 transcriptional targets and interferes with p53-mediated growth arrest and apoptosis (Li et al., 2008; Li and Martinez, 2011; Logan et al., 2009). These studies mechanistically showed that HSF1 could modulate the activity of wild-type p53, firstly, by directly mediating the activation of p53 (Logan et al., 2009) and secondly, by regulating p53 nuclear translocation (Li et al., 2008; Li and Martinez, 2011). The results from these studies suggest an interesting concept that HSF1 activation could lead to the enhancement of wild-type p53 activity, beneficial in cancer treatment. However, the actual biological consequences of HSF1 activation in cancer containing wild-type p53 requires further investigation.

In contrast to the effect observed in cells with wild-type p53, expression of HSF1 is required for the stability of mutant p53 proteins. Li et al. (2010) demonstrated that mutant p53 proteins in human cancer cells are stabilized by HSP90. The HSP90 chaperone complex protects the mutant p53 proteins from ubiquitination and subsequent degradation mediated by Mdm2 and CHIP E3 ligase. Consequently, knockdown of HSP90 by shRNA or inhibition by HSP90 inhibitors liberates mutant p53 proteins from the HSP90-p53 complex, thereby reactivating p53 degradation. As HSF1 regulates HSP90 expression, knockdown of HSF1 consequently leads to a reduction in mutant p53 levels. Since most cancers rely on hyper-stable mutant p53 isoforms for survival and proliferation, the reduction of mutant p53 stability by targeting HSF1 or HSP90 has been shown to significantly reduce tumour growth (Li et al., 2011b).

To date, apart from the study by Dai et al. (2007) showing that HSF1 is required for tumourigenesis induced by the hot-spot mutant $p53^{R172H}$ in mice, there has been no other studies which investigate the association between HSF1 and activity of mutant

p53 proteins, and their links that to cancer cell biological effects and/or patient outcomes. However, with the accumulating evidence suggesting a role of HSF1 in p53 pathway modulation, it is likely that HSF1 may exert its cancer promoting effects via mutant p53 activity. Future investigations into the mechanisms between these molecules are likely to lead to research outcomes that have substantial clinical relevance to the cancer patient.

1.10.5. Objectives

The specific aims of this study are:

1. To investigate and compare the effects of HSF1 activation upon cell biology and gene expression in normal mammary epithelial cells and in oncogenic Ras transformed mammary epithelial cells.

2. To investigate the effects of HSF1 activation on breast cancer cell lines with differing p53 status and its role in the activities of wild-type and mutant p53.

3. To investigate the effects of HSF1 knockdown within differing cellular contexts of breast cancer.

4. To initially develop a cell-based screening model for HSF1 inhibitor identification.

CHAPTER 2

MATERIALS AND METHODS

2.1. MOLECULAR CLONING

2.1.1. Bacterial cultures

Bacteria were cultured in LB broth (1% tryptone, 0.5% yeast extract and 1% NaCl). The LB broth was sterilized by autoclaving at 121^{o}C for 20 minutes. For colony selection, bacteria were cultured on LB agar plate comprising of 20ml of LB agar (LB broth with 1% Bacto Agar) and appropriate antibiotics to a final concentration of 100µg/ml for ampicillin and kanamycin, and 50µg/ml for zeocin. Bacteria cultures were grown at 37^{o}C. Liquid bacteria cultures were grown with agitation in an orbital shaker at 225rpm.

2.1.2. Bacteria transformation

2.1.2.1. Preparation of competent bacteria

Competent bacteria were prepared by the calcium chloride method described previously (Nakata et al., 1997). Briefly, TOP10B *Escherichia coli* cells were grown overnight in 5ml LB broth. Two ml of the cell culture was used to inoculate 100ml of fresh LB broth and the culture was grown at 37^{o}C with agitation (225rpm) until the OD reached 0.4-0.6 (approximately 2-3 hours), followed by incubation on ice for 20 minutes. The cells were then pelleted at 5000rpm for 5 minutes, resuspended in 50ml ice-cold 100mM and further incubated on ice for 30 minutes. After the incubation, the cells were centrifuged again and resuspended in 50ml of ice-cold 100mM $CaCl_2$, followed by incubation on ice for 1 hour. After centrifugation, the cells were resuspended into 5ml sterile ice-cold storage solution (100mM $CaCl_2$ and 15% glycerol). The competent bacteria were stored in 100µl aliquots at -80^{o}C for use up to three months.

2.1.2.2. Plasmid Transformation

To transform plasmids into bacterial cells, up to 100ng of plasmid was added to 100µl of TOP10B Calcium Chloride competent bacterial cells and the mixture was incubated on ice for 10 minutes. The cells were heat shocked for 45 seconds at 42^{o}C and

immediately cooled on ice. Two hundred µl of LB broth was added to dilute the bacteria cells and 100µl of the diluted transformed bacterial solution was plated onto an LB agar plate containing appropriate antibiotics. The plate was inverted and incubated at 37^{o}C overnight until colonies were visible.

2.1.3. Bacteria glycerol stock

Bacteria cultures were inoculated into 5ml of LB broth containing appropriate antibiotics and grown overnight at 37^{o}C with agitation (225rpm). Eight hundred µl of that culture was mixed with 200µl of 75% sterile glycerol in a 2ml cryotube by gentle vortexing and stored at -80^{o}C.

2.1.4. Plasmid extraction

Crude plasmid extraction was performed to isolate plasmids for diagnostic digestion, which identified bacterial colonies that contain the plasmids of interest. Ten colonies of the transformed bacteria were selected and each was cultured overnight in a sterile Falcon tube containing 5ml LB broth at 37^{o}C with agitation (225rpm). For each tube, bacterial cells were pelleted at 5000rpm for 10 minutes, resuspended in 200µl of resuspension buffer P1 (50mM Tris HCl pH 8.0, 10mM ethylenediaminetetraacetic acid (EDTA), 100µg/ml RNaseA) and then transferred into a microfuge tube. To lyse the cells, 250µl of lysis buffer P2 (200nM NaOH, 1% SDS) was added and mixed thoroughly by inverting the tubes 5 times, followed by incubation at room temperature for 5 minutes. The solution was neutralized by the addition of 350µl of precipitation buffer P3 (3M KOAc, pH 5.5) with gentle mixing. To collect the plasmids, the mixture was centrifuged at 13,000rpm for 10 minutes and 700µl of the supernatant was collected into a new microfuge tube containing 490µl isopropanol. The solution was mixed by inverting the tube and then centrifuged at 14,000rpm for 30 minutes to pellet the plasmid DNA. The DNA pellet was then rinsed with 500µl of 70% ethanol, centrifuged and resuspended in 100µl of TE buffer (10mM Tris-Cl, pH 8.0).

For large-scale isolation of plasmids with high purity for transfection, plasmids were extracted using a Plasmid Midi-prep kit (Invitrogen, California, USA) as per the manufacturer's instructions.

2.1.5. Restriction enzyme digestion

Restriction enzyme digestion was used for cloning or for diagnosis purposes. For diagnostic digestions that confirmed the identity of the plasmid, 1µg DNA was used in a total 10µl reaction. For cloning, 10µg DNA was used in a total 50µl reaction. Each reaction mixture contained DNA, appropriate buffer, bovine serum albumin (BSA), restriction enzymes and water to make up to the total volume. Double digestion was performed in a common buffer in which each restriction enzyme had at least 80% efficiency. The digestion mix was incubated at 37^{o}C for 1 hour. The DNA samples were subjected to agarose electrophoresis as described in 2.1.7.

2.1.6. Ligation reaction

Ligation reactions were performed in a 10µl reaction containing the plasmids and inserts at a 1:3 ratio, ligation buffer and T4 DNA ligase (New England Biolabs, Massachusetts, USA). The reaction mixture was incubated at room temperature for 30 minutes then 4µl was used to transform into bacterial cells for plasmid selection.

2.1.7. Agarose gel electrophoresis

Agarose gel was prepared in 1X TAE buffer (40 mM tris-acetate, 1 mM EDTA, pH 8.3) at 1% or 2% for DNA larger or smaller than 500 base pairs, respectively. Sybr Safe DNA gel stain (Invitrogen, California, USA) was added for DNA visualisation. DNA samples were mixed with 6x loading buffer (0.125% w/v xylen cyanol, 0.125% w/v bromophenol phenol blue, 15% glycerol) before loading onto the gel. One plus DNA ladder (Invitrogen, California, USA) was loaded for size estimation of the DNA samples. The gel was electrophoresed at 110V for 40-50 minutes and visualized using UV transillumination.

2.1.8. DNA sequencing

Sequencing was performed to confirm the correct gene sequences of the plasmids of interest. Each sequencing PCR mixture contained 300ng DNA, 1µl Big Dye Terminator (BDT), 2µl 10X PCR buffer, 4pmoles sequencing primer and water was added to adjust the final volume to 20µl. The reaction was carried out with an initial denaturation at 95^{o}C (2 minutes), followed by 30 cycles of denaturation (95^{o}C, 15 seconds), annealing and extension (60^{o}C, 4 minutes) and a final extension phase (72^{o}C, 7 minutes). When the reaction was complete, 5µl 125mM EDTA and 60µl 100% ethanol were added to

the PCR product. The solution was mixed well by gently flicking of the tube and then centrifuged at 14,000rpm for 30 minutes to isolate the DNA. The DNA pellet was rinsed with 200µl 70% ethanol and air dried. The dried DNA pellet was sent to Micromon (Monash University, Victoria, Australia) for sequencing electrophoresis.

2.1.9. Generation of expression constructs

Sequences of all plasmids and genes were analysed using Vector NTI software (Invitrogen, California, USA). The software was also used to devise cloning strategies and design cloning primers.

2.1.9.1. Generation of retroviral vector expressing HSF1wt and HSF1ΔRDT

HSF1wt cDNA was amplified from MCF10A cDNA by PCR using HSF1_Fwd and EcoR1_HSF1_Rev primers. The reaction was carried out by denaturation at 95°C (2 minutes), 30 cycles of denaturation (95°C, 15 seconds), annealing (60°C, 30 seconds) and extension (72°C, 2 minutes) and a final extension phase (72°C, 7 minutes). PCR products were electrophoresed in agarose gel to confirm the size (1.6k base pairs) and then purified using Qiagen DNA gel extraction kit, followed by digestion with EcoRI. The digested cDNA was ligated with the linearized pBABEpuro IRES EGFP (L. Miguel

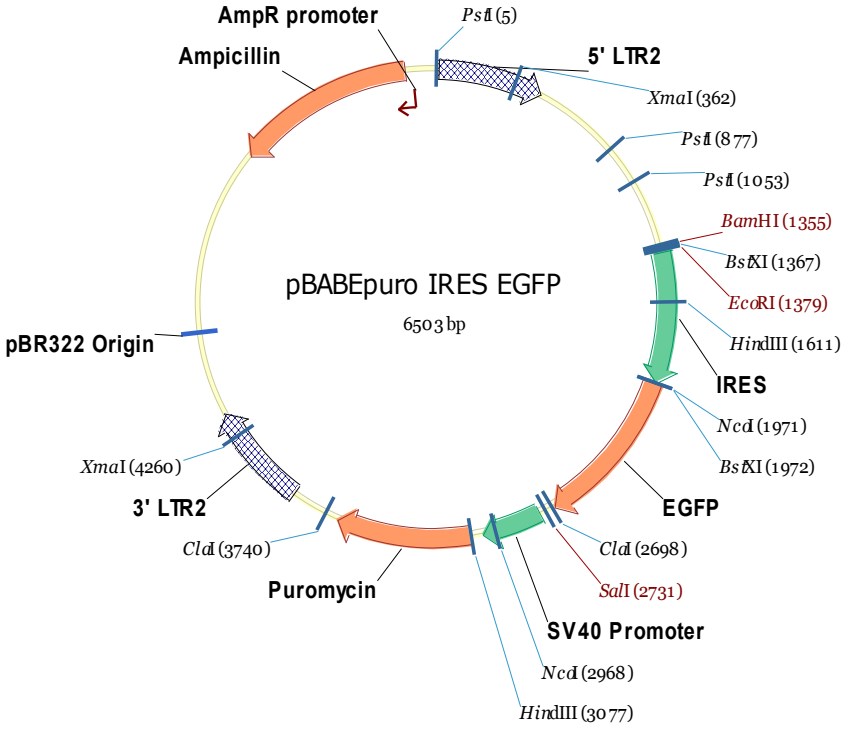


Figure 2.1. Schematic map of pBABEpuro IRES EGFP retroviral construct

Martins; Adgene, Massachusetts, USA, Fig.2.1), which was digested with BamHI, refilled by T4 DNA polymerase to generate blunt ends and then digested again with EcoRI enzyme. The resultant vector was called pBABE HSF1wt IRES EGF (Fig.2.2).

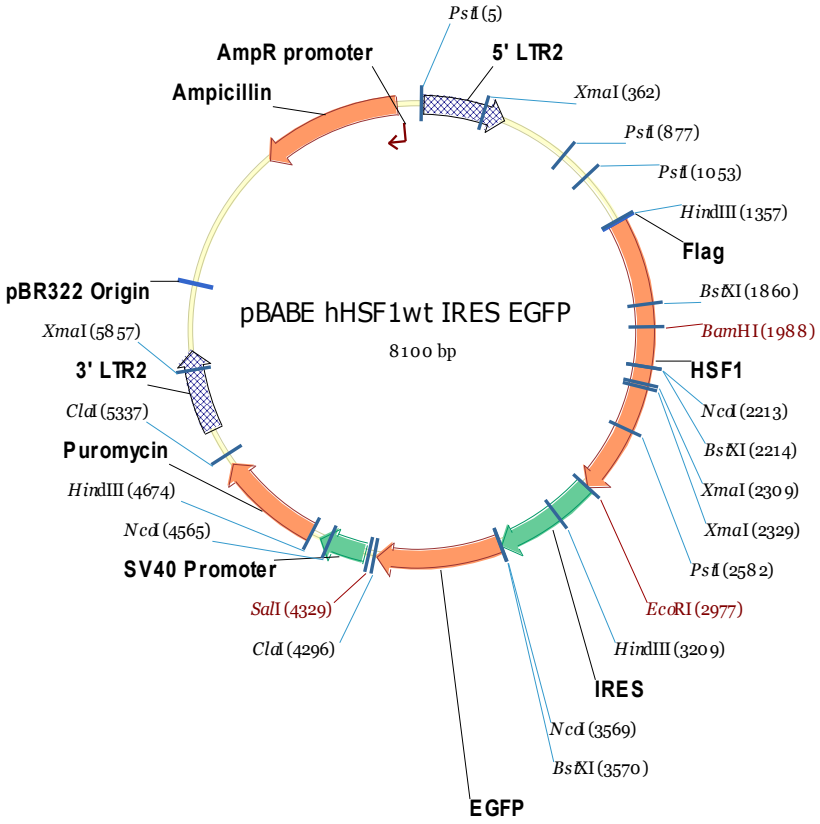


Figure 2.2. Schematic map of pBABE hHSF1wt IRES EGFP retroviral construct

The HSF1ΔRDT was generated by deletion of the regulatory domain and substitution of leucine 395 with glutamic acid (L395E) thereby facilitating active HSF1 trimer formation. HSF1ΔRDT cDNA was synthesized from HSF1wt cDNA using PCR site-directed mutagenesis method described previously (Fujimoto et al., 2005; Hutchison et al., 1978). Briefly, two DNA fragments were synthesized from HSF1wt cDNA: one using the Flag_HSF1_Fwd and HSF1ΔRD_Rev primers, the other fragment was generated using the HSF1ΔRD_Fwd and EcoRI_HSF1_Rev primers. The two fragments were mixed and used as a template to generate full-length HSF1ΔRD via PCR using the Flag-HSF1_Fwd and EcoRI HSF1_Rev primers. To introduce an additional mutation in the HSF1ΔRD and generate HSF1ΔRDT, full length HSF1ΔRD

cDNA was then used as a template for PCR site-directed mutagenesis using a HSF1_T primer pair containing Leucine 394 to Glutamic acid (394L→E) mutation. The generated HSF1ΔRDT cDNA was then inserted into the BamHI-EcoRI site of pBABEpuro IRES EGFP by similar method used to insert HSF1wt cDNA. The resulted vector was called pBABE HSF1ΔRDT IRES EGFP (Fig.2.3).

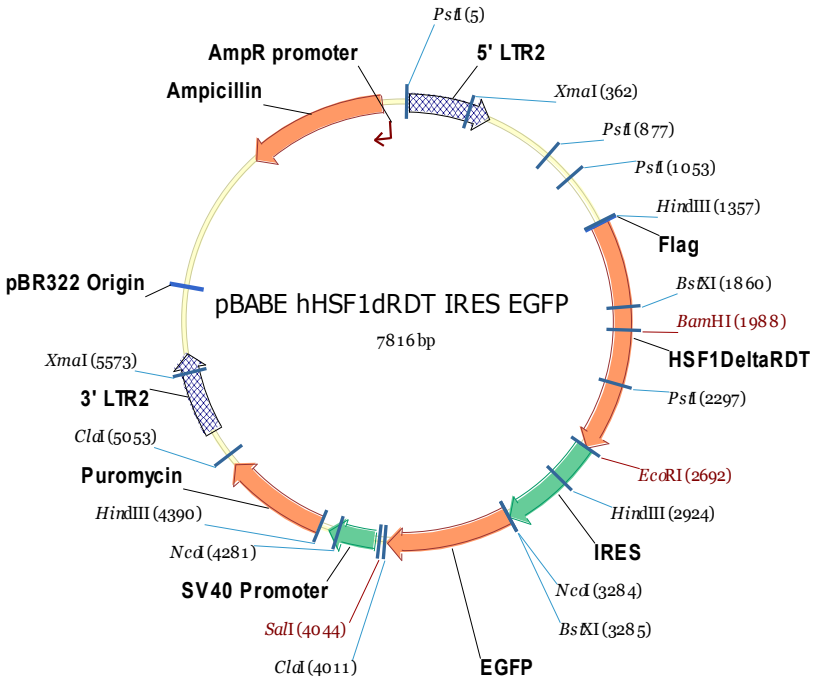


Figure 2.3. Schematic map of pBABE hHSF1ΔRDT IRES EGFP retroviral construct

2.1.9.2. Generation of pBABEpuro IRES mCherry vector

The pBABEpuro_IRES_ mCherry was generated to use as a retroviral expression vector expressing genes linked to mCherry expression. mCherry cDNA was amplified from pRSET-B mCherry vector and was kindly provided by Roger Tsien (UC San Diego, CA, USA) using BstXI_mCherry_Fwd and SalI_mCherry_Rev primers, followed by digestion with BstXI and SalI. pBABEpuro-IRES-EGFP vector was digested with EcoRI and SalI into two fragments. The fragment that contained IRES-EGFP was further digested with BstXI and the IRES fragment with EcoRI and BstXI overhangs was collected. A ligation reaction was performed to ligate the three fragments: pBABEpuro with EcoRI and SalI overhangs, IRES with EcoRI and BstXI overhangs and mCherry with BstXI and SalI overhangs. The pBABEpuro_IRES_mCherry vector was confirmed by diagnostic digestions (Fig.2.4).

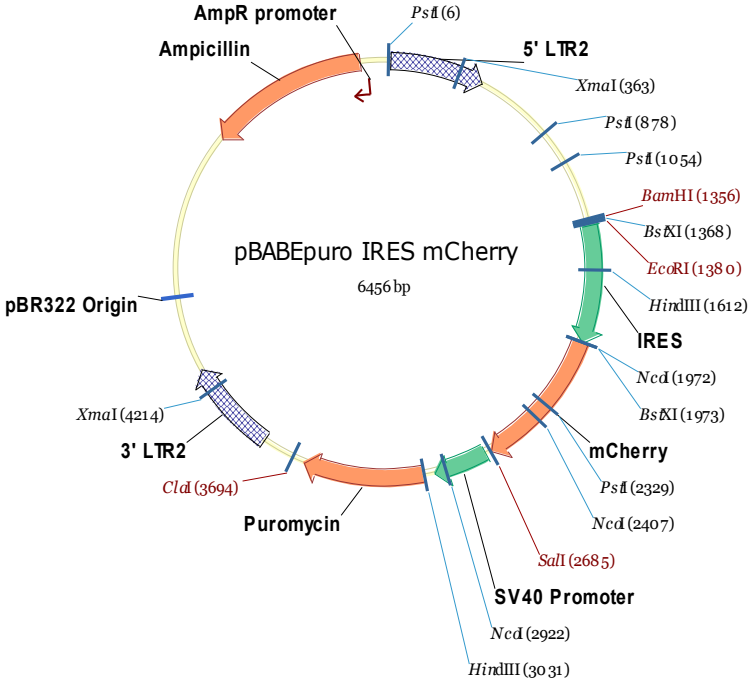


Figure 2.4. Schematic map of pBABEpuro IRES mCherry retroviral construct

2.1.9.3. Generation of retroviral vector expressing mutant p53^{R273H}

Mutant p53^{R273H} gene was excised from the vector pSUPER- p53^{R273H} kindly provided by Prof Ygal Haupt (Peter MacCallum Cancer Center, Victoria, Australia) by digestion

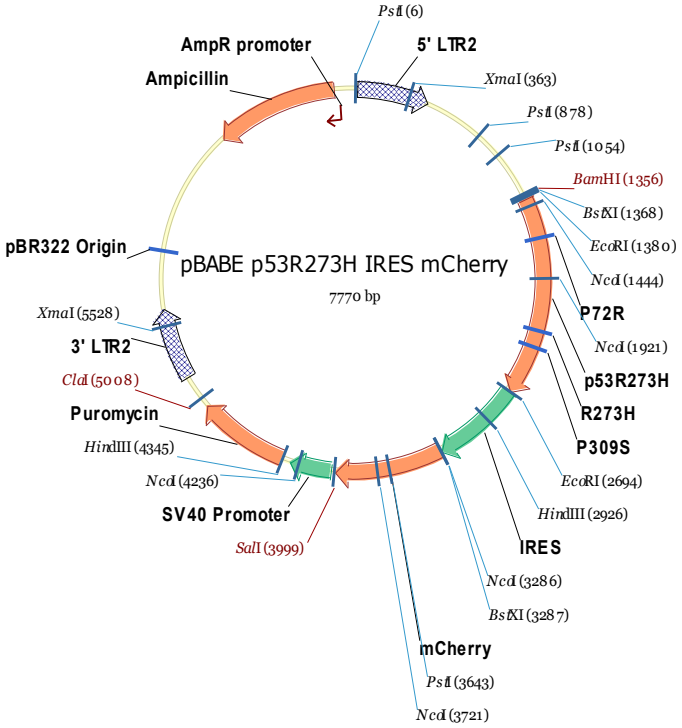


Figure 2.5. Schematic map of pBABE p53^{R273H} IRES mCherry retroviral construct

with EcoRI and inserted into the EcoRI site of pBABEpuro IRES mCherry vector. The ligated vector was called pBABE $p53^{R273H}$ IRES mCherry and was sequenced to confirm correct orientation and the nucleotide sequences of the mutant $p53^{R273H}$ gene (Fig.2.5).

2.1.9.4. Generation of vector expressing mCherry under HSE promoter

HSP70B promoter containing HSE was excised from HSE-Luc plasmid by digestion with BglII and HindIII restriction enzymes. mCherry cDNA was amplified from pRSET-B mCherry vector by PCR with HindIII mCherry Fwd and SalI mCherry Rev primers, followed by digestion with HindIII and SalI enzymes. pcDNA3.1(+) was used as the backbone vector. The CMV promoter was removed from this vector by digestion with BglII and XhoI restriction enzymes (XhoI has compatible end to SalI end. Three fragments: HSE promoter, mCherry and the vector backbone were ligated and the resultant vector was transformed into TOP10B competent *Escherichia coli*. The final vector was confirmed by diagnostic digestion and called pHSE-mCherry (Fig.2.6).

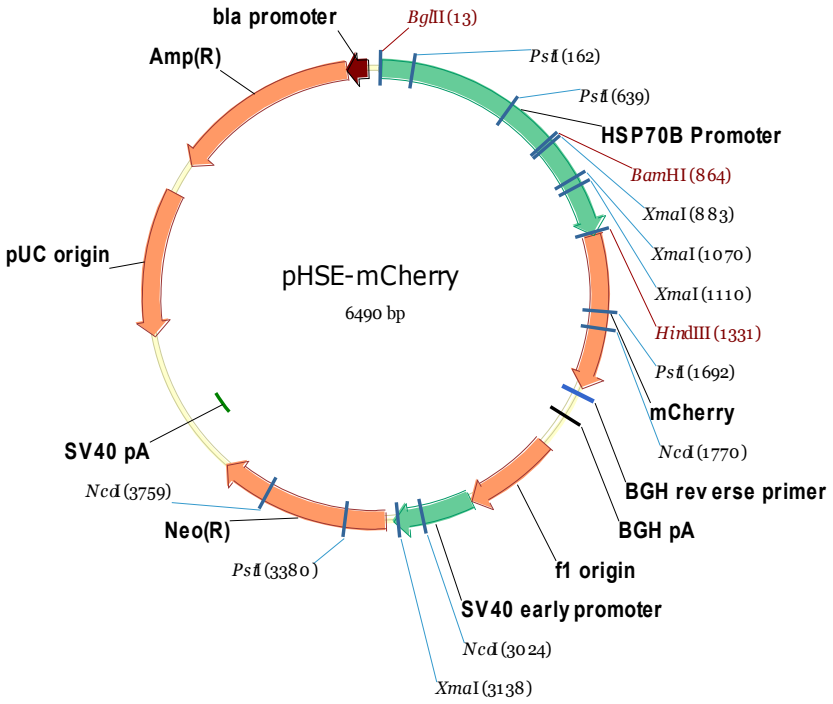


Figure 2.6. Schematic map of pHSE-mCherry construct

2.1.9.6. Generation of retroviral vector expressing shRNAmir targeting HSF1

HSF1 targeted shRNAmir vectors were constructed as described previously (Paddison et al., 2004). Briefly, 21-mer siRNA sequences targeting HSF1 were designed using Biopredsi siRNA design tool (http://www.biopredsi.org/start.html). Five siRNA sequences with the highest Biopred scores were selected and run through Cold Spring Harbor Laboratory website to generate five 97-mer shRNAmir oligos (http://katahdin.cshl.org:9331/siRNA/RNAi.cgi?type=shRNA. Table 2.10). The oligos were synthesized using a commercial oligo synthesis service (Sigma Aldrich, Missouri, USA) and amplified with miR30 Fwd and mirR30 Rev primers containing XhoI and EcoRI sites to clone into the MSCV-LMP vector. Two clones of each MSCV-LMP HSF1 shRNAmir vector were isolated and sequenced. Three constructs with the most effective knockdown efficiency were selected for further experiments (shRNAmir2, shRNAmir3 and shRNAmir4).

Table 2.1. *HSF1 shRNAmir sequences*

Construct	Hairpin shRNAmir Sequence (sense , loop, antisense)	Target site on HSF1 mRNA
pMSCV-LMP HSF1 shRNAmir1	TGCTGTTGACAGTGAGCGCCAGCGTAGCCTGCCTGGAC AATAGTGAAGCCACAGATGTATTGTCCAGGCAGGCTAC GCTGATGCCTACTGCCTCGGA	1292-1312
pMSCV-LMP HSF1 shRNAmir2	TGCTGTTGACAGTGAGCGACACATTCCATGCCCAAGTA TATAGTGAAGCCACAGATGTATATACTTGGGCATGGAA TGTGCTGCCTACTGCCTCGGA	826-846
pMSCV-LMP HSF1 shRNAmir3	TGCTGTTGACAGTGAGCGCGCCCAAGTACTTCAAGCAC AATAGTGAAGCCACAGATGTATTGTGCTTGAAGTACTT GGGCATGCCTACTGCCTCGGA	341-361
pMSCV-LMP HSF1 shRNAmir4	TGCTGTTGACAGTGAGCGACAGGTTGTTCATAGTCAGA ATTAGTGAAGCCACAGATGTAATTCTGACTATGAACAA CCTGCTGCCTACTGCCTCGGA	2010-2030 (3'UTR)
pMSCV-LMP HSF1 shRNAmir5	TGCTGTTGACAGTGAGCGAAGGCAGAGATCTATAAACA GATAGTGAAGCCACAGATGTATCTGTTTATAGATCTCT GCCTGTGCCTACTGCCTCGGA	2118-2138 (5'UTR)

2.2. CELL CULTURE

2.2.1. Routine culturing of cell lines

MCF10A cell line was obtained from the American Type Culture Collection (ATCC) and was routinely cultured as described previously (Debnath et al., 2003). The cell line was maintained in monolayer culture in Dulbecco's modified Eagle Medium / Ham's nutrient mixture F12 (DMEM/F12, Gibco Invitrogen, California, USA) supplemented with 5% horse serum (Invitrogen, California, USA), 10µg/ml bovine pancreas insulin (Sigma Aldrich, Missouri, USA), 10ng/ml EGF (BD Biosciences, California, USA), 10ng/ml cholera toxin (Sigma Aldrich, Missouri, USA), 5µg/ml hydrocortisone (Sigma Aldrich, Missouri, USA) and 1% antibiotic/antimycotic (Invitrogen, California, USA). MCF10A cells were grown in T75 flasks and passaged every 3-4 days once confluent. For passaging, growth media was removed and the cell monolayer was washed once with 10ml PBS. Two ml of trypsin was added to cover the cells and aspirated immediately to leave only a thin layer of trypsin. The cells were then incubated at 37^{o}C for 15 minutes to detach and then resuspended in 5ml of resuspension media (DMEM/F12 supplemented with 20% horse serum and antibiotic/antimycotic). Cells were then pelleted at 150g for 5 minutes and resuspended in growth media. Approximately one million cells were seeded into a new T75 flask containing 10ml fresh growth media.

T47D cells and SkBr3 cells were cultured in RMPI and McCoy's 5A media respectively. Hs578T, HEK293 and HEK293T cells were cultured in DMEM. All media were supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic. Once confluent, cells were washed with 10ml phosphate bufferred saline (PBS), lifted in 1 ml trypsin and resuspended in their growth media. A proportion of the cells were used to seed fresh flasks. All cells were grown in a 5% CO_2 humidified incubator at 37^{o}C.

2.2.2. Cryopreservation of cell lines

All cell lines in this study were stored in liquid nitrogen at low passage. Cells were cultured in T75 flasks to 70-80% confluence and then lifted as described in 2.3.1, followed by centrifugation to pellet at 150g (or 1500rpm) for 5 minutes. Cell pellets were resuspended in 4ml of ice cold storage media. MCF10A cells were stored in

media containing 50% growth media, 40% horse serum and 10% dimethyl sulfoxide (DMSO). All other cell lines were stored in media containing 90% FBS and 10% DMSO. Cells were transferred as 1ml aliquots into cryotubes and incubated on ice for 5-10 minutes. The tubes were kept in a Cryo 1^{o} freezing container at -80^{o}C overnight before being transferred into liquid nitrogen for long-term storage.

2.2.3. Generation of stable cell lines

2.2.3.1. Virus production

All stable cell lines in this study were generated by retroviral or lentiviral transduction as previously described (Debnath et al., 2003) using HEK293T as a packaging cell line. Briefly, HEK293T cells ($3x10^7$) were seeded into 10-cm cell culture dishes one day before transfection. On the day of transfection, culture media was replaced with fresh media without antibiotic/antimycotic. To produce retroviruses, cells were transfected with 7µg Ampho vector (packaging plasmid) and 7µg appropriate retroviral vector expressing the gene of interest per one 10cm cell culture dish. To produce lentiviruses, 5µg psPAX.2, 2.5µg pMD2.g and 7µg appropriate lentiviral vector expressing the gene of interest were used. Plasmids were delivered into the cells using Lipofectamine LTX with PLUS transfection reagents (Invitrogen, Caliornia, USA) according to the manufacturer's instructions. The transfection media was removed after ~16 hours and replaced by 6ml of harvesting media (growth media of the virus recipient cells). Culture supernatants containing virus particles were collected 48 hours after transfection and filtered through 0.4µm size pore filters. Viruses were immediately used to infect recipient cells or stored at -80^{o}C.

2.3.3.2. Generation of stable cell lines expressing HSF1

Three retroviral vectors were used to produce retroviruses including pBABEpuro IRES EGFP, pBABE HSF1wt IRES EGFP and pBABE HSF1ΔRDT IRES EGFP as in 2.3.3.1. The virus recipient cells ($1.5x10^6$) were seeded into T25 flasks the day before infection. On the day of infection, the culture media was replaced with 2ml of retrovirus media combined with 2ml of fresh media. Polybrene (hexadimethrine bromide) was added to a final concentration of 10µg/ml. Cells were incubated with the viruses overnight, followed by 24-hour recovery in fresh media. After two rounds of infection, cells were selected by Fluorescence Activated Cell Sorting (FACS) using an Influx cell

sorter (BD Biosciences, California, USA) to isolate cells expressing EGFP, indicative of successfully transduced cells. Selected cells were grown and sorted again to ensure that all cells contained the viral construct. Approximately 30% of cells were expressing GFP after the infection (Multiple of infection (MOI) of 0.3) and at least $2.5x10^6$ cells were selected after each sort. The ectopic expression of HSF1 was later confirmed by western blot analysis.

2.2.3.2. Generation of stable cells expressing H-Ras^{V12}

MCF10A cells expressing GFP control, HSF1wt or HSF1ΔRDT were transduced with mCherry control or H-Ras^{V12} retroviruses produced from MSCV-mCherry and MSCV H-Ras^{V12} mCherry retroviral vectors kindly provided by Dr. Patrick Humbert (Peter McCallum Cancer Center, Victoria, Australia) (Dow et al., 2008). The virus infection was also done twice in the presence of 10µg/ml polybrene. The cells were sorted twice by FACS to select for the cells expressing both EGFP and mCherry. Western blot analysis was performed to confirm the ectopic expression of H-Ras^{V12}.

2.2.3.3. Generation of stable MCF10A expressing p53^{R273H}

MCF10A cells expressing GFP control, HSF1wt or HSF1ΔRDT were transduced with retroviruses expressing mCherry control or p53^{R273H} produced from pBABEpuro IRES mCherry and pBABE p53^{R273H} IRES mCherry, respectively. Stable cells were sorted by FACS and analysed by western blot analysis.

2.2.3.4. Generation of stable HSF1 knockdown cell lines

Five retroviral MSCV-LMP vectors generated as in 2.1.9.5 were used to produce retroviruses expressing HSF1 shRNAmir. MCF10A cells were infected once with the viruses in the presence of 10µg/ml polybrene. Western blot analysis was performed to determine the knock down efficiency of each HSF1 shRNAmir. The two most efficient HSF1 shRNAmirs were selected for HSF1 knockdown experiments. Cells infected with viruses expressing HSF1 shRNAmir constructs were sorted twice by FACS to select for cells with highest GFP expression.

2.2.3.4 Generation of stable p53 knockdown cell lines

Stable p53 knocked-down cell lines were generated by lentiviral transduction. Lentiviuses expressing p53 shRNAimir were produced as in 2.2.3.1 using the p53 shRNAi pGIPZ lentiviral construct set purchased from Open Biosystems, California, USA (Cat. No. RHS4531). The set contains 6 pGIPZ lentiviral constructs which were labelled from 1 to 6 (Table 2.11). The virus titre was determined by examining the percentage of GFP expressing cells by flow cytometry when various concentrations of viruses expressing p53 shRNAmir(1) were infected into MCF10A cells (Table 2.2). Concentrations that gave a final 10% to 20% of cells expressing GFP were used to determine virus titre. Virus titre (transfection unit (TU)/ml) was calculated by:

$$Virus\ titer\ (TU/ml) = \frac{\%\ of\ GFP\ cells \times Total\ cells\ infected}{Volume\ of\ viruses}$$

Since p53 shRNAmir were expressed in cells already expressing EGFP as the fluorescence marker for HSF1, cells expressing p53shRNAmir could not be sorted by FACS. The cells were therefore infected at MOI of 2 and immediately used for experiments without any selection.

Table 2.2. *p53 shRNAmir sequences*

Construct	**Hairpin shRNAmir Sequence (sense , loop, antisense)**	**Target site on p53 mRNA (transcript variant 1)**
pGIPZ p53 shRNAmir1	TGCTGTTGACAGTGAGCGCCGAGATGTTCCGAGAGCTG AATAGTGAAGCCACAGATGTATTCAGCTCTCGGAACAT CTCGATGCCTACTGCCTCGGA	1211-1231
pGIPZ p53 shRNAmir2	TGCTGTTGACAGTGAGCGCCCACTACAACTACATGTGT AATAGTGAAGCCACAGATGTATTACACATGTAGTTGTA GTGGATGCCTACTGCCTCGGA	893-913
pGIPZ p53 shRNAmir3	TGCTGTTGACAGTGAGCGCCCGCGCCATGGCCATCTAC AATAGTGAAGCCACAGATGTATTGTAGATGGCCATGGC GCGGATGCCTACTGCCTCGGA	668-688
pGIPZ p53 shRNAmir4	TGCTGTTGACAGTGAGCGCGGAGGATTTCATCTCTTGT ATTAGTGAAGCCACAGATGTAATACAAGAGATGAAAT CCTCCATGCCTACTGCCTCGGA	2057-2077 (3'UTR)
pGIPZ p53 shRNAmir5	TGCTGTTGACAGTGAGCGCCCGGCGCACAGAGGAAGAG AATAGTGAAGCCACAGATGTATTCTCTTCCTCTGTGCG CCGGTTGCCTACTGCCTCGGA	1041-1059
pGIPZ p53 shRNAmir6	TGCTGTTGACAGTGAGCGCAAGAAATGTTCTTGCAGTT AATAGTGAAGCCACAGATGTATTAACTGCAAGAACATT TCTTATGCCTACTGCCTCGGA	1639-1659 (3'UTR)

Table 2.3. *p53 shRNAmir1 Virus Titre*

Volume of virus (µl)	Percentage of GFP expressing cells	Virus titre (TU/ml)
50	5%	$1x10^6$
100	20%	**$2x10^6$**
200	40%	**$2x10^6$**
500	55%	$1.1x10^6$
1000	70%	$0.7x10^6$

2.3.4. Three dimensional (3-D) culture of cells on reconstituted basement membrane

MCF10A cells were grown in 3-D Matrigel culture by an overlay method as described previously (Debnath et al., 2003). Growth factor reduced matrigel was obtained from BD Biosciences, thawed on ice in 4^{o}C room overnight and stored at -20^{o}C in 500µl aliquots. Forty µl of ice-cold Matrigel was added to each well of an eight-well chamber glass slide (Millipore, Massachusetts, USA) and spread evenly. The Matrigel was allowed to solidify by incubating at 37^{o}C for 20 minutes. MCF10A cells were grown to 50-70% in growth media, lifted as in 2.3.1 and then resuspended in assay media (DMEM/F12 supplemented with 2% horse serum, 10µg/ml insulin from bovine pancreas, 5µl/ml hydrocortisone and 1% antibiotic/antimycotic) at 25,000 cells/ml. The cell suspension was mixed with stock assay media containing 10ng/ml EGF and 4% Matrigel at a 1:1 ratio to obtain cells in final assay media containing 5ng/ml EGF and 2% Matrigel. The mixture (400µl/5,000 cells) was added on top of the solidified Matrigel layer in each well of the chamber slide. Cells were allowed to grow in a 5% CO_2 humidified incubator at 37^{o}C for 10 to 12 days. Assay media containing 5ng/ml EGF and 2% Matrigel was replenished every 4 days.

2.3. *IN VITRO* ASSAYS

2.3.1. Observation of Cell Morphology

Cells were grown to 70-80% confluency in growth media and the morphology was viewed under bright field and/or fluorescence on the Olympus IX71 microscope (Olympus, Tokyo, Japan). The images were captured with a SPOT camera (CCD Direct, Holland, Florida) utilising the SPOT-advanced software.

2.3.2. Proliferation assay

Cell proliferation was examined in 96-well plates using the Sulforhodamine B (SRB) colorimetric assay as previously described (Skehan et al., 1990). Cells were seeded at $2x10^4$ – $5x10^4$ cells/well in 100µl culture medium in triplicate, grown and fixed every day for 5 days in 50% trichloroacetic acid (TCA) at 4^oC for 1 hour, followed by five washes in distilled water. The plates were air-dried at room temperature overnight and then stained by adding 100µl of 1% acetic acid, 0.4% (w/v) SRB (Sigma Aldrich, Missouri, USA) solution to the each well and incubated at room temperature for 10 minutes. The amount of SRB bound to the well is proportionate to the number of cells in each well. The plates were then washed with 1% acetic acid and air-dried. To dissolve the SRB, 150µl of 10mM Tris-HCl, pH 10.5 was added. Absorbance at 550nm was measured by spectrophotometry using a Multiskan FC Absorbance Plate Reader (Thermo-LabSystems, Massachusetts, USA).

2.3.3. Anchorage-dependent clonogenic survival and growth assay

The anchorage-dependent clonogenic survival and growth assay was performed as described previously by Kattan and co-workers to assess the ability of single cells to form colonies on a solid surface (Kattan et al., 2008). Briefly, cells were seeded at low density in 6-well cell culture plates and grown in standard conditions until defined colonies were evident. MCF10A cells were plated at 100 cells/well and grown for 8 days. T47D cells were plated at $5x10^2$ cells/well and grown for 3 weeks while SkBr3 cells were plated at $2x10^3$ cells/well and grown for 4 weeks. Growth media was replenished every week. Colonies were fixed with 100% methanol for 2 minutes and stained with Diff-Quick dyes (Fronine Lab Supplies, New South Wales, Australia). Plates were washed with distilled water and air-dried at room temperature overnight. The wells with cell colonies were imaged using a Nikon scanner and total number of colonies were counted using ImageJ software (public domain NIH Image program developed at the U.S. National Institutes of Health, USA).

2.3.4. Soft-agar anchorage-independent clonogenic survival and growth assay

The anchorage-independent clonogenic survival and growth assay was assessed by examining the ability of cells to form colonies in soft agar. Cells were grown to 50-70% confluency then lifted and resuspended in growth media. The cells were counted and

added into liquid agar media kept at 40°C (0.8% agar for MCF10A, 0.7% for T47D and SkBr3). The agar cell mixture (1.5ml) was plated in triplicate on top of a pre-hardened bottom agar layer comprising of 2 ml agar media (2% agar for MCF10A, 1% for T47D and SkBr3) in 6-well plates. MCF10A cells were plated at $5x10^3$cells/well; T47D cells were plated at $1x10^4$ cells/well and SkBr3 cells were plated at $3x10^4$ cells/well. The agar was allowed to set at room temperature for 30 minutes. One ml of growth media was then added on top of the solidified agar layers. Cells were grown for 3-4 weeks under standard conditions with growth media on top of the two agar layers being replenished every 4 days. Colonies were stained with 1ml of 0.005% crystal violet stain (Sigma Aldrich, Missouri, USA) for 15 minutes at room temperature with gentle shaking, followed by soaking in water overnight. Plates were imaged with a Nikon scanner and colonies were counted using ImageJ software.

2.3.5. Microchemotaxis Migration Assay

Cell migration was examined using 48-well microchemotaxis chamber assay (Neuro Probe, Maryland, USA) as described previously by Kouspou and Price (Kouspou and Price, 2011). Briefly, cells were lifted by trypsinization, resuspended and incubated in growth media for 45-60 minutes to recover. Cells were then washed 3 times in media containing 0.1% BSA and resuspended in that media at $5x10^6$-$2x10^7$cells/ml. Cells (56µl) were loaded in triplicates into wells of the upper chamber which separated to the wells containing chemoattractants (29µl) of the lower chamber by an 8 or 12 µm pore polyvinylpyrrolidone (PVP)-free polycarconate membrane coated with collagen (Neuro Probe, Maryland, USA). The chamber was incubated at 37°C in a humidified atmosphere of 5% CO_2 to allow cells to migrate from the wells of the upper chamber through the membrane pores to the wells containing chemoattractants. After 2-4 hours of migration, the membrane was dissembled from the chamber, soaked in 100% methanol for 2 minutes, Diff-Quick red stain for 1 minute and Diff-Quick purple stain for 2 minutes, followed by soaking in distilled water to remove all the excess stains. The membrane was then mounted onto a microscope slide with the side facing the chemoattracttants attaching to the slide. A damp Kimwipe (Kimberley-Clark Professional, New South Wales, Australia) was used to wipe away all non-migratory cells attached on the top of the membrane. Migrated cells were viewed using an Olympus CKX41 microscope (Olympus, Tokyo, Japan) and photographed with the

SPOT camera at x100 or x200 magnification. At least 4 fields of each triplicate well were imaged and the number of cells in each field was counted manually using the point tool in ImageJ software (NIH, Maryland, USA).

In this assay, membranes were coated overnight at room temperature with agitation in 40ml collagen type I solution ((20µg/mL in 10mM acetic acid; Sigma-Aldrich, Missouri, USA) or collagen type IV solution (40µg/mL in 200mM acetic acid; Sigma-Aldrich, Missouri, USA). The membranes were air-dried prior to use and were stored at room temperature for up to 1 month. The chemoattractants used in this study were Fibroblast conditioned media (FbCM) and EGF (20ng/ml for MCF10A, 10ng/ml for SkBr3 and MDA-MB-361).

2.3.6. Wound healing assay

MCF10A cells ($4x10^6$-$5x10^6$) were seeded in 6-well plates and grown to 100% confluence. The cell monolayer was wounded using a p10 pipet tip and washed once with PBS to remove dislodged cells. The cells were then maintained in assay media (DMEM/F12 supplemented with 2% horse serum, 10µg/ml insulin from bovine pancreas, 5µl/ml hydrocortisone and 1% antibiotic/antimycotic) containing 5ng/ml EGF. Mitomycin C was added to a final concentration of 500ng/ml to inhibit cell proliferation. Images of the wounds were taken every 30 minutes for 36 hours using a live cell imaging Leica AF6000 LX microscope (Leica Microsystems, Illinois, USA). Wound closure was quantified by measuring the size of the wound using ImageJ software. The percentage of wound closure was calculated by:

$$\%\ wound\ closure\ at\ time\ x = \frac{Size\ of\ wound\ at\ time\ 0\ - Size\ of\ wound\ at\ time\ x}{Size\ of\ would\ at\ time\ 0}$$

2.3.7. Flow cytometry

Cells were grown in monolayer to 60-70% confluency, lifted by trypsin and resuspended in 1X PBS at approximately $1x10^7$cells/ml before subjected to the fluorescence analysis using a FACSDIVA (BD, California, USA). Data were analysed using FlowJo software (TreeStar Inc, Oregon, USA).

2.4. EXPRESSION ANALYSIS

2.4.1. Protein extraction and quantification

Cells were grown in 10-cm cell culture dishes or 6-well plates to 50-70% confluency, then washed once with ice-cold PBS and lysed in modified RIPA buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 0.1 % SDS, 0.5 % Sodium Deoxycholate, 1% NP40, 5mM EDTA) containing a cocktail of protease (100mM 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride, 80µM Aprotinin, 2mM Leupeptin, 4mM Bestatin, 1.5mM Pepstatin A, 1.4mM E-64; Sigma-Aldrich, Missouri, USA) and phosphatase inhibitors (Sodium Orthovanadate, Sodium Molybdate, Sodium Tartrate, Imidazole; Sigma-Aldrich, Missouri, USA). Cell lysates were sonicated four times for 30 seconds with 30-60 second cooling intervals on ice, followed by centrifugation at 13,000rpm for 15 minutes at 4^{o}C.

Protein concentrations were quantified using the BCA protein assay kit as per manufacturer's instructions (Pierce Biotechnology, Illinois, USA). Briefly, to generate a standard curve, Bovine Serum Albumin (BSA) was diluted in water to final standard concentrations of 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.03125 mg/ml. Eight µl aliquots of the standards and protein samples were loaded into a 96 well plate in triplicate and 150µl of BCA reagent was added to each well. The plate was incubated at 37^{o}C for 30 minutes for colour development. Absorbance of each sample was determined at 540nm using a Multiskan FC Absorbance Plate Reader (Thermo-LabSystems, Massachusetts, USA). Protein concentrations of the samples were determined by referencing to the standard curve.

2.4.2. Western blot analysis

Equal aliquots of proteins (10-30µg) were combined with 4X loading buffer (Invitrogen, California, USA) containing NUPAGE sample reducing agent (Invitrogen, California, USA) and then denatured at 95^{o}C for 5 minutes. Protein samples were loaded into a 10 or 20 well NUPAGE Novex 10% or 4-12% Bis-Tris pre-cast gel (Invitrogen, California, USA) and electrophoresed at 200V for 50 minutes in NUPAGE MOPS or MES SDS running buffer containing anti-oxidants (Invitrogen, California, USA). Protein standards were run alongside the samples for size determination.

Following electrophoresis, proteins were transferred onto a PVDF membrane (Millipore, Massachusetts, USA) using TE42 standard protein transfer apparatus (Hoefer, Massachusetts, USA). Briefly, the membrane was cut to the size of the gel, soaked in methanol for 2 minutes and in Towbin transfer buffer (25mM Tris-HCl, 190mM Glycine, 0.1% SDS, pH 8.5, 20% methanol) for 5 minutes. The gel and membrane were sandwiched in between two stacks of filter paper that had been pre-soaked with transfer buffer. The transfer apparatus was assembled according to the manufacturer's instruction. The transfer tank was filled with 5L of transfer buffer and the transfer was performed at 90V for 2 hours at 4^{o}C.

For immunoblotting, membranes were blocked in Tris buffered saline (TBST, 50mM Tris-HCl, 150mM NaCl, 0.1% Tween-20, pH 7.4) containing 3% skim milk for 30 minutes at room temperature, followed by incubation with primary antibodies diluted in blocking solution or TBST overnight at 4^{o}C with rotation.

After overnight incubation with primary antibody, membranes were washed three times with TBST (9 minutes each) and then incubated with horseradish-conjugated secondary antibodies diluted at $1{:}1x10^{5}$ for 1 hour, followed by 3 washes in TBST (9 minutes each). To develop luminescence, membranes were soaked in Chemoluminescence Luminol reagent (Pierce, Illinois, USA) for 7 minutes. Protein bands were visualized on high-performance chemiluminescence film (GE Healthcare, Pennsylvania, USA) developed by the Kodak X-OMAT UV automatic developing system (Carestream Health, New York, USA).

2.4.3. Immunofluorescence staining of MCF10A acini cultured in Matrigel

Expression and localization of proteins in MCF10A acini grown on Matrigel were visualized by indirect immunofluorescence staining method as previously described by Debnath and co-workers (Debnath et al., 2003). Briefly, assay media in each well of the glass chamber slide was removed and the acini were fixed with 4% paraformaldehyde at room temperature for 20 minutes, followed by a 5 minute wash in 500µl PBS. To permeabilize cells, acini were incubated at room temperature for 10 minutes in 500µl PBS containing 0.5% Triton X-100 and then washed three times (10 minutes each) in PBS/Glycine (130mM NaCl, 7mM Na_2HPO4, 3.5mM NaH_2PO4, 100mM Glycine) at room temperature. For blocking, acini were incubated with 200µl/well IF buffer

(130mM NaCl, 7mM Na_2HPO4, 3.5mM NaH_2PO4, 0.1% BSA, 0.2% Triton X-100, 0.05% Tween-20) containing 10% horse serum for 1 hour at room temperature. Primary antibodies diluted in blocking solution at 1:100 or 1:200 dilutions were added and incubated at 4^{o}C overnight.

After overnight incubation with primary antibody, acini were rinsed at least five times with IF buffer (10 minutes each) at room temperature, then incubated with Alexa fluorescence conjugated secondary antibody diluted at 1:1000 dilution in IF buffer containing 10% horse serum for 1 hour at room temperature, followed by at least five times washes in IF buffer (10 minutes each). To counterstain nulei, acini were incubated with 0.5ng/ml 4'6-diamidino-2-phenylindole (DAPI; Sigma Aldrich, Missouri, USA) for 10 minutes and then washed in PBS for 10 minutes.

Slides were mounted with Vector Shield Hardset mounting media (Vector Laboratories Inc., California, USA) and allowed to dry overnight at room temperature. Once dried, slides were viewed under a Nikon C1 confocal microscope (Nikon, Tokyo, Japan). Images of the acini were taken using NIS Elements software (Nikon, Tokyo, Japan).

2.4.4. RT-qPCR

2.4.4.1. RNA extraction

Cells were grown in 10cm cell culture dishes to 50-70% confluency and total RNA was extracted using Qiagen RNA extraction kit (Qiagen, California, USA) according to the manufacturer's instruction. Briefly, growth media was removed and cells were lysed in 600µl of RLT buffer containing 1% β-mercaptoethanol. Cell lysate was collected using a cell scrapper and transferred to a microfuge tube. The cells were further lysed by passing the lysates 10 times through a 19g syringe needle. 700µl of 70% ethanol was added and mixed with the lysate by pipetting. The mixture was transferred onto a Qiagen RNeasy spin column and centrifuged for 15 seconds at 13,000rpm for the RNA to bind to the column. RW1 buffer (350µl) was added and the column was centrifuged at 13,000rpm for 15 seconds. On-column DNA digestion was performed by adding 80µl of DNase I in HDD buffer (Qiagen, California, USA) onto the column and incubated at room temperature for 15 minutes. Another 350µl of RW1 buffer was added and the column was centrifuged at 13,000rpm for 15 seconds. The column was then washed twice with 500µl of RPE buffer. RNA was eluted by adding 60µl of RNase free water to

the column and centrifuged at 14,000rpm for 1 minute. RNA samples were stored at -80°C for up to one year.

2.4.4.2. cDNA systhesis

cDNA was synthesised using SuperScript VILO cDNA synthesis kit (Invitrogen, California, USA). Briefly, 1-2µg RNA was combined with 4µl of 5X VILO reaction mix, 2µl of 10X SuperSript enzyme mix and water in a total 20µl reaction. The mixture was incubated at 25°C for 10 minutes for primer extension, followed by 60 minutes at 42°C for cDNA synthesis and 5 minutes at 85°C for reaction termination. For qPCR, the concentration of all RNA samples was normalized prior to cDNA synthesis. The synthesized cDNA was diluted to final concentration of 20ng/µl of input RNA and 1µl of the diluted cDNA was used for a final 20µl qPCR reaction.

2.4.4.3. qPCR

qPCR primers were designed using the NCBI primer designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 2.3). Each qPCR amplification mixture (20µl) contained 20ng cDNA, 10µl PerfeCTa Sybr Green Supermix (Quanta Biosciences, Maryland, USA) and 250nM forward and reverse primers. Reactions were run on a Rotor-gene 3000 Light Cycler (Corbett Life Sciences, Qiagen, Californis, USA). The cycling conditions comprised of 2 minute denaturation at 95°C and 40 cycles of denaturation at 95°C for 15 seconds and elongation at 60°C for 45 seconds. The final products were analysed by a melting curve analysis with temperature increasing 0.5°C/sec from 72°C to 95°C to check for contamination and primer dimer.

Table 2.4. *List of RT-qPCR primers*

Genes		Primer sequences	Primer length	Start position	Product length
p21	Forward	AGCAGAGGAAGACCATGTGGACCT	24	550	145
	Reverse	GGAGTGGTAGAAATCTGTCATGCTGG	26		
Bax	Forward	CACAGTGGTGCCCTCTCCCCAT	22	656	132
	Reverse	TCAAGGTCACAGTGAGGTCAGGGG	24		
PIG3	Forward	ACCCACCTCCAGGAGCCAGC	20	645	139
	Reverse	TACTGAGCCTGGCCCCCACC	20		
Mdm2	Forward	TGTTTGGCGTGCCAAGCTTCT	21	279	132
	Reverse	GGTGACACCTGTTCTCACTCACAG	24		
p53	Forward	GCCAGACTGCCTTCCGGGTCACT	23	172	150

	Reverse	CATCCATTGCTTGGGACGGCAAGGG	25		
RPL32	Forward	CAGGGTTCGTAGAAGATTCAAGGG	24	223	190
	Reverse	CTTGGAGGAAACATTGTCAGCGATC	25		

2.5.4..4. Data analysis

Raw data were exported to Excel and then analysed by LinRegPCR software (HFRC, Amsterdam, Netherlands) to determine PCR efficiency (E) and threshold cycle value (Ct) (Ruijter et al., 2009). Expressions of genes of interest (sample) were expressed as relative to expression of the house keeping ribosomal protein RPL32 (reference) (RE). Differences in gene expression among samples were expressed as ratio of relative gene expression of the treated sample versus that of the control sample. Equations used in the analysis of qPCR data were:

$$Relative\ expression\ (RE) = \frac{E_{Reference}{}^{Ct}}{E_{Sample}{}^{Ct}}$$

$$Ratio = \frac{RE_{Treated\ sample}}{RE_{Control\ sample}}$$

2.4.5. Microarray gene analysis

Total RNA was extracted from acini using Qiagen RNA extraction kit as in 2.5.4.1 with the Matrigel being dissolved in RLT buffer. RNA was diluted to 50ng/µl and submitted to Agilent Technologies (The Ramaciotti Center, New South Wales, Australia) for microarray processing. For determination of the most significant gene ontology pathways altered between the samples, Metacore™ bioinformatics software (GeneGo, Thompson Reuters, USA) was utilised.

2.5. STATISTICAL ANALYSIS

Assays were performed at least three times and data combined and presented as mean ± standard deviation (SD). Student's t-tests were conducted to determine whether the treatment group was statistically significant compared to the control. Significance is represented as * $P< 0.05$, ** $P<0.01$ and *** $P<0.001$.

2.6. MATERIALS

2.1.1. Plasmids

Table 2.5. *List of plasmids*

PLAMSIDS	SOURCE
HSE-luc	Richard Voellmy , University of Miami, Florida, USA
MSCV Ha-Ras^{V12} mCherry	Patrick Humbert, Peter MacCallum Cancer Center, Victoria, Australia
MSCV mCherry	Patrick Humbert, Peter MacCallum Cancer Center, Victoria, Australia
MSCV-LTRmiR30-PIG (LMP)	Open Biosystems, California, USA
MSCV-LMP HSF1 shRNAmir1	This study
MSCV-LMP HSF1 shRNAmir2	This study
MSCV-LMP HSF1 shRNAmir3	This study
MSCV-LMP HSF1 shRNAmir4	This study
MSCV-LMP HSF1 shRNAmir5	This study
pSUPERp53^{R273H}	Ygal Haupt, Peter MacCallum Cancer Center, Victoria, Australia
pVpack-Ampho	Agilent Technologies, California, USA
pBABE hHSF1wt IRES EGFP	This study
pBABE hHSF1ΔRDT IRES EGFP	This study
pBABEpuro Luc2 IRES mCherry	This study
pBABE p53^{R273H} IRES mCherry	This study
pBABE puro IRES EGFP	Addgene (plasmid 14430), Massachusetts, USA
pBABE puro IRES mCherry	This study
pcDNA3.1(+)	Invitrogen, California, USA
pGIPZ p53 shRNAmir1	Open Biosystems, California, USA
pHSE-mCherry	This study
pGIPZ p53 shRNAmir2	Open Biosystems, California, USA
pGIPZ p53 shRNAmir3	Open Biosystems, California, USA
pGIPZ p53 shRNAmir4	Open Biosystems, California, USA
pGIPZ p53 shRNAmir5	Open Biosystems, California, USA
pGIPZ p53 shRNAmir6	Open Biosystems, California, USA
pHSE-mCherry	This study
pRSET-B mCherry	Roger Tsien, University of California San Diego, California, USA
psPAX	Open Biosystems, California, USA
pDGM2.4	Open Biosystems, California, USA

2.1.2. Cloning primers

Table 2.6. *List of cloning primers*

PRIMER	SEQUENCE
BamHI Luc2 Fwd	ATGC**GGATCC**ACCATGGAAGATGCCAAAAA
BstXI mCherry Fwd	AT**CCA**CAACCA**TGG**TGAGCAAGGGC
EcoR1 Luc2 Rev	ATGC**GAATTC**TTACACGGCCGATCTTGCCGC
Flag HSF1 Fwd	AGCTTATGGACTACAAGGACGACGATGACAAGGATCTG CCCGTGGGCCCCGGC
HindIII mCherry Fwd	AA**AAGCTT**CAGCCATGGTGAGCAAGGGC
HSF1 EcoRI Rev	AAT**GAATTC**CTCGGAGACAGTGGGGTCCTT
HSF1 Fwd	ATGGATCTGCCCGTGGGCCCCGGC
HSF1 T Fwd	TTGGATGCTATGGACTCCAACGAGGATAAC
HSF1 T Rev	GTTATCCTCGTTGGAGTCCATAGCCATCCAA
HSF1ΔRD Fwd	GACAGTGGCTCAGCACATGGGCGCCCATCTTCCGTG
HSF1ΔRD Rev	CACGGAAGATGGGCGCCCATGTGCTGAGCCACTGTC
mirR30 Fwd	CAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAG CG
mirR30 Rev	CTAAAGTAGCCCCTTGAATTCCGAGGCAGTAGGCA
pBABE sequencing Fwd	CTCAATCCTCCCTTTATCCAG
pcDNA3.1(+) sequencing Fwd	GAGAACCCACTGCTTACTGGCTTATCG
SalI mCherry Rev	GGCG**GTCGAC**TTACTTGTACAGCTCG

2.1.3. Cloning reagents

Table 2.7. *List of cloning reagents*

ITEM	SUPPLIER
10mM dNTP	New England Biolabs, Massachusetts, USA
1kb DNA ladder	Invitrogen, California, USA
Ampicillin	Sigma-Aldrich, Missouri, USA
Bacto Tryptone (Pancreatic Digest of Casein)	BD Biosciences, California, USA
Bacto Yeast Extract	BD Biosciences, California, USA
High-grade DNA agarose	Invitrogen, California, USA
Kanamycin	Sigma-Aldrich, Missouri, USA
LB Agar	Merck, New Jersey, USA
Pfu high fidelity polymerase	GE Healthcare
Restriction Enzymes: BamHI, EcoRI, SalI, XhoI, BglII, HindIII, BstXI.	New England Biolabs, Massachusetts, USA
Sequencing reagents	
SYBR Safe DNA Gel Stain	Invitrogen, California, USA
T4 DNA Ligase	New England Biolabs, Massachusetts, USA

T4 DNA polymerase	New England Biolabs, Massachusetts, USA
Zeocin	Sigma-Aldrich, Missouri, USA

2.1.4. Reagents for cell culture and *In Vitro* assays

Table 2.8. *List of reagents for cell culture and in vitro assays*

ITEM	SUPPLIER
100X Antibiotic/Antimycotic	Gibco Invitrogen, California, USA
Bovine Serum Albumin (BSA)	Sigma-Aldrich, Missouri, USA
Cholera toxin	Sigma-Aldrich, Missouri, USA
Collagen I	Sigma-Aldrich, Missouri, USA
Collagen IV	Sigma-Aldrich, Missouri, USA
Crystal Violet	Sigma-Aldrich, Missouri, USA
Diff Quick Dyes	Fronine Lab Supplies, New South Wales, Australia
Dulbecco's modified Eagle Medium (DMEM)	Gibco Invitrogen, California, USA
Dulbecco's modified Eagle Medium (DMEM)/ Ham's nutrient mixture F12 (DMEM/F12)	Gibco Invitrogen, California, USA
Epidermal Growth Factor (EGF)	BD Biosciences, California, USA
Foetal Bovine Serum (FBS)	Thermo Scientific, California, USA
Horse Serum	Gibco Invitrogen, California, USA
HSF1 Inhibitors:	
KNK437	Calbiochem, California, USA
Triptolide	Calbiochem, California, USA
Quercetin	Calbiochem, California, USA
Hydrocortisone	Sigma-Aldrich, Missouri, USA
Insulin from Bovine Pancreas	Sigma-Aldrich, Missouri, USA
LipofectAMINE LTX	Invitrogen, California, USA
Matrigel	BD Biosciences, California, USA
McCoy's 5A Medium	Gibco Invitrogen, California, USA
Mitomycin C	Sigma-Aldrich, Missouri, USA
Puromycin	Sigma-Aldrich, Missouri, USA
RPMI Medium	Gibco Invitrogen, California, USA
Sulforhodamide B (SRB)	Sigma-Aldrich, Missouri, USA
Terg-a-Zyme	Alconox Inc., New York, USA
Trichloroacetic Acid (TCA)	Sigma-Aldrich, Missouri, USA
TrypLE Express (stable trypsin replacement)	Gibco Invitrogen, California, USA

2.1.5. Reagents for protein and mRNA expression analysis

Table 2.9. *List of reagents for protein and mRNA expression analysis*

ITEM	SUPPLIER
10x Reducing Agent	Invitrogen, California, USA
4x Loading Buffer	Invitrogen, California, USA
Antioxidant	Invitrogen, California, USA
Coomassie Brilliant Blue R-250	Bio-Rad, California, USA
MES Buffer	Invitrogen, California, USA
MOPS Buffer	Invitrogen, California, USA
NUPAGE 20-well 10% Gel	Invitrogen, California, USA
NUPAGE 20-well 4-12% Gel	Invitrogen, California, USA
Perfecta Sybr Green Supermix	Quanta Biosciences, Maryland, USA
Phosphatase Inhibitor	Sigma-Aldrich, Missouri, USA
Protease Inhibitor	Sigma-Aldrich, Missouri, USA
PVDF Membrane	Millipore, Massachusetts, USA
Restore Western Blot Stripping Buffer	Thermo Scientific, Massachusetts, USA
Seeblue Plus 2 Prestained Protein Marker	Invitrogen, California, USA
Skim Milk Powder	Diploma, Victoria, Australia
Sodium Dodecyl Sulfate (SDS)	Astral Scientific, New South Wales, Australia
X-ray Film	Amersham Biosciences, Upssala, Sweden

2.1.6. General Reagents

Table 2.10. *List of general reagents*

ITEM	SUPPLIER
Acetic Acid	BDH AnalaR, Poole, England
Calcium Chloride	Astral Scientific, New South Wales, Australia
Di-sodium Hydrogen Phosphate	Astral Scientific, New South Wales, Australia
DMSO (Dimethyl Sulfoxide)	Sigma-Aldrich, Missouri, USA
EDTA (Ethylenediaminetetra Acetic Acid Disodium Salt)	BDH AnalaR, Poole, England
Ethanol	Merck, New Jersey, USA
Glycerol	BDH AnalaR, Poole, England
Glycine	Amresco, Ohio, USA
Hydrochloric Acid (HCl)	BDH AnalaR, Poole, England
Isopropanol Alcohol	Merck, New Jersey, USA
Magnesium Chloride	Astral Scientific, New South Wales,

	Australia
Methanol	Merck, New Jersey, USA
NP-40	Sigma-Aldrich, Missouri, USA
Paraformaldehyde	BDH AnalaR, Poole, England
Potassium Acetate	Astral Scientific, New South Wales, Australia
Potassium Phosphate	Astral Scientific, New South Wales, Australia
Sodium Acetate	Astral Scientific, New South Wales, Australia
Sodium Chloride (NaCl)	Astral Scientific, New South Wales, Australia
Sodium Dioxycholate	Sigma-Aldrich, Missouri, USA
Sodium Hydrogen Phosphate	Astral Scientific, New South Wales, Australia
Sodium Hydroxide (NaOH)	Merck, New Jersey, USA
Tris-HCl (Tris (hydroxymethyl) aminomethane)	Astral Scientific, New South Wales, Australia
Triton-X100 (t-octylphenoxypoly-ethoxyethanol)	Sigma-Aldrich, Missouri, USA
Tween-20 (Polyoxyethylene sorbitanmonolaurate)	Sigma-Aldrich, Missouri, USA

2.1.7. Commercial Kits

Table 2.11. *List of commercial kits*

ITEM	SUPPLIER
BCA Protein Assay Kit	Pierce Biotechnology, Illinois, USA
Chemiluminescence Luminol	Pierce Biotechnology, Illinois, USA
Cytoplasmic And Nuclear Protein Extraction Kit	Pierce Biotechnology, Illinois, USA
HiPure Plasmid Midi Prep Kit	Invitrogen, California, USA
Plasmid Midi Kit	Qiagen, California, USA
QIAquick DNA Purification Kit	Qiagen, California, USA
RNeasy Mini Kit	Qiagen, California, USA
VILO cDNA Synthesis Kit	Invitrogen, California, USA

2.1.8. Primary Antibodies

Table 2.12. *List of primary antibodies*

ITEM	DILUTION FOR WESTERN BLOT ANALYSIS	SECONDARY ANTIBODY	SUPPLIER
Actin – pan	1:5000	Mouse	Neomarkers, California, USA
Akt	1:1000	Rabbit	Cell Signalling, Massachusetts, USA
Apaf-1	1:1000	Mouse	BD Pharmigen, California, USA
Bad	1:1000	Mouse	BD Pharmigen, California, USA
Bax	1:1000	Mouse	BD Pharmigen, California, USA
Bcl-2	1:3000	Mouse	BD Pharmigen, California, USA
Bcl-xL	1:1000	Mouse	BD Pharmigen, California, USA
Beta-Catenin	N/A	Rabbit	Cell Signalling, Massachusetts, USA
Cleaved Caspase-3	N/A	Rabbit	Cell Signalling, Massachusetts, USA
EGFR (Epidermal growth factor receptor)	1:1000	Rabbit	Cell Signalling, Massachusetts, USA
ERK	1:1000	Mouse	BD Biosciences, California, USA
HSF1 (Heat Shock Factor 1)	1:5000	Rabbit	Stressgen, Michigan, USA
HSP105/110	1:5000	Rabbit	Santa Cruz, California, USA
HSP27	1:50000	Mouse	Stressgen, Michigan, USA
HSP70i	1:10000	Mouse	Epitomics Inc, California, USA
HSP90	1:5000	Rat	Stressgen, Michigan, USA
Laminin V	1:5000	Mouse	Millipore, Massachusetts, USA
p21	1:1000	Mouse	BD Pharmigen, California, USA
p53	1:1000	Mouse	BD Pharmigen, California, USA
pAkt (Ser473)	1:1000	Rabbit	Cell Signalling, MA, USA
pERK (Thr202/Tyr204)	1:1000	Mouse	BD Biosciences, California, USA
pHSF1 (Ser326)	1:50,000	Rabbit	Epitomics Inc, California, USA
PLCγ1 (Phospholipase lipase Cγ1)	1:1000	Rabbit	Cell Signalling, Massachusetts, USA
pPLCγ1 (Tyr783)	1:1000	Rabbit	Cell Signalling, Massachusetts, USA
Ras	1:5000	Rabbit	Millipore, Massachusetts, USA
XIAP	1:1000	Mouse	BD Pharmigen, California, USA

2.1.9. Secondary Antibodies

Table 2.13. *List of secondary antibodies*

ITEM	SUPPLIER
Donkey Anti-Rabbit IgG+IgM, (H+L), Alexa-Fluor 647	Invitrogen, California, USA
Goat Anti-Mouse IgG+IgM, (H+L), Peroxidase conjugated	Pierce Biotechnology, Illinois, USA
Goat Anti-Rabbit IgG, (H+L), Peroxidase conjugated	Pierce Biotechnology, Illinois, USA
Goat Anti-Rat IgG, (H+L), Peroxidase conjugated	Pierce Biotechnology, Illinois, USA
Mouse Anti-Goat IgG, (H+L), Peroxidase conjugated	Pierce Biotechnology, Illinois, USA
Rabbit Anti-Mouse IgG+IgM, (H+L), Alexa-Fluor 647	Invitrogen, California, USA

CHAPTER 3

THE EFFECT OF HSF1 UPON THE PROGRESSION OF CANCER CELLS WITH RESPECT TO ONCOGENIC RAS

3.1. INTRODUCTION

Cancer cells characteristically exhibit a "stress phenotype" as a result of continuous exposure to a variety of extrinsic and intrinsic stresses. The stress-induced expression of heat shock proteins (HSPs), which function primarily as molecular chaperones that maintain cellular protein homeostasis, have been shown to be one of the major contributors to the maintenance and progression of tumourigenesis (Calderwood, 2010; Calderwood and Ciocca, 2008). Consistent with this, the master transcription factor of heat shock proteins, HSF1, is elevated and activated in many high grade cancers and a high level of HSF1 protein expression is positively associated with cancer aggressiveness (Santagata et al., 2011). Interestingly, in addition to regulating the expression of HSPs, HSF1 also directly promotes cancer progression through the regulation of many distinct transcriptional networks that support multiple malignant phenotypes such as proteasomal degradation (Lecomte et al., 2010), migration (O'Callaghan-Sunol and Sherman, 2006), apoptosis (Jacobs and Marnett, 2009), glucose metabolism (Dai et al., 2007; Zhao et al., 2009), protein translation (Dai et al., 2007) and oncogenic transformation (Dai et al., 2007; Mendillo et al., 2012). However, although it has been shown that cancer cells are dependent on HSF1 for their 'fitness', the mechanisms by which HSF1 achieves this are relatively unknown.

It has been reported that HSF1 is required for the proper functioning of many oncogenes and mutated tumour suppressors such as Ras (Dai et al., 2007), ErbB2 (Meng et al., 2010), Heregulin β1 (Khaleque et al., 2005), PDGF-B and p53 (Dai et al., 2007) in initiating tumourigenesis and/or promoting cancer progression. As a result, inhibition of HSF1 leads to the reduction of multiple malignant phenotypes induced by these oncogenes and mutated tumour suppressors. Conversely, the hypothesis of this study is

that within the context of the cancer cell, activation of HSF1 exerts a cancer promoting affect via modulation/support of oncogene activity and mutated tumour suppressor function. Therefore, the activities of HSF1 in cancer growth and progression are dependent upon the presence of these proteins, thus leading to unique effects of HSF1 activation within the context of the cancer cell.

Among oncogenes, members of the Ras family are the most frequently mutated genes in cancer with approximately 90% of pancreatic cancers, 70% of malignant neoplasms and 30% of all human cancers containing an active oncogenic Ras isoform. Human cells contain four highly homologous 21 kDa Ras proteins which are H-Ras, N-Ras, K-Ras4A and K-Ras4B (Graham and Olson, 2007). These proteins function as secondary messenger molecules that are activated upon stimulation of receptor tyrosine kinases (RTKs). Upon their activation they transmit their signals to downstream transduction pathways that regulate essential cellular processes such as cell cycle progression, survival and differentiation. Dysregulation of Ras in cancer promotes cell proliferation, neoplastic transformation, tumourigenesis and metastasis (Bos, 1989).

Previous studies have shown that HSF1 is required for Ras mediated transformation. In *hsf1-/-* mouse embryonic fibroblasts (MEFs), the lack of HSF1 results in the reduction of malignant phenotypes compared to their wild-type counterparts when transformed by the activated mutant H-Ras^{V12}. In addition, HSF1 depletion protects mice from tumour formation induced by the extopic expression of activated Ras (Dai et al., 2007). This chapter seeks to investigate the association of HSF1 and Ras activity in regulating the malignant phenotype through examining the impact of HSF1 activation upon the cell biology of both normal human mammary epithelial cells (HMEC), H-Ras^{V12} transformed HMEC and an established breast cancer cell line.

The two cell lines utilised in this study were the non tumorigenic immortalized breast cell line, MCF10A, and the breast cancer cell line SkBr3. MCF10A is a well characterized immortalized cell line derived from the mammary tissue of a patient with fibrocystic disease and was thought to be immortalized by the loss of the p16 locus associated with t(3;9) translocation (Cowell et al., 2005). The cell line does not form tumours in nude mice or colonies in soft-agar andimilar to normal breast epithelial cells, this cell line exhibits a dome structure in *in vitro* tissue culture and produces mammary spheroid growth in 3-D culture (Debnath et al., 2003). These characteristics of the

MCF10A cell line make it a model of choice for use as a 'normal' control in breast cancer progression studies.

The SkBr3 cell line was derived from a pleural effusion from an adenocarcinoma originating in the breast of a 43 year old female. The cancer cell line over-expresses HER2, leading to a constitutive activation of Ras and its downstream signalling pathways (Kroll et al., 2002). This cell line was thus selected to study the effect of HSF1 activation in cancer cells with activated Ras.

The aims of this chapter are:

- To investigate the impact of ectopic expression of HSF1 upon the cell biology of the human mammary epithelial cell line MCF10A and of isogenically- matched H-Ras^{V12} transformed cells.
- To examine the changes in gene expression caused by ectopic expression of activated HSF1 in normal and in H-Ras^{V12} transformed MCF10A cells.
- To investigate the impact of ectopic expression of activated HSF1 upon cell biology of the established breast cancer cell line SkBr3.

3.2. RESULTS

3.2.1. Generation of stable mCherry control non-transformed and the H-Ras^{V12} transformed MCF10A cells ectopically expressing HSF1.

To examine the impact of HSF1 activation upon the cell biology of normal HMECs, HSF1 was activated in MCF10A cells to levels similar to those in high-grade breast cancer cells. To achieve this, MCF10A cells were transduced by retroviruses that contained vectors with GFP control, wild-type HSF1 (HSF1wt) or a constitutively activated mutant HSF1, HSF1ΔRDT. HSF1ΔRDT is a mutated form of HSF1 which lacks the regulatory domain and has leucine 395 substituted by glutamic acid (Fujimoto et al., 2005). These mutations prevent the formation of the inactive monomeric structure and thereby facilitate active trimer formation. To examine the effect of HSF1 activation upon the oncogenicity of activated Ras, MCF10A cells expressing GFP control, HSF1wt or HSF1ΔRDT were transduced by retroviral vectors to stably express either mCherry control or the activated oncogene H-Ras^{V12}. Cells were transduced at a multiplicity of infection (MOI) of approximately 0.3 to ensure that each transduced cell

contained only one copy of the viral constructs. Stable cells were selected by fluorescence-activated cell sorting (FACS) based on GFP and mCherry expression with the gates for cell population selection chosen to equalise the levels HSF1 and H-Ras^{V12} ectopically expressed among the transduced cells (Appendix 1). Western blot analysis confirmed that HSF1wt, HSF1ΔRDT and Ras were successfully expressed (Fig.3.1). As expected, ectopic expression of HSF1wt or HSF1ΔRDT resulted in increased levels of HSPs such as HSP27 and HSP110 (Fig. 3.1). Consistent with previous reports, ectopic expression of H-Ras^{V12} altered expression of proteins such as epidermal growth factor

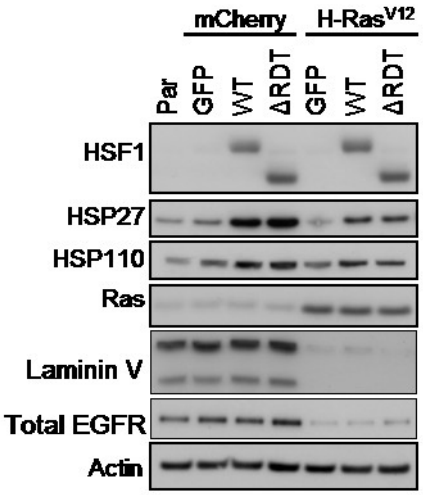


Figure 3.1. Western blot analysis demonstrating the successful generation of stable mCherry control and H-Ras^{V12} transformed MCF10A cells ectopically expressing GFP, HSF1wt or HSF1ΔRDT

Western blot analysis revealed that cells with HSF1 ectopic expression expressed increased levels of heat shock proteins such as HSP110 and HSP27. Cells transformed by H-Ras^{V12} expressed reduced levels of EGFR and Laminin V. In addition, ectopic expression of H-Ras^{V12} also caused a reduction in the levels of both basal and induced HSP expression.

receptor (EGFR) and Laminin V (Derer et al., 2012; Zinn et al., 2006). However, H-Ras^{V12} also reduced both the basal and induced expression of HSPs upon HSF1 ectopic expression (Stanhill et al., 2006); Fig.3.1).

3.2.2. Activation of HSF1 does not affect cell morphology or 2-D proliferation of non-transformed and H-Ras^{V12} transformed MCF10A.

MCF10A cells when cultured under 2-D conditions exhibit a cuboidal, cobblestone morphology characteristic of epithelial cells (Debnath et al., 2003). When transformed

with H-Ras^{V12}, the cells undergo an epithelial to mesenchymal transition (EMT) that results in the cells adopting a scattered and spindle-like morphology (Basolo et al., 1991; Wang et al., 1997). As a change in cellular morphology can indicate the progression of a cancer cell to a more migratory phenotype, the generated stable cell lines were examined with respect to their morphology upon HSF1 ectopic expression. As illustrated in Fig.3.2, as was expected, the mCherry control MCF10A cells exhibited an epithelial morphology while the H-Ras^{V12} transformed cells displayed a classical mesenchymal morphology. HSF1 activation was observed to not affect cell morphology in either the non-transformed or the H-Ras^{V12} transformed MCF10A cells (Fig. 3.2).

To investigate the impact of HSF1 activation upon cell proliferation, cell growth was examined using a two-dimensional (2-D) anchorage-dependent cell proliferation assay. Consistent with previous reports, as illustrated in Fig.3.3, MCF10A cells transformed with H-Ras^{V12} proliferated at a similar rate to the non-transformed cells in full growth media; however, they are able to proliferate in limiting media conditions (2% horse serum, 5ng/ml EGF) when the non-transformed cells have stopped proliferating (Basolo et al., 1991; Wang et al., 1997). Ectopic expression of HSF1wt or HSF1ΔRDT did not

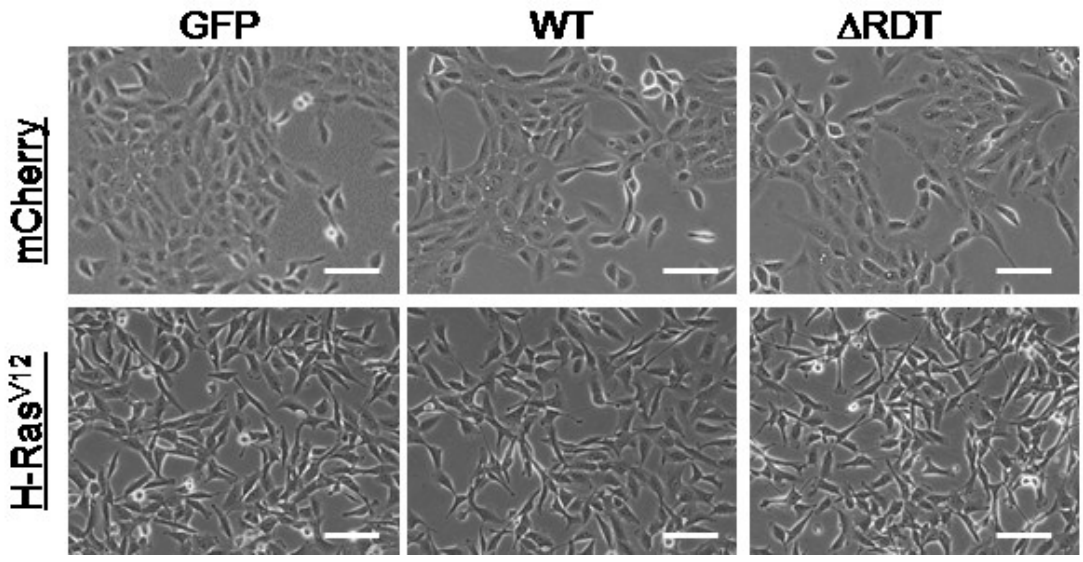


Figure 3.2. Ectopic expression of HSF1 does not impact upon cell morphology of both the mCherry non-transformed and H-Ras^{V12} transformed MCF10A.
When cultured in 2-D conditions, the mCherry control non-transformed MCF10A cells exhibited a cobblestone epithelial morphology while the H-Ras^{V12} transformed cells exhibited a spindle-like mesenchymal morphology. Ectopic expression of HSF1 did not cause any alteration in cell morphology. Scale bar - 100μM.

cause any significant alteration in proliferation rate of both the non-transformed and H-Ras^{V12} transformed MCF10A in either full growth media or in limiting media condition.

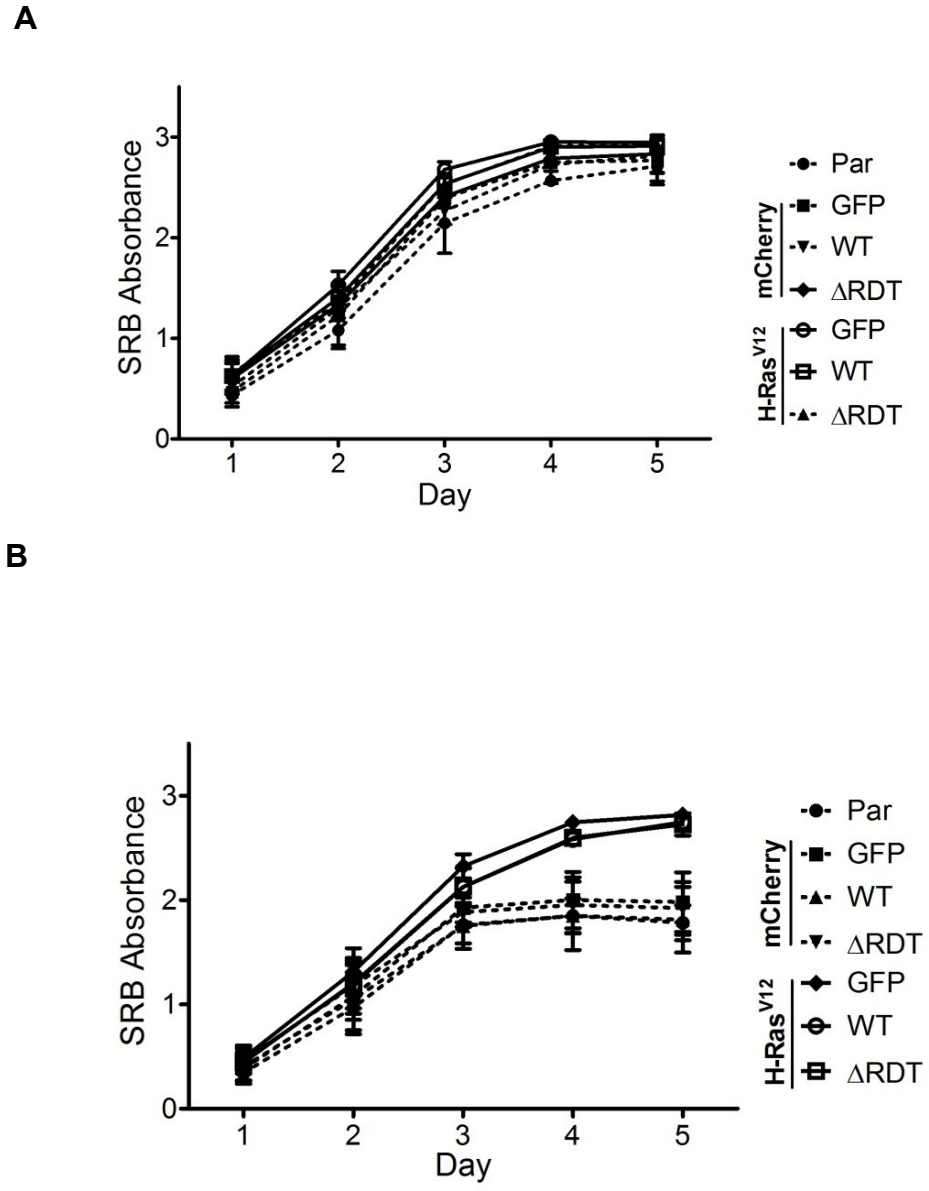


Figure 3.3. Ectopic expression of HSF1 does not impact upon 2-D cell proliferation of both the mCherry non-transformed and H-Ras^{V12} transformed MCF10A in either full or limiting media condition.

(A) In full media conditions, neither ectopic ecpression of HSF1 nor H-Ras^{V12} had a significant impact upon proliferation rate. **(B)** In limiting media conditions, the H-Ras^{V12} transformed cells were still able to proliferate when the non-transformed mCherry cells have stopped proliferating. Ectopic expression of HSF1 did not affect the proliferation rate of eiher the mCherry non-transformed or H-Ras^{V12} transformed MCF10A cells in either full or limiting media condition.

3.2.3. HSF1 activation does not impact upon acini formation in non-transformed MCF10A but promotes highly disorganized growth in H-Ras^{V12} transformed cells in 3-D culture conditions.

When cultured in 3-D reconstituted basement membrane (Matrigel), normal MCF10A cells undergo a defined program of proliferation, differentiation and apoptosis to form organized hollow spherical acini that resemble the acinar structure of the mammary lobules *in vivo* (Debnath et al., 2003). The development and maintenance of this polarized structure is critical for the normal function of the cells *in vivo*. In addition, one of the pathological hallmarks of epithelial carcinomas is the disruption of this intact, well-organized structure. To investigate whether activation of HSF1 leads to an alteration in the growth of either the mCherry non-transformed or the H-Ras^{V12} transformed MCF10A cells in 3-D culture condition, cells were grown on top of a thin Matrigel layer in liquid media containing 2% Matrigel. Observation of cell morphology under a bright-field microscope revealed that consistent with previous reports, both the non-transformed and H-Ras^{V12} transformed GFP control cells formed organized acini structures (Fig.3.4A) (Dow et al., 2008). HSF1 activation did not change the 3-D cell morphology or the growth of the untransformed cells (Fig.3.4A); however, interestingly, H-Ras^{V12} transformed cells expressing HSF1wt or HSF1ΔRDT did not form organized acini but exhibited highly invasive and disorganized growth with significantly higher acini area (Fig.3.4A), indicating that activation of HSF1 in the H-Ras^{V12} transformed cell context promoted the loss of cell architecture in 3-D culture conditions.

The 3-D cell structures were further examined by immuno-staining with laminin V and β-catenin antibodies and images of the equatorial cross section of the acini were taken by a Nikon C1 confocal microscope to examine the luminal space of the acini. Laminin V is normally deposited at the basal surface of the acini while β-catenin primarily localizes at the cell periphery (Debnath et al., 2003). As illustrated in Fig.3.4B, non-transformed GFP control, HSF1wt and HSF1ΔRDT MCF10A cells exhibited normal hollow acini structures with laminin V deposited at the basal surface and β-catenin localizing at the cell periphery (Fig.3.4B). Consistent with previous reports, H-Ras^{V12} transformed GFP control cells formed organized acini structures with filled lumen. Consistent with the western blot analysis showing that laminin V is significantly

reduced in H-Ras^{V12} transformed cells (Fig.3.1), the acini structures formed by these cells lack laminin V at the basal surface. In addition, although H-Ras^{V12} cells underwent EMT and exhibited a mesenchymal phenotype in 2-D culture, β-catenin still localized at the cell periphery and ectopic expression of HSF1 did not affect this localisation

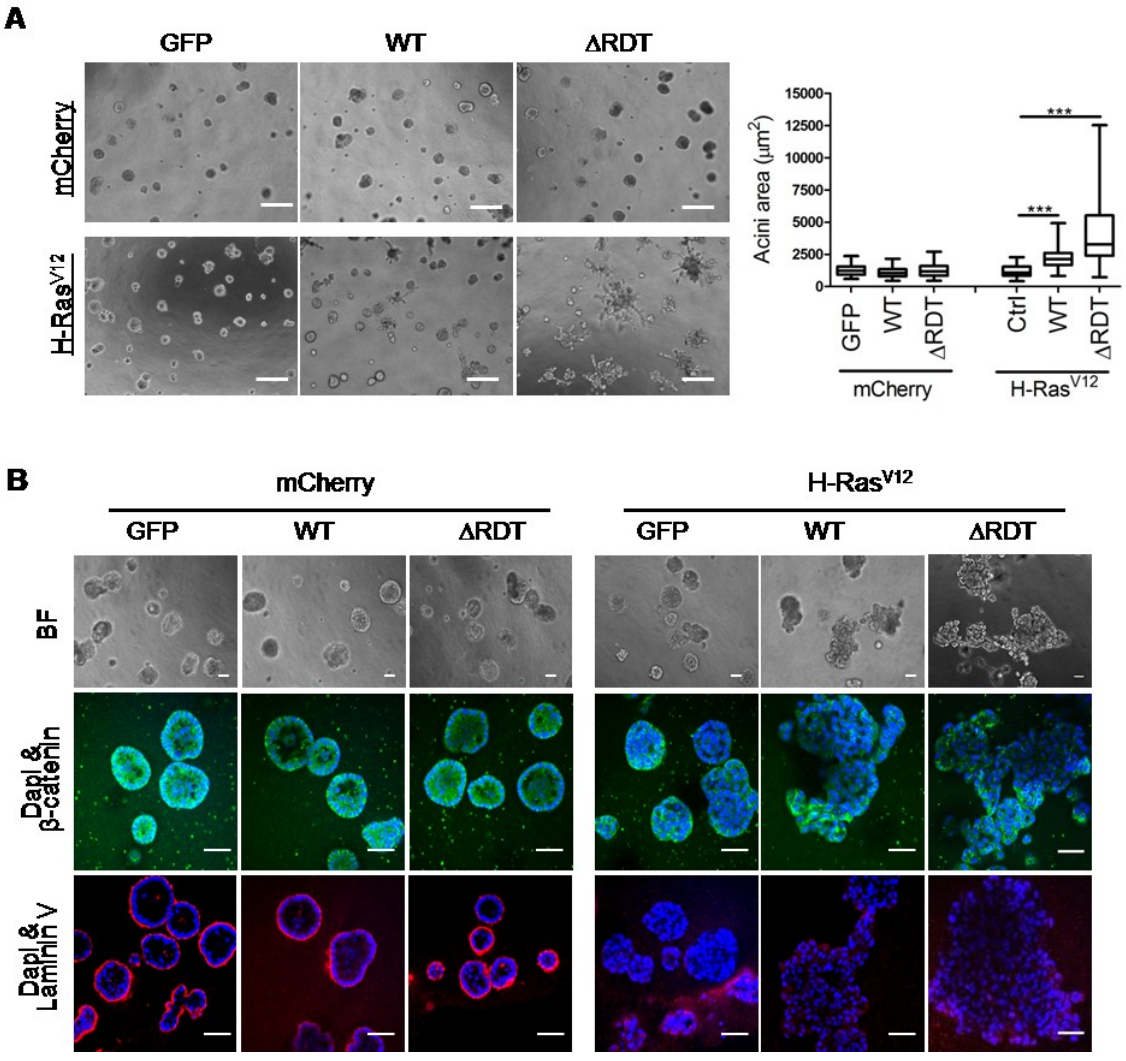


Figure 3.4.Ectopic expression of HSF1 promotes disorganized growth of H-Ras^{V12} transformed cells in 3-D growth conditions.

(A) When cultured in 3-D reconstituted basement membrane (Matrigel), both the MCF10A non-transformed and H-Ras^{V12} transformed GFP control cells formed organized acini structures observed under bright-field microscope. Ectopic expression of HSF1 in the non-transformed cells did not affect the acini structures. In contrast, H-Ras^{V12} transformed MCF10A cells over-expressing HSF1 (WT and ΔRDT) exhibited highly disorganized invasive growth observed under bright-field microscope, with significantly increased acini equatorial area. Scale bar - 200μM. **(B)** Observation of acini structures under confocal microscopy with images of acini taken at the equatorial section revealed that the mCherry non-transformed GFP control cells formed acini with hollow lumen whereas acini formed by H-Ras^{V12} transformed GFP control cells had filled lumen. Ectopic expression of HSF1 did not affect the morphology of non-transformed MCF10A cells but promoted disorganized growth of the H-Ras^{V12} transformed cells. Blue – DAPI, Red - β-catenin, Green – LamininV. Scale bar - 50μM.

(Fig.3.4B), indicating that HSF1 activation promotes disorganized growth of H-Ras^{V12} transformed MCF10A cells through mechanisms other than changing localisation of proteins regulating cell structure such as β-catenin. Altogether, the results suggest that HSF1 by itself has little effect upon cell growth in 3-D conditions; however, when co-operating with activated Ras, it enables the cells to have a greatly enhanced ability to invade and grow in the surrounding matrix.

3.2.4. HSF1 overexpression does not affect cell migration and wound healing ability of the non-transformed MCF10A but significantly enhances these parameters in H-Ras^{V12} transformed MCF10A.

The migratory and chemotactic properties of a cancer cell are indicative of its invasive and metastatic potential, to further investigate the effect of HSF1 ectopic expression upon these *in vitro* measures of metastatic propensity, the impact of HSF1 ectopic expression upon cell migration of both the non-transformed and H-Ras^{V12} transformed MCF10A cells were examined. Cells were assayed for their migratory ability using a standard 48 well microchemotaxis assay as described previously (Kouspou and Price, 2011). Migration of cells toward 0.1% BSA represents the basal un-directional migratory capacity of cells while migration toward 20ng/ml EGF represents the chemotactic migratory capacity of cells in response to external stimuli. As illustrated in Fig.3.5A, activation of HSF1 did not impact upon the migratory ability of the non-transformed MCF10A cells toward either 0.1% BSA or 20ng/ml EGF. However, activation of HSF1 in the H-Ras^{V12} transformed MCF10A cells significantly enhanced both the basal and chemotactic migration (Fig.3.5B), indicating that HSF1 plays a role in cell migration and this role of HSF1 is dependent on the oncogenic transformation status of the cell.

To further investigate the role of HSF1 in cell migration, both the non-transformed and H-Ras^{V12} transformed MCF10A cells were examined for their wound closure ability. Similar to the findings from the microchemotaxis assay, activation of HSF1 in the non-transformed MCF10A cells did not impact upon the wound closure ability of these cells whereas activation of HSF1 in the H-Ras^{V12} transformed cells significantly enhanced the wound closure rate (Fig.3.6). This further confirmed that HSF1 activation enhanced cell migration of cells that also expressed the activated mutant H-Ras^{V12}.

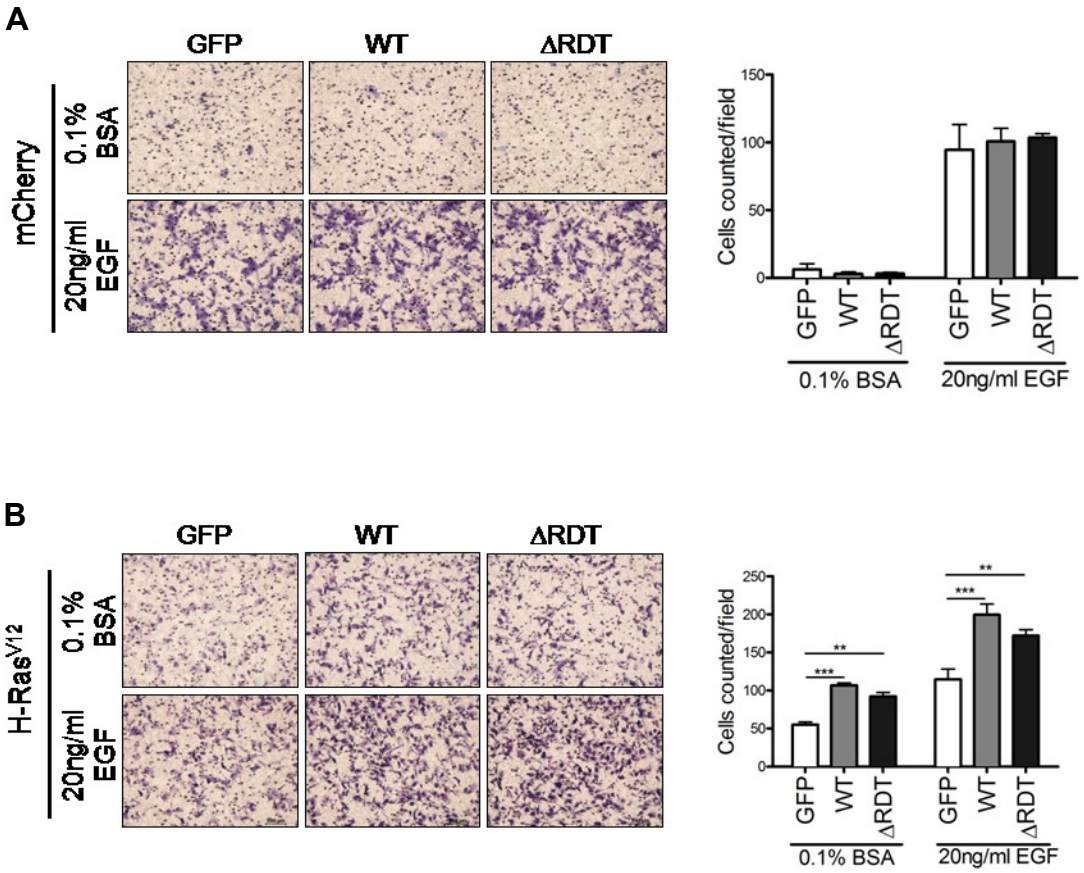


Figure 3.5. Ectopic expression of HSF1 promotes both basal and EGF stimulated cell migration of H-Ras^{V12} transformed MCF10A.

Cells were lifted by trypsin, resuspended and incubated in growth media for 1 hour to recover. Following the recovery, cells were then washed three times in DMEM/F12+0.1% BSA and resuspended in that media at 1x10^6 cells/ml. An aliquot of cell suspension was loaded in triplicate into a Boyden microchemotatic chamber. Cell migration toward DMEM/F12+0.1% BSA and 10ng/ml EGF was analysed after 4-5 hours. Cell migration was quantified by taking the average number of cell counted in four fields at X200 magnification of each triplicate well. Representative images of the migration membranes are shown. The number of cells migrated are represented as the mean±sd. The results are representative of at least three independent experiments. **A.** Ectopic expression of HSF1 has no impact upon cell migration of mCherry untransformed MCF10A cells. **B.** Ectopic expression of HSF1sgnificantly enhances both basal and EGF-simulated cell migration of H-Ras^{V12} transformed MCF10A cells.

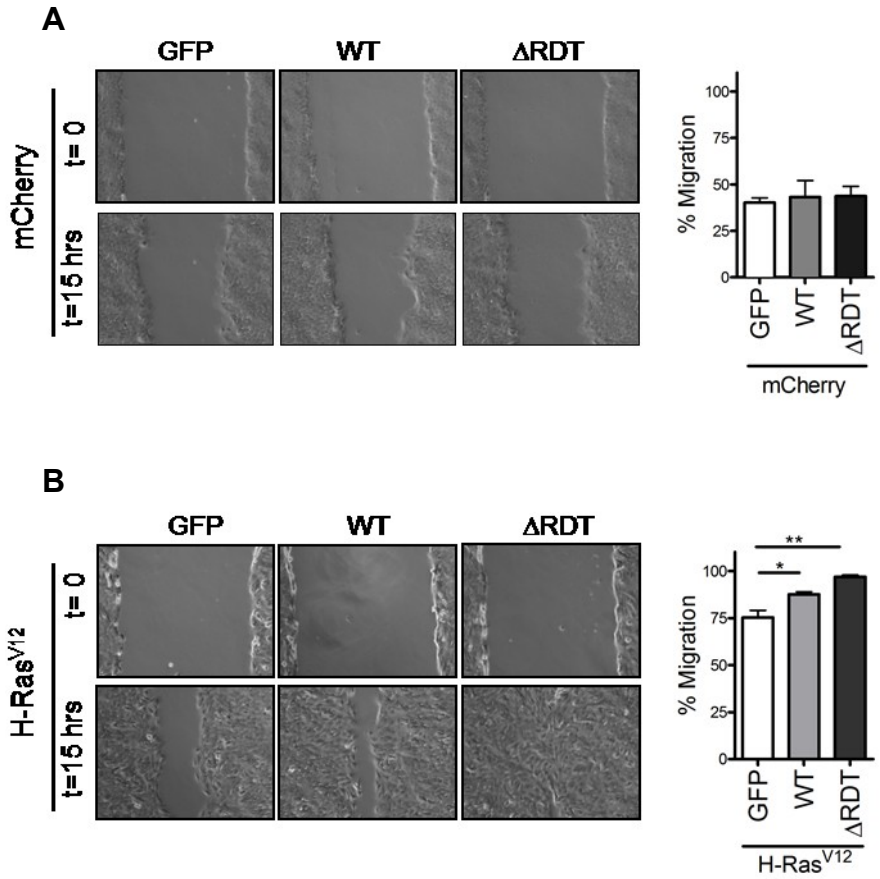


Figure 3.6. Ectopic expression of HSF1 enhances wound healing ability of H-Ras^{V12} transformed MCF10A.

(A) Ectopic expression of HSF1 has no impact upon the wound closure rate of the mCherry non-transformed MCF10A cells. **(B)** Ectopic expression of HSF1 significantly enhances the wound closure rate of the H-Ras^{V12} transformed MCF10A cells. Representative images of the wounds at the start and after 15 hours are shown. Percentage of wound closure was quantified after 15 hours.

3.2.5. HSF1 activation promotes 3-D growth and chemotactic migration of HER2 overexpressing SkBr3 cells.

To determine whether HSF1 activation could promote the malignant phenotype of breast cancer cells which express activated Ras, GFP control, HSF1wt or HSF1ΔRDT retroviral constructs were introduced into the breast cancer cell line SkBr3 which is known to express high levels of HER2, leading to the constitutive activation of Ras.

Stable cells that expressed the retroviral constructs as determined by GFP expression were selected by FACS. Western blot analysis confirmed the successful generation of stable GFP control, HSF1wt and HSF1ΔRDT SkBr3 expressing cells. Consistent with the role of HSF1 in regulating the heat shock response, HSF1wt and HSF1ΔRDT SkBr3 cells expressed high levels of HSPs including HSP27 and HSP110 (Fig.3.7A). Similar to the findings from ectopic expression of HSF1 in the MCF10A cell line models, HSF1 activation in SkBr3 cells did not alter the cell morphology or the proliferation rate in a 2-D growth assay (Fig.3.7B and 3.7C).

The impact of HSF1 activation upon migration and 3-D growth of SkBr3 cells was then

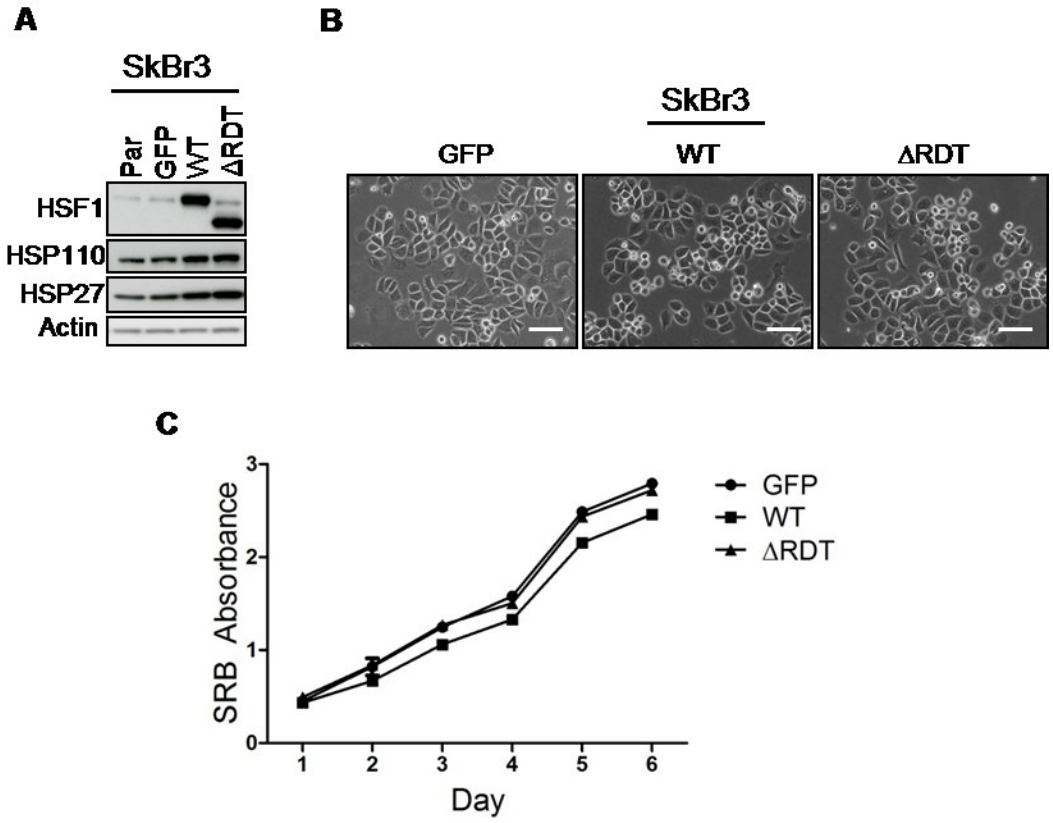


Figure 3.7. Ectopic expression of HSF1 has no impact upon cell morphology or 2-D growth of SkBr3 cells

(A) Western blot analysis confirmed the successful generation of stable SkBr3 cells expressing HSF1wt and HSF1ΔRDT. Ectopic expression of HSF1 increased expression of HSPs including HSP27 and HSP110. **(B)** SkBr3 cells exhibited an epithelial morphology when grown in 2-D conditions. Ectopic expression of HSF1 had no impact upon the morphology of SkBr3 cells. Scale bar - 100μM. (C) Ectopic expression of HSF1 also had no impact upon cell proliferation in 2-D growth condition.

examined. When grown in 3-D culture, SkBr3 cells have been reported to exhibit grape-like structures (Kenny et al., 2007). Ectopic expression of GFP had no impact upon the morphology of the cells when cultured in Matrigel. However, cells that expressed HSF1wt or HSF1ΔRDT grew better in Matrigel, forming larger 3-D structures when compared to the GFP control cells (Fig.3.8A). In addition, SkBr3 cells expressing HSF1wt or HSF1ΔRDT were significantly more migratory toward Fibroblast condition media (FbCM) than the GFP control cells (Fig. 3.8B). The results indicate that similar to the findings from ectopic expression of HSF1 in the H-Ras^{V12} transformed MCF10A cells, activation of HSF1 in cells expressing activated Ras significantly enhanced the 3-D growth and migration of these cells.

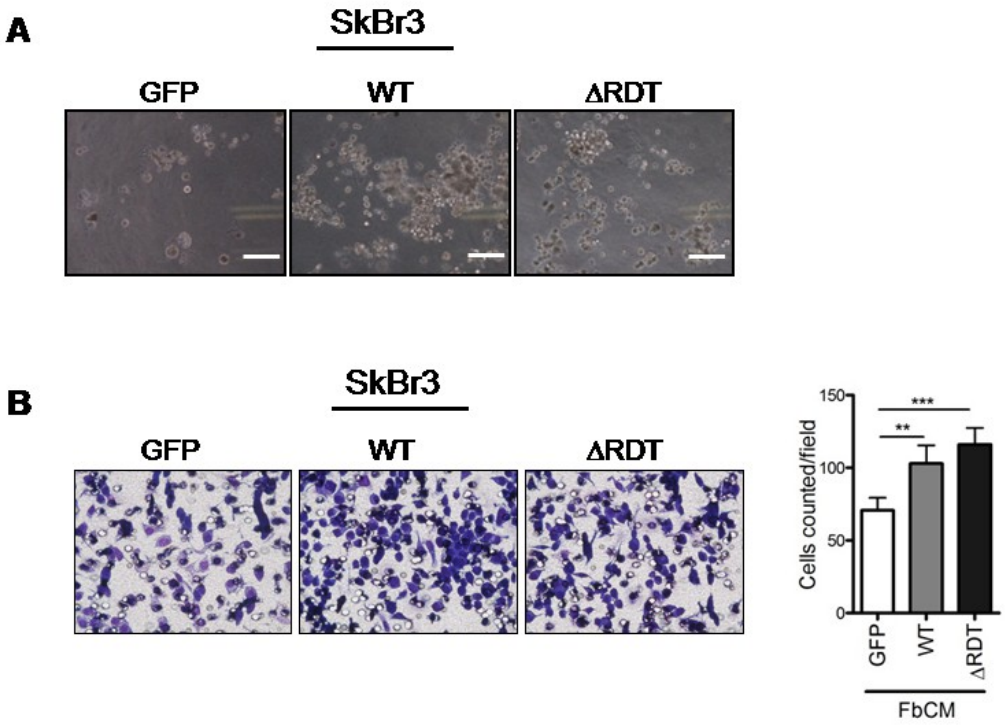


Figure 3.8. Ectopic expression of HSF1 enhances 3-D growth and cell migration of SkBr3 cells

(A) Ectopic expression of HSF1 enhances growth of SkBr3 cells in 3-D conditions. Representative images of acini at 100X magnification are shown. Scale bar – 200μM. **(B)** Ectopic expression of HSF1 significantly enhances cell migration of SkBr3 toward FbCM. Representative images of the migration membranes at 400x magnification are shown. The number of cells migrated are represented as the mean±sd. The results are representative of at least three independent experiments.

3.2.6. HSF1 activation does not alter signalling pathways downstream of Ras or EGFR.

To explain the effect of HSF1 activation upon 3-D growth and cell migration, we examined whether HSF1 activation could enhance Ras downstream signalling as previous studies have indicated that HSF1 contributes to signal transduction pathway integrity (Lo et al., 2000; Xi et al., 2012). Major Ras downstream signalling pathways include the Raf/MEK/Erk and the PI3K/Akt pathways, thus the signalling molecules

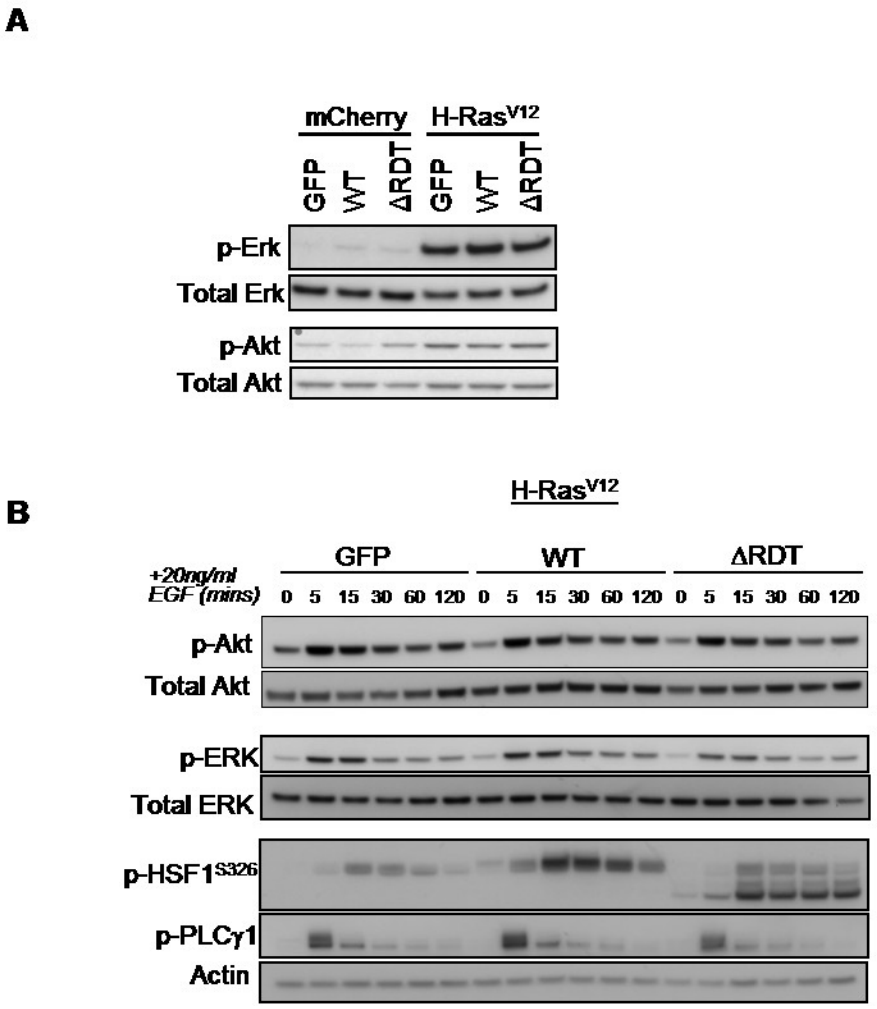


Figure 3.9. Western blot analysis demonstrating that ectopic expression of HSF1 has no impact signalling pathways downstream of Ras.

(A) Ectopic expression of H-Ras^{V12} up-regulated levels of phosphorylated Erk and Akt while ectopic expression of HSF1 had no impact upon the total and phosphorylated levels of these proteins in both the mCherry non-transformed and H-Ras^{V12} transformed cells. **(B)** Ectopic expression of HSF1 had no impact upon levels of total and phosphorylated Erk, Akt and PLCγ of the H-Ras^{V12} transformed cells following EGF treatment, which also activates HSF1 phosphorylation.

Erk and Akt were examined by western blot for levels of total protein expression and levels of phosphorylation. As illustrated in Fig.3.9A, as expected, western blot analysis showed that ectopic expression of H-Ras^{V12} increased levels of phosphorylated Erk1/2 and Akt. Ectopic expression of HSF1wt or HSF1ΔRDT, however, did not impact upon Erk1/2 or Akt activation or protein expression.

In addition, to examine if HSF1 enhanced the activation of Ras downstream signalling pathways after growth factor stimulation, the H-Ras^{V12} transformed MCF10A were serum starved overnight, stimulated with serum or epidermal growth factor (EGF) and then protein lysates were analysed by western blot analysis to determine levels of phosphorylated Erk1/2 and Akt. As illustrated in Fig.3.9B, EGF treatment induced HSF1 activating phosphorylation on serine 326. However, ectopic expression of HSF1 did not impact upon the Erk1/2 and Akt signalling pathways downstream of Ras, indicating that the enhanced migration and invasion observed in cells with ectopic expressions of both HSF1 and H-Ras^{V12} were not due to enhanced Ras signal transduction through these signalling pathways.

Previously, Kouspou (2009) demonstrated that inhibition of HSF1 reduces phospholipase C γ1 (PLC γ1) signal transduction pathway in TNBC cells following EGF stimulationtion. Similar to Ras, PLCγ1 is also a second messenger molecule that is activated by RTKs and transfers signals to downstream pathways that regulate many processes involved in cancer progression. However, western blot analysis revealed that ectopic expression of HSF1 also had no impact upon the activation of PLCγ1 in the H-Ras^{V12} cells following EGF stimulation (Fig.3.9B). This indicates that the enhanced migration toward EGF of the H-Ras^{V12} cells with ectopic expression of HSF1 was also not due to enhanced PLCγ1 signal transduction.

3.2.7. Ectopic expression of HSF1ΔRDT had unique impact upon gene expression in the H-Ras^{V12} transformed MCF10A cells compared to that in the mCherry non-transformed cells.

To further investigate the mechanism by which HSF1 may exert its cancer promoting effects in Ras transformed cells, gene expression microarray analysis was performed to examine changes at the mRNA level upon ectopic expression of HSF1ΔRDT in the mCherry non-transformed and in the H-Ras^{V12} transformed MCF10A cells. Cells were

cultured in 3-D growth conditions as in 3.2.3 for 12 days and total RNA was extracted. The RNA samples were analysed by a gene expression microarray. The array was conducted in triplicate using three different RNA samples for each sample, with the significant difference for each gene being determined by combining the data from the three arrays. Genes that were up-regulated and down-regulated by at least 2 fold in the HSF1ΔRDT cells compared to GFP control cells were identified and listed (Appendix 3). In the non-transformed cell context, a total of 252 and 220 (472 in total) genes with known identification and functions were found to be up- and down-regulated by at least 2 fold respectively in HSF1ΔRDT cells compared to GFP control cells. Interestingly, the number of genes altered by at least 2 fold due to HSF1ΔRDT ectopic expression was much higher in the H-Ras^{V12} transformed cells, with 428 and 556 (984 in total) genes identified to be up- and down regulated respectively (Appendix 3). This indicates an increase in magnitude of the impact of HSF1 activation upon gene expressions in cells with activated Ras compared to that in the non-transformed cells. In addition, comparision between the two lists of genes that were altered by ectopic expression of HSF1ΔRDT in the mCherry non-transformed and H-Ras^{V12} transformed MCF10A cells revealed 98 genes that were in common (Fig.3.10). This indicates that HSF1 exerted both common and unique effects upon gene expressions in each of these cell contexts.

The lists of genes up- and down-regulated identified by the microarray upon ectopic expression of HSF1ΔRDT were examined using Metacore^{TM} bioinformatics software (GenGo Inc., Thomson Reuters, USA) for the most significantly altered signalling pathways. Analysis of genes that were altered upon ectopic expression of HSF1ΔRDT in the non-transformed cells revealed that the cell-adhesion_extracellular matrix (ECM) remodelling pathway was the most significant pathway altered in these cells (p-value of 0.001588, Fig.3.11A). Four genes of this pathway were found to be down regulated in HSF1ΔRDT cells, which were HB-EGF, SERPINE2, Kallikrein 1 and Kallikrein 3 (PSA) and one gene was found to be up-regulated, which was Collagen II (Fig.3.12). Other cell adhesion pathways were also reduced, such as tight and gap junctions. Genes that were up-regulated upon expression of HSF1ΔRDT included those involved in cytoskeletal remodelling pathway such as myosin light chain (MELC), actin, myosin regulatory light chain (MRLC). In addition, several immune response pathways were also found to be affected upon HSF1 activation (Fig.3.11A, Table 3.1 and Appendix 4).

Analysis of genes that were altered upon ectopic expression of HSF1ΔRDT in the H-Ras^{V12} transformed cells revealed similar affected pathways that were identified in the non-transformed cells, with the most significant one also being the cell adhesion_ECM remodelling pathway and other pathways that were less significantly altered included many immune response and cytoskeletal remodelling pathways. However, the numbers of genes altered in each pathway were much higher compared to those in the non-transformed cell context, leading to higher p values and ratios of genes altered to the total number of genes in each pathway. For example, while only 5 genes out of the 52 genes currently identidied of the ECM remodelling pathway were found to have altered expressions upon ectopic expression of HSF1ΔRDT in the non-transformed cells (Fig.3.12), 12 genes were found to have altered expression upon ectopic expression of HSF1ΔRDT in the H-Ras^{V12} transformed cells (Fig.3.13). In addition to the increase in numbers of genes affected in each pathway, expression of HSF1ΔRDT in context of H-Ras^{V12} expression also led to an alteration in other novel pathways such as astrocyte conditioned media (ACM) regulation of nerve impulse and cytokine production (Fig.3.11B, Table 3.2 and Appendix 4).

Taken together, microarray analysis revealed that HSF1 activation led to a global alteration in gene expression. Major alterations in pathways identified include down-regulation of cell adhesion, up-regulation of cytoskeletal remodelling and up and down-regulation of multiple immune response pathways. Interestingly, the impact of the activation of HSF1 upon gene expression in the H-Ras^{V12} transformed context was much greater compared to that in the non-transformed context. Activation of HSF1 in the H-Ras^{V12} transformed cells also led to alterations in expression of genes of novel pathways that were not altered when HSF1 was activated in the non-transformed cells.

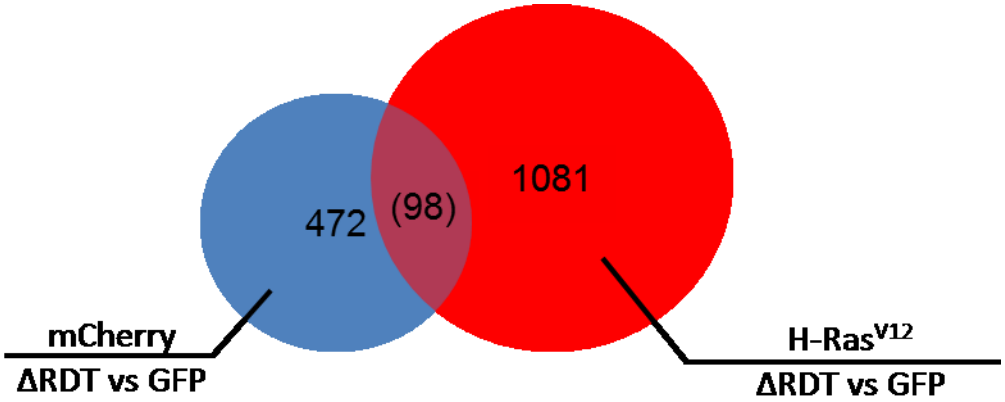


Figure 3.10. Comparison of the numbers of genes with known functions altered by at least 2 folds upon ectopic expression of HSF1ΔRDT between the mCherry non-transformed and H-Ras^{V12} transformed MCF10A cells

Gene expression microarray analysis using Metacore™ software revealed that expression of a total of 472 genes with known functions were altered upon ectopic expression of HSF1ΔRDT in mCherry untransformed cells whereas a total of 984 genes were found to be altered in H-Ras^{V12} transformed cells. Comparison between the two lists revealed 98 common genes that were altered in both cell contexts.

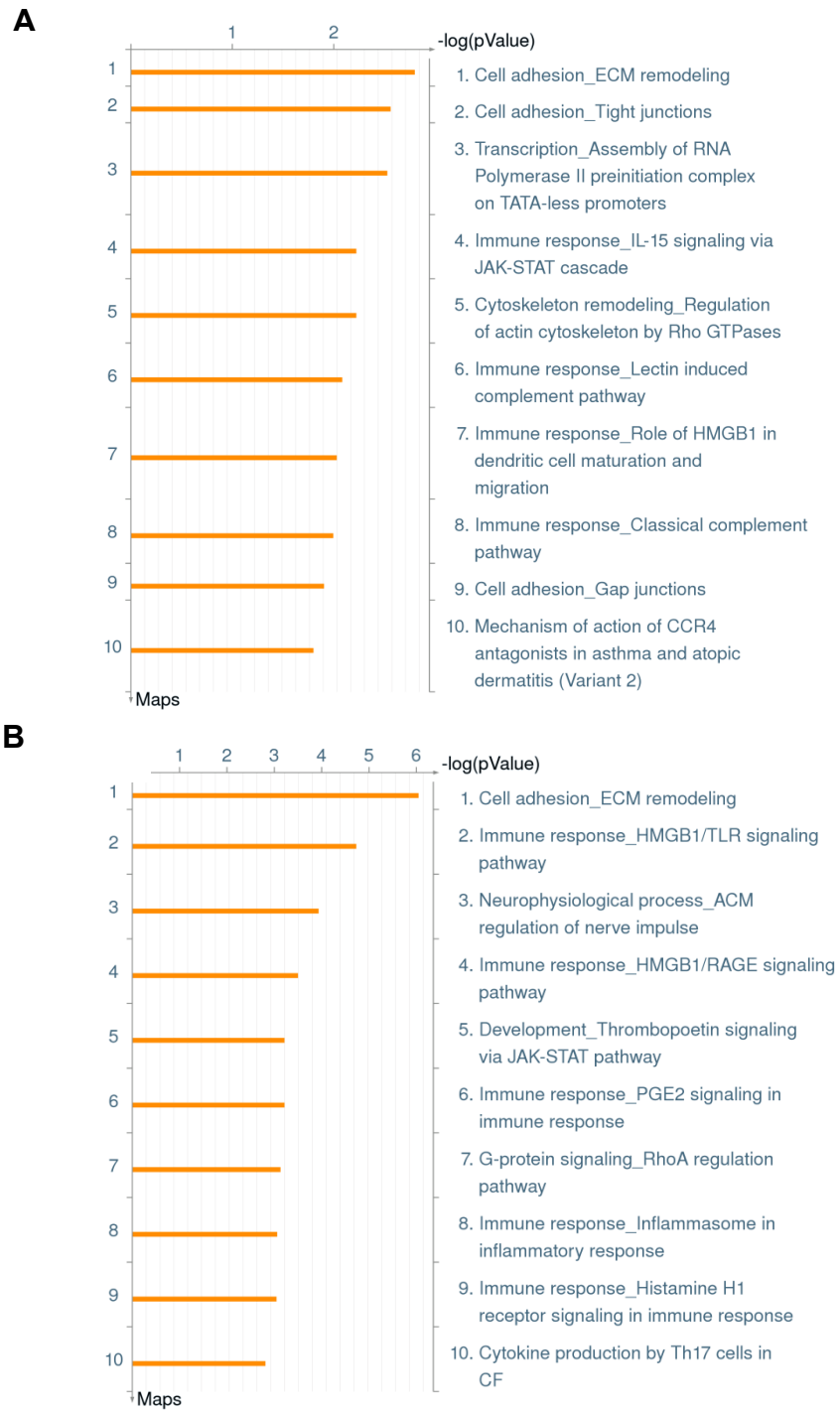


Figure 3.11. Gene array analysis by Metacore™ revealed that ectopic expression of HSF1ΔRDT in H-Ras^{V12} transformed MCF10A cells has greater impact on gene expression than in mCherry untransformed cells.

(A) Gene array analysis revealed that most significant pathway maps affected by ectopic expression of HSF1ΔRDT in mCherry untransformed MCF10A cells were cell adhesion, immune response and cytoskeletal remodelling pathways. **(B)** Gene array analysis revealed that the most significant pathway maps affected by ectopic expression of HSF1ΔRDT in H-Ras^{V12} transformed MCF10A cells were also cell adhesion and the immune responses, however, with higher p value (reflective of more genes in these pathways altered). Unique pathways were also altered such as the neurophysiological process_Astrocyte conditioned media (ACM) regulation.

A

Table 3.1. *Ten most significant pathway maps altered upon ectopic expression of HSF1ΔRDT in mCherry untransformed MCF10A cells identified by Metacore™*

mCherry untransformed cell context				
	Maps	**p values**	**Ratios**	**Genes affected**
1	Cell adhesion_ECM remodeling	1.588E-03	5/52	HB-EGF, SERPINE2, Kallikrein 1, Collagen II, Kallikrein 3 (PSA)
2	Cell adhesion_Tight junctions	2.777E-03	4/36	Claudin-2, ZO-3, Actin, JAM3
3	Transcription_Assembly of RNA Polymerase II preinitiation complex on TATA-less promoters	2.993E-03	3/18	IGFRB, p15, AML1 (RUNX1)
4	Immune response_IL-15 signaling via JAK-STAT cascade	6.116E-03	3/23	IL-2R beta chain, sIL-15RA, IL-15RA
5	Cytoskeleton remodeling_Regulation of actin cytoskeleton by Rho GTPases	6.116E-03	3/23	MELC, Actin, MRLC
6	Immune response_Lectin induced complement pathway	8.472E-03	4/49	C2, C1 inhibitor, C2b, C2a
7	Immune response_Role of HMGB1 in dendritic cell maturation and migration	9.628E-03	3/27	MHC class II, CD40(TNFRSF5), RAGE
8	Immune response_Classical complement pathway	1.042E-02	4/52	C2, C1 inhibitor, C2b, C2a
9	Cell adhesion_Gap junctions	1.289E-02	3/30	ZO-3, Actin, Connexin 43
10	B Mechanism of action of CCR4 onists in asthma and atopic dermatitis (Variant 2)	1.650E-02	1/1	CCR4

Table 3.2. *Ten most significant pathway maps altered upon ectopic expression of HSF1ΔRDT in H-Ras^{V12} transformed MCF10A cells identified by Metacore™*

H-Ras^{V12} transformed cell context				
	Maps	**p values**	**Ratios**	**Genes affected**
1	Cell adhesion_ECM remodeling	8.978E-07	12/52	HB-EGF, MMP-13, Stromelysin-2, Kallikrein 1, MMP-1, Stromelysin-1, IL-8, LAMA4, IGF-2, PLAU (UPA), Kallikrein 3 (PSA), Laminin 5
2	Immune response_HMGB1/TLR signaling pathway	1.959E-05	8/36	IRF7, IRAK1/2, IL1RN, MIP-1-alpha, IL-8, TLR4, TLR2, RAGE
3	Neurophysiological process_ACM regulation of nerve impulse	1.262E-04	8/46	PKC, N-type Ca(II) channel alpha1B, P/Q-type calcium channel alpha-1A subunit, CACNA1G, G-protein alpha-i family, PKA-cat (cAMP-dependent), G-protein alpha-o, ACM3
4	Immune response_HMGB1/RAGE signaling pathway	3.497E-04	8/53	IL1RN, ICAM1, MIP-1-alpha, IL-8, Secretogranin II, TLR4, TLR2, RAGE
5	Development_Thrombopoetin signaling via JAK-STAT pathway	6.794E-04	5/22	Oncostatin M, TAP1 (PSF1), SHPS-1, STAT5, Thrombopoietin
6	Immune response_PGE2 signaling in immune response	6.800E-04	7/45	COX-2 (PTGS2), PGE2R2, COX-1 (PTGS1), IL-8, GM-CSF, PKA-cat (cAMP-dependent), SLC21A2
7	G-protein signaling_RhoA regulation pathway	8.289E-04	6/34	LyGDI, Fyn, BMX, PLD1, RhoGDI gamma, GRAF
8	Immune response_Inflammasome in inflammatory response	9.727E-04	6/35	CARD7, P2X7, Nod2 (CARD15), CARD5, TLR4, NALP3
9	Immune response_Histamine H1 receptor signaling in immune response	1.012E-03	7/48	MMP-13, ICAM1, MMP-1, Stromelysin-1, IL-8, GM-CSF, NF-AT2(NFATC1)
10	Cytokine production by Th17 cells in CF	1.747E-03	6/39	GRO-1, ICAM1, IL23A, IL-8, TLR4, GM-CSF

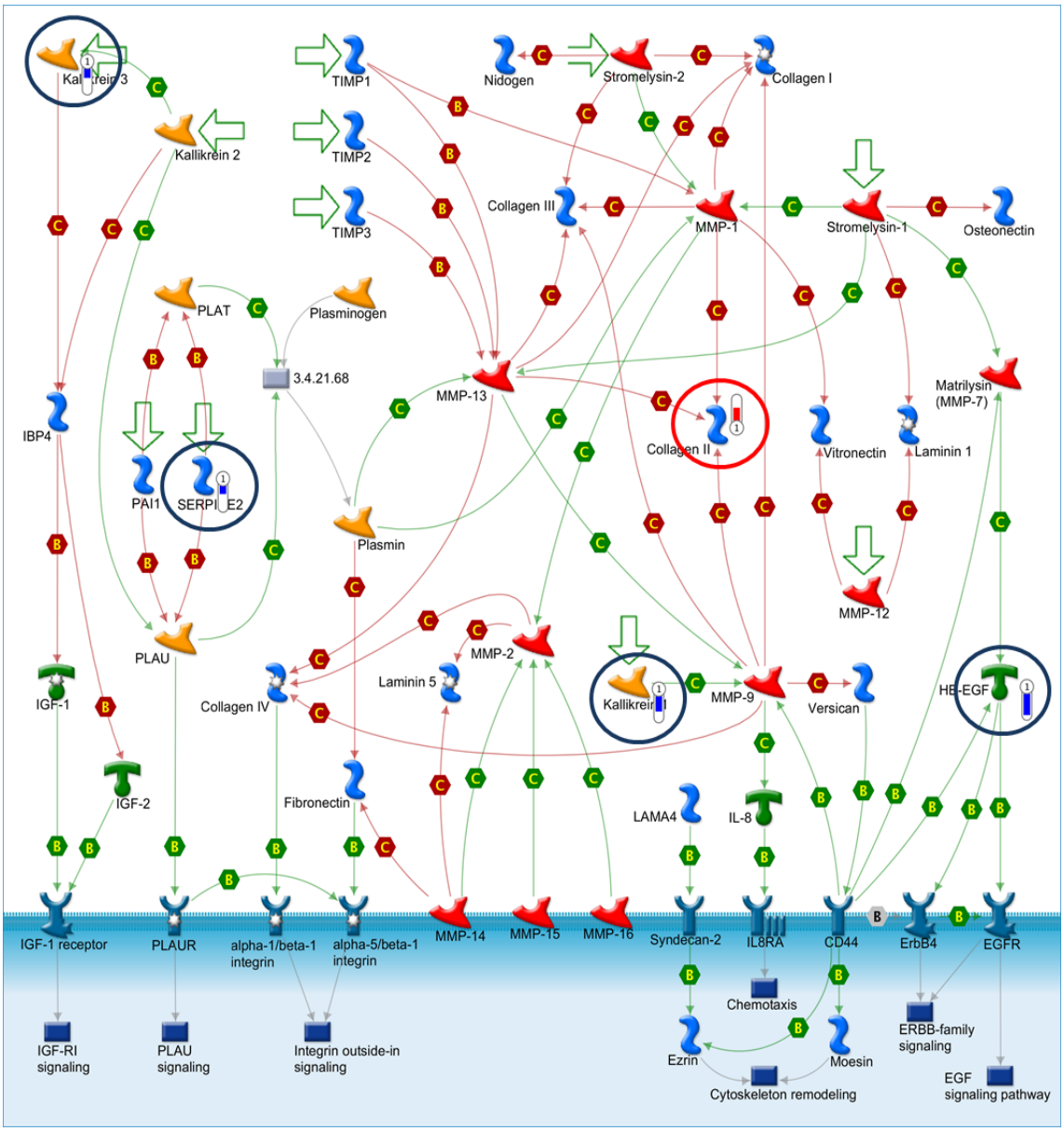


Figure 3.12. Gene analysis using Metacore™ software revealed that the most significantly altered pathway map affected upon ectopic expression of HSF1ΔRDT in the mCherry non-transformed MCF10A cells was cellular adhesion_ECM remodelling

Analysis by Metacore™ software found that HB-EGF, SERPINE2, Kallikrein 1 and Kallikrein 3 were down regulated while Collagen II was up-regulated in the cellular adhesion pathway map upon ectopic expression of HSF1ΔRDT in the non-transformed MCF10A cells. Red and blue gauges present next to gene demonstrate its up-regulation and down-regulation in the pathway, respectively. B-binding. C-Cleavage.

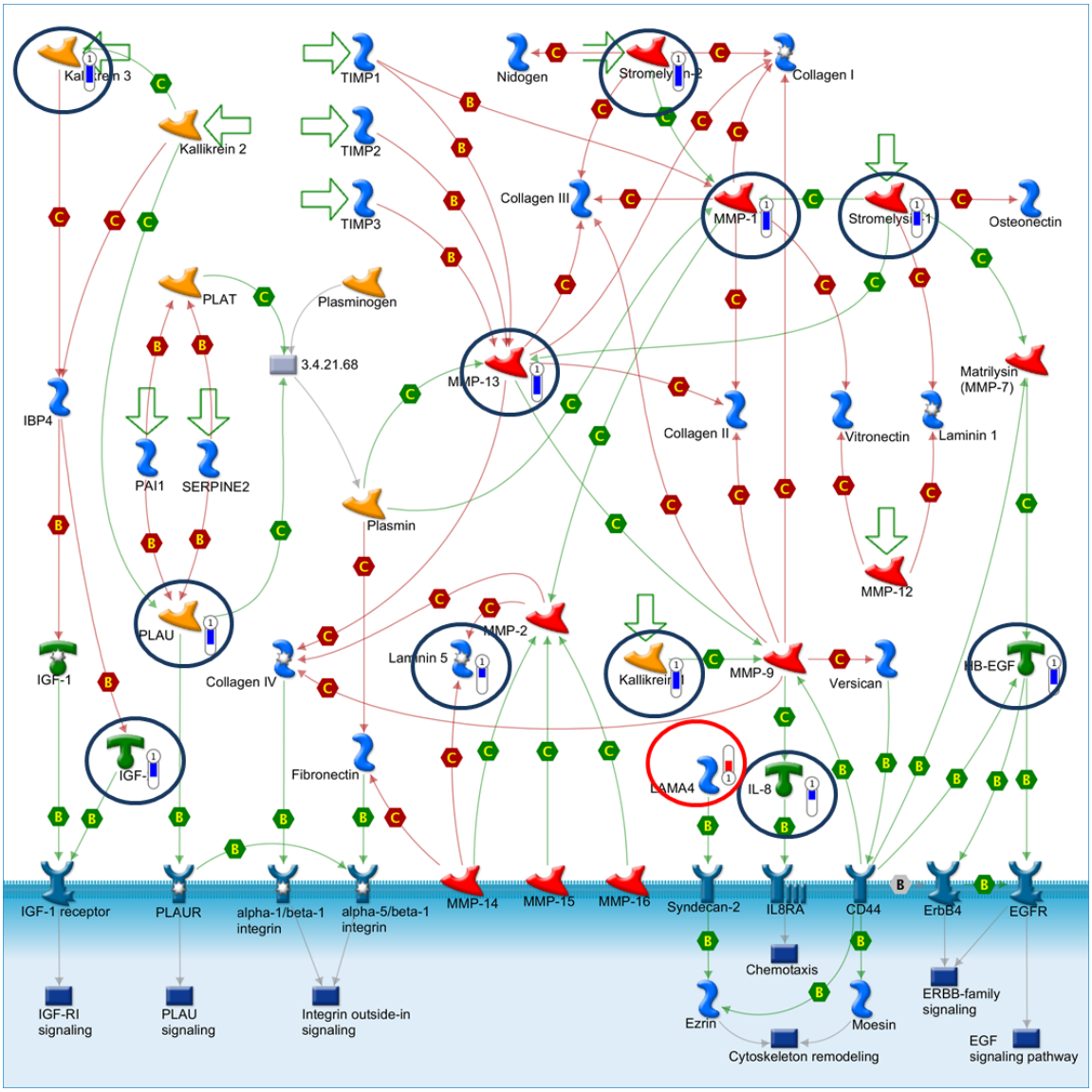


Figure 3.13. Gene analysis using Metacore™ software revealed that the most significantly altered pathway map affected upon ectopic expression of HSF1ΔRDT in the H-Ras^{V12} transformed MCF10A cells was Cellular adhesion_ECM remodelling.

Analysis by Metacore™ software identified 12 genes of the ECM remodelling pathway affected by ectopic expression of HSF1ΔRDT in the H-Ras^{V12} transformed MCF10A cells. Eleven genes were down regulated, which were Laminin 5, MMP-13, Stromelysin-2, MMP-1, Stromelysin-1, IL-8, IGF-2, PLAU (UPA), Kallikrein 1, HB-EGF and Kallikrein 3 (PSA) and 1 gene was up-regulated, which was LAMA4,. Red and blue gauges present next to gene demonstrate its up-regulation and down-regulation in the pathway, respectively. B-binding. C-Cleavage.

3.3. DISCUSSION

HSF1 has been identified as a powerful multifaceted modulator of cancer through regulating transcriptional networks distinct to many malignant states. As the mechanisms for the roles of HSF1 in cancer are relatively unknown, this study presented work which demonstrated that HSF1 co-operates with oncogenic Ras to activate a variety of transcriptional networks that promote tumourigenesis and cancer progression, especially with respect to enhancing cell migration and invasion, highlighting the context dependency of the role of HSF1 in cancer.

3.3.1. HSF1 activation does not affect cell morphology and proliferation.

Changes in morphology and proliferation are important factors contributing to tumourigenesis and cancer progression. In particular, the morphological switch from epithelial to mesenchymal (EMT) allows cells to escape the growth control of neighbouring cells through cell-cell contact and enhances cell motility (Larue and Bellacosa, 2005). Many activated oncogenes induce cancer initiation and progression through the promotion of morphological changes enabling escape from growth suppression and supporting unlimited proliferation (Drasin et al., 2011). Consistent with this, the current study shows that expression of activated Ras in MCF10A cells induces the EMT and enables cells to grow in limiting media conditions.

It has been shown that inhibition of HSF1 in cancer cells reduces cell proliferation and HSF1 knockdown cells exhibit reduced EMT induced by TGFβ and the ectopic expression of ErbB2 (Nakamura et al., 2010; Xi et al., 2012), it was expected that ectopic expression of HSF1 would enhance EMT and cell proliferation in a 2-D growth assay of MCF10A cells. However, the present study shows that HSF1 activation does not have a marked impact upon the morphology and 2-D growth of both the non-transformed and H-Ras^{V12} transformed cells. Similar results were also observed when active HSF1 was expressed in the breast cancer cell line SkBr3. This demonstrated that activation of HSF1 is not sufficient to induce EMT and enhance growth of cancer cells within these contexts. Together with previous studies, it is suggested that HSF1 is required for EMT and growth induced by activated oncogenes; however, when insufficient signalling from the appropriate pathways exists; ectopic expression of HSF1 has no impact on these phenotypes. This is consistent with the notion that HSF1 is not

an oncogene *per se* but rather functions as an enhancer of cancer progression by supporting the maintenance of malignant phenotypes induced by other genetic and epigenetic alterations within the tumour cells.

3.3.2. HSF1 activation enhances cell migration of cells with activated Ras

Cell migration is an essential and highly regulated process required for normal physiological conditions such as tissue formation during embryonic development, wound healing and the immune response (Ridley et al., 2003). This process involves a continuous cyclic process, which is initiated by the sensing of a chemotactic gradient from the microenvironment that promotes cell polarisation and the formation of membrane protrusions via actin polymerisation. The protrusions extend towards the desired direction of cell movement defined by the chemogradient and attach onto the ECM fibers, creating new contacts called focal adhesions. The cell cytoplasm then contracts and promotes the disassembly of the focal contacts at the trailing edge allowing the cells to move forward (Ridley et al., 2003). In cancer, the acquisition of cell motility is an important step in tumour progression. The increased ability of tumour cells to migrate is strongly associated with cancer aggressiveness as it allows the cells to evade the surrounding tissues, and thereby intravasate into the circulation and metastasise to distant organs. Understanding the molecular mechanisms behind this process has been a major focus of cancer studies to identify therapeutic targets which can inhibit cancer metastasis.

To date, there have been several studies that demonstrated that HSF1 is required for cell migration. MEFs from HSF1 knockout mice exhibit a reduced ability for wound closure in basal and in EGF-induced conditions when compared to their wild-type counterparts (O'Callaghan-Sunol and Sherman, 2006). Inhibition of HSF1 by pharmacological inhibitors or expression of a dominant negative HSF1 (HSF1-DN) reduces cell migration of TNBC cells (Kouspou, 2009). In hepatocellular carcinoma (HCC), knockdown of HSF1 by shRNAmir reduces cell migration *in vitro* and metastasis *in vivo* (Fang et al., 2011). The present study supports and extends these findings by illustrating for the first time that activation of HSF1 by ectopic expression of HSF1wt or HSF1ΔRDT enhances cell migration in both basal and EGF induced conditions as well as wound closure of breast transformed epithelial cells. In addition to this, this study is also first to demonstrate divergent effects of HSF1 on migration between the non-

transformed and Ras transformed cellular contexts, indicating the context dependence of HSF1 activities in enhancing cell migration. These results also confirm the notion that activation of HSF1 during cancer progression may foster the malignant phenotype and increase cancer aggressiveness.

Cells migrate in response to specific chemo-attractant signals such as growth factors and chemokines. These chemo-attractants facilitate directional cell migration by binding and activating cell surface receptors and promoting intracellular signal transduction pathways that regulate molecules involved in cytoskeletal rearrangement. Specifically, EGF has been shown to induce cell migration through activation of Erk, PLCγ1 and PI3K/Akt pathways (Jiang et al., 2006; Li et al., 2009; Shien et al., 2004). These signalling pathways are mediated through Ras (Li and Sparano, 2003). Consistent with this, the present study shows that activation of Ras by ectopic expression of H-Ras^{V12}, which leads to the constitutive activation of these signalling pathways, enabled cell migration and significantly enhanced wound closure of MCF10A cells.

To explain the role of HSF1 in cellular migration, it was previously reported that *hsf1-/-* MEF cells have reduced basal and EGF-stimulated cell migration due to the reduced activation of the MAPK/Erk signalling pathway downstream of EGF (O'Callaghan-Sunol and Sherman, 2006). Recently, Dai et al. (2012) also reported that HSF1 deficiency in mice impeded neurofibromatosis type 1 (NF1)-associated carcinogenesis by attenuating oncogenic Ras/MAPK signalling (Dai et al., 2012). In addition, Xi et al. (2012) reported that deletion of HSF1 in mice overexpressing ErbB2/Neu significantly reduces mammary tumorigenesis and metastasis as the HSF1 knockout cells did not exhibit activated Erk1/2 and showed reduced EMT in the presence of TGFβ (Xi et al., 2012). However, in contrast to these findings, the present studies reveals that ectopic expression of HSF1 in both the non-transformed and H-Ras^{V12} transformed MCF10A cells does not alter the levels of phosphorylation of Erk1/2 and Akt at basal condition or following EGF stimulation. This is in line with the study by Kouspou (2009) that shows no significant difference in Erk1/2 and Akt signalling pathways between MDA-MB-231 cells expressing HSF-DN and vector control cells following EGF treatment (Kouspou, 2009). The reason for the discrepancy between the results in this study and other previous studies will requires

further investigation. However, a possible explanation is that the impact of HSF1 upon signal transduction cascades is likely to be cell line and context specific.

Although Kouspou (2009) reported that HSF1 does not regulate the Erk1/2 and Akt signalling pathways in human breast cancer cells, HSF1 inhibition was shown to reduce the level of phosphorylated PLCγ1 in MDA-MB-231 cells after EGF stimulation. Activation of PLCγ1 pathway is known to promote cell migration and invasion as it plays a key role in facilitating actin polymerization required for cell motility (Mouneimne et al., 2004; van Rheenen et al., 2007). Although the present study reveals that ectopic expression of HSF1 does not affect the level of phosphorylated PLCγ1 in the H-Ras^{V12} MCF10A transformed cells, HSF1 in fact enhances PLCγ1 signalling pathway in SkBr3 cells (Appendix 2). It is therefore further confirmed that activity of HSF1 upon signalling transduction cascades is cell line specific and the enhancement effect of HSF1 upon cell migration and invasion would be mediated partly through the modulation of the PLCγ1 signalling in some cancer cell lines.

3.3.3. HSF1 activation promotes disorganized 3-D growth of cancer cells with activated Ras

Local microenvironments or niches play an important role in regulating cell behaviour *in vivo* (Lu et al., 2012). A major component of the cell microenvironment is the ECM, which is composed of a complex mixture of biochemically distinct components including collagen, non-collagenous glycoproteins and proteoglycans. These components are produced by the resident cells and secreted into the ECM by exocytosis (Lu et al., 2011). The ECM exists in two forms: the interstitial matrix that fills the intercellular space and the basement membrane which is a thin layer of ECM gel that forms at the basal surface of many cell types, including epithelial cells. Basement membranes are tightly cross-linked networks of four major components, which are type IV collagen, laminin, nidogen/entactin, and perlecan. In contrast, the interstitial matrix is less compact and is mainly composed of fibrillar collagens, proteoglycans, and various glycoproteins such as tenascin, fibronectin and vitronectin. Normal ECM dynamics are essential for the maintenance of tissue integrity and homeostasis. The ECM can also provide attachment sites for cell surface receptors and serves as a reservoir of cytokines and growth factors. Additionally, as components of the ECM may function as a barrier, anchorage site or movement track, the ECM can

exert both negative and positive roles in cell migration (Werb, 2010; Werb and Chin, 1998)

Upon dissociation and culture on plastic substrata in standard 2-D tissue culture conditions, non-malignant cells rapidly lose many aspects of their differentiated states and express phenotypes that otherwise characterise tumour cells *in vivo*. The functional and morphological differentiation of the cells can be largely restored when cells are grown in a reconstituted basement membrane such as Matrigel, which provides crucial cues of the ECM that cells normally respond to *in vivo*. In this 3-D tissue culture context, non-malignant cells normally undergo a small number of cell divisions and then organize into polarised, growth-arrested colonies with defined organized architectures (Debnath et al., 2003). In contrast, malignant cells, including both established cell lines and cells from primary tumours, adopt a variety of colony morphologies, which are common in the loss of tissue polarity, a disorganized architecture and a failure to arrest growth (Kenny et al., 2007). Consistent with these, the present study demonstrates that MCF10A cells when grown in Matrigel, formed well-defined spherical acini with hollow lumen. Upon expression of activated Ras, the cells form spherical acini structures with a filled lumen. As cells grown in this 3-D growth context better reflects the actual behaviours of cells *in vivo*, this tissue culture technique is a reliable method to study cell invasion and progression *in vitro* (Kenny et al., 2007; Lee et al., 2007).

Previously, HSF1 has been postulated to promote invasion by its association with the metastasis associated protein MTA1 and the transcriptional repression of anti-metastatic estrogen responsive genes (Khaleque et al., 2008; Khaleque et al., 2005). In addition, Kouspou (2009) demonstrated that MDA-MB-231 cell line expressing HSF1-DN exhibited decreased lung metastases compared to the wild-type cells in a xenograft model (Kouspou, 2009). Moreover, Fang el al. (2011) demonstrated that HSF1 shRNA expression reduced cell invasion and metastasis of HCC (Fang et al., 2011). While all previous studies demonstrating the effect of HSF1 on cell invasion by knocking down HSF1, the present study extends these findings by showing that HSF1 activation promotes invasive growth in the 3-D reconstituted basement membrane. Importantly, the present study is the first to demonstrate that HSF1 co-operates with Ras to promote invasive growth.

3.3.4. HSF1 co-operates with activated Ras activation to regulate gene expression

HSF1 is known to regulate diverse networks of genes that function in a variety of biological processes (Mendillo et al., 2012; Page et al., 2006). In agreement with this, the present study has identified many genes that were altered upon ectopic expression of HSF1ΔRDT. In addition, the present study has also revealed the differential impact of ectopic expression of HSF1 upon gene expression within different cellular contexts. While HSF1 appears to not exert its phenotypic effects through the alteration of a number of Ras downstream signalling pathways, microarray gene expression analysis revealed that the observed impact of HSF1 activation upon cell migration and growth in 3-D in association with activated Ras, may be due to its role in regulating gene expressions.

3.3.4.1. HSF1 represses the expression of genes involved in ECM remodelling.

The ECM is a highly dynamic structure, which constantly undergoes a tightly regulated remodelling process where the ECM components are deposited, degraded or modified. This is achieved by redundant mechanisms that regulate the expression and function of ECM modifying enzymes including the matrix metalloproteases (MMPs). As ECM remodelling is an important mechanism whereby cell morphogenesis and differentiation can be regulated (Fata et al., 2004); in cancer, deregulation of ECM remodelling can promote cell transformation and hyperplasia, cancer progression, angiogenesis, tumour cell invasion and migration, as well as the establishment of tumours at distant sites (Lu et al., 2012).

From the microarray analysis in the present study, HSF1 activation suppresses the gene expression of several MMPs which mediate the degradation of ECM components, especially collagen. Although high levels of MMPs have been shown to be associated with poor prognosis in cancer patients as MMP-mediated ECM remodelling promotes cell migration, invasion and angiogenesis (Tetu et al., 2006), most MMP inhibitors have failed to advance to clinical stage treatments (Coussens et al., 2002). This indicates that the decrease in ECM remodelling due to the reduction in MMPs may also contribute to malignancy. In fact, an increase in collagen deposition or ECM stiffening is a characteristic of tumour stroma and has been exploited to detect cancer (Butcher et al., 2009; Sinkus et al., 2000). Breast tumours were found to predominantly arise from

dense regions that are collagen rich (Ursin et al., 2005). The ECM rigidity can cause increase in tension leading to the disruption of tissue morphogenesis. In agreement with this, it has been shown that reducing ECM tension represses malignant behaviours of mammary epithelial cells in culture (Paszek et al., 2005) and ECM stiffness can enhance cancer cell growth, survival and cell migration (Lo et al., 2000). In addition, artificially increasing collagen crosslinking in mouse mammary stroma leads to increase in ECM stiffness and promotes growth and invasion of normally non-invasive mammary epithelial cells (Levental et al., 2009). Moreover, a recent study demonstrated that with increased ECM stiffness, cells shift from contact inhibited to contact-independent growth, and the increased ECM stiffness can promote disorganized growth by disrupting the maturation of cell-cell contacts through reducing of the recruitment of E-cadherin and ZO-1 to cell junctions (Kim and Asthagiri, 2011).

Recently, Mendillo et al. (2012) has shown that HSF1 regulates genes involved in multiple processes to support many malignant phenotypes. Microarray analysis in this study has also revealed a number of genes that function in ECM remodelling; however, the exact role of this regulation in supporting cancer malignancy is currently unknown. The present study demonstrates that HSF1 may reduce ECM remodelling that would promote ECM stiffness as well as cancer cell invasion and growth and that this effect of HSF1 is significantly enhanced upon oncogenic transformation.

3.3.4.2. HSF1 up-regulates the expression of genes involved in cytoskeleton remodelling.

Aside from the reduced ECM remodelling observed at the level of gene expression, HSF1ΔRDT cells were found to up-regulate genes that are involved in cytoskeletal remodelling through the RhoA-GTPase pathway. This is in line with findings from a previous study by Kouspou et al, which demonstrated that HSF1 regulated cytoskeletal remodelling genes such as Rac1, cortactin and cofilin 1 (Kouspou, 2009). Actin cytoskeleton remodelling is a driving force that facilitates the formation of membrane protrusions, leading to increased cell migration and invasion. While it is possible that HSF1 directly regulates genes mediating cytoskeleton remodelling, additionally, it is also possible that the increase in RhoA signalling is due to a stiffer matrix caused by reduced ECM remodelling. In support of this, previous studies have shown that RhoA activity is increased in cells growing on stiffer 2-D substrate (Heck et al., 2012) and

increased tension from a stiffer matrix induces integrin clustering, the development of focal adhesions and the activation of many downstream signalling pathways including RhoA (Paszek et al., 2005). The increase in the activity of HSF1 would thus promote cancer cell invasion and metastasis via enhancing the intrinsic cell migratory capacity.

3.3.4.3. HSF1 controls the expression of genes involved in immune response pathways.

HSF1 has been reported to regulate many genes of the immune response and is a key molecule linking inflammation to cancer (Rokavec et al., 2012; Takii et al., 2010). Consistent with this, the current study shows that cells expressing HSF1ΔRDT have altered expression of several molecules mediating the immune response. While Metacore^{TM} analysis identified several pathways affected, in each pathway, HSF1 ectopic expression caused both up-and down-regulation of genes. However, as HSF1 regulated genes play roles in several biological processes in addition to the roles in the immune response pathways, although Metacore^{TM} software has identified several immune-response pathway regulated by HSF1, these pathways may have little effects in the cellular contexts of this study. The impact of the regulatory roles of HSF1 in immune response pathways upon cancer cell biology thus requires further empirical validation.

3.*3.4.4. HSF1 co-operates with Ras to control the expression of unique genes and pathways*

Microarray analysis has also identified other genes and pathways that are uniquely affected upon ectopic expression of HSF1ΔRDT in the H-Ras^{V12} transformed MCF10A. Some of the most significantly affected pathways were the neurophysiological process_ACM regulation of nerve impulse and the cytokine production pathway. Metacore^{TM} analysis has also revealed unique gene networks affected (Appendix 5). The activities of HSF1 in these pathways and networks have been documented in many previous studies (Jin et al., 2011; Uchida et al., 2011; Wirth et al., 2004); however, understanding the roles of HSF1 in these pathways upon cancer progression remains limited. As these pathways and networks extend beyond the scope of this study, further study investigating the role of HSF1 in these pathways and networks would give more insights into the role and impact of HSF1 activation in cancer, especially in the context of oncogenic Ras.

3.3.4.5. *HSF1 in different cellular contexts*

While this chapter focuses on the novel co-operation between HSF1 and Ras in supporting cancer progression, especially in cell migration and invasion, the fact that HSF1 also promotes cancer progression via its regulation and/or co-operation activities with other oncogenic proteins cannot be excluded. For example, in chapter 4 of this thesis, HSF1 is demonstrated to function as an enhancer of p53 activities. Among the cell models of this chapter, MCF10A contains wild-type p53 while SkBr3 contains a mutated p53 gene ($p53^{R175H}$). This chapter has shown that HSF1 promotes cell migration and invasion of both wild-type (MCF10A) and mutant p53 cells (SkBr3). In addition, this chapter also showed that HSF1 activation only facilitates cell migration in cells with activated Ras while having no effect on normal MCF10A cells. It is thus possible that the co-operation between HSF1 and Ras signalling pathways is the main factor contributing to the cell migration and invasion enhancement effect of HSF1.

3.4. CONCLUSION

Previous studies have shown that HSF1 regulates transcriptional program distinct from heat shock to support many malignant phenotypes in cancer. While the mechanisms are relatively unknown that enable the multifaceted role of HSF1 in cancer, the present study identifies the novel co-operation between HSF1 and Ras in supporting cancer progression. This was demonstrated through ectopic expression of the activated mutant HSF1ΔRDT in the normal mammary epithelial cell line MCF10A, in the MCF10A cells transformed with activated Ras and in the breast cancer cell line SkBr3 which is known to have up-regulated Ras activity. The current study is the first to demonstrate that HSF1 co-operates with activated Ras in the regulation of genes promoting cell migration and invasive growth in the 3-D context. This highlights the context dependency of HSF1 function, which has important implications in the targeting of HSF1 in cancer treatment

DECLARATION FOR THESIS CHAPTER FOUR

In the case of Chapter **Four**, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
- Participated in project hypothesis - Designed and performed all experimental procedure except figure 4G and 4H - Analysed data - Prepared and wrote the manuscript	75%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	**Nature of contribution**	**Extent of contribution (%) for student co-authors only**
Ben Lang	Performed experiments (Figure 2), contributed to writing of manuscript, refinements to manuscript	8
Michelle Kouspou	Participated in development of project hypothesis	
Ryan Chai	Provided technical support, contributed to refinements to manuscript	2
Jessica Vieusseux	Provided technical support, contributed to refinements to manuscript	
John Price	Supervision; project co-ordination and development of hypothesis; contributed to writing and refinements to manuscript	

Candidate's Signature	[redacted]	**Date 24/01/2013**

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

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John Price		6/12/12.

CHAPTER 4

THE EFFECT OF HSF1 UPON MAMMARY EPITHELIAL AND CANCER CELL CLONOGENICITY WITH RESPECT TO P53 STATUS

PAPER TITLE:

Heat Shock Factor 1 Impacts Both Positively and Negatively Upon Mammary Epithelial and Cancer Cell Clonogenicity Depending Upon p53 Status*

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*Running title: *HSF1 regulates clonogenicity via p53*

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Keywords: HSF1; p53; mutant; clonogenic; breast; epithelial.

ABSTRACT

HSF1 is the master regulator of the heat shock response; however, it is also activated by cancer-associated stresses and supports cellular transformation and cancer progression. We examined the role of HSF1 in relation to cancer cell clonogenicity, an important attribute of metastatic cancer cells. Ectopic expression or knockdown of HSF1 demonstrated that HSF1 positively regulated breast cancer cell clonogenicity. Furthermore, knockdown of mutant p53 indicated that HSF1 mediated its actions via a mutant p53 dependent mechanism. To more specifically examine this relationship we ectopically co-expressed mutant $p53^{R273H}$ and HSF1 in the human mammary epithelial cell line, MCF10A. Surprisingly, within this cellular context, HSF1 inhibited clonogenicity. However, when endogenous wild-type p53 was specifically knocked-down leaving mutant $p53^{R273H}$ expression intact, HSF1 greatly enhanced clonogenicity indicating that HSF1 suppressed clonogenicity via wild-type p53 actions. To confirm this we ectopically expressed HSF1 in non-transformed and $H\text{-}Ras^{V12}$ transformed MCF10A cells. As expected, HSF1 significantly reduced clonogenicity and altered p53 target gene expression levels consistent with an increased activity of p53. In line with HSF1 acting via wild-type p53 to suppress clonogenicity, knockdown of wild-type p53 rescued the inhibitory effects of HSF1. We thus show that HSF1 impacts upon clonogenicity in a context dependent manner, and more specifically can act via both mutant p53 and wild-type p53 to bring about divergent effects upon clonogenic growth. These findings have important implications for understanding HSF1's role in cancer cell growth and survival, its relationship with mutant and wild-type p53, and the potential consequences of its therapeutic targeting in differing cellular contexts

INTRODUCTION

Heat shock factor 1 (HSF1) is transcriptionally activated by cells in response to a variety of extrinsic and intrinsic stresses, including heat shock, oxidative stress, nutrient deprivation and oncogene activation (1). Its activation results in the expression of the highly conserved family of heat shock proteins (HSPs), which upon acute and chronic forms of stress, function as molecular chaperones, maintaining intracellular protein homeostasis, as well as providing cytoprotection to limit stress-induced cell death. Consistent with this role, the action of HSF1 in malignancy has long been seen as indirect, via its transcriptional regulation of HSP's and its provision of cytoprotection, however, it has recently emerged that HSF1 can directly co-ordinate a vast number of transcriptional networks that are unique to the malignant state and are distinct from the heat shock response (2). Although the exact mechanisms by which HSF1 may achieve this control are still to be fully elucidated, it is thought that the cellular context, and the unique interactions of HSF1 therein, may be an important determinant in eliciting the unique transcriptional networks.

The actions of HSF1 in regulating these networks results in the support of fundamental processes within the cancer cell that maintains its 'fitness', such as protein translation, glucose metabolism, cell cycle control and ribosome biogenesis (2,3). Consistent with this HSF1 has been shown to support and promote the oncogenic activity of a number of oncogenes such as Ras, ErbB2, Heregulin β1 and PDGF-B (3-5). Previous studies have also demonstrated that HSF1 is required for lymphoma development in p53 knockout mice and protects mice from tumours induced by oncogenic $p53^{R172H}$ (6).

Although decreased levels of HSF1 are implicated in aging and protein folding diseases, such as neurodegenerative diseases (1), consistent with a role in tumourigenesis and cancer progression, HSF1 has been shown to be increased in expression in a

number of cancer types and has been strongly associated with cancer progression and poor prognosis (2,7,8).

A feature of the malignant cancer cell is its ability to survive and grow in isolation, or its clonogenicity, marking the cancer cells ability for unlimited proliferation (9,10). This feature has been associated with cancer 'stem-like' properties that allow for increased tumour initiating and metastasis initiating capacities (10-13). It has also long been recognised that many factors can positively or negatively impact upon cancer cell clonogenicity. Amongst these, wild-type p53 has been shown to negatively impact upon clonogenicity while mutated forms of the tumour suppressor is known to increase the clonogenic capacity of cancer cells (14-16).

Wild-type p53 mediates its tumour suppressor actions via transcriptional pathways that regulate the expression of genes involved in DNA damage repair, cell-cycle arrest and apoptosis (17,18). Mutation of p53 is the most frequent genetic change identified in cancer, with more than 50% of all cancers exhibiting a loss or mutation of the gene. Expression of mutant p53 is not simply equivalent to p53 loss but can exert 'gain-of-function' properties that have been shown to be important at key stages of metastatic progression, via the promotion of cancer cell migration, invasion, survival and chemoresistance (17).

Although it has been suggested that HSF1 is required for mutant p53 activity, during genotoxic stress, HSF1 is known to mediate pro-apoptotic actions by the modulation of wild-type p53. However, previous studies have provided conflicting reports on the effect of HSF1 depletion on wild-type p53. While some studies demonstrated that HSF1 regulates wild-type p53 proteasomal degradation, leading to the increase in p53 levels and activity upon HSF1 depletion (19,20), other studies have reported that HSF1 depletion abrogates wild-type p53 activity, as HSF1 is required for normal wild-type p53 transactivation activity and nuclear translocation (18,21,22). Therefore, the full molecular and biological consequences of HSF1 activity upon wild-type p53 within cancer are still to be fully elucidated. Moreover, knowledge regarding the positive impact of HSF1 upon p53 mutant isoform action is limited.

With HSF1 emerging as an attractive therapeutic target in cancer (23), it is important to determine whether altered HSF1 activity can positively or negatively regulate clonogenicity of cancer cells, the direct or indirect downstream targets of HSF1 that mediate these actions and how these may relate to cellular context.

Herein, with both knockdown and ectopic expression approaches we demonstrate that HSF1 can positively regulate breast cancer cell line clonogenicity *in vitro*. Moreover, we demonstrate that this occurs via a mutant p53 dependent mechanism. Conversely, we show that HSF1 can also positively regulate wild-type p53, thereby inhibiting clonogenicity in both non-transformed and H-Ras^{V12} transformed human mammary epithelial cells. Our study demonstrates that HSF1 can have divergent effects upon cell clonogenicity depdendent upon the cellular status of p53 and has implications for the targeting of HSF1 in differing cellular contexts.

MATERIALS AND METHODS

Generation and Sources of Plasmid constructs.

HSF1wt cDNA was amplified from MCF10A cDNA by PCR using Flag_HSF1_Fwd (AGCTTATGGACTACAAGGACGACGATGACAAGGATCTGCCCGTGGGCCCCGGC) and EcoRI_HSF1_Rev (AATGAATTCCTCGGAGACAGTGGGGTCCTT) primers. HSF1ΔRDT cDNA was synthesized from HSF1wt cDNA using a PCR site-directed mutagenesis method as described previously (24). The HSF1wt and HSF1ΔRDT cDNAs were then cloned into the BamHI-EcoRI site of pBABEpuro IRES EGFP kindly supplied by L. Miguel Martins (Plasmid #14430, Addgene, MA, USA). The pBABEpuro_IRES_mCherry was generated by the ligation of 3 fragments: pBABEpuro-IRES-EGFP vector digested with EcoRI and SalI, an IRES with EcoRI and BstX1 overhangs and an mCherry with BstXI and SalI overhangs. Mutant p53^{R273H} gene was

excised from the vector pSUPER- $p53^{R273H}$ kindly provided by Ygal Haupt (Peter MacCallum Cancer Center, Victoria, Australia) by digestion with EcoRI and cloned into the EcoRI site of the pBABEpuro IRES mCherry vector. MSCV_mCherry and MSCV_H-Ras^{V12}_mCherry plasmids were kindly provided by Patrick Humbert (Peter MacCallum Cancer Center, Victoria, Australia) (25). All expression vector sequences were confirmed by DNA sequencing (Micromon DNA Sequencing Facility, Monash University). HSF1 targeted shRNAmir vectors were constructed as described previously (26). pGIPZ lentiviral vectors expressing shRNAmir's targeting p53 were purchased from Open Biosystems (CA, USA).

Cell lines and cell cultures.

The MCF10A cell line was obtained from ATCC and cultured as described previously (27). T47D cells were grown in RPMI 1640 media, SkBr3 cells were grown in McCoys'5A media, and HEK293T and Hs578T cells were grown in DMEM. The media was supplemented with 10% FCS and 1% Penicillin/Streptomycin. All stable cell lines were generated by retroviral or lentiviral transduction as described previously (27). Viral stocks were generated by transient transfection of appropriate viral packaging vectors into the HEK293T cell line as previously described (28).

Two-dimensional Standard Growth Assay.

Cell proliferation was examined in 96-well plates using the Sulforhodamine B (SRB) colorimetric assay as previously described (29). Briefly, cells were seeded at $2x10^4$ – $5x10^4$ cells/well in 100µl culture medium in triplicates, grown and fixed each day for 5 days in 50% trichloroacetic acid (TCA) at 4°C for 1 hour, followed by five washes in distilled water. Cells were stained with SRB (Sigma Aldrich, USA) and solubilized in 150µl of 10mM Tris-HCl, pH 10.5. Absorbance at 550nm was measured by spectrophotometry using a Multiskan FC Absorbance Plate Reader (Thermo-LabSystems, MA, USA).

Two-dimensional Clonogenic Growth Assay.

The assay was performed as described previously by Kattan et al. (30). MCF10A cells were plated at 100 cells/well and grown for 8 days. T47D cells were plated at 500 cells/well and grown for 3 weeks while SkBr3 cells were plated at $2x10^3$ cells/well and grown for 4 weeks.

Three-dimensional Adhesion-Independent Clonogenic Growth Assay.

Cells were suspended in 1.5ml of growth media containing Bacto agar (top agar) and added over a pre-hardened base agar layer (bottom agar) comprising of Bactor agar and 2ml of growth media in 6-well plates. MCF10A cells were grown in 0.4% top agar and 1% bottom agar while T47D and SkBr3 cells were grown in 0.35% top agar and 0.5% bottom agar. One ml of the appropriate cell growth media for each cell line was added to the plates and was replenished every 4 days. Cultures were stained with 0.005% crystal violet and colonies were counted using ImageJ software.

Western Blot Analysis and Antibodies.

Generation of protein lysates from cells and subsequent western blot analysis were performed as previously described (31). All blots were incubated with primary antibodies overnight at 4°C and with peroxidase-conjugated secondary antibodies for 1 hour. Protein bands were visualized by chemiluminescence (ECL, Amersham Biosciences, NJ, USA). All antibodies were purchased from commercial sources, and included anti-HSF1, anti-HSP70, anti-HSP27, anti-HSP90 (Stressgen, MI, USA), anti-HSP105/110 (Santa Cruz, CA, USA), anti-Ras (Millipore, MA, USA), anti-Pan-Actin (Neo-markers, CA, USA), anti-p53, anti-CDKN1A (p21), anti Bcl-2, anti-BAX, anti-BAD, anti-Bcl-XL and anti-XIAP (BD Pharmigen, CA, USA).

RT-qPCR and primers.

RT-qPCR was performed as previously described (28). Briefly, total RNA was isolated using the Qiagen RNeasy kit

according to the manufacturer's instructions (Qiagen, CA, USA). One to two micrograms of total RNA was used to synthesize cDNA using the superscript VILO cDNA synthesis kit (Invitrogen, CA, USA). The synthesized cDNAs underwent qPCR using the Perfecta SybrGreen SuperMix (Quanta Biosciences, MD, USA) and was performed in a Rotogene 3000 light cycler (Corbett Research, Cambridge, UK). Raw data was exported to Excel and then analysed by LinRegPCR software (HFRC, Amsterdam, Netherlands) to determine PCR efficiency (E) and threshold cycle value (Ct) (32). The level of expression of target genes was represented as relative to the expression of the housekeeping ribosomal protein gene RPL32. Differences in gene expression between samples were expressed as a ratio of the relative gene expression of the treated sample versus that of the control sample. RT-qPCR primers were designed using NCBI primer design website (www.ncbi.nlm.nih.gov/tools/primer-blast/) such that each amplicon was between 100-150 base pairs and spanned at least one intron/exon boundary. The primers used in this study were: NKDN1A_Fwd: AGCAGAGGAAGACCATGTGGACCT, NKDN1A_Rev: GGAGTGGTAGAAATCTGTCATGCTGG, BAX-Fwd: CACAGTGGTGCCCTCTCCCCAT, BAX_Rev: TCAAGGTCACAGTGAGGTCAGGGG, PIG3_Fwd: ACCCACCTCCAGGAGCCAGC, PIG3_Rev: TACTGAGCCTGGCCCCCACC, Mdm2_Fwd: TGTTTGGCGTGCCAAGCTTCT Mdm2_Rev: GGTGACACCTGTTCTCACTCACAG, TP53_Fwd: GCCAGACTGCCTTCCGGGTCACT TP53_Rev: CATCCATTGCTTGGGACGGCAAGGG, PRL32_Fwd: CAGGGTTCGTAGAAGATTCAAGGG and PRL32_Rev: CTTGGAGGAAACATTGTCAGCGATC.

Statistical analysis.

All cell biology assays were performed at least three times and data combined. Data are presented as mean ± SD. Student's t-tests were conducted to determine whether the treatment group was statistically significant compared to control. Significance is represented as * P< 0.05, ** P<0.01 and *** P<0.001.

RESULTS

Ectopic expression of HSF1 promotes the clonogenicity of breast cancer cell lines- As recent studies have demonstrated that HSF1 expression and activation are correlated with a more advanced cancer phenotype (2,7,8), we wanted to determine the effect of ectopic expression of HSF1 in less aggressive breast cancer cell lines that contained lower levels of active HSF1. To achieve this, we utilised retroviral constructs to express HSF1 (HSF1WT) or a constitutively activated HSF1 mutant (HSF1ΔRDT) in the T47D (Fig. 1A) and SkBr3 (Fig.1F) cell lines to levels comparable to more aggressive breast cancer cell lines. The HSF1ΔRDT was generated by deletion of the regulatory domain and substitution of leucine 395 with glutamic acid (L395E) thereby facilitating active HSF1 trimer formation. Consistent with increased expression and activation of HSF1, expression of both HSF1wt and HSF1ΔRDT resulted in increased levels of HSP expression levels (Fig. 1A and 1F). Within both T47D and SkBr3 cells the ectopically expressed HSF1wt was activated to levels similar to that of the constitutively activated mutant (Fig. 1A and 1F). Although HSF1 has been shown to have a role in cell proliferation, we observed no effect of HSF1 either upon cell morphology (Fig.1B and 1G) or in standard 2-D cell proliferation assays (Fig.1C and 1H). However, when the cells were examined for their ability to survive and grow to form colonies, ectopic expression of HSF1wt and HSF1ΔRDT significantly increased clonogenicity in both 2-D (Fig.1D and 1I) and 3-D (Fig.1E and 1J) assays in both cell types. This indicates that HSF1 may be more importantly required for mediating the establishment and growth of viable cell colonies during stringent and stressed growth conditions rather than in proliferation *per se*.

Knockdown of HSF1 reduces clonogenicity of breast cancer cells- To further examine the impact of HSF1 upon

clonogenic growth, we examined the consequences of HSF1 knockdown in the triple negative breast cancer cell line, Hs578T, which expresses high levels of activated HSF1. Western blot analysis revealed that knockdown of HSF1 in Hs578T cells reduced HSP27 and HSP90 (Fig.2A). Consistent with previous findings, knockdown of HSF1 in Hs578T cells did not affect morphology of the cells in 2-D conditions (Fig.2B) but significantly reduced their clonogenicity (Fig.2C).

HSF1 impacts upon clonogenicity via mutant p53 activity- HSF1 is known to regulate the expression of genes beyond that of HSPs to promote cancer progression; however, the exact mechanism whereby HSF1 achieves this is relatively unknown. Previously, it has been shown that HSF1 knockdown can reduce mutant p53 levels due to the reduction of HSP90, a required molecular chaperone for mutant p53 stability. Furthermore, and as previously discussed, mutant p53 has been shown to also have a role in cancer cell clonogenicity (14-16). As the breast cancer cell lines being examined contained mutant p53, we wanted to test whether mutant p53 acts in conjunction with HSF1 to enhance clonogenicity by initially examining downstream targets of mutant p53. Consistent with previous studies, knockdown of HSF1 reduced protein levels of mutant $p53^{V175F}$ in the Hs578T cell line (Fig.3A). Moreover, knockdown of HSF1 increased levels of CDKN1A, a wild-type p53 target, thus suggesting that HSF1 knockdown relieved the suppressing effect of mutant p53 (Fig. 3A).

We then examined the impact of ectopic expression of HSF1 upon protein level and downstream targets of mutant $p53^{L194F}$ in the T47D cells. This mutant isoform is known to suppress p53 targets such as CDKN1A, TP53I3, and Gadd45, while enhancing Bcl-2 (33,34), however, it also retains some wild-type p53 functions (35). Ectopic expression of HSF1 resulted in the reduction of CDKN1A and increased Bcl-2 protein levels, indicating that HSF1 supported mutant p53 activities (Fig.3B). Consistent with this, mRNA levels of CDKN1A were also decreased while TP53I3 mRNA levels were increased (Fig.3C). Interestingly, HSF1 also enhanced the retained wild-type activity of mutant $p53^{L194F}$ such that BAX and BAD were increased upon HSF1 expression. Critically, HSF1 expression did not significantly increase the protein level of mutant $p53^{L194F}$ in T47D cells or substantially increase HSP90 levels (Fig. 3B), indicating that HSF1 supports mutant p53 activities beyond that of enhancing its stability. To mechanistically confirm a novel relationship between HSF1 and mutant p53 in relation to clonogenicity, mutant p53 was knocked-down in the T47D model (Fig.3D). Once mutant p53 expression was reduced, HSF1 no longer possessed an enhancing affect upon clonogenecity (Fig. 3E and 3F), thus demonstrating that HSF1 acted via a mutant p53-dependent pathway.

HSF1 divergently affects clonogenicity via wild-type and mutant p53- To further confirm the involvement of mutant p53 in mediating HSF1 effects upon enhancing clonogenicity, we stably expressed mutant $p53^{R273H}$ in GFP control and HSF1ΔRDT expressing MCF10A cells. The MCF10A cell line expresses wild-type p53 endogenously (Fig.4A). Mutant $p53^{R273H}$ is one of the most common point mutants in breast cancer and can act in a dominant negative manner in relation to wild-type p53 function. However, it also possesses 'gain-of-function' activities that are emerging as important contributors to the metastatic phenotype of cancers (17). The expression of $p53^{R273H}$ enabled MCF10A cells to grow in 3-D clonogenic growth assays consistent with its known oncogenic activity (36) (Fig 4B). However, surprisingly, expression of HSF1ΔRDT suppressed 3-D clonogenicity within the context of $p53^{R273H}$ expression and endogenous wild-type p53 (Fig.4B). To determine whether endogenous wild-type p53 was a cause of this effect, specific knockdown of wild-type p53 was performed through shRNAmir targeting of the 5'UTR of p53, leaving ectopically expressed $p53^{R273H}$ mRNA intact. The clonogenicity defect mediated by HSF1ΔRDT expression was rescued and consistent with previous findings, HSF1ΔRDT was then able to promote clonogenicity in this cellular context (Fig. 4B). Furthermore, this result suggested that HSF1 could support the 'gain of function' activities of $p53^{R273H}$ in the absence of wild-

type p53, yet inhibit clonogenicity via a wild-type p53 dependent mechanism (Fig. 4B).

Ectopic expression of HSF1 reduces clonogenic survival and growth of cells via the actions of wild-type p53- To examine this latter point, that is the impact of HSF1 upon the clonogenicity of cells with a wild-type p53 background, we ectopically expressed HSF1wt or HSF1ΔRDT in non-transformed MCF10A cells (Fig. 5A). To determine whether the effects of HSF1 upon wild-type p53 actions were altered in a transformed cellular context, ectopic expression of HSF1wt or HSF1ΔRDT was performed in isogenic matched H-Ras^{V12} transformed MCF10A cells (Fig. 5A). Consistent with increased expression and activation of HSF1, expression of HSF1wt and HSF1ΔRDT resulted in increased levels of HSP expression (Fig. 5A). The expression of H-Ras^{V12} in the MCF10A cell line induced morphological changes consistent with an epithelial-mesenchymal transition (EMT) (Fig. 5B), enhanced 2-D growth in limiting media conditions (Fig. 5D) as well as increasing 2-D clonogenicity (Fig. 5E). H-Ras^{V12} expression also enabled MCF10A cells to grow in the 3-D clonogenic anchorage-independent soft agar growth assay (Fig.5C), consistent with their transformed phenotype. Consistent with previous findings in this study, HSF1wt or HSF1ΔRDT expression did not significantly impact upon the cell morphology (Fig.5B) or alter proliferation rates in 2-D standard growth assays for both the non-transformed and MCF10A H-Ras^{V12} transformed cells (Fig. 5C and 5D). Ectopic expression of HSF1wt or HSF1ΔRDT was also not sufficient in supporting MCF10A growth in the 3-D clonogenic growth assays (Fig.5F), providing evidence that HSF1 is not a '*bone-fide*' oncogene. Interestingly, consistent with the notion that HSF1 acts through wild-type p53 to inhibit clonogenicity, expression of HSF1 in both the non-transformed and MCF10A H-Ras^{V12} transformed cells significantly reduced clonogenicity under both 2-D (Fig.5E) and 3-D conditions (Fig. 5F).

HSF1 impacts upon clonogenicity through wild-type p53 activity- To determine whether HSF1 acts through wild-type p53 activity in the MCF10A cell line models, leading to the reduced clonogenicity, we initially examined p53 target expression at the protein and mRNA levels in the MCF10A cell line models. Analysis of mRNA expression of p53 and its target genes by RT-qPCR demonstrated that HSF1 expression did not alter p53 mRNA levels (Fig.6A and 6B), however, despite this, its expression significantly increased the mRNA levels of a panel of p53 positively regulated transcriptional target genes, namely CDKN1A, Mdm2, TP53I3, and BAX, in both non-transformed and H-Ras^{V12} transformed MCF10A cells (Fig.6A and 6B). In agreement with previously published findings (19,20), cells expressing HSF1wt or HSF1ΔRDT had lower levels of p53 in comparison to GFP control cells, most notably in the non-transformed MCF10A cells (Fig. 6C). However, despite this reduction, HSF1wt and HSF1ΔRDT expression still increased the levels of the p53 transcriptional target, CDKN1A (p21), in line with the RT-qPCR results, and reduced the levels of anti-apoptotic proteins, Bcl-2, XIAP and Bcl-xL (Fig. 6A), which are suppressed by wild-type p53 activity (37). Expression of HSF1wt and HSF1ΔRDT in the H-Ras^{V12} transformed cells produced similar effects upon p53 target protein levels (Fig.6C).

To mechanistically determine whether p53 is a mediator of the HSF1 inhibitory affect upon clonogenicity, we knocked-down p53 with two independent shRNAmirs in both the GFP control and HSF1ΔRDT H-Ras^{V12} transformed cells (Fig. 7A). Knockdown of p53 in the GFP control cells did not significantly increase the clonogenicity of MCF10A cells (Fig. 7B and 7C), however, p53 knockdown negated the inhibitory affect of HSF1ΔRDT expression upon clonogenicity to levels similar to that of MCF10A control cells (Fig. 7B and 7C). These results indicate that HSF1 acts via wild-type p53 to reduce clonogenicity in both non-transformed and H-Ras^{V12} human mammary epithelial cells, and also suggests that within these cellular contexts requires mutant p53 'gain-of-function' activities to enhance clonogenicity.

DISCUSSION

HSF1 acts as a master regulator of the heat shock response, however, it also facilitates malignant transformation, cell survival and proliferation by mediating distinct transcriptional networks within cancer cells (1,3-5). In addition, it is emerging that HSF1 also supports malignant progression (8,38). Consistent with this, increased HSF1 expression, activation and its nuclear localization have been associated with more advanced disease, metastasis and poorer patient outcomes {Mendillo, 2012 #43;Calderwood, 2012 #59;Santagata, 2011 #41(8).

Within this study we examined whether HSF1 impacted upon an attribute of highly malignant cancer cells, that of clonogenic growth and survival. This feature is associated with cancer 'stem-like' properties allowing for increased tumour initiating and metastasis initiating capacities (10-13). In line with the hypothesis that HSF1 supports a more advanced cancer phenotype, we identified that in a number of breast cancer cell lines, and the human mammary epithelial cell line, MCF10A, within the cellular context of mutant p53, HSF1 positively regulated clonogenicity. However, interestingly within the cellular context of wild-type p53, HSF1 actually inhibited clonogenicity.

Although HSF1 is known to have multifaceted roles in cancer and that HSF1, either directly or indirectly, regulates distinct transcriptional networks, many of the mediators required for HSF1's cell biological actions are not known. In seeking to identify mediators of HSF1 action upon clonogenicity, we identified that HSF1 impacted upon the action of wild-type p53 and mutant p53 isoforms to negatively or positively regulate clonogenicity, respectively.

These findings indicate that HSF1 may enhance tumour progression and metastasis by promoting mutant p53 isoform actions, especially with respect to their 'gain-of-function' attributes that are emerging as important contributors to the metastatic phenotype (17,36). However, paradoxically, HSF1 may also promote the actions of wild-type p53 to inhibit tumour progression. In line with our findings, Logan el al. (2009) previously reported that co-expression of HSF1 with wild-type p53 in cancer cell lines caused a significant increase in p53 activity upon genotoxic stress, impacting upon the efficacy of growth inhibition by genotoxic agents such as doxorubicin (18). Furthermore, heat shock and HSF1 activation have been shown to enhance the expression of DNA damage response and pro-apoptotic proteins upon doxorubicin treatment, as well as support p53 mediated apoptosis (39,40). Therefore, we have not only demonstrated the novel finding that HSF1 mediates its affect via a mutant p53-dependent pathway to promote clonogenicity, but we have extended the understanding that in addition to genotoxic stress, HSF1 can act to support wild-type p53 actions in also abrogating clonogenicity. The latter finding suggests that activation of HSF1 within a wild-type p53 cellular context may be beneficial in combination cancer treatment regimes. Moreover, it could be hypothesized that due to its action on wild-type p53, tumours with highly activated HSF1 may be associated with p53 mutation status.

The accumulating evidence of an important role of HSF1 in cancer growth and progression has seen it emerge as an attractive therapeutic target, however, intriguingly, both activators of HSF1, such as withaferin A and celastrol, as well as HSF1 inhibitors, such as KNK437 and Triptolide, exhibit anticancer effects (23,41). Our results indicate that the p53 status of the tumour may directly impact upon the therapeutic efficacy of such HSF1 activators or inhibitors in cancer treatment. More importantly, this should be a consideration for the future testing and development of such agents. Moreover, our results point towards the potential inhibition of HSF1 as providing a way of indirectly therapeutically targeting the diverse range of mutant p53 proteins that exist by a single targeted approach.

Although we have shown a clear functional association of HSF1 with both wild-type and mutant p53 pathways, the precise mechanism by which HSF1 achieves this still requires elucidation. However, previous studies have indicated that HSF1 can impact upon wild-type p53 activity by enhancing its translocation to the nucleus (22), which may be achieved indirectly by FKBP52, a transcriptional target of HSF1, which links p53 to dynein and the microtubule network leading to p53 nuclear transport (42). A direct

interaction between HSF1 and wild-type p53 has also been shown during genotoxic stress. This complex is then co-operatively recruited to p53-responsive genes where HSF1 enhances p53-mediated transcription (18).

Contrasting with the increased activity of wild-type p53, we observed that expression of HSF1 reduced the steady state levels of wild-type p53 in the MCF10A non-transformed cells. Wild-type p53 is a very labile protein and its level within the cell is regulated by the rate of its proteasomal degradation. In support of our findings, knockdown of HSF1 has previously been shown to increase p53 protein levels (19,20) due to a reduction of αB-crystallin, a HSF1 transcriptional target. The αB-crystallin interacts with Fbx4 ubiquitin ligase, targeting p53 for degradation and thus a reduction in its steady state levels (19). Moreover, HSF1 and HSF2 complexes have been shown to transcriptionally regulate proteasomal subunits, such as Psmb5 and gankyrin, which are also involved in p53 degradation (20). Thus, although HSF1 decreases wild-type p53 levels it also increases its transcriptional activity suggesting a complex interplay between the transcription factors.

With respect to the actions of HSF1 upon mutant p53, it has been shown that mutant p53 forms a complex with the HSF1 transcriptional target, HSP90, and this interaction stabilizes mutant p53, protecting it from Mdm2 and CHIP E3 ligase mediated proteasomal degradation (21). Consistent with this, we found that HSF1 depletion in the Hs578T cells leads to a significant reduction in mutant p53 levels. However, a concordance between the decrease in HSP90 levels and the concomitant decrease in mutant p53 levels and increase in CDKN1A levels was not clearly evident in a number of cell lines in this study. Moreover, increased HSF1 expression in the T47D cell line leads to minimal increases in HSP90 and mutant p53 levels, suggesting that the role for HSF1 in mediating mutant p53 activity extends beyond that of mutant p53 stabilization. Whether there is a direct interaction between mutant p53 isoforms and HSF1 as with wild-type p53 is currently unknown.

Whether these or additional mechanisms are utilized by HSF1 in relation to wild-type p53 and mutant p53 functionality are still to be determined.

In conclusion, this study provides novel compelling evidence of an important interplay between HSF1 and mutant and wild-type p53 in mediating disparate clonogenicity, and highlights the importance of cellular context for HSF1 mediated actions.

REFERENCES

1. Akerfelt, M., Morimoto, R. I., and Sistonen, L. (2010) *Nat Rev Mol Cell Biol* **11**, 545-555
2. Mendillo, M. L., Santagata, S., Koeva, M., Bell, G. W., Hu, R., Tamimi, R. M., Fraenkel, E., Ince, T. A., Whitesell, L., and Lindquist, S. (2012) *Cell* **150**, 549-562
3. Dai, C., Whitesell, L., Rogers, A. B., and Lindquist, S. (2007) *Cell* **130**, 1005-1018
4. Meng, L., Gabai, V. L., and Sherman, M. Y. (2010) *Oncogene* **29**, 5204-5213
5. Calderwood, S. K. (2012) *Curr Mol Med*
6. Min, J. N., Huang, L., Zimonjic, D. B., Moskophidis, D., and Mivechi, N. F. (2007) *Oncogene* **26**, 5086-5097
7. Santagata, S., Hu, R., Lin, N. U., Mendillo, M. L., Collins, L. C., Hankinson, S. E., Schnitt, S. J., Whitesell, L., Tamimi, R. M., Lindquist, S., and Ince, T. A. (2011) *Proc Natl Acad Sci U S A* **108**, 18378-18383
8. Fang, F., Chang, R., and Yang, L. (2012) *Cancer* **118**, 1782-1794
9. Talmadge, J. E., and Fidler, I. J. (2010) *Cancer Res* **70**, 5649-5669
10. Baccelli, I., and Trumpp, A. (2012) *J Cell Biol* **198**, 281-293
11. Li, L., Price, J. E., Fan, D., Zhang, R. D., Bucana, C. D., and Fidler, I. J. (1989) *J Natl Cancer Inst* **81**, 1406-1412
12. Guo, L., Fan, D., Zhang, F., Price, J. E., Lee, J. S., Marchetti, D., Fidler, I. J., and Langley, R. R. (2011) *Am J Pathol* **178**, 2357-2366
13. van den Hoogen, C., van der Horst, G., Cheung, H., Buijs, J. T., Lippitt, J. M., Guzman-Ramirez, N., Hamdy, F. C., Eaton, C. L., Thalmann, G. N., Cecchini, M. G., Pelger, R. C., and van der Pluijm, G. (2010) *Cancer Res* **70**, 5163-5173
14. Vassilev, L. T., Vu, B. T., Graves, B., Carvajal, D., Podlaski, F., Filipovic, Z., Kong, N., Kammlott, U., Lukacs, C., Klein, C., Fotouhi, N., and Liu, E. A. (2004) *Science* **303**, 844-848

15.Gerwin, B. I., Spillare, E., Forrester, K., Lehman, T. A., Kispert, J., Welsh, J. A., Pfeifer, A. M., Lechner, J. F., Baker, S. J., Vogelstein, B., and et al. (1992) *Proc Natl Acad Sci U S A* **89**, 2759-2763

16.Bartussek, C., Naumann, U., and Weller, M. (1999) *Exp Cell Res* **253**, 432-439

17.Muller, P. A., Vousden, K. H., and Norman, J. C. (2011) *J Cell Biol* **192**, 209-218

18.Logan, I. R., McNeill, H. V., Cook, S., Lu, X., Meek, D. W., Fuller-Pace, F. V., Lunec, J., and Robson, C. N. (2009) *Nucleic acids research* **37**, 2962-2973

19.Jin, X., Moskophidis, D., Hu, Y., Phillips, A., and Mivechi, N. F. (2009) *J Cell Biochem* **107**, 504-515

20.Lecomte, S., Desmots, F., Le Masson, F., Le Goff, P., Michel, D., Christians, E. S., and Le Drean, Y. (2010) *Oncogene* **29**, 4216-4224

21.Li, D., Marchenko, N. D., Schulz, R., Fischer, V., Velasco-Hernandez, T., Talos, F., and Moll, U. M. (2011) *Molecular cancer research : MCR* **9**, 577-588

22.Li, Q., and Martinez, J. D. (2011) *Molecular carcinogenesis* **50**, 143-152

23.Whitesell, L., and Lindquist, S. (2009) *Expert Opin Ther Targets* **13**, 469-478

24.Fujimoto, M., Takaki, E., Hayashi, T., Kitaura, Y., Tanaka, Y., Inouye, S., and Nakai, A. (2005) *J Biol Chem* **280**, 34908-34916

25.Dow, L. E., Elsum, I. A., King, C. L., Kinross, K. M., Richardson, H. E., and Humbert, P. O. (2008) *Oncogene* **27**, 5988-6001

26.Paddison, P. J., Cleary, M., Silva, J. M., Chang, K., Sheth, N., Sachidanandam, R., and Hannon, G. J. (2004) *Nat Methods* **1**, 163-167

27.Debnath, J., Muthuswamy, S. K., and Brugge, J. S. (2003) *Methods* **30**, 256-268

28.Lang, B. J., Nguyen, L., Nguyen, H. C., Vieusseux, J. L., Chai, R. C., Christophi, C., Fifis, T., Kouspou, M. M., and Price, J. T. (2012) *Cell Stress Chaperones*

29.Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., Bokesch, H., Kenney, S., and Boyd, M. R. (1990) *J. Natl. Cancer Inst.* **82**, 1107-1112

30.Kattan, Z., Minig, V., Leroy, P., Dauca, M., and Becuwe, P. (2008) *Breast cancer research and treatment* **108**, 203-215

31.Price, J. T., Quinn, J. M., Sims, N. A., Vieusseux, J., Waldeck, K., Docherty, S. E., Myers, D., Nakamura, A., Waltham, M. C., Gillespie, M. T., and Thompson, E. W. (2005) *Cancer Res* **65**, 4929-4938

32.Ruijter, J. M., Ramakers, C., Hoogaars, W. M., Karlen, Y., Bakker, O., van den Hoff, M. J., and Moorman, A. F. (2009) *Nucleic acids research* **37**, e45

33.Pratt, M. A., White, D., Kushwaha, N., Tibbo, E., and Niu, M. Y. (2007) *Apoptosis : an international journal on programmed cell death* **12**, 657-669

34.Vikhanskaya, F., Lee, M. K., Mazzoletti, M., Broggini, M., and Sabapathy, K. (2007) *Nucleic acids research* **35**, 2093-2104

35.Kato, S., Han, S. Y., Liu, W., Otsuka, K., Shibata, H., Kanamaru, R., and Ishioka, C. (2003) *Proc Natl Acad Sci U S A* **100**, 8424-8429

36.Rivlin, N., Brosh, R., Oren, M., and Rotter, V. (2011) *Genes Cancer* **2**, 466-474

37.Menendez, D., Inga, A., and Resnick, M. A. (2009) *Nature reviews. Cancer* **9**, 724-737

38.Xi, C., Hu, Y., Buckhaults, P., Moskophidis, D., and Mivechi, N. F. (2012) *J Biol Chem*

39.Salmand, P. A., Jungas, T., Fernandez, M., Conter, A., and Christians, E. S. (2008) *Biology of reproduction* **79**, 1092-1101

40.Vedam, K., Nishijima, Y., Druhan, L. J., Khan, M., Moldovan, N. I., Zweier, J. L., and Ilangovan, G. (2010) *Am J Physiol Heart Circ Physiol* **298**, H1832-1841

41.Santagata, S., Xu, Y. M., Wijeratne, E. M., Kontnik, R., Rooney, C., Perley, C. C., Kwon, H., Clardy, J., Kesari, S., Whitesell, L., Lindquist, S., and Gunatilaka, A. A. (2012) *ACS Chem Biol* **7**, 340-349

42.Galigniana, M. D., Harrell, J. M., O'Hagen, H. M., Ljungman, M., and Pratt, W. B. (2004) *J Biol Chem* **279**, 22483-22489

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FOOTNOTES

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[3]The abbreviations used are: 5'UTR, 5' untranslated region; BAD, Bcl-2-associated death promoter; BAX, Bcl-2 associated X; Bcl-2, B-cell lymphoma 2; Bcl-xL, B-cell lymphoma-extra large; CDKN1A, cyclin-dependent kinase inhibitor 1A; CHIP, carboxyl-terminus of hsp70 interacting protein; EMT, epithelial to mesenchymal transition; Fbx4, F-box only protein 4; HSF1, heat shock factor 1; FKBP52, FK506-binding Protein; HSP, heat shock protein; Mdm2, murine double minute 2; P53I3, p53 inducible 3; Psmb5, proteasome (prosome, macropain) subunit beta type 5; SRB, Sulforhodamine B; TCA, trichloroacetic acid; XIAP, X-linked inhibitor of apoptosis protein; WT, wild-type.

FIGURE LEGENDS

FIGURE 1. Ectopic expression of HSF1 promotes clonogenic survival and growth of breast cancer cells. Western blot analysis confirmed the expression of wild-type (WT) and constitutively active (ΔRDT) HSF1 in low aggressive breast cancer cell lines, (A) T47D and (F) SkBr3. Expression of HSF1 (WT and ΔRDT) did not alter (B and G) cell morphology and (C and H) proliferation in standard 2-D growth assays. However, both T47D and SkBr3 cells expressing HSF1 exhibited a significant increase in (D and I) 2-D and (E and J) 3-D clonogenic survival and growth when compared to GFP controls.

FIGURE 2. HSF1 knockdown reduces clonogenic survival and growth of triple negative breast cancer cell line Hs578T. Western blot analysis confirmed the knockdown of HSF1 in Hs578T cells (A). Knockdown of HSF1 did not impact upon (B) cell morphology but significantly reduced clonogenic survival and growth in (C) 2-D condition of Hs578T cells.

FIGURE 3. HSF1 stimulates mutant p53 activities. Knockdown of HSF1 in Hs578T cells reduced steady state levels of mutant p53 and enhanced expression of wild-type p53 transcriptional target CDKN1A. HSF1 expression in T47D cells altered the expression of p53 regulated targets that was consistent with HSF1 enhancing both wild-type and mutant activities of the mutant $p53^{L194F}$. (C) RT-qPCR demonstrated that HSF1 regulated the activity of mutant p53 at the transcriptional level in T47D cells with decreased expression of *CDKN1A* and increased expression of *TP53I3*. (D) Knockdown of mutant p53 in T47D GFP and T47D HSF1ΔRDT cells negated the HSF1 effects upon T47D (E and F) 2-D and (G) 3-D clonogenic growth and survival.

FIGURE 4. HSF1 stimulates both wild-type and mutant p53 activities. (A) Western blot analysis of MCF10A GFP and MCF10A HSF1ΔRDT cells expressing $TP53^{R273H}$. (B) Analysis of 3-D clonogenic survival and growth revealed that HSF1ΔRDT expression reduced clonogenicity of cells that contained both wild-type and mutant p53. With the specific knockdown of wild-type p53, HSF1ΔRDT then stimulated the 'gain-of-function' activity of mutant $p53^{R273H}$ demonstrated by the enhanced clonogenic survival and growth.

FIGURE 5. HSF1 ectopic expression reduces clonogenic survival and growth of cells with wild-type p53. (A) Western blot analysis confirmed the expression of HSF1wt and HSF1ΔRDT HSF1 in the non-transformed (mCherry) and H-Ras^{V12} transformed MCF10A cells. (B) mCherry control MCF10A cells under 2-D conditions exhibit a cuboidal, cobblestone morphology characteristic of epithelial cells. When transformed with H-Ras^{V12}, the cells underwent EMT that resulted in cells adopting a scattered and spindle-like morphology. Expression of HSF1 did not alter the cell morphology of either the mCherry control or the H-Ras^{V12} MCF10A cells. (C) mCherry control and H-Ras^{V12} MCF10A cells exhibited similar 2-D standard growth in full media conditions. (D) In limiting media, H-Ras^{V12} MCF10A cells were still able to grow after 3 days when the mCherry control cells have stopped proliferating. Ectopic expression of HSF1 did not alter proliferation of either the MCF10A mCherry control or the MCF10A H-Ras^{V12} cells in either full or limiting media conditions. MCF10A cells expressing HSF1 (WT and ΔRDT) exhibited a significant reduction in (E) 2-D and (F) 3-D clonogenic survival and growth when compared to GFP controls.

FIGURE 6. HSF1 mediates clonogenic survival and growth via modulating wild-type p53 activities.
RT-qPCR of (A) MCF10A and (B) MCF10A H-Ras^{V12} cells revealed that HSF1 expression did not alter p53 mRNA levels but enhanced p53 target gene expression, including *CKDN1A*, *Mdm2*, *TP53I3* and *BAX*. (C) Western blot analysis of mCherry control and H-Ras^{V12} transformed MCF10A cells revealed that HSF1 expression decreased steady state levels of p53 yet results in an overall increased activity of p53, demonstrated by increased protein expression of the p53 positively regulated transcriptional target, CDKN1A (p21), and a reduction in the levels of p53 negatively regulated targets XIAP, Bcl-2 and Bcl-xL.

FIGURE 7. Knockdown of wild-type p53 negated the HSF1 mediated inhibition of clonogenic survival and growth. (A) Western blot analysis confirmed the knockdown of wild-type p53 in GFP control and HSF1ΔRDT expressing MCF10A H-Ras^{V12} cells. Knockdown of wtp53 negated the capacity of HSF1 to suppress (B) 2-D and (C) 3-D clonogenic survival and growth in the MCF10A H-Ras^{V12} cell line.

FIGURES

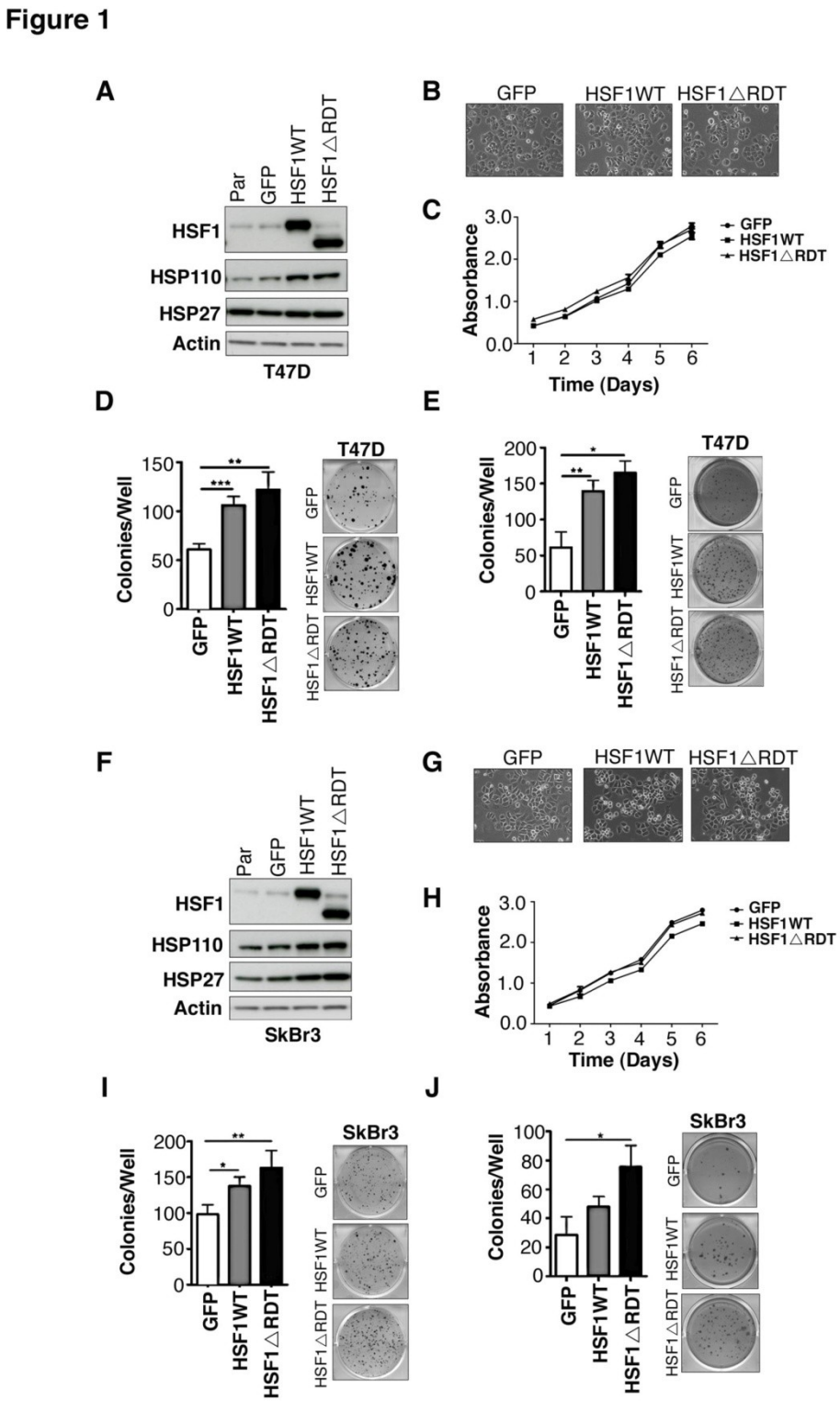


Figure 4.1. Ectopic expression of HSF1 promotes clonogenic survival and growth of breast cancer cells.

Figure 2

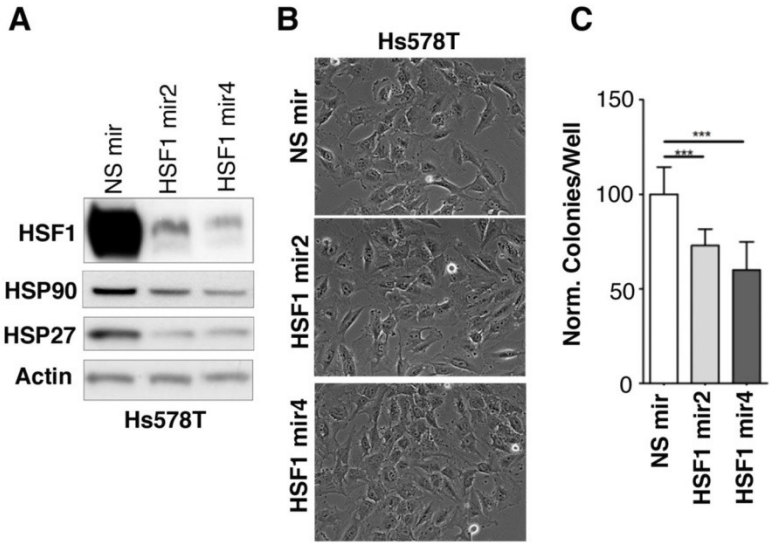


Figure 4.2. HSF1 knockdown reduces clonogenic survival and growth of triple negative breast cancer cell line Hs578T.

Figure 3

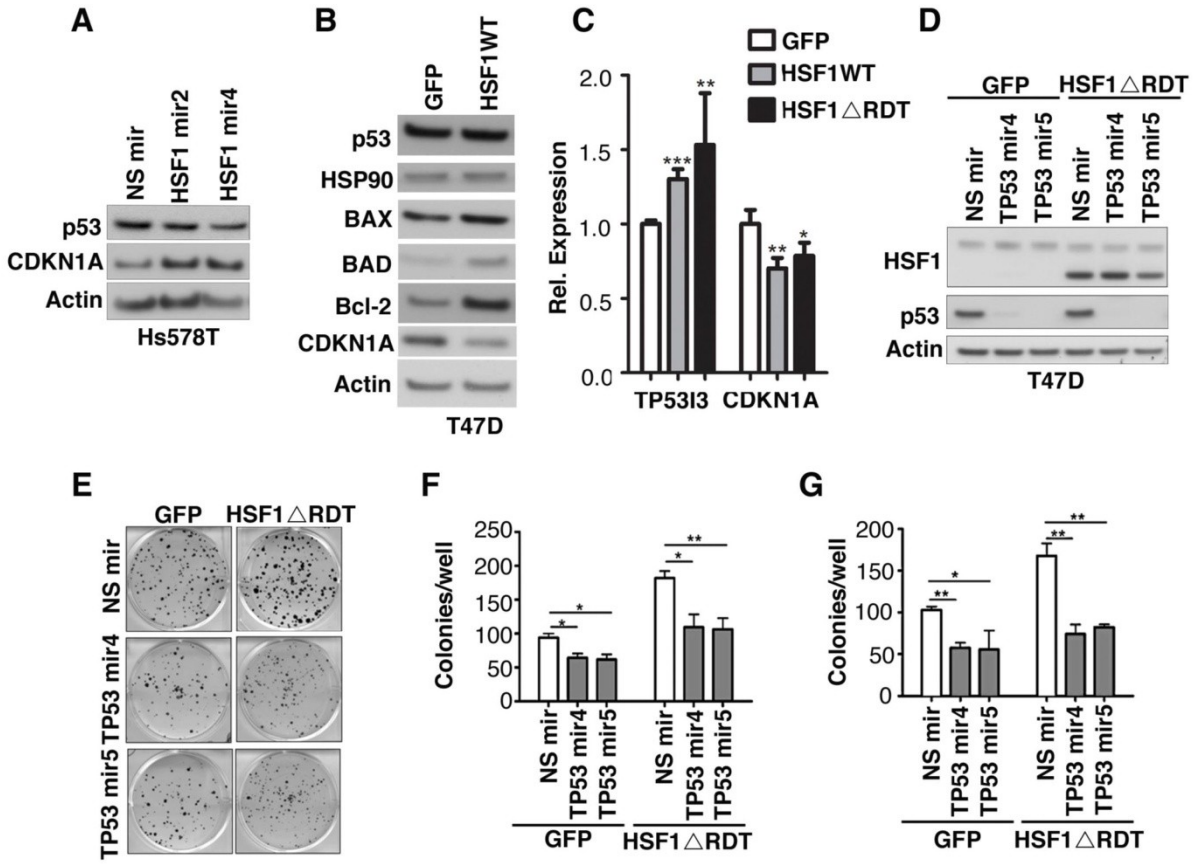


Figure 4.3. HSF1 stimulates mutant p53 activities.

Figure 4

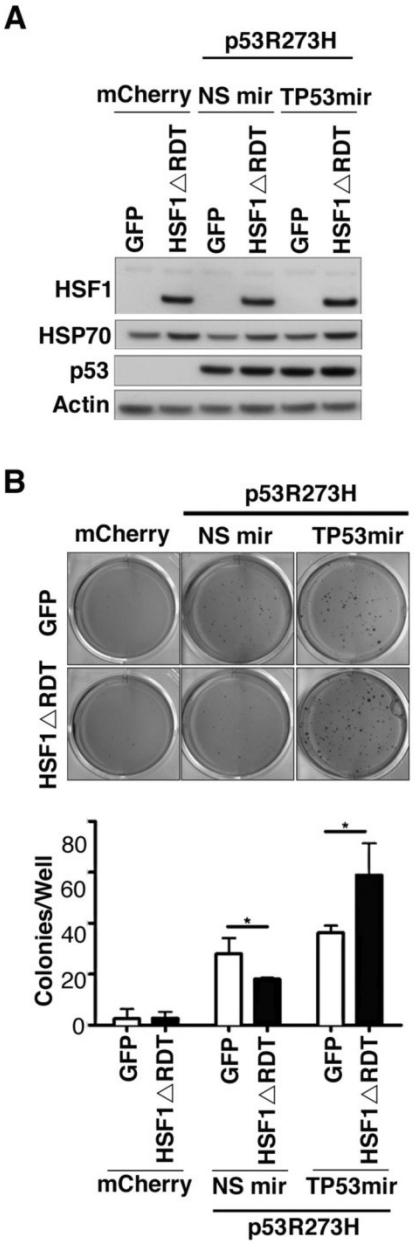


Figure 4.4. HSF1 stimulates both wild-type and mutant p53 activities

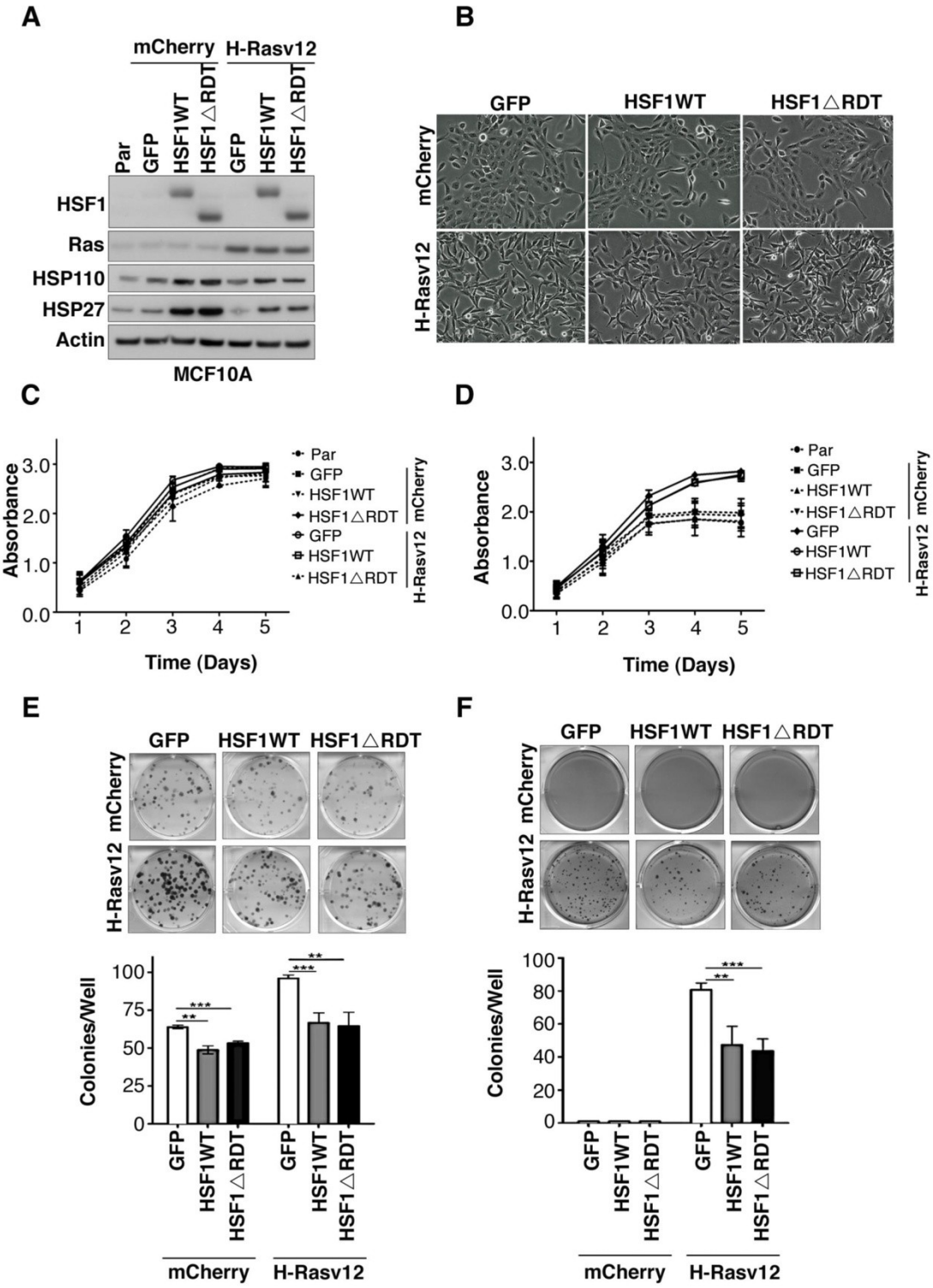


Figure 4.5. HSF1 ectopic expression reduces clonogenic survival and growth of cells with wild-type p53

Figure 6

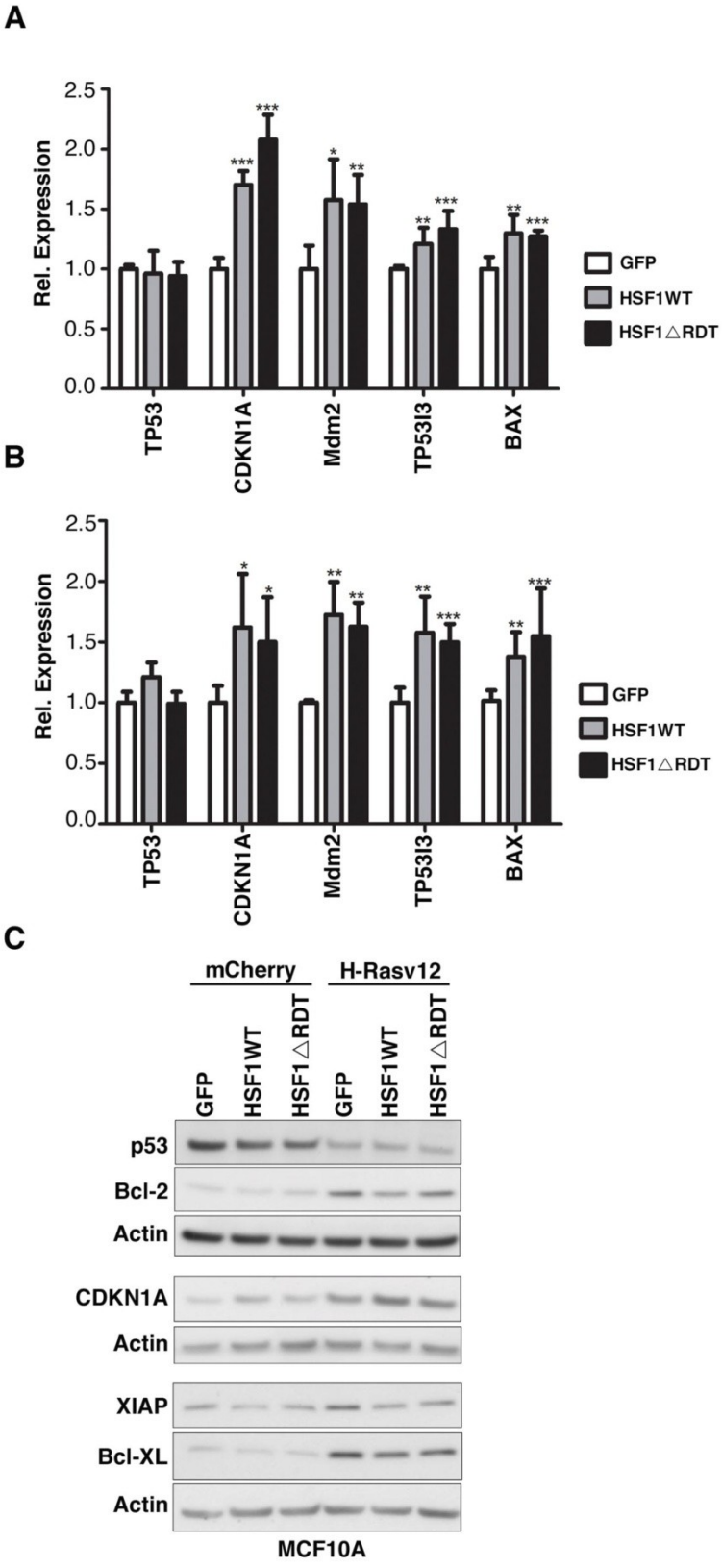


Figure 4.6. HSF1 mediates clonogenic survival and growth via modulating wild-type p53 activities.

Figure 7

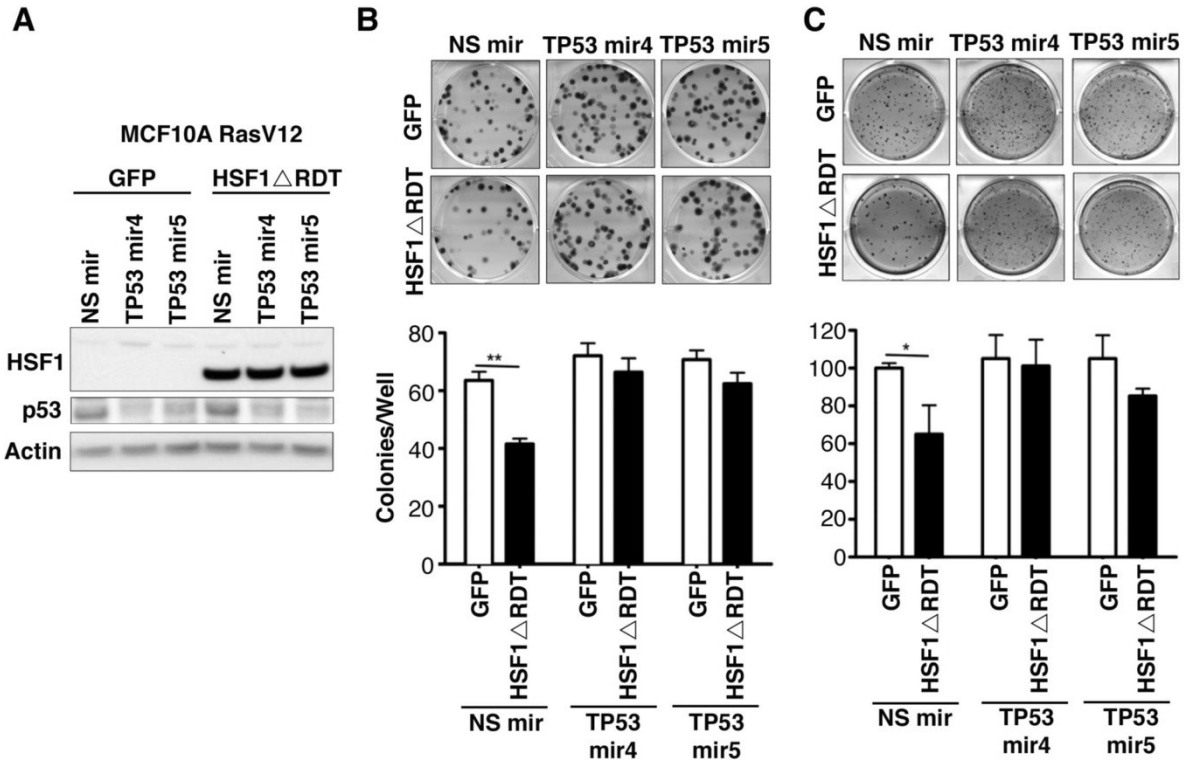


Figure 4.7. Knockdown of wild-type p53 negated the HSF1 mediated inhibition of clonogenic survival and growth

CHAPTER 5

THE EFFECT OF HSF1 KNOCKDOWN ON CELL BIOLOGY AND THE DEVELOPMENT OF AN HSF1 INHIBITOR SCREENING MODEL

5.1. INTRODUCTION

HSF1 is the transcription factor that regulates the heat shock response and several other biological processes promoting tumourigenesis, cancer progression and metastasis (Calderwood, 2012b; Calderwood and Gong, 2011). Although HSF1 is not an oncogene *per se*, many cancer cells are found to rely on the factor for survival, proliferation and the regulation of many other cellular functions, a phenomenon known as 'non-oncogene addiction' (Solimini et al., 2007). With accumulating evidence demonstrating the importance of HSF1 activity in cancer, inhibition of HSF1 has emerged as a potential strategy for cancer treatment (Whitesell and Lindquist, 2009). However, as this study has shown context dependent aspects for the roles of HSF1 in cancer biology, further investigation into the effects of HSF1 inhibition within different cellular contexts is required to both understand the full role of HSF1 and the potential efficacy of future HSF1 inhibitors as potential anticancer therapies. Although studies have identified many HSF1 inhibitors as anticancer therapeutic candidates, these compounds exhibit high toxicity and/or lack of specificity (Whitesell and Lindquist, 2009). The identification and development of more efficacious HSF1 inhibitors are therefore required in order to successfully therapeutically target HSF1.

In breast cancer, Santagata et al. (2011) revealed that high protein HSF1 expression is more likely to be found in high-grade human breast tumours and that high protein expression of HSF1 is significantly correlated with cancer aggressiveness (Santagata et al., 2011). Consistent with this, a study by Kouspou has demonstrated that inhibition of HSF1 by pharmacological compounds or expression of a dominant negative (DN) form of HSF1 in highly aggressive, triple negative breast cancer cell lines abrogates several malignant properties of the cancer cells, both *in vitro* and *in vivo* (Kouspou, 2009). In addition to this, further studies by Price and colleagues have recently revealed that

breast cancer cell lines with HSF1 knockdown by shRNAmir exhibited reduced malignancy (manuscripts under preparation). To further examine the impact of the loss of HSF1 activity in normal breast tissue compared to transformed tissue, this chapter investigates the effects of HSF1 knockdown by HSF1shRNAmir in the 'normal' breast cell line, MCF10A and its isogenic matched H-Ras^{V12} transformed cells. As this study has demonstrated in chapter 3 that HSF1 activation co-operates with active oncogenic Ras to activate distinct transcriptional programs linked to cancer cell migration and invasion, this chapter investigates whether HSF1 knockdown can negate the effect of oncogenic transformation of the MCF10A cell line that are induced by the expression of H-Ras^{V12}.

In a normal, unstressed state, HSF1 exists in the cell in an inactive monomeric conformation. Upon stress, the factor oligomerises to form an active trimer conformation that binds to heat shock elements (HSEs) within the promoter region of target genes leading to altered gene expression. To screen for inhibitor compounds of HSF1, previous studies have most commonly employed a reporter system containing a HSP70 promoter-luciferase construct whereby HSF1 inhibitors were identified as compounds that were able to inhibit the induced luciferase expression in the reporter cells following heat shock (Westerheide et al., 2006; Yoon et al., 2011). This strategy has led to the discovery of several HSF1 inhibitors, however, currently, none of these compounds exhibits potent and specific HSF1 inhibition. Therefore, in addition to investigating the impact of the loss of HSF1 activity in both normal and transformed human mammary cells, this chapter also aims at develop a cell-based reporter model for HSF1 inhibitors which can be used in future large scale screening that is not dependent upon the administration of an external heat-shock or stress.

Altogether, the aims of this chapter were:

1. To determine the effects of HSF1 knockdown on the cell biology of normal non-transformed MCF10A cells and MCF10A cells transformed with H-Ras^{V12}.
2. To generate a novel HSF1 inhibitor cell-line based model that would provide proof-of-concept studies for the future development of a reliable screening tool for the identification of compounds capable of directly inhibiting HSF1.

5.2. RESULTS: IMPACT OF HSF1 KNOCKDOWN UPON THE CELL BIOLOGY OF THE NON-TRANSFORMED AND H-RAS^{V12} TRANSFORMED MCF10A EPITHELIAL CELL LINE

5.2.1. Generation and selection of shRNAmir retroviral constructs against HSF1

In order to knockdown HSF1 mRNA expression in the cell lines examined, five shRNAmir retroviral constructs were designed and generated (see Chapter 2, section 2.1.9.6). To select the two most effective HSF1 shRNAmir constructs for further experiments, all five HSF1 shRNAmir constructs (1-5) were introduced into MCF10A cells by retroviral transduction and the transduced cells were examined by western blot analysis. All five HSF1 shRNAmir constructs were shown to reduce HSF1 expression (Fig.5.1). Among these, HSF1 shRNAmir2, 3 and 4 gave the highest levels of HSF1 knockdown. Cells transduced with these HSF1 shRNAmir constructs or scrambled control shRNAmir were sorted by FACS, with cells exhibiting high GFP expression, which is an indication of stably transduced cells, being selected (Fig.5.1B). Western blot analysis of cells after FACS revealed that cells expressing shRNAmir2 and shRNAmir3 exhibited the highest HSF1 knockdown (Fig.5.1C). These two shRNAmir constructs were thus selected for further experiments. The use of two independent HSF1 shRNAmir constructs was to control against off-target silencing effects. Scrambled control shRNAmir containing RNA sequences that did not bind to any known vertebrate genes was also used in further experiments as a negative control.

5.2.2. Generation of stable HSF1 knockdown cell lines

To examine and compare the impact of HSF1 knockdown upon the cell biology of normal and transformed cells, HSF1 shRNAmir2/3 and scrambled non-silencing control shRNAmir were introduced into the MCF10A mammary epithelial cell line. These cells were further transduced with retroviral vectors that contained the activated mutated oncogene H-Ras^{V12} or a mCherry control. Cells were sorted by FACS such that cells with high GFP and mCherry expression were selected. With FAC analysis, the levels of mCherry and H-Ras^{V12} transduction were found to be similar between the scramble control and HSF1 knockdown cells (data not shown). Western blot analysis was performed to confirm the successful generation of the stable cell lines. As expected, expression of HSF1 was reduced in HSF1 shRNAmir2 and HSF1 shRNAmir3 cells.

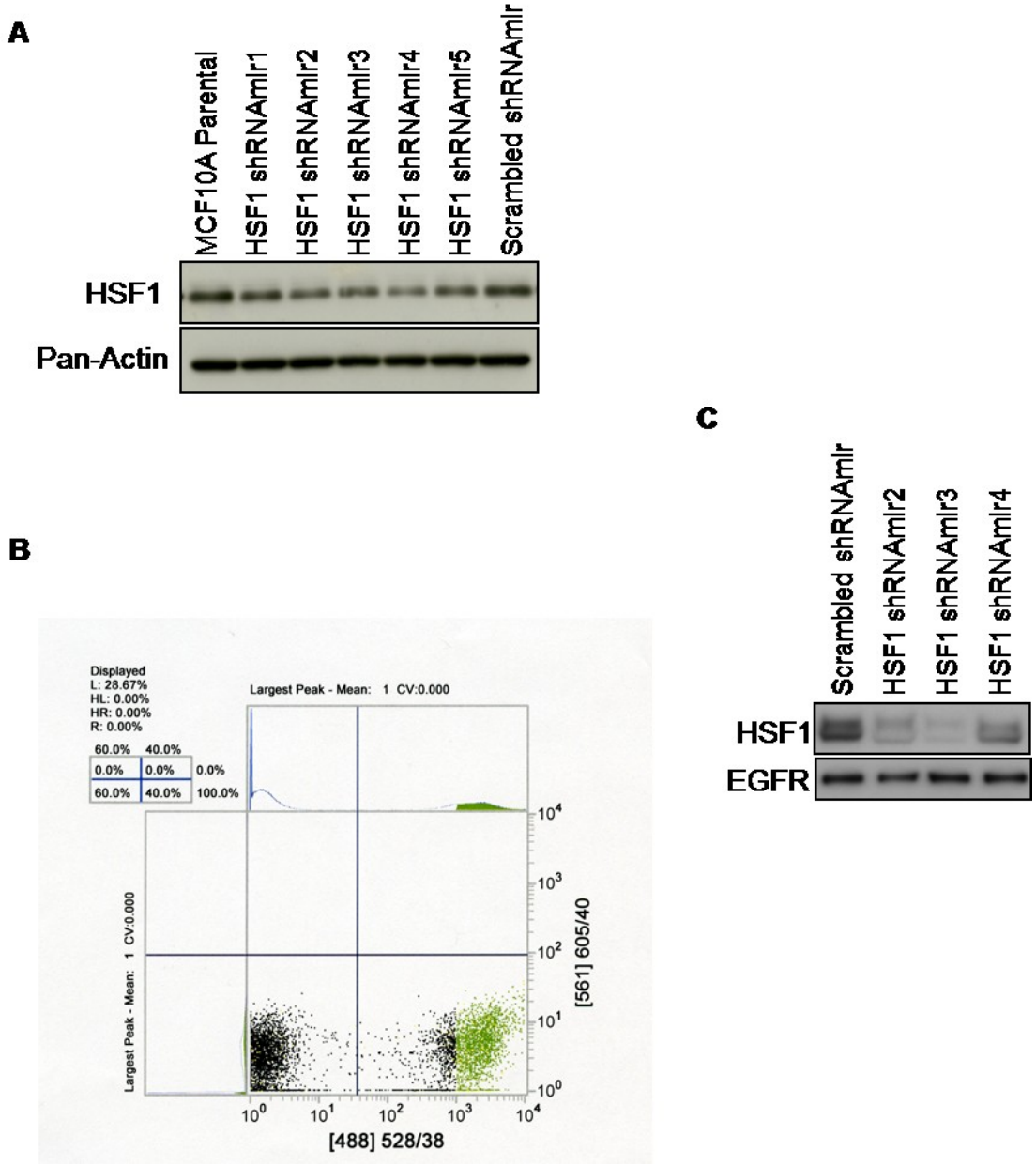


Figure 5.1. Selection of two most effective HSF1 shRNAmir constructs

(A) Western blot analysis of MCF10A cells transduced with retroviral constructs containing HSF1 shRNAmir or scramble shRNAmir before FACS revealed that cells expressing HSF1 shRNAmir2, HSF1 shRNAmir3 or HSF1 shRNAmir4 exhibited much lower HSF1 levels compared to cells expressing the scramble shRNAmir. **(B)** Stable cells were selected by FACS. Representative image of cell analysis by fluorescence flow cytometry was shown. Green region indicates the cell population that was gated and sorted with high levels of EGFP. **(C)** Western blot analysis of cells after FACS revealed that HSF1 shRNAmir2 and HSF1 shRNAmir3 were two most effective shRNAmir constructs in knocking-down HSF1.

Consistent with a role of HSF1 in regulating HSP expression, protein expression of HSP27 and HSP110 were lower in cells with HSF1 knockdown. Ectopic expression of H-Ras^{V12} reduced the expression of the epidermal growth factor receptor EGFR (Fig.5.2A). Additionally, conistent with a previous report, the H-Ras^{V12} transformed cells exhibited reduced levels of HSF1 and HSPs compared to the mChery control cells (Stanhill et al., 2006). Furthermore, consistent with the results obtained from HSF1 activation studies in chapter 3 that altering HSF1 level does not affect signalling pathways downstream of Ras, western blot analysis showed that both the non-transformed and H-Ras^{V12} transformed MCF10A cells expressing HSF1 shRNAmir2 or

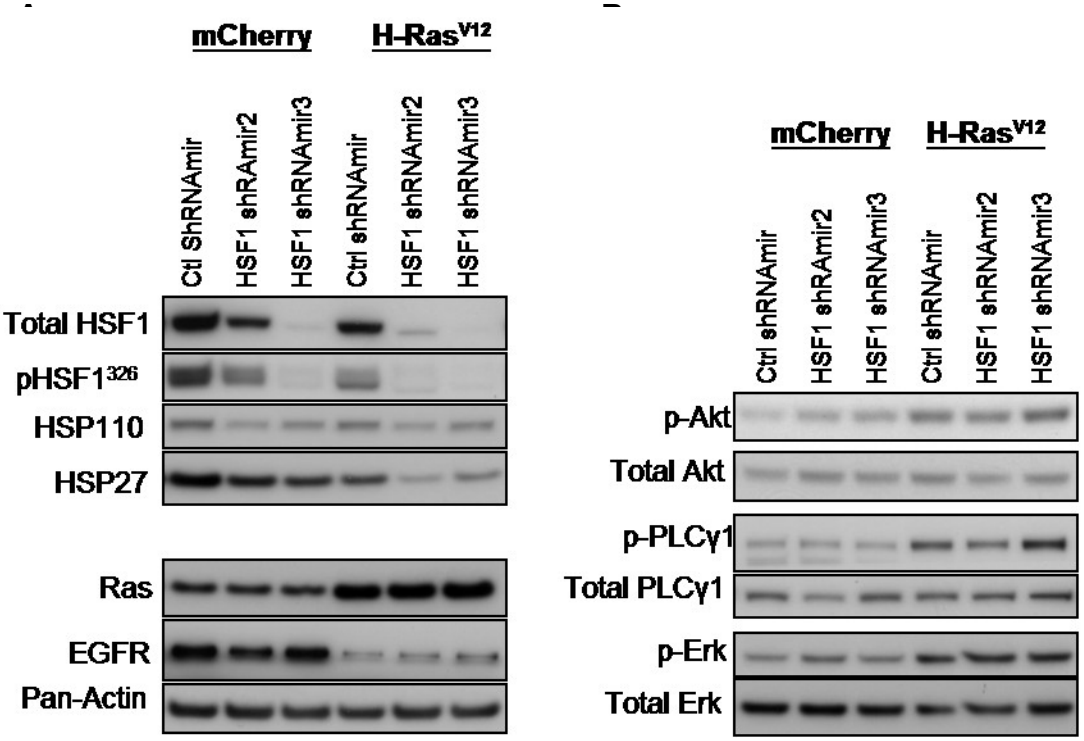


Figure 5.2. Western blot analysis demonstrating the successful generation of stable mCherry control and H-Ras^{V12} transformed MCF10A cells with HSF1 knockdown

(A) Western blot analysis revealed that cells with HSF1 knockdown expressed reduced levels of heat shock proteins such as HSP110 and HSP27. Cells transformed by H-Ras^{V12} expressed reduced level of EGFR. **(B)** Western blot analysis also indicated that HSF1 knockdown did not impact upon levels of total and phosphorylation levels of signalling molecules such as Akt, Erk and PLCγ1 compared to the control shRNAmir cells.

HSF1shRNAmir3 exhibited similar levels of total and phosphorylated signalling molecules such as Akt, Erk and PLCγ1, compared to the control shRNAmir cells (Fig.5.2B).

5.2.3. HSF1 knockdown does not affect cell morphology or proliferation of either the non-transformed or MCF10A H-Ras^{V12} transformed cells.

As it has been reported that HSF1 knockout MEFs exhibited reduced EMT in response to TGFβ (Xi et al., 2012), the impact of HSF1 knockdown upon cell morphology of the non-transformed and H-Ras^{V12} transformed MCF10A cells was examined. Similar to the findings reported in chapter 3, the non-transformed mCherry MCF10A cells exhibited a cobblestone epithelial morphology while H-Ras^{V12} transformed MCF10A cells exhibited a spindle-like mesenchymal morphology when grown in 2-D monolayer. However, in contrast to the previous report (Xi et al., 2012), HSF1 knockdown by shRNAmir did not affect the cell morphology of both the non-transformed and H-Ras^{V12}

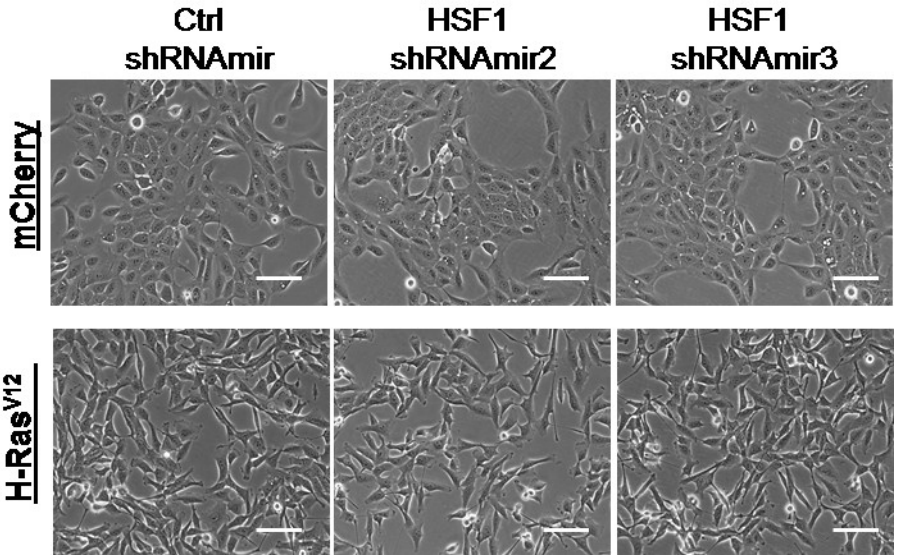


Figure 5.3. H-Ras^{V12} expression induced epithelial to mesenchymal transition (EMT) while HSF1 knockdown did not affect cell morphology of both mCherry control and H-Ras^{V12} cells.

Non-transformed mCherry MCF10A cells grown in 2-D monolayer exhibited a cobble stone morphology characteristic of an epithelial phenotype whereas MCF10A H-Ras^{V12} transformed cells exhibited a scattered spindle-like morphology characteristic of a messenchymal phenotype. Scale bar - 100μM

transformed cells (Fig.5.3).

In addition, as previous studies have demonstrated that HSF1 inhibition decreases cell proliferation of cancer cells (Nakamura et al., 2010) , the impact of HSF1 knockdown upon cell proliferation of the non-transformed and H-Ras^{V12} transformed MCF10A cells was examined using a two-dimensional (2D) anchorage-dependent growth assay. Consistent with the results in chapter 3, expression of H-Ras^{V12} did not alter cellular

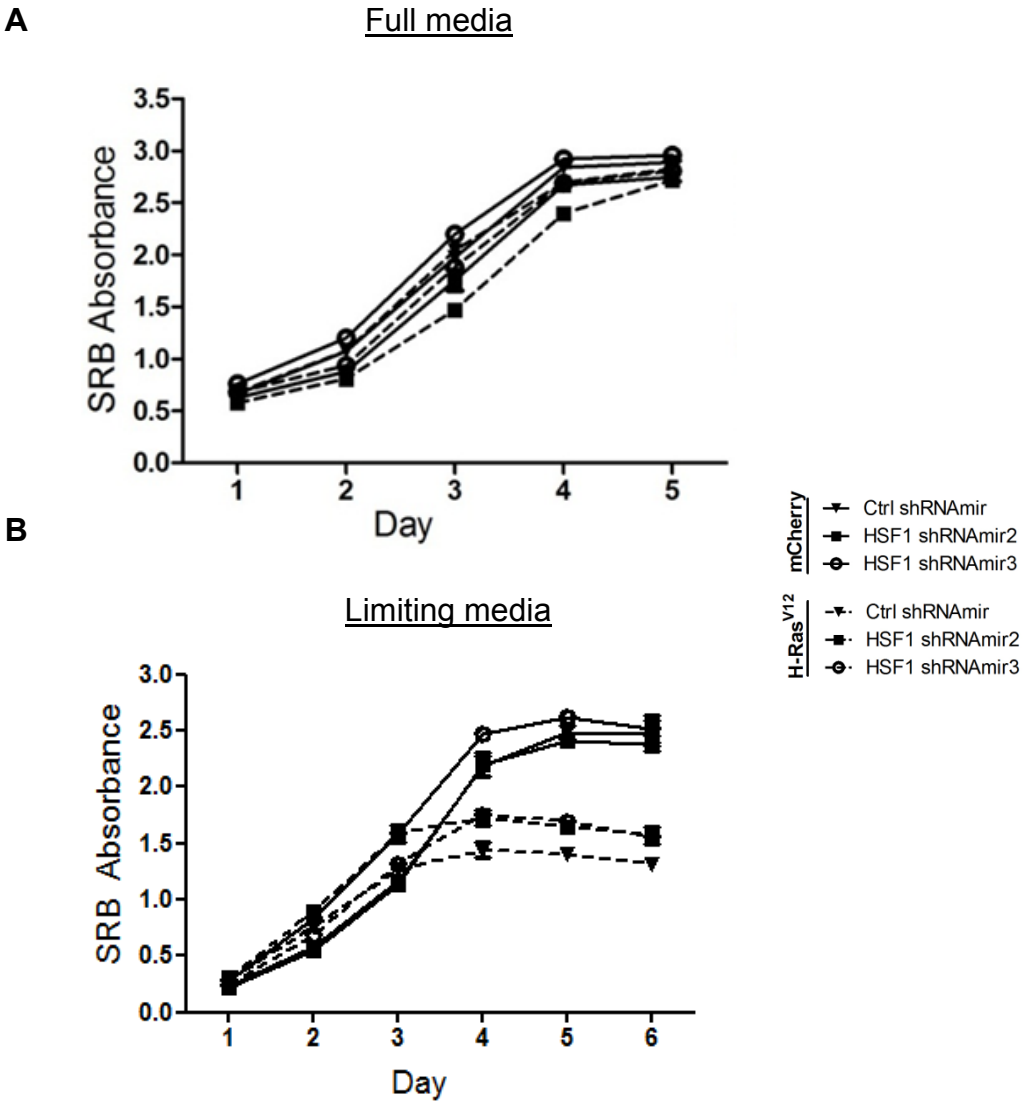


Figure 5.4. H-Ras^{v12} overexpression enabled cells to grow in limiting condition media while HSF1 knockdown has no effect on cell growth

(A) In full media conditions, all cells proliferated at similar rate. **(B)** In limiting media conditions, H-Ras^{V12} transformed cells were still able to proliferate when the non-transformed mCherry cells have stopped proliferating. Knockdown of HSF1 did not affect the proliferation rate of either the mCherry or H-Ras^{V12} transformed MCF10A cells in either growth condition.

proliferation in full media but enabled cells to grow in limiting media conditions (low horse serum, low EGF) where the mCherry control cells had become contact inhibited in their growth (Fig.5.4). HSF1 knockdown did not impact upon cell proliferation of either the non-transformed mCherry or the H-Ras^{V12} cells in either full or limiting media conditions (Fig.5.4).

5.2.4. HSF1 knockdown does not affect cell migration and 3-D growth of both the non-transformed and MCF10A H-Ras^{V12} transformed cells.

Cell migration is a fundamental property of cancer that allows the tumour cells to migrate from the primary site, through the extracellular matrix into the circulation, promoting metastasis at distant organs. The migratory and chemotactic ability of a cancer cell can thus reflect its invasive and metastatic potential. In chapter 3 of the current study it was shown that activation of HSF1 in H-Ras^{V12} transformed cells promoted both intrinsic and chemotactic cell migration. To determine whether HSF1 knockdown can cause the opposing effect, control shRNAmir and HSF1 shRNAmir MCF10A cells were examined for their migratory ability using a two-chamber-migration assay (Kouspou and Price, 2011). In contrast to previous studies demonstrating that cells with HSF1 depletion exhibited reduced migratory ability, HSF1 knockdown did not reduce cell migration of either the non-transformed or the H-Ras^{V12} transformed cells toward either 0.1% BSA or 20ng/ml EGF (Fig.5.5 and 5.6). This finding indicates that HSF1 knockdown by shRNAmir is not sufficient to abrogate cell migration of non-transformed cells or the enhanced migratory phenotype caused by the ectopic expression of H-Ras^{V12}.

Previously, it has been reported that heat shock can cause an increase in cell migration (Lang et al., 2012), to examine whether this was also true in the MCF10A cell line , the migratory potential of the Ras-transformed MCF10A cells was measured following recovery after heat shock. Cells were incubated at 42^{o}C for 30 minutes and then returned to standard growth conditions (37^{o}C, 5% CO_2) to recover overnight. It was found that heat shock did indeed induce a significant increase in cell migration (Fig.5.6); however, consistent with findings from Lang et al. (2012), HSF1 knockdown was unable to negate the heat-shock enhanced migration effect in the H-Ras^{V12} transformed MCF10A.

In addition to cell migration, the current study has also demonstrated in chapter 3 that HSF1 can co-operate with H-Ras^{V12} to promote the disorganized, invasive growth of cells in a 3-D reconstituted basement membrane (Matrigel). The morphology of the cells within the 3-D reconstituted basement membrane reflects their invasive potential.

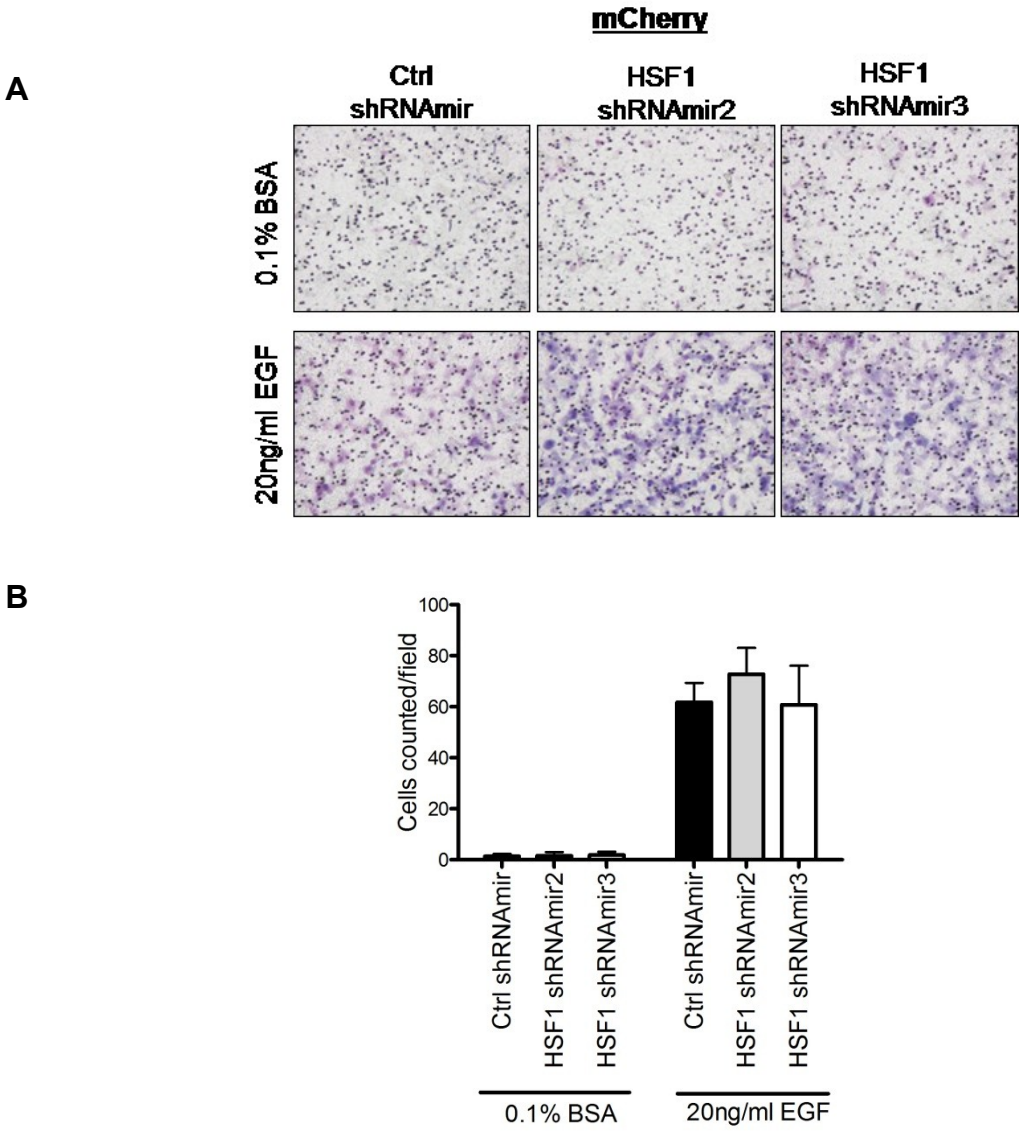


Figure 5.5. HSF1 knockdown does not affect either basal or EGF-induced cell migration mCherry control MCF10A cells.

(A) Representative images of the migration membranes of the non-transformed mCherry cells toward 0.1% BSA and 20ng/ml EGF at 100x magnification. **(B)** The number of cells migrated are represented as the mean±sd. Non-transformed cells expressing HSF1 shRNAmir exhibited similar migratory and chemotactic ability compared to control shRNAmir cells. The results are representative of at least three independent experiments. EGF treatment facilitates chemotactic cell migration of the non-transformed mCherry MCF10A cell. Cells expressing HSF1 shRNAmir exhibited similar migratory ability to cells expressing control shRNAmir.

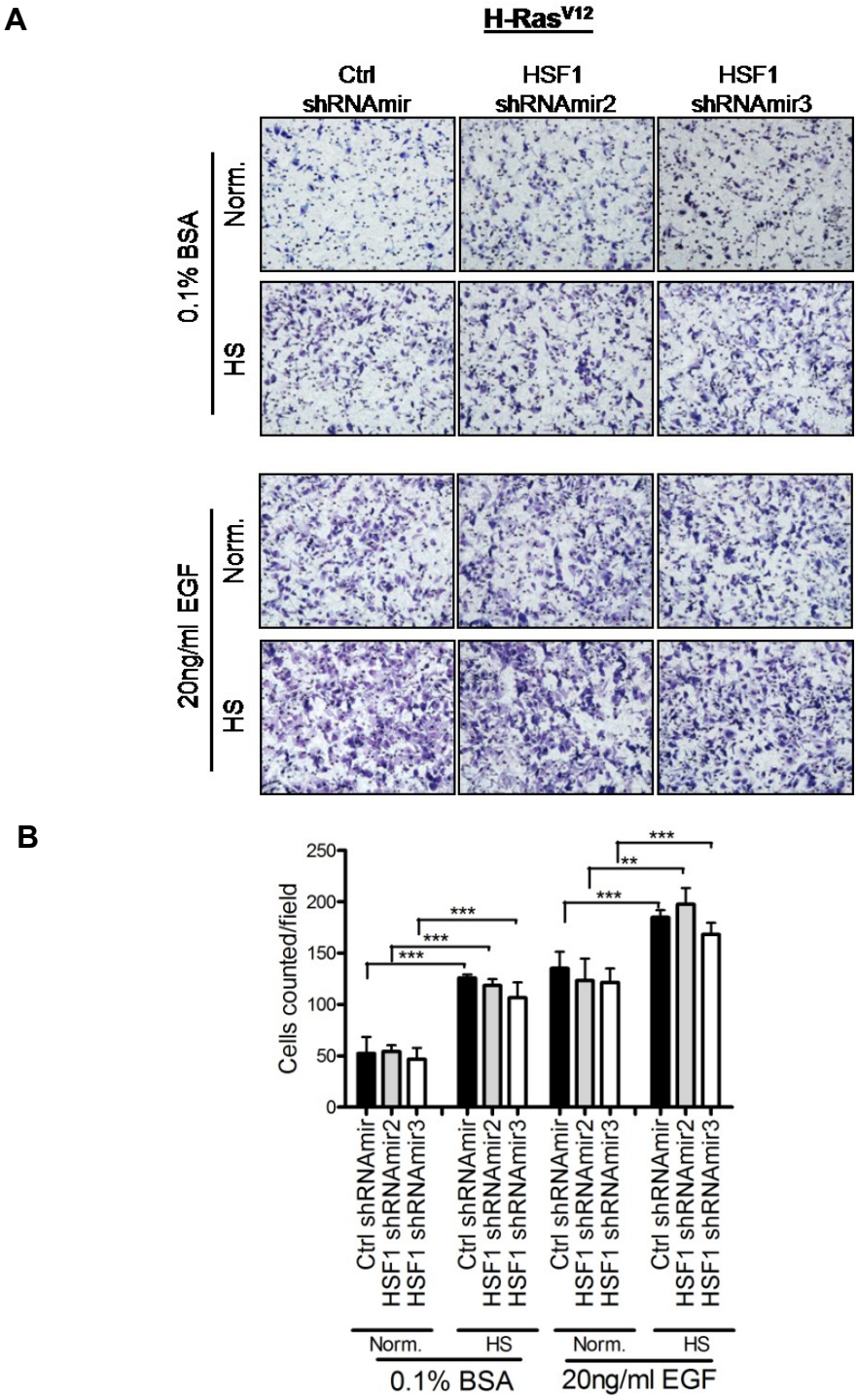


Figure 5.6. HSF1 knockdown does not affect either the basal cell or heat-shock induced cell migration of the H-Ras^{V12} transformed MCF10A.

(A) Representative images of the migration membranes of the H-Ras^{V12} transformed cells toward 0.1% BSA and 20ng/ml EGF at normal condition and following heat shock. **(B)** The number of cells migrated are represented as the mean±sd. Cells expressing HSF1 shRNAmir exhibited similar migratory ability to the control shRNAmir cells at either basal condition or following heat-shock. The results are representative of at least three independent experiments. EGF treatment facilitates cell migration Statistical analysis was performed on one experiment using the Student's t-test where $p<0.05$ is denoted by *, $p<0.01$ by ** and $p<0.001$ by *** when compared to cells at normal conditions.

To further investigate the impact of HSF1 upon cell invasion, the non-transformed and H-Ras^{V12} transformed MCF10A cells with HSF1 knockdown were examined for their growth in Matrigel. Consistent with findings from previous studies and those in chapter 3, the non-transformed MCF10A cells formed defined hollow acinar structures with lamininV deposited at the basement membrane (Fig. 5.7). Consistent with the transformed phenotype, MCF10A H-Ras^{V12} transformed cells formed acini structures with filled lumen and lacking laminin V. Thus, expression of HSF1 shRNAmir2 or shRNAmir3 was found to have no impact upon cell architecture of both the non-transformed and H-Ras^{V12} transformed MCF10A cells when grown in 3-D conditions (Fig.5.7). HSF1 knockdown therefore is not sufficient to negate the alteration in morphology of cells in 3-D culture induced by ectopic expression of H-Ras^{V12}.

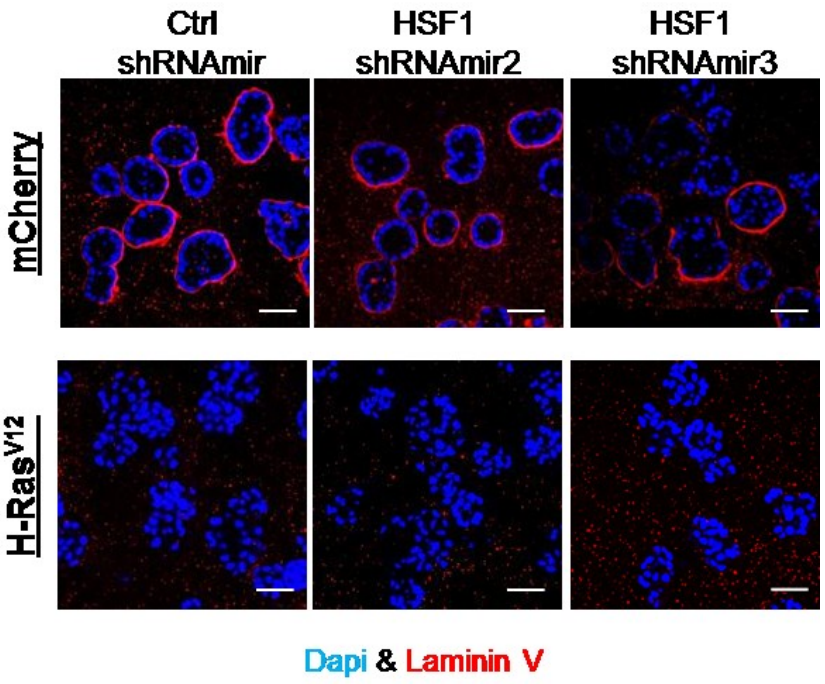


Figure 5.7. HSF1 knockdown does not affect cell growth in 3-D reconstituted basement membrane.

Non-transformed mCherry MCF10A cells formed spherical acini with hollow lumen and laminin V was deposited at the basement membrane around the edge of the acini. H-Ras^{V12} transformed MCF10A cells formed spherical acini with filled lumen and have almost absence of laminin V staining. Expression of HSF1 shRNAmir did not affect the morphology of either the non-transformed or H-Ras^{V12} transformed cells in 3-D growth conditions. Images of acini structures at the equatorial section are shown (Blue – Dapi and Red – Laminin V). Scale bar - 50µM.

5.2.5. HSF1 knockdown reduces clonogenic growth of both the non-transformed and H-Ras^{V12} transformed MCF10A

Another feature of the cancer cell is its ability to survive and grow in the isolation of any neibouring cell, which is referred to as clonogenicity. This feature reflects the cancer cells' ability of unlimited proliferation as well as the tumour initiating and metastasis initiating potential. To investigate the impact that HSF1 knockdown has upon the clonogenic survival and growth of both the non-transformed and H-Ras^{V12} transformed MCF10A cells, cells were examined for their ability to form colonies from single cells in 2-D and 3-D soft-agar conditions. In 2-D conditions, it was found that both the non-transformed and H-Ras^{V12} transformed cells expressing HSF1 shRNAmir exhibited reduced clonogenicity compared to control shRNAmir cells (Fig.5.8A). Consistent with the transformed phenotype, H-Ras^{V12} transformed cells were able to form colonies in the 3-D soft-agar conditions. Consistent with the reduced colonogenicity in 2-D condition, H-Ras^{V12} transformed cells with HSF1 knockdown also exhibited reduced clonogenic survival and growth in soft-agar (Fig.5.8B). These findings show that knockdown of HSF1 abrogates the clonogenicity of both the non-transformed and H-Ras^{V12} transformed MCF10A cells in both 2-D and 3-D conditions.

5.2.6. HSF1 knockdown increases the level and transactivation activity of wild-type p53.

As it has been demonstrated in chapter 4 that HSF1 impacts upon clonogenic growth via modulating p53 activity; western blot analysis was performed to examine the effect of HSF1 knockdown upon the levels of p53 and its transcriptional targets. Consistent with the role of HSF1 in regulating p53 degradation, levels of p53 were elevated in both the non-transformed and H-Ras^{V12} transformed HSF1 shRNAmir2 and shRNAmir3 expressing cells. Although it has been demonstrated in chapter 4 that HSF1 enhances wild-type p53 activity, results here showed that HSF1 knockdown did not reduce wild-type p53 transactivation activity but in contrast, enhanced it. Cells with HSF1 knockdown were found to express higher protein levels of p53 and p53 transcriptional targets including CDKN1A (p21) and BAX (Fig.5.9). This indicates that the reduced clonogenicity caused by HSF1 knockdown would also partly be due to the increased wild-type p53 level and activity.

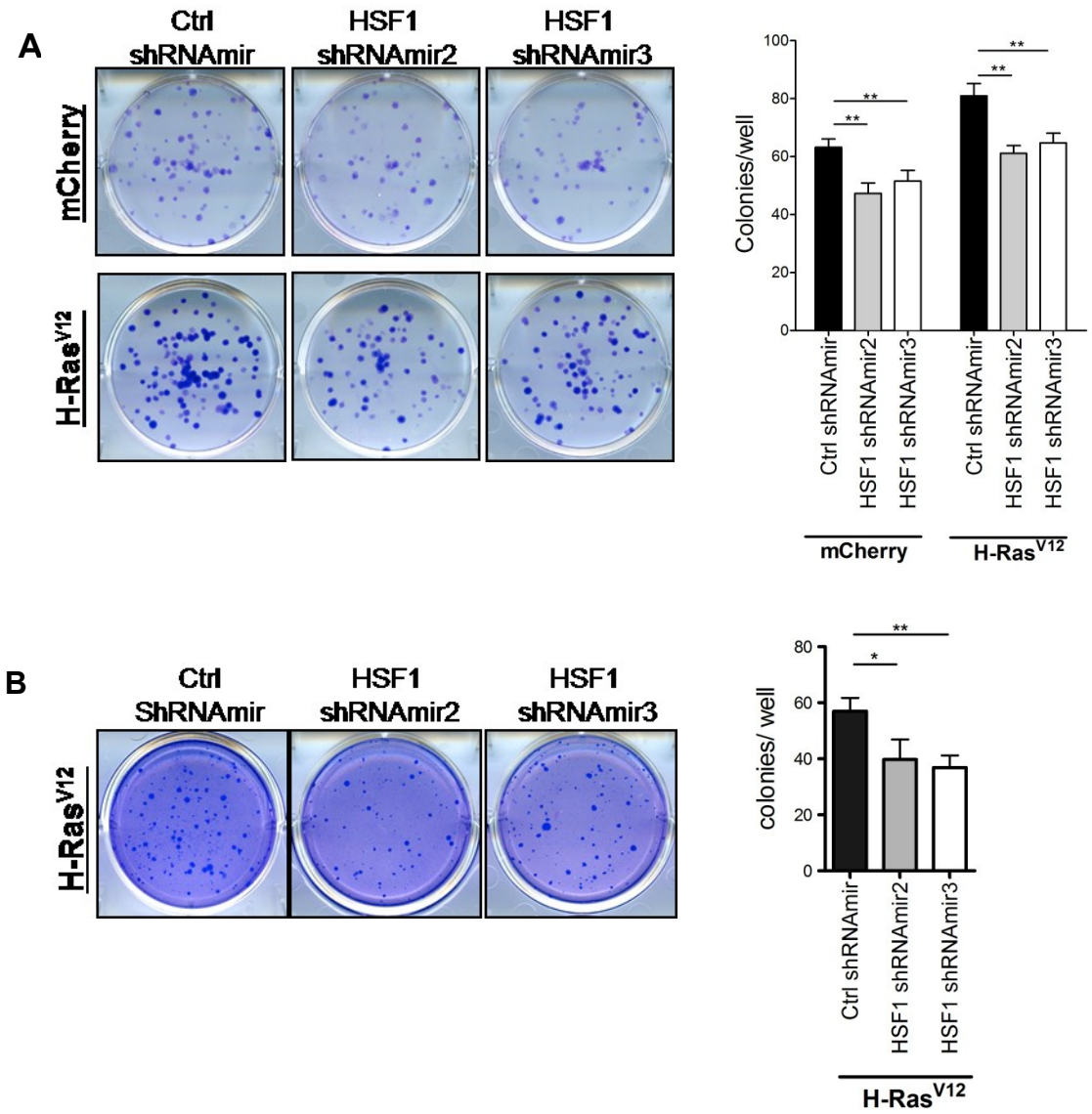


Figure 5.8. HSF1 knockdown reduces both the 2-D and 3-D clonogenic survival and growth of mCherry untransformed and H-Ras^{V12} transformed MCF10A cells. **(A)** 2-D clonogenic survival and growth assay revealed that both the non-transformed and H-Ras^{V12} transformed MCF10A cells expressing HSF1 shRNAmir exhibited a reduced ability to form colonies from single cells in anchorage-dependent conditions. **(B)** 3-D soft-agar clonogenic survival and growth assay revealed that consistent with the transformed phenotype, the H-Ras^{V12} MCF10A cells were able to form colonies in soft-agar. HSF1 knockdown significantly reduced the clonogenicity of the H-Ras^{V12} transformed cells in this 3-D condition. The number of colonies formed in each well was counted manually and is represented as mean ±sd calculated from the means of three independent experiments. Statistical analysis was performed using the Student's t-test where $p<0.05$ is denoted by *, $p<0.01$ by ** and $p<0.001$ by *** when compared to the control shRNAmir cells.

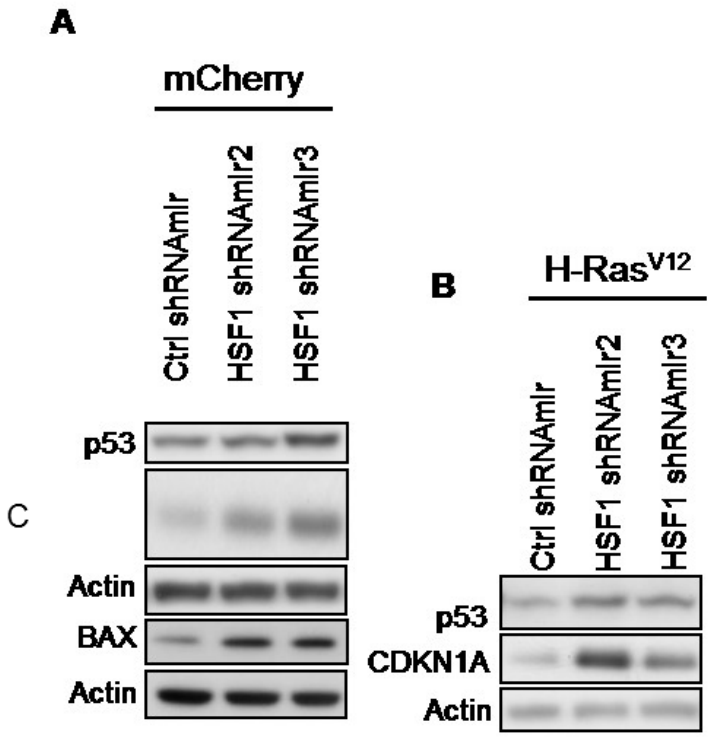


Figure 5.9. HSF1 knockdown increases wild-type p53 level and transactivation activity

Western blot analysis of the **(A)** non-transformed mCherry and **(B)** H-Ras^{V12} transformed MCF10A cells revealed that cells expressing HSF1 shRNAmir exhibited increased level of wild-type p53 and its transcriptional targets such as CKDN1A (p21) and BAX.

5.3. RESULTS: DEVELOPMENT OF STABLE HEK293 REPORTER CELL LINE FOR HSF1 INHIBITOR SCREENING

5.3.1. Generation of HSF1 inhibitor screening model

HSF1 supports many malignant phenotypes in cancer and studies have also highlighted the potential benefits of HSF1 inhibitors in cancer treatment. In order to address the current lack of specific HSF1 inhibitors, the current study sought to develop a cell-based reporter model which could potentially be used for large scale screening of compounds to identify novel HSF1 inhibitors. The strategy of generating the reporter cell model was to develop a stable cell line that constitutively expresses two fluorescent proteins: one is HSF1 regulated and the other is non-HSF1 regulated. Compounds that can reduce the HSF1-regulated fluorescent protein while exerting no effect upon the level of the non-HSF1 regulated fluorescent protein would be identified as specific HSF1 inhibitors.

To generate the reporter cell model, HEK293 cells were transduced with the bicistronic retroviral vectors, pBABE_HSF1wt_IRES_EGFP or pBABE_ΔRDT_IRES_EGFP. These vectors contain HSF1wt or the HSF1 constitutively active mutant, HSF1ΔRDT, respectively, which is co-expressed at the gene level with an EGFP separated by an internal ribosomal entry site (IRES). Cells with stable expression of HSF1wt or HSF1ΔRDT and EGFP were selected by puromycin (1μg/ml) treatment for 2 weeks and were further transfected with a pHSE-mCherry vector in which mCherry expression is under the control of the inducible HSP70 (HSP70i) promoter (Chapter 2, section 2.1.9.4). The high level of HSF1wt or HSF1ΔRDT in these cells led to the constitutive expression of mCherry (Fig.5.10A). Stable cells containing HSE-mCherry construct were selected by G418 treatment (1μg/ml) for 2 weeks. Subsequently, cells with high levels of EGFP and mCherry were sorted by FACS (Fig.5.10B). Flow cytometry and western blot analysis revealed that high expression of HSPs and mCherry was achieved at much higher levels in cells expressing HSF1ΔRDT compared to that of cells expressing HSF1wt. HSF1ΔRDT cells containing HSE-mCherry construct were thus chosen as the final reporter cell model (Fig.5.10C). Using this model, compounds that can reduce the mCherry level while leaving the EGFP level unaffected would be identified as potential HSF1 inhibitors.

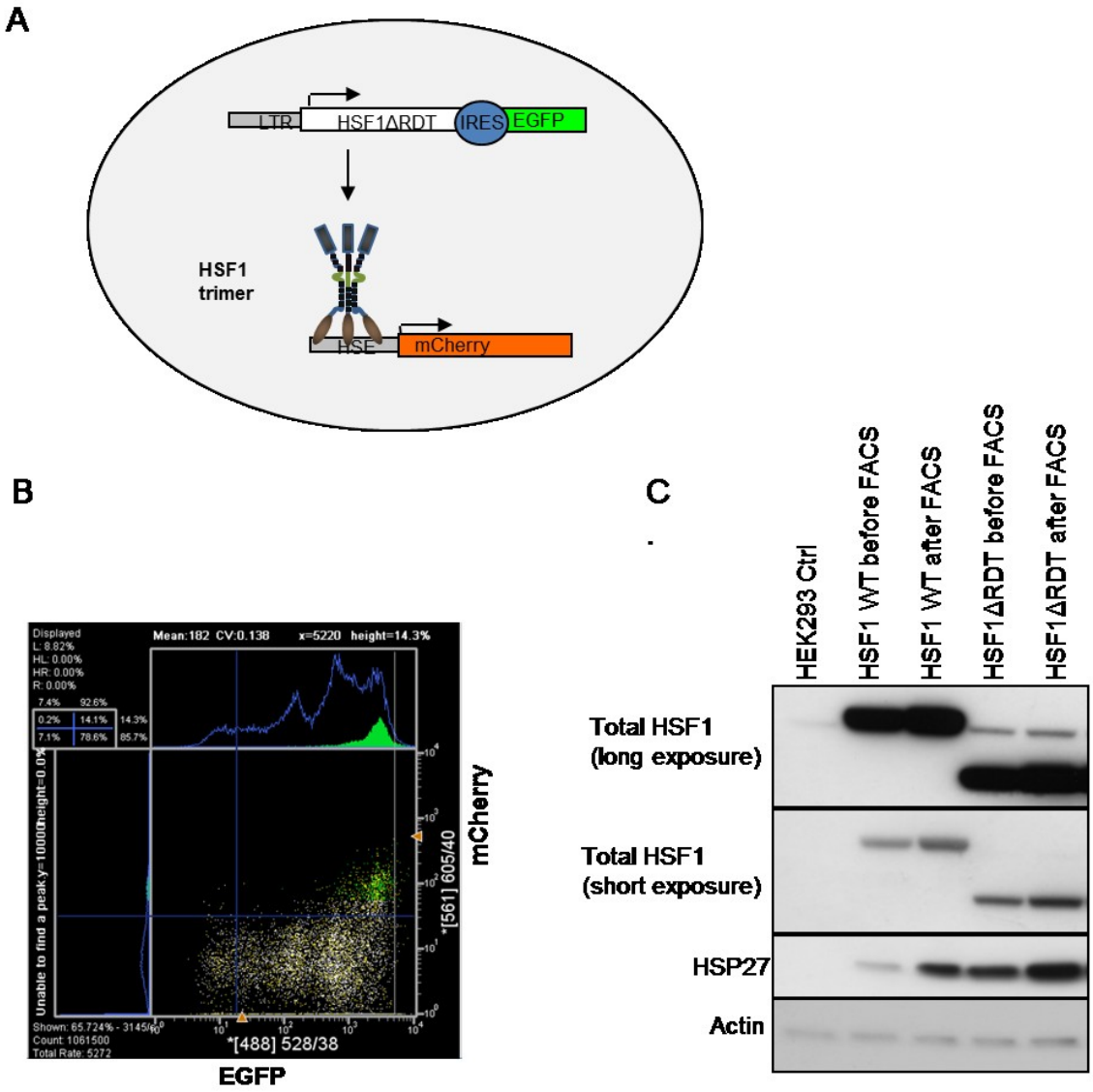


Figure 5.10. Generation of HSF1 inhibitor reporter cell line

(A) The HSF1 inhibitor reporter cell line was designed to express a bicistronic construct that contains the activated HSF1, HSF1ΔRDT, and EGFP connected by an internal ribosomal entry site (IRES). It was also designed to express an HSE-mCherry construct that is transcriptionally regulated by HSF1. Under basal conditions, the reporter cells constitutively express three proteins: activated HSF1, EGFP and mCherry. **(B)** HEK293 cells stably expressed the two designed constructs were selected sequentially by puromycin and G418 for 2 weeks each and then sorted by FACS, which selected cells with high EGFP and high mCherry levels. Cell analysis by FACS prior to cell sorting is shown. Green region indicates the cell population that was gated and sorted with high levels of EGFP and mCherry. **(C)** Western blot analysis revealed that HEK293 cells expressing HSF1ΔRDT, which were selected by FACS exhibited the highest HSP27 levels, indicating high HSF1 activity. This cell line was the chosen as the reporter cell model.

5.3.2. Validating reporter cell line by HSF1 knockdown using HSF1 siRNA

The reporter cell line was first tested for its functionality through examining its mCherry and EGFP expression levels after HSF1 knockdown by siRNAs. Two different HSF1 siRNA sequences, siRNA3 and siRNA4, were transfected into the cells at 25pmol and 50pmol per well in a 6-well tissue culture dish. The efficiency of the knockdown was examined by western blot analysis. As expected, siRNA transfection reduced the levels of both the endogenous HSF1 and HSF1ΔRDT, which consequently led to the reduction in protein expression of HSP110 and HSP27 (Fig.5.11A). Fluorescence microscopy was then utilised to observe if there were any changes in the levels of EGFP and mCherry. After 48hrs of siRNA transfection, a reduction in both EGFP and mCherry fluorescence was observed. As HSF1 and EGFP are expressed by the same mRNA molecule due to the IRES system, it can be expected that the reporter cells with HSF1 knockdown would also exhibit a marked reduction in EGFP levels. The level of mCherry fluorescence was also found to be reduced upon siRNA transfection indicating that the mCherry expression was sensitive to modulation of HSF1 activity (Fig.5.11B).

To further examine the reporter cells following HSF1 siRNA transfection, cells were then analysed by flow cytometry. In this assay, changes in fluorescence levels of cells are expressed as a shift in fluorescence intensity on a logarithmic scale. As shown in Fig.5.12, cells transfected with the siRNAs HSF1 siRNA3 and HSF1siRNA4 50pmol/well exhibited reduced EGFP and mCherry fluorescence intensity levels. Consistent with the western blot analysis demonstrating that HSF1 siRNA3 was less effective in reducing HSF1 and HSP levels than HSF1 siRNA4, flow cytometry analysis showed that HSF1 siRNA4 transfection caused a greater reduction in both EGFP and mCherry levels than HSF1 siRNA3 transfection (Fig.5.12).

Taken together, these results indicated that the reporter system functioned as designed. With the level of mCherry reducing upon HSF1 knockdown by siRNAs, it is confirmed that mCherry can be an indicator of HSF1 activity. Additionally, EGFP cannot only act as a control for general protein synthesis but can also reflect expression of the ectopically expressed HSF1ΔRDT at the mRNA level.

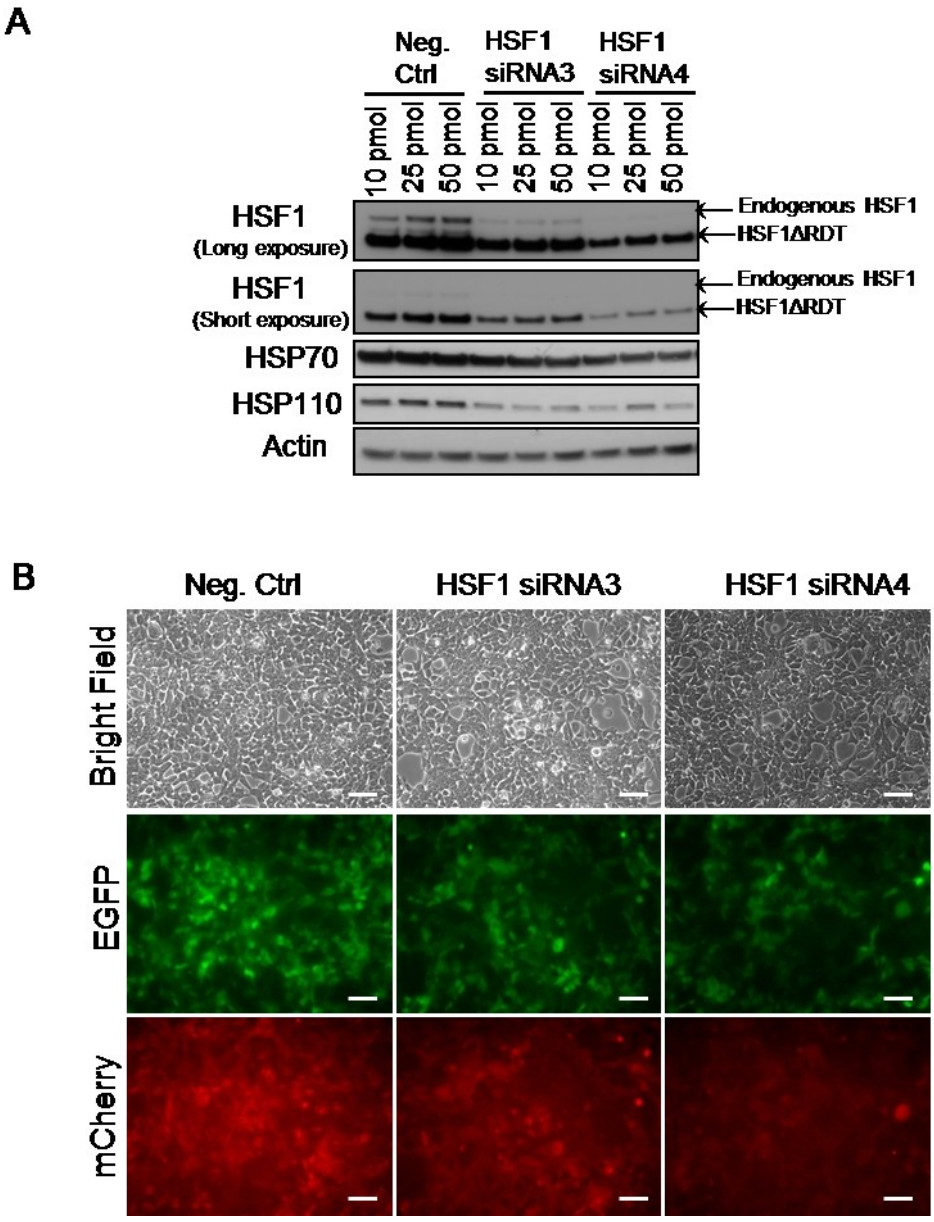


Figure 5.11. Validating the reporter cell line by HSF1 knockdown using HSF1 siRNA
(A) Western blot analysis revealed that both HSF1 siRNA3 and HSF1 siRNA4 reduced the levels of both the endogenous HSF1 and HSF1ΔRDT, which led to the reduction in the expression of HSP70 and HSP110. All concentrations of siRNA examined caused similar HSF1 knockdown effect**. (B)** Observation of cells under bright-field and fluorescence microscopy revealed that both EGFP and mCherry levels were reduced upon HSF1 siRNA transfection. Representative images of cells transfected with 50pmol of siRNA are shown. Scale bar - 100µm.

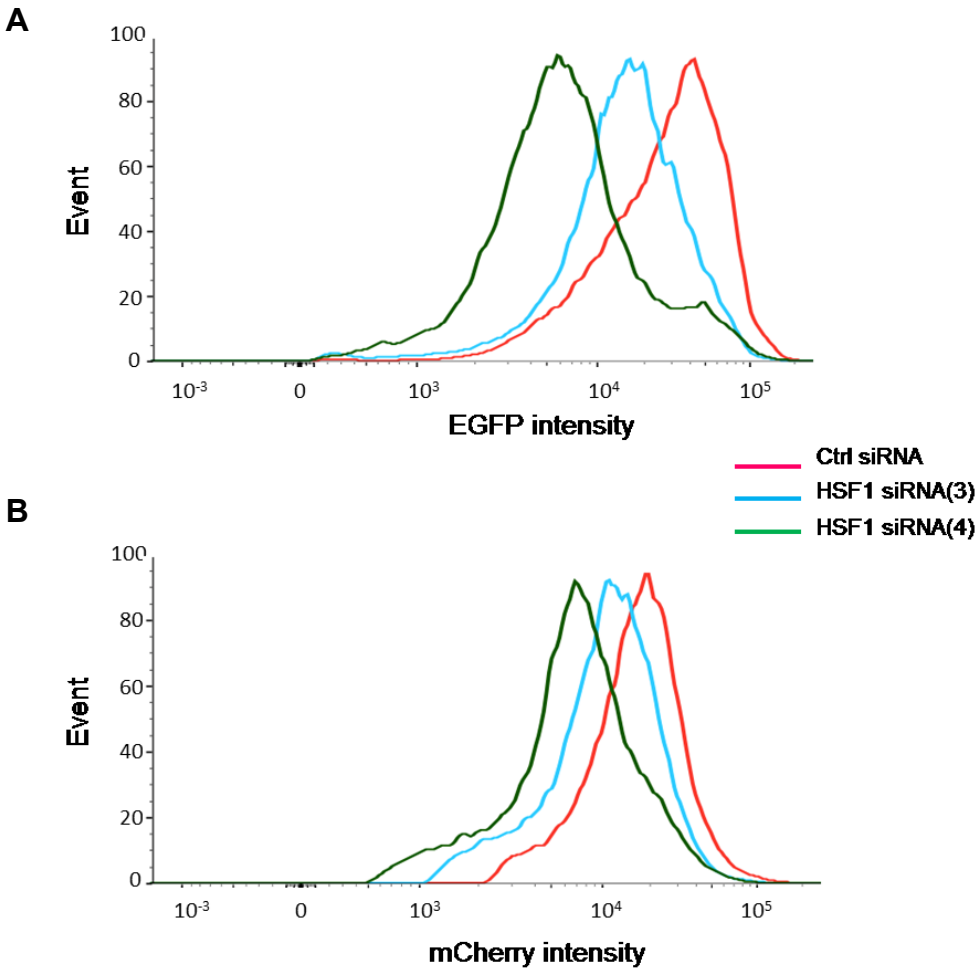


Figure 5.12. Flow cytometry analysis validating the reporter cell line following siRNA transfection

Overlayed histograms of events vs. fluorescence intensity on EGFP **(A)** and mCherry **(B)** of reporter cells transfected with control siRNA or HSF1 siRNAs revealed that cells transfected with HSF1 siRNA3 or siRNA4 exhibited lower levels of both EGFP and mCherry compared to cells transfected with the control siRNA. siRNA4 was more potent in knocking down both EGFP, which corresponded to a lower mCherry level of cells transfected with siRNA4 compared to cells transfected with siRNA3.

5.3.3. Investigating the effect of known HSF1 inhibitors on the reporter cell line.

The reporter cell line model was further validated through the use of known HSF1 inhibitors such as triptolide, KNK437 and quercetin. Cells were treated with different concentrations of these compounds and then after 24 hours, examined for EGFP and mCherry levels. The effect of the HSF1 inhibitors was also examined by western blot analysis. As shown in Fig.5.13A, western blot analysis revealed that triptolide and KNK437 treatments both caused a marked reduction in the total and phosphorylated levels of HSF1ΔRDT (Fig.5.13A). At concentrations of 15μM and 30μM, quercetin

also reduced HSF1ΔRDT protein levels, although to a lesser extent to that of triptolide and KNK437. However, none of these compounds reduced the endogenous wild-type HSF1 levels. In addition, at the concentration of 30μM, in contrast to its HSF1 inhibition function, triptolide induced HSF1 activation, which was evident by an increase in the level of serine-326 phosphorylated HSF1 and a small shift in HSF1 molecular weight (Fig.5.13A). Western blot analysis of the expression of HSPs revealed that consistent with previous studies demonstrating that these known HSF1 inhibitors cause varying effects on expression of HSPs in different cell lines, among the compounds examined; only KNK437 effectively reduced the protein expression of HSP110, HSP27 and HSP70i. Triptolide caused a reduction in HSP27 and HSP110 levels but did not reduce HSP70i. Quercetin appeared to only effectively reduce HSP27 expression, while leaving HSP70i and HSP110 levels unaffected (Fig.5.13A).

The reporter cells treated with the vehicle control DMSO or the HSF1 inhibitors were observed under bright-field and fluorescence microscopy (Fig. 5.13B). As triptolide is highly toxic, cells treated with this compound were much less confluent compared to the DMSO treated cells after 24 hours of treatment. KNK437 and quercetin were relatively non-toxic to the cells at the concentrations tested. Observation of cells using fluorescence microscopy revealed that cells treated with triptolide or KNK437 had much lower EGFP fluorescence levels compared to those treated with DMSO control. Cells treated with quercetin also appeared to express slightly less EGFP, indicating the inhibition of universal protein synthesis. Changes in mCherry levels were not obvious and difficult to detect by fluorescence microscopy, however, it was observed that KNK437 had caused a reduction in mCherry level (Fig. 5.13B).

Flow cytometry was then performed to further examine the levels of EGFP and mCherry in the reporter cells upon HSF1 inhibitor treatments. Consistent with the results observed by fluorescence microscopy, the intensity of EGFP was reduced in cells treated with triptolide, KNK437 or quercetin, with KNK437 causing the highest reduction in EGFP compared to the DMSO control (Fig.5.14A). Consistent with levels of HSP70 protein expression measured by western blot analysis, KNK437 treatment indeed led to a reduction in mCherry intensity while both triptolide and quercetin treatments did not alter the level of this fluorescent protein (Fig.5.14B).

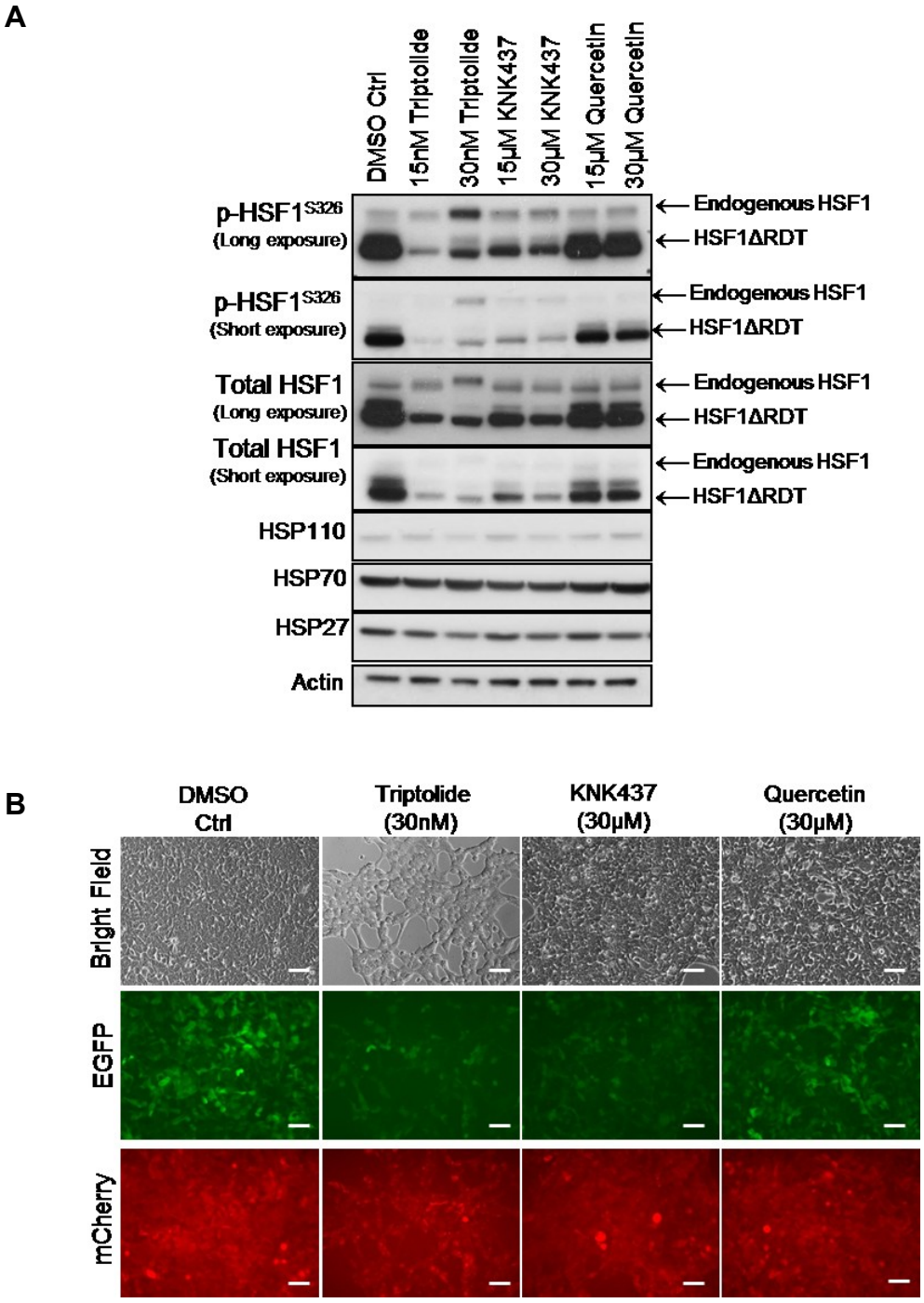


Figure 5.13. Effects of known HSF1 inhibitors on the reporter cell line

The reporter cell line was treated with various concentrations of HSF1 inhibitors, Triptolide, KNK437 and Quercetin. **(A)** Western blot analysis revealed that both Triptolide and KNK437 significantly reduced the levels of HSF1ΔRDT and HSPs such as HSP27and HSP110. However, while KNK437 treatment reduced the protein level of HSP70, Triptolide had minial effect on theexpression of this protein. Quercetin also reduced HSF1 and HSP27 levels but had little effect on the levels of HSP70 and HSP110. **(B)** Observation of cells under bright-field and fluorescence microscope revealed that either EGFP or mCherry levels were significantly reduced upon Triptolide and KNK437 treatments. Quercertin did not appear to alter the levels of both EGFP and mCherry. Representative images of cells transfected with highest concentration of HSF1 inhibitors are shown. Scale bar -100μm.

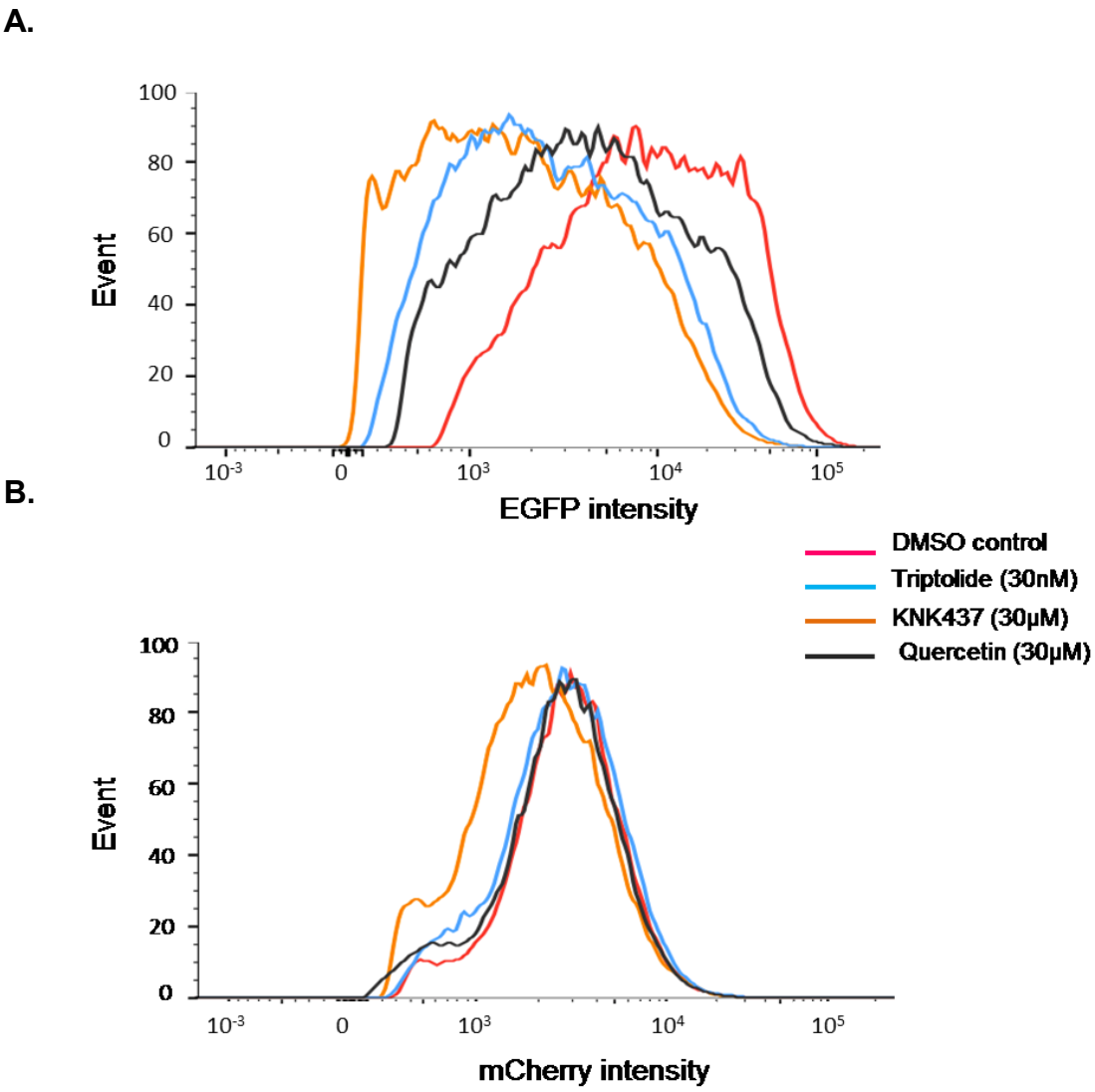


Figure 5.14. Flow cytometry analysis validating the reporter cell line following treatments with known HSF1 inhibitor

Overlayed histograms of event vs. EGFP **(A)** and mCherry **(B)** fluorescence intensities of reporter cells treated with vehicle control or HSF1 inhibitors revealed that all compounds tested caused a shift in EGFP intensity in the reporter cells, with KNK437 causing the greatest shift. However, only KNK437 reduced mCherry fluorescence intensity of the cells following 24 hours of treatment Triptolide or Quercetin did not cause any alteration in the level of mCherry fluorescence.

Taken together, the results from the initial testing of the reporter cell line with known HSF1 inhibitors further confirmed that the reporter cell line is sensitive to modulation of HSF1 activity by compounds and concentrations that also reduce HSP expression. The reduction in EGFP fluorescence by current HSF1 inhibitors such as triptolide, KNK437 and quercetin demonstrated that while these compounds may reduce expression of HSPs, the action of these compounds is not HSF1 specific.

5.4. DISCUSSION

HSF1 has been identified as an attractive anticancer therapeutic target with previous studies demonstrating that HSF1 knockout, knockdown or inhibition by therapeutic compounds reduces many malignant phenotypes (Calderwood, 2012a; Fang et al., 2011; Kouspou, 2009; Santagata et al., 2011; Wang et al., 2004b). This chapter presents work that investigates and compares the impact of HSF1 knockdown upon the cell biology of the 'normal' mammary epithelial cell line MCF10A and isogenically matched H-Ras^{V12} transformed MCF10A cells, thereby further identifying the activities of HSF1 in differing cellular contexts. The current study has also established a reliable cell-based screening model which can allow for the identification of specific HSF1 inhibitors.

5.4.1. HSF1 inhibition and cell proliferation

Previous studies have demonstrated that HSF1 inhibition reduces cancer cell proliferation. Silencing of HSF1 by shRNAi has been shown to decrease cell proliferation of human melanoma cell lines (Nakamura et al., 2010). In breast cancer, triple negative cell lines expressing a dominant negative mutant form of HSF1 exhibited reduced cell growth both *in vitro* and *in vivo* (Kouspou, 2009). This has been explained by the fact that HSF1 regulates the expression of cell cycle molecules such as cyclin D1 and cyclin B1 (Kouspou, 2009; Wang et al., 2004b). Moreover, HSF1 plays a direct regulatory role in the cell cycle and mitotic exit, as the factor is phosphorylated and localised to the centrosomes during mitosis, especially to the spindle poles in metaphase (Kim et al., 2005). Consistent with this, a null mutant or knockdown of HSF1 causes defective mitotic progression and enhances cell apoptosis upon UV irradiation (Chang et al., 2012b; Lee et al., 2008a). In contrast to these studies providing evidence for a role of HSF1 in cell growth and mitosis, the current study demonstrated that knockdown of HSF1 did not cause any significant alteration in the cellular proliferation rate of both untransformed and H-Ras^{V12} transformed MCF10A in full growth media or during limiting media conditions. This finding suggests that normal cells and cells transformed by an oncogenic Ras do not rely on HSF1 for cell proliferation. Alternatively, as demonstrated in previous studies, HSF1 would be required for normal cell cycle and cell proliferation in some particular stress conditions or in the context of some high-grade cancer cells such as TNBC cells and melanoma cells used in previous studies (Kouspou, 2009; Nakamura et al., 2010).

5.4.2. HSF1 inhibition and cancer cell migration and invasion

In contrast to previous studies which demonstrated that inhibition of HSF1 can abrogate cancer cell migration and invasion (Fang et al., 2011; Khaleque et al., 2008; Kouspou, 2009; O'Callaghan-Sunol and Sherman, 2006), the present study demonstrated that knockdown of HSF1 in both the non-transformed and H-Ras^{V12} transformed MCF10A does not affect the overall migratory ability of these cells. Moreover, inhibition of HSF1 also does not affect the enhanced migration induced by heat-shock. This result is consistent with a recent study which has demonstrated that heat shock and other proteotoxic stresses induce cell migration through a HSF1 independent mechanism (Lang et al., 2012). In addition, the current study has demonstrated in chapter 3 that activation of HSF1 lead to enhanced migration and invasion only in the context of H-Ras^{V12} transformation while exerting no effect on cell migration of the non-transformed mCherry control cells, these findings indicate that HSF1 does not enable cell migration and invasion in isolation but rather functions as a modulator or downstream effector of activated oncogenes and/or mutated tumour suppressor genes in the overall regulation of these processes. The H-Ras^{V12} transformed MCF10A cells represent an "early stage" transformation model (Basolo et al., 1991; Spandidos, 1987). HSF1 knockdown was shown not to impact upon migration and invasion in this model while previous studies demonstrated that HSF1 knockdown can abrogate cell migration and invasion of aggressive cancer cells such as TNBC cells (Kouspou, 2009) and HCC cancer cells (Fang et al., 2011). This would suggests that cancer cells may rely on HSF1 to maintain malignant phenotype at the later stages of carcinogenesis, where they have acquired multiple genetic and epigenetic alterations that drive cancer progression.

Taken together, the results in this chapter confirm the context dependency of HSF1 activity in cancer which was demonstrated in chapter 3 and 4. As HSF1 has been shown to directly regulate distinct transcriptional programs that are unique to the malignant state (Mendillo et al., 2012), it is suggested that interactions of HSF1 and transcriptional co-regulators that are unique in cancer cells are important determinants for the cancer promoting properties of HSF1. Inhibition of HSF1 therefore would be more effective against high-grade cancer but may only cause minimal cytotoxic effect to normal cells, a desired feature for targeted cancer therapy. The data presented in this study suggest that inhibition of HSF1 in early stage cancer may prevent cancer progression but would

not completely eradicate tumours, which is demonstrated by the fact that HSF1 knockdown does not protect cells from malignant phenotypes induced by activated H-Ras^{V12}. This is also consistent with the notion that low-grade tumours do not exhibit high levels and activity of HSF1 (Calderwood, 2012a; Calderwood, 2012b; Santagata et al., 2011).

5.4.3. HSF1 knockdown decreases clonogenicity and results in increased wild-type p53 levels and activity

Although the current study has demonstrated that activation of HSF1 reduces the clonogenic survival and growth of both non-transformed and H-Ras^{V12} transformed MCF10A via enhancing activities of the wild-type p53 in these cells (see chapter 4), this chapter showed that knockdown of HSF1 by shRNAmir can also reduce the clonogenicity of both non-transformed and H-Ras^{V12} transformed MCF10A cells. This finding is consistent with the fact that knockdown of HSF1 reduces the expression of HSPs, which function as molecular chaperones that can restore cellular protein homeostasis upon stress exposure and protect cells from apoptosis and stress-induced cell death (Calderwood and Ciocca, 2008; Calderwood and Gong, 2011). Aside from the reduction of HSP expression, this chapter has also demonstrated that HSF1 knockdown leads to an increased p53 level, which then facilitates expression of some p53 transcriptional targets such as CDKN1A and BAX. The increase in wild-type p53 levels and its activity could contribute to the reduced clonogenicity of the MCF10A cells upon HSF1 depletion.

Previous studies have reported conflicting data of the effect of HSF1 knockdown on the activity of the wild-type p53 protein. Small HSPs regulated by HSF1, such as HSP27 and αB-Crystallin, are responsible for targeting wild-type p53 protein for proteasomal degradation (Jin et al., 2009). In addition, HSF1 and HSF2 complexes regulate the expression of proteasome subunits, including Psmb5 and Gankyrin which are required for p53 degradation. Therefore, HSF1 knockdown can impair p53 degradation, thus leading to an increase in wild-type p53 levels and activity (Lecomte et al., 2010). While increasing wild-type p53 sensitizes cells to DNA damaging agents such as Etoposide and Doxorubixin, HSF1 depletion indeed has been shown to enhance cell sensitivity to these agents (Jin et al., 2009). In contrast, other studies have shown HSF1 knockdown to enhance p53 activity, for example, Logan et al. (2009) demonstrated that HSF1

knockdown by siRNA reduced the expression of several wild-type p53 targets and rendered cancer cells more resistant to DNA damaging agents. Additionally, Li et al. (2008) demonstrated that suppression of HSF1 by quercetin or by siRNA can reduce p53 nuclear importation and inhibited p53 mediated expression of CDKN1A. While this chapter supports the finding that HSF1 depletion enhances wild-type p53 activity, these opposing findings can be explained by a number of possibilities. Firstly, the experiments in Li et al.'s studies (2008 and 2011) were conducted on temperature sensitive mutant p53 proteins that might not be regulated in a similar manner to wild-type p53 (Li et al., 2008; Li and Martinez, 2011). In support of this, western blot analysis performed in the current study has shown that overexpression or knockdown of HSF1 did not impact upon the level of p53 protein in the cytoplasmic and nuclear protein fractions isolated from MCF10A cells (Appendix 6). In addition, in contrast to the current study, Logan et al. (2009) demonstrated that HSF1 knockdown by siRNA did not exhibit increased wild-type p53levels. This may be due to the transient nature of the HSF1 knockdown effect by siRNA while wild-type p53 accumulated slowly and therefore was not yet elevated at the time when cells were examined.

Taken together, the current study demonstrated an interesting concept; that either overexpression or knockdown of HSF1 can lead to an increase in overall wild-type p53 activity and a subsequent decrease in clonogenic survival and growth. While activation of HSF1 increases wild-type p53 activity by supporting its transcriptional activity, knockdown of HSF1 can increase wild-type p53 activity by increasing its level of protein expression. These findings are supported by previous studies which showed that HSF1 inhibitors such as triptolide can co-operate with DNA damaging agents that activate wild-type p53 such as Cisplatin to induce apoptosis in pancreatic and gastric cancer (Li et al., 2012a; Zhu et al., 2012). In addition, triptolide can also inhibit cancer cells' proliferation by induction of G1 phase arrest through up-regulation of CDKN1A (p21) (Liu et al., 2012a). The current study thus suggests that HSF1 inhibitors could be beneficial for the treatment of cancers expressing wild-type p53 protein or in combination with anticancer therapies that activate activities of wild-type p53.

5.4.4. Generation of the HSF1 inhibitor screening cell line model

5.4.4.1. The current study provides an alternate and potentially better reporter cell line model for the identification of HSF1 inhibitors than previous luciferase reporter models.

HSF1 inhibitors have been previously identified through use of a dual luciferase assay in which cells are co-transfected with a HSF1 inducible HSE-firefly luciferase reporter construct and a constitutively expressed renilla luciferase construct (Westerheide et al., 2006; Yoon et al., 2011). Compounds that have been identified as HSF1 inhibitors in these models are those that can inhibit the firefly luciferase expression in the reporter cells upon heat-shock while leaving the renilla luciferase level unaffected (Westerheide et al., 2006; Yoon et al., 2011). Levels of the two luciferases have been measured by a two-reaction assay in which cells are lysed; the substrates for renilla luciferase and firefly luciferase are added sequentially to the samples and the luminescence generated by each luciferase catalysed reaction is measured and compared to the vector control cells. This method has led to the identification of several HSF1 inhibitor compounds such as triptolide, KNK437 and quercetin (Westerheide et al., 2006; Yoon et al., 2011).

Although many known HSF1 inhibitors have been shown to be able to inhibit heat-shock induced expressions of HSPs, none of these compounds exhibit a potent and specific HSF1 inhibition. A potential explanation for this is that heat-shock stimulates a vast array of alterations in the activity of multiple signalling cascades that involve many HSF1 regulatory molecules (Calderwood et al., 2010). Consequently, compounds identified to inhibit the heat-shock induced expressions of HSPs might target these molecules instead of HSF1. In addition, heat-shock can also increase the expression of HSPs by altering mRNA metabolism and cellular protein translational control such that only the heat shock mRNAs plus a small number of pre-existing mRNAs are translated (Storti et al., 1980). Compounds that were identified to inhibit HSP expressions upon heat shock are also likely to inhibit protein translation. Indeed, it has been reported in previous studies that HSF1 inhibitors such as triptolide and KNK437 inhibit general protein expression by regulating mRNA stability (Sun et al., 2011; Yokota et al., 2000). Triptolide has also been shown to function as a potent RNA polymerase II inhibitor (Titov et al., 20011). As heat-shock may cause alterations in expressions of HSPs

independently to HSF1, the use of heat-shock is thus not optimal for the identification of inhibitors of HSP expression that specifically target HSF1.

The current study has provided an initial proof-of-concept model of a novel reporter cell line, which provides a better methodology for the identification of specific HSF1 inhibitors. In this reporter system, HSF1 is constitutively activated by the overexpression of the activated HSF1 mutant, HSFΔRDT, which facilitates the constitutive formation of the active HSF1 trimer, leading to a constitutive expression of mCherry. As intrinsic activation of HSF1 is the only factor contributing to mCherry expression in this model, compounds identified that reduce mCherry expression would therefore have a greater likelihood in specifically targeting HSF1 activity. In addition, to control for the general inhibition of universal cellular protein synthesis, EGFP is constitutively expressed in the reporter cells. As the activity of HSF1 is measured through the changes in fluorescence levels of intact cells following compound treatments, the approach validated in the present study is more straightforward compared to the luciferase-based approach utilised by previous studies as it omits the cell lysing and substrate addition steps.

5.4.4.2. Current HSF1 inhibitors are non-specific towards HSF1

Through the reporter model generated in the present study, the activity and specificity of the known HSF1 inhibitors, triptolide, KNK437 and quercetin were re-assessed. Consistent with previous reports, triptolide and KNK437 did not alter the expression of the endogenous HSF1 in the reporter cells (Westerheide et al., 2006; Yokota et al., 2000); however, these compounds reduced the expression of both HSF1ΔRDT and EGFP which were ectopically expressed by the same bicistronic mRNA molecule. As previous studies have reported that both triptolide and KNK437 can inhibit heat-shock induced protein expression at the mRNA level and Triptolide has been shown as a potent RNA polymerase II inhibitor, these compounds thus would have reduced the stability or the protein translation of the mRNA molecules containing HSF1ΔRDT and EGFP in the reporter cells (Sun et al., 2011; Yokota et al., 2000; Titov et al.,2011. This further confirms that activities of both triptolide and KNK437 are non-specific towards HSF1.

Although both triptolide and KNK437 reduced the HSF1ΔRDT and EGFP levels, only KNK437 reduced HSP70i level and the HSF1-inducible mCherry expression in the reporter cells. This would be partly due to the fact that triptolide activated the endogenous HSF1 and the remaining HSF1ΔRDT. This was demonstrated by western blot analysis which showed that triptolide increased HSF1 phosphorylation at serine 326. The activated HSF1 may have induced some HSP70i expression. In addition, triptolide is known to be highly toxic at nanomolar concentration (Mak et al., 2009; Whitesell and Lindquist, 2009). Consistent with this, the reporter cells appeared to be very stressed following triptolide treatment, which was evident by the significant reduction in cell growth and the appearance of cell death. It is thus possible that the surviving cells had activated alternative pathways that up-regulate HSF1-independent expression of HSP70 to support cell survival while cells that have reduced expression of HSP70i and mCherry might have mostly died following the treatment. As the aim of cancer treatment is to target cancer cells while leaving normal cells relatively intact, the finding from this chapter also confirms that triptolide would not be suitable for cancer treatment due to its highly toxic nature towards normal cells.

Similar to triptolide and KNK437, quercetin also reduced the levels of HSF1ΔRDT and EGFP in the reporter cells. However, its effect was minimal at the concentrations tested. Consistent with this, previous studies have shown that quercetin can only work effectively at concentrations greater than 100μM (Harwood et al., 2007). As potent inhibitors are those effective in the nanomolar range (Whitesell and Lindquist, 2009), this indicates that quercetin has very low potency.

Taken together, the data presented here demonstrates that the known HSF1 inhibitors are non-specific and lack potency. The development of better HSF1 inhibitors for cancer treatment is thus necessary. The reporter system developed in this study could be a better tool for the future identification of HSF1 inhibitors.

5.4.4.3. Limitations and further considerations of the reporter cell model

Although the reporter cell line model that has been generated in this study has been shown to function as designed, it can be further improved. As mCherry and EGFP have the half-life of approximately 24 hours (Barrow et al., 2005; Maye et al., 2011; Shaner et al., 2004), the highest reduction in fluorescence can only be measured after 1-2 days

following compound treatment. During the development of the system, the present study has investigated the use of destabilised DsRed and ZsGreen as the fluorescence markers, which have a half-life of only 1-2 hours (Clontech, California, USA). However, these proteins were too unstable within the HEK293 cell line. The fluorescence levels in cells with these short-lived proteins were not high enough to be properly detected. This could also be due to the fact that the cells were too efficient in degrading these proteins. Nevertheless, it is possible that the reporter system can be improved by changing cell type or by using different shorter-lived fluorescent proteins (Wang et al., 2008b).

There are many ways whereby small chemical compounds could inhibit HSF1 activity. They may directly associate with HSF1 at the transactivation domain, the DNA binding domain or the heptad repeat regions necessary for trimerization and prevent the factor from its correct function. HSF1 inhibitor compounds could also inhibit HSF1 function by binding to HSE and prevent HSF1-DNA binding. As the mechanism for HSF1 inhibition may vary, any compounds identified to inhibit HSF1 activity using the reporter system developed from the present study would thus require further investigation to validate the precise mechanism of action. Additionally, it has to be noted that the activated HSF1ΔRDT expressed in the reporter cells lacks the HSF1 regulatory domain and contains a point mutation in the heptad repeat C (HR-C) domain. Compounds identified from the reporter system would require further validation in normal cells expressing wild-type HSF1.

Another potential limitation of the reporter cell line model is that when HSF1 is knocked-down, HSF2 may compensate for its activity. However, studies have shown that HSF2 DNA binding activity is dependent on HSF1 expression (Ostling, 2007; Sandqvist, 2009). In addition, HSF2 has limited transcriptional activity and does not independently activate HSP expression following a heat shock (Lecomte, 2013). More importantly, HSF2 levels have been shown to be reduced upon HSF1 knockdown (Sandqvist, 2009). Although previous studies would suggest that the activities of HSF2 would not affect the current reporter cell line model, further experiments that investigate HSF2 levels and its activity in the reporter cell line are warranted.

5.4.4.4. Potential use of the reporter cell model to identify novel HSF1 inhibitors

By generating the novel reporter cell line, the present study aims at performing high-throughput HSF1 inhibitor screening from small compound libraries using an automatic fluorescence plate reader. As the resources were not available at the time of this study, this experiment has yet to be performed. Future study is thus required to fully explore the system to identify novel HSF1 inhibitors.

5.5. CONCLUSION

HSF1 inhibition has been considered as a potential therapy in anticancer treatment. The present study showed that unlike high-grade cancer cells, which rely on HSF1 to maintain their malignant phenotypes, knockdown of HSF1 in normal breast cells and early stage breast cancer cells does not affect cell morphology, growth and migration. These results are consistent with previous observations that normal cells and low-grade breast cancer cells do not express high levels of HSF1. It is thus suggested that HSF1 inhibition would abrogate tumour growth and metastasis of high-grade cancer but exert little effect on normal tissues. In addition, the present study also reported that inhibition of HSF1 would also be beneficial in cancer cells with wild-type p53 protein, as HSF1 knockdown increases wild-type p53 level and activity.To address the lack of effective and specific HSF1 inhibitors, the present study has described a novel reporter cell line that may be developed for large-scale HSF1 inhibitor screening.

CHAPTER 6

FINAL DISCUSSION

6.1. INTRODUCTION

The heat shock transcription factor, HSF1, has been identified as a powerful modulator of the malignant phenotype in many cancer types (Dai et al., 2007; Mendillo et al., 2012; Santagata et al., 2012). As inhibition of HSF1 has emerged as a potential anticancer therapeutic strategy (Whitesell and Lindquist, 2009), understanding the functional mechanisms of HSF1 in cancer will be important for the design of suitable therapeutic agents and regimes to target and inhibit HSF1's actions for the treatment of different types of cancer. To this end, this thesis describes work that elucidates the activities of HSF1 in different breast cancer cellular contexts, thereby highlighting a context dependent nature of HSF1 activity, especially with respect to the activation and mutation status of oncogenic Ras and the tumour suppressor p53. In addition, this work also describes the development of a novel cellular screening model for the identification of more specific HSF1 inhibitors.

6.1.1. Roles of Ras and p53 in cancer

Cancer is enabled mainly by the accumulation of genetic and epigenetic alterations, which result in the activation of oncogenes and the inactivation of tumour suppressors (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). Among the oncogenes, members of the Ras family are amongst the most frequently mutated (Bos, 1989). Ras is a family of small GTPase's which function as secondary messenger molecules that relay signals between trans-membrane tyrosine kinase receptors and intracellular signalling pathways, thereby regulating many essential biological processes such as cell cycle, proliferation, survival and migration (Downward, 2003; Drosten et al., 2010). Deregulation of Ras in cancer leads to the activation of multiple downstream signal transduction pathways, which ultimately result in tumourigenesis, cancer progression and metastasis (Graham and Olson, 2007).

In addition to Ras, aberrant activity of the tumour suppressor p53 is common in all types of cancers (Vousden and Lane, 2007). Wild-type p53 is normally activated in response to stress and functions as "the guardian of the genome", conserving the genome by multiple mechanisms such as activating DNA damage repair responses or inducing apoptosis in cells that contain irreparable DNA damage (Menendez et al., 2009; Riley et al., 2008). Mutations in the TP53 gene which encodes p53, lead to the production of mutant p53 proteins which not only lose their wild-type tumour suppressing functions but can also gain additional properties that promote cancer progression, metastasis and chemo-resistance, a phenomenon called 'gain-of-function' (Oren and Rotter, 2010; Solomon et al., 2011). Therefore, while retaining wild-type p53 activity is a mechanism by which tumour growth can be abrogated, inhibition of mutant p53 proteins is also a focus of current anticancer treatments (Chen et al., 2010; Wang and Sun, 2010).

6.1.2. Summary of findings from the present study

6.1.2.1. HSF1 exerts cancer promoting effects via co-operating with activated Ras

While activation of Ras initiates tumourigenesis and promotes cancer progression, mice that are HSF1 null are protected from tumour formation induced by activated Ras (Dai et al., 2007). Previous studies have suggested that HSF1 is required for signal transduction pathways downstream of Ras as MEF cells null for HSF1 exhibit reduced levels of MAPK signalling, which leads to reduced cell migration, clonogenic survival and growth, as well as overall carcinogenesis in comparison to normal wild-type cells (Dai et al., 2012; O'Callaghan-Sunol and Sherman, 2006; Xi et al., 2012). The present study extends previous findings by demonstrating that the increased expression and activation of HSF1, achieved by ectopic expression of wild-type HSF1 or a constitutively activated form of HSF1, HSF1ΔRDT, does not impact upon normal non-transformed mammary epithelial cell biology; however, it significantly enhances the cell migration and invasion of cells transformed with the activated H-Ras^{V12}. Although previous studies have demonstrated that HSF1 is required for signalling pathways downstream of Ras (Dai et al., 2012; O'Callaghan-Sunol and Sherman, 2006; Xi et al., 2012), this study has extended these findings and shown that HSF1 activation impacts upon a diverse range of gene expression networks that are consistent with the effects observed upon the cell biology of MCF10A cells containing activated Ras. In particular,

the number of genes that were altered upon ectopic expression of HSF1ΔRDT in the H-Ras^{V12} transformed MCF10A cells was much higher than in the mCherry non-transformed MCF10A cells. Pathway analysis using Metacore^{TM} software revealed that the most significant alterations were the down-regulation of ECM remodelling pathways and the up-regulation of cytoskeleton remodelling pathways in cells ectopically expressing HSF1ΔRDT. This effect was significantly enhanced in cells that were transformed through the expression of activated Ras, with the number of genes altered in each of these pathways being much higher when compared to the non-transformed cellular context. As both the down-regulation of ECM remodelling pathways and the up-regulation of cytoskeleton remodelling pathways have been shown to support cancer cell migration and invasion (Levental et al., 2009; Lo et al., 2000; Yamaguchi and Condeelis, 2007), the present study indicates that HSF1 functions through these pathways to promote the migratory and invasion abilities of cancer cells containing activated Ras.

6.1.2.2. HSF1 modulates activities of both wild-type and mutant p53, leading to divergent effects of HSF1 in cancer

HSF1 has been reported to modulate the activity of wild-type p53. Co-expression of HSF1 and wild-type p53 in Hela cells has been shown to cause a significant increase in p53 activity upon DNA damage compared to the expression of wild-type p53 alone (Logan et al., 2009). Consistent with this, heat-shock and HSF1 activation enhance the protein expression of members of the DNA damage response proteins upon Doxorubicin treatment (Salmand et al., 2008). In addition to these findings, the current study has demonstrated that HSF1 can enhance the activity of both wild-type and mutant p53 in cancer cells, leading to divergent effects of HSF1 upon clonogenic survival and growth depending upon cellular p53 status. In particular, activation of HSF1 in cells with wild-type p53 promoted the p53-regulated expression of genes involved in cell cycle arrest and apoptosis, thereby reducing clonogenic survival and growth of these cells. In contrast, activation of HSF1 in cells with mutant p53 causes a mutant p53-dependent increase in clonogenicity. While many studies have identified HSF1 as a positive regulator of cancer progression, the current study proposes that HSF1 may enhance the tumour suppressing activities of wild type p53 as originally postulated by Logan *et al.* (Logan et al., 2009). It is therefore feasible that HSF1 may

initially act to prevent tumour onset in healthy tissue that contains wild type p53; however, in the context of advanced tumours, HSF1 may act via mutant p53 to promote cancer progression.

6.1.2.3. Knockdown of HSF1by shRNAmir reduces clonogenicity of normal and H-Ras^{V12} transformed MCF10A

HSF1 is found to enhance the activity of wild-type p53 protein and this leads to the reduced clonogenicity of wild-type p53 containing cells upon HSF1 activation. However, the present study also found that the knockdown of HSF1 by shRNAmir could also reduce the clonogenic survival and growth of both normal and H-Ras^{V12} transformed MCF10A which contain wild-type p53. This is also consistent with previous studies demonstrating that HSF1 knockdown or inhibition by pharmacological compounds abrogates the ability of many cancer cell types to form colonies in both 2-D and 3-D *in vitro* growth conditions (Dai et al., 2007; Khaleque et al., 2005; Kouspou, 2009). Although this appears to be at odds with our finding that overexpression of HSF1 can also reduce clonogenicity, this can be explained by the fact that upon the reduction of HSF1 by shRNAmir, the protein expression of HSPs is reduced, rendering the cells more susceptible to cell death upon stringent conditions. In addition, the present study also shows that consistent with previous reports (Jin et al., 2009; Lecomte et al., 2010), HSF1 knockdown increases wild-type p53 protein levels in both the non-transformed and H-Ras^{V12} transformed MCF10A as HSF1 up-regulates the expression of proteins responsible for the degradation of wild-type p53 (Fig.6.1). Inhibition of HSF1 would thus also be beneficial in the treatment of cancer cells containing wild-type p53.

6.1.3. HSF1 in tumourigenesis and cancer progression

Previous studies have reported that HSF1 functions in co-operation with oncogenic proteins to support cancer progression. For example, Min et al. (2007) reported that mice that are HSF1 null exhibited an altered spectrum of tumours arising from p53 loss. Dai et al. (2009) reported that mice null for HSF1 are protected from tumours induced by the activated H-Ras^{V12} and the mutated p53^{R172H}. In addition, Khaleque et al. (2005) reported that the highly malignant factor heregulin β1 induces a more malignant phenotype in tumour cells via the activation of HSF1. More recently, Xi et al. (2012) reported that HSF1 co-operates with ErbB2 to promote mammary tumourigenesis and

metastasis. Moreover, Dai et al. (2012) showed that HSF1 depletion impedes neurofibromatosis type 1 (NF1)-associated carcinogenesis. Taken together, these studies and the present study suggest that while the acquisition of genetic and epigenetic alterations is required for cancer cells to survive and proliferate, HSF1 appears to foster malignant phenotypes uniquely in these cancer cells through co-operating and/or facilitating the activated oncogenes and mutated tumour suppressors. This is consistent with the notion that different to that of normal cells, cancer cells are more dependent on HSF1 to prosper, a phenomenon known as non-oncogenic addiction (Solimini et al., 2007). By regulating and/or co-operating with several activated oncogenes and mutated tumour suppressors, as previously reported, HSF1 can thus regulate a diverse range of transcriptional networks in cancer cells distinct to that of heat-shock and plays a multifaceted role in tumourigenesis and cancer progression (Dai et al., 2007; Mendillo et al., 2012; Santagata et al., 2011). As such, HSF1 emerges as a unique and most likely universal therapeutic target to inhibit multiple oncogenic proteins in cancer.

6.1.3. The novel HSF1 inhibitor screening model

Although several studies have identified HSF1 as a potential anticancer therapeutic target, there are currently no specific and/or potent HSF1 inhibitors available. Aside from investigating the activity of HSF1 within different cellular contexts of breast cancer, the present study also developed a novel HSF1 inhibitor cell reporter model which would allow for the identification of more specific HSF1 inhibitors. This reporter system constitutively expresses an mCherry gene under the control of an HSF1 inducible promoter and an EGFP gene under the control of a non-inducible promoter. HSF1 is activated in the reporter cells by the ectopic expression of HSF1ΔRDT to drive the expression of the mCherry. This reporter system thus has the potential to detect HSF1 inhibitors as the chemical compounds that can specifically reduce the mCherry levels within the reporter cell lines while leaving the levels of EGFP unaffected. While previous studies have commonly identified HSF1 inhibitors by dual luciferase approaches,wherein HSF1 inhibitors were compounds that could lower the levels of HSF1 inducible luciferase upon heat-shock (Westerheide et al., 2006; Yoon et al., 2011), this HSF1 inhibitor reporter model avoids the need for heat-shock and controls for the fact that many compounds inhibit protein translation rather than HSF1 '*per se*'. As such, it is hoped that this would prove a more effective approach to identify

compounds that directly interfered with HSF1 function. One caveat to the system is that the use of a mutated HSF1 molecule may result in the isolation of compounds that would only inhibit mutated HSF1 rather than wild-type HSF1. To control for this, subsequent screens would also incorporate the use of wild-type HSF1 within the assay. This system would thus enable large-scale screening for HSF1 inhibitors from available compound libraries using a high-throughput fluorescence plate reader.

6.2. INHIBITION OF HSF1 IN CANCER TREATMENT

HSPs are important factors in tumourigenesis and cancer progression (Calderwood and Ciocca, 2008; Calderwood et al., 2006). The fact that the expression of these proteins can be inhibited by therapeutically targeting HSF1 makes the transcription factor an attractive anticancer therapeutic target. Moreover, with increasing evidence that HSF1 contributes to cancer tumourigenesis and progression by other unique mechanisms not related to its role in HSP expression, the therapeutic targeting of HSF1 in cancer has gained special interest in recent years (Whitesell and Lindquist, 2009). Consistent with this, the present study has further confirmed that HSF1 is a valid therapeutic target for cancer treatment by demonstrating the regulation of HSF1 upon many aspects of cancer. However, as the activity of HSF1 appears to be context dependent, it is postulated that the effectiveness of HSF1 targeting therapies may vary in differing cancer types and contexts. In particular, inhibition of HSF1 in normal cells and in cells at an early stage of transformation does not drastically affect the cell biology of these cells. However, activation of HSF1 promotes cancer progression by enhancing the oncogenic activities of activated oncogenes and mutated tumour suppressors. Together with previous studies which have demonstrated that HSF1 depletion can abrogate the malignant phenotype, it is suggested that inhibition of HSF1 would cause unique and specific anticancer effects on high-grade tumours while having minimal toxicity upon normal cells. In low-grade cancer, an HSF1 inhibitor by itself may not be a powerful therapeutic treatment but within the neo-adjuvant or adjuvant setting may aid in delaying or preventing cancer progression.

Additionally, consistent with previous studies demonstrating that HSF1 regulates wild-type p53 degradation (Jin et al., 2009; Lecomte et al., 2010; Meng et al., 2010), this study has shown that knockdown of HSF1 can increase wild-type p53 levels and

activity, thus decreasing clonogenic survival and growth. Moreover, inhibition of HSF1 in high-grade cancer cells that possess mutant p53 also abrogates clonogenic survival and growth. This is most likely due to the fact that the molecule not only promotes the activity of mutant p53 but also regulates the expression of HSP90, which is the main chaperone required for mutant p53 stabilization (Li et al., 2011b). The present study thus suggests that inhibition of HSF1 in either wild-type or mutant p53 containing tumour cells would be beneficial in cancer treatment. In addition, HSF1 inhibitors could act synergistically with therapies that target the p53 pathway, thus enhancing their efficacy. The combination of HSF1 inhibitors with p53 targeting therapies, especially in the treatment of low-grade cancers may therefore prove more beneficial.

6.3. ACTIVATION OF HSF1 IN CANCER TREATMENT

Although HSF1 activation has been shown to promote cancer progression, activation of HSF1 has also been used in cancer treatment regimes. As cancer cells are continuously exposed to numerous extrinsic and intrinsic stresses, further activation of the HSR by HSF1 activation is thought to heighten stress levels beyond the cells capacity to compensate and thus the cells undergo apoptosis (Santagata et al., 2012). Consistent with this, hyperthermia (heat therapy) has been widely used as an adjunct to other forms of cancer therapies such as radiation therapy and chemotherapy, being shown to effectively sensitise cancer cells to these therapies (Torigoe et al., 2009). Consistent with this, HSF1 activators such as celastrol and withaferin A have also been reported to exhibit potent anticancer properties (Hahm et al., 2011; Kannaiyan et al., 2011; Li et al., 2012c; Zhang et al., 2012b). In addition to this, the current study presents an interesting concept that in contrast to many studies that point to high levels of HSF1 expression or activation to promote tumour progression, HSF1 activation in the context of wild-type p53 may be beneficial, leading to increased apoptosis and decreased tumour growth due to HSF1 enhancing wild-type p53 activity. This raises a potential use for HSF1 activators as agents that potentiate DNA damaging therapeutics by enhancing the activity of p53. Indeed, recent studies have shown celastrols and withaferin A can induce p53-dependent apoptosis (Hahm et al., 2011; Sung et al., 2010). Consistent with this, withaferin A and celastrols have been shown to enhance apoptosis induced by X-ray irradiation (Devi and Kamath, 2003; Yang et al., 2011a). Altogether, while HSF1 activation is associated with cancer aggressiveness and metastasis, HSF1 activators,

when utilised within the correct cellular context, may also be an effective anticancer treatment against certain types of tumours. However, the use of such agents requires a greater understanding of the role of HSF1 in relation to the genetic and epigenetic contexts of cancer cells.

6.4. HSF1 AS A BIOMARKER TOOL TO PREDICT PATIENT OVERALL SURVIVAL AND RESPONSE TO THERAPEUTIC INTERVENTION

HSF1 has been shown to be an independent prognostic indicator associating with poorer overall survival of breast cancer. High levels of HSF1 are more likely to be found in high-grade tumours, especially in ER-positive breast cancer (Santagata et al., 2011). As HSF1 is expressed in all cell types, with increasing evidence of the cancer promoting roles of HSF1 in other types of cancers, it is emerging that HSF1 could be used as a biomarker tool to predict patient overall survival in several cancer types (Calderwood and Gong, 2011). In addition to this, the findings of this present study suggest that combining the HSF1 status with the activation status of Ras and/or mutation status of p53 may provide more reliable biomarker tools for prediction of overall patient survival.

Additionally, while resistance to chemotherapy remains a major obstacle to the successful management of many human cancers, HSF1 would also be an indicator of poor patient response. As well as the elevated levels of HSPs caused by HSF1 activation that would confer cancer cells resistance to drug-induce cell death, HSF1 also regulates the expression of the multidrug resistance protein MDR-1 (Kioka et al., 1992) (Chin et al., 1990; Miyazaki et al., 1992; Vilaboa et al., 2000). Besides, as mutant p53 proteins have been shown to confer chemo-resistance in cancer cells *in vitro* (Strano et al., 2007a), HSF1 would also act through these proteins to enhance resistance. Therefore, examining both HSF1 and p53 in patients would ultimately provide a better predictor of therapeutic response than either alone.

6.5. CONCLUSION AND FUTURE WORK

The findings in this thesis have extended our current understanding of the mechanisms by which HSF1 may promote tumourigenesis and cancer progression. Further studies that would extend these findings include:

- The present study has demonstrated that HSF1 co-operates with activated Ras to promote cancer cell migration and invasion, with microarray analysis revealing that HSF1 activates distinct transcriptional networks promoting cancer progression especially pathways regulating the immune response in cells with Ras activation. Future experiments therefore should be conducted to assess the roles of HSF1 in these processes in relation to Ras activity.
- Although the present study has shown that the impact of HSF1 activation upon cell migration and 3-D growth in cancer cells with activated Ras is enhanced, the exact mechanism of the association between HSF1 activity and activation status of Ras is yet to be characterised. Western blot analysis in the present study suggest that Ras activation does not increase HSF1 activation and/or synthesis but in contrast, reduces both HSF1 levels and activity in the induction of HSP expressions (Fig.3.1 and 5.2). This is consistent with a previous study demonstrating that expression of activated Ras reduced HSF1-induced expression of HSP70 in MEF cells (Stanhill et al., 2006). Downstream signalling pathways of Ras are known to regulate HSF1 both positively and negatively. For example, HSF1 can be inactivated by MAPK kinase 2 (MK2) and ribosomal S6 kinase 2 (RSK2), which are activated following Ras activation. In contrast, HSF1 can be activated by the phosphorylation activity of protein kinase A (PKA), which is a downstream effector of Ras (Murshid et al., 2010). HSF1 can also be activated by the PI3K/Akt signalling pathway downstream of Ras due to the ability of Akt to phosphorylate and inhibit GSK3β, which is a repressor of HSF1 activity (He et al., 1998; Xavier et al., 2000). Therefore, one possibility is that activated Ras can alter the activity of HSF1 by altering the activity of these kinases. Another possibility is that HSF1 may associate with proteins regulated by activated Ras and this may be a mode of regulation of HSF1 activity. Further investigations upon these potential modes of HSF1 regulation may further reveal HSF1 based mechanisms in cancer.
- The current study has also demonstrated that the effect of HSF1 upon cancer cell clonogenicity is via a p53 dependent mechanism, although the precise mechanism by which this association occurs remains to be elucidated. However, as HSF1 has been found to interact with wild-type p53 following heat-shock (Logan et al., 2009), it is thus suggested that HSF1 and p53 may engage in a common transcriptional complex that is formed and activated only during certain specific conditions. Studies that investigate interactions of HSF1 with p53 in different conditions are therefore

suggested. In addition, HSF1 could potentially increase or decrease p53 acetylation, in that HSF1 transcriptionally controls Strap (Stress-responsive activator of p300), a co-factor that aids in wild-type p53 acetylation via p300/CBP77 (Xu and La Thangue, 2008; Xu et al., 2008). Moreover, HSF1 can also control global deacetylation via the regulation of HDAC1 and HDAC2 activity (Fritah et al., 2009). Studies that investigate the effect of HSF1 upon post-translational modifications of wild-type and mutant p53 such as phosphorylation, acetylation, ubiquitination and overall stability would further elucidate the association of HSF1 and the p53 pathway.

- As each mutant p53 protein can confer differing 'gain-on-function' capabilities that promote cancer progression, studies that investigate the effect of HSF1 on cell biology and chemo-resistance of cancer cells with differing mutant p53 proteins would further elucidate the mechanisms and the multifaceted roles of HSF1 in cancer, as well as its cellular context dependency.
- The present study has demonstrated that HSF1 enhances the activities of mutant p53 proteins that go beyond protein stabilization. There are several mechanisms that enable mutant p53 'gain-of-function' activities. One of the mechanisms is by its interaction with other transcription factors and its stimulation (e.g. NF-Y, NFκB; 'pro-life') or inhibition of their activities (TAp63, TAp73; 'pro-death') ((Freed-Pastor and Prives, 2012). Therefore, further studies are suggested that investigate whether HSF1 can directly interact with mutant p53 or enhance mutant p53 interactions with NF-Y, NFκB, STAT1, E2F1, TAp63 and TAp73.
- As HSF1 appears to exert its cancer promoting effects via modulating the activity of activated oncogenes and mutated tumour suppressors, studies that investigate the interaction between HSF1 with oncogenic proteins other than Ras and mutant p53 are thus suggested. For example, the oncogene cMyc is known to regulate HSP (Kingston et al., 1984). In addition, a crosstalk between the oncogenic β-catenin/Wnt pathway and the heat shock response has been identified in highly metastatic breast tumours (Fanelli et al., 2008). It is possible that HSF1 also plays supporting roles in these oncogenic pathways.
- With the development of a novel HSF1 inhibitor screening model, this study suggests that a large scale screen for novel HSF1 inhibitors from available compound libraries should be pursued.

In conclusion, the current study provides highly relevant and novel molecular mechanistic insights into the role for HSF1 in cancer. These findings have provided additional evidence to support the notion that HSF1 inhibition is an attractive strategy for cancer therapies. Importantly, this work also identifies HSF1 as a therapeutic target by which activated oncogenes such as Ras and mutated tumour suppressor such as p53 could be inhibited. Although the focus of this study is upon breast cancer, these findings also have wider relevance to other cancers where activated Ras and mutant p53 proteins are major contributors. While identification of potent and specific HSF1 inhibitors is still challenging, this work also proposes a novel methodology for future screening studies

APPENDIX 1

EXAMPLES OF SELECTION OF CELLS USING FLUORESCENCE-ACTIVATED CELL SORTING

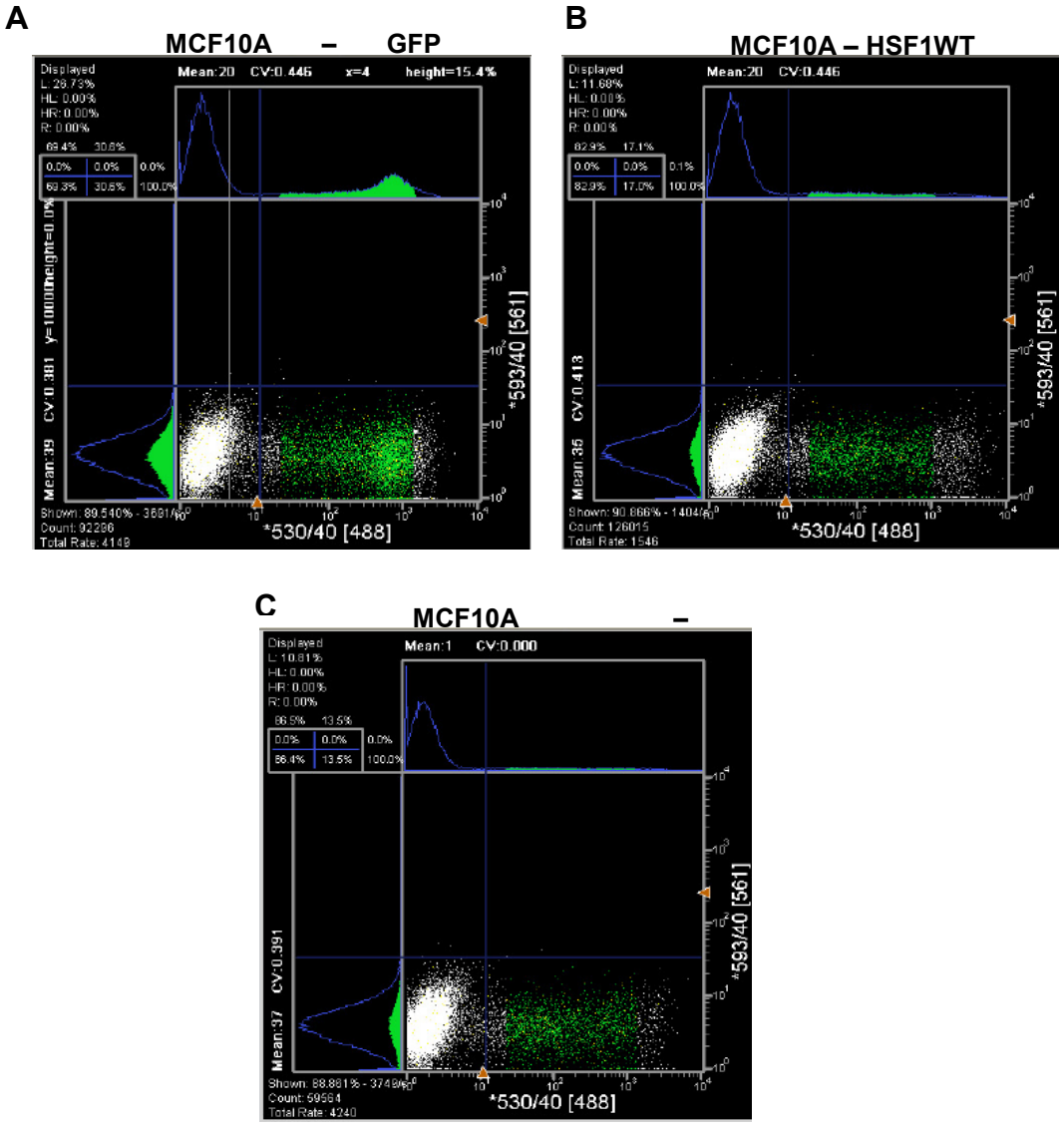


Figure A1. Flow cytometry analysis and selection gates for FACS for the selection of stably transduced MCF10A cells after viral transduction to stably express HSF1.

low cytometry analysis of MCF10A cells transduced with retroviral constructs xpressing **(A)** EGFP control, **(B)** HSF1WT-IRES-EGFP and **(C)** HSF1ΔRDT-IRES-GFP revealed that 15-30% of the cells were successfully transduced, which xpressed EGFP. Green cells are cells selected by FACS. The same selection gates ere chosen for all cell types to ensure similar levels of ectopic gene expressions mong the cell types.

APPENDIX 2

ECTOPIC EXPRESSION OF HSF1 IN SkBr3 CELLS ENHANCES PLCγ1 SIGNAL TRANSDUCTION

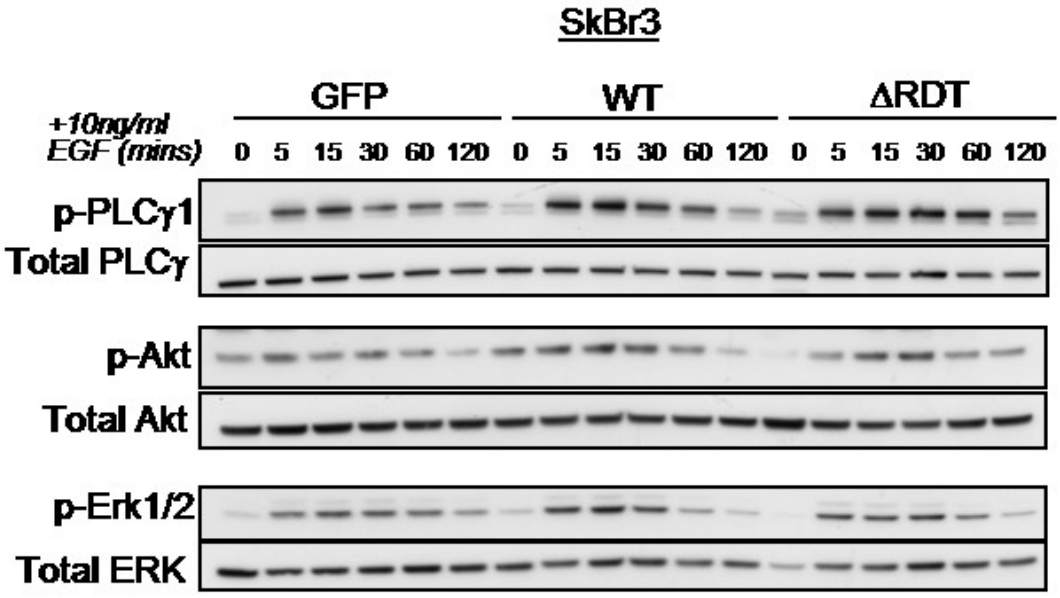


Figure A2. Ectopic expression of HSF1 enhances PLCγ1 signalling pathway of SkBr3 cells following EGF treatment.

SkBr3 cells expressing wild-type HSF1 (WT) or HSF1ΔRDT (ΔRDT) exhibited increased levels of phosphorylated PLCγ1 compared to the GFP control cells. Ectopic expression of HSF1 not impact upon the total and phosphorylated levels of Erk1/2 and Akt of SkBr3 cells after EGF stimulation.

APPENDIX 3

LISTS OF GENES ALTERED UPON ECTOPIC EXPRESSION OF HSF1ΔRDT IN MCF10A CELLS

Table A1. *List of genes down-regulated upon ectopic expression of HSF1ΔRDT in the non-transformed mCherry MCF10A cells*

No.	Gene symbol	Gene name	LogFC
1	ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2 (ABCG2), mRNA [NM_004827]	-3.0306
2	ABHD10	abhydrolase domain containing 10 (ABHD10), mRNA [NM_018394]	-1.0054
3	ACOT4	acyl-CoA thioesterase 4 (ACOT4), mRNA [NM_152331]	-1.2597
4	ACTR3C	mRNA; cDNA DKFZp686O24114 (from clone DKFZp686O24114). [BX640643]	-1.4312
5	ADAM19	ADAM metallopeptidase domain 19 (ADAM19), mRNA [NM_033274]	-1.4194
6	ADD2	adducin 2 (beta) (ADD2), transcript variant 2, mRNA [NM_017482]	-2.0893
7	ADIPOQ	adiponectin, C1Q and collagen domain containing (ADIPOQ), transcript variant 2, mRNA [NM_004797]	-1.3616
8	AGR2	anterior gradient homolog 2 (Xenopus laevis) (AGR2), mRNA [NM_006408]	-1.2019
9	ALOX5AP	arachidonate 5-lipoxygenase-activating protein (ALOX5AP), transcript variant 1, mRNA [NM_001629]	-3.1002
10	AMIGO2	adhesion molecule with Ig-like domain 2 (AMIGO2), transcript variant 2, mRNA [NM_181847]	-1.3507
11	ANO7	anoctamin 7 (ANO7), transcript variant NGEP-L, mRNA [NM_001001891]	-1.3822
12	APCDD1	adenomatosis polyposis coli down-regulated 1 (APCDD1), mRNA [NM_153000]	-1.0409
13	ARHGAP28	Rho GTPase activating protein 28 (ARHGAP28), mRNA [NM_001010000]	-1.2719
14	ASIP	agouti signaling protein (ASIP), mRNA [NM_001672]	-1.1001
15	ATAD3C	ATPase family, AAA domain containing 3C (ATAD3C), mRNA [NM_001039211]	-1.4282
16	ATP10B	ATPase, class V, type 10B (ATP10B), mRNA [NM_025153]	-1.067
17	ATP2A1	ATPase, Ca++ transporting, cardiac muscle, fast twitch 1 (ATP2A1), transcript variant b,	-1.3621
18	ATP8B4	ATPase, class I, type 8B, member 4 (ATP8B4), mRNA [NM_024837]	-1.4896
19	BMP7	bone morphogenetic protein 7 (BMP7), mRNA [NM_001719]	-1.3541
20	BPIL3	bactericidal/permeability-increasing protein-like 3 (BPIL3), mRNA [NM_174897]	-5.2008
21	BTC	betacellulin (BTC), mRNA [NM_001729]	-1.1422
22	C10orf55	chromosome 10 open reading frame 55 (C10orf55), mRNA [NM_001001791]	-1.1424
23	C11orf34	chromosome 11 open reading frame 34 (C11orf34), mRNA [NM_001145024]	-1.0542
24	C12orf70	chromosome 12 open reading frame 70 (C12orf70), mRNA [NM_001145010]	-1.2254
25	C15orf48	chromosome 15 open reading frame 48 (C15orf48), transcript variant 2, mRNA [NM_032413]	-1.2007
26	C1orf114	chromosome 1 open reading frame 114 (C1orf114), mRNA [NM_021179]	-1.0276
27	C1orf86	chromosome 1 open reading frame 86 (C1orf86), transcript variant 1, mRNA [NM_001146310]	-1.2826
28	C1QTNF2	C1q and tumor necrosis factor related protein 2 (C1QTNF2), mRNA [NM_031908]	-1.1681
29	C2	complement component 2 (C2), transcript variant 3, mRNA [NM_001178063]	-1.251
30	C2orf84	chromosome 2 open reading frame 84 (C2orf84), mRNA [NM_001040710]	-1.0719
31	CACNA1A	calcium channel, voltage-dependent, P/Q type, alpha 1A subunit (CACNA1A),	-1.0365
32	CACNA1F	calcium channel, voltage-dependent, L type, alpha 1F subunit (CACNA1F), mRNA [NM_005183]	-1.0192
33	CACNG1	calcium channel, voltage-dependent, gamma subunit 1 (CACNG1), mRNA [NM_000727]	-1.0648
34	CALB1	calbindin 1, 28kDa (CALB1), mRNA [NM_004929]	-1.2893
35	CAMK1	calcium/calmodulin-dependent protein kinase I (CAMK1), mRNA [NM_003656]	-1.0203
36	CCDC87	coiled-coil domain containing 87 (CCDC87), mRNA [NM_018219]	-1.5372
37	CD19	CD19 molecule (CD19), transcript variant 2, mRNA [NM_001770]	-1.1638
38	CD40	CD40 molecule, TNF receptor superfamily member 5 (CD40), transcript variant 1, mRNA [NM_001250]	-1.5115
39	CDC14B	CDC14 cell division cycle 14 homolog B (S. cerevisiae) (CDC14B), transcript variant 2, mRNA [NM_033331]	-1.0928
40	CDC6	cell division cycle 6 homolog (S. cerevisiae) (CDC6), mRNA [NM_001254]	-1.1275
41	CHIC1	cysteine-rich hydrophobic domain 1 (CHIC1), mRNA [NM_001039840]	-1.1536
42	CHST11	carbohydrate (chondroitin 4) sulfotransferase 11 (CHST11), transcript variant 1, mRNA [NM_018413]	-1.393
43	CLDN2	claudin 2 (CLDN2), transcript variant 2, mRNA [NM_001171092]	-1.0596
44	CNTNAP3	contactin associated protein-like 3 (CNTNAP3), mRNA [NM_033655]	-1.1729
45	CPEB1	cytoplasmic polyadenylation element binding protein 1 (CPEB1), transcript variant 1, mRNA [NM_030594]	-1.3096
46	CPN1	carboxypeptidase N, polypeptide 1 (CPN1), mRNA [NM_001308]	-1.1152
47	CRABP2	cellular retinoic acid binding protein 2 (CRABP2), transcript variant 1, mRNA [NM_001878]	-1.0473
48	CRCT1	cysteine-rich C-terminal 1 (CRCT1), mRNA [NM_019060]	-1.469
49	CROCC	ciliary rootlet coiled-coil, rootletin (CROCC), mRNA [NM_014675]	-1.0464
50	CRX	cone-rod homeobox (CRX), mRNA [NM_000554]	-1.278
51	CTNND2	catenin (cadherin-associated protein), delta 2 (neural plakophilin-related arm-repeat protein) (CTNND2), mRNA [NM_001332]	-1.0947
52	CYMP	chymosin pseudogene (CYMP), non-coding RNA [NR_003599]	-1.2643
53	CYP4F11	cytochrome P450, family 4, subfamily F, polypeptide 11 (CYP4F11), transcript variant 1, mRNA [NM_021187]	-1.0201

54	DAPL1	death associated protein-like 1 (DAPL1), mRNA [NM_001017920]	-1.3102
55	DCLK2	doublecortin-like kinase 2 (DCLK2), transcript variant 1, mRNA [NM_001040260]	-1.4164
56	DLX6-AS1	DLX6 antisense RNA 1 (non-protein coding) (DLX6-AS1), non-coding RNA [NR_015448]	-1.3357
57	DNAH2	dynein, axonemal, heavy chain 2 (DNAH2), mRNA [NM_020877]	-1.3881
58	DNAJC14	DnaJ (Hsp40) homolog, subfamily C, member 14 (DNAJC14), mRNA [NM_032364]	-1.2198
59	DNASE1L3	deoxyribonuclease I-like 3 (DNASE1L3), mRNA [NM_004944]	-1.191
60	DPP6	dipeptidyl-peptidase 6 (DPP6), transcript variant 3, mRNA [NM_001039350]	-1.0825
61	DUOXA1	dual oxidase maturation factor 1 alpha (DUOXA1) mRNA, complete cds, alternatively spliced. [EU927394]	-1.1922
62	EFHB	cDNA clone IMAGE:5295205, with apparent retained intron. [BC043212]	-1.3033
63	EML6	microtubule associated protein like 6 [Source:HGNC Symbol;Acc:35412] [ENST00000490828]	-1.2171
64	ESR1	estrogen receptor 1 (ESR1), transcript variant 1, mRNA [NM_000125]	-1.1607
65	EXOC3L2	exocyst complex component 3-like 2 (EXOC3L2), mRNA [NM_138568]	-1.2452
66	EYS	eyes shut homolog (Drosophila) (EYS), transcript variant 1, mRNA [NM_001142800]	-1.1395
67	FAM127C	family with sequence similarity 127, member C (FAM127C), mRNA [NM_001078173]	-1.0263
68	FAM132B	sequence similarity 132, member B [Source:HGNC Symbol;Acc:26727] [ENST00000481917]	-1.2024
69	FAM183A	family with sequence similarity 183, member A (FAM183A), mRNA [NM_001101376]	-3.5713
70	FAM57A	family with sequence similarity 57, member A (FAM57A), mRNA [NM_024792]	-1.6117
71	FBLL1	fibrillarin-like 1 (FBLL1), non-coding RNA [NR_024356]	-1.0439
72	FBXO43	F-box protein 43 (FBXO43), transcript variant 2, mRNA [NM_001029860]	-1.0643
73	FGD3	FYVE, RhoGEF and PH domain containing 3 (FGD3), transcript variant 2, mRNA [NM_033086]	-1.0551
74	FLJ40453	omo sapiens hypothetical protein LOC100288254 (LOC100288254), mRNA [XM_002342572]	-1.6517
75	FLJ43944	cDNA FLJ43944 fis, clone TESTI4014392. [AK125932]	-2.4363
76	FOXA1	forkhead box A1 (FOXA1), mRNA [NM_004496]	-1.2804
77	FST	follistatin (FST), transcript variant FST344, mRNA [NM_013409]	-1.0758
78	GATA4	GATA binding protein 4 (GATA4), mRNA [NM_002052]	-1.0039
79	GATS	GATS, stromal antigen 3 opposite strand (GATS), transcript variant 1, mRNA [NM_178831]	-1.1668
80	GCNT4	glucosaminyl (N-acetyl) transferase 4, core 2 (GCNT4), mRNA [NM_016591]	-1.1321
81	GIMAP2	GTPase, IMAP family member 2 (GIMAP2), mRNA [NM_015660]	-1.0794
82	GJA1	gap junction protein, alpha 1, 43kDa (GJA1), mRNA [NM_000165]	-1.9638
83	GLDC	glycine dehydrogenase (decarboxylating) (GLDC), nuclear gene encoding mitochondrial protein, mRNA [NM_000170]	-1.0147
84	GPR110	G protein-coupled receptor 110 (GPR110), transcript variant 1, mRNA [NM_153840]	-1.0908
85	GRIP1	glutamate receptor interacting protein 1 (GRIP1), transcript variant 1, mRNA [NM_021150]	-1.1337
86	GZMH	granzyme H (cathepsin G-like 2, protein h-CCPX) (GZMH), mRNA [NM_033423]	-1.0862
87	H2BFM	H2B histone family, member M (H2BFM), mRNA [NM_001164416]	-1.1624
88	H2BFXP	H2B histone family, member X, pseudogene (H2BFXP), non-coding RNA [NR_003238]	-1.1025
89	HAS3	hyaluronan synthase 3 (HAS3), transcript variant 1, mRNA [NM_005329]	-1.3998
90	HBEGF	heparin-binding EGF-like growth factor (HBEGF), mRNA [NM_001945]	-2.8706
91	HLA-DQA1	major histocompatibility complex, class II, DQ alpha 1 (HLA-DQA1), mRNA [NM_002122]	-1.3957
92	HLA-DRB4	major histocompatibility complex, class II, DR beta 4 (HLA-DRB4), mRNA [NM_021983]	-1.0547
93	HMGCLL1	3-hydroxymethyl-3-methylglutaryl-CoA lyase-like 1 (HMGCLL1), transcript variant 1, mRNA [NM_019036]	-1.1371
94	HOPX	HOP homeobox (HOPX), transcript variant 2, mRNA [NM_139211]	-1.6193
95	HRG	histidine-rich glycoprotein (HRG), mRNA [NM_000412]	-1.6336
96	HSD11B1	hydroxysteroid (11-beta) dehydrogenase 1 (HSD11B1), transcript variant 2, mRNA [NM_181755]	-1.429
97	HSD17B3	hydroxysteroid (17-beta) dehydrogenase 3 (HSD17B3), mRNA [NM_000197]	-1.1692
98	IL15RA	interleukin 15 receptor, alpha (IL15RA), transcript variant 2, mRNA [NM_172200]	-1.2805
99	IL17F	interleukin 17F (IL17F), mRNA [NM_052872]	-1.0004
100	IQCF3	IQ motif containing F3 (IQCF3), transcript variant 1, mRNA [NM_001085479]	-1.1875
101	KIF3C	kinesin family member 3C (KIF3C), mRNA [NM_002254]	-1.194
102	KIR2DS2	killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 2 (KIR2DS2), mRNA [NM_012312]	-1.0194
103	KLHL6	kelch-like 6 (Drosophila) (KLHL6), mRNA [NM_130446]	-1.0096
104	KLK1	kallikrein 1 (KLK1), mRNA [NM_002257]	-1.2036
105	KLK11	kallikrein-related peptidase 11 (KLK11), transcript variant 2, mRNA [NM_144947]	-1.204
106	KLK7	kallikrein-related peptidase 7 (KLK7), transcript variant 1, mRNA [NM_005046]	-2.1142
107	KLK8	kallikrein-related peptidase 8 (KLK8), transcript variant 2, mRNA [NM_144505]	-1.192
108	KRT1	keratin 1 (KRT1), mRNA [NM_006121]	-1.7969
109	KRT39	keratin 39 (KRT39), mRNA [NM_213656]	-1.2242
110	KRT80	keratin 80 (KRT80), transcript variant 1, mRNA [NM_182507]	-1.5968
111	KRTDAP	keratinocyte differentiation-associated protein (KRTDAP), mRNA [NM_207392]	-1.4341
112	LAG3	lymphocyte-activation gene 3 (LAG3), mRNA [NM_002286]	-1.0542
113	LANCL2	LanC lantibiotic synthetase component C-like 2 (bacterial) (LANCL2), mRNA [NM_018697]	-1.6404
114	LCN2	lipocalin 2 (LCN2), mRNA [NM_005564]	-2.0184
115	LCT	lactase (LCT), mRNA [NM_002299]	-1.1263
116	LMBRD2	LMBR1 domain containing 2 (LMBRD2), mRNA [NM_001007527]	-1.0189
117	LOC100128361	hypothetical LOC100128361 (LOC100128361), non-coding RNA [NR_036505]	-1.3181
118	LOC100128429	cDNA FLJ41329 fis, clone BRAMY2047676. [AK123323]	-1.5842
119	LOC100132529	omo sapiens hypothetical LOC100132529 (LOC100132529), partial miscRNA [XR_109319]	-1.1003
120	LOC100292427	omo sapiens hypothetical protein LOC100292427 (LOC100292427), mRNA [XM_002346075]	-1.3705
121	LOC284009	cDNA FLJ36671 fis, clone UTERU2004039. [AK093990]	-1.0849
122	LOC285740	hypothetical LOC285740 (LOC285740), non-coding RNA [NR_027114]	-1.0619
123	LOC339442	hypothetical LOC339442 (LOC339442), non-coding RNA [NR_038928]	-1.294

124	LOC389634	hypothetical LOC389634 (LOC389634), non-coding RNA [NR_024420]	-1.2253
125	LOC400685	hypothetical LOC400685 (LOC400685), non-coding RNA [NR_033982]	-1.1894
126	LOC401052	hypothetical LOC401052 (LOC401052), mRNA [NM_001008737]	-1.0052
127	LOC440934	omo sapiens hypothetical LOC440934 (LOC440934), miscRNA [XR_108436]	-1.8001
128	LOC541467	hypothetical LOC541467, mRNA (cDNA clone IMAGE:4830703), partial cds. [BC045815]	-1.0996
129	LOC729970	hCG2028352-like (LOC729970), non-coding RNA [NR_033998]	-1.0173
130	LRFN2	leucine rich repeat and fibronectin type III domain containing 2 (LRFN2), mRNA [NM_020737]	-1.107
131	LRIT1	leucine-rich repeat, immunoglobulin-like and transmembrane domains 1 (LRIT1), mRNA [NM_015613]	-1.412
132	LRRC52	leucine rich repeat containing 52 (LRRC52), mRNA [NM_001005214]	-1.3101
133	LTF	lactotransferrin (LTF), transcript variant 1, mRNA [NM_002343]	-2.284
134	MAGEC1	melanoma antigen family C, 1 (MAGEC1), mRNA [NM_005462]	-1.9302
135	MAPK10	mitogen-activated protein kinase 10 (MAPK10), transcript variant 3, mRNA [NM_138980]	-1.0489
136	MARK1	MAP/microtubule affinity-regulating kinase 1 (MARK1), mRNA [NM_018650]	-1.4556
137	MGLL	monoglyceride lipase (MGLL), transcript variant 1, mRNA [NM_007283]	-1.0931
138	MOXD2P	monooxygenase, DBH-like 2, pseudogene (MOXD2P), non-coding RNA [NR_024346]	-1.116
139	MUCL1	mucin-like 1 (MUCL1), mRNA [NM_058173]	-2.2869
140	MYOZ3	myozenin 3 (MYOZ3), transcript variant 2, mRNA [NM_133371]	-2.7619
141	NCRNA00311	non-protein coding RNA 311 (NCRNA00311), non-coding RNA [NR_038859]	-1.0976
142	NGEF	nine nucleotide exchange factor [Source:HGNC Symbol;Acc:7807] [ENST00000409079]	-1.7814
143	NLRP5	NLR family, pyrin domain containing 5 (NLRP5), mRNA [NM_153447]	-1.1895
144	NPSR1	neuropeptide S receptor 1 (NPSR1), transcript variant 2, mRNA [NM_207173]	-1.0452
145	NPY	neuropeptide Y (NPY), mRNA [NM_000905]	-1.2147
146	NR2F1	nuclear receptor subfamily 2, group F, member 1 (NR2F1), mRNA [NM_005654]	-1.3294
147	NUFIP1	nuclear fragile X mental retardation protein interacting protein 1 (NUFIP1), mRNA [NM_012345]	-1.2187
148	OAS3	2'-5'-oligoadenylate synthetase 3, 100kDa (OAS3), mRNA [NM_006187]	-1.2145
149	ODZ2	odz, odd Oz/ten-m homolog 2 (Drosophila) (ODZ2), mRNA [NM_001122679]	-1.1336
150	OR2T5	olfactory receptor, family 2, subfamily T, member 5 (OR2T5), mRNA [NM_001004697]	-1.6911
151	OR51B6	olfactory receptor, family 51, subfamily B, member 6 (OR51B6), mRNA [NM_001004750]	-1.0279
152	OR7A17	olfactory receptor, family 7, subfamily A, member 17 (OR7A17), mRNA [NM_030901]	-1.0381
153	ORM2	orosomucoid 2 (ORM2), mRNA [NM_000608]	-1.2566
154	OTUD5	OTU domain containing 5 (OTUD5), transcript variant 1, mRNA [NM_017602]	-3.2862
155	PAPSS2	3'-phosphoadenosine 5'-phosphosulfate synthase 2 (PAPSS2), transcript variant 2, mRNA [NM_001015880]	-1.243
156	PDE2A	phosphodiesterase 2A, cGMP-stimulated (PDE2A), transcript variant 1, mRNA [NM_002599]	-1.164
157	PDE6A	phosphodiesterase 6A, cGMP-specific, rod, alpha (PDE6A), mRNA [NM_000440]	-1.3206
158	PER3	period homolog 3 (Drosophila) (PER3), mRNA [NM_016831]	-1.3603
159	PLA2G2A	phospholipase A2, group IIA (platelets, synovial fluid) (PLA2G2A), transcript variant 1, mRNA [NM_000300]	-1.1332
160	PLGLB1	plasminogen-like B1 (PLGLB1), mRNA [NM_001032392]	-1.2425
161	PLVAP	plasmalemma vesicle associated protein (PLVAP), mRNA [NM_031310]	-1.3228
162	PP12613	hypothetical LOC100192379 (PP12613), non-coding RNA [NR_024365]	-1.065
163	PPEF1	protein phosphatase, EF-hand calcium binding domain 1 (PPEF1), transcript variant 1, mRNA [NM_006240]	-1.0248
164	PPP1R2P3	protein phosphatase 1, regulatory (inhibitor) subunit 2 pseudogene 3 (PPP1R2P3), non-coding RNA [NR_002168]	-1.2847
165	PROX2	prospero homeobox 2 (PROX2), transcript variant 1, mRNA [NM_001243007]	-1.1067
166	PRY2	PTPN13-like, Y-linked 2 (PRY2), mRNA [NM_001002758]	-1.4473
167	PTPN22	protein tyrosine phosphatase, non-receptor type 22 (lymphoid) (PTPN22), transcript variant 1, mRNA [NM_015967]	-1.5067
168	RBM14	mRNA for RNA binding motif protein 14 variant protein. [AB209007]	-4.7799
169	RBPJL	recombination signal binding protein for immunoglobulin kappa J region-like (RBPJL), mRNA [NM_014276]	-1.0537
170	RFPL4A	ret finger protein-like 4A (RFPL4A), mRNA [NM_001145014]	-1.2729
171	RNF186	ring finger protein 186 (RNF186), mRNA [NM_019062]	-1.3428
172	RPS4Y2	ribosomal protein S4, Y-linked 2 (RPS4Y2), mRNA [NM_001039567]	-1.1973
173	RUNX1	runt-related transcription factor 1 (RUNX1), transcript variant 3, mRNA [NM_001122607]	-1.4101
174	S100A7	S100 calcium binding protein A7 (S100A7), mRNA [NM_002963]	-1.855
175	SCARA3	scavenger receptor class A, member 3 (SCARA3), transcript variant 2, mRNA [NM_182826]	-1.2114
176	SCARA5	scavenger receptor class A, member 5 (putative) (SCARA5), mRNA [NM_173833]	-1.479
177	SCEL	sciellin (SCEL), transcript variant 1, mRNA [NM_144777]	-4.1215
178	SDK1	sidekick homolog 1, cell adhesion molecule (chicken) (SDK1), transcript variant 1, mRNA [NM_152744]	-1.3087
179	SEMA5B	sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5B (SEMA5B), transcript variant 1, mRNA [NM_001031702]	-1.0677
180	SERP1	iated endoplasmic reticulum protein 1 [Source:HGNC Symbol;Acc:10759] [ENST00000479209]	-1.0642
181	SERPINB3	serpin peptidase inhibitor, clade B (ovalbumin), member 3 (SERPINB3), mRNA [NM_006919]	-2.0989
182	SERPINB4	serpin peptidase inhibitor, clade B (ovalbumin), member 4 (SERPINB4), mRNA [NM_002974]	-1.5655
183	SERPINE2	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2 (SERPINE2)	-1.1402
184	SHROOM2	shroom family member 2 (SHROOM2), mRNA [NM_001649]	-1.353
185	SIRPB1	signal-regulatory protein beta 1 (SIRPB1), transcript variant 1, mRNA [NM_006065]	-1.1275
186	SLC14A1	solute carrier family 14 (urea transporter), member 1 (Kidd blood group) (SLC14A1)	-2.0504
187	SLC22A2	solute carrier family 22 (organic cation transporter), member 2 (SLC22A2), mRNA [NM_003058]	-1.6898
188	SLC35D3	solute carrier family 35, member D3 (SLC35D3), mRNA [NM_001008783]	-1.4514
189	SLC5A10	solute carrier family 5 (sodium/glucose cotransporter), member 10 (SLC5A10)	-1.1134
190	SMPD3	sphingomyelin phosphodiesterase 3, neutral membrane (neutral sphingomyelinase II) (SMPD3)	-1.0126
191	SNORA53	small nucleolar RNA, H/ACA box 53 (SNORA53), small nucleolar RNA [NR_003015]	-1.0075
192	SOBP	sine oculis binding protein homolog (Drosophila) (SOBP), mRNA [NM_018013]	-1.3298

193	SPDYA	speedy homolog A (Xenopus laevis) (SPDYA), transcript variant 2, mRNA [NM_001008779]	-1.3348
194	SPRED2	sprouty-related, EVH1 domain containing 2 (SPRED2), transcript variant 1, mRNA [NM_181784]	-1.0621
195	SPRR2A	small proline-rich protein 2A (SPRR2A), mRNA [NM_005988]	-1.8041
196	SPRR2E	small proline-rich protein 2E (SPRR2E), mRNA [NM_001024209]	-1.0438
197	SSH1	slingshot homolog 1 (Drosophila) (SSH1), transcript variant 3, mRNA [NM_001161331]	-1.0108
198	SULF1	sulfatase 1 (SULF1), transcript variant 3, mRNA [NM_015170]	-1.3806
199	TBL1X	transducin (beta)-like 1X-linked (TBL1X), transcript variant 1, mRNA [NM_005647]	-1.1619
200	TEX22	testis expressed 22 (TEX22), mRNA [NM_001195082]	-3.4591
201	TGM2	transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase) (TGM2)	-1.074
202	THEM5	thioesterase superfamily member 5 (THEM5), mRNA [NM_182578]	-1.7651
203	TJP3	tight junction protein 3 (zona occludens 3) (TJP3), mRNA [NM_014428]	-1.6635
204	TMEM108	transmembrane protein 108 (TMEM108), transcript variant 1, mRNA [NM_023943]	-1.1406
205	TMEM35	transmembrane protein 35 (TMEM35), mRNA [NM_021637]	-1.282
206	TNXB	tenascin XB (TNXB), transcript variant XB, mRNA [NM_019105]	-1.2057
207	TPRXL	tetra-peptide repeat homeobox-like (TPRXL), non-coding RNA [NR_002223]	-1.454
208	TPTE2P3	transmembrane phosphoinositide 3-phosphatase and tensin homolog 2 pseudogene 3 (TPTE2P3),	-1.3285
209	TRIB2	tribbles homolog 2 (Drosophila) (TRIB2), transcript variant 1, mRNA [NM_021643]	-1.8339
210	TRIM24	tripartite motif containing 24 [Source:HGNC Symbol;Acc:11812] [ENST00000378381]	-1.2946
211	TRPV1	transient receptor potential cation channel, subfamily V, member 1 (TRPV1), transcript variant 3	-1.2771
212	UBD	ubiquitin D (UBD), mRNA [NM_006398]	-2.8255
213	VLDLR	very low density lipoprotein receptor (VLDLR), transcript variant 1, mRNA [NM_003383]	-1.0491
214	WFDC5	WAP four-disulfide core domain 5 (WFDC5), mRNA [NM_145652]	-1.402
215	WFDC9	WAP four-disulfide core domain 9 (WFDC9), mRNA [NM_147198]	-1.3457
216	XKR6	primary neuroblastoma cDNA, clone:Nbla00437, full insert sequence. [AB073660]	-1.6707
217	ZBED2	zinc finger, BED-type containing 2 (ZBED2), mRNA [NM_024508]	-1.0424
218	ZNF148	zinc finger protein 148 (ZNF148), mRNA [NM_021964]	-3.5666
219	ZNF285	zinc finger protein 285 (ZNF285), mRNA [NM_152354]	-1.4507
220	ZNF850	zinc finger protein 850 (ZNF850), mRNA [NM_001193552]	-1.0304

Table A2. *List of genes up-regulated upon ectopic expression of HSF1ΔRDT in the non-transformed mCherry MCF10A cells*

No.	Symbol	Gene name	LogFC
1	ABCC13	ATP-binding cassette, sub-family C (CFTR/MRP), member 13, pseudogene (ABCC13), transcript variant D, non-coding RNA [NR_003088]	2.1397
2	ABCC2	ATP-binding cassette, sub-family C (CFTR/MRP), member 2 [Source:HGNC Symbol;Acc:53] [ENST00000370434]	1.4978
3	ACAD9	acyl-CoA dehydrogenase family, member 9 (ACAD9), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA [NM_014049]	2.001
4	ACPP	acid phosphatase, prostate (ACPP), transcript variant 1, mRNA [NM_001099]	1.1361
5	ACTG2	actin, gamma 2, smooth muscle, enteric (ACTG2), transcript variant 1, mRNA [NM_001615]	2.3828
6	ACTL9	actin-like 9 (ACTL9), mRNA [NM_178525]	1.0334
7	ADORA2A	adenosine A2a receptor (ADORA2A), mRNA [NM_000675]	1.6231
8	AGER	advanced glycosylation end product-specific receptor (AGER), transcript variant 9, mRNA [NM_001206966]	1.3965
9	AKAP5	A kinase (PRKA) anchor protein 5 (AKAP5), mRNA [NM_004857]	1.9067
10	AKD1	adenylate kinase domain containing 1 [Source:HGNC Symbol;Acc:33814] [ENST00000368948]	1.2569
11	ANKRD30A	ankyrin repeat domain 30A (ANKRD30A), mRNA [NM_052997]	1.0376
12	ARHGAP44	Rho GTPase activating protein 44 (ARHGAP44), mRNA [NM_014859]	4.1946
13	ARHGDIG	Rho GDP dissociation inhibitor (GDI) gamma (ARHGDIG), mRNA [NM_001176]	1.1479
14	ARSA	arylsulfatase A (ARSA), transcript variant 1, mRNA [NM_000487]	1.5267
15	ATHL1	ATH1, acid trehalase-like 1 (yeast) (ATHL1), mRNA [NM_025092]	2.1961
16	BST2	bone marrow stromal cell antigen 2 [Source:HGNC Symbol;Acc:1119] [ENST00000252593]	1.3577
17	BST2	bone marrow stromal cell antigen 2 (BST2), mRNA [NM_004335]	1.3131
18	C20orf197	chromosome 20 open reading frame 197 (C20orf197), mRNA [NM_173644]	1.0952
19	C20orf26	chromosome 20 open reading frame 26 (C20orf26), transcript variant 1, mRNA [NM_015585]	2.1965
20	C22orf26	chromosome 22 open reading frame 26 (C22orf26), mRNA [NM_018280]	2.7334
21	C22orf31	chromosome 22 open reading frame 31 (C22orf31), mRNA [NM_015370]	1.0032
22	C3orf74	chromosome 3 open reading frame 74 (C3orf74), non-coding RNA [NR_027331]	1.1553
23	C5orf13	chromosome 5 open reading frame 13 (C5orf13), transcript variant 1, mRNA [NM_004772]	2.1459
24	C6orf164	chromosome 6 open reading frame 164 (C6orf164), non-coding RNA [NR_026784]	1.4129
25	C7orf46	chromosome 7 open reading frame 46 (C7orf46), transcript variant 1, mRNA [NM_199136]	1.2091
26	C7orf51	chromosome 7 open reading frame 51 (C7orf51), mRNA [NM_173564]	1.1611
27	C8orf84	chromosome 8 open reading frame 84 (C8orf84), mRNA [NM_153225]	1.4875
28	CACNA1G	calcium channel, voltage-dependent, T type, alpha 1G subunit (CACNA1G), transcript variant 1, mRNA [NM_018896]	1.2227
29	CCR4	chemokine (C-C motif) receptor 4 (CCR4), mRNA [NM_005508]	2.5444
30	CD302	CD302 molecule (CD302), transcript variant 1, mRNA [NM_014880]	2.5265
31	CDKN2B	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4) (CDKN2B), transcript variant 1, mRNA [NM_004936]	1.382
32	CER1	cerberus 1, cysteine knot superfamily, homolog (Xenopus laevis) (CER1), mRNA [NM_005454]	2.2109
33	CERKL	ceramide kinase-like (CERKL), transcript variant 3, mRNA [NM_001030312]	1.6862
34	CFTR	cystic fibrosis transmembrane conductance regulator (ATP-binding cassette sub-family C, member 7) (CFTR), mRNA [NM_000492]	1.5716
35	CHGA	chromogranin A (parathyroid secretory protein 1) (CHGA), mRNA [NM_001275]	1.1312

36	CLIC2	chloride intracellular channel 2 (CLIC2), mRNA [NM_001289]	2.2751
37	CLIC3	chloride intracellular channel 3 (CLIC3), mRNA [NM_004669]	1.4051
38	COL12A1	collagen, type XII, alpha 1 (COL12A1), transcript variant long, mRNA [NM_004370]	1.6673
39	COL2A1	collagen, type II, alpha 1 (COL2A1), transcript variant 1, mRNA [NM_001844]	1.7558
40	COL5A3	collagen, type V, alpha 3 (COL5A3), mRNA [NM_015719]	1.6566
41	COPZ2	coatomer protein complex, subunit zeta 2 (COPZ2), mRNA [NM_016429]	1.8723
42	CPT1C	carnitine palmitoyltransferase 1C (CPT1C), transcript variant 3, mRNA [NM_001199752]	1.23
43	CRYAB	crystallin, alpha B (CRYAB), mRNA [NM_001885]	1.4888
44	CSRP2	cysteine and glycine-rich protein 2 (CSRP2), mRNA [NM_001321]	1.7415
45	CTAG1A	cancer/testis antigen 1A (CTAG1A), mRNA [NM_139250]	3.2841
46	CXorf36	chromosome X open reading frame 36 (CXorf36), transcript variant 2, mRNA [NM_024689]	1.1549
47	CXorf36	chromosome X open reading frame 36 (CXorf36), transcript variant 1, mRNA [NM_176819]	1.0208
48	CXXC4	CXXC finger protein 4 (CXXC4), mRNA [NM_025212]	1.231
49	DCN	decorin (DCN), transcript variant A1, mRNA [NM_001920]	1.6621
50	DCN	decorin (DCN), transcript variant E, mRNA [NM_133507]	1.5631
51	DHRS7C	dehydrogenase/reductase (SDR family) member 7C (DHRS7C), transcript variant 1, mRNA [NM_001220493]	2.0183
52	DISC1	disrupted in schizophrenia 1 (DISC1), transcript variant S, mRNA [NM_001012959]	1.0059
53	DNAH12	dynein, axonemal, heavy chain 12 (DNAH12), transcript variant 1, mRNA [NM_178504]	1.3455
54	DNAH7	dynein, axonemal, heavy chain 7 [Source:HGNC Symbol;Acc:18661] [ENST00000410072]	1.0391
55	EDNRB	endothelin receptor type B (EDNRB), transcript variant 2, mRNA [NM_003991]	1.6313
56	EGR4	early growth response 4 (EGR4), mRNA [NM_001965]	5.1765
57	ELOVL3	ELOVL fatty acid elongase 3 (ELOVL3), mRNA [NM_152310]	1.2427
58	EPB41	erythrocyte membrane protein band 4.1 (elliptocytosis 1, RH-linked) (EPB41), transcript variant 4, mRNA [NM_203342]	1.1443
59	EPYC	epiphycan (EPYC), mRNA [NM_004950]	1.1501
60	FABP3	fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor) (FABP3), mRNA [NM_004102]	1.0938
61	FAM151B	family with sequence similarity 151, member B (FAM151B), mRNA [NM_205548]	1.0215
62	FAM47B	family with sequence similarity 47, member B (FAM47B), mRNA [NM_152631]	1.4705
63	FAM66C	family with sequence similarity 66, member C (FAM66C), non-coding RNA [NR_026788]	1.5975
64	FBF1	Fas (TNFRSF6) binding factor 1 (FBF1), mRNA [NM_001080542]	1.4787
65	FCGR1B	Fc fragment of IgG, high affinity Ib, receptor (CD64) (FCGR1B), transcript variant 1, mRNA [NM_001017986]	2.2311
66	FCRL3	Fc receptor-like 3 (FCRL3), mRNA [NM_052939]	1.498
67	FEZ1	fasciculation and elongation protein zeta 1 (zygin I) (FEZ1), transcript variant 1, mRNA [NM_005103]	1.5728
68	FLJ37638	omo sapiens hypothetical LOC400660 (FLJ37638), partial miscRNA [XR_110158]	1.1559
69	FLJ42709	hypothetical LOC441094 (FLJ42709), non-coding RNA [NR_021490]	1.2195
70	FOXL2	forkhead box L2 (FOXL2), mRNA [NM_023067]	1.592
71	FTCD	formiminotransferase cyclodeaminase (FTCD), transcript variant A, mRNA [NM_206965]	2.6377
72	GALC	galactosylceramidase [Source:HGNC Symbol;Acc:4115] [ENST00000445021]	1.0694
73	GLIS1	GLIS family zinc finger 1 (GLIS1), mRNA [NM_147193]	1.0987
74	GLYATL2	glycine-N-acyltransferase-like 2 (GLYATL2), mRNA [NM_145016]	1.039
75	GNRHR	gonadotropin-releasing hormone receptor (GNRHR), transcript variant 1, mRNA [NM_000406]	1.3989
76	GPR157	G protein-coupled receptor 157 (GPR157), mRNA [NM_024980]	1.6691
77	GREB1L	growth regulation by estrogen in breast cancer-like (GREB1L), mRNA [NM_001142966]	1.0235
78	GRM5	glutamate receptor, metabotropic 5 (GRM5), transcript variant b, mRNA [NM_000842]	4.7676
79	HCN1	hyperpolarization activated cyclic nucleotide-gated potassium channel 1 (HCN1), mRNA [NM_021072]	1.9016
80	HCP5P10	HLA complex P5 pseudogene 10 (HCP5P10), non-coding RNA [NR_031762]	1.2076
81	HHATL	hedgehog acyltransferase-like (HHATL), transcript variant 1, mRNA [NM_020707]	1.1499
82	HIST1H2BA	histone cluster 1, H2ba (HIST1H2BA), mRNA [NM_170610]	1.3363
83	HIST1H4G	histone cluster 1, H4g (HIST1H4G), mRNA [NM_003547]	1.4502
84	HMCN2	hemicentin-2-like (LOC100292387), mRNA [XM_002346203]	1.9698
85	HOXB13	homeobox B13 (HOXB13), mRNA [NM_006361]	1.1511
86	HS3ST2	heparan sulfate (glucosamine) 3-O-sulfotransferase 2 (HS3ST2), mRNA [NM_006043]	1.3616
87	IGF2BP1	insulin-like growth factor 2 mRNA binding protein 1 (IGF2BP1), transcript variant 1, mRNA [NM_006546]	1.2551
88	IGSF5	immunoglobulin superfamily, member 5 (IGSF5), mRNA [NM_001080444]	2.2535
89	IL28RA	interleukin 28 receptor, alpha (interferon, lambda receptor) (IL28RA), transcript variant 1, mRNA [NM_170743]	1.0436
90	IL2RB	interleukin 2 receptor, beta (IL2RB), mRNA [NM_000878]	1.2438
91	INMT	indolethylamine N-methyltransferase (INMT), transcript variant 2, mRNA [NM_001199219]	1.1659
92	JAM3	junctional adhesion molecule 3 (JAM3), transcript variant 1, mRNA [NM_032801]	2.4711
93	KCNIP4	Kv channel interacting protein 4 (KCNIP4), transcript variant 5, mRNA [NM_001035003]	1.5276
94	KIAA0125	KIAA0125 (KIAA0125), non-coding RNA [NR_026800]	1.2011
95	KLHL15	kelch-like 15 (Drosophila) (KLHL15), mRNA [NM_030624]	1.0476
96	KLRK1	killer cell lectin-like receptor subfamily K, member 1 (KLRK1), mRNA [NM_007360]	1.8735
97	KREMEN2	kringle containing transmembrane protein 2 (KREMEN2), transcript variant 4, mRNA [NM_172229]	1.8758
98	KRTAP9-1	keratin associated protein 9-1 (KRTAP9-1), mRNA [NM_001190460]	1.9
99	KRTCAP3	keratinocyte associated protein 3 (KRTCAP3), transcript variant 2, mRNA [NM_173853]	2.5535
100	LBH	limb bud and heart development homolog (mouse) [Source:HGNC Symbol;Acc:29532] [ENST00000404397]	2.5359
101	LEPR	leptin receptor (LEPR), transcript variant 1, mRNA [NM_002303]	1.7372
102	LHCGR	luteinizing hormone/choriogonadotropin receptor (LHCGR), mRNA [NM_000233]	1.4744
103	LMCD1	LIM and cysteine-rich domains 1 (LMCD1), mRNA [NM_014583]	1.7074
104	LOC100128064	hypothetical protein LOC100128064 (LOC100128064), mRNA [XM_001725877]	1.5363
105	LOC100128239	hypothetical LOC100128239 (LOC100128239), non-coding RNA [NR_027276]	1.582

106	LOC100128885	cDNA FLJ43440 fis, clone OCBBF2030517. [AK125429]	2.313
107	LOC100131289	hypothetical LOC100131289 (LOC100131289), non-coding RNA [NR_038929]	2.6877
108	LOC100131738	hypothetical LOC100131738 (LOC100131738), partial miscRNA [XR_108808]	1.3578
109	LOC100133920	hypothetical LOC100133920 (LOC100133920), non-coding RNA [NR_024443]	1.0114
110	LOC100192378	hypothetical LOC100192378 (LOC100192378), non-coding RNA [NR_024360]	1.1956
111	LOC100507431	hypothetical protein LOC100507431 (LOC100507431), mRNA [XM_003118983]	1.1852
112	LOC100509805	putative mucosal pentraxin homolog (LOC100509805), mRNA [XM_003119758]	1.8087
113	LOC150185	hypothetical LOC150185 (LOC150185), non-coding RNA [NR_024381]	2.3783
114	LOC220980	hypothetical LOC220980 (LOC220980), non-coding RNA [NR_033842]	2.4205
115	LOC254057	cDNA: FLJ21000 fis, clone CAE03359. [AK024653]	2.3213
116	LOC283481	hypothetical LOC283481 (LOC283481), non-coding RNA [NR_036487]	2.2445
117	LOC284630	cDNA FLJ39065 fis, clone NT2RP7014721. [AK096384]	2.1251
118	LOC285375	hypothetical LOC285375 (LOC285375), non-coding RNA [NR_027103]	1.3424
119	LOC286063	cDNA FLJ33573 fis, clone BRAMY2010798. [AK090892]	1.264
120	LOC339400	cDNA FLJ31869 fis, clone NT2RP7002151. [AK056431]	1.1346
121	LOC339666	hypothetical LOC339666 (LOC339666), non-coding RNA [NR_038918]	1.7826
122	LOC401022	hypothetical LOC401022 (LOC401022), non-coding RNA [NR_033979]	2.0606
123	LOC645195	cDNA FLJ41456 fis, clone BRSTN2012320. [AK123450]	1.2458
124	LOC645434	hypothetical LOC645434 (LOC645434), non-coding RNA [NR_033919]	2.4364
125	LOC646034	cDNA FLJ43185 fis, clone FCBBF3021940. [AK125175]	1.0712
126	LOC648149	cDNA FLJ41355 fis, clone BRAWH2016724. [AK123349]	1.4826
127	LOC652215	omo sapiens ER lumen protein retaining receptor-like (LOC652215), mRNA [XM_941595]	1.1092
128	LOC728978	hypothetical LOC728978 (LOC728978), non-coding RNA [NR_038453]	1.0384
129	LOC729867	cDNA FLJ35980 fis, clone TESTI2013546. [AK093299]	1.0367
130	LOC730441	trypsin X3 pseudogene (LOC730441), non-coding RNA [NR_036483]	1.4601
131	LOXHD1	lipoxygenase homology domains 1 [Source:HGNC Symbol;Acc:26521] [ENST00000335730]	1.6283
132	LRIT2	leucine-rich repeat, immunoglobulin-like and transmembrane domains 2 (LRIT2), mRNA [NM_001017924]	1.3718
133	MAGEA12	melanoma antigen family A, 12 (MAGEA12), transcript variant 3, mRNA [NM_005367]	1.0004
134	MIA2	melanoma inhibitory activity 2 (MIA2), mRNA [NM_054024]	1.6248
135	MLC1	megalencephalic leukoencephalopathy with subcortical cysts 1 (MLC1), transcript variant 1, mRNA [NM_015166]	2.8458
136	MTMR8	myotubularin related protein 8 (MTMR8), mRNA [NM_017677]	1.1633
137	MYEOV2	myeloma overexpressed 2 (MYEOV2), transcript variant 1, mRNA [NM_138336]	1.1297
138	MYL9	myosin, light chain 9, regulatory (MYL9), transcript variant 2, mRNA [NM_181526]	1.4408
139	NAP1L5	nucleosome assembly protein 1-like 5 (NAP1L5), mRNA [NM_153757]	1.8778
140	NBPF6	neuroblastoma breakpoint family, member 6 (NBPF6), transcript variant 2, mRNA [NM_001143988]	1.1833
141	NCRNA00157	non-protein coding RNA 157 (NCRNA00157), non-coding RNA [NR_024354]	2.7398
142	NECAB2	N-terminal EF-hand calcium binding protein 2 (NECAB2), mRNA [NM_019065]	1.0257
143	NHEDC1	Na+/H+ exchanger domain containing 1 (NHEDC1), transcript variant 1, mRNA [NM_139173]	1.4987
144	NME5	non-metastatic cells 5, protein expressed in (nucleoside-diphosphate kinase) (NME5), mRNA [NM_003551]	2.5605
145	NMUR1	neuromedin U receptor 1 (NMUR1), mRNA [NM_006056]	1.0114
146	NOSTRIN	nitric oxide synthase trafficker (NOSTRIN), transcript variant 1, mRNA [NM_052946]	1.1037
147	NOTCH4	notch 4 (NOTCH4), mRNA [NM_004557]	2.0054
148	NPNT	nephronectin (NPNT), transcript variant 2, mRNA [NM_001033047]	3.7174
149	NTS	neurotensin (NTS), mRNA [NM_006183]	2.3518
150	NXN	nucleoredoxin, mRNA (cDNA clone IMAGE:4689777), complete cds. [BC104634]	1.6423
151	NXPH1	neurexophilin 1 (NXPH1), mRNA [NM_152745]	1.2015
152	O3FAR1	omega-3 fatty acid receptor 1 (O3FAR1), transcript variant 1, mRNA [NM_181745]	1.6036
153	OGDHL	oxoglutarate dehydrogenase-like (OGDHL), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA [NM_018245]	1.09
154	OR4N5	olfactory receptor, family 4, subfamily N, member 5 (OR4N5), mRNA [NM_001004724]	1.2408
155	OR51I2	olfactory receptor, family 51, subfamily I, member 2 (OR51I2), mRNA [NM_001004754]	1.581
156	OR8A1	olfactory receptor, family 8, subfamily A, member 1 (OR8A1), mRNA [NM_001005194]	2.3921
157	OR8G5	olfactory receptor, family 8, subfamily G, member 5 (OR8G5), mRNA [NM_001005198]	1.096
158	PAPD5	PAP associated domain containing 5 (PAPD5), transcript variant 1, mRNA [NM_001040284]	2.6107
159	PARVB	parvin, beta (PARVB), transcript variant 1, mRNA [NM_001003828]	1.8815
160	PCDH15	mRNA; cDNA DKFZp667A1711 (from clone DKFZp667A1711). [AL834134]	2.0356
161	PCDHA4	protocadherin alpha 4 (PCDHA4), transcript variant 2, mRNA [NM_031500]	1.8172
162	PCDHB4	protocadherin beta 4 (PCDHB4), mRNA [NM_018938]	1.6396
163	PCDHGB7	protocadherin gamma subfamily B, 7 (PCDHGB7), transcript variant 2, mRNA [NM_032101]	1.5111
164	PDE7B	phosphodiesterase 7B (PDE7B), mRNA [NM_018945]	1.3811
165	PDE9A	phosphodiesterase 9A (PDE9A), transcript variant 1, mRNA [NM_002606]	1.0045
166	PDZD7	PDZ domain containing 7 (PDZD7), transcript variant 2, mRNA [NM_024895]	1.1311
167	PHTF2	putative homeodomain transcription factor 2 (PHTF2), transcript variant 5, mRNA [NM_001127360]	1.0875
168	PI15	peptidase inhibitor 15 (PI15), mRNA [NM_015886]	1.2613
169	PIKFYVE	phosphoinositide kinase, FYVE finger containing (PIKFYVE), transcript variant 4, mRNA [NM_001178000]	1.1375
170	PKIB	protein kinase (cAMP-dependent, catalytic) inhibitor beta (PKIB), transcript variant 1, mRNA [NM_181795]	1.6784
171	PLA2G16	phospholipase A2, group XVI (PLA2G16), transcript variant 1, mRNA [NM_007069]	2.0381
172	PLEKHB1	pleckstrin homology domain containing, family B (evectins) member 1 (PLEKHB1), transcript variant 1, mRNA [NM_021200]	1.011
173	PLEKHH2	pleckstrin homology domain containing, family H (with MyTH4 domain) member 2 (PLEKHH2), mRNA [NM_172069]	1.0732

174	PLXNC1	plexin C1 (PLXNC1), transcript variant 1, mRNA [NM_005761]	1.1351
175	PMCH	pro-melanin-concentrating hormone (PMCH), mRNA [NM_002674]	2.089
176	PNLIPRP1	pancreatic lipase-related protein 1 (PNLIPRP1), mRNA [NM_006229]	1.261
177	PODXL	podocalyxin-like (PODXL), transcript variant 1, mRNA [NM_001018111]	1.3085
178	POF1B	premature ovarian failure, 1B (POF1B), mRNA [NM_024921]	2.4696
179	POM121L10P	POM121 membrane glycoprotein-like 10, pseudogene (POM121L10P), non-coding RNA [NR_024593]	1.317
180	POU5F2	POU domain class 5, transcription factor 2 (POU5F2), mRNA [NM_153216]	1.9269
181	PPIL4	peptidylprolyl isomerase (cyclophilin)-like 4 (PPIL4), mRNA [NM_139126]	1.031
182	PRAMEF10	PRAME family member 10 (PRAMEF10), mRNA [NM_001039361]	1.321
183	PRAMEF12	PRAME family member 12 (PRAMEF12), mRNA [NM_001080830]	1.516
184	PRAMEF3	PRAME family member 3 (PRAMEF3), mRNA [NM_001013692]	1.8769
185	PROM2	prominin 2 (PROM2), transcript variant 3, mRNA [NM_144707]	1.3415
186	PROM2	prominin 2 (PROM2), transcript variant 1, mRNA [NM_001165978]	1.3204
187	PRORSD1P	prolyl-tRNA synthetase associated domain containing 1, pseudogene (PRORSD1P), non-coding RNA [NR_027258]	2.1062
188	PRSS58	protease, serine, 58 (PRSS58), mRNA [NM_001001317]	2.1515
189	PTCH1	patched 1 (PTCH1), transcript variant 1a, mRNA [NM_001083602]	2.395
190	PTGDS	prostaglandin D2 synthase 21kDa (brain) (PTGDS), mRNA [NM_000954]	1.1279
191	PTGER2	prostaglandin E receptor 2 (subtype EP2), 53kDa (PTGER2), mRNA [NM_000956]	1.0976
192	QPCT	glutaminyl-peptide cyclotransferase (QPCT), mRNA [NM_012413]	1.0119
193	RAB25	RAB25, member RAS oncogene family (RAB25), mRNA [NM_020387]	1.1315
194	RASGRP3	RAS guanyl releasing protein 3 (calcium and DAG-regulated) (RASGRP3), transcript variant 1, mRNA [NM_001139488]	1.0572
195	RHBDL3	rhomboid, veinlet-like 3 (Drosophila) (RHBDL3), mRNA [NM_138328]	2.5747
196	RINL	Ras and Rab interactor-like (RINL), transcript variant 1, mRNA [NM_001195833]	1.1303
197	RNF17	ring finger protein 17 (RNF17), transcript variant 1, mRNA [NM_031277]	6.8572
198	RPL39L	ribosomal protein L39-like (RPL39L), mRNA [NM_052969]	2.6086
199	RRAD	Ras-related associated with diabetes (RRAD), transcript variant 2, mRNA [NM_004165]	2.3119
200	SAMD13	sterile alpha motif domain containing 13 (SAMD13), transcript variant 1, mRNA [NM_001010971]	1.4353
201	SEPHS1P	selenophosphate synthetase pseudogene (SEPHS1P), non-coding RNA [NR_002789]	1.1281
202	SERPINB7	serpin peptidase inhibitor, clade B (ovalbumin), member 7 (SERPINB7), transcript variant 2, mRNA [NM_001040147]	1.2853
203	SERPING1	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1 (SERPING1), transcript variant 1, mRNA [NM_000062]	1.3329
204	SHANK2	SH3 and multiple ankyrin repeat domains 2 (SHANK2), transcript variant 1, mRNA [NM_012309]	1.149
205	SLC13A3	solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3 (SLC13A3), transcript variant 2, mRNA [NM_001011554]	1.5282
206	SLC22A18	solute carrier family 22, member 18 (SLC22A18), transcript variant 2, mRNA [NM_183233]	1.3347
207	SLC2A5	solute carrier family 2 (facilitated glucose/fructose transporter), member 5 (SLC2A5), transcript variant 1, mRNA [NM_003039]	1.2477
208	SLC38A11	solute carrier family 38, member 11 (SLC38A11), transcript variant 2, mRNA [NM_173512]	2.2531
209	SLITRK3	SLIT and NTRK-like family, member 3 (SLITRK3), mRNA [NM_014926]	1.4446
210	SMAP1	small ArfGAP 1 [Source:HGNC Symbol;Acc:19651] [ENST00000370442]	1.1267
211	SNORA81	small nucleolar RNA, H/ACA box 81 (SNORA81), small nucleolar RNA [NR_002989]	2.9067
212	SNX20	sorting nexin 20 (SNX20), transcript variant 1, mRNA [NM_182854]	2.5486
213	SPINLW1	serine peptidase inhibitor-like, with Kunitz and WAP domains 1 (eppin) (SPINLW1), mRNA [NM_020398]	1.2289
214	SPOCD1	SPOC domain containing 1 (SPOCD1), mRNA [NM_144569]	1.6838
215	SPON2	spondin 2, extracellular matrix protein (SPON2), transcript variant 1, mRNA [NM_012445]	1.5391
216	STS	steroid sulfatase (microsomal), isozyme S (STS), mRNA [NM_000351]	1.6377
217	SYT12	synaptotagmin XII (SYT12), transcript variant 1, mRNA [NM_177963]	1.9493
218	SYT14	synaptotagmin XIV (SYT14), transcript variant 1, mRNA [NM_001146261]	1.5423
219	TAC1	tachykinin, precursor 1 (TAC1), transcript variant beta, mRNA [NM_003182]	1.0257
220	TDO2	tryptophan 2,3-dioxygenase (TDO2), mRNA [NM_005651]	1.573
221	TEKT5	tektin 5 (TEKT5), mRNA [NM_144674]	1.098
222	TFDP3	transcription factor Dp family, member 3 (TFDP3), mRNA [NM_016521]	1.3663
223	TLR10	toll-like receptor 10 (TLR10), transcript variant 1, mRNA [NM_030956]	1.8851
224	TMC5	transmembrane channel-like 5 (TMC5), transcript variant 3, mRNA [NM_024780]	1.1039
225	TMEM150C	transmembrane protein 150C (TMEM150C), mRNA [NM_001080506]	1.0133
226	TMEM216	transmembrane protein 216 (TMEM216), transcript variant 2, mRNA [NM_001173990]	1.2868
227	TMEM56	transmembrane protein 56 (TMEM56), transcript variant 1, mRNA [NM_001199679]	2.0147
228	TMEM56	transmembrane protein 56 (TMEM56), transcript variant 2, mRNA [NM_152487]	1.4862
229	TMEM71	transmembrane protein 71 (TMEM71), transcript variant 1, mRNA [NM_144649]	1.9274
230	TMPRSS11F	transmembrane protease, serine 11F (TMPRSS11F), mRNA [NM_207407]	1.0617
231	TMPRSS12	transmembrane (C-terminal) protease, serine 12 (TMPRSS12), mRNA [NM_182559]	1.1615
232	TOX2	TOX high mobility group box family member 2 (TOX2), transcript variant 3, mRNA [NM_032883]	2.498
233	TRIM17	tripartite motif containing 17 (TRIM17), transcript variant 4, mRNA [NM_001134855]	1.0378
234	TRMT61A	tRNA methyltransferase 61 homolog A (S. cerevisiae) (TRMT61A), mRNA [NM_152307]	1.7365
235	TSPAN11	tetraspanin 11 (TSPAN11), mRNA [NM_001080509]	1.594
236	TSPY3	testis specific protein, Y-linked 3 (TSPY3), mRNA [NM_001077697]	1.9594
237	TTL	tubulin tyrosine ligase (TTL), transcript variant TTL-B2, non-coding RNA [NR_024507]	1.5364
238	UBA5	ubiquitin-like modifier activating enzyme 5 (UBA5), transcript variant 1, mRNA [NM_024818]	1.8459
239	UBR4	ubiquitin protein ligase E3 component n-recognin 4 [Source:HGNC Symbol;Acc:30313] [ENST00000375218]	1.1977

240	ULBP1	UL16 binding protein 1 (ULBP1), mRNA [NM_025218]	1.5289
241	USP45	ubiquitin specific peptidase 45 [Source:HGNC Symbol;Acc:20080] [ENST00000369232]	1.947
242	WDR19	WD repeat domain 19 (WDR19), mRNA [NM_025132]	1.941
243	WDR49	WD repeat domain 49 (WDR49), mRNA [NM_178824]	1.5009
244	WFDC2	WAP four-disulfide core domain 2 (WFDC2), mRNA [NM_006103]	1.4506
245	ZNF100	zinc finger protein 100 [Source:HGNC Symbol;Acc:12880] [ENST00000358296]	1.0375
246	ZNF221	zinc finger protein 221 (ZNF221), mRNA [NM_013359]	1.167
247	ZNF382	zinc finger protein 382 (ZNF382), mRNA [NM_032825]	1.3768
248	ZNF396	zinc finger protein 396 (ZNF396), mRNA [NM_145756]	1.4875
249	ZNF561	zinc finger protein 561 (ZNF561), mRNA [NM_152289]	1.0426
250	ZNF594	zinc finger protein 594 (ZNF594), mRNA [NM_032530]	1.2865
251	ZNF711	zinc finger protein 711 (ZNF711), mRNA [NM_021998]	1.5355
252	ZNF81	zinc finger protein 81 (ZNF81), mRNA [NM_007137]	1.0431

***Table A3**. List of genes down-regulated upon ectopic expression of HSF1ΔRDT in the H-Ras^{V12} transformed MCF10A cells*

No.	Symbol	Gene name	LogFC
1	ABAT	4-aminobutyrate aminotransferase (ABAT), nuclear gene encoding mitochondrial protein, transcript variant 2, mRNA [NM_000663]	-1.102
2	ABCC5	ATP-binding cassette, sub-family C (CFTR/MRP), member 5 (ABCC5), transcript variant 1, mRNA [NM_005688]	-2.7391
3	ABCC5	ATP-binding cassette, sub-family C (CFTR/MRP), member 5 (ABCC5), transcript variant 2, mRNA [NM_001023587]	-2.2257
4	ABCG1	ATP-binding cassette, sub-family G (WHITE), member 1 (ABCG1), transcript variant 5, mRNA [NM_207627]	-1.0144
5	ABI3BP	ABI family, member 3 (NESH) binding protein (ABI3BP), mRNA [NM_015429]	-1.5019
6	ABLIM2	actin binding LIM protein family, member 2 (ABLIM2), transcript variant 1, mRNA [NM_001130083]	-1.1142
7	ACBD3	acyl-CoA binding domain containing 3 (ACBD3), mRNA [NM_022735]	-1.2353
8	ACOXL	acyl-CoA oxidase-like (ACOXL), mRNA [NM_001142807]	-1.0199
9	ACVRL1	activin A receptor type II-like 1 (ACVRL1), transcript variant 1, mRNA [NM_000020]	-1.8773
10	ADAM19	ADAM metallopeptidase domain 19 (ADAM19), mRNA [NM_033274]	-1.0853
11	ADAM22	ADAM metallopeptidase domain 22 (ADAM22), transcript variant 5, mRNA [NM_021721]	-1.3775
12	ADAMTS4	ADAM metallopeptidase with thrombospondin type 1 motif, 4 (ADAMTS4), mRNA [NM_005099]	-1.8197
13	ADARB2	adenosine deaminase, RNA-specific, B2 (ADARB2), mRNA [NM_018702]	-1.3453
14	ADORA2A	adenosine A2a receptor (ADORA2A), mRNA [NM_000675]	-1.7983
15	AGER	advanced glycosylation end product-specific receptor (AGER), transcript variant 8, mRNA [NM_001206954]	-1.7779
16	AGPAT9	1-acylglycerol-3-phosphate O-acyltransferase 9 (AGPAT9), mRNA [NM_032717]	-1.5955
17	AK7	adenylate kinase 7 (AK7), mRNA [NM_152327]	-1.1453
18	AKAP2	A kinase (PRKA) anchor protein 2 (AKAP2), transcript variant 1, mRNA [NM_001004065]	-1.1208
19	AKAP9	A kinase (PRKA) anchor protein (yotiao) 9, mRNA (cDNA clone IMAGE:3914749), complete cds. [BC015533]	-1.2473
20	ALDH2	aldehyde dehydrogenase 2 family (mitochondrial) (ALDH2), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA [NM_000690]	-1.5109
21	ALMS1	Alstrom syndrome 1 (ALMS1), mRNA [NM_015120]	-1.6351
22	ANGPT2	angiopoietin 2 (ANGPT2), transcript variant 1, mRNA [NM_001147]	-1.8078
23	ANKHD1	ankyrin repeat and KH domain containing 1 (ANKHD1), transcript variant 3, mRNA [NM_024668]	-1.1079
24	ANKS4B	ankyrin repeat and sterile alpha motif domain containing 4B (ANKS4B), mRNA [NM_145865]	-1.504
25	AOC3	amine oxidase, copper containing 3 (vascular adhesion protein 1) (AOC3), mRNA [NM_003734]	-1.4778
26	APAF1	apoptotic peptidase activating factor 1 (APAF1), transcript variant 3, mRNA [NM_181861]	-1.025
27	APOL6	apolipoprotein L, 6 (APOL6), mRNA [NM_030641]	-1.2068
28	ARF4	ADP-ribosylation factor 4 (ARF4), mRNA [NM_001660]	-1.1414
29	ARHGAP21	Rho GTPase activating protein 21 [Source	-1.2735
30	ARHGAP23	Rho GTPase activating protein 23 (ARHGAP23), mRNA [NM_001199417]	-1.0446
31	ARHGAP33	Rho GTPase activating protein 33 [Source	-1.7249
32	ARHGDIG	Rho GDP dissociation inhibitor (GDI) gamma (ARHGDIG), mRNA [NM_001176]	-1.4185
33	ARHGEF10	Rho guanine nucleotide exchange factor (GEF) 10 [Source	-1.0301
34	ARHGEF7	Rho guanine nucleotide exchange factor (GEF) 7 (ARHGEF7), transcript variant 2, mRNA [NM_145735]	-1.0497
35	ARL4C	ADP-ribosylation factor-like 4C (ARL4C), mRNA [NM_005737]	-1.2221
36	ARMCX3	armadillo repeat containing, X-linked 3 (ARMCX3), transcript variant 1, mRNA [NM_016607]	-1.3699
37	ARNT2	aryl-hydrocarbon receptor nuclear translocator 2 (ARNT2), mRNA [NM_014862]	-1.6914
38	ARRDC4	arrestin domain containing 4 (ARRDC4), mRNA [NM_183376]	-1.161
39	AS3MT	arsenic (+3 oxidation state) methyltransferase (AS3MT), mRNA [NM_020682]	-1.6396
40	ASPN	asporin (ASPN), transcript variant 2, mRNA [NM_001193335]	-1.0886
41	ATF3	activating transcription factor 3 (ATF3), transcript variant 4, mRNA [NM_001040619]	-1.3544
42	ATP2A1	ATPase, Ca++ transporting, cardiac muscle, fast twitch 1 (ATP2A1), transcript variant b, mRNA [NM_173201]	-1.1409
43	ATP7A	ATPase, Cu++ transporting, alpha polypeptide [Source:HGNC Symbol;Acc:869] [ENST00000355691]	-1.3725
44	ATP8B2	ATPase, class I, type 8B, member 2 (ATP8B2), transcript variant 2, mRNA [NM_001005855]	-1.3016
45	BAIAP3	BAI1-associated protein 3 (BAIAP3), transcript variant 1, mRNA [NM_003933]	-1.1324
46	BATF	basic leucine zipper transcription factor, ATF-like (BATF), mRNA [NM_006399]	-1.1696
47	BCAT1	branched chain amino-acid transaminase 1, cytosolic (BCAT1), transcript variant 1, mRNA [NM_005504]	-2.2842
48	BCL11B	B-cell CLL/lymphoma 11B (zinc finger protein) (BCL11B), transcript variant 1, mRNA [NM_138576]	-1.5799
49	BHLHE40	basic helix-loop-helix family, member e40 (BHLHE40), mRNA [NM_003670]	-1.0148
50	BMF	Bcl2 modifying factor (BMF), transcript variant 1, mRNA [NM_001003940]	-1.0048
51	BMP2	bone morphogenetic protein 2 (BMP2), mRNA [NM_001200]	-1.5309

52	BMP6	bone morphogenetic protein 6 (BMP6), mRNA [NM_001718]	-1.1738
53	BNIPL	BCL2/adenovirus E1B 19kD interacting protein like (BNIPL), transcript variant 1, mRNA [NM_138278]	-1.1785
54	BNIPL	BCL2/adenovirus E1B 19kD interacting protein like (BNIPL), transcript variant 2, mRNA [NM_001159642]	-1.1065
55	BRPF3	bromodomain and PHD finger containing, 3 (BRPF3), mRNA [NM_015695]	-1.7538
56	BTBD11	BTB (POZ) domain containing 11 (BTBD11), transcript variant a, mRNA [NM_001018072]	-1.0022
57	BTK	Bruton agammaglobulinemia tyrosine kinase (BTK), mRNA [NM_000061]	-1.2468
58	BVES	blood vessel epicardial substance (BVES), transcript variant B, mRNA [NM_147147]	-1.37
59	C11orf66	chromosome 11 open reading frame 66 (C11orf66), transcript variant 1, mRNA [NM_145017]	-1.1975
60	C12orf68	chromosome 12 open reading frame 68 (C12orf68), mRNA [NM_001013635]	-1.0609
61	C12orf70	chromosome 12 open reading frame 70 (C12orf70), mRNA [NM_001145010]	-1.1125
62	C15orf48	chromosome 15 open reading frame 48 (C15orf48), transcript variant 2, mRNA [NM_032413]	-1.6694
63	C15orf5	chromosome 15 open reading frame 5 (C15orf5), non-coding RNA [NR_026813]	-1.0358
64	C16orf79	chromosome 16 open reading frame 79 (C16orf79), mRNA [NM_182563]	-1.072
65	C19orf77	chromosome 19 open reading frame 77 (C19orf77), mRNA [NM_001136503]	-1.7383
66	C1orf70	chromosome 1 open reading frame 70 (C1orf70), mRNA [NM_001114748]	-1.1163
67	C1orf9	chromosome 1 open reading frame 9 (C1orf9), transcript variant 2, mRNA [NM_016227]	-1.0259
68	C1QL4	complement component 1, q subcomponent-like 4 (C1QL4), mRNA [NM_001008223]	-1.1015
69	C1QTNF4	C1q and tumor necrosis factor related protein 4 (C1QTNF4), mRNA [NM_031909]	-1.4879
70	C20orf195	chromosome 20 open reading frame 195 (C20orf195), mRNA [NM_024059]	-1.0527
71	C21orf71	chromosome 21 open reading frame 71 (C21orf71), non-coding RNA [NR_024092]	-1.8987
72	C2orf52	chromosome 2 open reading frame 52 (C2orf52), non-coding RNA [NR_024079]	-1.0769
73	C2orf84	chromosome 2 open reading frame 84 (C2orf84), mRNA [NM_001040710]	-1.7088
74	C3orf55	chromosome 3 open reading frame 55 (C3orf55), transcript variant 3, mRNA [NM_001099777]	-1.3372
75	C3P1	complement component 3 precursor pseudogene (C3P1), non-coding RNA [NR_027300]	-2.2069
76	C4orf49	chromosome 4 open reading frame 49 (C4orf49), mRNA [NM_032623]	-1.3366
77	C5orf22	chromosome 5 open reading frame 22 (C5orf22), mRNA [NM_018356]	-1.1291
78	C6orf204	chromosome 6 open reading frame 204 (C6orf204), transcript variant 2, mRNA [NM_206921]	-1.1991
79	C8orf4	chromosome 8 open reading frame 4 (C8orf4), mRNA [NM_020130]	-2.2971
80	CA3	carbonic anhydrase III, muscle specific (CA3), mRNA [NM_005181]	-1.6343
81	CABP1	calcium binding protein 1 (CABP1), transcript variant 3, mRNA [NM_001033677]	-1.5283
82	CABP2	calcium binding protein 2 (CABP2), mRNA [NM_016366]	-1.2953
83	CABP7	calcium binding protein 7 (CABP7), mRNA [NM_182527]	-1.1987
84	CACNG6	calcium channel, voltage-dependent, gamma subunit 6 (CACNG6), transcript variant 1, mRNA [NM_145814]	-1.8287
85	CAMK1G	calcium/calmodulin-dependent protein kinase IG (CAMK1G), mRNA [NM_020439]	-2.7164
86	CASP10	caspase 10, apoptosis-related cysteine peptidase (CASP10), transcript variant 2, mRNA [NM_032974]	-1.3122
87	CBFA2T2	core-binding factor, runt domain, alpha subunit 2; translocated to, 2 [Source:HGNC Symbol;Acc:1536] [ENST00000397798]	-1.1466
88	CCDC11	coiled-coil domain containing 11 (CCDC11), mRNA [NM_145020]	-1.3887
89	CCDC147	coiled-coil domain containing 147 [Source	-1.5249
90	CCDC147	coiled-coil domain containing 147 (CCDC147), mRNA [NM_001008723]	-1.7566
91	CCDC165	coiled-coil domain containing 165 (CCDC165), mRNA [NM_015210]	-1.131
92	CCDC33	coiled-coil domain containing 33 [Source:HGNC Symbol;Acc:26552] [ENST00000321288]	-2.3281
93	CCL3	chemokine (C-C motif) ligand 3 (CCL3), mRNA [NM_002983]	-1.0075
94	CCL5	chemokine (C-C motif) ligand 5 (CCL5), mRNA [NM_002985]	-2.6531
95	CCNA1	cyclin A1 (CCNA1), transcript variant 1, mRNA [NM_003914]	-1.0537
96	CCT8L2	chaperonin containing TCP1, subunit 8 (theta)-like 2 (CCT8L2), mRNA [NM_014406]	-1.8513
97	CD247	CD247 molecule (CD247), transcript variant 1, mRNA [NM_198053]	-1.5124
98	CD300C	CD300c molecule (CD300C), mRNA [NM_006678]	-1.6461
99	CD40	CD40 molecule, TNF receptor superfamily member 5 (CD40), transcript variant 1, mRNA [NM_001250]	-1.38
100	CD99	CD99 molecule [Source	-1.0738
101	CDH1	cadherin 1, type 1, E-cadherin (epithelial) (CDH1), mRNA [NM_004360]	-2.4314
102	CDH15	cadherin 15, type 1, M-cadherin (myotubule) (CDH15), mRNA [NM_004933]	-1.6729
103	CDKN2A	cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4) (CDKN2A), transcript variant 1, mRNA [NM_000077]	-1.0002
104	CDR2L	cerebellar degeneration-related protein 2-like (CDR2L), mRNA [NM_014603]	-1.2191
105	CDX4	caudal type homeobox 4 (CDX4), mRNA [NM_005193]	-2.0406
106	CG030	hypothetical CG030 (CG030), non-coding RNA [NR_026928]	-1.1596
107	CLCA1	chloride channel accessory 1 (CLCA1), mRNA [NM_001285]	-1.3706
108	CLIC3	chloride intracellular channel 3 (CLIC3), mRNA [NM_004669]	-1.2121
109	CNOT4	CCR4-NOT transcription complex, subunit 4 [Source:HGNC Symbol;Acc:7880] [ENST00000315544]	-1.2594
110	CNST	consortin, connexin sorting protein (CNST), transcript variant 2, mRNA [NM_001139459]	-1.068
111	CNTRL	centriolin (CNTRL), mRNA [NM_007018]	-1.1473
112	COL20A1	collagen, type XX, alpha 1 (COL20A1), mRNA [NM_020882]	-1.7582
113	COL6A1	collagen, type VI, alpha 1 (COL6A1), mRNA [NM_001848]	-1.8853
114	COL6A2	collagen, type VI, alpha 2 (COL6A2), transcript variant 2C2a, mRNA [NM_058174]	-2.5457
115	COL6A2	collagen, type VI, alpha 2 (COL6A2), transcript variant 2C2a', mRNA [NM_058175]	-1.546
116	COL6A3	collagen, type VI, alpha 3 (COL6A3), transcript variant 1, mRNA [NM_004369]	-1.2
117	CSF2	colony stimulating factor 2 (granulocyte-macrophage) (CSF2), mRNA [NM_000758]	-2.8034
118	CSGALNACT2	chondroitin sulfate N-acetylgalactosaminyltransferase 2 (CSGALNACT2), mRNA [NM_018590]	-1.0529
119	CSNK2A1	casein kinase 2, alpha 1 polypeptide (CSNK2A1), transcript variant 1, mRNA [NM_177559]	-1.2834
120	CTSS	cathepsin S (CTSS), transcript variant 1, mRNA [NM_004079]	-1.0225
121	CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha) (CXCL1), mRNA [NM_001511]	-1.2022

122	CXCL2	chemokine (C-X-C motif) ligand 2 (CXCL2), mRNA [NM_002089]	-1.2499
123	CXCL3	chemokine (C-X-C motif) ligand 3 (CXCL3), mRNA [NM_002090]	-1.5028
124	CXCR4	chemokine (C-X-C motif) receptor 4 (CXCR4), transcript variant 1, mRNA [NM_001008540]	-1.2427
125	CXCR7	chemokine (C-X-C motif) receptor 7 (CXCR7), mRNA [NM_020311]	-1.0169
126	CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1), mRNA [NM_000499]	-1.0934
127	CYP27B1	cytochrome P450, family 27, subfamily B, polypeptide 1 (CYP27B1), nuclear gene encoding mitochondrial protein, mRNA [NM_000785]	-1.2556
128	CYTH4	cytohesin 4 (CYTH4), mRNA [NM_013385]	-2.0027
129	CYTIP	cytohesin 1 interacting protein (CYTIP), mRNA [NM_004288]	-1.6645
130	DAB2	disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila) (DAB2), mRNA [NM_001343]	-1.1585
131	DCAF5	DDB1 and CUL4 associated factor 5 [Source	-1.586
132	DCLK2	doublecortin-like kinase 2 (DCLK2), transcript variant 1, mRNA [NM_001040260]	-1.2366
133	DDAH2	dimethylarginine dimethylaminohydrolase 2 (DDAH2), mRNA [NM_013974]	-1.0433
134	DDIT3	DNA-damage-inducible transcript 3 (DDIT3), transcript variant 5, mRNA [NM_004083]	-1.2688
135	DDRGK1	DDRGK domain containing 1 [Source	-1.0826
136	DDX3Y	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked (DDX3Y), transcript variant 1, mRNA [NM_001122665]	-1.2381
137	DDX60	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60 (DDX60), mRNA [NM_017631]	-1.3481
138	DENND3	DENN/MADD domain containing 3 [Source	-1.0724
139	DKFZP586B0319	mRNA; cDNA DKFZp586B0319 (from clone DKFZp586B0319) [AL050097]	-1.2947
140	DKK4	dickkopf homolog 4 (Xenopus laevis) (DKK4), mRNA [NM_014420]	-1.1764
141	DMKN	dermokine (DMKN), transcript variant 1, mRNA [NM_001035516]	-1.749
142	DMKN	dermokine (DMKN), transcript variant 2, mRNA [NM_033317]	-1.4284
143	DMRTA1	DMRT-like family A1 (DMRTA1), mRNA [NM_022160]	-1.0345
144	DNAH6	dynein, axonemal, heavy chain 6 (DNAH6), mRNA [NM_001370]	-1.1778
145	DNAJB2	DnaJ (Hsp40) homolog, subfamily B, member 2 (DNAJB2), transcript variant 1, mRNA [NM_001039550]	-1.0018
146	DNAJC28	DnaJ (Hsp40) homolog, subfamily C, member 28 (DNAJC28), transcript variant 1, mRNA [NM_017833]	-1.1294
147	DNHD1	dynein heavy chain domain 1 (DNHD1), transcript variant 1, mRNA [NM_144666]	-1.51
148	DPPA5	developmental pluripotency associated 5 (DPPA5), mRNA [NM_001025290]	-1.9153
149	DRD4	dopamine receptor D4 (DRD4), mRNA [NM_000797]	-1.8736
150	DSC2	desmocollin 2 (DSC2), transcript variant Dsc2a, mRNA [NM_024422]	-1.2521
151	DUSP16	dual specificity phosphatase 16 (DUSP16), mRNA [NM_030640]	-1.0478
152	DUSP5	dual specificity phosphatase 5 (DUSP5), mRNA [NM_004419]	-1.4787
153	EAF2	ELL associated factor 2 (EAF2), mRNA [NM_018456]	-1.0971
154	ECM1	extracellular matrix protein 1 (ECM1), transcript variant 1, mRNA [NM_004425]	-1.2373
155	EFR3B	EFR3 homolog B (S. cerevisiae) (EFR3B), mRNA [NM_014971]	-1.1174
156	EGR3	early growth response 3 (EGR3), transcript variant 1, mRNA [NM_004430]	-1.2922
157	EGR4	early growth response 4 (EGR4), mRNA [NM_001965]	-1.0827
158	EIF2AK3	eukaryotic translation initiation factor 2-alpha kinase 3 (EIF2AK3), mRNA [NM_004836]	-1.1172
159	ELOVL7	ELOVL fatty acid elongase 7 (ELOVL7), transcript variant 1, mRNA [NM_024930]	-1.8324
160	EML6	echinoderm microtubule associated protein like 6 (EML6), mRNA [NM_001039753]	-1.5978
161	ENTHD1	ENTH domain containing 1 (ENTHD1), mRNA [NM_152512]	-1.3731
162	ERAS	ES cell expressed Ras (ERAS), mRNA [NM_181532]	-1.0464
163	ERC2	ELKS/RAB6-interacting/CAST family member 2 (ERC2), mRNA [NM_015576]	-2.603
164	ERP44	endoplasmic reticulum protein 44 (ERP44), mRNA [NM_015051]	-1.3765
165	ETNK1	ethanolamine kinase 1 (ETNK1), transcript variant 2, mRNA [NM_001039481]	-1.2601
166	EVC2	Ellis van Creveld syndrome 2 (EVC2), transcript variant 2, mRNA [NM_001166136]	-1.0309
167	FADS2	fatty acid desaturase 2 (FADS2), mRNA [NM_004265]	-1.4618
168	FAM117B	family with sequence similarity 117, member B [Source:HGNC Symbol;Acc:14440] [ENST00000481658]	-1.064
169	FAM118A	full-length cDNA clone CS0DI044YI19 of Placenta Cot 25-normalized of Homo sapiens (human). [CR624528]	-1.105
170	FAM126A	family with sequence similarity 126, member A (FAM126A), mRNA [NM_032581]	-1.3361
171	FAM129A	family with sequence similarity 129, member A (FAM129A), transcript variant 2, mRNA [NM_052966]	-2.1984
172	FAM132A	family with sequence similarity 132, member A (FAM132A), mRNA [NM_001014980]	-1.437
173	FAM167A	family with sequence similarity 167, member A (FAM167A), mRNA [NM_053279]	-1.249
174	FAM174B	family with sequence similarity 174, member B (FAM174B), mRNA [NM_207446]	-1.0537
175	FAM19A1	family with sequence similarity 19 (chemokine (C-C motif)-like), member A1 (FAM19A1), mRNA [NM_213609]	-1.4871
176	FAM27L	family with sequence similarity 27-like (FAM27L), non-coding RNA [NR_028336]	-1.3451
177	FAM47A	family with sequence similarity 47, member A (FAM47A), mRNA [NM_203408]	-1.4024
178	FAM49A	family with sequence similarity 49, member A (FAM49A), mRNA [NM_030797]	-1.7457
179	FAM83B	family with sequence similarity 83, member B (FAM83B), mRNA [NM_001010872]	-1.5103
180	FAM99A	family with sequence similarity 99, member A (FAM99A), non-coding RNA [NR_026643]	-1.06
181	FBLL1	fibrillarin-like 1 (FBLL1), non-coding RNA [NR_024356]	-1.1694
182	FBXO32	F-box protein 32 (FBXO32), transcript variant 1, mRNA [NM_058229]	-2.0895
183	FCHSD2	FCH and double SH3 domains 2 (FCHSD2), mRNA [NM_014824]	-1.8351
184	FES	feline sarcoma oncogene (FES), transcript variant 1, mRNA [NM_002005]	-2.0409
185	FLCN	folliculin (FLCN), transcript variant 1, mRNA [NM_144997]	-1.4193
186	FLJ25694	cDNA FLJ46084 fis, clone TESTI2006543. [AK127969]	-1.2861
187	FLJ25917	cDNA FLJ25917 fis, clone CBR04926. [AK098783]	-1.2446
188	FLJ31104	hypothetical LOC441072 (FLJ31104), partial miscRNA [XR_108600]	-1.8595
189	FLJ35024	hypothetical LOC401491 (FLJ35024), non-coding RNA [NR_015375]	-2.1484
190	FLJ37786	hypothetical LOC642691 (FLJ37786), miscRNA [XR_108343]	-1.3084
191	FOXA1	forkhead box A1 (FOXA1), mRNA [NM_004496]	-1.1605
192	FST	follistatin (FST), transcript variant FST344, mRNA [NM_013409]	-1.9069

193	FTCD	formiminotransferase cyclodeaminase (FTCD), transcript variant A, mRNA [NM_206965]	-1.2878
194	FYN	FYN oncogene related to SRC, FGR, YES (FYN), transcript variant 1, mRNA [NM_002037]	-1.1168
195	G0S2	G0/G1switch 2 (G0S2), mRNA [NM_015714]	-1.3873
196	GAL3ST1	galactose-3-O-sulfotransferase 1 (GAL3ST1), mRNA [NM_004861]	-1.5026
197	GEM	GTP binding protein overexpressed in skeletal muscle (GEM), transcript variant 1, mRNA [NM_005261]	-1.4107
198	GEMC1	geminin coiled-coil domain-containing protein 1 (GEMC1), mRNA [NM_001146686]	-1.0829
199	GFPT1	glutamine--fructose-6-phosphate transaminase 1 (GFPT1), mRNA [NM_002056]	-1.1281
200	GHITM	growth hormone inducible transmembrane protein (GHITM), mRNA [NM_014394]	-1.1556
201	GIGYF2	GRB10 interacting GYF protein 2 [Source:HGNC Symbol;Acc:11960] [ENST00000458528]	-1.8012
202	GNAO1	guanine nucleotide binding protein (G protein), alpha activating activity polypeptide O (GNAO1), transcript variant 2, mRNA [NM_138736]	-1.2658
203	GOLT1B	golgi transport 1B (GOLT1B), mRNA [NM_016072]	-1.0831
204	GPCPD1	glycerophosphocholine phosphodiesterase GDE1 homolog (S. cerevisiae) (GPCPD1), mRNA [NM_019593]	-1.5974
205	GPR56	G protein-coupled receptor 56 (GPR56), transcript variant 3, mRNA [NM_201525]	-1.0097
206	GPR6	G protein-coupled receptor 6 (GPR6), mRNA [NM_005284]	-1.2863
207	GPRC6A	G protein-coupled receptor, family C, group 6, member A (GPRC6A), mRNA [NM_148963]	-1.1883
208	GRB10	growth factor receptor-bound protein 10 (GRB10), transcript variant 4, mRNA [NM_001001555]	-1.1292
209	GRM1	glutamate receptor, metabotropic 1 (GRM1), transcript variant 1, mRNA [NM_000838]	-1.2441
210	GSDMB	gasdermin B (GSDMB), transcript variant 3, mRNA [NM_001165958]	-1.4941
211	GTPBP2	GTP binding protein 2 (GTPBP2), mRNA [NM_019096]	-1.091
212	GUCA1B	guanylate cyclase activator 1B (retina) (GUCA1B), mRNA [NM_002098]	-1.0823
213	H1F0	H1 histone family, member 0 (H1F0), mRNA [NM_005318]	-1.0134
214	HBEGF	heparin-binding EGF-like growth factor (HBEGF), mRNA [NM_001945]	-1.9278
215	HDAC8	histone deacetylase 8 (HDAC8), transcript variant 3, mRNA [NM_001166419]	-1.136
216	HES4	hairy and enhancer of split 4 (Drosophila) (HES4), transcript variant 2, mRNA [NM_021170]	-1.1274
217	HHIPL1	HHIP-like 1 (HHIPL1), transcript variant 1, mRNA [NM_001127258]	-1.1973
218	HIPK2	homeodomain interacting protein kinase 2 [Source	-1.5149
219	HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble) (HMGCS1), transcript variant 2, mRNA [NM_002130]	-1.0348
220	HMOX1	heme oxygenase (decycling) 1 (HMOX1), mRNA [NM_002133]	-2.0071
221	HNF1A	HNF1 homeobox A	-1.0547
222	HOOK1	hook homolog 1 (Drosophila) (HOOK1), mRNA [NM_015888]	-1.0891
223	HOXB6	homeobox B6 (HOXB6), mRNA [NM_018952]	-1.0608
224	HOXB9	homeobox B9 (HOXB9), mRNA [NM_024017]	-1.6213
225	HPCA	hippocalcin (HPCA), mRNA [NM_002143]	-2.0445
226	HS3ST2	heparan sulfate (glucosamine) 3-O-sulfotransferase 2 (HS3ST2), mRNA [NM_006043]	-1.2285
227	HTR3C	5-hydroxytryptamine (serotonin) receptor 3, family member C (HTR3C), mRNA [NM_130770]	-4.0994
228	HUS1B	HUS1 checkpoint homolog b (S. pombe) (HUS1B), mRNA [NM_148959]	-1.1518
229	ICAM1	intercellular adhesion molecule 1 (ICAM1), mRNA [NM_000201]	-1.4006
230	ICAM2	intercellular adhesion molecule 2 (ICAM2), transcript variant 5, mRNA [NM_000873]	-1.7746
231	IFIH1	interferon induced with helicase C domain 1 (IFIH1), mRNA [NM_022168]	-1.0884
232	IGF2	insulin-like growth factor 2 (somatomedin A) (IGF2), transcript variant 1, mRNA [NM_000612]	-1.7179
233	IGSF3	immunoglobulin superfamily, member 3 (IGSF3), transcript variant 1, mRNA [NM_001542]	-1.3774
234	IKZF5	IKAROS family zinc finger 5 (Pegasus) (IKZF5), mRNA [NM_022466]	-1.7033
235	IL1RN	interleukin 1 receptor antagonist (IL1RN), transcript variant 4, mRNA [NM_173843]	-1.5778
236	IL22	interleukin 22 (IL22), mRNA [NM_020525]	-2.2595
237	IL23A	interleukin 23, alpha subunit p19 (IL23A), mRNA [NM_016584]	-1.2755
238	IL24	interleukin 24 (IL24), transcript variant 3, mRNA [NM_001185156]	-2.6106
239	IL4I1	interleukin 4 induced 1 (IL4I1), transcript variant 2, mRNA [NM_172374]	-1.4302
240	IL8	interleukin 8 (IL8), mRNA [NM_000584]	-1.2856
241	IL8	interleukin 8	-1.1568
242	IP6K3	inositol hexakisphosphate kinase 3 (IP6K3), transcript variant 1, mRNA [NM_054111]	-1.1031
243	IQCF1	IQ motif containing F1 (IQCF1), mRNA [NM_152397]	-1.4612
244	IRAK2	interleukin-1 receptor-associated kinase 2 (IRAK2), mRNA [NM_001570]	-1.2805
245	IRF7	interferon regulatory factor 7 (IRF7), transcript variant d, mRNA [NM_004031]	-1.3902
246	IRGM	immunity-related GTPase family, M [Source:HGNC Symbol;Acc:29597] [ENST00000520549]	-2.5148
247	ISG20	interferon stimulated exonuclease gene 20kDa (ISG20), mRNA [NM_002201]	-1.1031
248	ISLR2	immunoglobulin superfamily containing leucine-rich repeat 2 (ISLR2), transcript variant 2, mRNA [NM_020851]	-1.3134
249	ISYNA1	inositol-3-phosphate synthase 1 (ISYNA1), transcript variant 1, mRNA [NM_016368]	-1.0075
250	ITGAX	integrin, alpha X (complement component 3 receptor 4 subunit) (ITGAX), mRNA [NM_000887]	-1.7298
251	ITGB3	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61) (ITGB3), mRNA [NM_000212]	-2.4503
252	ITPKB	inositol-trisphosphate 3-kinase B [Source	-1.1662
253	KCND1	potassium voltage-gated channel, Shal-related subfamily, member 1 (KCND1), mRNA [NM_004979]	-1.8225
254	KCNE1	potassium voltage-gated channel, Isk-related family, member 1 (KCNE1), transcript variant 2, mRNA [NM_000219]	-1.0225
255	KCNK6	potassium channel, subfamily K, member 6 (KCNK6), mRNA [NM_004823]	-1.7249
256	KCNMA1	potassium large conductance calcium-activated channel, subfamily M, alpha member 1 (KCNMA1), transcript variant 2, mRNA [NM_002247]	-1.8062
257	KCNS3	potassium voltage-gated channel, delayed-rectifier, subfamily S, member 3 (KCNS3), mRNA [NM_002252]	-1.1645
258	KDELR3	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 3 (KDELR3), transcript variant 2, mRNA [NM_016657]	-1.08
259	KDM2B	lysine (K)-specific demethylase 2B [Source	-1.2594

260	KIAA1244	KIAA1244 (KIAA1244), mRNA [NM_020340]	-1.2665
261	KIAA1644	KIAA1644 (KIAA1644), mRNA [NM_001099294]	-1.1608
262	KIAA1984	KIAA1984 (KIAA1984), mRNA [NM_001039374]	-1.2992
263	KIF3C	kinesin family member 3C (KIF3C), mRNA [NM_002254]	-1.0287
264	KITLG	KIT ligand (KITLG), transcript variant b, mRNA [NM_000899]	-1.232
265	KLC3	kinesin light chain 3 (KLC3), mRNA [NM_177417]	-1.4635
266	KLC4	kinesin light chain 4 (KLC4), transcript variant 4, mRNA [NM_138343]	-1.0113
267	KLF2	Kruppel-like factor 2 (lung) (KLF2), mRNA [NM_016270]	-1.6448
268	KLF7	Kruppel-like factor 7 (ubiquitous) (KLF7), mRNA [NM_003709]	-1.423
269	KLHDC1	kelch domain containing 1 (KLHDC1), mRNA [NM_172193]	-1.1548
270	KLHDC7B	kelch domain containing 7B (KLHDC7B), mRNA [NM_138433]	-1.9025
271	KLHL30	kelch-like 30 (Drosophila) (KLHL30), mRNA [NM_198582]	-1.0783
272	KLK1	kallikrein 1 (KLK1), mRNA [NM_002257]	-2.2166
273	KLK10	kallikrein-related peptidase 10 (KLK10), transcript variant 1, mRNA [NM_002776]	-1.0291
274	KRT6B	keratin 6B (KRT6B), mRNA [NM_005555]	-1.5694
275	KRTCAP3	keratinocyte associated protein 3 (KRTCAP3), transcript variant 2, mRNA [NM_173853]	-1.0971
276	LAMB3	laminin, beta 3 (LAMB3), transcript variant 2, mRNA [NM_001017402]	-1.1321
277	LAMC2	laminin, gamma 2 (LAMC2), transcript variant 1, mRNA [NM_005562]	-1.2591
278	LAMC2	laminin, gamma 2 (LAMC2), transcript variant 2, mRNA [NM_018891]	-1.1332
279	LAMTOR3	late endosomal/lysosomal adaptor, MAPK and MTOR activator 3 (LAMTOR3), transcript variant 1, mRNA [NM_021970]	-1.3348
280	LBH	limb bud and heart development homolog (mouse) (LBH), mRNA [NM_030915]	-1.4106
281	LCP1	lymphocyte cytosolic protein 1 (L-plastin) (LCP1), mRNA [NM_002298]	-1.9491
282	LDB2	LIM domain binding 2 (LDB2), transcript variant 1, mRNA [NM_001290]	-1.547
283	LEPREL1	leprecan-like 1 (LEPREL1), transcript variant 1, mRNA [NM_018192]	-1.6241
284	LGI4	leucine-rich repeat LGI family, member 4 (LGI4), mRNA [NM_139284]	-1.2906
285	LIF	leukemia inhibitory factor (cholinergic differentiation factor) (LIF), mRNA [NM_002309]	-1.1875
286	LILRB3	leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3 (LILRB3), transcript variant 2, mRNA [NM_006864]	-1.3396
287	LIPG	lipase, endothelial (LIPG), mRNA [NM_006033]	-1.3808
288	LOC100128402	cDNA FLJ42583 fis, clone BRACE3009090. [AK124574]	-1.2272
289	LOC100128950	cDNA FLJ42204 fis, clone THYMU2035400. [AK124198]	-1.7331
290	LOC100129973	hypothetical LOC100129973 (LOC100129973), partial miscRNA [XR_109194]	-1.2211
291	LOC100130097	kinesin-like protein family member 6-like (LOC100130097), mRNA [XM_001717121]	-1.3542
292	LOC100130357	hypothetical LOC100130357 (LOC100130357), mRNA [NM_001242698]	-1.5834
293	LOC100130372	cDNA FLJ45625 fis, clone BRTHA3028505. [AK127532]	-1.3298
294	LOC100130428	IGYY565 (LOC100130428), miscRNA [XR_110533]	-1.4256
295	LOC100130865	cDNA FLJ41135 fis, clone BRACE2028970. [AK123130]	-1.0248
296	LOC100131825	hypothetical LOC100131825 (LOC100131825), non-coding RNA [NR_037870]	-1.077
297	LOC100132764	cDNA FLJ16434 fis, clone BRACE3015829. [AK131376]	-1.3963
298	LOC100271836	602363146F1 NIH_MGC_90 Homo sapiens cDNA clone IMAGE:4471450 5', mRNA sequence [BG250912]	-1.1186
299	LOC100505564	hypothetical protein LOC100505564 (LOC100505564), mRNA [XM_003118688]	-1.0821
300	LOC100505585	hypothetical LOC100505585 (LOC100505585), partial miscRNA [XR_109536]	-2.0241
301	LOC100506136	hypothetical LOC100506136 (LOC100506136), non-coding RNA [NR_038948]	-1.6989
302	LOC100509927	histone H2A type 1-like (LOC100509927), mRNA [XM_003119551]	-1.2411
303	LOC100510454	integrin alpha-X-like, transcript variant 2 (LOC100510454), mRNA [XM_003120366]	-1.7176
304	LOC152225	hypothetical LOC152225 (LOC152225), non-coding RNA [NR_026934]	-1.4749
305	LOC283174	hypothetical LOC283174 (LOC283174), non-coding RNA [NR_024344]	-1.2912
306	LOC283856	hypothetical LOC283856 (LOC283856), non-coding RNA [NR_027078]	-1.0833
307	LOC284669	cDNA FLJ38794 fis, clone LIVER2003854. [AK096113]	-1.0788
308	LOC338694	hypothetical protein LOC338694, mRNA (cDNA clone IMAGE:5168969), partial cds. [BC043531]	-1.0069
309	LOC389634	hypothetical LOC389634, mRNA (cDNA clone IMAGE:4157715). [BC037255]	-2.4929
310	LOC399715	hypothetical LOC399715 (LOC399715), non-coding RNA [NR_040079]	-1.3026
311	LOC442421	hypothetical LOC442421 (LOC442421), non-coding RNA [NR_024496]	-1.6582
312	LOC642587	NPC-A-5 (LOC642587), mRNA [NM_001104548]	-1.0715
313	LOC643770	hypothetical LOC643770 (LOC643770), non-coding RNA [NR_038383]	-3.302
314	LOC644083	hypothetical LOC644083 (LOC644083), miscRNA [XR_112044]	-1.0136
315	LOC645195	cDNA FLJ41456 fis, clone BRSTN2012320. [AK123450]	-1.3194
316	LOC728392	hypothetical protein LOC728392 (LOC728392), mRNA [NM_001162371]	-1.5396
317	LPCAT2	lysophosphatidylcholine acyltransferase 2 (LPCAT2), mRNA [NM_017839]	-1.2536
318	LRRC18	leucine rich repeat containing 18 (LRRC18), mRNA [NM_001006939]	-1.054
319	LRRC55	leucine rich repeat containing 55 (LRRC55), mRNA [NM_001005210]	-1.1452
320	LRRC8C	leucine rich repeat containing 8 family, member C (LRRC8C), mRNA [NM_032270]	-1.4723
321	LTB	lymphotoxin beta (TNF superfamily, member 3) (LTB), transcript variant 1, mRNA [NM_002341]	-1.1586
322	LTN1	cDNA clone IMAGE:5172245, containing frame-shift errors. [BC031633]	-1.997
323	LYPD3	LY6/PLAUR domain containing 3 (LYPD3), mRNA [NM_014400]	-1.0736
324	LYPD5	LY6/PLAUR domain containing 5 (LYPD5), transcript variant B, mRNA [NM_182573]	-1.1093
325	MACROD2	MACRO domain containing 2 (MACROD2), transcript variant 1, mRNA [NM_080676]	-1.4293
326	MAGEA11	melanoma antigen family A, 11 (MAGEA11), transcript variant 2, mRNA [NM_001011544]	-1.5647
327	MAGEB5	igen family B, 5 [Source:HGNC Symbol;Acc:23795] [ENST00000379029]	-1.1245
328	MAGEB6	melanoma antigen family B, 6 (MAGEB6), mRNA [NM_173523]	-1.4369
329	MAN1A1	mannosidase, alpha, class 1A, member 1 (MAN1A1), mRNA [NM_005907]	-1.0673

330	MAP1B	microtubule-associated protein 1B (MAP1B), mRNA [NM_005909]	-1.3656
331	MARK1	MAP/microtubule affinity-regulating kinase 1 (MARK1), mRNA [NM_018650]	-1.0236
332	MCHR2	melanin-concentrating hormone receptor 2 (MCHR2), transcript variant 1, mRNA [NM_001040179]	-1.1817
333	MDGA1	MAM domain containing glycosylphosphatidylinositol anchor 1 (MDGA1), mRNA [NM_153487]	-2.5901
334	MEI1	meiosis inhibitor 1 (MEI1), mRNA [NM_152513]	-1.2963
335	METRNL	meteorin, glial cell differentiation regulator-like (METRNL), mRNA [NM_001004431]	-2.6467
336	METTL9	methyltransferase like 9 (METTL9), transcript variant 1, mRNA [NM_016025]	-1.1903
337	MGLL	monoglyceride lipase (MGLL), transcript variant 1, mRNA [NM_007283]	-1.0591
338	MICALCL	MICAL C-terminal like (MICALCL), mRNA [NM_032867]	-1.8341
339	MMP1	matrix metallopeptidase 1 (interstitial collagenase) (MMP1), transcript variant 1, mRNA [NM_002421]	-1.2669
340	MMP10	matrix metallopeptidase 10 (stromelysin 2) (MMP10), mRNA [NM_002425]	-2.4792
341	MMP3	matrix metallopeptidase 3 (stromelysin 1, progelatinase) (MMP3), mRNA [NM_002422]	-2.6441
342	MON2	MON2 homolog (S. cerevisiae) (MON2), mRNA [NM_015026]	-1.7724
343	MPRIP	phatase Rho interacting protein [Source	-1.0523
344	MPV17L	MPV17 mitochondrial membrane protein-like (MPV17L), nuclear gene encoding mitochondrial protein, transcript variant 2, mRNA [NM_173803]	-1.1162
345	MSH4	mutS homolog 4 (E. coli) (MSH4), mRNA [NM_002440]	-1.2266
346	MT1DP	metallothionein 1D, pseudogene (MT1DP), transcript variant 1, non-coding RNA [NR_003658]	-1.0154
347	MT1F	metallothionein 1F (MT1F), mRNA [NM_005949]	-1.5784
348	MTAP	methylthioadenosine phosphorylase (MTAP), mRNA [NM_002451]	-1.2399
349	MTHFR	methylenetetrahydrofolate reductase (NAD(P)H) (MTHFR), mRNA [NM_005957]	-1.0253
350	MUSTN1	musculoskeletal, embryonic nuclear protein 1 (MUSTN1), mRNA [NM_205853]	-1.2492
351	MYO1D	myosin ID (MYO1D), mRNA [NM_015194]	-1.1807
352	MYO7A	myosin VIIA (MYO7A), transcript variant 1, mRNA [NM_000260]	-1.5708
353	NANOS1	nanos homolog 1 (Drosophila) (NANOS1), mRNA [NM_199461]	-2.243
354	NCKAP5L	NCK-associated protein 5-like [Source	-1.1455
355	NDOR1	NADPH dependent diflavin oxidoreductase 1 (NDOR1), transcript variant 1, mRNA [NM_001144026]	-1.0467
356	NEURL3	neuralized homolog 3 (Drosophila) pseudogene (NEURL3), non-coding RNA [NR_026875]	-1.066
357	NFATC1	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 (NFATC1), transcript variant 3, mRNA [NM_172387]	-1.0885
358	NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta (NFKBIZ), transcript variant 1, mRNA [NM_031419]	-1.0603
359	NHSL1	NHS-like 1 (NHSL1), transcript variant 1, mRNA [NM_020464]	-1.2207
360	NLRP1	NLR family, pyrin domain containing 1 (NLRP1), transcript variant 1, mRNA [NM_033004]	-1.4476
361	NLRP3	NLR family, pyrin domain containing 3 (NLRP3), transcript variant 3, mRNA [NM_001079821]	-1.3815
362	NLRP3	NLR family, pyrin domain containing 3 (NLRP3), transcript variant 1, mRNA [NM_004895]	-1.2675
363	NOD2	nucleotide-binding oligomerization domain containing 2 (NOD2), mRNA [NM_022162]	-1.1126
364	NOTCH1	notch 1 (NOTCH1), mRNA [NM_017617]	-1.0118
365	NPAS1	neuronal PAS domain protein 1 (NPAS1), mRNA [NM_002517]	-1.5245
366	NPC1	Niemann-Pick disease, type C1 (NPC1), mRNA [NM_000271]	-1.0347
367	NR4A1	nuclear receptor subfamily 4, group A, member 1 (NR4A1), transcript variant 1, mRNA [NM_002135]	-1.0969
368	NRADDP	neurotrophin receptor associated death domain, pseudogene (NRADDP), non-coding RNA [NR_024046]	-1.2713
369	NRIP3	nuclear receptor interacting protein 3 (NRIP3), mRNA [NM_020645]	-1.7049
370	NRN1L	neuritin 1-like (NRN1L), mRNA [NM_198443]	-1.7245
371	NSAP11	nervous system abundant protein 11 (NSAP11), miscRNA [XR_110862]	-1.1851
372	NTN1	netrin 1 (NTN1), mRNA [NM_004822]	-1.1939
373	NTRK2	neurotrophic tyrosine kinase, receptor, type 2 (NTRK2), transcript variant c, mRNA [NM_001018064]	-2.1942
374	OASL	2'-5'-oligoadenylate synthetase-like (OASL), transcript variant 1, mRNA [NM_003733]	-1.5819
375	OBSCN	obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF [Source:HGNC Symbol;Acc:15719] [ENST00000366706]	-1.2144
376	ODZ1	odz, odd Oz/ten-m homolog 1 (Drosophila) (ODZ1), transcript variant 1, mRNA [NM_001163278]	-2.0751
377	OR10H4	olfactory receptor, family 10, subfamily H, member 4 (OR10H4), mRNA [NM_001004465]	-1.5509
378	OR2A42	olfactory receptor, family 2, subfamily A, member 42 (OR2A42), mRNA [NM_001001802]	-1.1472
379	OR4F4	olfactory receptor, family 4, subfamily F, member 4 (OR4F4), mRNA [NM_001004195]	-1.2812
380	OSBPL8	oxysterol binding protein-like 8 (OSBPL8), transcript variant 1, mRNA [NM_020841]	-1.6297
381	OXCT2	3-oxoacid CoA transferase 2 (OXCT2), mRNA [NM_022120]	-1.0058
382	P2RX4	purinergic receptor P2X, ligand-gated ion channel, 4 (P2RX4), mRNA [NM_002560]	-1.1408
383	P2RX7	purinergic receptor P2X, ligand-gated ion channel, 7 (P2RX7), transcript variant 1, mRNA [NM_002562]	-1.04
384	PABPC4L	poly(A) binding protein, cytoplasmic 4-like (PABPC4L), mRNA [NM_001114734]	-1.2659
385	PAEP	progestagen-associated endometrial protein (PAEP), transcript variant 2, mRNA [NM_002571]	-1.2487
386	PAGE2B	P antigen family, member 2B (PAGE2B), mRNA [NM_001015038]	-1.2198
387	PANX2	pannexin 2 (PANX2), transcript variant 1, mRNA [NM_052839]	-1.5193
388	PARG	poly (ADP-ribose) glycohydrolase (PARG), mRNA [NM_003631]	-1.1322
389	PCDHA1	protocadherin alpha 1 (PCDHA1), transcript variant 2, mRNA [NM_031410]	-1.0385
390	PCDHB6	protocadherin beta 6 (PCDHB6), mRNA [NM_018939]	-1.2685
391	PDE1C	phosphodiesterase 1C, calmodulin-dependent 70kDa (PDE1C), transcript variant 4, mRNA [NM_005020]	-2.0939
392	PDE1C	phosphodiesterase 1C, calmodulin-dependent 70kDa (PDE1C), transcript variant 2, mRNA [NM_001191057]	-5.7672
393	PDE1C	phosphodiesterase 1C, calmodulin-dependent 70kDa [Source:HGNC Symbol;Acc:8776] [ENST00000396184]	-5.4788
394	PDE4DIP	phosphodiesterase 4D interacting protein (PDE4DIP), transcript variant 9, mRNA [NM_001198834]	-2.3473
395	PDIA2	protein disulfide isomerase family A, member 2 (PDIA2), mRNA [NM_006849]	-1.1239
396	PDLIM3	PDZ and LIM domain 3 (PDLIM3), transcript variant 1, mRNA [NM_014476]	-1.8695
397	PDLIM5	PDZ and LIM domain 5 (PDLIM5), transcript variant 4, mRNA [NM_001011515]	-1.3156

398	PDXK	yridoxine, vitamin B6) kinase [Source:HGNC Symbol;Acc:8819] [ENST00000476313]	-1.1224
399	PDZRN4	PDZ domain containing ring finger 4 (PDZRN4), transcript variant 2, mRNA [NM_013377]	-1.104
400	PGLYRP4	peptidoglycan recognition protein 4 (PGLYRP4), mRNA [NM_020393]	-1.813
401	PGM2L1	phosphoglucomutase 2-like 1 (PGM2L1), mRNA [NM_173582]	-1.8849
402	PHC1	polyhomeotic homolog 1 (Drosophila) (PHC1), mRNA [NM_004426]	-1.0474
403	PHYHD1	phytanoyl-CoA dioxygenase domain containing 1 (PHYHD1), transcript variant 2, mRNA [NM_174933]	-1.0812
404	PIK3IP1	phosphoinositide-3-kinase interacting protein 1 (PIK3IP1), transcript variant 1, mRNA [NM_052880]	-1.0953
405	PIKFYVE	phosphoinositide kinase, FYVE finger containing (PIKFYVE), transcript variant 2, mRNA [NM_015040]	-1.6868
406	PIM1	pim-1 oncogene (PIM1), mRNA [NM_002648]	-1.019
407	PLAU	plasminogen activator, urokinase (PLAU), transcript variant 1, mRNA [NM_002658]	-1.5721
408	PLAU	plasminogen activator, urokinase (PLAU), transcript variant 2, mRNA [NM_001145031]	-1.8978
409	PLBD1	phospholipase B domain containing 1 (PLBD1), mRNA [NM_024829]	-1.8654
410	PLD1	phospholipase D1, phosphatidylcholine-specific (PLD1), transcript variant 1, mRNA [NM_002662]	-1.1565
411	PLEKHF1	pleckstrin homology domain containing, family F (with FYVE domain) member 1 (PLEKHF1), mRNA [NM_024310]	-1.2857
412	POLM	cDNA FLJ35482 fis, clone SMINT2008133. [AK092801]	-1.1877
413	POLR1A	RNA) I polypeptide A, 194kDa [Source:HGNC Symbol;Acc:17264] [ENST00000486964]	-1.0689
414	PP14571	hypothetical LOC100130449 (PP14571), non-coding RNA [NR_024014]	-1.1801
415	PPP1R15A	protein phosphatase 1, regulatory (inhibitor) subunit 15A (PPP1R15A), mRNA [NM_014330]	-1.0024
416	PRKCZ	protein kinase C, zeta (PRKCZ), transcript variant 1, mRNA [NM_002744]	-1.0604
417	PRR16	proline rich 16 (PRR16), mRNA [NM_016644]	-1.1771
418	PRSS1	protease, serine, 1 (trypsin 1) (PRSS1), mRNA [NM_002769]	-1.0975
419	PRSS35	protease, serine, 35 (PRSS35), transcript variant 2, mRNA [NM_153362]	-1.0039
420	PRUNE	prune homolog (Drosophila) (PRUNE), mRNA [NM_021222]	-1.2071
421	PSME4	proteasome (prosome, macropain) activator subunit 4 [Source:HGNC Symbol;Acc:20635] [ENST00000488687]	-1.7233
422	PTGIR	prostaglandin I2 (prostacyclin) receptor (IP) (PTGIR), mRNA [NM_000960]	-1.3223
423	PTGS1	prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase) (PTGS1), transcript variant 1, mRNA [NM_000962]	-1.2332
424	PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) (PTGS2), mRNA [NM_000963]	-1.2116
425	PTPRH	protein tyrosine phosphatase, receptor type, H (PTPRH), transcript variant 1, mRNA [NM_002842]	-1.1262
426	PTPRN2	protein tyrosine phosphatase, receptor type, N polypeptide 2 (PTPRN2), transcript variant 1, mRNA [NM_002847]	-1.2646
427	PYCARD	PYD and CARD domain containing (PYCARD), transcript variant 1, mRNA [NM_013258]	-1.0638
428	RAB11FIP4	RAB11 family interacting protein 4 (class II) (RAB11FIP4), mRNA [NM_032932]	-2.6619
429	RAG1	recombination activating gene 1 (RAG1), mRNA [NM_000448]	-1.0275
430	RASSF5	Ras association (RalGDS/AF-6) domain family member 5 (RASSF5), transcript variant 1, mRNA [NM_182663]	-1.3775
431	RASSF5	Ras association (RalGDS/AF-6) domain family member 5 (RASSF5), transcript variant 2, mRNA [NM_182664]	-1.3174
432	RBCK1	RanBP-type and C3HC4-type zinc finger containing 1 (RBCK1), transcript variant 2, mRNA [NM_031229]	-1.154
433	RBCK1	RanBP-type and C3HC4-type zinc finger containing 1 (RBCK1), transcript variant 1, mRNA [NM_006462]	-1.0231
434	RBM12	RNA binding motif protein 12 (RBM12), transcript variant 1, mRNA [NM_006047]	-1.0043
435	RCAN2	regulator of calcineurin 2 (RCAN2), mRNA [NM_005822]	-1.0014
436	RFPL3-AS1	RFPL3 antisense RNA 1 (non-protein coding) (RFPL3-AS1), antisense RNA [NR_001450]	-1.0927
437	RGS9	regulator of G-protein signaling 9 (RGS9), transcript variant 1, mRNA [NM_003835]	-1.2837
438	RHCE	Rh blood group, CcEe antigens (RHCE), transcript variant 1, mRNA [NM_020485]	-1.44
439	RHCG	Rh family, C glycoprotein (RHCG), mRNA [NM_016321]	-1.2818
440	RIMBP3	RIMS binding protein 3 (RIMBP3), mRNA [NM_015672]	-2.0328
441	RIMS3	regulating synaptic membrane exocytosis 3 (RIMS3), mRNA [NM_014747]	-1.0888
442	RND3	Rho family GTPase 3 (RND3), mRNA [NM_005168]	-1.8253
443	RNF152	ring finger protein 152 (RNF152), mRNA [NM_173557]	-1.0264
444	RNU11	BP873537 Sugano cDNA library, embryonal kidney Homo sapiens cDNA clone HKR13896, mRNA sequence [BP873537]	-1.7513
445	RNU4ATAC	HHAGE001732 Human liver regeneration after partial hepatectomy Homo sapiens cDNA, mRNA sequence [DW419002]	-1.2681
446	RORA	RAR-related orphan receptor A (RORA), transcript variant 2, mRNA [NM_134260]	-1.1539
447	RP1	retinitis pigmentosa 1 (autosomal dominant) (RP1), mRNA [NM_006269]	-1.2484
448	RPL31	full-length cDNA clone CS0DI015YG06 of Placenta Cot 25-normalized of Homo sapiens (human). [CR595074]	-1.4645
449	RSAD2	radical S-adenosyl methionine domain containing 2 (RSAD2), mRNA [NM_080657]	-1.0618
450	RTN4R	reticulon 4 receptor (RTN4R), mRNA [NM_023004]	-2.1844
451	RTP3	receptor (chemosensory) transporter protein 3 (RTP3), mRNA [NM_031440]	-1.602
452	SC4MOL	sterol-C4-methyl oxidase-like (SC4MOL), transcript variant 1, mRNA [NM_006745]	-1.1961
453	SCG5	secretogranin V (7B2 protein) (SCG5), transcript variant 2, mRNA [NM_003020]	-1.0064
454	SCGB1D1	secretoglobin, family 1D, member 1 (SCGB1D1), mRNA [NM_006552]	-1.3905
455	SCXA	scleraxis homolog A (mouse) (SCXA), mRNA [NM_001008271]	-1.1648
456	SEC13	SEC13 homolog (S. cerevisiae) [Source	-1.0552
457	SEC24A	SEC24 family, member A (S. cerevisiae), mRNA (cDNA clone MGC:12985 IMAGE:3355949), complete cds. [BC019341]	-1.3372
458	Sep-05	septin 5 (SEPT5), transcript variant 2, mRNA [NM_001009939]	-1.382
459	Sep-08	septin 8 (SEPT8), transcript variant 1, mRNA [NM_001098811]	-1.0709
460	SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2 (SERPINB2), transcript variant 2, mRNA [NM_002575]	-1.0745
461	SERPINB3	serpin peptidase inhibitor, clade B (ovalbumin), member 3 (SERPINB3), mRNA [NM_006919]	-2.2229

462	SERPINB4	serpin peptidase inhibitor, clade B (ovalbumin), member 4 (SERPINB4), mRNA [NM_002974]	-1.9934
463	SEZ6L2	seizure related 6 homolog (mouse)-like 2 (SEZ6L2), transcript variant 2, mRNA [NM_201575]	-1.3708
464	SFN	stratifin (SFN), mRNA [NM_006142]	-1.1294
465	SH2D3C	SH2 domain containing 3C (SH2D3C), transcript variant 1, mRNA [NM_170600]	-1.0438
466	SHC4	SHC (Src homology 2 domain containing) family, member 4 (SHC4), mRNA [NM_203349]	-2.2984
467	SHF	Src homology 2 domain containing F (SHF), mRNA [NM_138356]	-1.1617
468	SIGLEC7	sialic acid binding Ig-like lectin 7 (SIGLEC7), transcript variant 1, mRNA [NM_014385]	-1.2472
469	SIKE1	suppressor of IKBKE 1 (SIKE1), transcript variant 1, mRNA [NM_001102396]	-1.2235
470	SIRPA	signal-regulatory protein alpha (SIRPA), transcript variant 1, mRNA [NM_001040022]	-1.4705
471	SLC13A3	solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3 (SLC13A3), transcript variant 2, mRNA [NM_001011554]	-1.3126
472	SLC16A6	solute carrier family 16, member 6 (monocarboxylic acid transporter 7) (SLC16A6), transcript variant 2, mRNA [NM_004694]	-3.534
473	SLC1A4	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4 (SLC1A4), transcript variant 1, mRNA [NM_003038]	-1.6231
474	SLC26A9	solute carrier family 26, member 9 (SLC26A9), transcript variant 1, mRNA [NM_052934]	-1.2785
475	SLC27A6	solute carrier family 27 (fatty acid transporter), member 6 (SLC27A6), transcript variant 2, mRNA [NM_001017372]	-1.0414
476	SLC2A14	solute carrier family 2 (facilitated glucose transporter), member 14 [Source:HGNC Symbol;Acc:18301] [ENST00000431042]	-1.4169
477	SLC2A3	solute carrier family 2 (facilitated glucose transporter), member 3 (SLC2A3), mRNA [NM_006931]	-1.2214
478	SLC4A5	solute carrier family 4, sodium bicarbonate cotransporter, member 5 (SLC4A5), transcript variant c, mRNA [NM_133478]	-1.4189
479	SLC5A10	solute carrier family 5 (sodium/glucose cotransporter), member 10 (SLC5A10), transcript variant 1, mRNA [NM_152351]	-1.1488
480	SLC7A8	solute carrier family 7 (amino acid transporter light chain, L system), member 8 (SLC7A8), transcript variant 2, mRNA [NM_182728]	-1.0959
481	SMAP1	small ArfGAP 1 [Source	-1.2492
482	SNX31	sorting nexin 31 (SNX31), mRNA [NM_152628]	-1.0885
483	SP8	Sp8 transcription factor (SP8), transcript variant 2, mRNA [NM_198956]	-2.3366
484	SPINK1	serine peptidase inhibitor, Kazal type 1 (SPINK1), mRNA [NM_003122]	-1.3099
485	SPINK6	serine peptidase inhibitor, Kazal type 6 (SPINK6), transcript variant 1, mRNA [NM_205841]	-2.625
486	SPRR2A	small proline-rich protein 2A (SPRR2A), mRNA [NM_005988]	-1.4207
487	SPRR2D	small proline-rich protein 2D (SPRR2D), mRNA [NM_006945]	-2.3407
488	SPRY2	sprouty homolog 2 (Drosophila) (SPRY2), mRNA [NM_005842]	-1.2952
489	SRSF12	serine/arginine-rich splicing factor 12 (SRSF12), mRNA [NM_080743]	-1.039
490	ST6GALNAC2	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 2 (ST6GALNAC2), mRNA [NM_006456]	-2.1197
491	STAT5A	signal transducer and activator of transcription 5A (STAT5A), mRNA [NM_003152]	-1.3407
492	STC1	stanniocalcin 1 (STC1), mRNA [NM_003155]	-1.1378
493	STIL	SCL/TAL1 interrupting locus (STIL), transcript variant 1, mRNA [NM_001048166]	-1.1244
494	STRA6	stimulated by retinoic acid gene 6 homolog (mouse) (STRA6), transcript variant 8, mRNA [NM_001199042]	-1.5632
495	STX3	syntaxin 3 (STX3), transcript variant 1, mRNA [NM_004177]	-1.4849
496	SUV420H1	suppressor of variegation 4-20 homolog 1 (Drosophila) (SUV420H1), transcript variant 2, mRNA [NM_016028]	-1.1782
497	SVOP	SV2 related protein homolog (rat) (SVOP), mRNA [NM_018711]	-1.0032
498	SYT17	synaptotagmin XVII (SYT17), mRNA [NM_016524]	-1.2056
499	SYT7	synaptotagmin VII (SYT7), mRNA [NM_004200]	-1.879
500	TAGLN	transgelin (TAGLN), transcript variant 1, mRNA [NM_001001522]	-1.0956
501	TAP1	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP) (TAP1), mRNA [NM_000593]	-1.2451
502	TAS2R14	taste receptor, type 2, member 14 (TAS2R14), mRNA [NM_023922]	-1.1146
503	TBX21	T-box 21 (TBX21), mRNA [NM_013351]	-1.5144
504	TCF7L1	transcription factor 7-like 1 (T-cell specific, HMG-box) (TCF7L1), mRNA [NM_031283]	-1.4485
505	TDRKH	tudor and KH domain containing (TDRKH), transcript variant 1, mRNA [NM_001083965]	-1.3498
506	TEKT3	tektin 3 (TEKT3), mRNA [NM_031898]	-1.7392
507	TFPI2	tissue factor pathway inhibitor 2 (TFPI2), mRNA [NM_006528]	-1.0109
508	THAP5	THAP domain containing 5 (THAP5), transcript variant 2, mRNA [NM_182529]	-1.1534
509	THPO	thrombopoietin (THPO), transcript variant 1, mRNA [NM_000460]	-1.2435
510	THRB	thyroid hormone receptor, beta (erythroblastic leukemia viral (v-erb-a) oncogene homolog 2, avian) (THRB), transcript variant 3, mRNA [NM_001128177]	-1.1549
511	TJP2	tight junction protein 2 (zona occludens 2) (TJP2), transcript variant 2, mRNA [NM_201629]	-1.5773
512	TLE4	transducin-like enhancer of split 4 (E(sp1) homolog, Drosophila) (TLE4), mRNA [NM_007005]	-1.1784
513	TLR2	toll-like receptor 2 (TLR2), mRNA [NM_003264]	-1.09
514	TM4SF19	transmembrane 4 L six family member 19 (TM4SF19), transcript variant 1, mRNA [NM_138461]	-1.7518
515	TMEM132B	transmembrane protein 132B (TMEM132B), mRNA [NM_052907]	-1.3634
516	TMEM158	transmembrane protein 158 (gene/pseudogene) (TMEM158), mRNA [NM_015444]	-1.5102
517	TMEM2	transmembrane protein 2 (TMEM2), transcript variant 1, mRNA [NM_013390]	-1.2666
518	TMEM40	transmembrane protein 40 (TMEM40), mRNA [NM_018306]	-3.0319
519	TMEM45B	transmembrane protein 45B (TMEM45B), mRNA [NM_138788]	-1.0757
520	TMEM88	transmembrane protein 88 (TMEM88), mRNA [NM_203411]	-1.1305
521	TMPRSS11BNL	TMPRSS11B N terminal-like (TMPRSS11BNL), mRNA [NM_001129907]	-1.2529
522	TNFAIP3	tumor necrosis factor, alpha-induced protein 3 (TNFAIP3), mRNA [NM_006290]	-1.4007
523	TNFAIP6	tumor necrosis factor, alpha-induced protein 6 (TNFAIP6), mRNA [NM_007115]	-1.0671
524	TNFAIP8	tumor necrosis factor, alpha-induced protein 8 (TNFAIP8), transcript variant 1, mRNA [NM_014350]	-1.714

525	TNIK	TRAF2 and NCK interacting kinase (TNIK), transcript variant 1, mRNA [NM_015028]	-1.0003
526	TNNT1	troponin T type 1 (skeletal, slow) (TNNT1), transcript variant 1, mRNA [NM_003283]	-1.0422
527	TP53INP2	tumor protein p53 inducible nuclear protein 2 (TP53INP2), mRNA [NM_021202]	-1.1395
528	TPPP	tubulin polymerization promoting protein (TPPP), mRNA [NM_007030]	-1.792
529	TPPP3	tubulin polymerization-promoting protein family member 3 (TPPP3), mRNA [NM_016140]	-1.3569
530	TRAF1	TNF receptor-associated factor 1 (TRAF1), transcript variant 1, mRNA [NM_005658]	-1.4257
531	TRAF3IP2-AS1	TRAF3IP2 antisense RNA 1 (non-protein coding) (TRAF3IP2-AS1), transcript variant 1, non-coding RNA [NR_034108]	-1.4149
532	TRIM15	tripartite motif containing 15 (TRIM15), mRNA [NM_033229]	-1.4793
533	TRIM24	tripartite motif containing 24 [Source	-1.4331
534	TSEN15	tRNA splicing endonuclease 15 homolog (S. cerevisiae) (TSEN15), transcript variant 3, non-coding RNA [NR_023349]	-1.3802
535	UAP1L1	UDP-N-acteylglucosamine pyrophosphorylase 1-like 1 (UAP1L1), mRNA [NM_207309]	-1.2231
536	UBE4B	ubiquitination factor E4B (UFD2 homolog, yeast) [Source:HGNC Symbol;Acc:12500] [ENST00000377153]	-1.0824
537	UNC5B	unc-5 homolog B (C. elegans) (UNC5B), mRNA [NM_170744]	-1.2787
538	VAMP4	vesicle-associated membrane protein 4 (VAMP4), transcript variant 1, mRNA [NM_003762]	-1.0841
539	VGLL3	vestigial like 3 (Drosophila) (VGLL3), mRNA [NM_016206]	-1.0333
540	WDR33	WD repeat domain 33 (WDR33), transcript variant 2, mRNA [NM_001006622]	-1.2907
541	WNT5A	wingless-type MMTV integration site family, member 5A (WNT5A), mRNA [NM_003392]	-1.1928
542	WT1-AS	WT1 antisense RNA (non-protein coding) (WT1-AS), non-coding RNA [NR_023920]	-1.932
543	YPEL2	yippee-like 2 (Drosophila) (YPEL2), mRNA [NM_001005404]	-1.008
544	ZCCHC6	zinc finger, CCHC domain containing 6 [Source:HGNC Symbol;Acc:25817] [ENST00000375948]	-1.0334
545	ZCCHC6	zinc finger, CCHC domain containing 6 [Source:HGNC Symbol;Acc:25817] [ENST00000375947]	-2.2818
546	ZCCHC6	zinc finger, CCHC domain containing 6 (ZCCHC6), transcript variant 1, mRNA [NM_024617]	-1.8406
547	ZFP82	zinc finger protein 82 homolog (mouse) (ZFP82), mRNA [NM_133466]	-1.0215
548	ZNF229	zinc finger protein 229 (ZNF229), mRNA [NM_014518]	-1.4968
549	ZNF280A	zinc finger protein 280A (ZNF280A), mRNA [NM_080740]	-1.4605
550	ZNF433	zinc finger protein 433 (ZNF433), mRNA [NM_001080411]	-5.1659
551	ZNF474	zinc finger protein 474 (ZNF474), mRNA [NM_207317]	-1.0432
552	ZNF566	zinc finger protein 566 (ZNF566), transcript variant 3, mRNA [NM_032838]	-1.2884
553	ZNF700	zinc finger protein 700 (ZNF700), mRNA [NM_144566]	-1.5259
554	ZNF81	zinc finger protein 81 (ZNF81), mRNA [NM_007137]	-1.1905
555	ZP4	zona pellucida glycoprotein 4 (ZP4), mRNA [NM_021186]	-1.4811
556	ZSCAN5D	zinc finger and SCAN domain containing 5D (ZSCAN5D), mRNA [XM_001725568]	-1.7321

Table A4. *List of genes up-regulated upon ectopic expression of HSF1ΔRDT in the H-Ras^{V12} transformed MCF10A cells*

1	ACY3	aspartoacylase (aminocyclase) 3 (ACY3), mRNA [NM_080658]	1.1949
2	ADAM21	ADAM metallopeptidase domain 21 (ADAM21), mRNA [NM_003813]	1.525
3	ADH1A	alcohol dehydrogenase 1A (class I), alpha polypeptide (ADH1A), mRNA [NM_000667]	1.9418
4	ADM	adrenomedullin (ADM), mRNA [NM_001124]	1.6764
5	AIFM2	apoptosis-inducing factor, mitochondrion-associated, 2 [Source:HGNC Symbol;Acc:21411] [ENST00000373248]	1.1483
6	ALDH1L1	aldehyde dehydrogenase 1 family, member L1 (ALDH1L1), mRNA [NM_012190]	1.3508
7	ALDH3A1	aldehyde dehydrogenase 3 family, member A1 (ALDH3A1), transcript variant 1, mRNA [NM_001135168]	1.1386
8	ALKBH8	alkB, alkylation repair homolog 8 (E. coli) (ALKBH8), mRNA [NM_138775]	1.1526
9	ALX4	ALX homeobox 4 (ALX4), mRNA [NM_021926]	1.0224
10	AMIGO1	adhesion molecule with Ig-like domain 1 (AMIGO1), mRNA [NM_020703]	1.3748
11	ANGPT1	angiopoietin 1 (ANGPT1), transcript variant 1, mRNA [NM_001146]	2.0381
12	ANKRD2	ankyrin repeat domain 2 (stretch responsive muscle) (ANKRD2), transcript variant 1, mRNA [NM_020349]	1.3316
13	ANKRD35	ankyrin repeat domain 35 (ANKRD35), mRNA [NM_144698]	1.0648
14	ANO4	anoctamin 4 (ANO4), mRNA [NM_178826]	1.0739
15	ANXA10	annexin A10 (ANXA10), mRNA [NM_007193]	1.1754
16	APC2	adenomatosis polyposis coli 2 (APC2), mRNA [NM_005883]	1.0282
17	APLN	apelin (APLN), mRNA [NM_017413]	1.1328
18	APLNR	apelin receptor (APLNR), transcript variant 1, mRNA [NM_005161]	1.008
19	ARHGAP26	Rho GTPase activating protein 26 (ARHGAP26), transcript variant 1, mRNA [NM_015071]	1.1876
20	ARHGAP44	Rho GTPase activating protein 44 (ARHGAP44), mRNA [NM_014859]	2.8696
21	ARHGDIB	Rho GDP dissociation inhibitor (GDI) beta (ARHGDIB), mRNA [NM_001175]	1.5449
22	ARL15	ADP-ribosylation factor-like 15 (ARL15), mRNA [NM_019087]	1.5885
23	ASB9	ankyrin repeat and SOCS box containing 9 (ASB9), transcript variant 1, mRNA [NM_001031739]	1.897
24	ASPHD2	aspartate beta-hydroxylase domain containing 2 (ASPHD2), mRNA [NM_020437]	1.0367
25	ATAD3C	ATPase family, AAA domain containing 3C (ATAD3C), mRNA [NM_001039211]	1.0297
26	ATP1A2	ATPase, Na+/K+ transporting, alpha 2 polypeptide (ATP1A2), mRNA [NM_000702]	2.2004
27	ATP6V0A4	ATPase, H+ transporting, lysosomal V0 subunit a4 (ATP6V0A4), transcript variant 1, mRNA [NM_020632]	1.0449
28	ATP6V1G3	ATPase, H+ transporting, lysosomal 13kDa, V1 subunit G3 (ATP6V1G3), transcript variant 2, mRNA [NM_133326]	1.3231

29	BAIAP2L2	BAI1-associated protein 2-like 2 (BAIAP2L2), mRNA [NM_025045]	1.0445
30	BCL11A	B-cell CLL/lymphoma 11A (zinc finger protein) (BCL11A), transcript variant 1, mRNA [NM_022893]	2.1709
31	BCL11A	B-cell CLL/lymphoma 11A (zinc finger protein) (BCL11A), transcript variant 3, mRNA [NM_138559]	1.1041
32	BCL2	B-cell CLL/lymphoma 2 (BCL2), nuclear gene encoding mitochondrial protein, transcript variant alpha, mRNA [NM_000633]	1.5551
33	BDNF-AS1	BDNF antisense RNA 1 (non-protein coding) (BDNF-AS1), transcript variant BT2B, non-coding RNA [NR_002832]	1.0047
34	BMX	BMX non-receptor tyrosine kinase (BMX), transcript variant 2, mRNA [NM_001721]	1.073
35	BST2	bone marrow stromal cell antigen 2 [Source:HGNC Symbol;Acc:1119] [ENST00000252593]	1.7219
36	BST2	bone marrow stromal cell antigen 2 (BST2), mRNA [NM_004335]	1.7037
37	BTBD16	BTB (POZ) domain containing 16 (BTBD16), mRNA [NM_144587]	1.3688
38	BTN1A1	butyrophilin, subfamily 1, member A1 (BTN1A1), mRNA [NM_001732]	1.1253
39	C11orf52	chromosome 11 open reading frame 52 (C11orf52), mRNA [NM_080659]	1.1153
40	C11orf63	chromosome 11 open reading frame 63 (C11orf63), transcript variant 2, mRNA [NM_199124]	1.1024
41	C11orf94	chromosome 11 open reading frame 94 (C11orf94), mRNA [NM_001080446]	1.3846
42	C12orf48	chromosome 12 open reading frame 48 (C12orf48), mRNA [NM_017915]	1.1318
43	C14orf99	DKFZp434E1423_r1 434 (synonym: htes3) Homo sapiens cDNA clone DKFZp434E1423 5', mRNA sequence [AL043142]	1.307
44	C16orf72	PRO0149 protein, mRNA (cDNA clone IMAGE:5172419), containing frame-shift errors. [BC029878]	1.1716
45	C17orf66	chromosome 17 open reading frame 66 (C17orf66), mRNA [NM_152781]	1.7572
46	C17orf67	chromosome 17 open reading frame 67 (C17orf67), mRNA [NM_001085430]	1.5608
47	C17orf87	chromosome 17 open reading frame 87 (C17orf87), mRNA [NM_207103]	1.2825
48	C1orf114	chromosome 1 open reading frame 114 (C1orf114), mRNA [NM_021179]	1.0028
49	C1orf145	chromosome 1 open reading frame 145, mRNA (cDNA clone IMAGE:5204063). [BC027909]	1.0952
50	C1orf227	chromosome 1 open reading frame 227 (C1orf227), mRNA [NM_001024601]	1.1483
51	C20orf26	chromosome 20 open reading frame 26 (C20orf26), transcript variant 1, mRNA [NM_015585]	3.3228
52	C21orf122	chromosome 21 open reading frame 122 (C21orf122), non-coding RNA [NR_027292]	1.6087
53	C21orf30	chromosome 21 open reading frame 30 (C21orf30), miscRNA [XR_109680]	1.067
54	C21orf67	chromosome 21 open reading frame 67 (C21orf67), transcript variant 2, non-coding RNA [NR_027129]	1.5573
55	C22orf31	chromosome 22 open reading frame 31 (C22orf31), mRNA [NM_015370]	1.0184
56	C2orf15	chromosome 2 open reading frame 15 (C2orf15), mRNA [NM_144706]	1.3748
57	C2orf55	chromosome 2 open reading frame 55 (C2orf55), mRNA [NM_207362]	1.4787
58	C3orf72	chromosome 3 open reading frame 72 (C3orf72), mRNA [NM_001040061]	1.1593
59	C4orf19	chromosome 4 open reading frame 19 (C4orf19), transcript variant 2, mRNA [NM_018302]	1.2807
60	C5orf47	chromosome 5 open reading frame 47 (C5orf47), mRNA [NM_001144954]	1.2315
61	C6orf163	chromosome 6 open reading frame 163 (C6orf163), mRNA [NM_001010868]	1.1059
62	C6orf168	chromosome 6 open reading frame 168 (C6orf168), mRNA [NM_032511]	1.1
63	C6orf176	chromosome 6 open reading frame 176 (C6orf176), transcript variant 1, non-coding RNA [NR_026860]	2.5021
64	C7orf29	chromosome 7 open reading frame 29 (C7orf29), mRNA [NM_138434]	1.6063
65	C7orf58	chromosome 7 open reading frame 58 (C7orf58), transcript variant 2, mRNA [NM_001105533]	1.9191
66	C8orf47	chromosome 8 open reading frame 47 (C8orf47), transcript variant 1, mRNA [NM_173549]	1.4737
67	C8orf48	chromosome 8 open reading frame 48 (C8orf48), mRNA [NM_001007090]	1.2695
68	C9orf47	chromosome 9 open reading frame 47 (C9orf47), transcript variant 1, mRNA [NM_001001938]	1.0711
69	C9orf93	chromosome 9 open reading frame 93 (C9orf93), mRNA [NM_173550]	1.0598
70	CA8	carbonic anhydrase VIII (CA8), mRNA [NM_004056]	1.6384
71	CACNA1B	calcium channel, voltage-dependent, N type, alpha 1B subunit (CACNA1B), mRNA [NM_000718]	1.2878
72	CACNA1G	calcium channel, voltage-dependent, T type, alpha 1G subunit (CACNA1G), transcript variant 1, mRNA [NM_018896]	1.0005
73	CACNB2	calcium channel, voltage-dependent, beta 2 subunit (CACNB2), transcript variant 1, mRNA [NM_000724]	1.6042
74	CAPS2	calcyphosine 2 [Source:HGNC Symbol;Acc:16471] [ENST00000328705]	1.1704
75	CCDC102B	coiled-coil domain containing 102B (CCDC102B), transcript variant 2, mRNA [NM_024781]	1.2885
76	CCDC103	coiled-coil domain containing 103 (CCDC103), mRNA [NM_213607]	1.2721
77	CCDC74B	coiled-coil domain containing 74B (CCDC74B), mRNA [NM_207310]	1.0344
78	CD244	CD244 molecule, natural killer cell receptor 2B4 (CD244), transcript variant 2, mRNA [NM_001166663]	1.0726
79	CD34	CD34 molecule (CD34), transcript variant 1, mRNA [NM_001025109]	1.3258
80	CD79B	CD79b molecule, immunoglobulin-associated beta (CD79B), transcript variant 3, mRNA [NM_001039933]	1.1907
81	CDH12	cadherin 12, type 2 (N-cadherin 2) (CDH12), mRNA [NM_004061]	1.426
82	CDH16	cadherin 16, KSP-cadherin (CDH16), transcript variant 1, mRNA [NM_004062]	1.9508
83	CDHR4	cadherin-related family member 4 (CDHR4), mRNA [NM_001007540]	1.1876
84	CDRT8	qf35h11.x1 Soares_testis_NHT Homo sapiens cDNA clone IMAGE:1752069 3', mRNA sequence [AI150443]	1.3542
85	CERS1	ceramide synthase 1 (CERS1), transcript variant 2, mRNA [NM_198207]	1.7669
86	CFD	complement factor D (adipsin) (CFD), mRNA [NM_001928]	1.1399
87	CHDH	choline dehydrogenase (CHDH), nuclear gene encoding mitochondrial protein, mRNA [NM_018397]	2.1434
88	CHRM3	cholinergic receptor, muscarinic 3 (CHRM3), mRNA [NM_000740]	1.036
89	CHRNA5	cholinergic receptor, nicotinic, alpha 5 (CHRNA5), mRNA [NM_000745]	1.0233
90	CLEC3B	C-type lectin domain family 3, member B (CLEC3B), mRNA [NM_003278]	1.0833
91	CLIC2	chloride intracellular channel 2 (CLIC2), mRNA [NM_001289]	2.0931
92	CLU	clusterin (CLU), transcript variant 2, mRNA [NM_203339]	1.6583
93	CNTF	ciliary neurotrophic factor (CNTF), mRNA [NM_000614]	1.9712
94	COBL	cordon-bleu homolog (mouse) (COBL), mRNA [NM_015198]	1.1965
95	COL17A1	collagen, type XVII, alpha 1 (COL17A1), mRNA [NM_000494]	1.0878
96	COL24A1	collagen, type XXIV, alpha 1 (COL24A1), mRNA [NM_152890]	1.0277

97	COL28A1	collagen, type XXVIII, alpha 1 (COL28A1), mRNA [NM_001037763]	1.256
98	COL9A3	collagen, type IX, alpha 3 (COL9A3), mRNA [NM_001853]	1.0164
99	CPNE6	copine VI (neuronal) (CPNE6), mRNA [NM_006032]	1.0193
100	CRYAB	crystallin, alpha B (CRYAB), mRNA [NM_001885]	3.9905
101	CSAG2	CSAG family, member 2 (CSAG2), transcript variant 2, mRNA [NM_004909]	1.5081
102	CSRP2	cysteine and glycine-rich protein 2 (CSRP2), mRNA [NM_001321]	1.3744
103	CTAG1A	cancer/testis antigen 1A (CTAG1A), mRNA [NM_139250]	2.2689
104	CTGF	connective tissue growth factor (CTGF), mRNA [NM_001901]	1.7568
105	CTSC	cathepsin C (CTSC), transcript variant 2, mRNA [NM_148170]	2.1563
106	CTSC	cathepsin C (CTSC), transcript variant 3, mRNA [NM_001114173]	1.988
107	CTSC	cathepsin C (CTSC), transcript variant 1, mRNA [NM_001814]	1.1744
108	CUBN	cubilin (intrinsic factor-cobalamin receptor) (CUBN), mRNA [NM_001081]	1.6321
109	CYP2S1	cytochrome P450, family 2, subfamily S, polypeptide 1 (CYP2S1), mRNA [NM_030622]	1.3566
110	CYP4B1	cytochrome P450, family 4, subfamily B, polypeptide 1 (CYP4B1), transcript variant 2, mRNA [NM_000779]	2.2272
111	CYP4F2	cytochrome P450, family 4, subfamily F, polypeptide 2 (CYP4F2), mRNA [NM_001082]	1.2546
112	DBP	D site of albumin promoter (albumin D-box) binding protein (DBP), mRNA [NM_001352]	1.1632
113	DBX1	developing brain homeobox 1 (DBX1), mRNA [NM_001029865]	1.136
114	DDC	dopa decarboxylase (aromatic L-amino acid decarboxylase) (DDC), transcript variant 2, mRNA [NM_000790]	1.6849
115	DDR2	discoidin domain receptor tyrosine kinase 2 (DDR2), transcript variant 1, mRNA [NM_001014796]	1.2308
116	DDX43	DEAD (Asp-Glu-Ala-Asp) box polypeptide 43 (DDX43), mRNA [NM_018665]	1.8544
117	DEPDC7	DEP domain containing 7 (DEPDC7), transcript variant 2, mRNA [NM_139160]	1.2448
118	DERL3	Der1-like domain family, member 3 (DERL3), transcript variant 3, mRNA [NM_198440]	1.4754
119	DISC1	disrupted in schizophrenia 1 (DISC1), transcript variant L, mRNA [NM_018662]	2.1215
120	DKK1	dickkopf homolog 1 (Xenopus laevis) (DKK1), mRNA [NM_012242]	1.4473
121	DLEU2L	deleted in lymphocytic leukemia 2-like (DLEU2L), non-coding RNA [NR_002771]	1.2398
122	DLG2	discs, large homolog 2 (Drosophila) (DLG2), transcript variant 3, mRNA [NM_001142700]	1.5745
123	DLX4	distal-less homeobox 4 (DLX4), transcript variant 1, mRNA [NM_138281]	1.3804
124	DNAH3	dynein, axonemal, heavy chain 3 [Source:HGNC Symbol;Acc:2949] [ENST00000396036]	1.5447
125	DOK2	docking protein 2, 56kDa (DOK2), mRNA [NM_003974]	1.1463
126	DPRX	divergent-paired related homeobox (DPRX), mRNA [NM_001012728]	1.1221
127	DPYD	dihydropyrimidine dehydrogenase (DPYD), transcript variant 2, mRNA [NM_001160301]	1.0814
128	EEPD1	endonuclease/exonuclease/phosphatase family domain containing 1 (EEPD1), mRNA [NM_030636]	1.2676
129	EFHB	EF-hand domain family, member B (EFHB), mRNA [NM_144715]	1.1973
130	EHD3	EH-domain containing 3 (EHD3), mRNA [NM_014600]	1.3535
131	ENHO	energy homeostasis associated (ENHO), mRNA [NM_198573]	1.0132
132	EPCAM	epithelial cell adhesion molecule (EPCAM), mRNA [NM_002354]	2.223
133	EPHB1	EPH receptor B1 [Source:HGNC Symbol;Acc:3392] [ENST00000467013]	1.1353
134	EPHB6	EPH receptor B6 (EPHB6), mRNA [NM_004445]	1.2232
135	EPN3	epsin 3 (EPN3), mRNA [NM_017957]	1.2947
136	ERI2	ERI1 exoribonuclease family member 2 (ERI2), transcript variant 2, mRNA [NM_080663]	1.4384
137	ETNK2	ethanolamine kinase 2 (ETNK2), mRNA [NM_018208]	1.2831
138	FABP6	fatty acid binding protein 6, ileal (FABP6), transcript variant 1, mRNA [NM_001040442]	1.6368
139	FAM101B	family with sequence similarity 101, member B (FAM101B), mRNA [NM_182705]	1.6811
140	FAM198B	family with sequence similarity 198, member B (FAM198B), transcript variant 2, mRNA [NM_016613]	2.1462
141	FAM59B	family with sequence similarity 59, member B (FAM59B), transcript variant 2, mRNA [NM_001191033]	1.3247
142	FAM83D	family with sequence similarity 83, member D (FAM83D), mRNA [NM_030919]	1.2732
143	FAM95B1	family with sequence similarity 95, member B1 (FAM95B1), non-coding RNA [NR_026759]	1.0091
144	FANK1	fibronectin type III and ankyrin repeat domains 1 (FANK1), mRNA [NM_145235]	1.272
145	FBXL13	F-box and leucine-rich repeat protein 13 (FBXL13), transcript variant 1, mRNA [NM_145032]	1.0159
146	FBXO15	F-box protein 15 (FBXO15), transcript variant 1, mRNA [NM_152676]	1.1155
147	FETUB	fetuin B (FETUB), mRNA [NM_014375]	1.394
148	FIGNL1	fidgetin-like 1 (FIGNL1), transcript variant 1, mRNA [NM_001042762]	1.6803
149	FIGNL2	fidgetin-like 2 (FIGNL2), mRNA [NM_001013690]	1.1039
150	FLJ41278	hypothetical LOC400046 (FLJ41278), non-coding RNA [NR_033988]	1.774
151	FLJ41484	hypothetical LOC650669 (FLJ41484), miscRNA [XR_110591]	1.8397
152	FLJ44674	cDNA FLJ44674 fis, clone BRACE3007649. [AK128747]	1.4279
153	FOXL2	forkhead box L2 (FOXL2), mRNA [NM_023067]	3.5417
154	FRG2C	FSHD region gene 2 family, member C (FRG2C), mRNA [NM_001124759]	1.3321
155	FSIP2	fibrous sheath interacting protein 2 (FSIP2), mRNA [NM_173651]	1.3406
156	GABRB3	gamma-aminobutyric acid (GABA) A receptor, beta 3 (GABRB3), transcript variant 1, mRNA [NM_000814]	1.1308
157	GABRE	gamma-aminobutyric acid (GABA) A receptor, epsilon (GABRE), mRNA [NM_004961]	1.6774
158	GAGE7	G antigen 7 (GAGE7), mRNA [NM_021123]	3.9155
159	GAL	galanin prepropeptide (GAL), mRNA [NM_015973]	1.734
160	GARNL3	GTPase activating Rap/RanGAP domain-like 3 (GARNL3), mRNA [NM_032293]	1.0547
161	GCGR	glucagon receptor (GCGR), mRNA [NM_000160]	3.6096
162	GDA	guanine deaminase (GDA), transcript variant 2, mRNA [NM_004293]	2.1525
163	GEMIN8P4	gem (nuclear organelle) associated protein 8 pseudogene 4 (GEMIN8P4), non-coding RNA [NR_002830]	1.0149
164	GLRA2	glycine receptor, alpha 2 (GLRA2), transcript variant 3, mRNA [NM_001118886]	1.7793
165	GMPR	guanosine monophosphate reductase (GMPR), mRNA [NM_006877]	1.4276
166	GPER	G protein-coupled estrogen receptor 1 (GPER), transcript variant 3, mRNA [NM_001039966]	1.2527

167	GPM6B	glycoprotein M6B (GPM6B), transcript variant 1, mRNA [NM_001001995]	1.0126
168	GPR87	G protein-coupled receptor 87 (GPR87), mRNA [NM_023915]	1.0205
169	GRIN2C	glutamate receptor, ionotropic, N-methyl D-aspartate 2C (GRIN2C), mRNA [NM_000835]	1.4883
170	GRPR	gastrin-releasing peptide receptor (GRPR), mRNA [NM_005314]	1.0999
171	GSG2	germ cell associated 2 (haspin) (GSG2), mRNA [NM_031965]	1.3698
172	GSTM3	glutathione S-transferase mu 3 (brain) (GSTM3), transcript variant 1, mRNA [NM_000849]	1.3756
173	GTF2IRD2B	GTF2I repeat domain containing 2B [Source:HGNC Symbol;Acc:33125] [ENST00000394939]	1.4904
174	H2AFB2	H2A histone family, member B2 (H2AFB2), mRNA [NM_001017991]	1.267
175	HILS1	histone linker H1 domain, spermatid-specific 1 (HILS1), transcript variant 1, non-coding RNA [NR_024193]	1.1011
176	HIST1H1B	histone cluster 1, H1b (HIST1H1B), mRNA [NM_005322]	1.272
177	HIST1H2AI	histone cluster 1, H2ai (HIST1H2AI), mRNA [NM_003509]	1.1334
178	HIST2H3A	histone cluster 2, H3a (HIST2H3A), mRNA [NM_001005464]	1.058
179	HIST4H4	histone cluster 4, H4 (HIST4H4), mRNA [NM_175054]	1.17
180	HIVEP3	human immunodeficiency virus type I enhancer binding protein 3 [Source:HGNC Symbol;Acc:13561] [ENST00000372583]	1.188
181	HNMT	histamine N-methyltransferase (HNMT), transcript variant 2, mRNA [NM_001024074]	2.1113
182	HOPX	HOP homeobox (HOPX), transcript variant 2, mRNA [NM_139211]	2.3103
183	HOXC10	homeobox C10 (HOXC10), mRNA [NM_017409]	2.0229
184	HS2ST1	heparan sulfate 2-O-sulfotransferase 1 (HS2ST1), transcript variant 2, mRNA [NM_001134492]	1.1426
185	HS6ST3	heparan sulfate 6-O-sulfotransferase 3 (HS6ST3), mRNA [NM_153456]	1.1749
186	HSPA4L	heat shock 70kDa protein 4-like (HSPA4L), mRNA [NM_014278]	1.3655
187	HSPB8	heat shock 22kDa protein 8 (HSPB8), mRNA [NM_014365]	1.0268
188	ICA1L	islet cell autoantigen 1,69kDa-like (ICA1L), transcript variant 2, mRNA [NM_178231]	1.287
189	IGDCC4	immunoglobulin superfamily, DCC subclass, member 4 (IGDCC4), mRNA [NM_020962]	1.7089
190	IGFBP7	insulin-like growth factor binding protein 7 (IGFBP7), mRNA [NM_001553]	1.4807
191	IGKV1D-13	602502772F1 NIH_MGC_77 Homo sapiens cDNA clone IMAGE:4616320 5', mRNA sequence [BG482625]	1.0794
192	IGSF23	immunoglobulin superfamily, member 23 (IGSF23), mRNA [NM_001205280]	1.4168
193	IKZF1	IKAROS family zinc finger 1 (Ikaros) [Source:HGNC Symbol;Acc:13176] [ENST00000484847]	1.3234
194	IL7R	interleukin 7 receptor (IL7R), mRNA [NM_002185]	1.1273
195	INPP5D	inositol polyphosphate-5-phosphatase, 145kDa (INPP5D), transcript variant 1, mRNA [NM_001017915]	1.1371
196	JPH2	junctophilin 2 (JPH2), transcript variant 1, mRNA [NM_020433]	1.5024
197	KBTBD11	kelch repeat and BTB (POZ) domain containing 11 (KBTBD11), mRNA [NM_014867]	1.0507
198	KCNB1	potassium voltage-gated channel, Shab-related subfamily, member 1 (KCNB1), mRNA [NM_004975]	1.4529
199	KCNE3	potassium voltage-gated channel, Isk-related family, member 3 (KCNE3), mRNA [NM_005472]	1.9746
200	KCNK2	potassium channel, subfamily K, member 2 (KCNK2), transcript variant 1, mRNA [NM_001017424]	1.6079
201	KIAA0125	KIAA0125 (KIAA0125), non-coding RNA [NR_026800]	1.4875
202	KIAA1432	KIAA1432 (KIAA1432), transcript variant 2, mRNA [NM_001135920]	1.1089
203	KIAA1908	hypothetical LOC114796 (KIAA1908), transcript variant 1, non-coding RNA [NR_027329]	1.252
204	KIF26B	kinesin family member 26B (KIF26B), mRNA [NM_018012]	1.3921
205	KIF7	kinesin family member 7 (KIF7), mRNA [NM_198525]	1.941
206	KLHL3	kelch-like 3 (Drosophila) (KLHL3), mRNA [NM_017415]	2.7773
207	KLK3	kallikrein-related peptidase 3 (KLK3), transcript variant 6, mRNA [NM_001030050]	1.122
208	KREMEN2	kringle containing transmembrane protein 2 (KREMEN2), transcript variant 4, mRNA [NM_172229]	1.2937
209	KRT222	keratin 222 (KRT222), mRNA [NM_152349]	2.8797
210	KRTAP10-4	keratin associated protein 10-4 (KRTAP10-4), mRNA [NM_198687]	1.3705
211	KRTAP2-4	keratin associated protein 2-4 (KRTAP2-4), mRNA [NM_033184]	1.2737
212	KRTAP25-1	keratin associated protein 25-1 (KRTAP25-1), mRNA [NM_001128598]	1.0212
213	LAMA4	laminin, alpha 4 (LAMA4), transcript variant 5, mRNA [NM_001105209]	1.7733
214	LAMA4	laminin, alpha 4 (LAMA4), transcript variant 3, mRNA [NM_001105207]	1.2823
215	LIMCH1	LIM and calponin homology domains 1 (LIMCH1), transcript variant 1, mRNA [NM_014988]	1.0141
216	LMX1A	LIM homeobox transcription factor 1, alpha (LMX1A), transcript variant 1, mRNA [NM_177398]	1.4217
217	LOC100128064	hypothetical protein LOC100128064 (LOC100128064), mRNA [XM_001725877]	1.9919
218	LOC100128105	hypothetical protein LOC100128105 (LOC100128105), mRNA [XM_001721678]	1.1796
219	LOC100128881	hypothetical LOC100128881 (LOC100128881), non-coding RNA [NR_036480]	1.0856
220	LOC100129186	cDNA FLJ46336 fis, clone TESTI4046090. [AK128204]	1.7529
221	LOC100130938	hypothetical LOC100130938 (LOC100130938), partial miscRNA [XR_110148]	1.5105
222	LOC100131262	cDNA FLJ35102 fis, clone PLACE6006474, weakly similar to ADHESIVE PLAQUE MATRIX PROTEIN PRECURSOR. [AK092421]	1.247
223	LOC100131366	hypothetical LOC100131366 (LOC100131366), non-coding RNA [NR_033938]	1.1072
224	LOC100132529	hypothetical LOC100132529 (LOC100132529), partial miscRNA [XR_109319]	1.0175
225	LOC100133299	GALI1870 (LOC100133299), miscRNA [XR_108564]	1.5796
226	LOC100133669	hypothetical LOC100133669 (LOC100133669), non-coding RNA [NR_026913]	1.2237
227	LOC100507055	hypothetical LOC100507055 (LOC100507055), mRNA [NM_001195520]	1.4769
228	LOC284009	cDNA FLJ36671 fis, clone UTERU2004039. [AK093990]	1.5166
229	LOC348761	hypothetical LOC348761 (LOC348761), non-coding RNA [NR_033879]	1.0397
230	LOC349196	hypothetical LOC349196 (LOC349196), non-coding RNA [NR_027000]	1.3222
231	LOC440335	hypothetical LOC440335 (LOC440335), transcript variant 2, non-coding RNA [NR_029454]	1.2969
232	LOC441666	zinc finger protein 91 pseudogene (LOC441666), non-coding RNA [NR_024380]	1.2752
233	LOC553103	hypothetical LOC553103 (LOC553103), non-coding RNA [NR_037898]	1.0857
234	LOC613126	omo sapiens hypothetical LOC613126 (LOC613126), miscRNA [XR_108845]	1.1671
235	LOC642366	hypothetical LOC642366 (LOC642366), miscRNA [XR_108597]	1.1357
236	LOC644662	hypothetical protein LOC644662, transcript variant 2 (LOC644662), mRNA [XM_933903]	1.7908

237	LOC728093	putative POM121-like protein 1-like (LOC728093), mRNA [XM_003119959]	1.2274
238	LOC728660	full-length cDNA clone CS0DI026YN15 of Placenta Cot 25-normalized of Homo sapiens (human). [CR622587]	1.2613
239	LOC728723	hypothetical LOC728723 (LOC728723), non-coding RNA [NR_024398]	1.0368
240	LOC729444	hypothetical LOC729444 (LOC729444), non-coding RNA [NR_038388]	1.2875
241	LOC730091	hypothetical LOC730091 (LOC730091), non-coding RNA [NR_038387]	2.5936
242	LOC731779	hypothetical LOC731779 (LOC731779), non-coding RNA [NR_024441]	2.0274
243	LPHN2	latrophilin 2 (LPHN2), mRNA [NM_012302]	1.2513
244	LRG1	leucine-rich alpha-2-glycoprotein 1 (LRG1), mRNA [NM_052972]	1.334
245	LRRC33	leucine rich repeat containing 33 (LRRC33), mRNA [NM_198565]	1.1249
246	LRRTM4	leucine rich repeat transmembrane neuronal 4 (LRRTM4), transcript variant 1, mRNA [NM_001134745]	1.1771
247	LY6D	lymphocyte antigen 6 complex, locus D (LY6D), mRNA [NM_003695]	1.2131
248	LYNX1	Ly6/neurotoxin 1 (LYNX1), transcript variant 1, mRNA [NM_023946]	1.0573
249	LYPD1	LY6/PLAUR domain containing 1 (LYPD1), transcript variant 1, mRNA [NM_144586]	1.4379
250	LYPD6	LY6/PLAUR domain containing 6 (LYPD6), transcript variant 1, mRNA [NM_001195685]	1.4835
251	LYZL1	lysozyme-like 1 (LYZL1), mRNA [NM_032517]	1.3731
252	MAGEA12	melanoma antigen family A, 12 (MAGEA12), transcript variant 3, mRNA [NM_005367]	1.908
253	MAGEA2B	melanoma antigen family A, 2B (MAGEA2B), mRNA [NM_153488]	2.4718
254	MAGEA6	melanoma antigen family A, 6 (MAGEA6), transcript variant 2, mRNA [NM_175868]	3.0677
255	MAP3K13	mitogen-activated protein kinase kinase kinase 13 (MAP3K13), transcript variant 1, mRNA [NM_004721]	1.0027
256	MAT1A	methionine adenosyltransferase I, alpha (MAT1A), mRNA [NM_000429]	1.0131
257	MDH1B	malate dehydrogenase 1B, NAD (soluble) (MDH1B), mRNA [NM_001039845]	1.0335
258	MECOM	MDS1 and EVI1 complex locus (MECOM), transcript variant 6, mRNA [NM_001164000]	1.7847
259	METAP1D	methionyl aminopeptidase type 1D (mitochondrial) (METAP1D), nuclear gene encoding mitochondrial protein, mRNA [NM_199227]	1.5351
260	METTL7A	methyltransferase like 7A (METTL7A), mRNA [NM_014033]	1.4362
261	MGAT4A	mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isozyme A (MGAT4A), transcript variant 1, mRNA [NM_012214]	1.2366
262	MGC4294	hypothetical MGC4294 (MGC4294), miscRNA [XR_109628]	1.0611
263	MGP	matrix Gla protein (MGP), transcript variant 1, mRNA [NM_001190839]	2.3379
264	MGP	matrix Gla protein (MGP), transcript variant 2, mRNA [NM_000900]	1.8313
265	MKS1	Meckel syndrome, type 1 (MKS1), transcript variant 1, mRNA [NM_017777]	1.4286
266	MOSC1	MOCO sulphurase C-terminal domain containing 1 (MOSC1), nuclear gene encoding mitochondrial protein, mRNA [NM_022746]	1.1159
267	MST1R	macrophage stimulating 1 receptor (c-met-related tyrosine kinase) (MST1R), mRNA [NM_002447]	1.226
268	MUC1	mucin 1, cell surface associated (MUC1), transcript variant 1, mRNA [NM_002456]	1.2292
269	MYADML	myeloid-associated differentiation marker-like (MYADML), non-coding RNA [NR_003143]	1.068
270	MYBPC1	myosin binding protein C, slow type (MYBPC1), transcript variant 2, mRNA [NM_206819]	1.8281
271	MYH2	myosin, heavy chain 2, skeletal muscle, adult (MYH2), transcript variant 1, mRNA [NM_017534]	1.1849
272	MYO5C	myosin VC (MYO5C), mRNA [NM_018728]	1.3579
273	NACAD	NAC alpha domain containing (NACAD), mRNA [NM_001146334]	3.0396
274	NCAPG	non-SMC condensin I complex, subunit G (NCAPG), mRNA [NM_022346]	1.4483
275	NCRNA00168	non-protein coding RNA 168 (NCRNA00168), non-coding RNA [NR_033387]	1.4317
276	NCRNA00246A	non-protein coding RNA 246A (NCRNA00246A), non-coding RNA [NR_026595]	1.1767
277	NETO1	neuropilin (NRP) and tolloid (TLL)-like 1 (NETO1), transcript variant 3, mRNA [NM_138966]	1.2557
278	NIPSNAP3B	nipsnap homolog 3B (C. elegans) (NIPSNAP3B), mRNA [NM_018376]	1.2054
279	NLRP5	NLR family, pyrin domain containing 5 (NLRP5), mRNA [NM_153447]	1.0223
280	NME5	non-metastatic cells 5, protein expressed in (nucleoside-diphosphate kinase) (NME5), mRNA [NM_003551]	1.4755
281	NOXO1	NADPH oxidase organizer 1 (NOXO1), transcript variant c, mRNA [NM_172168]	1.2251
282	NR0B1	nuclear receptor subfamily 0, group B, member 1 (NR0B1), mRNA [NM_000475]	1.217
283	NR2F1	nuclear receptor subfamily 2, group F, member 1 (NR2F1), mRNA [NM_005654]	1.029
284	NRG2	neuregulin 2 (NRG2), transcript variant 3, mRNA [NM_013982]	1.5629
285	NRG4	neuregulin 4 (NRG4), mRNA [NM_138573]	1.268
286	NRGN	neurogranin (protein kinase C substrate, RC3) (NRGN), transcript variant 1, mRNA [NM_006176]	1.3089
287	NXN	nucleoredoxin (NXN), transcript variant 1, mRNA [NM_022463]	2.3746
288	OAS1	2',5'-oligoadenylate synthetase 1, 40/46kDa (OAS1), transcript variant 2, mRNA [NM_002534]	1.759
289	OGDHL	oxoglutarate dehydrogenase-like (OGDHL), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA [NM_018245]	1.6803
290	OLFM1	olfactomedin 1, mRNA (cDNA clone IMAGE:3351052), complete cds. [BC000189]	1.08
291	OPN1SW	opsin 1 (cone pigments), short-wave-sensitive (OPN1SW), mRNA [NM_001708]	1.1827
292	OR1E1	olfactory receptor, family 1, subfamily E, member 1 (OR1E1), mRNA [NM_003553]	1.3111
293	OR1Q1	olfactory receptor, family 1, subfamily Q, member 1 (OR1Q1), mRNA [NM_012364]	1.1746
294	OR2Z1	olfactory receptor, family 2, subfamily Z, member 1 (OR2Z1), mRNA [NM_001004699]	1.1564
295	OR51I1	olfactory receptor, family 51, subfamily I, member 1 (OR51I1), mRNA [NM_001005288]	1.2017
296	OR52A1	olfactory receptor, family 52, subfamily A, member 1 (OR52A1), mRNA [NM_012375]	1.1171
297	OR56B4	olfactory receptor, family 56, subfamily B, member 4 (OR56B4), mRNA [NM_001005181]	1.4882
298	OR5M1	olfactory receptor, family 5, subfamily M, member 1 (OR5M1), mRNA [NM_001004740]	1.1199
299	OSM	oncostatin M (OSM), mRNA [NM_020530]	1.519
300	P2RX6	purinergic receptor P2X, ligand-gated ion channel, 6 (P2RX6), transcript variant 1, mRNA [NM_005446]	2.3677
301	PAPPA	pregnancy-associated plasma protein A, pappalysin 1 (PAPPA), mRNA [NM_002581]	1.0277
302	PBX1	pre-B-cell leukemia homeobox 1 (PBX1), transcript variant 1, mRNA [NM_002585]	1.7266

303	PCDHB4	protocadherin beta 4 (PCDHB4), mRNA [NM_018938]	1.4772
304	PCDHGB4	protocadherin gamma subfamily B, 4 (PCDHGB4), transcript variant 2, mRNA [NM_032098]	2.1461
305	PCSK6	proprotein convertase subtilisin/kexin type 6 (PCSK6), transcript variant 3, mRNA [NM_138322]	1.6663
306	PDE11A	phosphodiesterase 11A (PDE11A), transcript variant 2, mRNA [NM_001077358]	1.6617
307	PDE6A	phosphodiesterase 6A, cGMP-specific, rod, alpha (PDE6A), mRNA [NM_000440]	1.175
308	PDE7A	phosphodiesterase 7A (PDE7A), transcript variant 3, mRNA [NM_001242318]	1.6217
309	PDE7B	phosphodiesterase 7B (PDE7B), mRNA [NM_018945]	2.0794
310	PDE9A	phosphodiesterase 9A (PDE9A), transcript variant 1, mRNA [NM_002606]	1.4219
311	PECAM1	platelet/endothelial cell adhesion molecule (PECAM1), mRNA [NM_000442]	1.0354
312	PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), transcript variant 1, mRNA [NM_004566]	1.0613
313	PHYHIP	phytanoyl-CoA 2-hydroxylase interacting protein (PHYHIP), transcript variant 2, mRNA [NM_014759]	1.087
314	PI15	peptidase inhibitor 15 (PI15), mRNA [NM_015886]	1.2995
315	PITX2	paired-like homeodomain 2 (PITX2), transcript variant 2, mRNA [NM_153426]	1.4133
316	PLA2G1B	phospholipase A2, group IB (pancreas) (PLA2G1B), mRNA [NM_000928]	1.0109
317	PLAC8	placenta-specific 8 (PLAC8), transcript variant 2, mRNA [NM_016619]	1.6241
318	PLCL2	phospholipase C-like 2 (PLCL2), transcript variant 2, mRNA [NM_015184]	1.063
319	PLCXD3	phosphatidylinositol-specific phospholipase C, X domain containing 3 (PLCXD3), mRNA [NM_001005473]	1.4936
320	PLD4	phospholipase D family, member 4 (PLD4), mRNA [NM_138790]	1.9778
321	PLEKHA6	pleckstrin homology domain containing, family A member 6 (PLEKHA6), mRNA [NM_014935]	1.5755
322	PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2), transcript variant 1, mRNA [NM_182943]	1.5973
323	PLXDC2	plexin domain containing 2 (PLXDC2), mRNA [NM_032812]	1.1297
324	PPP4R1L	protein phosphatase 4, regulatory subunit 1-like (PPP4R1L), non-coding RNA [NR_003505]	1.0602
325	PRKACB	se, cAMP-dependent, catalytic, beta [Source:HGNC Symbol;Acc:9381] [ENST00000370684]	1.4925
326	PROM2	prominin 2 (PROM2), transcript variant 1, mRNA [NM_001165978]	2.9707
327	PROM2	prominin 2 (PROM2), transcript variant 3, mRNA [NM_144707]	2.6719
328	PRTFDC1	phosphoribosyl transferase domain containing 1 [Source:HGNC Symbol;Acc:23333] [ENST00000376376]	1.0997
329	PRY2	PTPN13-like, Y-linked 2 (PRY2), mRNA [NM_001002758]	1.2567
330	PSMG4	proteasome (prosome, macropain) assembly chaperone 4 (PSMG4), transcript variant 3, mRNA [NM_001135750]	2.2986
331	PTGER2	prostaglandin E receptor 2 (subtype EP2), 53kDa (PTGER2), mRNA [NM_000956]	1.2091
332	PXMP2	peroxisomal membrane protein 2, 22kDa, mRNA (cDNA clone IMAGE:4098463), complete cds. [BC009836]	1.1397
333	QPCT	glutaminyl-peptide cyclotransferase (QPCT), mRNA [NM_012413]	1.494
334	RAB15	RAB15, member RAS onocogene family (RAB15), mRNA [NM_198686]	1.0879
335	RAMP1	receptor (G protein-coupled) activity modifying protein 1 (RAMP1), mRNA [NM_005855]	1.7177
336	RBP5	retinol binding protein 5, cellular (RBP5), mRNA [NM_031491]	1.1496
337	RBPMS2	RNA binding protein with multiple splicing 2 (RBPMS2), mRNA [NM_194272]	2.4941
338	RGAG4	retrotransposon gag domain containing 4 (RGAG4), mRNA [NM_001024455]	1.0275
339	RGPD1	RANBP2-like and GRIP domain containing 1 (RGPD1), mRNA [NM_001024457]	3.1371
340	RNU105C	RNA, U105C small nucleolar (RNU105C), small nucleolar RNA [NR_004385]	1.1815
341	ROPN1	rhophilin associated tail protein 1 (ROPN1), mRNA [NM_017578]	1.4576
342	RPL39L	ribosomal protein L39-like (RPL39L), mRNA [NM_052969]	1.853
343	RREB1	ras responsive element binding protein 1 (RREB1), transcript variant 4, mRNA [NM_001003700]	1.9271
344	RSPO2	R-spondin 2 (RSPO2), mRNA [NM_178565]	1.5158
345	RUNX1T1	runt-related transcription factor 1; translocated to, 1 (cyclin D-related) (RUNX1T1), transcript variant 1, mRNA [NM_004349]	1.7327
346	RYR2	ryanodine receptor 2 (cardiac) (RYR2), mRNA [NM_001035]	1.7639
347	S100A4	S100 calcium binding protein A4 (S100A4), transcript variant 1, mRNA [NM_002961]	1.3
348	S100B	S100 calcium binding protein B (S100B), mRNA [NM_006272]	1.4891
349	S1PR3	sphingosine-1-phosphate receptor 3 (S1PR3), mRNA [NM_005226]	1.3006
350	SALL2	sal-like 2 (Drosophila) (SALL2), mRNA [NM_005407]	2.7112
351	SAMD13	sterile alpha motif domain containing 13 (SAMD13), transcript variant 1, mRNA [NM_001010971]	1.0601
352	SCG2	secretogranin II (SCG2), mRNA [NM_003469]	1.6996
353	SCRG1	stimulator of chondrogenesis 1 (SCRG1), mRNA [NM_007281]	1.1465
354	SCUBE1	signal peptide, CUB domain, EGF-like 1 [Source:HGNC Symbol;Acc:13441] [ENST00000290460]	1.0465
355	SDPR	serum deprivation response (SDPR), mRNA [NM_004657]	1.5546
356	SEC14L3	SEC14-like 3 (S. cerevisiae) [Source:HGNC Symbol;Acc:18655] [ENST00000415957]	1.2758
357	SEPT6	septin 6 (SEPT6), transcript variant V, mRNA [NM_145802]	1.5534
358	SERINC4	serine incorporator 4 (SERINC4), mRNA [NM_001033517]	1.3263
359	SERP2	stress-associated endoplasmic reticulum protein family member 2 (SERP2), mRNA [NM_001010897]	1.2486
360	SERPINB11	serpin peptidase inhibitor, clade B (ovalbumin), member 11 (gene/pseudogene) (SERPINB11), mRNA [NM_080475]	1.634
361	SERPINF2	serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 2 (SERPINF2), transcript variant 1, mRNA [NM_000934]	1.1364
362	SFXN2	sideroflexin 2 (SFXN2), mRNA [NM_178858]	1.228
363	SH3RF2	SH3 domain containing ring finger 2 (SH3RF2), mRNA [NM_152550]	1.067
364	SHANK1	SH3 and multiple ankyrin repeat domains 1 (SHANK1), mRNA [NM_016148]	1.9852
365	SHROOM2	shroom family member 2 (SHROOM2), mRNA [NM_001649]	1.05
366	SIX2	SIX homeobox 2 (SIX2), mRNA [NM_016932]	1.1606
367	SLC12A7	solute carrier family 12 (potassium/chloride transporters), member 7 (SLC12A7), mRNA [NM_006598]	1.9715
368	SLC24A1	solute carrier family 24 (sodium/potassium/calcium exchanger), member 1 (SLC24A1), mRNA	1.0623

		[NM_004727]	
369	SLC26A7	solute carrier family 26, member 7 (SLC26A7), transcript variant 2, mRNA [NM_134266]	1.4755
370	SLC2A13	solute carrier family 2 (facilitated glucose transporter), member 13, mRNA (cDNA clone MGC:48624 IMAGE:5272386), complete cds. [BC047507]	1.581
371	SLC44A5	solute carrier family 44, member 5 (SLC44A5), transcript variant 1, mRNA [NM_152697]	1.3877
372	SLC47A1	solute carrier family 47, member 1 (SLC47A1), mRNA [NM_018242]	2.7336
373	SLC6A12	solute carrier family 6 (neurotransmitter transporter, betaine/GABA), member 12 (SLC6A12), transcript variant 1, mRNA [NM_003044]	1.2858
374	SLCO2A1	solute carrier organic anion transporter family, member 2A1 (SLCO2A1), mRNA [NM_005630]	1.5595
375	SLITRK5	SLIT and NTRK-like family, member 5 (SLITRK5), mRNA [NM_015567]	1.5395
376	SLITRK6	SLIT and NTRK-like family, member 6 (SLITRK6), mRNA [NM_032229]	1.1588
377	SNORA12	EST91069 Synovial sarcoma Homo sapiens cDNA 5' end, mRNA sequence [AA378382]	1.2865
378	SNORD22	AGENCOURT_6573317 NIH_MGC_124 Homo sapiens cDNA clone IMAGE:5732165 5', mRNA sequence [BM548627]	1.1467
379	SNTB1	syntrophin, beta 1 (dystrophin-associated protein A1, 59kDa, basic component 1) (SNTB1), mRNA [NM_021021]	1.0634
380	SNX10	sorting nexin 10 (SNX10), transcript variant 1, mRNA [NM_001199835]	1.0889
381	SOX10	SRY (sex determining region Y)-box 10 (SOX10), mRNA [NM_006941]	1.1508
382	SOX2OT	SOX2 overlapping transcript (non-protein coding) (SOX2OT), non-coding RNA [NR_004053]	1.0819
383	SPANXN3	SPANX family, member N3 (SPANXN3), mRNA [NM_001009609]	1.0799
384	SPP1	secreted phosphoprotein 1 (SPP1), transcript variant 1, mRNA [NM_001040058]	2.528
385	SPTLC3	serine palmitoyltransferase, long chain base subunit 3 (SPTLC3), mRNA [NM_018327]	1.8105
386	ST6GALNAC3	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 3 (ST6GALNAC3), transcript variant 2, mRNA [NM_001160011]	1.4392
387	ST8SIA1	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 1 (ST8SIA1), mRNA [NM_003034]	2.0474
388	STAC2	SH3 and cysteine rich domain 2 (STAC2), mRNA [NM_198993]	1.0188
389	STMN4	stathmin-like 4 (STMN4), mRNA [NM_030795]	1.4974
390	SULF1	sulfatase 1 (SULF1), transcript variant 3, mRNA [NM_015170]	1.1156
391	SYNPO	synaptopodin [Source:HGNC Symbol;Acc:30672] [ENST00000394243]	1.0231
392	SYT8	synaptotagmin VIII (SYT8), mRNA [NM_138567]	3.794
393	TAF7L	TAF7-like RNA polymerase II, TATA box binding protein (TBP)-associated factor, 50kDa (TAF7L), transcript variant 1, mRNA [NM_024885]	1.2429
394	TCF23	transcription factor 23 [Source:HGNC Symbol;Acc:18602] [ENST00000407815]	1.1411
395	TDRD9	tudor domain containing 9 (TDRD9), mRNA [NM_153046]	1.1497
396	TFF3	trefoil factor 3 (intestinal) (TFF3), mRNA [NM_003226]	1.0989
397	TGFBR3	transforming growth factor, beta receptor III (TGFBR3), transcript variant 1, mRNA [NM_003243]	2.134
398	TIMM13	translocase of inner mitochondrial membrane 13 homolog (yeast) (TIMM13), nuclear gene encoding mitochondrial protein, mRNA [NM_012458]	1.0984
399	TLR4	toll-like receptor 4 (TLR4), transcript variant 1, mRNA [NM_138554]	2.3692
400	TMCO5A	transmembrane and coiled-coil domains 5A (TMCO5A), mRNA [NM_152453]	1.3204
401	TMEM133	transmembrane protein 133 (TMEM133), mRNA [NM_032021]	1.0949
402	TMEM196	transmembrane protein 196 (TMEM196), mRNA [NM_152774]	1.3944
403	TMEM98	transmembrane protein 98 (TMEM98), transcript variant 1, mRNA [NM_015544]	1.1029
404	TMSB15A	thymosin beta 15a (TMSB15A), mRNA [NM_021992]	1.0664
405	TNFSF4	tumor necrosis factor (ligand) superfamily, member 4 (TNFSF4), mRNA [NM_003326]	1.1373
406	TNIP3	TNFAIP3 interacting protein 3 (TNIP3), transcript variant 1, mRNA [NM_024873]	1.9042
407	TNNI2	troponin I type 2 (skeletal, fast) (TNNI2), transcript variant 1, mRNA [NM_003282]	3.3076
408	TP53	tumor protein p53 (TP53), transcript variant 1, mRNA [NM_000546]	1.6146
409	TP53TG3	TP53 target 3 (TP53TG3), mRNA [NM_016212]	1.0744
410	TRDMT1	tRNA aspartic acid methyltransferase 1 (TRDMT1), mRNA [NM_004412]	2.2787
411	TTTY18	testis-specific transcript, Y-linked 18 (non-protein coding) (TTTY18), non-coding RNA [NR_001550]	1.5601
412	TYR	tyrosinase (oculocutaneous albinism IA) (TYR), mRNA [NM_000372]	1.644
413	VAV3	vav 3 guanine nucleotide exchange factor (VAV3), transcript variant 1, mRNA [NM_006113]	1.593
414	VSIG10	V-set and immunoglobulin domain containing 10 (VSIG10), mRNA [NM_019086]	1.2027
415	VTCN1	V-set domain containing T cell activation inhibitor 1 [Source:HGNC Symbol;Acc:28873] [ENST00000369456]	1.3265
416	WFDC11	WAP four-disulfide core domain 11 (WFDC11), mRNA [NM_147197]	2.4134
417	WISP2	WNT1 inducible signaling pathway protein 2 (WISP2), mRNA [NM_003881]	2.324
418	WWTR1	WW domain containing transcription regulator 1 (WWTR1), transcript variant 1, mRNA [NM_015472]	1.6528
419	ZC3H13	zinc finger CCCH-type containing 13 (ZC3H13), mRNA [NM_015070]	1.1434
420	ZCCHC23	cDNA FLJ45231 fis, clone BRCAN2021452. [AK127166]	3.4481
421	ZFYVE9	zinc finger, FYVE domain containing 9 (ZFYVE9), transcript variant 2, mRNA [NM_007323]	1.0546
422	ZIC1	Zic family member 1 (odd-paired homolog, Drosophila) [Source:HGNC Symbol;Acc:12872] [ENST00000474034]	1.6202
423	ZNF3	zinc finger protein 3 (ZNF3), transcript variant 1, mRNA [NM_017715]	1.8405
424	ZNF365	zinc finger protein 365 (ZNF365), transcript variant B, mRNA [NM_199450]	1.0506
425	ZNF709	zinc finger protein 709 (ZNF709), mRNA [NM_152601]	2.7979
426	ZNF711	zinc finger protein 711 (ZNF711), mRNA [NM_021998]	1.9116
427	ZNF816-ZNF321P	ZNF816-ZNF321P readthrough (ZNF816-ZNF321P), mRNA [NM_001202473]	1.4229
428	ZNF846	zinc finger protein 846 (ZNF846), mRNA [NM_001077624]	1.6864

APPENDIX 4

PATHWAYS ALTERED BY ECTOPIC EXPRESSION OF HSF1ΔRDT IDENTIFIED BY METACORE™ ANALYSIS

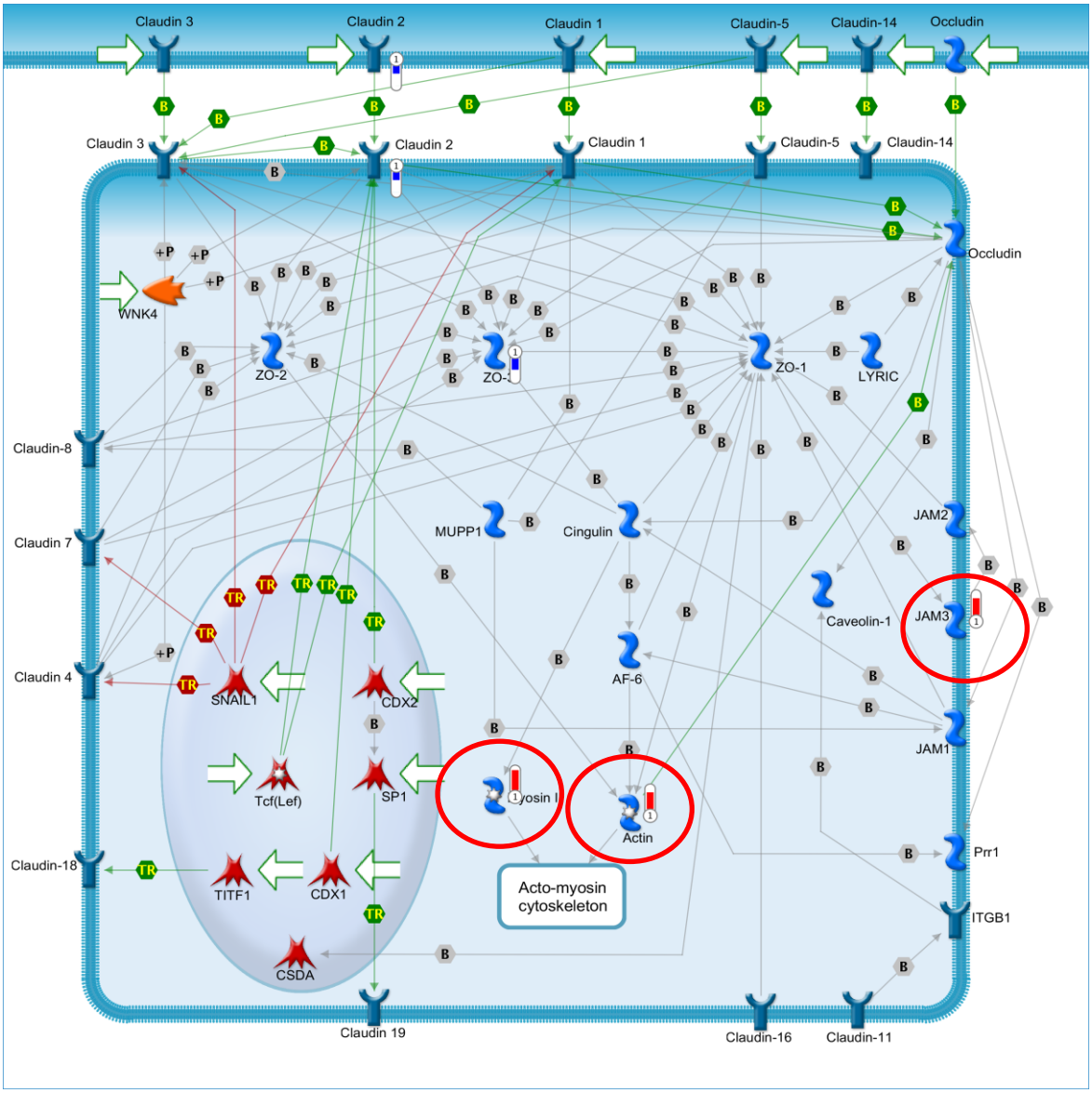


Figure A3. Metacore™ analysis revealed that a significantly altered pathway map affected upon ectopic expression of HSF1ΔRDT in the non-transformed mCherry MCF10A cells was the cellular adhesion – tight junction pathway.

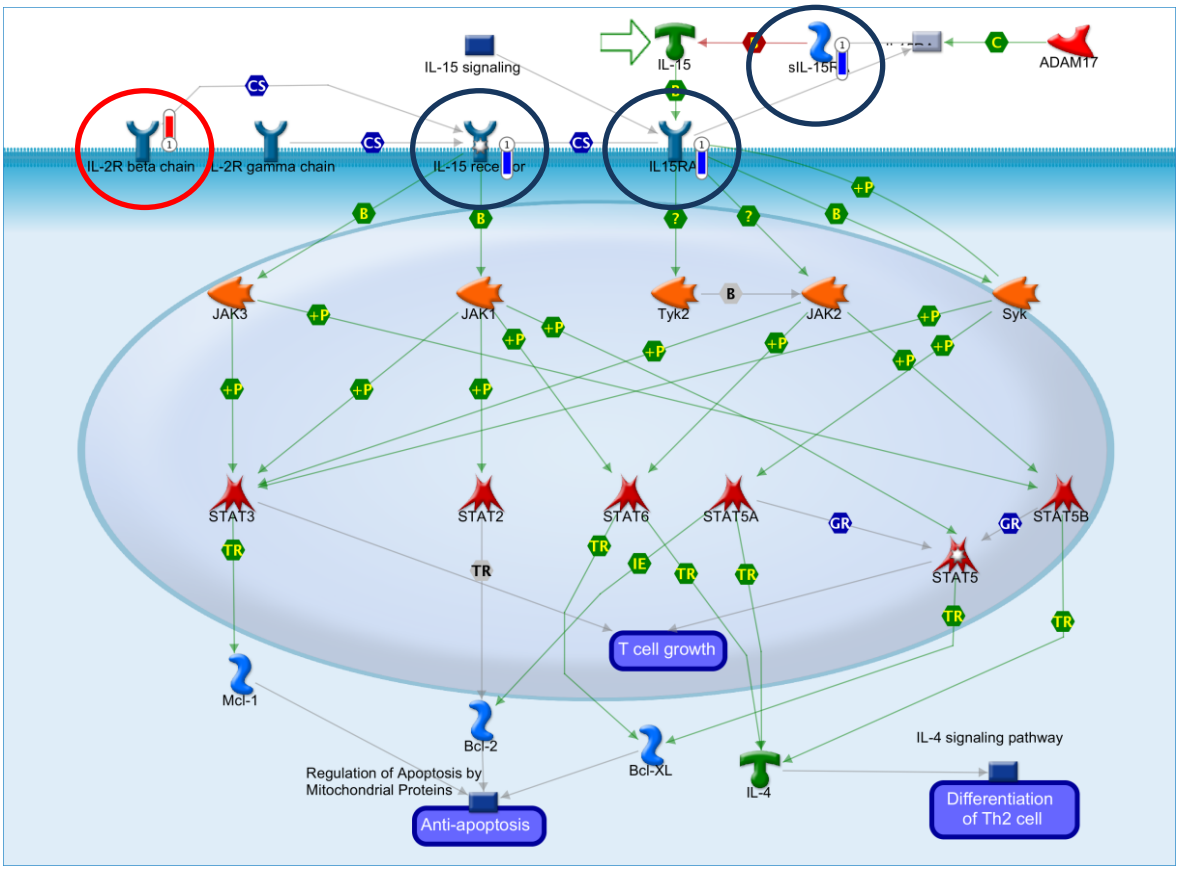


Figure A4. Metacore™ analysis revealed that a significantly altered pathway map affected upon ectopic expression of HSF1ΔRDT in the non-transformed mCherry MCF10A cells was the immune response – IL-15 signalling via JAK-STAT cascade.

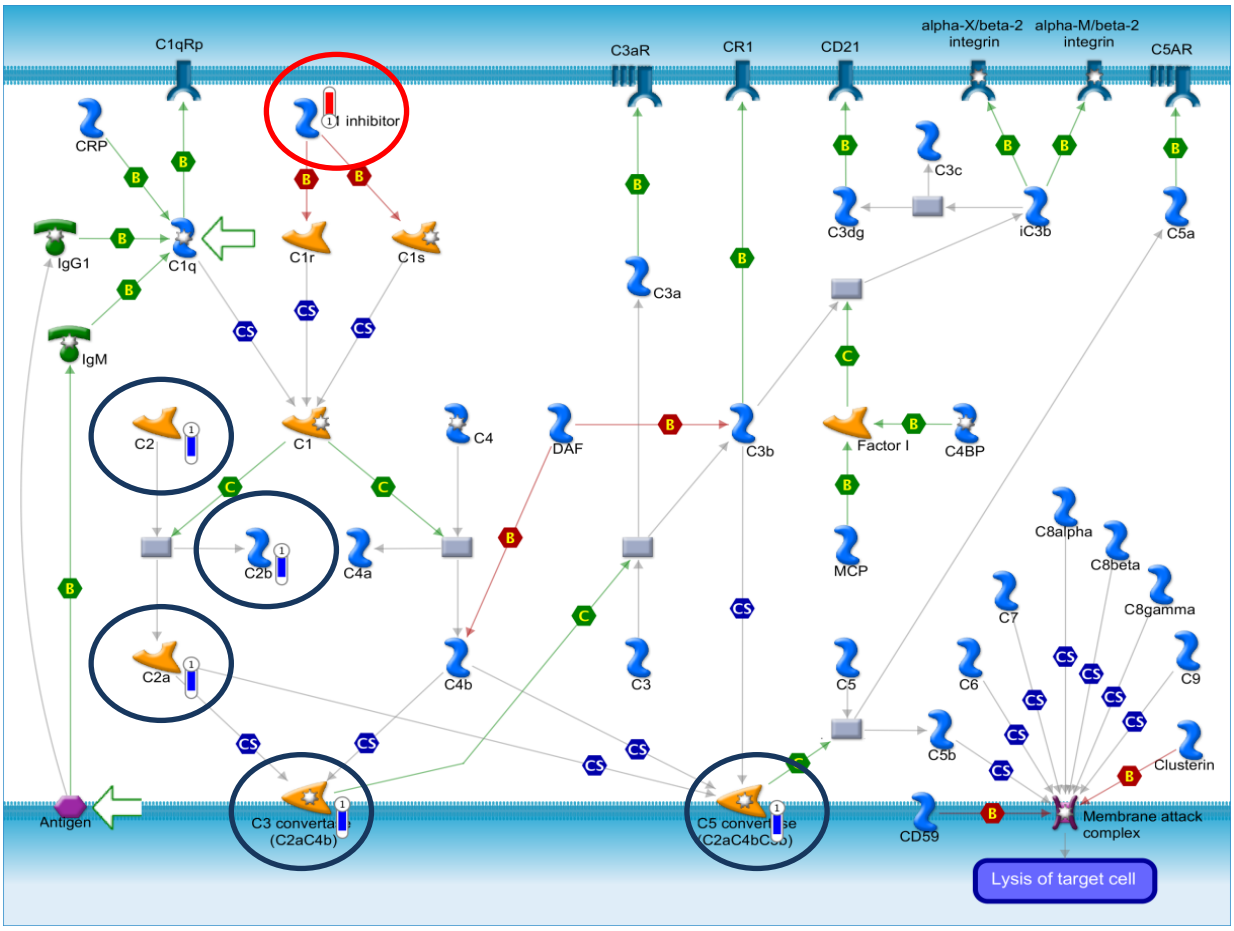


Figure A5. Metacore™ analysis revealed that a significantly altered pathway map affected upon ectopic expression of HSF1ΔRDT in the non-transformed mCherry MCF10A cells was the immune response – lectin induced complement pathway.

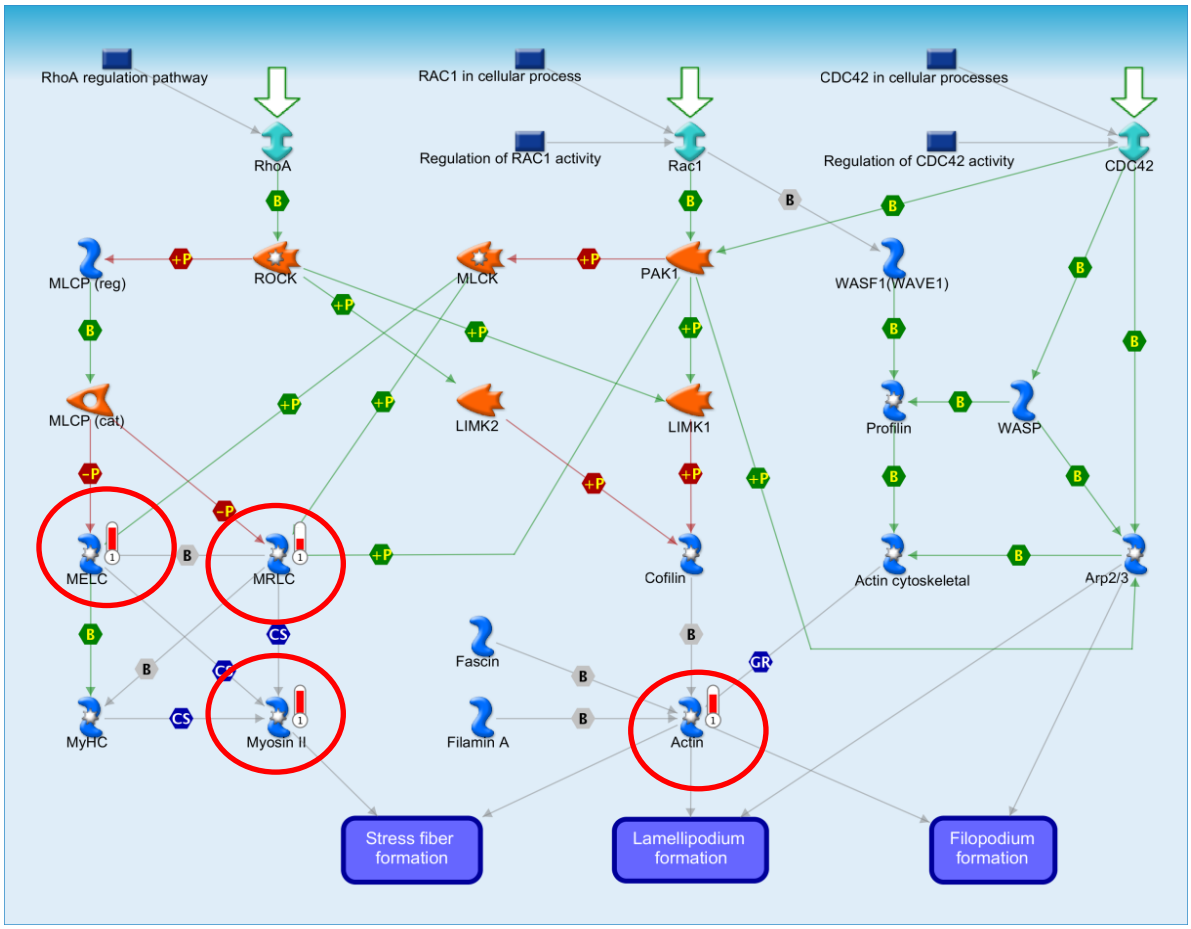


Figure A6. Metacore™ analysis revealed that a significantly altered pathway map affected upon ectopic expression of HSF1ΔRDT in the non-transformed mCherry MCF10A cells was the cytoskeletal remodeling - regulation of actin cytoskeleton by Rho GTPases pathway.

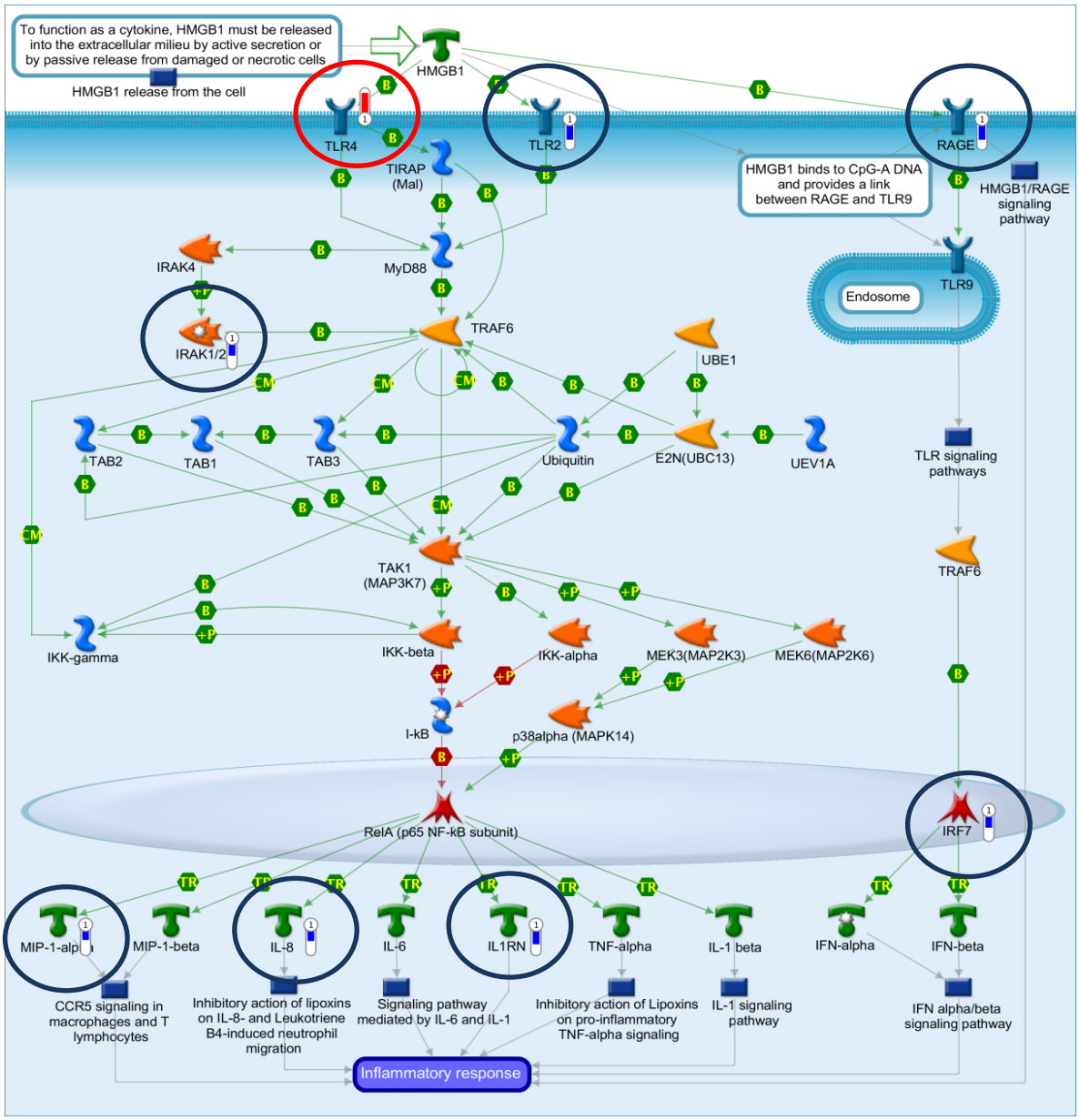


Figure A7. Metacore™ analysis revealed that a significantly altered pathway map affected upon ectopic expression of HSF1ΔRDT in the H-Ras^{V12} transformed MCF10A cells was the immune response – High mobility group box 1/Toll-like receptor (HMGB1/TLR) signaling pathway

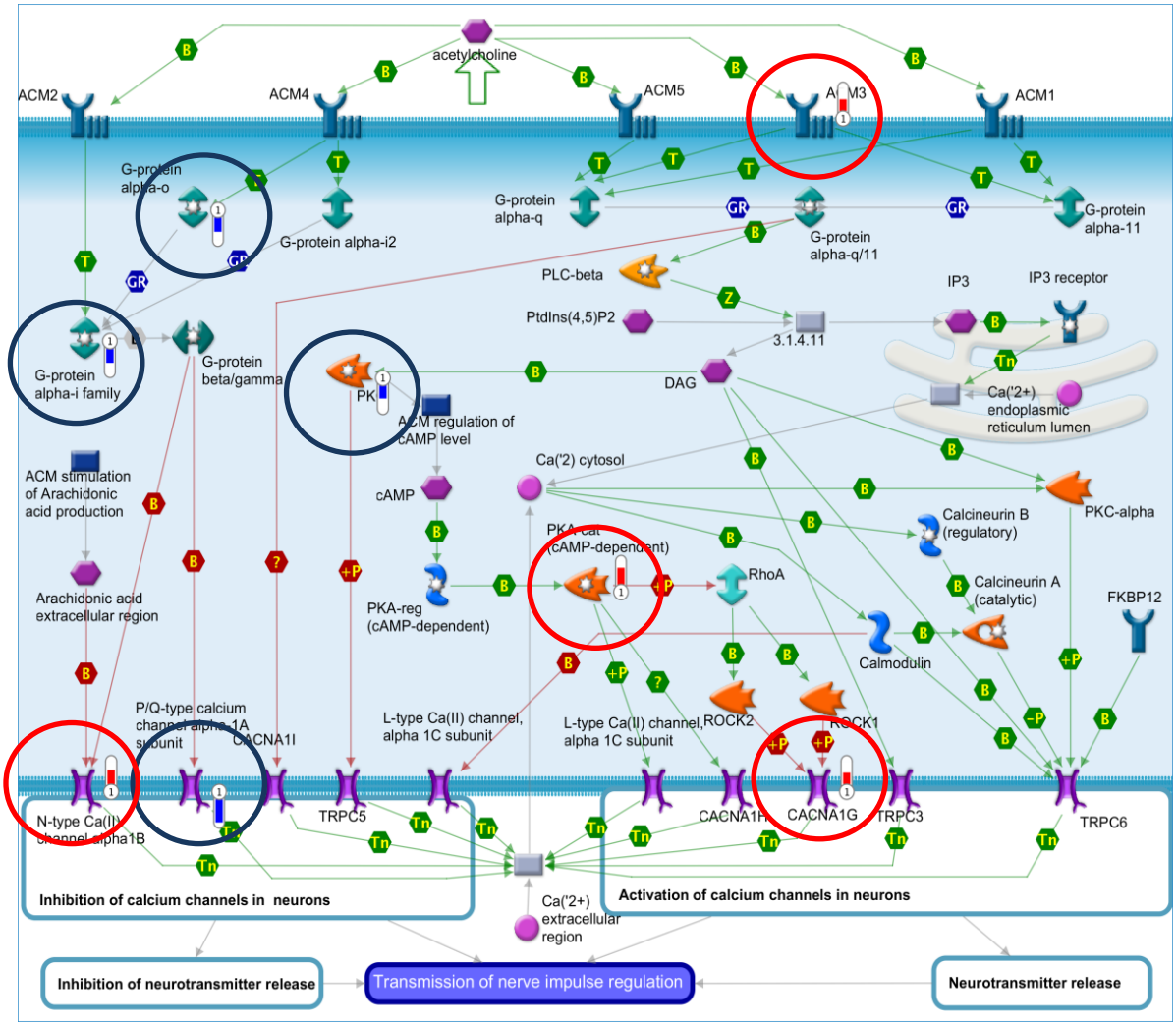


Figure A8. Metacore™ analysis revealed that a significantly altered pathway map affected upon ectopic expression of HSF1ΔRDT in the H-Ras^{V12} transformed MCF10A cells was the Neurophysiological process – Astrocyte-conditioned medium (ACM) regulation of nerve impulse pathway.

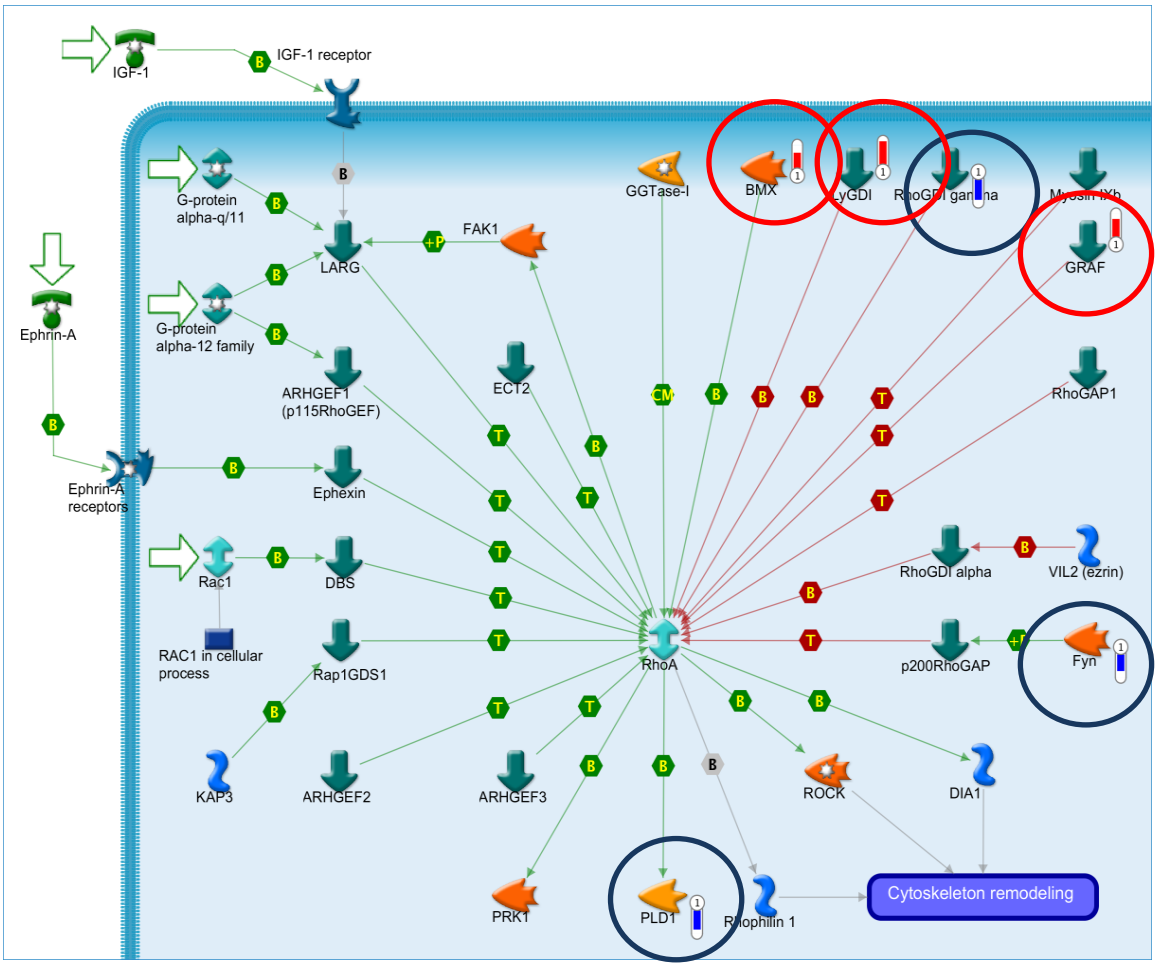


Figure A9. Metacore™ analysis revealed that a significantly altered pathway map affected upon ectopic expression of HSF1ΔRDT in the H-Ras^{V12} transformed MCF10A cells was the G-protein signalling - RhoA regulation pathway.

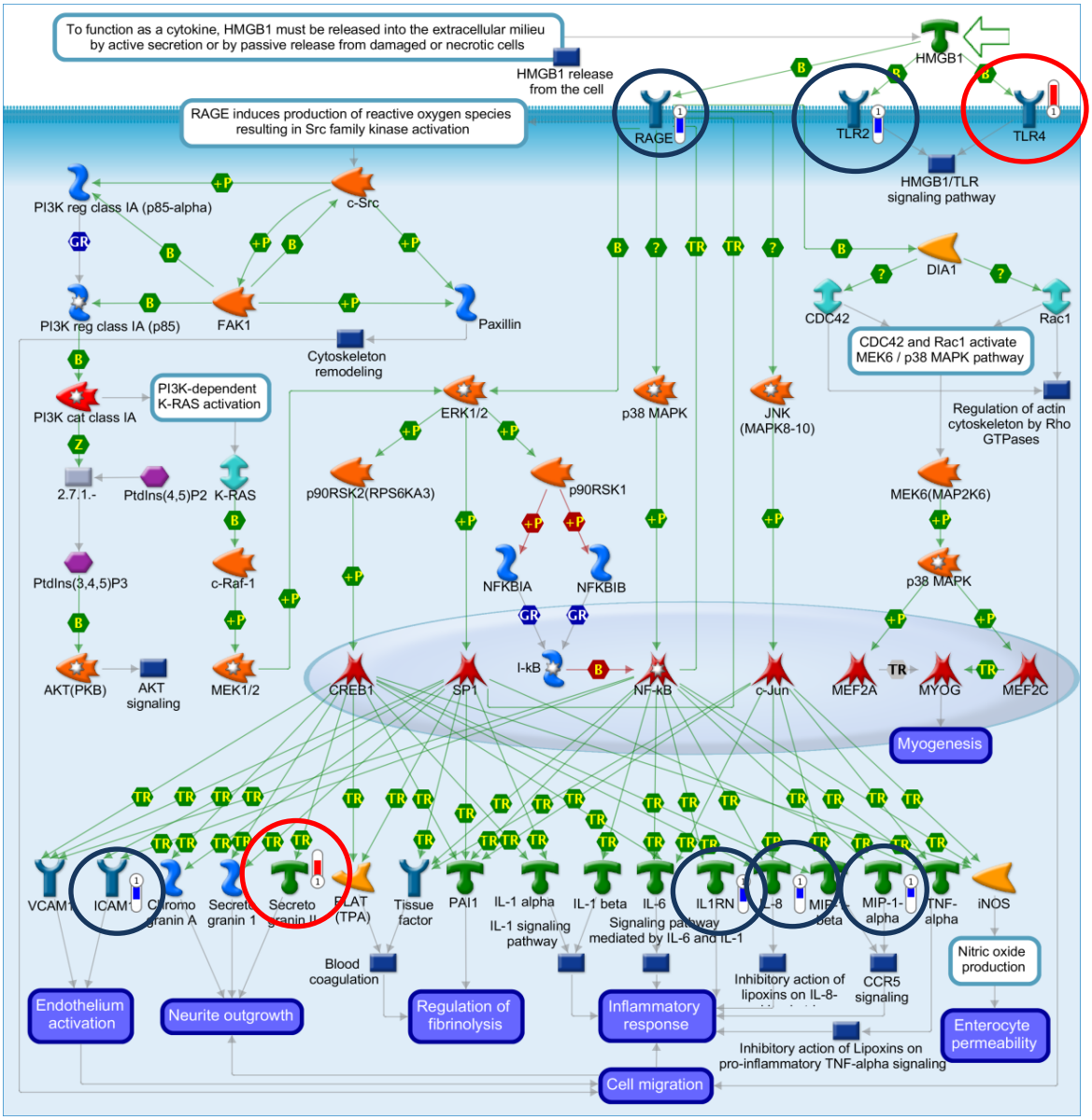


Figure A10. MetacoreTM analysis revealed that a significantly altered pathway map affected upon ectopic expression of HSF1ΔRDT in the H-RasV12 transformed MCF10A cells was the Immune response - HMGB1/Receptor for advanced glycation end products (RAGE) signaling pathway.

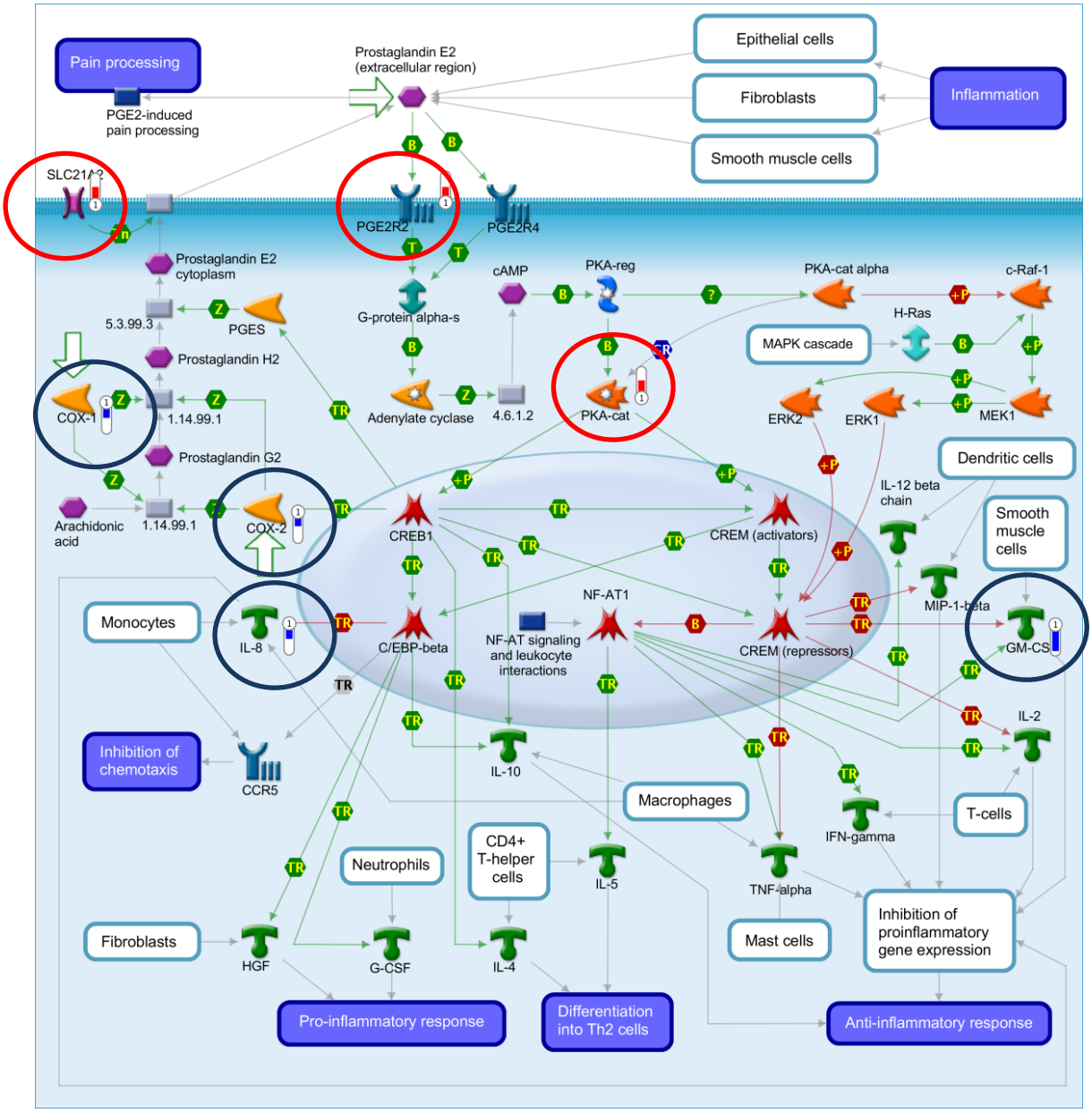
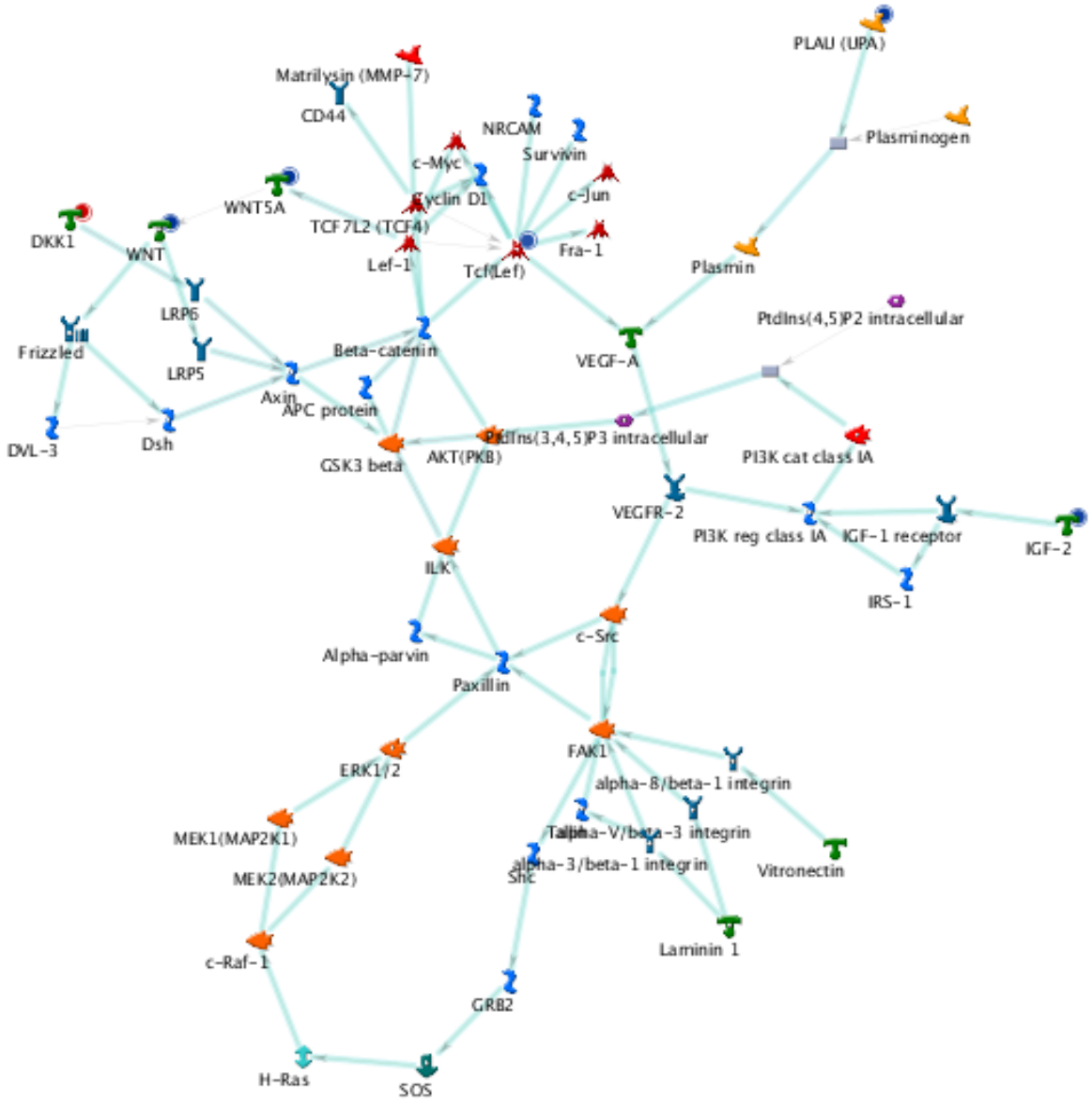


Figure A11. Metacore™ analysis revealed that a significantly altered pathway map affected upon ectopic expression of HSF1ΔRDT in the H-Ras^{V12} transformed MCF10A cells was the Immune response – Prostaglandin E2 (PGE2) signaling in immune response pathway.

APPENDIX 5

GENE NETWORKS UNIQUELY ALTERED UPON ECTOPIC EXPRESSION OF HSF1ΔRDT IN THE H-RAS^{V12} TRANSFORMED MCF10A CELLS IDENTIFIED



BY METACORE™ ANALYSIS

Figure A12. The top scored (by the number of pathways) network from unique genes altered upon the ectopic expression of HSF1ΔRDT in the H-Ras^{V12} transformed MCF10A cells.

Thick cyan lines indicate the fragments of canonical pathways. Up-regulated genes are marked with red circles; down-regulated with blue circles. The 'checkerboard' color indicates mixed expression for the gene between files or between multiple tags for the same gene.

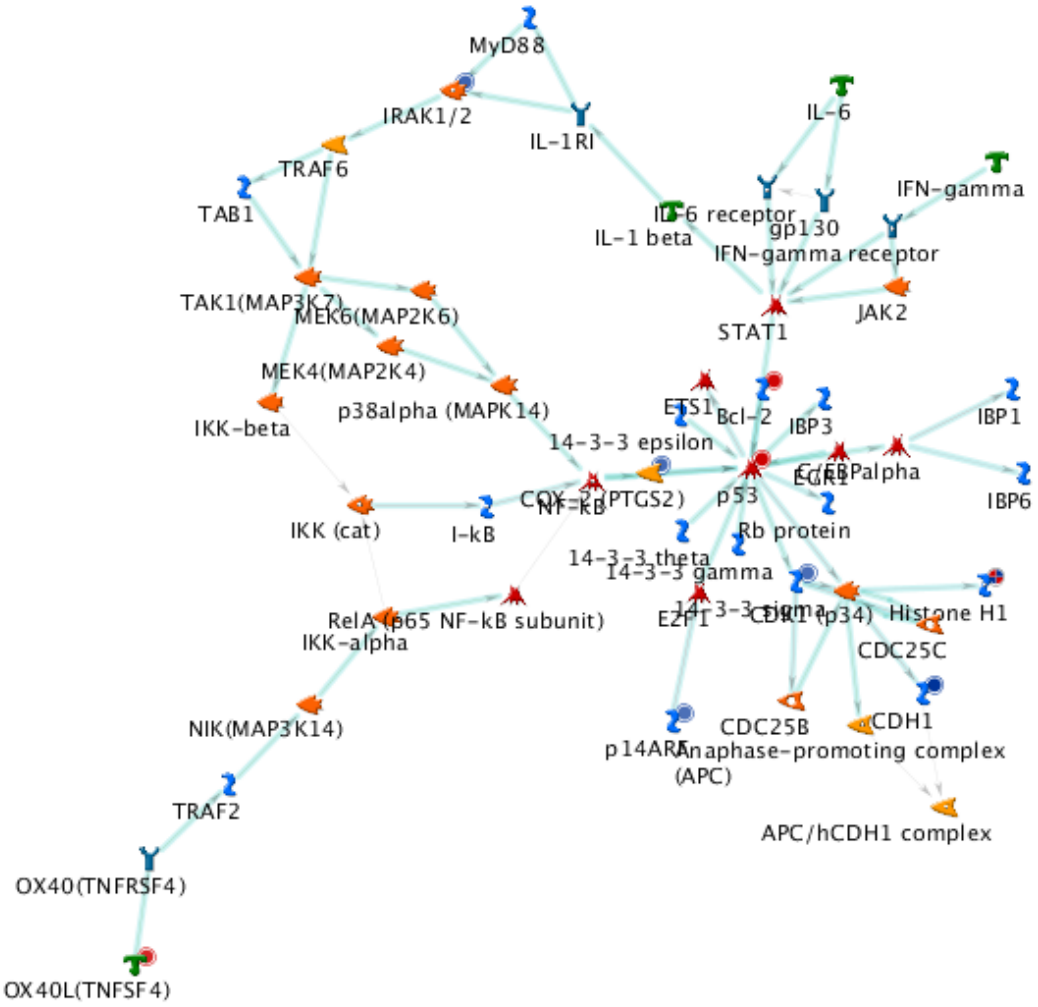


Figure A13. The second scored (by the number of pathways) network from unique genes altered upon the ectopic expression of HSF1ΔRDT in the H-RasV12 transformed MCF10A cells.

Thick cyan lines indicate the fragments of canonical pathways. Up-regulated genes are marked with red circles; down-regulated with blue circles. The 'checkerboard' color indicates mixed expression for the gene between files or between multiple tags for the same gene.

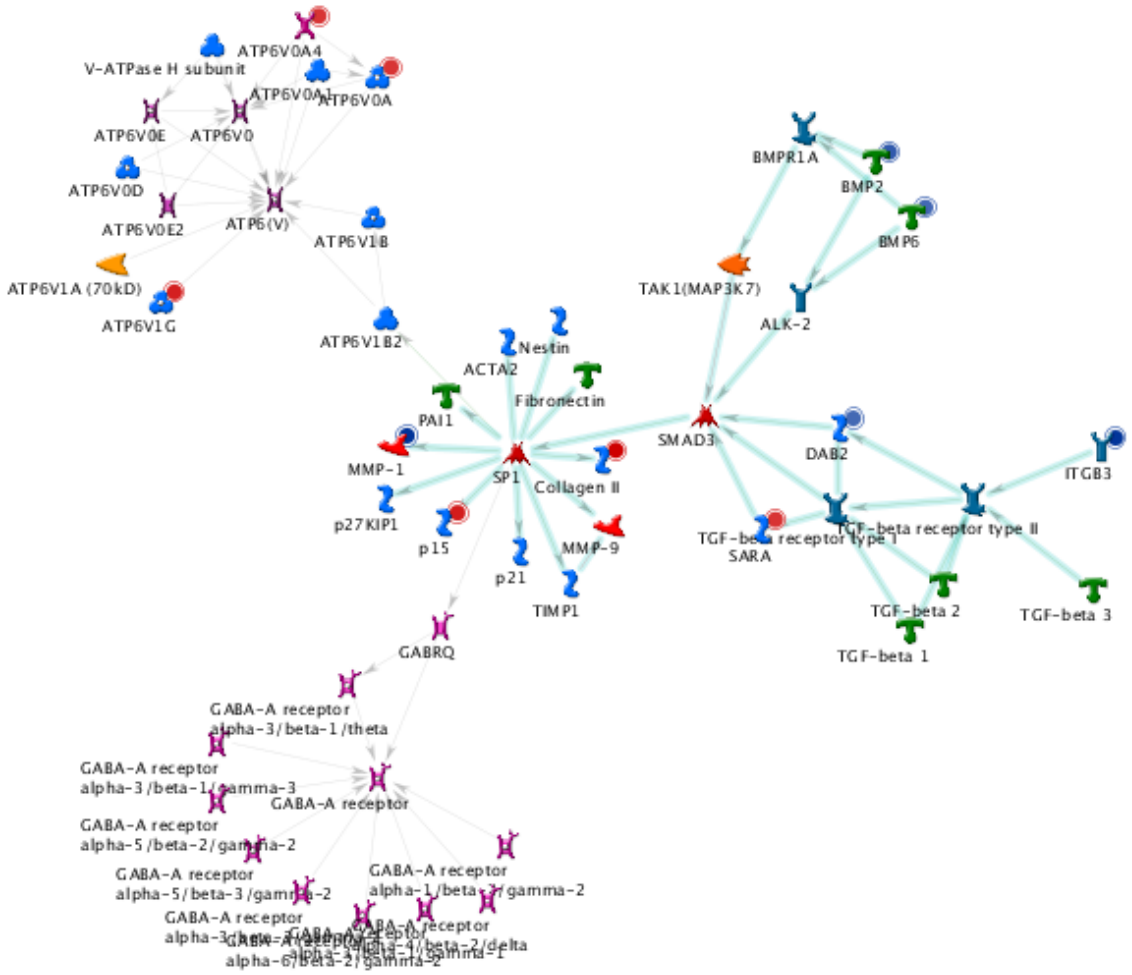


Figure A14. The second scored (by the number of pathways) network from unique genes altered upon the ectopic expression of HSF1ΔRDT in the H-RasV12 transformed MCF10A cells.

Thick cyan lines indicate the fragments of canonical pathways. Up-regulated genes are marked with red circles; down-regulated with blue circles. The 'checkerboard' color indicates mixed expression for the gene between files or between multiple tags for the same gene.

APPENDIX 6

WESTERN BLOT ANALYSIS SHOWING THAT HSF1 DOES NOT IMPACT UPON THE NUCLEAR LOCALIZATION OF BOTH THE WILD-TYPE AND MUTANT p53 PROTEINS

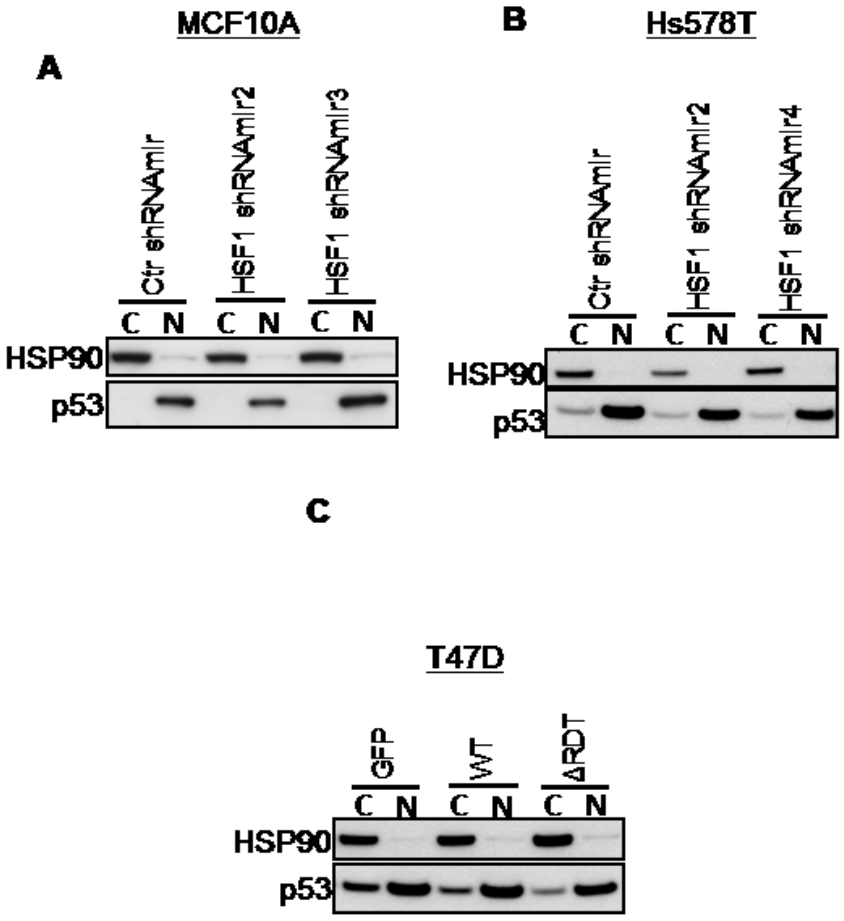


Figure A15. HSF1 does not impact upon the nuclear translocation of both wild-type and mutant p53 proteins.

(A) Western blot analysis of the cytoplasmic (C) and nuclear (N) protein fractions of MCF10A cells revealed that HSF1 knockdown by shRNAmir did not impact upon the nuclear translocation of wild-type p53. Western blot analysis also revealed that **(B)** knockdown of HSF1 in Hs578T cells or **(C)** ectopic expression of HSF1 in T47D cells did not impact upon the nuclear translocation of mutant p53 proteins in these cells.

REFERENCES

Abane, R., and Mezger, V. (2010). Roles of heat shock factors in gametogenesis and development. The FEBS journal *277*, 4150-4172.

Acunzo, J., Katsogiannou, M., and Rocchi, P. (2012). Small heat shock proteins HSP27 (HspB1), alphaB-crystallin (HspB5) and HSP22 (HspB8) as regulators of cell death. The international journal of biochemistry & cell biology.

Akerfelt, M., Morimoto, R. I., and Sistonen, L. (2010). Heat shock factors: integrators of cell stress, development and lifespan. Nat Rev Mol Cell Biol *11*, 545-555.

Akerfelt, M., Trouillet, D., Mezger, V., and Sistonen, L. (2007). Heat shock factors at a crossroad between stress and development. Ann N Y Acad Sci *1113*, 15-27.

Albor, A., Kaku, S., and Kulesz-Martin, M. (1998). Wild-type and mutant forms of p53 activate human topoisomerase I: a possible mechanism for gain of function in mutants. Cancer Res *58*, 2091-2094.

Altieri, D. C., Stein, G. S., Lian, J. B., and Languino, L. R. (2012). TRAP-1, the mitochondrial Hsp90. Biochim Biophys Acta *1823*, 767-773.

Amici, C., Sistonen, L., Santoro, M. G., and Morimoto, R. I. (1992). Antiproliferative prostaglandins activate heat shock transcription factor. Proc Natl Acad Sci U S A *89*, 6227-6231.

Ananthan, J., Goldberg, A. L., and Voellmy, R. (1986). Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes. Science *232*, 522-524.

Anckar, J., and Sistonen, L. (2011). Regulation of HSF1 function in the heat stress response: implications in aging and disease. Annu Rev Biochem *80*, 1089-1115.

Annibaldi, A., and Widmann, C. (2010). Glucose metabolism in cancer cells. Curr Opin Clin Nutr Metab Care *13*, 466-470.

Arrigo, A. P., Simon, S., Gibert, B., Kretz-Remy, C., Nivon, M., Czekalla, A., Guillet, D., Moulin, M., Diaz-Latoud, C., and Vicart, P. (2007). Hsp27 (HspB1) and alphaB-crystallin (HspB5) as therapeutic targets. FEBS Lett *581*, 3665-3674.

Arts, H. J., Hollema, H., Lemstra, W., Willemse, P. H., De Vries, E. G., Kampinga, H. H., and Van der Zee, A. G. (1999). Heat-shock-protein-27 (hsp27) expression in ovarian carcinoma: relation in response to chemotherapy and prognosis. International journal of cancer Journal international du cancer *84*, 234-238.

Asea, A., Kraeft, S. K., Kurt-Jones, E. A., Stevenson, M. A., Chen, L. B., Finberg, R. W., Koo, G. C., and Calderwood, S. K. (2000). HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. Nat Med *6*, 435-442.

Bagatell, R., Paine-Murrieta, G. D., Taylor, C. W., Pulcini, E. J., Akinaga, S., Benjamin, I. J., and Whitesell, L. (2000). Induction of a heat shock factor 1-dependent stress response alters the cytotoxic activity of Hsp90-binding agents. Clin Cancer Res *6*, 3312-3318.

Baler, R., Dahl, G., and Voellmy, R. (1993). Activation of human heat shock genes is accompanied by oligomerization, modification, and rapid translocation of heat shock transcription factor HSF1. Mol Cell Biol *13*, 2486-2496.

Balkwill, F., and Mantovani, A. (2001). Inflammation and cancer: back to Virchow? Lancet *357*, 539-545.

Barbie, D. A., Tamayo, P., Boehm, J. S., Kim, S. Y., Moody, S. E., Dunn, I. F., Schinzel, A. C., Sandy, P., Meylan, E., Scholl, C.*, et al.* (2009). Systematic RNA interference reveals that oncogenic KRAS-driven cancers require TBK1. Nature *462*, 108-112.

Barker, K. T., and Crompton, M. R. (1998). Ras-related TC21 is activated by mutation in a breast cancer cell line, but infrequently in breast carcinomas in vivo. Br J Cancer *78*, 296-300.

Barril, X., Beswick, M. C., Collier, A., Drysdale, M. J., Dymock, B. W., Fink, A., Grant, K., Howes, R., Jordan, A. M., Massey, A.*, et al.* (2006). 4-Amino derivatives of the Hsp90 inhibitor CCT018159. Bioorg Med Chem Lett *16*, 2543-2548.

Barrow, J., Bernardo, A. S., Hay, C. W., Blaylock, M., Duncan, L., Mackenzie, A., McCreath, K., Kind, A. J., Schnieke, A. E., Colman, A.*, et al.* (2005). Purification and Characterization of a Population of EGFP-Expressing Cells from the Developing Pancreas of a Neurogenin3/EGFP Transgenic Mouse. Organogenesis *2*, 22-27.

Basolo, F., Elliott, J., Tait, L., Chen, X. Q., Maloney, T., Russo, I. H., Pauley, R., Momiki, S., Caamano, J., Kleinszanto, A. J. P.*, et al.* (1991). transformation of human breast epithelial-cells by c-ha-ras oncogene. Mol Carcinog *4*, 25-35.

Biamonti, G. (2004). Nuclear stress bodies: a heterochromatin affair? Nat Rev Mol Cell Biol *5*, 493-498.

Biamonti, G., and Vourc'h, C. (2010). Nuclear stress bodies. Cold Spring Harb Perspect Biol *2*, a000695.

Birch-Machin, I., Gao, S., Huen, D., McGirr, R., White, R. A., and Russell, S. (2005). Genomic analysis of heat-shock factor targets in Drosophila. Genome Biol *6*, R63.

Biscardi, J. S., Ishizawar, R. C., Silva, C. M., and Parsons, S. J. (2000). Tyrosine kinase signalling in breast cancer: epidermal growth factor receptor and c-Src interactions in breast cancer. Breast cancer research : BCR *2*, 203-210.

Boellmann, F., Guettouche, T., Guo, Y., Fenna, M., Mnayer, L., and Voellmy, R. (2004). DAXX interacts with heat shock factor 1 during stress activation and enhances its transcriptional activity. Proc Natl Acad Sci U S A *101*, 4100-4105.

Borresen-Dale, A. L. (2003). TP53 and breast cancer. Hum Mutat *21*, 292-300.

Bos, J. L. (1989). ras oncogenes in human cancer: a review. Cancer Res *49*, 4682-4689.

Bouchier-Hayes, L., McBride, S., van Geelen, C. M., Nance, S., Lewis, L. K., Pinkoski, M. J., and Beere, H. M. (2010). Fas ligand gene expression is directly regulated by stress-inducible heat shock transcription factor-1. Cell Death Differ *17*, 1034-1046.

Bourdon, J. C., Deguin-Chambon, V., Lelong, J. C., Dessen, P., May, P., Debuire, B., and May, E. (1997). Further characterisation of the p53 responsive element--identification of new candidate genes for trans-activation by p53. Oncogene *14*, 85-94.

Boyault, C., Zhang, Y., Fritah, S., Caron, C., Gilquin, B., Kwon, S. H., Garrido, C., Yao, T. P., Vourc'h, C., Matthias, P., and Khochbin, S. (2007). HDAC6 controls major cell response pathways to cytotoxic accumulation of protein aggregates. Genes Dev *21*, 2172-2181.

Brandt, G. E., Schmidt, M. D., Prisinzano, T. E., and Blagg, B. S. (2008). Gedunin, a novel hsp90 inhibitor: semisynthesis of derivatives and preliminary structure-activity relationships. J Med Chem *51*, 6495-6502.

Bres, V., Yoh, S. M., and Jones, K. A. (2008). The multi-tasking P-TEFb complex. Curr Opin Cell Biol *20*, 334-340.

Brooks, C. L., Li, M., Hu, M., Shi, Y., and Gu, W. (2007). The p53--Mdm2--HAUSP complex is involved in p53 stabilization by HAUSP. Oncogene *26*, 7262-7266.

Brown, S. A., Weirich, C. S., Newton, E. M., and Kingston, R. E. (1998). Transcriptional activation domains stimulate initiation and elongation at different times and via different residues. The EMBO journal *17*, 3146-3154.

Bruce, J. L., Chen, C., Xie, Y., Zhong, R., Wang, Y. Q., Stevenson, M. A., and Calderwood, S. K. (1999). Activation of heat shock transcription factor 1 to a DNA binding form during the G(1)phase of the cell cycle. Cell Stress Chaperones *4*, 36-45.

Butcher, D. T., Alliston, T., and Weaver, V. M. (2009). A tense situation: forcing tumour progression. Nat Rev Cancer *9*, 108-122.

Cahill, C. M., Waterman, W. R., Xie, Y., Auron, P. E., and Calderwood, S. K. (1996). Transcriptional repression of the prointerleukin 1beta gene by heat shock factor 1. J Biol Chem *271*, 24874-24879.

Calderwood, S. K. (2010). Heat shock proteins in breast cancer progression--a suitable case for treatment? International journal of hyperthermia : the official journal of European Society for Hyperthermic Oncology, North American Hyperthermia Group *26*, 681-685.

Calderwood, S. K. (2012a). Elevated levels of HSF1 indicate a poor prognosis in breast cancer. Future Oncol *8*, 399-401.

Calderwood, S. K. (2012b). HSF1, a versatile factor in tumorogenesis. Curr Mol Med.

Calderwood, S. K., and Ciocca, D. R. (2008). Heat shock proteins: stress proteins with Janus-like properties in cancer. International journal of hyperthermia : the official journal of European Society for Hyperthermic Oncology, North American Hyperthermia Group *24*, 31-39.

Calderwood, S. K., and Gong, J. (2011). Molecular chaperones in mammary cancer growth and breast tumor therapy. J Cell Biochem.

Calderwood, S. K., Khaleque, M. A., Sawyer, D. B., and Ciocca, D. R. (2006). Heat shock proteins in cancer: chaperones of tumorigenesis. Trends Biochem Sci *31*, 164-172.

Calderwood, S. K., Theriault, J. R., and Gong, J. (2005). Message in a bottle: role of the 70-kDa heat shock protein family in anti-tumor immunity. Eur J Immunol *35*, 2518-2527.

Calderwood, S. K., Xie, Y., Wang, X., Khaleque, M. A., Chou, S. D., Murshid, A., Prince, T., and Zhang, Y. (2010). Signal Transduction Pathways Leading to Heat Shock Transcription. Signal transduction insights *2*, 13-24.

Carver, J. A., Rekas, A., Thorn, D. C., and Wilson, M. R. (2003). Small heat-shock proteins and clusterin: intra- and extracellular molecular chaperones with a common mechanism of action and function? IUBMB Life *55*, 661-668.

Cen, H., Zheng, S., Fang, Y. M., Tang, X. P., and Dong, Q. (2004). Induction of HSF1 expression is associated with sporadic colorectal cancer. World journal of gastroenterology : WJG *10*, 3122-3126.

Chadli, A., Felts, S. J., Wang, Q., Sullivan, W. P., Botuyan, M. V., Fauq, A., Ramirez-Alvarado, M., and Mer, G. (2010). Celastrol inhibits Hsp90 chaperoning of steroid receptors by inducing fibrillization of the Co-chaperone p23. J Bio Chem *285*, 4224-4231.

Chae, B. J., Bae, J. S., Lee, A., Park, W. C., Seo, Y. J., Song, B. J., Kim, J. S., and Jung, S. S. (2009). p53 as a specific prognostic factor in triple-negative breast cancer. Jpn J Clin Oncol *39*, 217-224.

Chalmin, F., Ladoire, S., Mignot, G., Vincent, J., Bruchard, M., Remy-Martin, J. P., Boireau, W., Rouleau, A., Simon, B., Lanneau, D*., et al.* (2010). Membrane-associated Hsp72 from tumor-derived exosomes mediates STAT3-dependent immunosuppressive function of mouse and human myeloid-derived suppressor cells. The Journal of clinical investigation *120*, 457-471.

Chang, W. W., Hu, F. W., Yu, C. C., Wang, H. H., Feng, H. P., Lan, C., Tsai, L. L., and Chang, Y. C. (2012a). Quercetin in elimination of tumor initiating stem-like and mesenchymal transformation property in head and neck cancer. Head Neck.

Chang, Z., Lu, M., Park, S. M., Park, H. K., Kang, H. S., Pak, Y., and Park, J. S. (2012b). Functional HSF1 requires aromatic-participant interactions in protecting mouse embryonic fibroblasts against apoptosis via G2 cell cycle arrest. Mol Cells *33*, 465-470.

Chen, C., Xie, Y., Stevenson, M. A., Auron, P. E., and Calderwood, S. K. (1997). Heat shock factor 1 represses Ras-induced transcriptional activation of the c-fos gene. J Biol Chem *272*, 26803-26806.

Chen, F., Wang, W., and El-Deiry, W. S. (2010). Current strategies to target p53 in cancer. Biochem Pharmacol *80*, 724-730.

Chen, M., Rose, A. E., Doudican, N., Osman, I., and Orlow, S. J. (2009a). Celastrol synergistically enhances temozolomide cytotoxicity in melanoma cells. Molecular cancer research : MCR *7*, 1946-1953.

Chen, T., Guo, J., Han, C., Yang, M., and Cao, X. (2009b). Heat shock protein 70, released from heat-stressed tumor cells, initiates antitumor immunity by inducing tumor cell chemokine production and activating dendritic cells via TLR4 pathway. J Immunol *182*, 1449-1459.

Chen, Z., Lu, W., Garcia-Prieto, C., and Huang, P. (2007). The Warburg effect and its cancer therapeutic implications. J Bioenerg Biomembr *39*, 267-274.

Chernorizov, C. K., and Svedas, V. K. (2010). Modeling of the Full-Size 3D Structure of Human Chaperone Hsp70 and Study of Its Interdomain Interactions. Acta Naturae *2*, 66-71.

Cheung, K. M., Matthews, T. P., James, K., Rowlands, M. G., Boxall, K. J., Sharp, S. Y., Maloney, A., Roe, S. M., Prodromou, C., Pearl, L. H*., et al.* (2005). The identification, synthesis, protein crystal structure and in vitro biochemical evaluation of a new 3,4-diarylpyrazole class of Hsp90 inhibitors. Bioorg Med Chem Lett *15*, 3338-3343.

Chin, K. V., Tanaka, S., Darlington, G., Pastan, I., and Gottesman, M. M. (1990). Heat shock and arsenite increase expression of the multidrug resistance (MDR1) gene in human renal carcinoma cells. J Biol Chem *265*, 221-226.

Chiosis, G. (2006). Discovery and development of purine-scaffold Hsp90 inhibitors. Curr Top Med Chem *6*, 1183-1191.

Chiosis, G., Huezo, H., Rosen, N., Mimnaugh, E., Whitesell, L., and Neckers, L. (2003). 17AAG: low target binding affinity and potent cell activity--finding an explanation. Mol Cancer Ther *2*, 123-129.

Choi, Y., Asada, S., and Uesugi, M. (2000). Divergent hTAFII31-binding motifs hidden in activation domains. J Bio Chem *275*, 15912-15916.

Chu, B., Soncin, F., Price, B. D., Stevenson, M. A., and Calderwood, S. K. (1996). Sequential phosphorylation by mitogen-activated protein kinase and glycogen synthase kinase 3 represses transcriptional activation by heat shock factor-1. J Biol Chem *271*, 30847-30857.

Chu, B., Zhong, R., Soncin, F., Stevenson, M. A., and Calderwood, S. K. (1998). Transcriptional activity of heat shock factor 1 at 37 degrees C is repressed through phosphorylation on two distinct serine residues by glycogen synthase kinase 3 and protein kinases Calpha and Czeta. J Biol Chem *273*, 18640-18646.

Cicero, M. P., Hubl, S. T., Harrison, C. J., Littlefield, O., Hardy, J. A., and Nelson, H. C. (2001). The wing in yeast heat shock transcription factor (HSF) DNA-binding domain is required for full activity. Nucleic Acids Res *29*, 1715-1723.

Ciocca, D. R., and Calderwood, S. K. (2005). Heat shock proteins in cancer: diagnostic, prognostic, predictive, and treatment implications. Cell Stress Chaperones *10*, 86-103.

Ciocca, D. R., Rozados, V. R., Cuello Carrion, F. D., Gervasoni, S. I., Matar, P., and Scharovsky, O. G. (2003). Hsp25 and Hsp70 in rodent tumors treated with doxorubicin and lovastatin. Cell Stress Chaperones *8*, 26-36.

Coleman, W. B., and Tsongalis, G. J. (1995). Multiple mechanisms account for genomic instability and molecular mutation in neoplastic transformation. Clin Chem *41*, 644-657.

Conde, R., Xavier, J., McLoughlin, C., Chinkers, M., and Ovsenek, N. (2005). Protein phosphatase 5 is a negative modulator of heat shock factor 1. J Bio Chem *280*, 28989-28996.

Cotto, J. J., Kline, M., and Morimoto, R. I. (1996). Activation of heat shock factor 1 DNA binding precedes stress-induced serine phosphorylation. Evidence for a multistep pathway of regulation. J Biol Chem *271*, 3355-3358.

Coussens, L. M., Fingleton, B., and Matrisian, L. M. (2002). Matrix metalloproteinase inhibitors and cancer: trials and tribulations. Science *295*, 2387-2392.

Coutant, C., Rouzier, R., Qi, Y., Lehmann-Che, J., Bianchini, G., Iwamoto, T., Hortobagyi, G. N., Symmans, W. F., Uzan, S., Andre, F*., et al.* (2011). Distinct p53 gene signatures are needed to predict prognosis and response to chemotherapy in ER-positive and ER-negative breast cancers. Clinical cancer research : an official journal of the American Association for Cancer Research *17*, 2591-2601.

da Silva, K. P., and Borges, J. C. (2011). The molecular chaperone Hsp70 family members function by a bidirectional heterotrophic allosteric mechanism. Protein Pept Lett *18*, 132-142.

Dai, C., Santagata, S., Tang, Z., Shi, J., Cao, J., Kwon, H., Bronson, R. T., Whitesell, L., and Lindquist, S. (2012). Loss of tumor suppressor NF1 activates HSF1 to promote carcinogenesis. The Journal of clinical investigation *122*, 3742-3754.

Dai, C., Whitesell, L., Rogers, A. B., and Lindquist, S. (2007). Heat shock factor 1 is a powerful multifaceted modifier of carcinogenesis. Cell *130*, 1005-1018.

Dai, R., Frejtag, W., He, B., Zhang, Y., and Mivechi, N. F. (2000). c-Jun NH2-terminal kinase targeting and phosphorylation of heat shock factor-1 suppress its transcriptional activity. J Biol Chem *275*, 18210-18218.

Dai, Y., DeSano, J. T., Meng, Y., Ji, Q., Ljungman, M., Lawrence, T. S., and Xu, L. (2009). Celastrol potentiates radiotherapy by impairment of DNA damage processing in human prostate cancer. Int J Radiat Oncol Biol Phys *74*, 1217-1225.

Dajas, F. (2012). Life or death: neuroprotective and anticancer effects of quercetin. J Ethnopharmacol *143*, 383-396.

Daly, R. J., Binder, M. D., and Sutherland, R. L. (1994). Overexpression of the Grb2 gene in human breast cancer cell lines. Oncogene *9*, 2723-2727.

Davenport, A., Frezza, M., Shen, M., Ge, Y., Huo, C., Chan, T. H., and Dou, Q. P. (2010a). Celastrol and an EGCG pro-drug exhibit potent chemosensitizing activity in human leukemia cells. Int J Mol Med *25*, 465-470.

Davenport, E. L., Zeisig, A., Aronson, L. I., Moore, H. E., Hockley, S., Gonzalez, D., Smith, E. M., Powers, M. V., Sharp, S. Y., Workman, P.*, et al.* (2010b). Targeting heat shock protein 72 enhances Hsp90 inhibitor-induced apoptosis in myeloma. Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, UK *24*, 1804-1807.

Davies, H., Bignell, G. R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M. J., Bottomley, W.*, et al.* (2002). Mutations of the BRAF gene in human cancer. Nature *417*, 949-954.

de Billy, E., Powers, M. V., Smith, J. R., and Workman, P. (2009). Drugging the heat shock factor 1 pathway: exploitation of the critical cancer cell dependence on the guardian of the proteome. Cell Cycle *8*, 3806-3808.

de la Cruz-Merino, L., Henao Carrasco, F., Vicente Baz, D., Nogales Fernandez, E., Reina Zoilo, J. J., Codes Manuel de Villena, M., and Pulido, E. G. (2011). Immune microenvironment in colorectal cancer: a new hallmark to change old paradigms. Clin Dev Immunol *2011*, 174149.

de Magalhaes, J. P. (2004). From cells to ageing: a review of models and mechanisms of cellular senescence and their impact on human ageing. Exp Cell Res *300*, 1-10.

De Thonel, A., Mezger, V., and Garrido, C. (2011). Implication of Heat Shock Factors in Tumorigenesis: Therapeutical Potential. Cancers *3*, 1158-1181.

DeBerardinis, R. J., Lum, J. J., Hatzivassiliou, G., and Thompson, C. B. (2008). The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. Cell Metab *7*, 11-20.

Debnath, J., Muthuswamy, S. K., and Brugge, J. S. (2003). Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. Methods *30*, 256-268.

Denegri, M., Chiodi, I., Corioni, M., Cobianchi, F., Riva, S., and Biamonti, G. (2001). Stress-induced nuclear bodies are sites of accumulation of pre-mRNA processing factors. Mol Biol Cell *12*, 3502-3514.

Denegri, M., Moralli, D., Rocchi, M., Biggiogera, M., Raimondi, E., Cobianchi, F., De Carli, L., Riva, S., and Biamonti, G. (2002). Human chromosomes 9, 12, and 15 contain the nucleation sites of stress-induced nuclear bodies. Mol Biol Cell *13*, 2069-2079.

Derer, S., Berger, S., Schlaeth, M., Schneider-Merck, T., Klausz, K., Lohse, S., Overdijk, M. B., Dechant, M., Kellner, C., Nagelmeier, I.*, et al.* (2012). Oncogenic KRAS impairs EGFR antibodies' efficiency by C/EBPbeta-dependent suppression of EGFR expression. Neoplasia *14*, 190-205.

DeSantis, C., Siegel, R., Bandi, P., and Jemal, A. (2011). Breast cancer statistics, 2011. CA Cancer J Clin *61*, 409-418.

Devi, P. U., and Kamath, R. (2003). Radiosensitizing effect of withaferin A combined with hyperthermia on mouse fibrosarcoma and melanoma. J Radiat Res (Tokyo) *44*, 1-6.

Di Como, C. J., and Prives, C. (1998). Human tumor-derived p53 proteins exhibit binding site selectivity and temperature sensitivity for transactivation in a yeast-based assay. Oncogene *16*, 2527-2539.

DiDomenico, B. J., Bugaisky, G. E., and Lindquist, S. (1982). The heat shock response is self-regulated at both the transcriptional and posttranscriptional levels. Cell *31*, 593-603.

Ding, X., Zhou, X., Zhang, H., Qing, J., Qiang, H., and Zhou, G. (2012). Triptolide augments the effects of 5-lipoxygenase RNA interference in suppressing pancreatic tumor growth in a xenograft mouse model. Cancer Chemother Pharmacol *69*, 253-261.

Dittmer, D., Pati, S., Zambetti, G., Chu, S., Teresky, A. K., Moore, M., Finlay, C., and Levine, A. J. (1993). Gain of function mutations in p53. Nat Genet *4*, 42-46.

Dokmanovic, M., Clarke, C., and Marks, P. A. (2007). Histone deacetylase inhibitors: overview and perspectives. Molecular cancer research : MCR *5*, 981-989.

Donnelly, A., and Blagg, B. S. (2008). Novobiocin and additional inhibitors of the Hsp90 C-terminal nucleotide-binding pocket. Curr Med Chem *15*, 2702-2717.

Donzelli, S., Biagioni, F., Fausti, F., Strano, S., Fontemaggi, G., and Blandino, G. (2008). Oncogenomic Approaches in Exploring Gain of Function of Mutant p53. Current genomics *9*, 200-207.

Dow, L. E., Elsum, I. A., King, C. L., Kinross, K. M., Richardson, H. E., and Humbert, P. O. (2008). Loss of human Scribble cooperates with H-Ras to promote cell invasion through deregulation of MAPK signalling. Oncogene *27*, 5988-6001.

Downward, J. (2003). Targeting ras signalling pathways in cancer therapy. Nature Reviews Cancer *3*, 11-22.

Drasin, D. J., Robin, T. P., and Ford, H. L. (2011). Breast cancer epithelial-to-mesenchymal transition: examining the functional consequences of plasticity. Breast cancer research : BCR *13*, 226.

Drosten, M., Dhawahir, A., Sum, E. Y., Urosevic, J., Lechuga, C. G., Esteban, L. M., Castellano, E., Guerra, C., Santos, E., and Barbacid, M. (2010). Genetic analysis of Ras signalling pathways in cell proliferation, migration and survival. The EMBO journal *29*, 1091-1104.

Du, Z. X., Zhang, H. Y., Meng, X., Gao, Y. Y., Zou, R. L., Liu, B. Q., Guan, Y., and Wang, H. Q. (2009). Proteasome inhibitor MG132 induces BAG3 expression through activation of heat shock factor 1. J Cell Physiol *218*, 631-637.

Duffy, M. J., McGowan, P. M., and Gallagher, W. M. (2008). Cancer invasion and metastasis: changing views. The Journal of pathology *214*, 283-293.

Duo, J., Ying, G. G., Wang, G. W., and Zhang, L. (2012). Quercetin inhibits human breast cancer cell proliferation and induces apoptosis via Bcl-2 and Bax regulation. Molecular medicine reports *5*, 1453-1456.

Eckert, L. B., Repasky, G. A., Ulku, A. S., McFall, A., Zhou, H., Sartor, C. I., and Der, C. J. (2004). Involvement of ras activation in human breast cancer cell signaling, invasion, and anoikis. Cancer Res *64*, 4585-4592.

Eckhardt, S. G., and Pluda, J. M. (1997). Development of angiogenesis inhibitors for cancer therapy. Invest New Drugs *15*, 1-3.

Eichenbaum, K. D., Rodriguez, Y., Mezei, M., and Osman, R. (2010). The energetics of the acetylation switch in p53-mediated transcriptional activation. Proteins *78*, 447-456.

Eiseman, J. L., Lan, J., Lagattuta, T. F., Hamburger, D. R., Joseph, E., Covey, J. M., and Egorin, M. J. (2005). Pharmacokinetics and pharmacodynamics of 17-demethoxy 17-[[(2-dimethylamino)ethyl]amino]geldanamycin (17DMAG, NSC 707545) in C.B-17 SCID mice bearing MDA-MB-231 human breast cancer xenografts. Cancer Chemother Pharmacol *55*, 21-32.

El-Deiry, W. S. (2003). The role of p53 in chemosensitivity and radiosensitivity. Oncogene *22*, 7486-7495.

el-Deiry, W. S., Kern, S. E., Pietenpol, J. A., Kinzler, K. W., and Vogelstein, B. (1992). Definition of a consensus binding site for p53. Nat Genet *1*, 45-49.

Enoki, Y., and Sakurai, H. (2011). Diversity in DNA recognition by heat shock transcription factors (HSFs) from model organisms. FEBS Lett *585*, 1293-1298.

Eriksson, M., Jokinen, E., Sistonen, L., and Leppa, S. (2000). Heat shock factor 2 is activated during mouse heart development. The International journal of developmental biology *44*, 471-477.

Eustace, B. K., Sakurai, T., Stewart, J. K., Yimlamai, D., Unger, C., Zehetmeier, C., Lain, B., Torella, C., Henning, S. W., Beste, G.*, et al.* (2004). Functional proteomic screens reveal an essential extracellular role for hsp90 alpha in cancer cell invasiveness. Nat Cell Biol *6*, 507-514.

Eymery, A., Souchier, C., Vourc'h, C., and Jolly, C. (2010). Heat shock factor 1 binds to and transcribes satellite II and III sequences at several pericentromeric regions in heat-shocked cells. Exp Cell Res *316*, 1845-1855.

Fanelli, M. A., Montt-Guevara, M., Diblasi, A. M., Gago, F. E., Tello, O., Cuello-Carrion, F. D., Callegari, E., Bausero, M. A., and Ciocca, D. R. (2008). P-cadherin and beta-catenin are useful prognostic markers in breast cancer patients; beta-catenin interacts with heat shock protein Hsp27. Cell Stress Chaperones 13, 207-220.

Fang, F., Chang, R., and Yang, L. (2011). Heat shock factor 1 promotes invasion and metastasis of hepatocellular carcinoma in vitro and in vivo. Cancer.

Fang, X., Yu, S. X., Lu, Y., Bast, R. C., Jr., Woodgett, J. R., and Mills, G. B. (2000). Phosphorylation and inactivation of glycogen synthase kinase 3 by protein kinase A. Proc Natl Acad Sci U S A *97*, 11960-11965.

Fata, J. E., Werb, Z., and Bissell, M. J. (2004). Regulation of mammary gland branching morphogenesis by the extracellular matrix and its remodeling enzymes. Breast cancer research : BCR *6*, 1-11.

Feige, U., and Polla, B. S. (1994). Hsp70--a multi-gene, multi-structure, multi-function family with potential clinical applications. Experientia *50*, 979-986.

Fernandez-Medarde, A., and Santos, E. (2011). Ras in cancer and developmental diseases. Genes Cancer *2*, 344-358.

Flores, E. R., Sengupta, S., Miller, J. B., Newman, J. J., Bronson, R., Crowley, D., Yang, A., McKeon, F., and Jacks, T. (2005). Tumor predisposition in mice mutant for p63 and p73: evidence for broader tumor suppressor functions for the p53 family. Cancer Cell *7*, 363-373.

Fossati, S., Formentini, L., Wang, Z. Q., Moroni, F., and Chiarugi, A. (2006). Poly(ADP-ribosyl)ation regulates heat shock factor-1 activity and the heat shock response in murine fibroblasts. Biochemistry and cell biology = Biochimie et biologie cellulaire *84*, 703-712.

Franceschelli, S., Rosati, A., Lerose, R., De Nicola, S., Turco, M. C., and Pascale, M. (2008). Bag3 gene expression is regulated by heat shock factor 1. J Cell Physiol *215*, 575-577.

Freed-Pastor, W. A., and Prives, C. (2012). Mutant p53: one name, many proteins. Genes Dev *26*, 1268-1286.

Fritah, S., Col, E., Boyault, C., Govin, J., Sadoul, K., Chiocca, S., Christians, E., Khochbin, S., Jolly, C., and Vourc'h, C. (2009). Heat-shock factor 1 controls genome-wide acetylation in heat-shocked cells. Mol Biol Cell *20*, 4976-4984.

Fujimoto, M., Takaki, E., Hayashi, T., Kitaura, Y., Tanaka, Y., Inouye, S., and Nakai, A. (2005). Active HSF1 significantly suppresses polyglutamine aggregate formation in cellular and mouse models. J Biol Chem *280*, 34908-34916.

Fujimoto, M., Hayashi, T., Katoh, T., Oshima, K., Shinkawa, T., Prakasam, R., Tan, K., Inouye, S., Takii, R., and Nakai, A. (2010). A novel mous HSF3 has the potential to activate nonclassical heat shock genes during heat-shock. Mol Biol Cell *21*, 106-116.

Fujiwara, T., Grimm, E. A., Mukhopadhyay, T., Cai, D. W., Owen-Schaub, L. B., and Roth, J. A. (1993). A retroviral wild-type p53 expression vector penetrates human lung cancer spheroids and inhibits growth by inducing apoptosis. Cancer Res *53*, 4129-4133.

Fulda, S., and Debatin, K. M. (2006). Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. Oncogene *25*, 4798-4811.

Funk, W. D., Pak, D. T., Karas, R. H., Wright, W. E., and Shay, J. W. (1992). A transcriptionally active DNA-binding site for human p53 protein complexes. Mol Cell Biol *12*, 2866-2871.

Gabai, V. L., Budagova, K. R., and Sherman, M. Y. (2005). Increased expression of the major heat shock protein Hsp72 in human prostate carcinoma cells is dispensable for their viability but confers resistance to a variety of anticancer agents. Oncogene *24*, 3328-3338.

Gabai, V. L., Meng, L., Kim, G., Mills, T. A., Benjamin, I. J., and Sherman, M. Y. (2012). Heat shock transcription factor Hsf1 is involved in tumor progression via regulation of hypoxia-inducible factor 1 and RNA-binding protein HuR. Mol Cell Biol *32*, 929-940.

Gabai, V. L., Meriin, A. B., Yaglom, J. A., Volloch, V. Z., and Sherman, M. Y. (1998). Role of Hsp70 in regulation of stress-kinase JNK: implications in apoptosis and aging. FEBS Lett *438*, 1-4.

Gabai, V. L., Yaglom, J. A., Waldman, T., and Sherman, M. Y. (2009). Heat shock protein Hsp72 controls oncogene-induced senescence pathways in cancer cells. Mol Cell Biol *29*, 559-569.

Gaiser, T., Becker, M. R., Meyer, J., Habel, A., and Siegelin, M. D. (2009). p53-mediated inhibition of angiogenesis in diffuse low-grade astrocytomas. Neurochem Int *54*, 458-463.

Garbe, J. C., Holst, C. R., Bassett, E., Tlsty, T., and Stampfer, M. R. (2007). Inactivation of p53 function in cultured human mammary epithelial cells turns the telomere-length dependent senescence barrier from agonescence into crisis. Cell Cycle *6*, 1927-1936.

Garrido, C., Brunet, M., Didelot, C., Zermati, Y., Schmitt, E., and Kroemer, G. (2006). Heat shock proteins 27 and 70: anti-apoptotic proteins with tumorigenic properties. Cell Cycle *5*, 2592-2601.

Ge, P., Ji, X., Ding, Y., Wang, X., Fu, S., Meng, F., Jin, X., Ling, F., and Luo, Y. (2010). Celastrol causes apoptosis and cell cycle arrest in rat glioma cells. Neurol Res *32*, 94-100.

Geng, Y., Fang, M., Wang, J., Yu, H., Hu, Z., Yew, D. T., and Chen, W. (2012). Triptolide down-regulates COX-2 expression and PGE2 release by suppressing the activity of NF-kappaB and MAP kinases in lipopolysaccharide-treated PC12 cells. Phytotherapy research : PTR *26*, 337-343.

Gerber, B., Freund, M., and Reimer, T. (2010). Recurrent breast cancer: treatment strategies for maintaining and prolonging good quality of life. Deutsches Arzteblatt international *107*, 85-91.

Girardini, J. E., Napoli, M., Piazza, S., Rustighi, A., Marotta, C., Radaelli, E., Capaci, V., Jordan, L., Quinlan, P., Thompson, A.*, et al.* (2011). A Pin1/mutant p53 axis promotes aggressiveness in breast cancer. Cancer Cell *20*, 79-91.

Goldstein, N. S., Decker, D., Severson, D., Schell, S., Vicini, F., Margolis, J., and Dekhne, N. S. (2007). Molecular classification system identifies invasive breast carcinoma patients who are most likely and those who are least likely to achieve a complete pathologic response after neoadjuvant chemotherapy. Cancer *110*, 1687-1696.

Gomez-Lazaro, M., Fernandez-Gomez, F. J., and Jordan, J. (2004). p53: twenty five years understanding the mechanism of genome protection. J Physiol Biochem *60*, 287-307.

Gomez, A. V., Galleguillos, D., Maass, J. C., Battaglioli, E., Kukuljan, M., and Andres, M. E. (2008). CoREST represses the heat shock response mediated by HSF1. Mol Cell *31*, 222-231.

Goodson, M. L., and Sarge, K. D. (1995). Heat-inducible DNA binding of purified heat shock transcription factor 1. J Bio Chem *270*, 2447-2450.

Gorska, M., Popowska, U., Sielicka-Dudzin, A., Kuban-Jankowska, A., Sawczuk, W., Knap, N., Cicero, G., and Wozniak, F. (2012). Geldanamycin and its derivatives as Hsp90 inhibitors. Frontiers in bioscience : a journal and virtual library *17*, 2269-2277.

Graham, K., and Olson, M. F. (2007). The ras signalling pathway as a target in cancer therapy. Recent results in cancer research Fortschritte der Krebsforschung Progres dans les recherches sur le cancer *172*, 125-153.

Green, D. R., and Chipuk, J. E. (2006). p53 and metabolism: Inside the TIGAR. Cell *126*, 30-32.

Green, M., Schuetz, T. J., Sullivan, E. K., and Kingston, R. E. (1995). A heat shock-responsive domain of human HSF1 that regulates transcription activation domain function. Mol Cell Biol *15*, 3354-3362.

Greenhough, A., Smartt, H. J., Moore, A. E., Roberts, H. R., Williams, A. C., Paraskeva, C., and Kaidi, A. (2009). The COX-2/PGE2 pathway: key roles in the hallmarks of cancer and adaptation to the tumour microenvironment. Carcinogenesis *30*, 377-386.

Gualberto, A., Aldape, K., Kozakiewicz, K., and Tlsty, T. D. (1998). An oncogenic form of p53 confers a dominant, gain-of-function phenotype that disrupts spindle checkpoint control. Proc Natl Acad Sci U S A *95*, 5166-5171.

Guerra, E., Vacca, G., Palombo, B., and Alberti, S. (2003). Prognostic value of mutations in TP53 and RAS genes in breast cancer. The International journal of biological markers *18*, 49-53.

Guettouche, T., Boellmann, F., Lane, W. S., and Voellmy, R. (2005). Analysis of phosphorylation of human heat shock factor 1 in cells experiencing a stress. BMC biochemistry *6*, 4.

Gurova, K. (2009). New hopes from old drugs: revisiting DNA-binding small molecules as anticancer agents. Future Oncol *5*, 1685-1704.

Gusev, N. B., Bogatcheva, N. V., and Marston, S. B. (2002). Structure and properties of small heat shock proteins (sHsp) and their interaction with cytoskeleton proteins. Biochemistry Biokhimiia *67*, 511-519.

Gyurkocza, B., Plescia, J., Raskett, C. M., Garlick, D. S., Lowry, P. A., Carter, B. Z., Andreeff, M., Meli, M., Colombo, G., and Altieri, D. C. (2006). Antileukemic activity of shepherdin and molecular diversity of hsp90 inhibitors. J Natl Cancer Inst *98*, 1068-1077.

Hadden, M. K., Hill, S. A., Davenport, J., Matts, R. L., and Blagg, B. S. (2009). Synthesis and evaluation of Hsp90 inhibitors that contain the 1,4-naphthoquinone scaffold. Bioorg Med Chem *17*, 634-640.

Hahm, E. R., Moura, M. B., Kelley, E. E., Van Houten, B., Shiva, S., and Singh, S. V. (2011). Withaferin A-induced apoptosis in human breast cancer cells is mediated by reactive oxygen species. PLoS ONE *6*, e23354.

Hahn, J. S., Hu, Z., Thiele, D. J., and Iyer, V. R. (2004). Genome-wide analysis of the biology of stress responses through heat shock transcription factor. Mol Cell Biol *24*, 5249-5256.

Hanahan, D., and Weinberg, R. A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

Hanahan, D., and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646-674.

Hansen, R. K., Oesterreich, S., Lemieux, P., Sarge, K. D., and Fuqua, S. A. (1997). Quercetin inhibits heat shock protein induction but not heat shock factor DNA-binding in human breast carcinoma cells. Biochem Biophys Res Commun *239*, 851-856.

Hargitai, J., Lewis, H., Boros, I., Racz, T., Fiser, A., Kurucz, I., Benjamin, I., Vigh, L., Penzes, Z., Csermely, P., and Latchman, D. S. (2003). Bimoclomol, a heat shock protein co-inducer, acts by the prolonged activation of heat shock factor-1. Biochem Biophys Res Commun *307*, 689-695.

Harrison, C. J., Bohm, A. A., and Nelson, H. C. (1994). Crystal structure of the DNA binding domain of the heat shock transcription factor. Science *263*, 224-227.

Harwood, M., Danielewska-Nikiel, B., Borzelleca, J. F., Flamm, G. W., Williams, G. M., and Lines, T. C. (2007). A critical review of the data related to the safety of quercetin and lack of evidence of in vivo toxicity, including lack of genotoxic/carcinogenic properties. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association *45*, 2179-2205.

Hashikawa, N., Mizukami, Y., Imazu, H., and Sakurai, H. (2006). Mutated yeast heat shock transcription factor activates transcription independently of hyperphosphorylation. J Biol Chem *281*, 3936-3942.

Haupt, S., di Agostino, S., Mizrahi, I., Alsheich-Bartok, O., Voorhoeve, M., Damalas, A., Blandino, G., and Haupt, Y. (2009). Promyelocytic leukemia protein is required for gain of function by mutant p53. Cancer Res *69*, 4818-4826.

Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997). Mdm2 promotes the rapid degradation of p53. Nature *387*, 296-299.

Hayes, D., Napoli, V., Mazurkie, A., Stafford, W. F., and Graceffa, P. (2009). Phosphorylation dependence of hsp27 multimeric size and molecular chaperone function. J Bio Chem *284*, 18801-18807.

He, B., Meng, Y. H., and Mivechi, N. F. (1998). Glycogen synthase kinase 3beta and extracellular signal-regulated kinase inactivate heat shock transcription factor 1 by facilitating the disappearance of transcriptionally active granules after heat shock. Mol Cell Biol *18*, 6624-6633.

He, H., Chen, C., Xie, Y., Asea, A., and Calderwood, S. K. (2000). HSP70 and heat shock factor 1 cooperate to repress Ras-induced transcriptional activation of the c-fos gene. Cell Stress Chaperones *5*, 406-411.

He, H., Soncin, F., Grammatikakis, N., Li, Y., Siganou, A., Gong, J., Brown, S. A., Kingston, R. E., and Calderwood, S. K. (2003). Elevated expression of heat shock factor (HSF) 2A stimulates HSF1-induced transcription during stress. J Biol Chem *278*, 35465-35475.

Heck, J. N., Ponik, S. M., Garcia-Mendoza, M. G., Pehlke, C. A., Inman, D. R., Eliceiri, K. W., and Keely, P. J. (2012). Microtubules regulate GEF-H1 in response to extracellular matrix stiffness. Mol Biol Cell *23*, 2583-2592.

Helton, E. S., and Chen, X. (2007). p53 modulation of the DNA damage response. J Cell Biochem *100*, 883-896.

Hernandez, M. P., Chadli, A., and Toft, D. O. (2002a). HSP40 binding is the first step in the HSP90 chaperoning pathway for the progesterone receptor. J Bio Chem *277*, 11873-11881.

Hernandez, M. P., Sullivan, W. P., and Toft, D. O. (2002b). The assembly and intermolecular properties of the hsp70-Hop-hsp90 molecular chaperone complex. J Bio Chem *277*, 38294-38304.

Hietakangas, V., Ahlskog, J. K., Jakobsson, A. M., Hellesuo, M., Sahlberg, N. M., Holmberg, C. I., Mikhailov, A., Palvimo, J. J., Pirkkala, L., and Sistonen, L. (2003). Phosphorylation of serine 303 is a prerequisite for the stress-inducible SUMO modification of heat shock factor 1. Mol Cell Biol *23*, 2953-2968.

Hightower, L. E. (1980). Cultured animal cells exposed to amino acid analogues or puromycin rapidly synthesize several polypeptides. J Cell Physiol *102*, 407-427.

Hirpara, K. V., Aggarwal, P., Mukherjee, A. J., Joshi, N., and Burman, A. C. (2009). Quercetin and its derivatives: synthesis, pharmacological uses with special emphasis on anti-tumor properties and prodrug with enhanced bio-availability. Anticancer Agents Med Chem *9*, 138-161.

Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. (1991). p53 mutations in human cancers. Science *253*, 49-53.

Holmberg, C. I., Hietakangas, V., Mikhailov, A., Rantanen, J. O., Kallio, M., Meinander, A., Hellman, J., Morrice, N., MacKintosh, C., Morimoto, R. I.*, et al.* (2001). Phosphorylation of serine 230 promotes inducible transcriptional activity of heat shock factor 1. The EMBO journal *20*, 3800-3810.

Holmberg, C. I., Illman, S. A., Kallio, M., Mikhailov, A., and Sistonen, L. (2000). Formation of nuclear HSF1 granules varies depending on stress stimuli. Cell Stress Chaperones *5*, 219-228.

Holmberg, C. I., Tran, S. E., Eriksson, J. E., and Sistonen, L. (2002). Multisite phosphorylation provides sophisticated regulation of transcription factors. Trends Biochem Sci *27*, 619-627.

Hong, S., Kim, S. H., Heo, M. A., Choi, Y. H., Park, M. J., Yoo, M. A., Kim, H. D., Kang, H. S., and Cheong, J. (2004). Coactivator ASC-2 mediates heat shock factor 1-mediated transactivation dependent on heat shock. FEBS Lett *559*, 165-170.

Hong, Y. L., Rogers, R., Matunis, M. J., Mayhew, C. N., Goodson, M., Park-Sarge, O. K., and Sarge, K. D. (2001). Regulation of heat shock transcription factor 1 by stress-induced SUMO-1 modification. J Biol Chem *276*, 40263-40267.

Hsiao, M., Low, J., Dorn, E., Ku, D., Pattengale, P., Yeargin, J., and Haas, M. (1994). Gain-of-function mutations of the p53 gene induce lymphohematopoietic metastatic potential and tissue invasiveness. The American journal of pathology *145*, 702-714.

Hsu, P. P., and Sabatini, D. M. (2008). Cancer cell metabolism: Warburg and beyond. Cell *134*, 703-707.

Hu, H., and Wulf, G. M. (2011). The amplifier effect: how Pin1 empowers mutant p53. Breast cancer research : BCR *13*, 315.

Huang, X., Yang, M., and Jin, J. (2012). Triptolide enhances the sensitivity of multiple myeloma cells to dexamethasone via microRNAs. Leuk Lymphoma *53*, 1188-1195.

Huber, H. J., Dussmann, H., Kilbride, S. M., Rehm, M., and Prehn, J. H. (2011). Glucose metabolism determines resistance of cancer cells to bioenergetic crisis after cytochrome-c release. Mol Syst Biol *7*, 470.

Hungness, E. S., Robb, B. W., Luo, G. J., Hershko, D. D., and Hasselgren, P. O. (2002). Hyperthermia-induced heat shock activates the transcription factor c/EBP-beta and augments IL-6 production in human intestinal epithelial cells. J Am Coll Surg *195*, 619-626.

Hussain, S. P., and Harris, C. C. (2000). Molecular epidemiology and carcinogenesis: endogenous and exogenous carcinogens. Mutat Res *462*, 311-322.

Hussain, S. P., and Harris, C. C. (2007). Inflammation and cancer: an ancient link with novel potentials. International journal of cancer Journal international du cancer *121*, 2373-2380.

Hutchison, C. A., Phillips, S., Edgell, M. H., Gillam, S., Jahnke, P., and Smith, M. (1978). Mutagenesis at a spcecific position in a DNA-sequence. J Biol Chem *253*, 6551-6560.

Hynes, N. E. (2000). Tyrosine kinase signalling in breast cancer. Breast cancer research : BCR *2*, 154-157.

Inoue, H., Uyama, T., Hayashi, J., Watanabe, A., Kobayashi, K., Tadokoro, T., and Yamamoto, Y. (2010). N-Formyl-3,4-methylenedioxy-benzylidene-gamma-butyrolaetam, KNK437 induces caspase-3 activation through inhibition of mTORC1 activity in Cos-1 cells. Biochem Biophys Res Commun *395*, 56-60.

Jacobs, A. T., and Marnett, L. J. (2009). HSF1-mediated BAG3 Expression Attenuates Apoptosis in 4-Hydroxynonenal-treated Colon Cancer Cells via Stabilization of Anti-apoptotic Bcl-2 Proteins. J Biol Chem *284*, 9176-9183.

Jakob, U., Gaestel, M., Engel, K., and Buchner, J. (1993). Small heat shock proteins are molecular chaperones. J Bio Chem *268*, 1517-1520.

Janes, P. W., Daly, R. J., deFazio, A., and Sutherland, R. L. (1994). Activation of the Ras signalling pathway in human breast cancer cells overexpressing erbB-2. Oncogene *9*, 3601-3608.

Jego, G., Hazoume, A., Seigneuric, R., and Garrido, C. (2010). Targeting heat shock proteins in cancer. Cancer Lett.

Jhaveri, K., Taldone, T., Modi, S., and Chiosis, G. (2012). Advances in the clinical development of heat shock protein 90 (Hsp90) inhibitors in cancers. Biochim Biophys Acta *1823*, 742-755.

Jiang, Q., Zhou, C., Bi, Z., and Wan, Y. (2006). EGF-induced cell migration is mediated by ERK and PI3K/AKT pathways in cultured human lens epithelial cells. Journal of ocular pharmacology and therapeutics : the official journal of the Association for Ocular Pharmacology and Therapeutics *22*, 93-102.

Jiang, Z., Jones, R., Liu, J. C., Deng, T., Robinson, T., Chung, P. E., Wang, S., Herschkowitz, J. I., Egan, S. E., Perou, C. M., and Zacksenhaus, E. (2011). RB1 and p53 at the crossroad of EMT and triple-negative breast cancer. Cell Cycle *10*, 1563-1570.

Jin, X., Moskophidis, D., Hu, Y., Phillips, A., and Mivechi, N. F. (2009). Heat Shock Factor 1 Deficiency Via its Downstream Target Gene alpha B-Crystallin (Hspb5) Impairs p53 Degradation. J Cell Biochem *107*, 504-515.

Jin, X., Moskophidis, D., and Mivechi, N. F. (2011). Heat Shock Transcription Factor 1 Is a Key Determinant of HCC Development by Regulating Hepatic Steatosis and Metabolic Syndrome. Cell Metab *14*, 91-103.

Jurivich, D. A., Sistonen, L., Kroes, R. A., and Morimoto, R. I. (1992). Effect of sodium salicylate on the human heat shock response. Science *255*, 1243-1245.

Jurivich, D. A., Sistonen, L., Sarge, K. D., and Morimoto, R. I. (1994). Arachidonate is a potent modulator of human heat shock gene transcription. Proc Natl Acad Sci U S A *91*, 2280-2284.

Kaghad, M., Bonnet, H., Yang, A., Creancier, L., Biscan, J. C., Valent, A., Minty, A., Chalon, P., Lelias, J. M., Dumont, X.*, et al.* (1997). Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. Cell *90*, 809-819.

Kairouz, R., and Daly, R. J. (2000). Tyrosine kinase signalling in breast cancer: modulation of tyrosine kinase signalling in human breast cancer through altered expression of signalling intermediates. Breast cancer research : BCR *2*, 197-202.

Kamal, A., Thao, L., Sensintaffar, J., Zhang, L., Boehm, M. F., Fritz, L. C., and Burrows, F. J. (2003). A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. Nature *425*, 407-410.

Kamath, S. G., Chen, N., Xiong, Y., Wenham, R., Apte, S., Humphrey, M., Cragun, J., and Lancaster, J. M. (2009). Gedunin, a novel natural substance, inhibits ovarian cancer cell proliferation. International journal of gynecological cancer : official journal of the International Gynecological Cancer Society *19*, 1564-1569.

Kannaiyan, R., Shanmugam, M. K., and Sethi, G. (2011). Molecular targets of celastrol derived from Thunder of God Vine: potential role in the treatment of inflammatory disorders and cancer. Cancer Lett *303*, 9-20.

Kaptain, S., Tan, L. K., and Chen, B. (2001). Her-2/neu and breast cancer. Diagnostic molecular pathology : the American journal of surgical pathology, part B *10*, 139-152.

Kastan, M. B., Zhan, Q., el-Deiry, W. S., Carrier, F., Jacks, T., Walsh, W. V., Plunkett, B. S., Vogelstein, B., and Fornace, A. J., Jr. (1992). A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. Cell *71*, 587-597.

Kato, S., Han, S. Y., Liu, W., Otsuka, K., Shibata, H., Kanamaru, R., and Ishioka, C. (2003). Understanding the function-structure and function-mutation relationships of p53 tumor suppressor protein by high-resolution missense mutation analysis. Proc Natl Acad Sci U S A *100*, 8424-8429.

Kattan, Z., Minig, V., Leroy, P., Dauca, M., and Becuwe, P. (2008). Role of manganese superoxide dismutase on growth and invasive properties of human estrogen-independent breast cancer cells. Breast Cancer Res Treat *108*, 203-215.

Kenny, P. A., Lee, G. Y., Myers, C. A., Neve, R. M., Semeiks, J. R., Spellman, P. T., Lorenz, K., Lee, E. H., Barcellos-Hoff, M. H., Petersen, O. W.*, et al.* (2007). The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. Mol Oncol *1*, 84-96.

Khaleque, M. A., Bharti, A., Gong, J., Gray, P. J., Sachdev, V., Ciocca, D. R., Stati, A., Fanelli, M., and Calderwood, S. K. (2008). Heat shock factor 1 represses estrogen-dependent transcription through association with MTA1. Oncogene *27*, 1886-1893.

Khaleque, M. A., Bharti, A., Sawyer, D., Gong, J. L., Benjamin, I. J., Stevenson, M. A., and Calderwood, S. K. (2005). Induction of heat shock proteins by heregulin beta 1 leads to protection from apoptosis and anchorage-independent growth. Oncogene *24*, 6564-6573.

Kieran, D., Kalmar, B., Dick, J. R., Riddoch-Contreras, J., Burnstock, G., and Greensmith, L. (2004). Treatment with arimoclomol, a coinducer of heat shock proteins, delays disease progression in ALS mice. Nat Med *10*, 402-405.

Kim, D. H., Shin, E. K., Kim, Y. H., Lee, B. W., Jun, J. G., Park, J. H., and Kim, J. K. (2009a). Suppression of inflammatory responses by celastrol, a quinone methide triterpenoid isolated from Celastrus regelii. Eur J Clin Invest *39*, 819-827.

Kim, D. Y., Park, J. W., Jeoung, D., and Ro, J. Y. (2009b). Celastrol suppresses allergen-induced airway inflammation in a mouse allergic asthma model. Eur J Pharmacol *612*, 98-105.

Kim, E.-H., Lee, Y.-J., Bae, S., Lee, J. S., Kim, J., and Lee, Y.-S. (2009c). Heat Shock Factor 1-Mediated Aneuploidy Requires a Defective Function of p53. Cancer Res *69*, 9404-9412.

Kim, G., Meriin, A. B., Gabai, V. L., Christians, E., Benjamin, I., Wilson, A., Wolozin, B., and Sherman, M. Y. (2012). The heat shock transcription factor Hsf1 is downregulated in DNA damage-associated senescence, contributing to the maintenance of senescence phenotype. Aging Cell *11*, 617-627.

Kim, H. R., Kang, H. S., and Kim, H. D. (1999). Geldanamycin induces heat shock protein expression through activation of HSF1 in K562 erythroleukemic cells. IUBMB Life *48*, 429-433.

Kim, J. H., and Asthagiri, A. R. (2011). Matrix stiffening sensitizes epithelial cells to EGF and enables the loss of contact inhibition of proliferation. J Cell Sci *124*, 1280-1287.

Kim, S.-A., Kwon, S.-M., Yoon, J.-H., and Ahn, S.-G. (2010). The antitumor effect of PLK1 and HSF1 double knockdown on human oral carcinoma cells. Int J Oncol *36*, 867-872.

Kim, S. A., Yoon, J. H., Lee, S. H., and Ahn, S. G. (2005). Polo-like kinase 1 phosphorylates heat shock transcription factor 1 and mediates its nuclear translocation during heat stress. J Biol Chem *280*, 12653-12657.

Kim, S. H., Hur, W. Y., Kang, C. D., Lim, Y. S., Kim, D. W., and Chung, B. S. (1997). Involvement of heat shock factor in regulating transcriptional activation of MDR1 gene in multidrug-resistant cells. Cancer Lett *115*, 9-14.

Kim, S. H., Yeo, G. S., Lim, Y. S., Kang, C. D., Kim, C. M., and Chung, B. S. (1998). Suppression of multidrug resistance via inhibition of heat shock factor by quercetin in MDR cells. Exp Mol Med *30*, 87-92.

Kingston, R. E., Baldwin, A. S., Jr., and Sharp, P. A. (1984). Regulation of heat shock protein 70 gene expression by c-myc. Nature *312*, 280-282.

Kioka, N., Yamano, Y., Komano, T., and Ueda, K. (1992). Heat-shock responsive elements in the induction of the multidrug resistance gene (MDR1). FEBS Lett *301*, 37-40.

Komarova, E. Y., Afanasyeva, E. A., Bulatova, M. M., Cheetham, M. E., Margulis, B. A., and Guzhova, I. V. (2004). Downstream caspases are novel targets for the antiapoptotic activity of the molecular chaperone hsp70. Cell Stress Chaperones *9*, 265-275.

Kouspou, M. M. (2009) The characterisation of the stress-related transcription factor, HSF1, in breast cancer biology, University of Melbourne.

Kouspou, M. M., and Price, J. T. (2011). Analysis of cellular migration using a two-chamber methodology. Methods in molecular biology (Clifton, NJ) *787*, 303-317.

Krishnamurthy, K., Vedam, K., Kanagasabai, R., Druhan, L. J., and Ilangovan, G. (2012). Heat shock factor-1 knockout induces multidrug resistance gene, MDR1b, and enhances P-

glycoprotein (ABCB1)-based drug extrusion in the heart. Proc Natl Acad Sci U S A *109*, 9023-9028.

Kroeger, P. E., and Morimoto, R. I. (1994). Selection of new HSF1 and HSF2 DNA-binding sites reveals difference in trimer cooperativity. Mol Cell Biol *14*, 7592-7603.

Kroll, T., Odyvanova, L., Clement, J. H., Platzer, C., Naumann, A., Marr, N., Hoffken, K., and Wolfl, S. (2002). Molecular characterization of breast cancer cell lines by expression profiling. J Cancer Res Clin Oncol *128*, 125-134.

Krukenberg, K. A., Bottcher, U. M., Southworth, D. R., and Agard, D. A. (2009). Grp94, the endoplasmic reticulum Hsp90, has a similar solution conformation to cytosolic Hsp90 in the absence of nucleotide. Protein science : a publication of the Protein Society *18*, 1815-1827.

Kubbutat, M. H., Jones, S. N., and Vousden, K. H. (1997). Regulation of p53 stability by Mdm2. Nature *387*, 299-303.

Kugel, J. F., and Goodrich, J. A. (2006). Beating the heat: A translation factor and an RNA mobilize the heat shock transcription factor HSF1. Mol Cell *22*, 153-154.

Lai, A. Y., and Wade, P. A. (2011). Cancer biology and NuRD: a multifaceted chromatin remodelling complex. Nat Rev Cancer *11*, 588-596.

Lane, D. P. (1992). Cancer. p53, guardian of the genome. Nature *358*, 15-16.

Lang, B. J., Nguyen, L., Nguyen, H. C., Vieusseux, J. L., Chai, R. C., Christophi, C., Fifis, T., Kouspou, M. M., and Price, J. T. (2012). Heat stress induces epithelial plasticity and cell migration independent of heat shock factor 1. Cell Stress Chaperones.

Langerod, A., Zhao, H., Borgan, O., Nesland, J. M., Bukholm, I. R., Ikdahl, T., Karesen, R., Borresen-Dale, A. L., and Jeffrey, S. S. (2007). TP53 mutation status and gene expression profiles are powerful prognostic markers of breast cancer. Breast cancer research : BCR *9*, R30.

Larue, L., and Bellacosa, A. (2005). Epithelial-mesenchymal transition in development and cancer: role of phosphatidylinositol 3' kinase/AKT pathways. Oncogene *24*, 7443-7454.

Lecomte, S., Desmots, F., Le Masson, F., Le Goff, P., Michel, D., Christians, E. S., and Le Drean, Y. (2010). Roles of heat shock factor 1 and 2 in response to proteasome inhibition: consequence on p53 stability. Oncogene *29*, 4216-4224.

Lee, B. S., Chen, J., Angelidis, C., Jurivich, D. A., and Morimoto, R. I. (1995). Pharmacological modulation of heat shock factor 1 by antiinflammatory drugs results in protection against stress-induced cellular damage. Proc Natl Acad Sci U S A *92*, 7207-7211.

Lee, G. Y., Kenny, P. A., Lee, E. H., and Bissell, M. J. (2007). Three-dimensional culture models of normal and malignant breast epithelial cells. Nat Methods *4*, 359-365.

Lee, Y. J., and Dewey, W. C. (1987). Induction of heat shock proteins in Chinese hamster ovary cells and development of thermotolerance by intermediate concentrations of puromycin. J Cell Physiol *132*, 1-11.

Lee, Y. J., Kim, E. H., Lee, J. S., Jeoung, D., Bae, S., Kwon, S. H., and Lee, Y. S. (2008a). HSF1 as a mitotic regulator: phosphorylation of HSF1 by Plk1 is essential for mitotic progression. Cancer Res *68*, 7550-7560.

Lee, Y. J., Lee, H. J., Lee, J. S., Jeoung, D., Kang, C. M., Bae, S., Lee, S. J., Kwon, S. H., Kang, D., and Lee, Y. S. (2008b). A novel function for HSF1-induced mitotic exit failure and genomic instability through direct interaction between HSF1 and Cdc20. Oncogene *27*, 2999-3009.

Levental, K. R., Yu, H., Kass, L., Lakins, J. N., Egeblad, M., Erler, J. T., Fong, S. F., Csiszar, K., Giaccia, A., Weninger, W.*, et al.* (2009). Matrix crosslinking forces tumor progression by enhancing integrin signaling. Cell *139*, 891-906.

Li, C. J., Chu, C. Y., Huang, L. H., Wang, M. H., Sheu, L. F., Yeh, J. I., and Hsu, H. Y. (2012a). Synergistic anticancer activity of triptolide combined with cisplatin enhances apoptosis in gastric cancer in vitro and in vivo. Cancer Lett *319*, 203-213.

Li, D., Marchenko, N. D., and Moll, U. M. (2011a). SAHA shows preferential cytotoxicity in mutant p53 cancer cells by destabilizing mutant p53 through inhibition of the HDAC6-Hsp90 chaperone axis. Cell Death Differ *18*, 1904-1913.

Li, D., Marchenko, N. D., Schulz, R., Fischer, V., Velasco-Hernandez, T., Talos, F., and Moll, U. M. (2011b). Functional Inactivation of Endogenous MDM2 and CHIP by HSP90 Causes Aberrant Stabilization of Mutant p53 in Human Cancer Cells. Mol Cancer Res *9*, 577-588.

Li, M., Brooks, C. L., Kon, N., and Gu, W. (2004). A dynamic role of HAUSP in the p53-Mdm2 pathway. Mol Cell *13*, 879-886.

Li, Q., Feldman, R. A., Radhakrishnan, V. M., Carey, S., and Martinez, J. (2008). Hsf1 is required for the nuclear translocation of p53 tumor suppressor. Neoplasia *10*, 1138-1145.

Li, Q., and Martinez, J. D. (2011). P53 is Transported Into the Nucleus Via an Hsf1-Dependent Nuclear Localization Mechanism. Mol Carcinog *50*, 143-152.

Li, S., Wang, Q., Wang, Y., Chen, X., and Wang, Z. (2009). PLC-gamma1 and Rac1 coregulate EGF-induced cytoskeleton remodeling and cell migration. Mol Endocrinol *23*, 901-913.

Li, S. Z., Li, K., Zhang, J. H., and Dong, Z. (2012b). The Effect of Quercetin On Doxorubicin Cytotoxicity in Human Breast Cancer Cells. Anticancer Agents Med Chem.

Li, T., and Sparano, J. A. (2003). Inhibiting Ras signaling in the therapy of breast cancer. Clin Breast Cancer *3*, 405-416; discussion 417-420.

Li, Z., Wu, X., Li, J., Yao, L., Sun, L., Shi, Y., Zhang, W., Lin, J., Liang, D., and Li, Y. (2012c). Antitumor activity of celastrol nanoparticles in a xenograft retinoblastoma tumor model. Int J Nanomedicine *7*, 2389-2398.

Lim, L. Y., Vidnovic, N., Ellisen, L. W., and Leong, C. O. (2009). Mutant p53 mediates survival of breast cancer cells. Br J Cancer *101*, 1606-1612.

Lin, Y. L., Sengupta, S., Gurdziel, K., Bell, G. W., Jacks, T., and Flores, E. R. (2009). p63 and p73 transcriptionally regulate genes involved in DNA repair. PLoS Genet *5*, e1000680.

Lindquist, S. (1986). The heat-shock response. Annu Rev Biochem *55*, 1151-1191.

Lindquist, S., and Craig, E. A. (1988). The heat-shock proteins. Annu Rev Genet *22*, 631-677.

Lis, J. T., Mason, P., Peng, J., Price, D. H., and Werner, J. (2000). P-TEFb kinase recruitment and function at heat shock loci. Genes Dev *14*, 792-803.

Littlefield, O., and Nelson, H. C. (1999). A new use for the 'wing' of the 'winged' helix-turn-helix motif in the HSF-DNA cocrystal. Nat Struct Biol *6*, 464-470.

Liu, J., Shen, M., Yue, Z., Yang, Z., Wang, M., Li, C., Xin, C., Wang, Y., Mei, Q., and Wang, Z. (2012a). Triptolide inhibits colon-rectal cancer cells proliferation by induction of G1 phase arrest through upregulation of p21. Phytomedicine : international journal of phytotherapy and phytopharmacology *19*, 756-762.

Liu, K., Bellam, N., Lin, H. Y., Wang, B., Stockard, C. R., Grizzle, W. E., and Lin, W. C. (2009). Regulation of p53 by TopBP1: a potential mechanism for p53 inactivation in cancer. Mol Cell Biol *29*, 2673-2693.

Liu, K., Ling, S., and Lin, W. C. (2011a). TopBP1 mediates mutant p53 gain of function through NF-Y and p63/p73. Mol Cell Biol *31*, 4464-4481.

Liu, K. C., Yen, C. Y., Wu, R. S., Yang, J. S., Lu, H. F., Lu, K. W., Lo, C., Chen, H. Y., Tang, N. Y., Wu, C. C., and Chung, J. G. (2012b). The roles of endoplasmic reticulum stress and mitochondrial apoptotic signaling pathway in quercetin-mediated cell death of human prostate cancer PC-3 cells. Environ Toxicol.

Liu, X., Qi, W., Cooke, L. S., Kithsiri Wijeratne, E. M., Xu, Y. M., Marron, M. T., Leslie Gunatilaka, A. A., and Mahadevan, D. (2011b). An analog of withaferin A activates the MAPK and glutathione "stress" pathways and inhibits pancreatic cancer cell proliferation. Cancer Invest *29*, 668-675.

Liu, Y., Song, F., Wu, W. K., He, M., Zhao, L., Sun, X., Li, H., Jiang, Y., Yang, Y., and Peng, K. (2012c). Triptolide inhibits colon cancer cell proliferation and induces cleavage and translocation of 14-3-3 epsilon. Cell Biochem Funct *30*, 271-278.

Lo, C. M., Wang, H. B., Dembo, M., and Wang, Y. L. (2000). Cell movement is guided by the rigidity of the substrate. Biophys J *79*, 144-152.

Logan, I. R., McNeill, H. V., Cook, S., Lu, X., Meek, D. W., Fuller-Pace, F. V., Lunec, J., and Robson, C. N. (2009). Heat shock factor-1 modulates p53 activity in the transcriptional response to DNA damage. Nucleic Acids Res *37*, 2962-2973.

Louie, A., Stein, D. S., Zack, J. Z., Liu, W., Conde, H., Fregeau, C., Vanscoy, B. D., and Drusano, G. L. (2011). Dose range evaluation of Mycograb C28Y variant, a human recombinant antibody fragment to heat shock protein 90, in combination with amphotericin B-desoxycholate for treatment of murine systemic candidiasis. Antimicrob Agents Chemother *55*, 3295-3304.

Lu, H., Ouyang, W., and Huang, C. (2006). Inflammation, a key event in cancer development. Molecular cancer research : MCR *4*, 221-233.

Lu, P., Takai, K., Weaver, V. M., and Werb, Z. (2011). Extracellular matrix degradation and remodeling in development and disease. Cold Spring Harb Perspect Biol *3*.

Lu, P., Weaver, V. M., and Werb, Z. (2012). The extracellular matrix: a dynamic niche in cancer progression. The Journal of cell biology *196*, 395-406.

Luck, A. A., Evans, A. J., Green, A. R., Rakha, E. A., Paish, C., and Ellis, I. O. (2008). The influence of basal phenotype on the metastatic pattern of breast cancer. Clin Oncol *20*, 40-45.

Lundy, J., Grimson, R., Mishriki, Y., Chao, S., Oravez, S., Fromowitz, F., and Viola, M. V. (1986). Elevated ras oncogene expression correlates with lymph node metastases in breast cancer patients. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *4*, 1321-1325.

Luo, J., Emanuele, M. J., Li, D., Creighton, C. J., Schlabach, M. R., Westbrook, T. F., Wong, K. K., and Elledge, S. J. (2009). A genome-wide RNAi screen identifies multiple synthetic lethal interactions with the Ras oncogene. Cell *137*, 835-848.

Ma, H., Gong, H., Chen, Z., Liang, Y., Yuan, J., Zhang, G., Wu, J., Ye, Y., Yang, C., Nakai, A., *et al.* (2012). Association of Stat3 with HSF1 plays a critical role in G-CSF-induced cardio-protection against ischemia/reperfusion injury. J Mol Cell Cardiol *52*, 1282-1290.

Malliri, A., van der Kammen, R. A., Clark, K., van der Valk, M., Michiels, F., and Collard, J. G. (2002). Mice deficient in the Rac activator Tiam1 are resistant to Ras-induced skin tumours. Nature *417*, 867-871.

Mambula, S. S., and Calderwood, S. K. (2006). Heat induced release of Hsp70 from prostate carcinoma cells involves both active secretion and passive release from necrotic cells. International journal of hyperthermia : the official journal of European Society for Hyperthermic Oncology, North American Hyperthermia Group *22*, 575-585.

Mansur, C. P. (1997). The regulation and function of the p53 tumor suppressor. Adv Dermatol *13*, 121-166.

Mathew, A., Mathur, S. K., Jolly, C., Fox, S. G., Kim, S., and Morimoto, R. I. (2001). Stress-specific activation and repression of heat shock factors 1 and 2. Mol Cell Biol *21*, 7163-7171.

Mathew, A., Mathur, S. K., and Morimoto, R. I. (1998). Heat shock response and protein degradation: regulation of HSF2 by the ubiquitin-proteasome pathway. Mol Cell Biol *18*, 5091-5098.

Matsumoto, Y., Machida, H., and Kubota, N. (2005). Preferential sensitization of tumor cells to radiation by heat shock protein 90 inhibitor geldanamycin. J Radiat Res (Tokyo) *46*, 215-221.

Matter, W. F., Brown, R. F., and Vlahos, C. J. (1992). The inhibition of phosphatidylinositol 3-kinase by quercetin and analogs. Biochem Biophys Res Commun *186*, 624-631.

Matthews, R. C., Rigg, G., Hodgetts, S., Carter, T., Chapman, C., Gregory, C., Illidge, C., and Burnie, J. (2003). Preclinical assessment of the efficacy of mycograb, a human recombinant antibody against fungal HSP90. Antimicrob Agents Chemother *47*, 2208-2216.

Maye, P., Fu, Y., Butler, D. L., Chokalingam, K., Liu, Y., Floret, J., Stover, M. L., Wenstrup, R., Jiang, X., Gooch, C., and Rowe, D. (2011). Generation and characterization of Col10a1-mcherry reporter mice. Genesis *49*, 410-418.

Mayer, M. P., and Bukau, B. (2005). Hsp70 chaperones: cellular functions and molecular mechanism. Cellular and molecular life sciences : CMLS *62*, 670-684.

Mazurov, V. V., Solovieva, M. E., Leshchenko, V. V., Kruglov, A. G., Edelweiss, E. F., Yakubovskaya, R. I., and Akatov, V. S. (2003). Small heat shock protein hsp27 as a possible mediator of intercellular adhesion-induced drug resistance in human larynx carcinoma HEp-2 cells. Biosci Rep *23*, 187-197.

McCready, J., Sims, J. D., Chan, D., and Jay, D. G. (2010). Secretion of extracellular hsp90alpha via exosomes increases cancer cell motility: a role for plasminogen activation. BMC Cancer *10*, 294.

McDonald, E., Jones, K., Brough, P. A., Drysdale, M. J., and Workman, P. (2006). Discovery and development of pyrazole-scaffold Hsp90 inhibitors. Curr Top Med Chem *6*, 1193-1203.

Meek, D. W., and Knippschild, U. (2003). Posttranslational modification of MDM2. Molecular cancer research : MCR *1*, 1017-1026.

Mendez, F., Sandigursky, M., Kureekattil, R. P., Kenny, M. K., Franklin, W. A., and Bases, R. (2003). Specific stimulation of human apurinic/apyrimidinic endonuclease by heat shock protein 70. DNA Repair *2*, 259-271.

Mendillo, M. L., Santagata, S., Koeva, M., Bell, G. W., Hu, R., Tamimi, R. M., Fraenkel, E., Ince, T. A., Whitesell, L., and Lindquist, S. (2012). HSF1 Drives a Transcriptional Program Distinct from Heat Shock to Support Highly Malignant Human Cancers. Cell *150*, 549-562.

Menendez, D., Inga, A., and Resnick, M. A. (2009). The expanding universe of p53 targets. Nature Reviews Cancer *9*, 724-737.

Meng, L., Gabai, V. L., and Sherman, M. Y. (2010). Heat-shock transcription factor HSF1 has a critical role in human epidermal growth factor receptor-2-induced cellular transformation and tumorigenesis. Oncogene *29*, 5204-5213.

Meng, L., Hunt, C., Yaglom, J. A., Gabai, V. L., and Sherman, M. Y. (2011). Heat shock protein Hsp72 plays an essential role in Her2-induced mammary tumorigenesis. Oncogene *30*, 2836-2845.

Mesa, R. A., Loegering, D., Powell, H. L., Flatten, K., Arlander, S. J., Dai, N. T., Heldebrant, M. P., Vroman, B. T., Smith, B. D., Karp, J. E.*, et al.* (2005). Heat shock protein 90 inhibition sensitizes acute myelogenous leukemia cells to cytarabine. Blood *106*, 318-327.

Mezger, V., Rallu, M., Morimoto, R. I., Morange, M., and Renard, J. P. (1994). Heat shock factor 2-like activity in mouse blastocysts. Dev Biol *166*, 819-822.

Minamoto, T., Buschmann, T., Habelhah, H., Matusevich, E., Tahara, H., Boerresen-Dale, A. L., Harris, C., Sidransky, D., and Ronai, Z. (2001). Distinct pattern of p53 phosphorylation in human tumors. Oncogene *20*, 3341-3347.

Miyazaki, M., Kohno, K., Uchiumi, T., Tanimura, H., Matsuo, K., Nasu, M., and Kuwano, M. (1992). Activation of human multidrug resistance-1 gene promoter in response to heat shock stress. Biochem Biophys Res Commun *187*, 677-684.

Molchadsky, A., Shats, I., Goldfinger, N., Pevsner-Fischer, M., Olson, M., Rinon, A., Tzahor, E., Lozano, G., Zipori, D., Sarig, R., and Rotter, V. (2008). p53 plays a role in mesenchymal differentiation programs, in a cell fate dependent manner. PLoS ONE *3*, e3707.

Morii, T., Ohtsuka, K., Ohnishi, H., Mochizuki, K., and Satomi, K. (2010). Inhibition of heat-shock protein 27 expression eliminates drug resistance of osteosarcoma to zoledronic acid. Anticancer Res *30*, 3565-3571.

Morimoto, R. I. (1998). Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. Genes Dev *12*, 3788-3796.

Morimoto, R. I., Kline, M. P., Bimston, D. N., and Cotto, J. J. (1997). The heat-shock response: regulation and function of heat-shock proteins and molecular chaperones. Essays in Biochemistry, Vol 32, 1997 *32*, 17-29.

Mosser, D. D., Kotzbauer, P. T., Sarge, K. D., and Morimoto, R. I. (1990). In vitro activation of heat shock transcription factor DNA-binding by calcium and biochemical conditions that affect protein conformation. Proc Natl Acad Sci U S A *87*, 3748-3752.

Mou, H., Zheng, Y., Zhao, P., Bao, H., Fang, W., and Xu, N. (2011). Celastrol induces apoptosis in non-small-cell lung cancer A549 cells through activation of mitochondria- and Fas/FasL-mediated pathways. Toxicology in vitro : an international journal published in association with BIBRA *25*, 1027-1032.

Moulin, E., Zoete, V., Barluenga, S., Karplus, M., and Winssinger, N. (2005). Design, synthesis, and biological evaluation of HSP90 inhibitors based on conformational analysis of radicicol and its analogues. J Am Chem Soc *127*, 6999-7004.

Mouneimne, G., Soon, L., DesMarais, V., Sidani, M., Song, X., Yip, S. C., Ghosh, M., Eddy, R., Backer, J. M., and Condeelis, J. (2004). Phospholipase C and cofilin are required for carcinoma cell directionality in response to EGF stimulation. The Journal of cell biology *166*, 697-708.

Munagala, R., Kausar, H., Munjal, C., and Gupta, R. C. (2011). Withaferin A induces p53-dependent apoptosis by repression of HPV oncogenes and upregulation of tumor suppressor proteins in human cervical cancer cells. Carcinogenesis *32*, 1697-1705.

Murshid, A., Chou, S. D., Prince, T., Zhang, Y., Bharti, A., and Calderwood, S. K. (2010). Protein kinase A binds and activates heat shock factor 1. PLoS ONE *5*, e13830.

Murshid, A., Gong, J., Stevenson, M. A., and Calderwood, S. K. (2011). Heat shock proteins and cancer vaccines: developments in the past decade and chaperoning in the decade to come. Expert Rev Vaccines *10*, 1553-1568.

Nadin, S. B., Vargas-Roig, L. M., Cuello-Carrion, F. D., and Ciocca, D. R. (2003). Deoxyribonucleic acid damage induced by doxorubicin in peripheral blood mononuclear cells: possible roles for the stress response and the deoxyribonucleic acid repair process. Cell Stress Chaperones *8*, 361-372.

Nagai, N., Nakai, A., and Nagata, K. (1995). Quercetin suppresses heat shock response by down regulation of HSF1. Biochem Biophys Res Commun *208*, 1099-1105.

Nagarsekar, A., Hasday, J. D., and Singh, I. S. (2005). CXC chemokines: a new family of heat-shock proteins? Immunol Invest *34*, 381-398.

Nagayama, Y., Shigematsu, K., Namba, H., Zeki, K., Yamashita, S., and Niwa, M. (2000). Inhibition of angiogenesis and tumorigenesis, and induction of dormancy by p53 in a p53-null thyroid carcinoma cell line in vivo. Anticancer Res *20*, 2723-2728.

Nakagawa, M., Bando, Y., Nagao, T., Morimoto, M., Takai, C., Ohnishi, T., Honda, J., Moriya, T., Izumi, K., Takahashi, M.*, et al.* (2011). Expression of p53, Ki-67, E-cadherin, N-cadherin and TOP2A in triple-negative breast cancer. Anticancer Res *31*, 2389-2393.

Nakamura, Y., Fujimoto, M., Hayashida, N., Takii, R., Nakai, A., and Muto, M. (2010). Silencing HSF1 by short hairpin RNA decreases cell proliferation and enhances sensitivity to hyperthermia in human melanoma cell lines. J Dermatol Sci *60*, 187-192.

Nakata, Y., Tang, X., and Yokoyama, K. K. (1997). Preparation of competent cells for high-efficiency plasmid transformation of Escherichia coli. Methods Mol Biol *69*, 129-137.

Neckers, L., and Workman, P. (2012). Hsp90 molecular chaperone inhibitors: are we there yet? Clinical cancer research : an official journal of the American Association for Cancer Research *18*, 64-76.

Neef, D. W., Jaeger, A. M., and Thiele, D. J. (2011). Heat shock transcription factor 1 as a therapeutic target in neurodegenerative diseases. Nat Rev Drug Discov *10*, 930-944.

Neef, D. W., Turski, M. L., and Thiele, D. J. (2010). Modulation of Heat Shock Transcription Factor 1 as a Therapeutic Target for Small Molecule Intervention in Neurodegenerative Disease. PLoS Biol *8*.

Negrini, S., Gorgoulis, V. G., and Halazonetis, T. D. (2010). Genomic instability--an evolving hallmark of cancer. Nat Rev Mol Cell Biol *11*, 220-228.

Newton, E. M., Knauf, U., Green, M., and Kingston, R. E. (1996). The regulatory domain of human heat shock factor 1 is sufficient to sense heat stress. Mol Cell Biol *16*, 839-846.

Newton, K., and Vucic, D. (2007). Ubiquitin ligases in cancer: ushers for degradation. Cancer Invest *25*, 502-513.

Neznanov, N., Gorbachev, A. V., Neznanova, L., Komarov, A. P., Gurova, K. V., Gasparian, A. V., Banerjee, A. K., Almasan, A., Fairchild, R. L., and Gudkov, A. V. (2009). Anti-malaria drug blocks proteotoxic stress response Anti-cancer implications. Cell Cycle *8*, 3960-3970.

Ni, Z., Saunders, A., Fuda, N. J., Yao, J., Suarez, J. R., Webb, W. W., and Lis, J. T. (2008). P-TEFb is critical for the maturation of RNA polymerase II into productive elongation in vivo. Mol Cell Biol *28*, 1161-1170.

Ni, Z., Schwartz, B. E., Werner, J., Suarez, J. R., and Lis, J. T. (2004). Coordination of transcription, RNA processing, and surveillance by P-TEFb kinase on heat shock genes. Mol Cell *13*, 55-65.

Nielsen, T. O., Andrews, H. N., Cheang, M., Kucab, J. E., Hsu, F. D., Ragaz, J., Gilks, C. B., Makretsov, N., Bajdik, C. D., Brookes, C.*, et al.* (2004). Expression of the insulin-like growth factor I receptor and urokinase plasminogen activator in breast cancer is associated with poor survival: potential for intervention with 17-allylamino geldanamycin. Cancer Res *64*, 286-291.

O'Brien, D. I., Nally, K., Kelly, R. G., O'Connor, T. M., Shanahan, F., and O'Connell, J. (2005). Targeting the Fas/Fas ligand pathway in cancer. Expert Opin Ther Targets *9*, 1031-1044.

O'Callaghan-Sunol, C., Gabai, V. L., and Sherman, M. Y. (2007). Hsp27 modulates p53 signaling and suppresses cellular senescence. Cancer Res *67*, 11779-11788.

O'Callaghan-Sunol, C., and Sherman, M. Y. (2006). Heat shock transcription factor (HSF1) plays a critical role in cell migration via maintaining MAP kinase signaling. Cell Cycle *5*, 1431-1437.

Oesterreich, S., Weng, C. N., Qiu, M., Hilsenbeck, S. G., Osborne, C. K., and Fuqua, S. A. (1993). The small heat shock protein hsp27 is correlated with growth and drug resistance in human breast cancer cell lines. Cancer Res *53*, 4443-4448.

Ohnishi, K., Yokota, S., Takahashi, A., and Ohnishi, T. (2006). Induction of radiation resistance by a heat shock protein inhibitor, KNK437, in human glioblastoma cells. Int J Radiat Biol *82*, 569-575.

Ohno, K., Fukushima, M., Fujiwara, M., and Narumiya, S. (1988). Induction of 68,000-dalton heat shock proteins by cyclopentenone prostaglandins. Its association with prostaglandin-induced G1 block in cell cycle progression. J Biol Chem *263*, 19764-19770.

Olsen, J. V., Blagoev, B., Gnad, F., Macek, B., Kumar, C., Mortensen, P., and Mann, M. (2006). Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. Cell *127*, 635-648.

Oommen, D., and Prise, K. M. (2012). KNK437, abrogates hypoxia-induced radioresistance by dual targeting of the AKT and HIF-1alpha survival pathways. Biochem Biophys Res Commun *421*, 538-543.

Oren, M., and Rotter, V. (2010). Mutant p53 gain-of-function in cancer. Cold Spring Harb Perspect Biol *2*, a001107.

Osaki, M., Oshimura, M., and Ito, H. (2004). PI3K-Akt pathway: its functions and alterations in human cancer. Apoptosis : an international journal on programmed cell death *9*, 667-676.

Osipiuk, J., Walsh, M. A., Freeman, B. C., Morimoto, R. I., and Joachimiak, A. (1999). Structure of a new crystal form of human Hsp70 ATPase domain. Acta Crystallogr D Biol Crystallogr *55*, 1105-1107.

Ostling, P., Bjork, J. K., Roos-Mattjus, P., Mezger, V., and Sistonen, L. (2007). Heat shock factor 2 (HSF2) contributes to inducible expression of hsp genes through interplay with HSF1. J Biol Chem *282*, 7077-7086.

Paddison, P. J., Cleary, M., Silva, J. M., Chang, K., Sheth, N., Sachidanandam, R., and Hannon, G. J. (2004). Cloning of short hairpin RNAs for gene knockdown in mammalian cells. Nat Methods *1*, 163-167.

Page, T. J., Sikder, D., Yang, L., Pluta, L., Wolfinger, R. D., Kodadek, T., and Thomas, R. S. (2006). Genome-wide analysis of human HSF1 signaling reveals a transcriptional program linked to cellular adaptation and survival. Mol Biosyst *2*, 627-639.

Panaretou, B., Prodromou, C., Roe, S. M., O'Brien, R., Ladbury, J. E., Piper, P. W., and Pearl, L. H. (1998). ATP binding and hydrolysis are essential to the function of the Hsp90 molecular chaperone in vivo. The EMBO journal *17*, 4829-4836.

Pandey, P., Saleh, A., Nakazawa, A., Kumar, S., Srinivasula, S. M., Kumar, V., Weichselbaum, R., Nalin, C., Alnemri, E. S., Kufe, D., and Kharbanda, S. (2000). Negative regulation of cytochrome c-mediated oligomerization of Apaf-1 and activation of procaspase-9 by heat shock protein 90. The EMBO journal *19*, 4310-4322.

Park, H. S., Lee, J. S., Huh, S. H., Seo, J. S., and Choi, E. J. (2001a). Hsp72 functions as a natural inhibitory protein of c-Jun N-terminal kinase. The EMBO journal *20*, 446-456.

Park, J. M., Kim, J. M., Kim, L. K., Kim, S. N., Kim-Ha, J., Kim, J. H., and Kim, Y. J. (2003). Signal-induced transcriptional activation by Dif requires the dTRAP80 mediator module. Mol Cell Biol *23*, 1358-1367.

Park, J. M., Werner, J., Kim, J. M., Lis, J. T., and Kim, Y. J. (2001b). Mediator, not holoenzyme, is directly recruited to the heat shock promoter by HSF upon heat shock. Mol Cell *8*, 9-19.

Paszek, M. J., Zahir, N., Johnson, K. R., Lakins, J. N., Rozenberg, G. I., Gefen, A., Reinhart-King, C. A., Margulies, S. S., Dembo, M., Boettiger, D.*, et al.* (2005). Tensional homeostasis and the malignant phenotype. Cancer Cell *8*, 241-254.

Paul, C., Manero, F., Gonin, S., Kretz-Remy, C., Virot, S., and Arrigo, A. P. (2002). Hsp27 as a negative regulator of cytochrome C release. Mol Cell Biol *22*, 816-834.

Pearl, L. H., and Prodromou, C. (2000). Structure and in vivo function of Hsp90. Curr Opin Struct Biol *10*, 46-51.

Pearl, L. H., and Prodromou, C. (2001). Structure, function, and mechanism of the Hsp90 molecular chaperone. Adv Protein Chem *59*, 157-186.

Perou, C. M., Sorlie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. A., Pollack, J. R., Ross, D. T., Johnsen, H., Akslen, L. A.*, et al.* (2000). Molecular portraits of human breast tumours. Nature *406*, 747-752.

Peteranderl, R., and Nelson, H. C. (1992). Trimerization of the heat shock transcription factor by a triple-stranded alpha-helical coiled-coil. Biochemistry (Mosc) *31*, 12272-12276.

Peteranderl, R., Rabenstein, M., Shin, Y. K., Liu, C. W., Wemmer, D. E., King, D. S., and Nelson, H. C. (1999). Biochemical and biophysical characterization of the trimerization domain from the heat shock transcription factor. Biochemistry (Mosc) *38*, 3559-3569.

Peters, J. M. (2006). The anaphase promoting complex/cyclosome: a machine designed to destroy. Nat Rev Mol Cell Biol *7*, 644-656.

Petitjean, A., Mathe, E., Kato, S., Ishioka, C., Tavtigian, S. V., Hainaut, P., and Olivier, M. (2007). Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database. Hum Mutat *28*, 622-629.

Peyssonnaux, C., and Eychene, A. (2001). The Raf/MEK/ERK pathway: new concepts of activation. Biology of the cell / under the auspices of the European Cell Biology Organization *93*, 53-62.

Pirkkala, L., Alastalo, T. P., Nykanen, P., Seppa, L., and Sistonen, L. (1999). Differentiation lineage-specific expression of human heat shock transcription factor 2. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *13*, 1089-1098.

Pirkkala, L., Nykanen, P., and Sistonen, L. (2001). Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *15*, 1118-1131.

Plescia, J., Salz, W., Xia, F., Pennati, M., Zaffaroni, N., Daidone, M. G., Meli, M., Dohi, T., Fortugno, P., Nefedova, Y.*, et al.* (2005). Rational design of shepherdin, a novel anticancer agent. Cancer Cell *7*, 457-468.

Price, J. T., Quinn, J. M., Sims, N. A., Vieusseux, J., Waldeck, K., Docherty, S. E., Myers, D., Nakamura, A., Waltham, M. C., Gillespie, M. T., and Thompson, E. W. (2005). The heat shock protein 90 inhibitor, 17-allylamino-17-demethoxygeldanamycin, enhances osteoclast formation and potentiates bone metastasis of a human breast cancer cell line. Cancer Res *65*, 4929-4938.

Price, J. T., and Thompson, E. W. (2002). Mechanisms of tumour invasion and metastasis: emerging targets for therapy. Expert Opin Ther Targets *6*, 217-233.

Prodromou, C. (2012). The 'active life' of Hsp90 complexes. Biochim Biophys Acta *1823*, 614-623.

Prodromou, C., Roe, S. M., O'Brien, R., Ladbury, J. E., Piper, P. W., and Pearl, L. H. (1997). Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. Cell *90*, 65-75.

Pylayeva-Gupta, Y., Grabocka, E., and Bar-Sagi, D. (2011). RAS oncogenes: weaving a tumorigenic web. Nat Rev Cancer *11*, 761-774.

Qin, Q., Baudry, M., Liao, G., Noniyev, A., Galeano, J., and Bi, X. (2009). A novel function for p53: regulation of growth cone motility through interaction with Rho kinase. The Journal of neuroscience : the official journal of the Society for Neuroscience *29*, 5183-5192.

Rabindran, S. K., Haroun, R. I., Clos, J., Wisniewski, J., and Wu, C. (1993). Regulation of heat shock factor trimer formation: role of a conserved leucine zipper. Science *259*, 230-234.

Rahko, E., Blanco, G., Soini, Y., Bloigu, R., and Jukkola, A. (2003). A mutant TP53 gene status is associated with a poor prognosis and anthracycline-resistance in breast cancer patients. Eur J Cancer *39*, 447-453.

Ramocki, M. B., White, M. A., Konieczny, S. F., and Taparowsky, E. J. (1998). A role for RalGDS and a novel Ras effector in the Ras-mediated inhibition of skeletal myogenesis. J Bio Chem *273*, 17696-17701.

Resnick, M. A., and Inga, A. (2003). Functional mutants of the sequence-specific transcription factor p53 and implications for master genes of diversity. Proc Natl Acad Sci U S A *100*, 9934-9939.

Ribatti, D., and Djonov, V. (2011). Angiogenesis in development and cancer today. The International journal of developmental biology *55*, 343-344.

Ridley, A. J., Schwartz, M. A., Burridge, K., Firtel, R. A., Ginsberg, M. H., Borisy, G., Parsons, J. T., and Horwitz, A. R. (2003). Cell migration: integrating signals from front to back. Science *302*, 1704-1709.

Riley, T., Sontag, E., Chen, P., and Levine, A. (2008). Transcriptional control of human p53-regulated genes. Nat Rev Mol Cell Biol *9*, 402-412.

Robinson, M., Jiang, P., Cui, J., Li, J., Wang, Y., Swaroop, M., Madore, S., Lawrence, T. S., and Sun, Y. (2003). Global genechip profiling to identify genes responsive to p53-induced growth arrest and apoptosis in human lung carcinoma cells. Cancer Biol Ther *2*, 406-415.

Rochlitz, C. F., Scott, G. K., Dodson, J. M., Liu, E., Dollbaum, C., Smith, H. S., and Benz, C. C. (1989). Incidence of activating ras oncogene mutations associated with primary and metastatic human breast cancer. Cancer Res *49*, 357-360.

Rodrigues, L. M., Chung, Y. L., Al Saffar, N. M., Sharp, S. Y., Jackson, L. E., Banerji, U., Stubbs, M., Leach, M. O., Griffiths, J. R., and Workman, P. (2012). Effects of HSP90 inhibitor 17-allylamino-17- demethoxygeldanamycin (17-AAG) on NEU/HER2 overexpressing mammary tumours in MMTV-NEUNT mice monitored by Magnetic Resonance Spectroscopy. BMC Res Notes *5*, 250.

Roemer, K. (1999). Mutant p53: gain-of-function oncoproteins and wild-type p53 inactivators. Biol Chem *380*, 879-887.

Roger, L., Gadea, G., and Roux, P. (2006). Control of cell migration: a tumour suppressor function for p53? Biology of the cell / under the auspices of the European Cell Biology Organization *98*, 141-152.

Rokavec, M., Schroth, W., Amaral, S. M., Fritz, P., Antoniadou, L., Glavac, D., Simon, W., Schwab, M., Eichelbaum, M., and Brauch, H. (2008). A polymorphism in the TC21 promoter associates with an unfavorable tamoxifen treatment outcome in breast cancer. Cancer Res *68*, 9799-9808.

Rokavec, M., Wu, W., and Luo, J. L. (2012). IL6-mediated suppression of miR-200c directs constitutive activation of inflammatory signaling circuit driving transformation and tumorigenesis. Mol Cell *45*, 777-789.

Rosati, A., Graziano, V., De Laurenzi, V., Pascale, M., and Turco, M. C. (2011). BAG3: a multifaceted protein that regulates major cell pathways. Cell Death Dis *2*, e141.

Rossi, A., Coccia, M., Trotta, E., Angelini, M., and Santoro, M. G. (2012). Regulation of cyclooxygenase-2 expression by heat: a novel aspect of heat shock factor 1 function in human cells. PLoS ONE *7*, e31304.

Rossi, A., Elia, G., and Santoro, M. G. (1998). Activation of the heat shock factor 1 by serine protease inhibitors. An effect associated with nuclear factor-kappaB inhibition. J Biol Chem *273*, 16446-16452.

Ruijter, J. M., Ramakers, C., Hoogaars, W. M. H., Karlen, Y., Bakker, O., van den Hoff, M. J. B., and Moorman, A. F. M. (2009). Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Res *37*.

Ryan, K. M., Phillips, A. C., and Vousden, K. H. (2001). Regulation and function of the p53 tumor suppressor protein. Curr Opin Cell Biol *13*, 332-337.

Sahin, E., Sahin, M., Sanlioglu, A. D., and Gumuslu, S. (2011). KNK437, a benzylidene lactam compound, sensitises prostate cancer cells to the apoptotic effect of hyperthermia. International journal of hyperthermia : the official journal of European Society for Hyperthermic Oncology, North American Hyperthermia Group *27*, 63-73.

Sakurai, H., Kitamoto, Y., Saitoh, J. I., Nonaka, T., Ishikawa, H., Kiyohara, H., Shioya, M., Fukushima, M., Akimoto, T., Hasegawa, M., and Nakano, T. (2005). Attenuation of chronic thermotolerance by KNK437, a benzylidene lactam compound, enhances thermal

radiosensitization in mild temperature hyperthermia combined with low dose-rate irradiation. Int J Radiat Biol *81*, 711-718.

Sakurai, H., and Takemori, Y. (2007). Interaction between heat shock transcription factors (HSFs) and divergent binding sequences: binding specificities of yeast HSFs and human HSF1. J Bio Chem *282*, 13334-13341.

Saleh, A., Srinivasula, S. M., Balkir, L., Robbins, P. D., and Alnemri, E. S. (2000). Negative regulation of the Apaf-1 apoptosome by Hsp70. Nat Cell Biol *2*, 476-483.

Salmand, P. A., Jungas, T., Fernandez, M., Conter, A., and Christians, E. S. (2008). Mouse Heat-Shock Factor 1 (HSF1) Is Involved in Testicular Response to Genotoxic Stress Induced by Doxorubicin. Biol Reprod *79*, 1092-1101.

Salminen, A., Lehtonen, M., Paimela, T., and Kaarniranta, K. (2010). Celastrol: Molecular targets of Thunder God Vine. Biochem Biophys Res Commun *394*, 439-442.

Sandqvist, A., Bjork, J. K., Akerfelt, M., Chitikova, Z., Grichine, A., Vourc'h, C., Jolly, C., Salminen, T. A., Nymalm, Y., and Sistonen, L. (2009). Heterotrimerization of heat-shock factors 1 and 2 provides a transcriptional switch in response to distinct stimuli. Mol Biol Cell *20*, 1340-1347.

Santagata, S., Hu, R., Lin, N. U., Mendillo, M. L., Collins, L. C., Hankinson, S. E., Schnitt, S. J., Whitesell, L., Tamimi, R. M., Lindquist, S., and Ince, T. A. (2011). High levels of nuclear heat-shock factor 1 (HSF1) are associated with poor prognosis in breast cancer. Proc Natl Acad Sci U S A *108*, 18378-18383.

Santagata, S., Xu, Y. M., Wijeratne, E. M., Kontnik, R., Rooney, C., Perley, C. C., Kwon, H., Clardy, J., Kesari, S., Whitesell, L.*, et al.* (2012). Using the heat-shock response to discover anticancer compounds that target protein homeostasis. ACS Chem Biol *7*, 340-349.

Santoro, N., Johansson, N., and Thiele, D. J. (1998). Heat shock element architecture is an important determinant in the temperature and transactivation domain requirements for heat shock transcription factor. Mol Cell Biol *18*, 6340-6352.

Santos, C. R., and Schulze, A. (2012). Lipid metabolism in cancer. The FEBS journal *279*, 2610-2623.

Sarge, K. D., Murphy, S. P., and Morimoto, R. I. (1993). Activation of heat shock gene transcription by heat shock factor 1 involves oligomerization, acquisition of DNA-binding activity, and nuclear localization and can occur in the absence of stress. Mol Cell Biol *13*, 1392-1407.

Sarge, K. D., Park-Sarge, O. K., Kirby, J. D., Mayo, K. E., and Morimoto, R. I. (1994). Expression of heat shock factor 2 in mouse testis: potential role as a regulator of heat-shock protein gene expression during spermatogenesis. Biol Reprod *50*, 1334-1343.

Satyal, S. H., Chen, D., Fox, S. G., Kramer, J. M., and Morimoto, R. I. (1998). Negative regulation of the heat shock transcriptional response by HSBP1. Genes Dev *12*, 1962-1974.

Sawai, A., Chandarlapaty, S., Greulich, H., Gonen, M., Ye, Q., Arteaga, C. L., Sellers, W., Rosen, N., and Solit, D. B. (2008). Inhibition of Hsp90 down-regulates mutant epidermal growth factor receptor (EGFR) expression and sensitizes EGFR mutant tumors to paclitaxel. Cancer Res *68*, 589-596.

Schmitt, E., Gehrmann, M., Brunet, M., Multhoff, G., and Garrido, C. (2007). Intracellular and extracellular functions of heat shock proteins: repercussions in cancer therapy. J Leukoc Biol *81*, 15-27.

Scholl, C., Frohling, S., Dunn, I. F., Schinzel, A. C., Barbie, D. A., Kim, S. Y., Silver, S. J., Tamayo, P., Wadlow, R. C., Ramaswamy, S.*, et al.* (2009). Synthetic lethal interaction between oncogenic KRAS dependency and STK33 suppression in human cancer cells. Cell *137*, 821-834.

Seliger, B. (2005). Strategies of tumor immune evasion. BioDrugs : clinical immunotherapeutics, biopharmaceuticals and gene therapy *19*, 347-354.

Seo, H. R., Chung, D. Y., Lee, Y. J., Lee, D. H., Kim, J. I., Bae, S., Chung, H. Y., Lee, S. J., Jeoung, D., and Lee, Y. S. (2006). Heat shock protein 25 or inducible heat shock protein 70 activates heat shock factor 1 - Dephosphorylation on serine 307 through inhibition of ERK1/2 phosphorylation. J Biol Chem *281*, 17220-17227.

Seo, M. S., Oh, S. Y., Park, M. J., Kim, S. M., Kim, M. Y., Han, S. I., Park, H. G., and Kang, H. S. (2005). Implication of reactive oxygen species, ERK1/2, and p38MAPK in sodium salicylate-induced heat shock protein 72 expression in C6 glioma cells. Int J Mol Med *16*, 841-849.

Shamovsky, I., Ivannikov, M., Kandel, E. S., Gershon, D., and Nudler, E. (2006). RNA-mediated response to heat shock in mammalian cells. Nature *440*, 556-560.

Shaner, N. C., Campbell, R. E., Steinbach, P. A., Giepmans, B. N., Palmer, A. E., and Tsien, R. Y. (2004). Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. Nat Biotechnol *22*, 1567-1572.

Sheng, Y., Saridakis, V., Sarkari, F., Duan, S., Wu, T., Arrowsmith, C. H., and Frappier, L. (2006). Molecular recognition of p53 and MDM2 by USP7/HAUSP. Nat Struct Mol Biol *13*, 285-291.

Sherman, M. (2010). Major heat shock protein Hsp72 controls oncogene-induced senescence. Ann N Y Acad Sci *1197*, 152-157.

Shien, T., Doihara, H., Hara, H., Takahashi, H., Yoshitomi, S., Taira, N., Ishibe, Y., Teramoto, J., Aoe, M., and Shimizu, N. (2004). PLC and PI3K pathways are important in the inhibition of EGF-induced cell migration by gefitinib ('Iressa', ZD1839). Breast Cancer *11*, 367-373.

Shunmei, E., Zhao, Y., Huang, Y., Lai, K., Chen, C., Zeng, J., and Zou, J. (2010). Heat shock factor 1 is a transcription factor of Fas gene. Mol Cells *29*, 527-531.

Sidera, K., Gaitanou, M., Stellas, D., Matsas, R., and Patsavoudi, E. (2008). A critical role for HSP90 in cancer cell invasion involves interaction with the extracellular domain of HER-2. J Bio Chem *283*, 2031-2041.

Siegel, R., Naishadham, D., and Jemal, A. (2012). Cancer statistics, 2012. CA Cancer J Clin *62*, 10-29.

Simioni, M. B., De Thonel, A., Hammann, A., Joly, A. L., Bossis, G., Fourmaux, E., Bouchot, A., Landry, J., Piechaczyk, M., and Garrido, C. (2009). Heat shock protein 27 is involved in SUMO-2/3 modification of heat shock factor 1 and thereby modulates the transcription factor activity. Oncogene *28*, 3332-3344.

Sims, J. D., McCready, J., and Jay, D. G. (2011). Extracellular heat shock protein (Hsp)70 and Hsp90alpha assist in matrix metalloproteinase-2 activation and breast cancer cell migration and invasion. PLoS ONE *6*, e18848.

Singh, I. S., Gupta, A., Nagarsekar, A., Cooper, Z., Manka, C., Hester, L., Benjamin, I. J., He, J. R., and Hasday, J. D. (2008). Heat shock co-activates interleukin-8 transcription. Am J Respir Cell Mol Biol *39*, 235-242.

Singh, I. S., He, J. R., Calderwood, S., and Hasday, J. D. (2002). A high affinity HSF-1 binding site in the 5'-untranslated region of the murine tumor necrosis factor-alpha gene is a transcriptional repressor. J Bio Chem *277*, 4981-4988.

Singh, K., Mogare, D., Giridharagopalan, R. O., Gogiraju, R., Pande, G., and Chattopadhyay, S. (2007). p53 target gene SMAR1 is dysregulated in breast cancer: its role in cancer cell migration and invasion. PLoS ONE *2*, e660.

Sinkus, R., Lorenzen, J., Schrader, D., Lorenzen, M., Dargatz, M., and Holz, D. (2000). High-resolution tensor MR elastography for breast tumour detection. Phys Med Biol *45*, 1649-1664.

Sircoulomb, F., Bekhouche, I., Finetti, P., Adelaide, J., Ben Hamida, A., Bonansea, J., Raynaud, S., Innocenti, C., Charafe-Jauffret, E., Tarpin, C*., et al.* (2010). Genome profiling of ERBB2-amplified breast cancers. BMC Cancer *10*, 539.

Skaggs, H. S., Xing, H., Wilkerson, D. C., Murphy, L. A., Hong, Y., Mayhew, C. N., and Sarge, K. D. (2007). HSF1-TPR interaction facilitates export of stress-induced HSP70 mRNA. J Bio Chem *282*, 33902-33907.

Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., Bokesch, H., Kenney, S., and Boyd, M. R. (1990). New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst *82*, 1107-1112.

Smith, V., Hobbs, S., Court, W., Eccles, S., Workman, P., and Kelland, L. R. (2002). ErbB2 overexpression in an ovarian cancer cell line confers sensitivity to the HSP90 inhibitor geldanamycin. Anticancer Res *22*, 1993-1999.

Solimini, N. L., Luo, J., and Elledge, S. J. (2007). Non-oncogene addiction and the stress phenotype of cancer cells. Cell *130*, 986-988.

Solomon, H., Madar, S., and Rotter, V. (2011). Mutant p53 gain of function is interwoven into the hallmarks of cancer. The Journal of pathology *225*, 475-478.

Somasundaram, K. (2000). Tumor suppressor p53: regulation and function. Frontiers in bioscience : a journal and virtual library *5*, D424-437.

Soncin, F., Zhang, X., Chu, B., Wang, X., Asea, A., Ann Stevenson, M., Sacks, D. B., and Calderwood, S. K. (2003). Transcriptional activity and DNA binding of heat shock factor-1 involve phosphorylation on threonine 142 by CK2. Biochem Biophys Res Commun *303*, 700-706.

Sondermann, H., Becker, T., Mayhew, M., Wieland, F., and Hartl, F. U. (2000). Characterization of a receptor for heat shock protein 70 on macrophages and monocytes. Biol Chem *381*, 1165-1174.

Song, H., Hollstein, M., and Xu, Y. (2007). p53 gain-of-function cancer mutants induce genetic instability by inactivating ATM. Nat Cell Biol *9*, 573-580.

Song, S., Kole, S., Precht, P., Pazin, M. J., and Bernier, M. (2010). Activation of heat shock factor 1 plays a role in pyrrolidine dithiocarbamate-mediated expression of the co-chaperone BAG3. The international journal of biochemistry & cell biology *42*, 1856-1863.

Sorger, P. K. (1990). Yeast heat shock factor contains separable transient and sustained response transcriptional activators. Cell *62*, 793-805.

Sorlie, T. (2004). Molecular portraits of breast cancer: tumour subtypes as distinct disease entities. Eur J Cancer *40*, 2667-2675.

Sotiriou, C., Neo, S. Y., McShane, L. M., Korn, E. L., Long, P. M., Jazaeri, A., Martiat, P., Fox, S. B., Harris, A. L., and Liu, E. T. (2003). Breast cancer classification and prognosis based on

gene expression profiles from a population-based study. Proc Natl Acad Sci U S A *100*, 10393-10398.

Spandidos, D. A. (1987). Oncogene activation in malignant transformation: a study of H-ras in human breast cancer. Anticancer Res *7*, 991-996.

Sprang, S. R. (1997). G protein mechanisms: insights from structural analysis. Annu Rev Biochem *66*, 639-678.

Sreeramulu, S., Gande, S. L., Gobel, M., and Schwalbe, H. (2009). Molecular mechanism of inhibition of the human protein complex Hsp90-Cdc37, a kinome chaperone-cochaperone, by triterpene celastrol. Angewandte Chemie *48*, 5853-5855.

Stamatakos, M., Stefanaki, C., Kontzoglou, K., Masouridi, S., Sakorafas, G., and Safioleas, M. (2010). Recapitulation of ras oncogene mutations in breast cancer. Onkologie *33*, 540-544.

Stanhill, A., Levin, V., Hendel, A., Shachar, I., Kazanov, D., Arber, N., Kaminski, N., and Engelberg, D. (2006). Ha-ras(val12) induces HSP70b transcription via the HSE/HSF1 system, but HSP70b expression is suppressed in Ha-ras(val12)-transformed cells. Oncogene *25*, 1485-1495.

Stankiewicz, A. R., Lachapelle, G., Foo, C. P., Radicioni, S. M., and Mosser, D. D. (2005). Hsp70 inhibits heat-induced apoptosis upstream of mitochondria by preventing Bax translocation. J Bio Chem *280*, 38729-38739.

Stanulis-Praeger, B. M. (1987). Cellular senescence revisited: a review. Mech Ageing Dev *38*, 1-48.

Storti, R. V., Scott, M. P., Rich, A., and Pardue, M. L. (1980). Translational control of protein synthesis in response to heat shock in D. melanogaster cells. Cell *22*, 825-834.

Strano, S., Dell'Orso, S., Di Agostino, S., Fontemaggi, G., Sacchi, A., and Blandino, G. (2007a). Mutant p53: an oncogenic transcription factor. Oncogene *26*, 2212-2219.

Strano, S., Dell'Orso, S., Mongiovi, A. M., Monti, O., Lapi, E., Di Agostino, S., Fontemaggi, G., and Blandino, G. (2007b). Mutant p53 proteins: between loss and gain of function. Head Neck *29*, 488-496.

Strumane, K., Rygiel, T. P., and Collard, J. G. (2006). The Rac activator Tiam1 and Ras-induced oncogenesis. Methods Enzymol *407*, 269-281.

Sullivan, E. K., Weirich, C. S., Guyon, J. R., Sif, S., and Kingston, R. E. (2001). Transcriptional activation domains of human heat shock factor 1 recruit human SWI/SNF. Mol Cell Biol *21*, 5826-5837.

Sun, L., Zhang, S., Jiang, Z., Huang, X., Wang, T., Li, H., and Zhang, L. (2011). Triptolide inhibits COX-2 expression by regulating mRNA stability in TNF-alpha-treated A549 cells. Biochem Biophys Res Commun *416*, 99-105.

Sung, B., Park, B., Yadav, V. R., and Aggarwal, B. B. (2010). Celastrol, a Triterpene, Enhances TRAIL-induced Apoptosis through the Down-regulation of Cell Survival Proteins and Up-regulation of Death Receptors. J Biol Chem *285*, 11498-11507.

Taba, K., Kuramitsu, Y., Ryozawa, S., Yoshida, K., Tanaka, T., Mori-Iwamoto, S., Maehara, S., Maehara, Y., Sakaida, I., and Nakamura, K. (2011). KNK437 downregulates heat shock protein 27 of pancreatic cancer cells and enhances the cytotoxic effect of gemcitabine. Chemotherapy *57*, 12-16.

Takii, R., Inouye, S., Fujimoto, M., Nakamura, T., Shinkawa, T., Prakasam, R., Tan, K., Hayashida, N., Ichikawa, H., Hai, T., and Nakai, A. (2010). Heat shock transcription factor 1 inhibits expression of IL-6 through activating transcription factor 3. J Immunol *184*, 1041-1048.

Taldone, T., and Chiosis, G. (2009). Purine-scaffold Hsp90 inhibitors. Curr Top Med Chem *9*, 1436-1446.

Tang, Y., Zhao, W., Chen, Y., Zhao, Y., and Gu, W. (2008). Acetylation is indispensable for p53 activation. Cell *133*, 612-626.

Taura, M., Eguma, A., Suico, M. A., Shuto, T., Koga, T., Komatsu, K., Komune, T., Sato, T., Saya, H., Li, J. D., and Kai, H. (2008). p53 regulates Toll-like receptor 3 expression and function in human epithelial cell lines. Mol Cell Biol *28*, 6557-6567.

Tavaria, M., Gabriele, T., Kola, I., and Anderson, R. L. (1996). A hitchhiker's guide to the human Hsp70 family. Cell Stress Chaperones *1*, 23-28.

Tchenio, T., Havard, M., Martinez, L. A., and Dautry, F. (2006). Heat shock-independent induction of multidrug resistance by heat shock factor 1. Mol Cell Biol *26*, 580-591.

Tedeschi, A., and Di Giovanni, S. (2009). The non-apoptotic role of p53 in neuronal biology: enlightening the dark side of the moon. EMBO Rep *10*, 576-583.

Teodoro, J. G., Evans, S. K., and Green, M. R. (2007). Inhibition of tumor angiogenesis by p53: a new role for the guardian of the genome. J Mol Med *85*, 1175-1186.

Teodoro, J. G., Parker, A. E., Zhu, X., and Green, M. R. (2006). p53-mediated inhibition of angiogenesis through up-regulation of a collagen prolyl hydroxylase. Science *313*, 968-971.

Tetu, B., Brisson, J., Wang, C. S., Lapointe, H., Beaudry, G., Blanchette, C., and Trudel, D. (2006). The influence of MMP-14, TIMP-2 and MMP-2 expression on breast cancer prognosis. Breast cancer research : BCR *8*, R28.

Theriault, J. R., Lambert, H., Chavez-Zobel, A. T., Charest, G., Lavigne, P., and Landry, J. (2004). Essential role of the NH2-terminal WD/EPF motif in the phosphorylation-activated protective function of mammalian Hsp27. J Bio Chem *279*, 23463-23471.

Theriault, J. R., Mambula, S. S., Sawamura, T., Stevenson, M. A., and Calderwood, S. K. (2005). Extracellular HSP70 binding to surface receptors present on antigen presenting cells and endothelial/epithelial cells. FEBS Lett *579*, 1951-1960.

Titov, D.V., Gilman, B., He, Q.L., Bhat, S., Low, W.K., Dang, Y., Smeaton, M., Demain, A.L., Miller, P.S., Kudel, J.F., Goodrich, J.A., and Liu, J.O. (2011). XPB, a subunit of TFIIH, is a target of the natural product triptolide. Nat Chem Biol *7,* 182-188.

Todryk, S. M., Melcher, A. A., Dalgleish, A. G., and Vile, R. G. (2000). Heat shock proteins refine the danger theory. Immunology *99*, 334-337.

Tonkiss, J., and Calderwood, S. K. (2005). Regulation of heat shock gene transcription in neuronal cells. International journal of hyperthermia : the official journal of European Society for Hyperthermic Oncology, North American Hyperthermia Group *21*, 433-444.

Torigoe, T., Tamura, Y., and Sato, N. (2009). Heat shock proteins and immunity: application of hyperthermia for immunomodulation. International journal of hyperthermia : the official journal of European Society for Hyperthermic Oncology, North American Hyperthermia Group *25*, 610-616.

Travers, J., Sharp, S., and Workman, P. (2012). HSP90 inhibition: two-pronged exploitation of cancer dependencies. Drug Discov Today *17*, 242-252.

Trinklein, N. D., Murray, J. I., Hartman, S. J., Botstein, D., and Myers, R. M. (2004). The role of heat shock transcription factor 1 in the genome-wide regulation of the mammalian heat shock response. Mol Biol Cell *15*, 1254-1261.

Trott, A., West, J. D., Klaic, L., Westerheide, S. D., Silverman, R. B., Morimoto, R. I., and Morano, K. A. (2008). Activation of heat shock and antioxidant responses by the natural product celastrol: Transcriptional signatures of a thiol-targeted molecule. Mol Biol Cell *19*, 1104-1112.

Tsujio, I., Tanaka, T., Kudo, T., Nishikawa, T., Shinozaki, K., Grundke-Iqbal, I., Iqbal, K., and Takeda, M. (2000). Inactivation of glycogen synthase kinase-3 by protein kinase C delta: implications for regulation of tau phosphorylation. FEBS Lett *469*, 111-117.

Tsutsumi, S., and Neckers, L. (2007). Extracellular heat shock protein 90: a role for a molecular chaperone in cell motility and cancer metastasis. Cancer Sci *98*, 1536-1539.

Tutar, Y. (2010). Therapeutic use of heat shock proteins and essential factors in prognosis, diagnosis and treatment of neurodegenerative and metabolic diseases. Curr Pharm Biotechnol *11*, 138.

Uchida, S., Hara, K., Kobayashi, A., Fujimoto, M., Otsuki, K., Yamagata, H., Hobara, T., Abe, N., Higuchi, F., Shibata, T.*, et al.* (2011). Impaired hippocampal spinogenesis and neurogenesis and altered affective behavior in mice lacking heat shock factor 1. Proc Natl Acad Sci U S A *108*, 1681-1686.

Ursin, G., Hovanessian-Larsen, L., Parisky, Y. R., Pike, M. C., and Wu, A. H. (2005). Greatly increased occurrence of breast cancers in areas of mammographically dense tissue. Breast cancer research : BCR *7*, R605-608.

van Rheenen, J., Song, X., van Roosmalen, W., Cammer, M., Chen, X., Desmarais, V., Yip, S. C., Backer, J. M., Eddy, R. J., and Condeelis, J. S. (2007). EGF-induced PIP2 hydrolysis releases and activates cofilin locally in carcinoma cells. The Journal of cell biology *179*, 1247-1259.

Vicent, S., Chen, R., Sayles, L. C., Lin, C., Walker, R. G., Gillespie, A. K., Subramanian, A., Hinkle, G., Yang, X., Saif, S.*, et al.* (2010). Wilms tumor 1 (WT1) regulates KRAS-driven oncogenesis and senescence in mouse and human models. The Journal of clinical investigation *120*, 3940-3952.

Vigh, L., Literati, P. N., Horvath, I., Torok, Z., Balogh, G., Glatz, A., Kovacs, E., Boros, I., Ferdinandy, P., Farkas, B.*, et al.* (1997). Bimoclomol: a nontoxic, hydroxylamine derivative with stress protein-inducing activity and cytoprotective effects. Nat Med *3*, 1150-1154.

Vilaboa, N. E., Galan, A., Troyano, A., de Blas, E., and Aller, P. (2000). Regulation of multidrug resistance 1 (MDR1)/P-glycoprotein gene expression and activity by heat-shock transcription factor 1 (HSF1). J Bio Chem *275*, 24970-24976.

von Lintig, F. C., Dreilinger, A. D., Varki, N. M., Wallace, A. M., Casteel, D. E., and Boss, G. R. (2000). Ras activation in human breast cancer. Breast Cancer Res Treat *62*, 51-62.

Vousden, K. H. (2006). Outcomes of p53 activation--spoilt for choice. J Cell Sci *119*, 5015-5020.

Vousden, K. H., and Lane, D. P. (2007). p53 in health and disease. Nat Rev Mol Cell Biol *8*, 275-283.

Wang, B., Soule, H. D., and Miller, F. R. (1997). Transforming and oncogenic potential of activated c-Ha-ras in three immortalized human breast epithelial cell lines. Anticancer Res *17*, 4387-4394.

Wang, J., He, H., Yu, L., Xia, H. H., Lin, M. C., Gu, Q., Li, M., Zou, B., An, X., Jiang, B., *et al.* (2006a). HSF1 down-regulates XAF1 through transcriptional regulation. J Bio Chem *281*, 2451-2459.

Wang, J., Wang, A., Zeng, H., Liu, L., Jiang, W., Zhu, Y., and Xu, Y. (2012a). Effect of triptolide on T-cell receptor beta variable gene mRNA expression in rats with collagen-induced arthritis. Anat Rec *295*, 922-927.

Wang, P., Heber, D., and Henning, S. M. (2012b). Quercetin increased the antiproliferative activity of green tea polyphenol (-)-epigallocatechin gallate in prostate cancer cells. Nutr Cancer *64*, 580-587.

Wang, R. E. (2011). Targeting heat shock proteins 70/90 and proteasome for cancer therapy. Curr Med Chem *18*, 4250-4264.

Wang, R. E., Pandita, R. K., Cai, J., Hunt, C. R., and Taylor, J. S. (2012c). Inhibition of heat shock transcription factor binding by a linear polyamide binding in an unusual 1:1 mode. Chembiochem : a European journal of chemical biology *13*, 97-104.

Wang, S., Xu, Y., Maine, E. A., Wijeratne, E. M., Espinosa-Artiles, P., Gunatilaka, A. A., and Molnar, I. (2008a). Functional characterization of the biosynthesis of radicicol, an Hsp90 inhibitor resorcylic acid lactone from Chaetomium chiversii. Chem Biol *15*, 1328-1338.

Wang, W., Li, X., Sun, W., Zhang, L., Zhang, M., Hong, B., and Lv, G. (2012d). Triptolide triggers the apoptosis of pancreatic cancer cells via the downregulation of Decoy receptor 3 expression. J Cancer Res Clin Oncol.

Wang, X., Asea, A., Xie, Y., Kabingu, E., Stevenson, M. A., and Calderwood, S. K. (2000). RSK2 represses HSF1 activation during heat shock. Cell Stress Chaperones *5*, 432-437.

Wang, X., Grammatikakis, N., Siganou, A., and Calderwood, S. K. (2003). Regulation of molecular chaperone gene transcription involves the serine phosphorylation, 14-3-3 epsilon binding, and cytoplasmic sequestration of heat shock factor 1. Mol Cell Biol *23*, 6013-6026.

Wang, X., Grammatikakis, N., Siganou, A., Stevenson, M. A., and Calderwood, S. K. (2004a). Interactions between extracellular signal-regulated protein kinase 1, 14-3-3epsilon, and heat shock factor 1 during stress. J Bio Chem *279*, 49460-49469.

Wang, X., Khaleque, M. A., Zhao, M. J., Zhong, R., Gaestel, M., and Calderwood, S. K. (2006b). Phosphorylation of HSF1 by MAPK-activated protein kinase 2 on serine 121, inhibits transcriptional activity and promotes HSP90 binding. J Bio Chem *281*, 782-791.

Wang, X. N., Wu, Q., Yang, X., Zhang, L. S., Wu, Y. P., and Lu, C. (2010a). Effects of Celastrol on growth inhibition of U937 leukemia cells through the regulation of the Notch1/NF-kappaB signaling pathway in vitro. Chinese journal of cancer *29*, 385-390.

Wang, Y., and Morgan, W. D. (1994). Cooperative interaction of human HSF1 heat shock transcription factor with promoter DNA. Nucleic Acids Res *22*, 3113-3118.

Wang, Y., Ngo, V. N., Marani, M., Yang, Y., Wright, G., Staudt, L. M., and Downward, J. (2010b). Critical role for transcriptional repressor Snail2 in transformation by oncogenic RAS in colorectal carcinoma cells. Oncogene *29*, 4658-4670.

Wang, Y., Shyy, J. Y., and Chien, S. (2008b). Fluorescence proteins, live-cell imaging, and mechanobiology: seeing is believing. Annu Rev Biomed Eng *10*, 1-38.

Wang, Y., Theriault, J. R., He, H., Gong, J., and Calderwood, S. K. (2004b). Expression of a dominant negative heat shock factor-1 construct inhibits aneuploidy in prostate carcinoma cells. J Bio Chem *279*, 32651-32659.

Wang, Z., and Sun, Y. (2010). Targeting p53 for Novel Anticancer Therapy. Transl Oncol *3*, 1-12.

Warburg, O. (1956). On respiratory impairment in cancer cells. Science *124*, 269-270.

Weinberg, R. A. (1995). The retinoblastoma protein and cell cycle control. Cell *81*, 323-330.

Wen, L., Chen, Y., Zeng, L. L., Zhao, F., Li, R., Liu, Y., and Zhang, C. (2012). Triptolide induces cell-cycle arrest and apoptosis of human multiple myeloma cells in vitro via altering expression of histone demethylase LSD1 and JMJD2B. Acta Pharmacol Sin *33*, 109-119.

Werb, Z. (2010). The extracellular matrix and disease: An interview with Zena Werb. Interviewed by Kristin H. Kain. Dis Model Mech *3*, 513-516.

Werb, Z., and Chin, J. R. (1998). Extracellular matrix remodeling during morphogenesis. Ann N Y Acad Sci *857*, 110-118.

Westerheide, S. D., Anckar, J., Stevens, S. M., Jr., Sistonen, L., and Morimoto, R. I. (2009). Stress-inducible regulation of heat shock factor 1 by the deacetylase SIRT1. Science *323*, 1063-1066.

Westerheide, S. D., Bosman, J. D., Mbadugha, B. N., Kawahara, T. L., Matsumoto, G., Kim, S., Gu, W., Devlin, J. P., Silverman, R. B., and Morimoto, R. I. (2004). Celastrols as inducers of the heat shock response and cytoprotection. J Bio Chem *279*, 56053-56060.

Westerheide, S. D., Kawahara, T. L., Orton, K., and Morimoto, R. I. (2006). Triptolide, an inhibitor of the human heat shock response that enhances stress-induced cell death. J Bio Chem *281*, 9616-9622.

Westerheide, S. D., and Morimoto, R. I. (2005). Heat shock response modulators as therapeutic tools for diseases of protein conformation. J Bio Chem *280*, 33097-33100.

Westwood, J. T., Clos, J., and Wu, C. (1991). Stress-induced oligomerization and chromosomal relocalization of heat-shock factor. Nature *353*, 822-827.

Whitesell, L., and Lindquist, S. (2009). Inhibiting the transcription factor HSF1 as an anticancer strategy. Expert Opin Ther Targets *13*, 469-478.

Whitesell, L., and Lindquist, S. L. (2005). HSP90 and the chaperoning of cancer. Nat Rev Cancer *5*, 761-772.

Whitesell, L., Sutphin P.D., Pucini E.J., Martinez J.D., and Cook P.H. (1998). The physical association of multiple molecular chaperone proteins with mutant p53 is altered by geldanamycin, an hsp-90 binding agent, Mol Cell Biol *18, 1517-1524.*

Wilkerson, D. C., Skaggs, H. S., and Sarge, K. D. (2007). HSF2 binds to the Hsp90, Hsp27, and c-Fos promoters constitutively and modulates their expression. Cell Stress Chaperones *12*, 283-290.

Will, K., Warnecke, G., Wiesmuller, L., and Deppert, W. (1998). Specific interaction of mutant p53 with regions of matrix attachment region DNA elements (MARs) with a high potential for base-unpairing. Proc Natl Acad Sci U S A *95*, 13681-13686.

Willis, A., Jung, E. J., Wakefield, T., and Chen, X. B. (2004). Mutant p53 exerts a dominant negative effect by preventing wild-type p53 from binding to the promoter of its target genes. Oncogene *23*, 2330-2338.

Wing, M. R., Bourdon, D. M., and Harden, T. K. (2003). PLC-epsilon: a shared effector protein in Ras-, Rho-, and G alpha beta gamma-mediated signaling. Mol Interv *3*, 273-280.

Wirth, D., Bureau, F., Melotte, D., Christians, E., and Gustin, P. (2004). Evidence for a role of heat shock factor 1 in inhibition of NF-kappaB pathway during heat shock response-mediated lung protection. Am J Physiol Lung Cell Mol Physiol *287*, L953-961.

Witkin, S. S. (2001). Heat shock protein expression and immunity: relevance to gynecologic oncology. Eur J Gynaecol Oncol *22*, 249-256.

Won, K. Y., Lim, S. J., Kim, G. Y., Kim, Y. W., Han, S. A., Song, J. Y., and Lee, D. K. (2012). Regulatory role of p53 in cancer metabolism via SCO2 and TIGAR in human breast cancer. Hum Pathol *43*, 221-228.

Wu, C. (1995). Heat shock transcription factors: Structure and regulation. Annu Rev Cell Dev Biol *11*, 441-469.

Wu, C. H., Yamaguchi, Y., Benjamin, L. R., Horvat-Gordon, M., Washinsky, J., Enerly, E., Larsson, J., Lambertsson, A., Handa, H., and Gilmour, D. (2003). NELF and DSIF cause promoter proximal pausing on the hsp70 promoter in Drosophila. Genes Dev *17*, 1402-1414.

Wu, Y. C., Yen, W. Y., Lee, T. C., and Yih, L. H. (2009). Heat shock protein inhibitors, 17-DMAG and KNK437, enhance arsenic trioxide-induced mitotic apoptosis. Toxicol Appl Pharmacol *236*, 231-238.

Xavier, I. J., Mercier, P. A., McLoughlin, C. M., Ali, A., Woodgett, J. R., and Ovsenek, N. (2000). Glycogen synthase kinase 3beta negatively regulates both DNA-binding and transcriptional activities of heat shock factor 1. J Bio Chem *275*, 29147-29152.

Xi, C., Hu, Y., Buckhaults, P., Moskophidis, D., and Mivechi, N. F. (2012). Heat Shock Factor Hsf1 Cooperates with ErbB2 (Her2/Neu) Protein to Promote Mammary Tumorigenesis and Metastasis. J Bio Chem *287*, 35646-35657.

Xia, Y., Liu, Y., Rocchi, P., Wang, M., Fan, Y., Qu, F., Iovanna, J. L., and Peng, L. (2012). Targeting heat shock factor 1 with a triazole nucleoside analog to elicit potent anticancer activity on drug-resistant pancreatic cancer. Cancer Lett *318*, 145-153.

Xia, Y., Liu, Y., Wan, J., Wang, M., Rocchi, P., Qu, F., Iovanna, J. L., and Peng, L. (2009). Novel triazole ribonucleoside down-regulates heat shock protein 27 and induces potent anticancer activity on drug-resistant pancreatic cancer. J Med Chem *52*, 6083-6096.

Xiao, H., Perisic, O., and Lis, J. T. (1991). Cooperative binding of Drosophila heat shock factor to arrays of a conserved 5 bp unit. Cell *64*, 585-593.

Xie, Y., Zhong, R., Chen, C., and Calderwood, S. K. (2003). Heat shock factor 1 contains two functional domains that mediate transcriptional repression of the c-fos and c-fms genes. J Bio Chem *278*, 4687-4698.

Xing, H., Mayhew, C. N., Cullen, K. E., Park-Sarge, O. K., and Sarge, K. D. (2004). HSF1 modulation of Hsp70 mRNA polyadenylation via interaction with symplekin. J Bio Chem *279*, 10551-10555.

Xu, D., and La Thangue, N. B. (2008). Strap - A versatile transcription co-factor. Cell Cycle *7*, 2456-2457.

Xu, D., Zalmas, L. P., and La Thangue, N. B. (2008). A transcription cofactor required for the heat-shock response. EMBO Rep *9*, 662-669.

Xu, W., Mimnaugh, E., Rosser, M. F., Nicchitta, C., Marcu, M., Yarden, Y., and Neckers, L. (2001). Sensitivity of mature Erbb2 to geldanamycin is conferred by its kinase domain and is mediated by the chaperone protein Hsp90. J Bio Chem *276*, 3702-3708.

Xu, Y. M., Marron, M. T., Seddon, E., McLaughlin, S. P., Ray, D. T., Whitesell, L., and Gunatilaka, A. A. (2009). 2,3-Dihydrowithaferin A-3beta-O-sulfate, a new potential prodrug of withaferin A from aeroponically grown Withania somnifera. Bioorg Med Chem *17*, 2210-2214.

Yadav, V. R., Sung, B., Prasad, S., Kannappan, R., Cho, S. G., Liu, M., Chaturvedi, M. M., and Aggarwal, B. B. (2010). Celastrol suppresses invasion of colon and pancreatic cancer cells through the downregulation of expression of CXCR4 chemokine receptor. J Mol Med *88*, 1243-1253.

Yaglom, J. A., Gabai, V. L., and Sherman, M. Y. (2007). High levels of heat shock protein Hsp72 in cancer cells suppress default senescence pathways. Cancer Res *67*, 2373-2381.

Yamaguchi, H., and Condeelis, J. (2007). Regulation of the actin cytoskeleton in cancer cell migration and invasion. Biochim Biophys Acta *1773*, 642-652.

Yamaguchi, Y., Takagi, T., Wada, T., Yano, K., Furuya, A., Sugimoto, S., Hasegawa, J., and Handa, H. (1999). NELF, a multisubunit complex containing RD, cooperates with DSIF to repress RNA polymerase II elongation. Cell *97*, 41-51.

Yamashita, H., Nishio, M., Toyama, T., Sugiura, H., Zhang, Z., Kobayashi, S., and Iwase, H. (2004). Coexistence of HER2 over-expression and p53 protein accumulation is a strong prognostic molecular marker in breast cancer. Breast cancer research : BCR *6*, R24-30.

Yan, Y. H., Shang, P. Z., Lu, Q. J., and Wu, X. (2012). Triptolide regulates T cell-mediated immunity via induction of CD11c(low) dendritic cell differentiation. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association *50*, 2560-2564.

Yang, A., Kaghad, M., Wang, Y., Gillett, E., Fleming, M. D., Dotsch, V., Andrews, N. C., Caput, D., and McKeon, F. (1998). p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. Mol Cell *2*, 305-316.

Yang, E. S., Choi, M. J., Kim, J. H., Choi, K. S., and Kwon, T. K. (2011a). Combination of withaferin A and X-ray irradiation enhances apoptosis in U937 cells. Toxicol In Vitro *25*, 1803-1810.

Yang, E. S., Choi, M. J., Kim, J. H., Choi, K. S., and Kwon, T. K. (2011b). Withaferin A enhances radiation-induced apoptosis in Caki cells through induction of reactive oxygen species, Bcl-2 downregulation and Akt inhibition. Chem Biol Interact *190*, 9-15.

Yang, H., Chen, D., Cui, Q. C., Yuan, X., and Dou, Q. P. (2006). Celastrol, a triterpene extracted from the Chinese "Thunder of God Vine," is a potent proteasome inhibitor and suppresses human prostate cancer growth in nude mice. Cancer Res *66*, 4758-4765.

Yang, J., Bridges, K., Chen, K. Y., and Liu, A. Y. (2008). Riluzole increases the amount of latent HSF1 for an amplified heat shock response and cytoprotection. PLoS ONE *3*, e2864.

Yano, A., Tsutsumi, S., Soga, S., Lee, M. J., Trepel, J., Osada, H., and Neckers, L. (2008). Inhibition of Hsp90 activates osteoclast c-Src signaling and promotes growth of prostate carcinoma cells in bone. Proc Natl Acad Sci U S A *105*, 15541-15546.

Yecies, J. L., and Manning, B. D. (2010). Chewing the fat on tumor cell metabolism. Cell *140*, 28-30.

Yih, L. H., Hsu, N. C., Kuo, H. H., and Wu, Y. C. (2012). Inhibition of the Heat Shock Response by PI103 Enhances the Cytotoxicity of Arsenic Trioxide. Toxicological sciences : an official journal of the Society of Toxicology *128*, 126-136.

Yin, Z., Henry, E. C., and Gasiewicz, T. A. (2009). (-)-Epigallocatechin-3-gallate is a novel Hsp90 inhibitor. Biochemistry (Mosc) *48*, 336-345.

Yokota, S., Kitahara, M., and Nagata, K. (2000). Benzylidene lactam compound, KNK437, a novel inhibitor of acquisition of thermotolerance and heat shock protein induction in human colon carcinoma cells. Cancer Res *60*, 2942-2948.

Yoon, Y. J., Kim, J. A., Shin, K. D., Shin, D. S., Han, Y. M., Lee, Y. J., Lee, J. S., Kwon, B. M., and Han, D. C. (2011). KRIBB11 inhibits HSP70 synthesis through inhibition of heat shock factor 1 function by impairing the recruitment of positive transcription elongation factor b to the hsp70 promoter. J Bio Chem *286*, 1737-1747.

Zaarur, N., Gabai, V. L., Porco, J. A., Jr., Calderwood, S., and Sherman, M. Y. (2006). Targeting heat shock response to sensitize cancer cells to proteasome and Hsp90 inhibitors. Cancer Res *66*, 1783-1791.

Zaidi, S., McLaughlin, M., Bhide, S. A., Eccles, S. A., Workman, P., Nutting, C. M., Huddart, R. A., and Harrington, K. J. (2012). The HSP90 inhibitor NVP-AUY922 radiosensitizes by abrogation of homologous recombination resulting in mitotic entry with unresolved DNA damage. PLoS ONE *7*, e35436.

Zhang, H., Zhu, W., Su, X., Wu, S., Lin, Y., Li, J., Wang, Y., Chen, J., Zhou, Y., Qiu, P.*, et al.* (2012a). Triptolide inhibits proliferation and invasion of malignant glioma cells. J Neurooncol.

Zhang, L., Yang, M., Wang, Q., Liu, M., Liang, Q., Zhang, H., and Xiao, X. (2011a). HSF1 regulates expression of G-CSF through the binding element for NF-IL6/CCAAT enhancer binding protein beta. Mol Cell Biochem *352*, 11-17.

Zhang, X., Samadi, A. K., Roby, K. F., Timmermann, B., and Cohen, M. S. (2012b). Inhibition of cell growth and induction of apoptosis in ovarian carcinoma cell lines CaOV3 and SKOV3 by natural withanolide Withaferin A. Gynecol Oncol *124*, 606-612.

Zhang, X., and Yee, D. (2000). Tyrosine kinase signalling in breast cancer: insulin-like growth factors and their receptors in breast cancer. Breast cancer research : BCR *2*, 170-175.

Zhang, Y., Chou, S. D., Murshid, A., Prince, T. L., Schreiner, S., Stevenson, M. A., and Calderwood, S. K. (2011b). The role of heat shock factors in stress-induced transcription. Methods Mol Biol *787*, 21-32.

Zhang, Y. Q., and Sarge, K. D. (2007). Celastrol inhibits polyglutamine aggregation and toxicity though induction of the heat shock response. J Mol Med *85*, 1421-1428.

Zhao, Y., Liu, H., Liu, Z., Ding, Y., LeDoux, S. P., Wilson, G. L., Voellmy, R., Lin, Y., Lin, W., Nahta, R.*, et al.* (2011). Overcoming Trastuzumab Resistance in Breast Cancer by Targeting Dysregulated Glucose Metabolism. Cancer Res *71*, 4585-4597.

Zhao, Y. H., Zhou, M., Liu, H., Ding, Y., Khong, H. T., Yu, D., Fodstad, O., and Tan, M. (2009). Upregulation of lactate dehydrogenase A by ErbB2 through heat shock factor 1 promotes breast cancer cell glycolysis and growth. Oncogene *28*, 3689-3701.

Zhong, M., Orosz, A., and Wu, C. (1998). Direct sensing of heat and oxidation by Drosophila heat shock transcription factor. Mol Cell *2*, 101-108.

Zhou, Y. X., and Huang, Y. L. (2009). Antiangiogenic effect of celastrol on the growth of human glioma: an in vitro and in vivo study. Chin Med J (Engl) *122*, 1666-1673.

Zhu, W., Li, J., Wu, S., Li, S., Le, L., Su, X., Qiu, P., Hu, H., and Yan, G. (2012). Triptolide Cooperates With Cisplatin to Induce Apoptosis in Gemcitabine-Resistant Pancreatic Cancer. Pancreas.

Zinn, M., Aumailley, M., Krieg, T., and Smola, H. (2006). Expression of laminin 5 by parental and c-Ha-ras-transformed HaCaT keratinocytes in organotypic cultures. Eur J Cell Biol *85*, 333-343.

Zou, J., Guo, Y., Guettouche, T., Smith, D. F., and Voellmy, R. (1998). Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. Cell *94*, 471-480.