

The master-regulators of EMT and E-cadherin constitute a novel pathway in malignant melanoma

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ABSTRACT

The master-regulators of an epithelial-mesenchymal transition (MR-EMT) have a pivotal role in the regulation of carcinoma development, promoting transformation and generating a migratory and invasive phenotype. Within epithelial cells, the ZEB proteins are co-regulated, jointly repressed by the miR-200 family of microRNAs. However, here it is demonstrated that the expression and regulation of the MR-EMT in malignant melanoma cell lines appears to be fundamentally different, with a hierarchical organisation identified. ZEB2 and SNAIL2 were found to be expressed in melanocytes, whilst ZEB1 and TWIST1 expression was acquired by a sub-set of malignant melanoma cell lines. Melanoma-initiating mutations within B-RAF and N-RAS were shown to reversibly promote expression of ZEB1 and TWIST1 at the expense of ZEB2 and SNAIL2. Additionally, ZEB2 and SNAIL2 were identified up-stream of ZEB1 and TWIST1 within the MAPK signalling cascade, with ZEB2 functioning as a repressor of ZEB1. Furthermore, ZEB2 and SNAIL2 were found to positively regulate expression of MITF, a marker of melanocyte differentiation. In contrast, ZEB1 repressed expression of MITF and was the primary transcriptional repressor of E-cadherin, an adhesion molecule vital for the interaction between differentiated melanocytes and keratinocytes. Previously, within epithelial cell lines, all the MR-EMT have been identified as transcriptional repressors of E-cadherin. However, ZEB2 and SNAIL2 were co-expressed with E-cadherin within melanocytes and melanoma cell lines and, along with TWIST1, were not able to independently induce E-cadherin re-activation following repression. Surprisingly, ZEB2 became a repressor of E-cadherin in conjunction with ZEB1. Finally, E-cadherin expression was also shown to be controlled in a ZEB1-dependent manner by the transcriptional co-repressor BRG1, the ATPase subunit of the SWI/SNF chromatin remodelling complex, and by the presence of DNA methylation at the E-cadherin promoter. Indeed, DNA methylation was identified as a possible factor controlling the success rate of metastatic colonisation in melanoma cells, allowing for the dynamic re-expression of E-cadherin at the secondary site. These data demonstrate that in malignant melanoma the expression and regulation of the MR-EMT is fundamentally different to that of epithelial tumours, with the MR-EMT structured hierarchically, with opposing regulatory functions.

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LIST OF ABBREVIATIONS

Amp	-	Ampicillin
α MSH	-	α -melanocyte-stimulating hormone
ANOVA	-	Analysis of variance
BCA	-	Bicinchoninic acid
bHLH	-	Basic helix-loop-helix
β -ME	-	β -mercaptoethanol
BMP	-	Bone morphogenetic protein
bp	-	Base pair
BRG1	-	Brahm-related protein 1
BRM	-	Brahm
BSA	-	Bovine serum albumin
BSP	-	Bisulphite specific PCR
Ca^{2+}	-	Calcium
CBD	-	C-terminal binding domain or CtBP-binding domain
cDNA	-	Complementary deoxyribonucleic acid
ChIP	-	Chromatin immunoprecipitation
Co-IP	-	Co-immunoprecipitation
CoREST	-	Co-repressor of RE1 silencing transcription factor/neural restrictive silencing factor
CpG	-	Cytosine-phosphate-guanine
CREB	-	cAMP-responsive element binding protein
CtBP	-	C-terminal binding protein
DAPI	-	4',6-diamidino-2-phenylindole
DMEM	-	Dulbecco's modified eagle media
DMSO	-	Dimethyl sulphoxide
DNA	-	Deoxyribonucleic acid
DNMT	-	DNA methyltransferase
DOX	-	Doxycycline
EC	-	Extracellular cadherin repeats
ECL	-	Enhanced chemiluminescence
EDTA	-	Ethylenediaminetetraacetic acid
EMT	-	Epithelial-mesenchymal transition
ERK1/2	-	Extracellular signal-regulated proteins 1 and 2
F	-	Filamentous
FBS	-	Fetal bovine serum
FGF	-	Fibroblast growth factor
FOXO3	-	Forkhead box D3
Fra1	-	Fos-related antigen 1
GAPDH	-	Glyceraldehyde-3-phosphate dehydrogenase
GFP	-	Green fluorescent protein
HD	-	Homeodomain
HDAC	-	Histone deacetylase
HEM	-	Human epidermal melanocytes
HMT	-	Histone methyltransferase
HRP	-	Horseradish peroxidase

IF	-	Immunofluorescence
Ig	-	Immunoglobulin
IP	-	Immunoprecipitation
ITF2	-	Immunoglobulin transcription factor 2
iPSC	-	Induced pluripotent stem cell
kb	-	Kilobase
kDa	-	Kilodalton
KLF4	-	Krueppel-like factor 4
LB	-	Luria broth
LEF-1	-	Lymphoid enhancing factor-1
Lgl2	-	Lethal giant larvae
M	-	Methylated
MAPK	-	Mitogen-activated protein kinase
MBD	-	Methyl-CpG binding domain proteins
MCR1	-	Melanocortin-receptor 1
MCS	-	Multiple cloning site
MDCK	-	Madin-Darby canine kidney (cells)
MEF	-	Mouse embryonic fibroblast
MEK1/2	-	MAPK/ERK kinase
mESC	-	Mouse embryonic stem cells
MET	-	Mesenchymal-epithelial transition
min	-	Minutes
miR	-	MicroRNA
MITF	-	Microphthalmia-associated transcription factor
MITF-M	-	Melanocyte-specific MITF
MPCD	-	Membrane proximal cytoplasmic domain
MPEC	-	Membrane proximal extracellular cadherin repeat
MR-EMT	-	Master regulators of epithelial-mesenchymal transitions
mRNA	-	Messenger ribonucleic acid
MSP	-	Methylation specific PCR
MS-SSCA	-	Methylation-specific single strand conformational analysis
MTA	-	Metastasis associated (protein family)
MW	-	Molecular weight
MYC	-	Myelocytomatosis viral oncogene
N/A	-	Not applicable
NaCl	-	Sodium chloride
NAT	-	Natural antisense transcription
NCAM1	-	Neural cell adhesion molecule 1
NEAA	-	Non-essential amino acid
NHEM	-	Neonatal human epidermal melanocytes
NuRD	-	Nucleosome remodelling and deacetylase
OCT4	-	Octamer-binding transcription factor 4
p16 ^{INK4A}	-	Cyclin-dependent kinase inhibitor 2A
PAX3	-	Paired box 3
PBS	-	Phosphate buffered saline
P/CAF	-	P300/CBP-associated factor
pCMV	-	CMV (cytomegalovirus) promoter

PCR	-	Polymerase chain reaction
p-Fra1	-	Phosphorylated fos-related antigen 1
PI3K	-	Phosphatidylinositol 3-kinases
P/S	-	Penicillin-streptomycin
PTEN	-	Phosphatase and tensin homolog
PVDF	-	Polyvinylidene fluoride
Rb	-	Retinoblastoma protein
RGP	-	Radial growth phase
RLU	-	Relative fluorescent units
RNA	-	Ribonucleic acid
RNAi	-	RNA interference
RNase	-	Ribonuclease
RPMI	-	Roswell Park Memorial Institute (medium)
RT-PCR	-	Reverse transcriptase polymerase chain reaction
SA- β -Gal	-	Senescence-associated acidic β -galactosidase
SDS	-	Sodium dodecyl sulphate
SDS-PAGE	-	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sec	-	Seconds
SEM	-	Standard error of the mean
SID	-	Smad-interacting domain
SIP1	-	Smad-interacting protein 1
siRNA	-	Short interfering ribonucleic acid
SOX2	-	SRY (sex determining region Y)-box 2
SOX10	-	SRY (sex determining region Y)-box 10
SNAIL1/2	-	Snail homolog 1/2
SWI/SNF	-	SWItch/Sucrose NonFermentable
SUMO	-	Small ubiquitin-like modifier
TAE	-	Tris acetic acid EDTA
<i>Taq</i>	-	<i>Thermus aquaticus</i>
TBE	-	Tris boric acid EDTA
TBS	-	Tris-buffered saline
TBS-T	-	Tris-buffered saline-Tween
TBX2/3	-	T-box transcription factor 2/3
TCF/LEF-1	-	T-cell factor/lymphoid enhancer factor
TE	-	Tris-EDTA
TEMED	-	Tetramethylethylenediamine
TET	-	Ten-eleven translocation
TGF- β	-	Transforming growth factor-beta
TM	-	Transmembrane domain
TRP-1	-	Tyrosinase-related protein 1
TRP-2	-	Tyrosinase-related protein 2
TWIST1/2	-	Twist-related protein 1/2
U	-	Unmethylated
UK	-	United Kingdom
UTR	-	Untranslated region
UV	-	Ultraviolet
UVR	-	Ultraviolet radiation

VDR	-	Vitamin D receptor
VGP	-	Vertical growth phase
VHL	-	von-Hippel-Lindau
v/v	-	Volume by volume
WB	-	Western blotting
w/v	-	Weight by volume
ZEB1	-	Zinc finger E-box-binding homeobox 1
ZEB2	-	Zinc finger E-box-binding homeobox 2
ZHX1	-	Zinc finger homeodomain 1
×g	-	Times gravity
5-azaC	-	5-aza-2'-deoxycytidine
5hmC	-	5-hydroxymethylcytosine
5mC	-	5-methylcytosine

CHAPTER 1 : INTRODUCTION

Throughout the following section, multiple cancer related concepts will be introduced. The process of tumourigenesis will be defined, with a focus applied to malignant melanoma. Melanoma-associated mutations within the mitogen-activated protein kinase (MAPK) signalling pathway, a key pathway deregulated during melanomagenesis, will be described, and additional prominent melanocytic regulators mentioned. Disruption of cellular adhesion is a prominent feature of cancer progression, and this will be examined in relation to the cadherin molecule, E-cadherin. The structural features of E-cadherin will be described and the role of E-cadherin-mediated adhesion will be discussed in normal melanocytes, with the tumourigenic loss of E-cadherin expression addressed. Loss of E-cadherin expression is a hallmark of an epithelial-mesenchymal transition (EMT), a process that converts epithelial cells into mesenchymal cells, which has been linked to the generation of highly migratory and invasive tumour cells. Characteristic features of an EMT will be outlined, with the master-regulators of EMT (MR-EMT) described. These transcription factors have been identified as repressors of E-cadherin and specific tumourigenic examples will be highlighted. The process of E-cadherin transcriptional regulation will be further investigated in relation to transcriptional co-repressors, which have previously been shown to function in conjunction with the MR-EMT. Finally, the role of transcriptional regulation by the presence of DNA methylation will be discussed in a general tumourigenic context and specifically in relation to the control of E-cadherin expression. The introduction aims to provide the foundation to link EMT-like processes to the regulation of E-cadherin expression, in relation to the MAPK signalling pathway, within the context of malignant melanoma.

1.1 CANCER

Carcinogenesis is a multi-step process that requires genetic and epigenetic alterations to occur within oncogenes and tumour suppressor genes. These modifications result in the progressive conversion of a normal cell into a transformed, malignant tumour cell. Acquisition of a common set of cancer traits occurs in all tumour types. These hallmarks of cancer include autocrine growth, avoidance of growth inhibition, evasion of apoptosis, acquisition of an infinite replicative potential, ability to stimulate

angiogenesis, and development of a migratory and invasive phenotype (Hanahan & Weinberg, 2000). Additionally, sustained energy production and avoidance of immune detection are characteristics required for tumour cell propagation. Understanding the molecular complexity of an evolving tumour cell is vitally important but this information needs to be considered in the context of the tumour microenvironment. Tumour cells not only interact with local stromal cells, both at the primary and the metastatic locations, but also inflammatory cells, especially at the tumour invasive front. These tumour-stromal cell interactions are able to promote invasion by multiple mechanisms, including enhanced degradation of the extracellular matrix (Hanahan & Weinberg, 2011).

Cancer-related death is predominantly caused by the metastatic spread of tumour cells. In order to establish a metastatic malignancy, tumour cells must undergo a series of sequential, rate-limiting steps. After the initial transformation, tumour cells migrate and invade into the local tissue. Following angiogenesis, tumour cells intravasate into lymphatic or circulatory systems and spread throughout the body. Within the circulation, a tumour cell must evade immune detection and be resistant to circulatory pressure. Once present within a capillary bed, tumour cells extravasate into a secondary organ, where they must survive and proliferate in order to successfully colonise (Fidler, 2002). The 'soil and seed hypothesis' was originally proposed by Stephen Paget in 1889 and suggests that tumour cells from specific primary tumours favour specific secondary locations to establish a metastatic growth. In conjunction with the established vascular network, the presence of a favourable microenvironment at the secondary location enhances the rate of metastatic colonisation (Fidler, 2003). Indeed, metastatic spread is a highly inefficient process, with the final colonisation step being the major rate limiting factor, highlighting the importance of a favourable microenvironment (Koop *et al.*, 1995).

1.1.1 MELANOMA

Melanoma originates from the malignant transformation of melanocytes. Melanomas can be divided into two categories, classified as either radial growth phase (RGP),

where the transformed melanocytes are restricted to the epidermis, or vertical growth phase (VGP), where the melanoma cells invade the dermis and ultimately metastasise throughout the body (Lin *et al.*, 2010b). In the UK in 2010, 12,818 new cases of malignant melanoma were diagnosed, with 2,203 malignant melanoma-related deaths. Melanoma is the fifth most common cancer within the UK, with rates gradually increasing since the 1970s (*Skin cancer incidence statistics : Cancer Research UK.*).

Melanocytes are normally present within the basal layer of the epidermis of the skin, closely associated with keratinocytes. Melanocytes produce melanin, and via dendritic processes, transfer the melanin to the keratinocytes within organelles known as melanosomes. Melanin protects the keratinocyte DNA from the harmful effects of ultraviolet radiation (UVR) by covering the upper surface of the keratinocyte nucleus. However, in doing so, melanocytes are exposed to the UVR, resulting in DNA damage (Markovic *et al.*, 2007). Risk factors associated with the development of melanoma include an individual or family history of melanoma, the presence of multiple benign naevi, immunosuppression, a sun sensitive phenotype and the degree of exposure to UVR. A sun sensitive phenotype occurs due to polymorphisms within the gene encoding for the melanocortin receptor 1 (MCR1), which is expressed on melanocytes and activated by binding of the α -melanocyte-stimulating hormone (α MSH). Activation of the MCR1 results in intracellular signalling and transcriptional activation of genes involved in melanin production. MCR1 polymorphisms associated with fair skinned individuals result in a less active form of the MCR1, reducing the amount of melanin produced and so increasing the risk of UVR-associated melanoma (Miller & Mihm, 2006). The melanoma-associated risk from UVR is most pronounced in cases of acute, intermittent sun exposure, with the potential risk increased by sun burn at a young age (Markovic *et al.*, 2007).

Melanocytes originate from a highly migratory, transient population of cells, known as neural crest cells. During embryogenesis, neural crest cells are formed from ectoderm at the periphery of the neural tube, which undergoes an epithelial-mesenchymal transition (EMT), allowing them to migrate throughout the body. Neural crest-derived cells are able to differentiate into components of the peripheral nervous system, the

craniofacial bones and cartilage, structures within the heart, and melanocytes within the skin. Transcriptional regulators such as forkhead-box transcription factor D3 (FOXD3), SRY (sex determining region Y)-box 10 (SOX10), paired box gene 3 (PAX3) and microphthalmia-associated transcription factor (MITF) are vital for the differentiation of melanocytes (Sommer, 2011). The developmental lineage of melanocytes may predispose them to a migratory and invasive phenotype, which is evident in the highly metastatic nature of melanoma. Re-activation of genetic profiles responsible for neural crest cell migration may occur during melanoma development. For example, immortalised, transformed human melanocytes have a highly metastatic phenotype when compared to transformed human fibroblasts and mammary cells, which have been transformed with the same set of oncogenes. This indicates that melanocytes are functionally predisposed to an enhanced metastatic capacity (Gupta *et al.*, 2005).

1.1.1.1 MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) SIGNALLING PATHWAY

The involvement of the mitogen-activated protein kinase (MAPK) signalling pathway in melanoma development is well established. The MAPK pathway consists of three levels of kinases, the MAPKs that are phosphorylated and activated by MAPK kinases (MAPKK), which, in turn, are activated by the MAPKK kinases (MAPKKK). The components of the MAPK signalling pathway of primary interest in melanoma include the MAPK extracellular signal-regulated proteins 1 and 2 (ERK1/2), the MAPKK MEK1/2, and the MAPKKK RAF (A-RAF, B-RAF, C-RAF). RAF kinases are activated by membrane-bound RAS GTPases, which are themselves activated following extracellular growth factor interaction with receptor tyrosine kinases. Ultimately, active ERK1/2 phosphorylate cytoplasmic and nuclear targets, including kinases, phosphatases, transcription factors and cytoskeletal proteins (Dhillon *et al.*, 2007). Mutations in the components of the MAPK signalling pathway enhance melanocyte proliferation. Mutations within N-RAS occur in approximately 15% of melanomas, whilst mutations within B-RAF occur in approximately 50% of cases, with mutational estimates varying between studies. It is interesting to note that mutations within N-RAS and B-RAF appear to be mutually exclusive, both resulting in the constitutive activation of MAPK signalling (Miller & Mihm, 2006). A specific B-RAF mutation predominates, a single nucleotide change, resulting in a valine to glutamic acid

substitution, which was originally identified at position 599 and later confirmed as position 600 (V600E). This mutation occurs within the activation loop of the enzyme, resulting in the constitutive RAS-independent activation of B-RAF, so promoting cellular transformation and identifying B-RAF as an oncogene (Davies *et al.*, 2002). Subsequently, multiple mutations within the MAPK signalling pathway have been identified, which when screened for, will allow for the development of personalised, targeted drug therapies (Dutton-Regester *et al.*, 2012).

In the B-RAF^{V600E} melanoma cell line WM-793, knockdown of B-RAF and B-RAF^{V600E} resulted in reduced proliferation, increased apoptosis and reduced cellular transformation. These changes were not detected in the fibrosarcoma cell line HT1080, which expresses wildtype B-RAF, indicating that the induction of proliferation, survival and transformation by B-RAF signalling are specific features of melanoma and implicated B-RAF^{V600E} inhibition as a potential therapeutic target in the treatment of melanoma (Hingorani *et al.*, 2003). Interestingly, B-RAF^{V600E} expression in normal human skin melanocytes results in oncogene-induced senescence, with increased expression of the tumour suppressor p16^{INK4A} and the senescence marker, senescence-associated acidic β -galactosidase (SA- β -Gal) activity. Furthermore, the B-RAF^{V600E} mutation is frequently observed within benign naevi, which are also senescent. This indicates that B-RAF^{V600E} naevi undergo oncogene-induced growth arrest, highlighting the requirement for additional genetic defects prior to melanoma progression (Michaloglou *et al.*, 2005). The therapeutic targeting of the B-RAF^{V600E}-specific mutation has been achieved and clinically validated for several small molecule protein kinase inhibitors, for example vemurafenib, which has improved clinical outcomes for the treatment of melanoma. However, in the majority of cases, patient relapse occurs (Zambon *et al.*, 2012). Such acquired resistance may be gained following aberrant alterations in cellular signalling, including elevation in C-RAF signalling (Montagut *et al.*, 2008); mutually exclusive upregulation of receptor tyrosine kinases or mutational activation of N-RAS (Nazarian *et al.*, 2010); or alternatively through MEK/ERK activation, with B-RAF bypass, by activation of alternative MAPKK kinases (Johannessen *et al.*, 2010).

1.1.1.2 MICROPHTHALMIA-ASSOCIATED TRANSCRIPTION FACTOR (MITF)

Microphthalmia-associated transcription factor (MITF) is a basic helix-loop-helix (bHLH) leucine zipper dimeric transcriptional regulator that is responsible for the differentiation and survival of melanocytes. The *MITF* gene structure is highly complex, with nine different promoter-exon combinations, which allow for tissue-specific expression. The promoter most proximal to the common downstream sequence is the melanocyte-specific MITF promoter (MITF-M) (Levy *et al.*, 2006). Transcriptional regulation of MITF-M occurs via promoter binding by a wide range of transcription factors that are also important during neural crest cell development. These include SOX10, PAX3, cAMP-responsive element binding protein (CREB), lymphoid enhancing factor-1 (LEF-1), immunoglobulin transcription factor 2 (ITF2), FOXD3 and the POU domain transcription factor Brn-2. Additionally, MITF is regulated by miR-148 and is heavily controlled by post-translational modifications, including phosphorylation and SUMOylation.

Activated MITF is able to bind to M-boxes within the promoters of the tyrosinase, tyrosinase-related protein 1 (TRP-1) and tyrosinase-related protein 2 (TRP-2) genes, resulting in their transcriptional up-regulation (Wan *et al.*, 2011). These enzymes are responsible for the production of melanin, via the catalytic transformation of tyrosine (Murisier & Beermann, 2006). Within the adult, MITF mutations are associated with defects in pigmentation, hearing and sight and have also been linked to Waardenburg syndrome type II (Vachtenheim & Borovansky, 2010).

MITF has been described as a melanoma-associated oncogene, being amplified in 10-20% of melanoma cases. However, down-regulation of MITF in an alternative sub-set of melanoma cases appears to occur in the later stages of disease progression, providing a possible growth advantage due to reduced pigment production and differentiation (Levy *et al.*, 2006). In addition to pigment production and within a tumourigenic context, MITF has pro-proliferative and anti-apoptotic functions and promotes invasion and metastasis (Vachtenheim & Borovansky, 2010). In half of melanoma cell lines tested, knockdown of MITF resulted in G₁ phase cell cycle arrest, with the remaining cell lines resistant to MITF loss. This indicates that the pro-

proliferative function of MITF is cell-type specific. In MITF-insensitive cells, combined knockdown of MITF and inhibition of B-RAF^{V600E} resulted in loss of proliferation, indicating that combined targeting of MITF and B-RAF^{V600E} could be therapeutically beneficial in the treatment of melanoma (Kido *et al.*, 2009).

1.2 E-CADHERIN

To maintain tissue architecture during embryonic morphogenesis and to ensure tissue integrity and homeostasis in the adult, appropriate cell contacts and adhesion are required. The zonula adherens is located on the apical side of the lateral membrane of polarised epithelial cells and is composed of adherens junctions, in which cadherin proteins play a central role (Figure 1-1) (Harris & Tepass, 2010). Cadherins are calcium (Ca^{2+})-dependent cell adhesion molecules. The prototypic type I member, E-cadherin (uvomorulin in mouse and L-CAM in chicken), is a 120 kDa transmembrane protein, expressed from the *CDH1* gene and predominantly found on epithelial cells, whereby it allows homophilic contacts between neighbouring cells (van Roy & Berx, 2008). The membrane-proximal region of the intracellular domain is occupied by p120-catenin, which stabilises and clusters cadherin molecules, whilst the distal domain interacts with β -catenin. In turn, β -catenin interacts with α -catenin, which is able to bind filamentous (F) actin and associated actin-binding proteins. These interactions thus link cadherins to the actin cytoskeleton (Perez-Moreno & Fuchs, 2006).

The importance of E-cadherin in maintaining appropriate cell contacts and cellular polarisation is apparent in E-cadherin homozygous null mice, which are unable to maintain proper development post-blastocyst, resulting in a non-viable embryo. Initial cell-cell interactions are achieved via a pool of maternal E-cadherin (Larue *et al.*, 1994; Riethmacher *et al.*, 1995). Expression of E-cadherin is also vital in the maintenance of the pluripotent, undifferentiated state of mouse embryonic stem cells (mESCs), with loss of E-cadherin inducing a cadherin switch, ultimately resulting in an EMT. Interestingly, E-cadherin is able to replace OCT4 in the Yamanaka cocktail (OCT4, SOX2, KLF4, c-MYC), which converts mouse embryonic fibroblasts (MEFs) into induced

pluripotent stem cells (iPSCs), akin to a mesenchymal-epithelial transition (MET) (Redmer *et al.*, 2011; Lowry, 2011).

E-cadherin is encoded by *CDH1*, which is located on chromosome 16q22.1, downstream of a gene coding for another classical cadherin, P-cadherin. The *CDH1* gene spans over 100 kb, including 16 exons and a large second intron. The identification of a CpG island at the 5'end of *CDH1* suggests DNA methylation as a means to epigenetically regulate expression of E-cadherin (Berx *et al.*, 1995b).

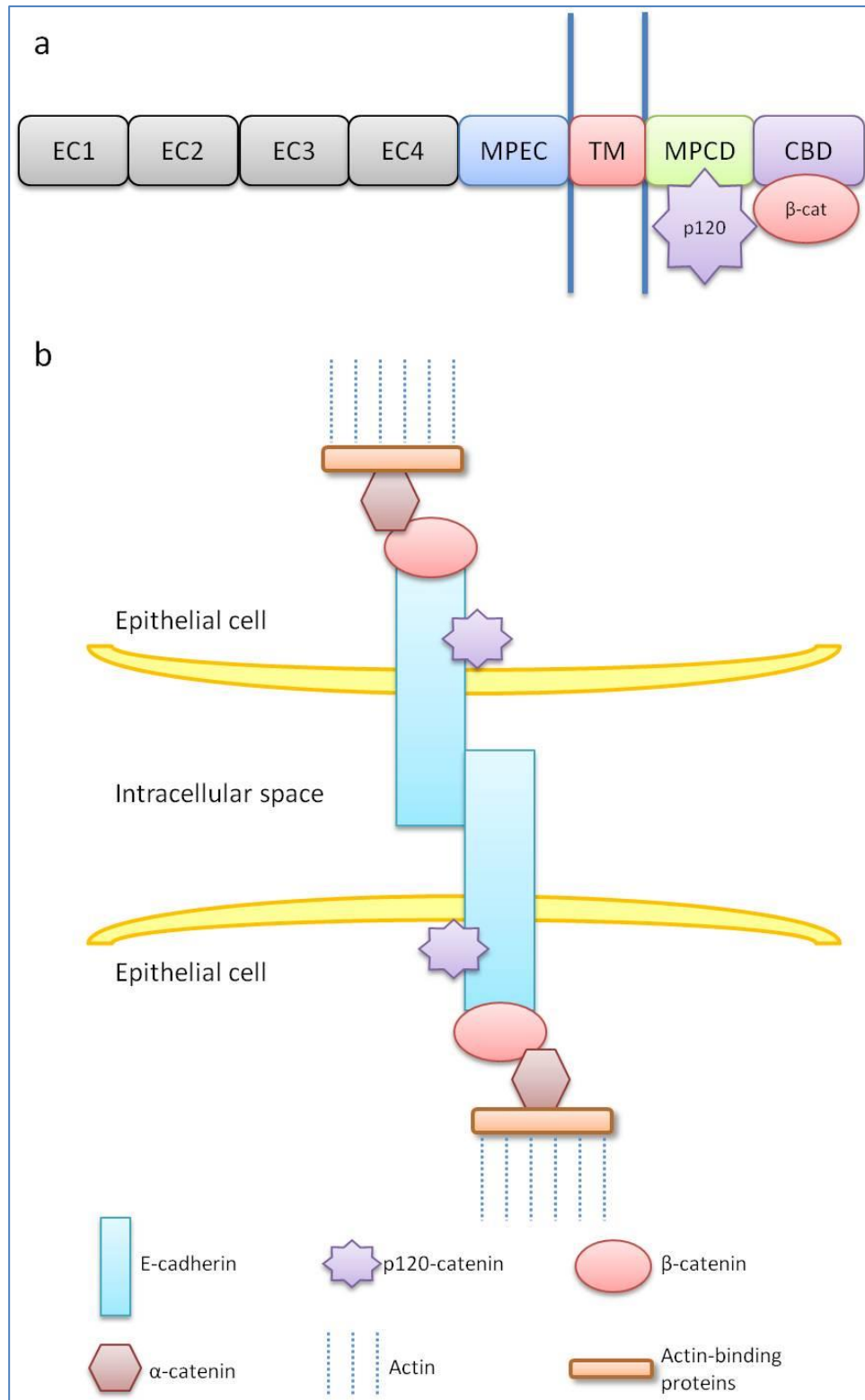


Figure 1-1: Structure of E-cadherin and adherens junctions

(a) E-cadherin consists of five extracellular cadherin repeats (EC) that bind calcium, with the fifth repeat known as the membrane-proximal cadherin repeat (MPEC). EC1 mediates homophilic adhesion between neighbouring cells. E-cadherin has a single-pass transmembrane domain (TM) and a cytoplasmic domain, including the membrane proximal cytoplasmic domain (MPCD), which binds p120-catenin, and the C-terminal binding domain (CBD), which interacts with β -catenin. (b) E-cadherin is localised to adherens junctions within epithelial cells. Through the interaction of E-cadherin bound β -catenin to α -catenin and actin-binding proteins, E-cadherin is linked to the actin cytoskeleton.

1.2.1 E-CADHERIN, SKIN MORPHOLOGY AND MELANOMA

Keratinocytes form a stratified epithelium within the epidermis of the skin, with both E- and P-cadherins establishing and maintaining the correct tissue structure. E-cadherin is expressed at the cell surface of keratinocytes within all layers of the epidermis, whereas expression of P-cadherin appears to be restricted to keratinocytes within the basal layer. When keratinocytes are maintained at low calcium concentration, no cell contacts are formed. However, after 24 hours of culture at high calcium concentration, a stratified structure is formed, with expression of both E- and P-cadherin. After the maturation of the stratified keratinocytes, E-cadherin expression is maintained throughout the layered structure, whereas P-cadherin is restricted to the basal layer. E- and P-cadherin are able to compensate for each other during stratification but stratification is prevented by loss of both cadherins (Wheelock & Jensen, 1992; Furukawa *et al.*, 1997; Jensen *et al.*, 1997).

Human melanocytes within the skin were also shown to express E- and P-cadherin, with the melanocyte-keratinocyte interaction mediated by both cadherins in a calcium and temperature sensitive manner. In a panel of melanoma cell lines, reduced E- and P-cadherin expression minimised the interaction with keratinocytes, promoting a migratory and invasive phenotype (Tang *et al.*, 1994). During tumour progression, melanoma cells undergo the cadherin switch, with lose E-cadherin and gain N-cadherin expression (Hsu *et al.*, 2000a). N-cadherin is an additional classical cadherin (Gumbiner, 1996), mapped to chromosome 18 in the human (Walsh *et al.*, 1990), later refined to 18q11.2, which consists of a 250kb region and includes 16 exons. N-cadherin was shown to be highly conserved between human and mouse, and to that of other classical cadherins (Wallis *et al.*, 1994). Developmentally, N-cadherin is required for the formation of the primitive streak and during gastrulation. However, N-cadherin has also been shown to be involved in tumour progression, implicated in the generation of migratory epithelial cells, so promoting invasion and metastasis (Gheldof & Berx, 2013).

Normal melanocytes communicate through gap junctions with keratinocytes, whereas melanoma cells communicate either with themselves or with fibroblasts. This change in communication is dependent upon cell sorting, mediated by expression of the appropriate cadherin molecule. Re-expression of E-cadherin in melanoma cells re-establishes interaction and communication with keratinocytes (Hsu *et al.*, 2000a). Indeed, the importance of E-cadherin expression in regulating the melanocyte phenotype was confirmed by re-expression of E-cadherin in an E-cadherin-negative melanoma cell line. This resulted in re-established interaction with keratinocytes, inducing growth control; reduced colony formation and tumourigenicity; loss of melanoma-associated antigens; and reduced invasion into the dermis when cultured in a three-dimensional reconstruction of the skin (Hsu *et al.*, 2000b; Herlyn *et al.*, 2000). Interaction between melanoma cells and keratinocytes was shown to require functional E-cadherin linkage to the actin cytoskeleton. When co-cultured at an initial melanoma cell to keratinocyte ratio of 1:5, functional E-cadherin repressed expression of the melanoma-associated antigen, MCAM. Expression of E-cadherin reduced monoculture melanoma cell growth by 40% and when cultured at a ratio (1:5) with keratinocytes, resulted in the maintenance of this fixed ratio. Release of β -catenin from the complex with E-cadherin appeared to increase melanoma proliferation, which was minimised by the establishment of β -catenin null adherens junctions. Overall, the control of melanocytes by keratinocytes is dependent upon cellular adhesion, with the growth inhibitory and tumour suppressive function of E-cadherin occurring independently of β -catenin regulation (Li *et al.*, 2004). Interestingly, re-expression of E-cadherin in E-cadherin-negative melanoma cell lines has also been shown to result in increased sensitivity to apoptotic stimuli (Kippenberger *et al.*, 2006).

However, the role of E-cadherin in melanoma progression *in vivo* is still controversial (Kuphal & Bosserhoff, 2011). Strong E-cadherin staining has been identified within *in vivo* melanocytes and nevus cells (Sanders *et al.*, 1999; Krengel *et al.*, 2004), yet E-cadherin-negative common naevi and dysplastic naevi have also been described (Danen *et al.*, 1996). Additionally, the strength of E-cadherin staining has been shown to decrease with melanoma progression to the VGP (Krengel *et al.*, 2004), whilst contradictory studies have identified consistent E-cadherin expression within

dysplastic naevi, RGP and VGP melanomas, with only a minor loss in metastatic VGP melanomas (Sanders *et al.*, 1999). Finally, additional studies have identified an increase in E-cadherin staining in malignant melanomas compared to naevi (Cowley & Smith, 1996), with E-cadherin present in a high proportion of VGP melanomas but absent in RGP melanomas (Silve *et al.*, 1998), with E-cadherin expression observed in 10-20% of advanced primary melanomas and melanoma metastases (Danen *et al.*, 1996).

The use of E-cadherin as a prognostic marker in human melanoma has not been conclusively confirmed but indications suggest that E-cadherin expression may be a positive prognostic feature. Higher levels of E-cadherin approached significance as a positive prognostic marker, with lower E-cadherin expression identified in metastatic lesions compared to the primary melanomas (Kreizenbeck *et al.*, 2008). The prognostic value of E-cadherin expression was further established by the identification of a significant reduction in E-cadherin in melanocytes from patients with metastatic melanoma compared to disease-free individuals (Tucci *et al.*, 2007).

1.2.2 E-CADHERIN AND CANCER

Loss of E-cadherin has been linked to the pathogenesis of cancers of epithelial origin, with reduced cellular adhesion promoting the formation of a dedifferentiated and invasive phenotype (Wijnhoven & Pignatelli, 1999). The connection between loss of E-cadherin and increased invasive capacity was first established in MDCK cells, where use of an E-cadherin-specific inhibitory antibody resulted in down-regulation of E-cadherin and acquisition of a fibroblast-like morphology, with increased invasion through collagen (Behrens *et al.*, 1989). Furthermore, E-cadherin expression was identified in differentiated carcinoma cells lines but was absent in carcinoma cell lines that had undergone dedifferentiation. Additionally, ectopic expression of E-cadherin in these E-cadherin-negative cell lines resulted in a reduction in invasiveness (Frixen *et al.*, 1991). Following the opposite approach, repression of E-cadherin in a non-invasive transformed epithelial cell line by an antisense approach resulted in the formation of a fibroblast-like morphology, with enhanced invasion (Vleminckx *et al.*, 1991). This

established E-cadherin as a positive regulator of the epithelial phenotype and an invasion suppressor gene.

Screening of tumour patient biopsies for expression of E-cadherin established a common pattern. E-cadherin expression tended to be higher in differentiated regions of a tumour, particularly within the tumour centre, but down-regulated in dedifferentiated tumour cells, especially evident around the tumour edge and invasive front. Such examples were identified in squamous cell carcinoma of the head and neck (Schipper *et al.*, 1991), colorectal carcinoma (Dorudi *et al.*, 1993), thyroid carcinoma (Brabant *et al.*, 1993), renal cell carcinoma (Katagiri *et al.*, 1995), stomach carcinoma (Shiozaki *et al.*, 1991), prostate carcinoma (Umbas *et al.*, 1992) and breast carcinoma (Oka *et al.*, 1993). The loss of E-cadherin appears essential for carcinogenesis to occur, increasing invasiveness and metastatic spread, and ultimately resulting in a poor patient prognosis (Tamura, 1997). Furthermore, loss of E-cadherin was shown to promote conversion of an adenoma to a carcinoma *in vivo* (Perl *et al.*, 1998).

Loss of E-cadherin within the primary tumour, with a corresponding increase in invasion and metastasis, is a well-established feature of carcinogenesis. However, an interesting feature of certain metastatic tumours is the re-expression of E-cadherin. For example, in primary colorectal carcinomas, E-cadherin expression is positively associated with differentiation, with E-cadherin absent in poorly differentiated tumour regions. However, in 35% of lymph node metastases, E-cadherin expression was identified (Dorudi *et al.*, 1993). Additionally, loss of E-cadherin, α -catenin and β -catenin in primary breast carcinomas was associated with increased lymph node metastases. However, strong homogenous re-expression of all three proteins was identified at the metastatic site in the majority of cases (Bukholm *et al.*, 2000; Ilyas, 2000). A similar situation was observed in the metastases from primary ovarian carcinomas, including E-cadherin, α -catenin, β -catenin and γ -catenin re-expression (Imai *et al.*, 2004). In cases of invasive ductal carcinoma of the breast, 55% of cases possessed normal E-cadherin expression, whereas at the metastatic site, this was as high as 70%. Additionally, E-cadherin staining at the metastatic site was comparable or stronger to the paired primary sample (Kowalski *et al.*, 2003). Another epithelial

marker, ZO-1, a component of tight junctions, was also shown to be re-expressed in liver metastasis originating from a colorectal carcinoma (Kaihara *et al.*, 2003).

Interaction of metastatic tumours cells with cells at the secondary site appears to be important in the control of E-cadherin re-expression. Primary prostate carcinomas frequently metastasize to the liver. When prostate carcinoma cells are co-cultured with hepatocytes, E-cadherin is re-expressed. Additionally, there was an increase in the epithelial marker, cytokeratin 18, indicating a reversal from a mesenchymal to an epithelial phenotype. Re-localisation of E-cadherin and β -catenin to the membrane connecting the prostate cells and hepatocytes indicated an interaction (Yates *et al.*, 2007a). Interaction between metastatic cells and cells at the secondary site was also identified following metastatic dissemination of infiltrating ductal carcinoma of the breast. At metastatic sites, including lung, liver and brain, 62% of cases showed higher E-cadherin expression compared to the paired primary tumours, with E-cadherin predominantly expressed at the tumour-host tissue interface, rather than within the tumour centre. Interestingly, co-culture of MDA-MB-231, a breast cancer cell line, with hepatocytes also resulted in E-cadherin re-expression. Co-culture with hepatocyte-derived culture media did not induce this effect, indicating that direct contact was required. Additionally, co-culture resulted in the conversion of the E-cadherin promoter from a hypermethylated to hypomethylated state (Chao *et al.*, 2010).

Mutations in the *CDH1* gene have been identified in lobular carcinoma of the breast (Berx *et al.*, 1995a; Vos *et al.*, 1997; Droufakou *et al.*, 2001), in diffuse gastric carcinoma (Becker *et al.*, 1994; Guilford *et al.*, 1998) and in gynaecological carcinomas (Risinger *et al.*, 1994). However, alternative mechanisms of E-cadherin deregulation must be present in cancer types lacking such inactivating E-cadherin mutations. Epithelial-specific expression from the *CDH1* promoter was confirmed with high promoter activity in epithelial cell lines, compared to greatly reduced expression in fibroblasts. Interestingly, *CDH1* promoter activity was also minimal in carcinoma cell lines lacking E-cadherin expression. This indicates that the *CDH1* promoter is controlled by the absence or presence of positive or negative transcriptional regulators (Girolodi *et al.*, 1997). The presence of multiple regulatory DNA sequences

upstream of the transcriptional start site of *CDH1* enables the expression of E-cadherin to be transcriptionally regulated. For instance, five Sp-1, three AML-1, two p300 and four HNF3 binding sites allow for expression to be positively induced. In contrast, four E-boxes inhibit E-cadherin expression when bound by appropriate transcriptional repressors, such as SNAIL1 (Liu *et al.*, 2005) (Figure 1-2). Indeed, targeted mutation of the E-boxes results in elevated promoter activity (Girolodi *et al.*, 1997). Due to the apparent rarity of mutational events targeting *CDH1*, the control of E-cadherin expression by transcriptional regulators has been focussed upon.

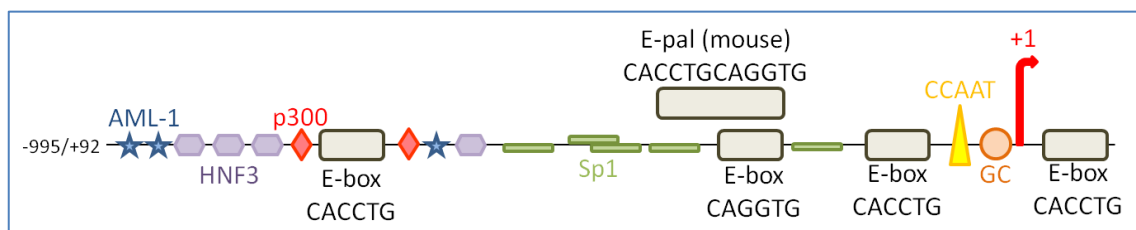


Figure 1-2: Structure of the E-cadherin promoter

Regulatory regions of the human E-cadherin promoter are presented, including sites that positively regulate transcription (AML-1, HNF3, p300 and Sp1), and E-boxes, sites of transcriptional repression. An interesting feature in the mouse genome is the E-pal site, consisting of two consecutive E-boxes, with only the second E-box conserved in the human genome. Additional regulatory structures include a CCAAT box and a GC rich region upstream of the transcriptional start site (Liu *et al.*, 2005; Peinado *et al.*, 2004).

The role of E-cadherin and the adherens junctions in mediating epithelial differentiation, by establishment of cellular adhesion and polarity, is well recognised. Additionally, E-cadherin has a role in signal transduction by restricting the cellular localisation of β -catenin. In normal epithelial cells, β -catenin is localised to the membrane, associated with E-cadherin. However, upon loss of E-cadherin, β -catenin can translocate to the nucleus, where it can function as a transcriptional activator with the TCF/LEF protein family, as part of Wnt signalling (Schmalhofer *et al.*, 2009). Release of β -catenin from the adherens junction results in increased expression of mesenchymal markers, such as N-cadherin, vimentin and fibronectin and is associated with increased invasion, avoidance of apoptosis and enhanced metastatic spread. However, β -catenin is not sufficient to induce all changes associated with E-cadherin signal transduction, indicating that E-cadherin is involved in additional, as yet unknown, regulatory networks (Onder *et al.*, 2008).

1.3 EPITHELIAL-MESENCHYMAL TRANSITION (EMT)

Epithelial cells are morphologically round and form compact clusters. These properties allow for the formation of layered structures, with highly organised cellular junctions connecting neighbouring cells. These include tight junctions, gap junctions, adherens junctions and desmosomes, whose positioning results in a defined apico-basolateral polarity. In contrast, mesenchymal cells have a spindle, fibroblast-like morphology, only focally contacting neighbouring cells, with the loss of organised cellular junctions. Additionally, mesenchymal cells have the potential to be highly migratory. An epithelial-mesenchymal transition (EMT) is a highly complex and dynamic process that converts epithelial cells into a mesenchymal cells, both at the morphological and transcriptional level. The reverse process, a mesenchymal-epithelial transition (MET), allows the reversion of mesenchymal cells to epithelial cells, highlighting the plastic nature of the cellular phenotype (Thiery & Sleeman, 2006) (Figure 1-3).

The process of EMT, originally identified in the chicken primitive streak, is vital at multiple stages of embryogenesis. Initially required for implantation, EMT within certain extra-embryonic structures is essential for placental formation and anchorage. During gastrulation, EMT is also required for the formation of the three germ layers from the primitive streak. This is initiated within the E-cadherin expressing epiblast layer, whereby cells undergo a programmed EMT and internalise. This results in the formation of the inner endoderm, middle mesoderm and outer ectoderm layers. Highly migratory neural crest cells are also generated from epithelial cells of the neuroectoderm lineage by an EMT. These neural crest cells disperse throughout the body, differentiating into a wide range of cell types, including melanocytes located within the skin (Kalluri & Weinberg, 2009; Acloque *et al.*, 2009).

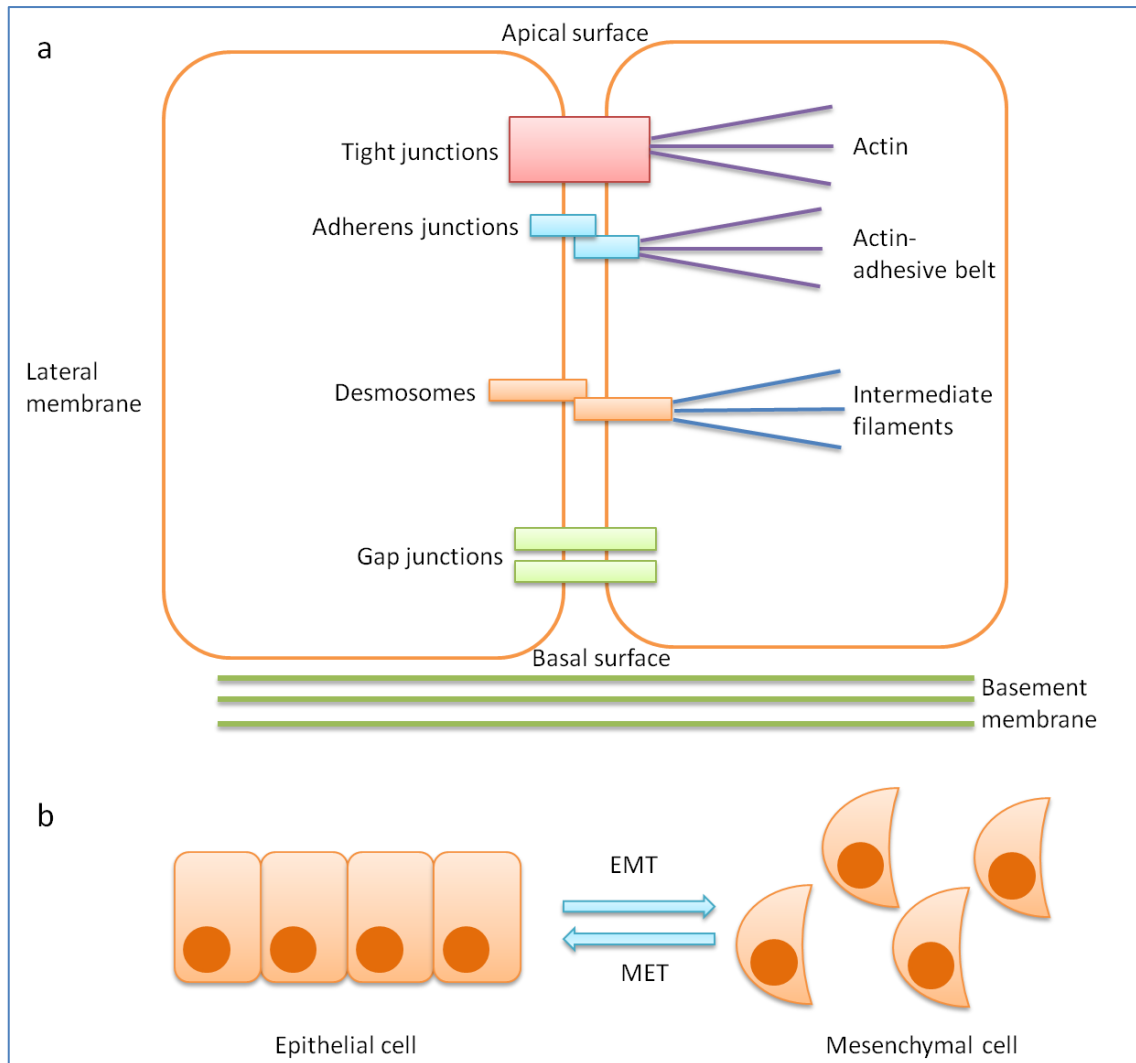


Figure 1-3: Epithelial junctional complexes and the processes of EMT

(a) The junctional complexes located within an epithelial cell result in the formation of a highly polarised cellular structure. Tight junctions, located to the most apical surface, form a seal around the top of the cell, connecting to the actin cytoskeleton. Adherens junctions are composed of E-cadherin in epithelial cells, also link neighbouring cells to the actin cytoskeleton, whilst desmosomes link cells to intermediate filaments. Finally, gap junctions are intercellular channels that allow the transfer of ions and small molecules between cells. (b) During the process of EMT, whereby an epithelial cell is converted into a mesenchymal cell, dramatic re-organisation of epithelial junctions occurs, including the loss of E-cadherin at adherens junctions. This results in the formation of migratory and invasive cells. The reverse process, a MET, results in reversion to the epithelial phenotype with re-establishment of the epithelial junctional complexes.

E-cadherin is the classical epithelial marker, with loss of E-cadherin being a hallmark of EMT. As previously mentioned, E-cadherin participates in the formation of the epithelial adherens junctions, which form a continuous belt around epithelial cells, connecting neighbouring cells to the intracellular actin cytoskeleton. Tight junctions, another important epithelial structure, are located on the most apical side of the lateral membrane and provide a seal around cells, preventing diffusion from the apical

surface to the baso-lateral membranes. These structures provide epithelial cells with a defined polarity (Christiansen & Rajasekaran, 2006). Junctional defects between neighbouring epithelial cells result in dramatic changes to cell proliferation and migration. Due to the polarised nature of epithelial cells, with the presence of tight junctions, growth factors present at their apical surface do not interact with baso-laterally positioned growth factor receptors. Therefore, a consequence of the loss of tight junctions is autocrine signalling, with increased proliferation. Additionally, junctional defects result in the loss of contact inhibition. In general, this results in the dedifferentiation of epithelial cells, with the acquisition of mesenchymal properties, characteristic features of an EMT (Wells *et al.*, 2008). In addition to the loss of epithelial markers, an EMT results in the expression of mesenchymal-related genes. These include the intermediate filament vimentin and smooth muscle actin and secretion of extracellular components, such as fibronectin and collagen (Christiansen & Rajasekaran, 2006).

The process of EMT is triggered by multiple extracellular factors, including transforming growth factor- β (TGF- β), Notch, fibroblast growth factor (FGF) and Wnt signalling. The majority of these signals converge on a set of transcriptional regulators, including the SNAIL (SNAIL1 and SNAIL2), ZEB (ZEB1 and ZEB2) and bHLH (TWIST1 and TWIST2) family members. These master regulators of EMT (MR-EMT) primarily promote progression through an EMT by repression of epithelial genes, such as E-cadherin (Moreno-Bueno *et al.*, 2008; Hill *et al.*, 2013). Interestingly, complex regulatory loops appear to function between the MR-EMT resulting in enhanced expression, occurring via increased transcription, mRNA stabilisation through repression of the miR-200 family of microRNAs, and increased protein stability. For example, induction of SNAIL1 was shown to enhance the activity of ZEB1 (Dave *et al.*, 2011).

Three types of EMT are recognised, each with different functional and pathological outcomes. A type 1 EMT relates to the normal process that occurs during embryogenesis, allowing for the controlled formation of mesenchymal, migratory cells. A type 2 EMT occurs during wound healing, tissue repair and fibrosis. In this

situation an EMT results in the formation of active fibroblasts that encourage tissue repair following injury or inflammation. However, if the inflammatory signal persists, tissue fibrosis may occur. A type 3 EMT is a cancer-related EMT and occurs in tumour cells with genetic and/or epigenetic defects in oncogenes and tumour suppressor genes. This type of EMT induces formation of highly migratory and invasive tumour cells, which have an increased metastatic potential. A cancer-related EMT is most prevalent within cells at the tumour invasive front (Kalluri & Weinberg, 2009). A type 3 cancer-related EMT has also been linked to the formation of tumour cells with stem-like qualities. These cells are not only highly migratory and invasive, but also have the capacity to self-renew and differentiate at the metastatic site, aiding in the formation of macro-metastases (Mani *et al.*, 2008). Importantly, these three types of EMT all result in the formation of migratory cells but are induced to occur by divergent mechanisms and promote different outcomes. For example, a type 1 EMT occurs within a genetically stable cell and is highly controlled. However, a type 3 cancer-related EMT occurs within a genetically unstable background and can induce additional cellular characteristics, such as stem-like qualities. Biomarkers are required to definitively identify the type of EMT that is occurring within a cellular population (Zeisberg & Neilson, 2009).

Progression through a cancer-related EMT does not have to result in the full conversion of an epithelial cell into a fully committed mesenchymal cell. A mesenchymal reversible 'metastable' EMT state may be achieved, whereby removal of the EMT-inducing signal results in the reversion to an epithelial phenotype. This 'metastable' population of cells is not 'epigenetically fixed', meaning that phenotypic changes can be rapidly and dynamically regulated by withdrawal of the EMT-inducing signal (Thomson *et al.*, 2011). Indeed, the ability of tumour cells to dynamically switch between an epithelial and mesenchymal state has been highlighted in a metastatic model of bladder cancer. Tumour cells undergoing an EMT at the primary site have a metastatic advantage, with the development of a migratory and invasive phenotype. However, following invasion and intravasation, tumour cells that have the ability to undergo a MET have an additional advantage, increasing the ability of the tumour cells to become established at the metastatic site (Chaffer *et al.*, 2006). This indicates that

an EMT is important for tumour cells during the initial metastatic steps, whereas an MET enhances the final colonisation stage. The ability of tumour cells to undergo both an EMT and MET is highly dependent upon signals from the microenvironment (Kalluri & Weinberg, 2009; Korpál *et al.*, 2011).

1.4 MASTER REGULATORS OF EMT (MR-EMT)

1.4.1 ZEB PROTEINS

ZEB1 (δ -EF1, Nil-2-a, TCF8 or Zfhx1a) and ZEB2 (SIP1 or Zfhx1b) are complex, multi-domain proteins, each containing two zinc-finger clusters, separated by a homeodomain (Figure 1-4). There is a high degree of sequence homology between the zinc-finger clusters of ZEB1 and ZEB2, with both N-terminal clusters containing four zinc-fingers and the C-terminal clusters containing three zinc-fingers (Vandewalle *et al.*, 2009; Sanchez-Tillo *et al.*, 2011). Mammalian ZEB1 and ZEB2 appear to have evolved following a gene duplication event, with the *Drosophila* genome encoding a single ZEB protein, ZFH-1, with a high degree of homology remaining between the *Drosophila* and mammalian zinc-fingers (Nelles *et al.*, 2003; Gheldof *et al.*, 2012). A high degree of homology was also identified when the zinc-finger clusters were compared between chicken, mouse, hamster and human (Sekido *et al.*, 1996). Due to the homologous nature of the ZEB1 and ZEB2 zinc-finger clusters, ZEB proteins interact with similar CACCT sequences, including E-boxes (CANNTG) (Sekido *et al.*, 1994; Remacle *et al.*, 1999). This suggests that ZEB1 and ZEB2 may regulate identical or overlapping gene sets (Remacle *et al.*, 1999).

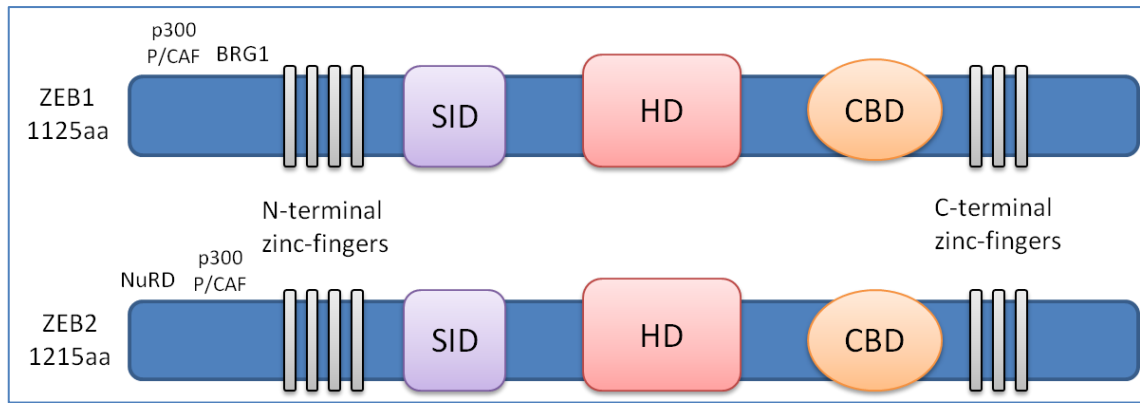


Figure 1-4: ZEB protein structure and binding sites

The main protein domains in the ZEB proteins include the N-terminal and C-terminal zinc-finger clusters, the CtBP-binding domain (CBD), the centrally located homeodomain (HD) and the Smad-interacting domain (SID) (major ZEB protein isoforms presented).

ZEB1 was originally named δ EF1 due to the observed interaction with the enhancer domain of the lens specific δ -crystallin (Funahashi *et al.*, 1991). The *ZEB1* gene was subsequently mapped on chromosome 10p11.2 (Copeland *et al.*, 1993). Cloning of *ZEB1* from the chicken identified a 50 kb coding sequence, consisting of nine exons. The N-terminal zinc-finger is encoded by exons 5-6, the C-terminal zinc-finger by exons 8-9, with a large exon 7 encoding the central region of ZEB1, including the homeodomain (Sekido *et al.*, 1996).

ZEB2 was identified in a two-hybrid yeast screen, due to ZEB2 interaction with receptor-activated Smads, resulting in the original ZEB2 name of Smad-interacting protein 1 (SIP1) (Verschuere *et al.*, 1999). Following the separate evolution of the ZEB proteins, *ZEB2* developed a divergent 5'UTR, which possesses a highly complex organisation, with three identified promoters regulating the expression of multiple, and alternatively spliced, transcripts. Analysis of the mouse *ZEB2* 5'UTR identified nine untranslated exons (U1-U9) upstream of the first translated exon. These untranslated exons were differentially spliced to the first commonly translated exon, but no additional upstream in-frame start codon was identified, indicating that translation of all resulting proteins would commence at the same site. Additionally, three potential promoters were identified, promoter 1 upstream of exon U1, promoter 2 upstream of exon U5 and promoter 3 upstream of exon 1. The promoter activity and production of the corresponding spliced products appeared to be cell type dependent. Interestingly,

a spliced anti-sense ZEB2 transcript (natural antisense transcription, NAT) was also identified (Nelles *et al.*, 2003).

Both ZEB1 and ZEB2 play an important role during embryonic development. Expression of ZEB1 during chicken embryogenesis was identified at the post-gastrulation stage in mesoderm, neuroectoderm and neural crest cell derivatives. ZEB1 was minimally expressed in the ectoderm and endoderm (Funahashi *et al.*, 1993). Subsequently, ZEB2 expression was shown in the neuroectoderm and neural crest derivatives in mouse embryos (Van de Putte *et al.*, 2003). Homozygous mice lacking the C-terminal ZEB1 zinc-finger have an aberrantly formed thymus, with corresponding defects in T-cell development (Higashi *et al.*, 1997). Following knockout of ZEB1, skeletal defects were also apparent, including defects in craniofacial, limb and vertebral regions. Heterozygous null ZEB1 mice are viable and fertile, whereas homozygotes develop to term but die perinatally (Takagi *et al.*, 1998). In contrast, knockout of ZEB2 in mice is embryonically lethal, with defects in neural tube closure, cranial neural crest cell migration and formation of shortened somites. In the mouse, ZEB1 and ZEB2 expression appears complementary with minimal overlap, yet they also have the potential to compensate for each other. In ZEB2 null mice, ZEB1 is up-regulated, indicating possible ZEB2-mediated repression of ZEB1. Compound ZEB1/ZEB2 null mice have a more severe phenotype; especially apparent are neural tube defects, with loss of SOX2 expression (Miyoshi *et al.*, 2006). Additionally, heterozygote ZEB2 mutations in humans have been implicated in the development of Mowat-Wilson syndrome, a hereditary condition associated with Hirschsprung disease and mental retardation (Van de Putte *et al.*, 2003), which include large-scale heterozygous truncating deletions or frame-shift mutations, resulting in loss-of-function (Amiel *et al.*, 2001; Cacheux *et al.*, 2001; Zweier *et al.*, 2002).

The central region of both ZEB1 and ZEB2 functions as a repressor domain, due to the presence of a CtBP-binding domain (CBD). This central region also contains the homeodomain and a Smad-interacting domain (SID). Both ZEB1 and ZEB2 were shown to interact with activated R-Smads, with the interaction stronger for ZEB2. Smad interaction with the ZEB proteins was induced by both TGF- β and bone morphogenetic

protein (BMP) signalling. Interestingly though, ZEB1-Smad signalling resulted in transcriptional activation of responsive promoters, the ZEB2-Smad complex resulted in transcriptional repression (Postigo, 2003). Even though ZEB1 and ZEB2 bind similar DNA sequences, opposing transcriptional function can be mediated via interaction with differing transcriptional co-activators and co-repressors. Initially, the N-terminal region of ZEB1, but not ZEB2, was shown to bind the histone acetyltransferases, p300 and P/CAF, which acetylate histones, so generating an active chromatin configuration (Postigo, 2003). Subsequently, ZEB2 was also shown to interact with p300 and P/CAF (van Grunsven *et al.*, 2006). In addition to a role in histone acetylation, P/CAF is able to acetylate several lysine residues located proximally to the ZEB1 CBD, preventing interaction of ZEB1 with CtBP and so converting ZEB1 from a transcriptional repressor to an activator (Postigo *et al.*, 2003).

ZEB1 and ZEB2 have both been identified as transcriptional repressors of E-cadherin (Grooteclaes & Frisch, 2000; Comijn *et al.*, 2001). Repression of *CDH1* by ZEB2 is mediated by zinc-finger binding to E-boxes located within the *CDH1* promoter. ZEB2 expression in epithelial MDCK cells resulted in the acquisition of an invasive phenotype, highlighting the potential role of ZEB2 in the induction of an EMT and the formation of migratory and invasive tumour cells (Comijn *et al.*, 2001). Induction of an EMT in an EpFosER mouse mammary cell line resulted in key morphological changes, including loss of apical-basal polarity, disruption of monolayer growth and conversion to a dedifferentiated, fibroblast-like phenotype. Following the induction of this EMT, ZEB1 expression was up-regulated, which inversely correlated with E-cadherin. Whilst up-regulation of ZEB2 and SNAIL1 in this model was slower than ZEB1 and, therefore, not responsible for the immediate repression of E-cadherin, ZEB1 directly repressed E-cadherin, via binding to the E-boxes. ZEB1 also mediated repression of the tight junction component, ZO-1, and the desmosome component, desmoplakin. In addition, ZEB1 expression resulted in the up-regulation of the mesenchymal markers, N-cadherin and vimentin. This study established ZEB1 as a major negative regulator of E-cadherin and the epithelial phenotype (Eger *et al.*, 2005). In another EMT model, treatment of NMuMG cells with TGF- β resulted in a shift to a mesenchymal morphology and induced a cadherin switch. TGF- β -induced repression of E-cadherin

was directly mediated by E-box binding of ZEB1 and ZEB2, in a Smad-independent manner. SNAIL1 and SNAIL2 were not involved in the repression of E-cadherin. Additionally, activation of mesenchymal markers, such as N-cadherin and vimentin, was not mediated by ZEB1 and ZEB2. TGF- β activation of ZEB1 and ZEB2 appeared to be indirect, possibly mediated via TGF- β activation of Ets (Shirakihara *et al.*, 2007). In a panel of non-small cell lung cancer cell lines, knockdown of ZEB1 and ZEB2 resulted in re-expression of E-cadherin but interestingly, the ability to re-activate E-cadherin was stronger following inhibition of ZEB1 and the repression of both ZEB1 and ZEB2 appeared synergistic, further enhancing E-cadherin re-expression (Gemmill *et al.*, 2011).

In a panel of human breast cancer cell lines, expression of ZEB1 and E-cadherin were inversely correlated, whilst incomplete correlation occurred between SNAIL1 and E-cadherin. Knockdown of ZEB1 in the breast cancer cell line MDA-MB-231, resulted in membranous E-cadherin re-expression and additional genome-wide transcriptional effects (Eger *et al.*, 2005). Many genes involved in epithelial differentiation were shown to be repressed by ZEB1, including epithelial cadherins; components of tight junctions, desmosomes and gap junctions; cell polarity genes; apically localised proteins; cell surface receptors; and genes involved in vascular transport. This ZEB1-dependent genome reprogramming resulted in enhanced migration in MDA-MB-231 cells, independently of E-cadherin expression. This indicates that ZEB1 has a functional role in tumourigenesis, inducing tumour cell migration and invasion (Aigner *et al.*, 2007). Meanwhile, induction of ZEB2 expression in the epithelial epidermoid carcinoma cell line A431 resulted in the conversion of the cells to a fibroblast-like morphology, with loss of E-cadherin and α -catenin and re-localisation of β -catenin to the cytoplasm. This conversion prevented cellular aggregation and enhanced the invasive capacity of the cells. These phenotypic changes were mediated by functional ZEB2 DNA-binding, dependent upon the zinc-finger clusters. Furthermore, ZEB2 was able to transcriptionally repress multiple epithelial cell adhesion components, including proteins associated with adherens junctions, gap junctions and desmosomes. ZEB2 was also able to induce the cadherin switch, with conversion from E- to N-cadherin (Vandewalle *et al.*, 2005). Additionally, induction of ZEB2 expression in A431

cells resulted in G₁ phase cell cycle arrest due to ZEB2-mediated transcriptional repression of cyclin D1, resulting in an increase in the level of hypo-phosphorylated and inactive Rb protein, so preventing progression through the cell cycle (Mejlvang *et al.*, 2007). ZEB2 has also been identified as a cell survival protein, protecting cells from UV-induced apoptosis and DNA fragmentation (Sayan *et al.*, 2009).

Immunohistochemical analysis of colon and breast cancer tissues revealed ZEB1 expression at the tumour edge, expressed within dedifferentiated tumour cells that have the capacity to invade the surrounding tissue (Aigner *et al.*, 2007). Additionally, in bladder carcinoma specimens, ZEB1 staining inversely correlates with E-cadherin, with ZEB2 expression a negative predictor of survival (Sayan *et al.*, 2009). Following the intrasplenic and tail-vein injection of the colorectal carcinoma cell line HCT116, in which ZEB1 expression was inhibited by RNA interference, comparable primary tumour size was formed but metastatic tumours were greatly reduced in both size and number when compared to ZEB1-expressing cells (Spaderna *et al.*, 2008). This indicates that ZEB1 does not regulate primary tumour formation but is important in the control of metastatic spread, partially through transcriptional repression of E-cadherin. However, an additional key ZEB1 target appears to be the polarity factor, lethal giant larvae (Lgl2), which regulates apical-basal epithelial polarity. Within tumour samples, ZEB1 and Lgl2 were inversely correlated, with high expression of ZEB1 and low expression of Lgl2 within dedifferentiated tumour cells at the invasive front. Lgl2 appears to be an important ZEB1 target, with ectopic expression inducing E-cadherin re-expression, cell-cell contacts and reducing metastatic capacity (Spaderna *et al.*, 2008).

1.4.2 SNAIL PROTEINS

The vertebrate family of SNAIL proteins includes three members, SNAIL1 (Snail homologue 1), SNAIL2 and SNAIL3. SNAIL family members are zinc-finger proteins, with 4 to 6 highly conserved zinc-fingers located within the C-terminal domain. These domains allow DNA binding, with interaction occurring at the E2-box sequence (C/A(CAGGTG)). Upon DNA binding, SNAIL proteins mediate transcriptional repression,

which is dependent upon the presence of the N-terminally located SNAG domain. The central serine-proline rich region of SNAIL proteins is subject to extensive post-translational modifications that mediate cellular localisation and protein stability and activity. Developmentally, SNAIL1 and SNAIL2 are both involved in mesoderm formation following gastrulation and the generation of the migratory neural crest cells (Peinado *et al.*, 2007).

SNAIL1 was the first repressor of E-cadherin to be identified. During mouse development, SNAIL1 and E-cadherin were shown to inversely correlate, with SNAIL1 expression predominately identified within mesodermal tissue and neural crest cells. In epithelial cell lines, such as MDCK cells, ectopic expression of SNAIL1 resulted in the formation of a mesenchymal morphology, with cellular extensions and loss of E-cadherin. Additionally, SNAIL1 induced expression of vimentin and fibronectin, with cells becoming migratory, invasive and tumourigenic. Indeed, SNAIL1 and E-cadherin were inversely correlated in a panel of carcinoma cell lines, highlighting the role of SNAIL1 in the malignant transformation of epithelial cells (Cano *et al.*, 2000). In the colon cancer cell line HT-26 M6, SNAIL1-mediated repression of E-cadherin was dependent upon functional SNAIL1 zinc-finger domains and E-boxes located within the *CDH1* promoter (Batlle *et al.*, 2000). Interestingly, induction of SNAIL1 in both MDCK cells and HT-26 M6 cells resulted in a delayed increase in ZEB1 expression, potentially due to transcriptional activation at the *ZEB1* promoter. It was suggested that SNAIL1 expression initiated the EMT, with ZEB1 expression maintaining the EMT-related transcriptional control. When the strength of transcriptional repression was tested, SNAIL1 was identified as a stronger E-cadherin repressor when compared to ZEB1. Furthermore, ZEB2 was identified as the weakest repressor, only functioning in a highly cell- and context-specific manner (Guaita *et al.*, 2002). Within melanoma, the role of SNAIL1 in the repression of E-cadherin appears to be contradictory, with no consistent correlation identified (Poser *et al.*, 2001; Tsutsumida *et al.*, 2004).

In the rat bladder cancer cell line NBT-11, ectopic SNAIL2 expression resulted in repression of the desmosomal markers, desmoplakin and desmoglein, with loss of epithelial morphology and cellular scattering. However, only minimal repression of E-

cadherin was observed (Savagner *et al.*, 1997). Furthermore, in conjunction with SNAIL1, SNAIL2 was identified as a transcriptional repressor of E-cadherin in E-cadherin positive breast cancer cell lines. In a panel of breast cancer cell lines, SNAIL2 was inversely correlated with E-cadherin, with no correlation for SNAIL1 revealed in this study (Hajra *et al.*, 2002). Interaction of SNAIL2 with the *CDH1* promoter was shown to be weaker when compared to SNAIL1, with SNAIL2 potentially functioning as a dimer or multimeric protein. Expression of SNAIL2 in MDCK cells resulted in repression of E-cadherin, though to a lesser extent when compared to SNAIL1. However, SNAIL2 was able to induce an EMT, with loss of E-cadherin and plakoglobin; gain of vimentin and fibronectin; and increase in cellular migration (Bolos *et al.*, 2003).

SNAIL2 expression has also been implicated in melanomagenesis, with SNAIL2 detected in benign naevi and in malignant melanoma. Knockdown of SNAIL2 in transformed melanocytes minimally reduced the size of the primary tumour but greatly decreased the number of metastases. This indicates that SNAIL2 is important in metastatic melanoma (Gupta *et al.*, 2005). In an alternative study, SNAIL2 expression was shown to be elevated in naevi when compared to primary and metastatic melanomas, with the melanocytes expressing E-cadherin, whereas melanoma cell lines had undergone the cadherin switch. No correlation was detected between SNAIL2 and E-cadherin expression, but SNAIL2 was shown to be positively correlated with MITF. Ectopic expression of SNAIL2 in melanocytes resulted in the minor repression of E-cadherin and induction of MITF, whereas ectopic SNAIL2 induced N-cadherin expression and increased migration in melanoma cell lines. These results indicate that SNAIL2 expression is elevated in melanocytes and naevi compared to melanoma, suggesting that SNAIL2 may be involved in the initial steps of transformation (Shirley *et al.*, 2012).

1.4.3 TWIST PROTEINS

TWIST proteins, TWIST1 and TWIST2, are bHLH transcription factors that have a vital role in embryonic developmental, essential for gastrulation, mesoderm formation and induction of migration in neural crest cells. Additionally, TWIST1 has been implicated

in the induction of a cancer-EMT (Vernon & LaBonne, 2004). The structure of bHLH proteins includes two parallel amphipathic α -helices joined by a loop, which is required for dimerisation. TWIST proteins are able to bind to DNA at E-box sequences, which occur following their homo- or heterodimerisation with an alternative HLH protein (Peinado *et al.*, 2007). In a mouse mammary cell line model of tumourigenesis, TWIST was identified as an inducer of metastatic spread, enhancing intravasation (Yang *et al.*, 2004). Ectopic expression of TWIST in MDCK cells induced an EMT, with cells becoming fibroblast-like, with down-regulation of E-cadherin and α -catenin and induction of fibronectin, N-cadherin and vimentin and enhanced migration. Indeed, in breast cancer cell lines, TWIST is expressed in the invasive and metastatic varieties and absent in the non-invasive types. Additionally, elevated expression of TWIST was identified in cases of invasive lobular breast carcinoma, which characteristically shows single cell migration and features of EMT (Yang *et al.*, 2004).

1.5 E-CADHERIN TRANSCRIPTIONAL CO-REPRESSORS

The functions of transcription factors can be modulated by interactions with either co-activators, such as p300, or co-repressors, such as CtBP. In colorectal carcinoma cases, the ability of ZEB1 to function as a transcriptional activator of the vitamin D receptor gene, *VDR*, was strongest in cases with high expression of p300. In comparison, ZEB1 mediated transcriptional repression of *CDH1* was most evident in cases with elevated expression of CtBP (Pena *et al.*, 2006).

1.5.1 C-TERMINAL BINDING PROTEIN (CtBP)

CtBP was originally identified as an important regulator of tumour progression due to interaction with the adenovirus E1A oncogene, which has a C-terminally located CtBP binding site. Loss of the E1A-CtBP interaction resulted in an increase in tumourigenicity, suggesting that E1A sequestered CtBP and prevented CtBP-mediated repression of epithelial genes, such as E-cadherin, desmoglein-2, and plakoglobin (Chinnadurai, 2009). CtBP1 appears to be expressed in both the embryo and the adult, whilst CtBP2 is restricted to embryogenesis. CtBP1 and CtBP2 are transcriptional co-repressors that are able to interact with proteins via PLDSL motifs. CtBP does not

contain a DNA-binding domain but is able to interact with ZEB1 via three PLDLSL motifs located proximally to the C-terminal side of the ZEB1 homeodomain. Interaction of ZEB1 with CtBP increases the repressive activity of ZEB1, with mutations within these motifs reducing the interaction (Furusawa *et al.*, 1999). CtBP has also been shown to interact with ZEB2 (Shi *et al.*, 2003) and SNAIL1 (Postigo & Dean, 1999). CtBP was identified as a global repressor of the epithelial phenotype and responsible for promoting anchorage-independent growth (Grooteclaes & Frisch, 2000). In embryonic development, homozygous CtBP1/2 null mice are non-viable, with major defects in cellular differentiation, whilst CtBP1/2 null embryonic mouse fibroblasts experience the up-regulation of multiple epithelial specific genes, such as E-cadherin, occludin and keratin-8. Additionally, knockout cells were hyper-sensitive to apoptosis, highlighting the role of CtBP in cell survival (Grooteclaes *et al.*, 2003).

The ability of CtBP to function as a transcriptional co-repressor was identified by co-immunoprecipitation of CtBP interacting proteins. CtBP was shown to interact with DNA-binding proteins, such as ZEB1 and ZEB2; histone modifiers, such as histone deacetylases (HDAC1 and HDAC2) and histone methyltransferase (HMTs); chromodomain-containing proteins (HPC2 and CDYL); and also an alternative transcriptional co-repressor, the CoREST (co-repressor of RE1 silencing transcription factor/neural restrictive silencing factor) complex (Shi *et al.*, 2003). The DNA binding proteins recognise specific DNA sequences and the histone modifiers can generate a fully repressed chromatin structure. Interestingly, knockdown of CtBP in an E-cadherin-negative cell line resulted in conversion of the repressed chromatin structure at the *CDH1* promoter into an active chromatin configuration. This confirmed that CtBP functions as an E-cadherin transcriptional co-repressor within the natural chromatin context (Shi *et al.*, 2003). In disagreement with these data, full-length ZEB1 and ZEB2 were shown to mediate repression of E-cadherin in a CtBP-independent manner, unaffected by mutation of the PLDLSL motifs (van Grunsven *et al.*, 2003).

The interaction between CtBP and ZEB1 appears to be controlled by the phosphorylation of CtBP, mediated by the MEK-ERK signalling pathway. Inhibition of the MEK-ERK pathway by the MEK inhibitor, U0126, results in reduced CtBP

phosphorylation and a reduction in the interaction between ZEB1 and CtBP. This regulation may be important in determining the functionality of ZEB1, with reduced interaction between ZEB1 and CtBP allowing ZEB1 to change from a transcriptional repressor into a transcriptional activator (Shirakihara *et al.*, 2011).

1.5.2 BRAHMA (BRM) AND BRAHMA RELATED GENE 1 (BRG1)

The SWItch/Sucrose NonFermentable (SWI/SNF) chromatin remodelling complex was originally identified in the yeast, *Saccharomyces cerevisiae*, and has since been shown to be conserved in eukaryotes. This multi-component complex can dynamically regulate chromatin structure, either generating a repressive or active chromatin configuration. Chromatin modification occurs in an ATP-dependent manner, mediated by the mutually exclusive ATPase subunits, BRG1 or BRM (Klochendler-Yeivin *et al.*, 2002). Developmentally, BRM^{-/-} mice are normal, with up-regulated expression of BRG1, indicating that these subunits may be functionally equivalent. Interestingly though, BRM null mice are 15% heavier, with BRM linked to the control of cellular proliferation (Reyes *et al.*, 1998). In comparison, BRG1^{-/-} mice die during the peri-implantation stage, whilst heterozygous mutants are predisposed to exencephaly and tumours. This identified that even though the BRM and BRG1 subunits may be interchangeable in certain situations, they also have unique roles (Bultman *et al.*, 2000). Identified as an important regulator in development and in adult tissue homeostasis, the SWI/SNF chromatin remodelling complex has also been implicated in cancer progression. Due to the multiple, interchangeable subunits, the SWI/SNF complex can mediate expression of a diverse set of genes, in a cell-specific manner, which has resulted in the BRM and BRG1 subunits being characterised as both tumour suppressors and oncogenes (Wu, 2012).

Multiple mutations in BRG1 were identified in a range of cancer cell lines, including prostate, lung, pancreatic and breast, with re-expression of BRG1 resulting in changes in cellular morphology, including cell flattening. Additionally, ectopic expression of BRG1 in the BRG1-negative breast cancer cell line ALAB resulted in G₁ phase cell cycle arrest (Wong *et al.*, 2000), whilst in cases of non-small cell lung cancer, loss of

BRG1/BRM was associated with poor patient survival (Reisman *et al.*, 2003). Furthermore, homozygous BRG1 knockout mice experience embryonic lethality; yet interesting data has been acquired using a mouse model with specific BRG1 knockout in lung epithelium. Heterozygote lung-specific BRG1 knockout results in an increase in the number and size of lung adenomas. However, homozygous BRG1 deletion did not induce this phenotype, indicating that homozygous loss of BRG1 may be detrimental to transformed cells, resulting in apoptosis (Glaros *et al.*, 2008). Taken together, all these results highlight the potential role of BRG1 as a tumour suppressor.

However, other studies have demonstrated the oncogenic potential of BRG1. In prostate cancer, BRG1 expression was shown to increase gradually from benign to metastatic disease. Interestingly, there appeared to be a BRM-BRG1 switch, with higher expression of BRM and lower expression of BRG1 in benign samples compared to the reverse situation in metastatic cases. High expression of BRG1 was associated with increased tumour size and invasiveness (Sun *et al.*, 2007). A similar situation in colorectal carcinoma was found, with increasing nuclear expression of BRG1 correlating with disease progression. Knockdown of BRG1 in colorectal carcinoma cell lines resulted in decreased cellular proliferation, with cells retained in G₁ phase. Additionally, cell scattering was observed, with reduced membranous E-cadherin and β -catenin. In comparison, PTEN expression was increased following knockdown of BRG1, with the consequent repression of the PI3K/AKT signalling pathway, providing an explanation for the deregulation of cell cycle progression (Watanabe *et al.*, 2011). The ability of BRG1 to function as a transcriptional co-repressor was established when BRG1 was shown to interact with ZEB1, leading to the enhanced repression of *CDH1* (Sanchez-Tillo *et al.*, 2010). In this study, ZEB1-mediated repression of E-cadherin was found to be dependent upon two co-repressors, CtBP and BRG1, which interact with different ZEB1 protein domains. BRG1-mediated repression was dependent upon ZEB1 binding to promoter based E-cadherin E-boxes and in the colorectal carcinoma cell line SW-480, knockdown of BRG1 resulted in enhanced E-cadherin expression and reduced levels of vimentin. Additionally, in colorectal carcinomas, ZEB1 and BRG1 were co-localised within cells at the invasive front of the tumour. These findings suggest that

BRG1 is a key co-repressor of E-cadherin and inducer of EMT (Sanchez-Tillo *et al.*, 2010).

Within melanoma, BRG1/BRM had modestly higher expression in a panel of melanoma cell lines compared to human skin melanocytes. BRG1/BRM were shown to positively regulate activity of the MITF-M promoter, in an ATP-dependent manner, with BRG1 shown to be the prominent co-activator. Loss of BRG1/BRM additionally resulted in growth inhibition and reduced survival, which may be a consequence of reduced MITF expression (Vachtenheim *et al.*, 2010). In an alternative study, expression of BRG1 was shown to significantly increase from dysplastic naevi to primary melanoma, indicating that BRG1 may enhance melanoma initiation. Again, knockdown of BRG1 in melanoma cell lines resulted in growth arrest, with cells retained in G₁ phase (Lin *et al.*, 2010a). In the BRG1-negative melanoma cell line SK-MEL-5, ectopic expression of BRG1 altered the expression of multiple genes coding for cell surface receptors, adhesion molecules and extracellular matrix remodelling enzymes, including activation of E-cadherin and the neural cell adhesion molecule 1 (NCAM1). However, E-cadherin function was compromised following expression of BRG1, with E-cadherin being redistributed from the membrane to the cytoplasm, which occurred in conjunction with the development of an invasive phenotype (Saladi *et al.*, 2010).

The information regarding the role of BRG1/BRM in tumour progression is controversial, with conflicting results in multiple studies. The function of BRG1/BRM appears highly dependent on cellular context and may vary between different malignancies. However, the wide range of BRG1/BRM targets suggests that an understanding of its deregulation in cancer may provide vital therapeutic benefits.

1.5.3 NUCLEOSOME REMODELLING AND DEACETYLASE (Mi-2/NuRD) COMPLEX

The Mi-2/NuRD complex combines ATPase-dependent chromatin remodelling and histone deacetylase activity, via the Mi-2 α /Mi-2 β and HDAC1/HDAC2 subunits, respectively (Denslow & Wade, 2007). Additional components of the Mi-2/NuRD

complex include the methyl CpG-binding domain (MBD) proteins, MBD2 and MBD3, with MBD2 recognising methylated DNA; structural subunits that assist in establishing protein interactions, including Rbbp4 and/or Rbbp7, and Gatad2a (p66) or Gatad2b (p68); and finally the metastasis associated (MTA) protein family, MTA1, MTA2 and/or MTA3. The combined attributes of the Mi-2/NuRD complex indicate a role in transcriptional repression of target genes, via the formation of hypo-acetylated histones, present within a densely packed nucleosome structure (Denslow & Wade, 2007). The role of the Mi-2 β subunit in development has been examined by conditional knock-out in mice Schwann cells, which resulted in their incomplete terminal differentiation and linked the Mi-2/NuRD complex to peripheral nerve myelination (Hung *et al.*, 2012).

In relation to the MR-EMT, ZEB2 was shown to interact, via an N-terminal domain, with components of the Mi-2/NuRD complex, including the Mi-2 β subunit. Interestingly, mutation of the Mi-2/NuRD-binding motif in ZEB2 partially alleviated ZEB2-mediated repression of E-cadherin, indicating that this complex may function as a ZEB2 transcriptional co-repressor. Additionally, defective interaction between ZEB2 and the Mi-2/NuRD complex has been implicated in the development of a mild or atypical Mowat-Wilson syndrome, with mutation of the first 24 amino acids of ZEB2 identified, resulting in loss of interaction with the Mi-2 β /NuRD complex. It was also suggested that the Mi-2/NuRD complex may interact with ZEB1, with the putative Mi-2/NuRD-binding motif conserved between both ZEB proteins (Verstappen *et al.*, 2008). Another MR-EMT, TWIST, has also been shown to interact with components of the Mi-2/NuRD complex, including MTA2, Rbbp4, Mi-2 β and HDAC, with Mi-2 β interacting with the N-terminal region of TWIST. The interaction between TWIST and the Mi-2/NuRD complex was shown to be important in TWIST-mediated repression of E-cadherin and induction of a migratory and invasive phenotype, with enhanced rates of intravasation and metastatic spread. Recruitment of the Mi-2/NuRD complex to the E-cadherin promoter resulted in transcriptional repression via histone modifications and chromatin remodelling (Fu *et al.*, 2011).

Interestingly, the MTA3 subunit has been shown to directly repress expression of SNAIL1 in mammary epithelial cell lines, preventing SNAIL1-mediated repression of E-cadherin. Activation of MTA3 is dependent upon estrogen receptor (ER) signalling, with abundant expression of MTA3 in ER-positive mammary cell lines (Fujita *et al.*, 2003). Subsequently, SNAIL1 has been shown to directly repress the *ESR1 locus*, the gene encoding for ER- α , resulting in induction of an EMT (Dhasarathy *et al.*, 2007). These results suggest that the ER and MTA3 subunit are regulators of the epithelial phenotype, whilst SNAIL1 promotes a mesenchymal conversion, and identifies the Mi-2/NuRD complex as an important component controlling progression through an EMT and MET.

1.6 EPIGENETICS

The term epigenetics was introduced by Conrad H. Washington in 1942 to describe phenotypic features that were the result of the genotype. A modern definition of the term relates to the cellular variations that modify genomic expression, other than changes in nucleotide sequence, which can be inherited through DNA replication and cellular division (Richards, 2006).

1.6.1 DNA METHYLATION

DNA methylation refers to the modification of cytosine residues by the covalent addition of a methyl group at carbon-5, producing 5-methylcytosine (5mC). This predominately occurs at CpG dinucleotides, which tend to be underrepresented within the genome as a whole, but are clustered within CpG islands located within promoter regions of some genes. Alterations in the pattern of DNA methylation have been implicated in imprinting defects such as Prader-Willi syndrome, Angelman syndrome and Beckwith-Wiedemann syndrome and also in the formation of tumours. The original defect identified in tumour cells was the loss of DNA methylation, known as hypomethylation, which results in the activation of repetitive DNA and oncogenes. Additionally, hypermethylation, an increase in DNA methylation, has been found to occur in the promoters of tumour suppressor genes, resulting in transcriptional repression (Feinberg & Tycko, 2004). DNA methylation results in transcriptional

repression by inhibition of transcription factor binding to methylated promoters. Additionally, methyl-CpG binding domain proteins (MBDs) interact with methylated cytosine residues, resulting in alterations to the chromatin structure and further inhibiting transcriptional activity (Robertson, 2005). A comparison of the DNA methylation pattern at single-base resolution was undertaken in embryonic stem cells and fibroblasts. This identified a higher level of cytosine methylation in the embryonic stem cells, which occurred due to methylation at non-CpG sites. This non-CpG methylation is a specific feature of embryonic stem cells, which is probably lost during differentiation (Lister *et al.*, 2009).

An additional base modification that has recently received attention is 5-hydroxymethylcytosine (5hmC), which is produced following the oxidation of 5-mC by the enzyme ten-eleven translocation 1 (TET1), which is able to recognise both fully methylated and hemi-methylated DNA (Tahiliani *et al.*, 2009). Subsequently, mouse TET1, TET2 and TET3 have all been shown to catalyse this conversion (Ito *et al.*, 2010). It has been theorised that 5hmC is the intermediary product produced during the active demethylation of the genome. However, 5hmC may also be an additional epigenetic modification that has a unique functional role (Branco *et al.*, 2011). The TET proteins have been linked to tumour formation, with TET1 found to be fused to the myeloid/lymphoid or mixed-lineage leukemia gene in acute myeloid leukemia patients that possess the t(10;11)(q22;q23) translocation (Lorsbach *et al.*, 2003). With hypomethylation being a frequently recognised event in tumourigenic cells, the role of the TET proteins in the oxidation of 5mC, and subsequent removal of DNA methylation from the genome, may be an important aspect of tumour progression.

1.6.2 DNA METHYLATION AND CANCER

An 'epimutation' is a heritable alteration in an epigenetic modification, which has been widely implicated in cancer development (Dobrovic & Kristensen, 2009).

The link between DNA methylation at the *CDH1* promoter and repression of E-cadherin expression was originally identified in a panel of carcinoma cell lines,

including colon, breast, liver, stomach, lung and bladder (Yoshiura *et al.*, 1995). In E-cadherin-negative cell lines, the *CDH1* promoter was hypermethylated, with re-expression of E-cadherin following treatment with the demethylating agent, 5-azacytidine. Re-expression of E-cadherin re-established epithelial cell-cell adhesion and a cobblestone-morphology (Yoshiura *et al.*, 1995). The inverse correlation between DNA methylation and E-cadherin expression was confirmed in breast and prostate carcinoma cell lines, with the use of 5-azacytidine again restoring E-cadherin expression, the extent of which was cell line dependent. Interestingly, *CDH1* promoter hypermethylation was linked to disease progression in primary breast carcinomas, with no detected promoter methylation in normal breast tissue (Graff *et al.*, 1995).

Following the development of the highly specific and sensitive methylation-specific PCR (MSP), DNA methylation was confirmed at the promoters of the cyclin-dependent kinase inhibitors p16^{INK4A} and p15, the von-Hippel-Lindau (VHL) tumour suppressor gene and E-cadherin (Herman *et al.*, 1996). In hepatocellular carcinoma samples, *CDH1* promoter hypermethylation was more frequently detected at later disease stages and positively correlated with loss of E-cadherin expression (Kanai *et al.*, 1997). Subsequently, E-cadherin repression mediated by *CDH1* hypermethylation has been linked to thyroid carcinoma (Graff *et al.*, 1998), acute leukaemia (Corn *et al.*, 2000), colorectal carcinoma (Wheeler *et al.*, 2001), renal cell carcinoma (Nojima *et al.*, 2001), hepatocellular carcinoma (Matsumura *et al.*, 2001), bladder cancer (Ribeiro-Filho *et al.*, 2002), cervical carcinoma (Chen *et al.*, 2003), and non-small cell lung cancer (Wang *et al.*, 2008). In a panel of melanoma cell lines, *CDH1* DNA methylation did not fully correlate with E-cadherin expression but DNA methylation was only detected in E-cadherin-negative cell lines (Tsutsumida *et al.*, 2004). In a comprehensive study of *CDH1* promoter methylation in the NCI-60 cell lines, DNA methylation at the level of individual CpG sites and the average level of DNA methylation within each cell line was distributed bimodally. This resulted in the clustering of cell lines, which tended to possess either 0-20% or 80-100% DNA methylation. E-cadherin expression inversely correlated with the presence of DNA methylation, forming an 'L-shaped' graph when the level of E-cadherin expression was plotted against the level of DNA methylation (Reinhold *et al.*, 2007).

The link between hypermethylation at the *CDH1* promoter, with loss of E-cadherin and a subsequent increase in the migratory and invasive nature of cells, was established in a prostate cancer cell line, TSUPr1 (Graff *et al.*, 2000). Invasion through an artificial membrane induced *CDH1* hypermethylation when compared to a monolayer culture. When grown in 3D culture, under conditions inducing sphere formation, the *CDH1* promoter became hypomethylated, with increased E-cadherin expression. This highlights the dynamic nature of DNA methylation, which allows flexible regulation of gene expression, depending upon cellular context (Graff *et al.*, 2000). The role of E-cadherin promoter methylation in breast cancer cells has been reported several times. The presence of *CDH1* promoter hypermethylation was detected in ductal breast carcinoma *in situ*, indicating that it occurs early in disease progression. Interestingly, the DNA methylation pattern appears heterogeneous within a sample, indicating that not all alleles are hypermethylated (Graff *et al.*, 2000; Nass *et al.*, 2000). Treatment of mice with 5-aza-2'-deoxycytidine prior to injection of the breast cancer cell line MDA-MB-436S, resulted in reduced growth of both the primary tumour and metastases, with re-expression of E-cadherin at the primary site. This indicates that removal of DNA methylation, with induction of E-cadherin expression, may reduce the invasive and metastatic nature of tumour cells (Nam *et al.*, 2004). The involvement of aberrant DNA methylation in cancer progression was highlighted by cases of hereditary diffuse gastric cancer, whereby individuals carry a single germline mutation in *CDH1*. Loss of the remaining wild type allele was shown to occur by *de novo* DNA methylation in 50% of cases, leaving the mutant allele unmethylated. This identified that DNA methylation may provide the 'second genetic hit' required for tumour cells to progress towards malignancy (Grady *et al.*, 2000). The combinatorial effect of DNA promoter methylation and the presence of a transcriptional repressor were identified in oral carcinoma cell lines. E-cadherin expression negatively correlated with *CDH1* hypermethylation but E-cadherin re-activation with 5-azacytidine was not successful in hypermethylated cell lines expressing high levels of ZEB2. This suggests that transcriptional control of E-cadherin is mediated by both DNA methylation and the presence of transcriptional repressors, with the potential for both mechanisms to dynamically control E-cadherin expression, depending upon demands from the microenvironment (Maeda *et al.*, 2007).

1.7 OVERVIEW

The majority of work conducted regarding the MR-EMT, in relation to the induction of EMT and repression of E-cadherin, has been conducted in the setting of carcinogenesis (Figure 1-5). Preliminary work has been undertaken examining the expression of the MR-EMT in melanomagenesis, however a more detailed analysis would be advantageous. Melanoma is a highly metastatic disease and a greater understanding of the signalling pathways involved in melanoma progression will help in the identification of additional druggable targets. Through the examination of the expression of the MR-EMT in melanoma cell lines and their ability to regulate E-cadherin, in conjunction with transcriptional co-repressors and DNA methylation, an understanding will be acquired as to whether the MR-EMT function in a comparable manner in melanomagenesis, as in carcinogenesis.

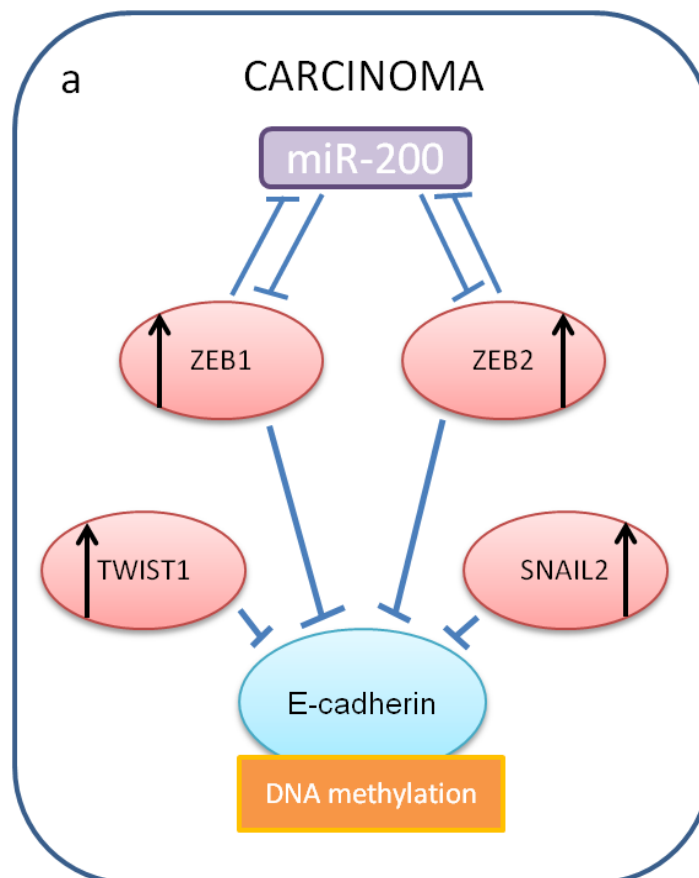


Figure 1-5 Regulation of E-cadherin and the MR-EMT during carcinogenesis

Expression of the MR-EMT increases during carcinogenesis, in a tumour specific manner, with all the MR-EMT identified as transcriptional repressors of E-cadherin, which is also regulated at the level of DNA methylation. Finally, a double negative feedback loop exists between the miR-200 family and the ZEB proteins.

1.8 AIMS AND OBJECTIVES

Primary aim: To investigate the relationship between the MR-EMT and E-cadherin during melanoma progression. In order to test this primary aim, the following areas will be investigated:

1. Aim: To investigate the expression profile of the MR-EMT and E-cadherin within melanoma cell lines.

Objectives: The expression patterns of the MR-EMT and E-cadherin will be analysed in human neonatal epidermal melanocytes and a panel of melanoma cell lines. The expression of the MR-EMT will then be investigated in relation to melanoma-initiating mutations within the MAPK signalling pathway.

2. Aim: To investigate how E-cadherin is transcriptionally regulated within melanoma cell lines, in relation to the MR-EMT, transcriptional co-repressors and DNA methylation

Objectives: Through the use of transient RNA interference, protein over-expression and reporter assays, the ability of the MR-EMT to control E-cadherin expression will be investigated. Additionally, through transient transfections, reporter assays and co-immunoprecipitation, the involvement of transcriptional co-repressors will be judged. Finally, the level of DNA methylation within the melanoma cell lines will be determined.

3. Aims: To investigate how the MR-EMT regulate melanocyte-specific markers of differentiation.

Objectives: MITF will be analysed in relation to the expression of the MR-EMT by the use of RNA interference and ectopic protein expression.

CHAPTER 2 : MATERIALS AND METHODS

2.1 CHEMICALS AND REAGENTS

Unless otherwise stated below, reagents were purchased from Sigma-Aldrich, Dorset, UK and solvents from Fisher Scientific, Loughborough, UK.

Table 2-1: Reagents

Reagent/ Kit	Company	Catalogue Number
α -Select Chemically Competent Cells Bronze and Gold efficiency	Bioline (London,UK)	BIO-85025 BIO-85027
BioMix Red	BioLine	BIO-25006
cOmplete, mini protease inhibitor cocktail tablets	Roche Applied Science (West Sussex, UK)	11 836 153 001
Dulbecco's modified eagle media (DMEM) high glucose without L-Glutamine	PAA (Pasching, Austria)	E15-009
Dulbecco's modified eagle media (DMEM) high glucose with L-Glutamine and Sodium Pyruvate	PAA	E15-843
Heat inactivated fetal bovine serum (FBS)	PAA	A15-152
Fluoromount G	Cambridge Bioscience (Cambridge, UK)	0100-01
HighRanger 1kb DNA Ladder	Geneflow (Staffordshire, UK)	L3-0020-S
Human Epidermal Melanocytes (HEM) growth medium package	TCS Cellworks (Buckingham, UK)	ZHM-1955
Human Epidermal Melanocytes (HEM) passage pack	TCS Cellworks	ZHR-9941
Ingenio® electroporation solution	Geneflow	E7-0516
Isoton® II diluents	Beckman Coulter Inc (High Wycombe, UK)	8448011
LB Agar, powder (Lennox L Agar)	Invitrogen (Paisley, UK)	22700-025
Lipofectamine™ 2000	Invitrogen	11668-027
Luciferase Assay System	Promega (Southampton, UK)	E4030
Marvel Dried Skimmed Milk Powder	Premier brans	N/A
100X MEM Non-essential amino acid (NEAA) without L-Glutamine	PAA	11140-035
Miller's LB Broth Base® (Luria Broth Base)	Invitrogen	12795-027
pcDNA™3.1/V5-His TOPO® TA Expression Kit	Invitrogen	K4800-01
PCR Ranger 100bp DNA Ladder	Geneflow	L3-0004-S
PD184352	Selleck Chemicals (Suffolk, UK)	S1020
Penicillin-Streptomycin (PS) (100X)	PAA	P11-010
PLX-4720	Selleck Chemicals	S1152
Protein G Sepharose beads	GE Healthcare (Buckinghamshire, UK)	17-0618-01
Precision plus protein prestained standards, dual colour	Bio-Rad (Hertfordshire, UK)	161-0374
RNeasy Plus Mini Kit	QIAGEN (West Sussex, UK)	74134
Roswell Park Memorial Institute (RPMI) 1640 with glutamine	PAA	E15-040
Sall HF	New England Biolabs (Hertfordshire, UK)	R3138S
Sodium Dodecyl Sulphate (SDS) (20%)	Geneflow	B9-0038

Trypsin EDTA UV inactivated (10X)	PAA	L11-659
U0126	Calbiochem (EMD Millipore) (Watford, UK)	662005
Ultra-pure water	Invitrogen	10977-049
Wizard SV Gel and PCR Clean-Up System	Promega	A9281
Zymo Research EZ DNA Methylation Gold Kit	Cambridge Bioscience	D5006
Zymo Research ZymoTaq™ PreMix	Cambridge Bioscience	E2004

Table 2-2: Buffers

Reagent Name	Components
B-galactosidase mastermix	66 µL 4 mg/ml ONPG (ortho nitrophenyl β-galactosidase) in 0.1 M NaPO ₄ , pH 7.5; 201 µL of 0.1 M NaPO ₄ , pH 7.5; 3 µL 4.5 M β-Me in 0.1 M MgCl ₂
DNA loading dye (6X)	0.25% (w/v) xylene cyanol or bromophenol blue; 30% glycerol (v/v) (25 mg xylene cyanol or bromophenol blue; 3 ml glycerol; 6.7 ml water)
Glycerol dye	0.5 mg/ml bromophenol blue; 50% (v/v) glycerol
Laemmli lysis buffer (cell lysis buffer) (4X)	200 mM Tris-HCl (pH 6.8), 8% SDS, 40% (v/v) glycerol
Lysis buffer	50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1mM EDTA; 1 mM EGTA; 50 mM NaF; 5 mM sodium pyrophosphate; 10% v/v glycerol; 1% v/v triton X-100; 1 mM NaVO ₄ ; 1 cOmplete mini protease inhibitor tablet per 10 mL of lysis buffer added immediately prior to use
Phosphate buffered saline (PBS) (g/L:- 8.0 sodium chloride; 0.2 potassium chloride; 1.15 di-sodium hydrogen phosphate; 0.2 potassium dihydrogen phosphate; pH 7.3± 0.2 at 25 °C)	Dissolve 10 PBS tablets/1 litre
Ponceau S staining solution	0.1% (w/v) Ponceau S; 5% (v/v) acetic acid
Protein loading buffer	0.2% (w/v) bromophenol blue; 1% (v/v) 2-mercaptoethanol
Protein running buffer	25 mM Tris-HCl; 192 mM glycine; 0.1% (w/v) SDS
Protein transfer buffer	25 mM Tris, 192 mM glycine, 20% (v/v) methanol; pH 8.3
RNA gel loading buffer	50 % (v/v) formamide; 20 % (v/v) formaldehyde; 10 % (v/v) 10x MOPS buffer; 20% (v/v) glycerol dye
Stripping Buffer	100 mM 2-mercaptoethanol, 2% SDS, 62.5mM tris-HCl pH 6.7
Tris-acetic acid ethylenediaminetetraacetic acid (EDTA) buffer (TAE)	40 mM Tris; 20 mM acetic acid; 1 mM EDTA
Tris-borate EDTA buffer (TBE)	(1X) 89 mM Tris; 89 mM boric acid; 2 mM EDTA
Tris-buffered saline with Tween (TBS-T)	50mM Tris-HCl (pH 8.0); 150 mM NaCl; 0.1% (v/v) Tween-20
TBS-T with bovine serum albumin (BSA)	TBS-T; 5% BSA
TBS-T with milk	TBS-T; 5% (w/v) Marvel dried milk powder
Tris-EDTA (TE) buffer	10 mM Tris-HCl (pH 7.5); 1 mM EDTA (pH 8.0)
Tris-SDS buffer	0.05 M Tris (pH 8); 0.1% SDS

Table 2-3: Primary antibodies

Antibody	Product no.	Clone no.	Lot no.	Company City/Country	Isotype	Migration in SDS/PAGE (kDa)	Dilution	Immunogen	References
α -tubulin	T5168	B-5-1-2	05IM4771	Sigma-Aldrich (Dorset, UK)	Mouse monoclonal IgG1	55	1:20000 WB	Raised against a C-terminal section	-
B-RAF	sc-5284	F-7	F1710	Santa Cruz Biotechnology, INC (Heidelberg, Germany)	Mouse monoclonal IgG2a	90	1:1000 WB	Raised against amino acids 12-156 of human B-Raf	-
BRG1 ¹	sc-10768	H-88	F1216	Santa Cruz Biotechnology, INC	Polyclonal rabbit IgG	185	1:1000 WB	Raised against N-terminally located amino acids 209-296 of human BRG1	-
CtBP	sc-17759	E-12	A2610	Santa Cruz Biotechnology, INC	Mouse monoclonal IgG1	48	1:500 WB	Raised against amino acids 1-440 of human CtBP1	-
E-cadherin	610181	36/E-Cadherin	70177	BD Transduction Laboratories™ (Oxford, UK)	Mouse monoclonal IgG2a, κ	120	1:2000 WB 1:100 IF	Raised against the cytoplasmic domain of E-cadherin	-
FLAG	F3165	M2	080M6034	Sigma-Aldrich	Mouse monoclonal IgG1	N/A	1:1000 WB	Binds FLAG fusion proteins	-
MITF	M3621	D5	10036747	Dako (Cambridgeshire, UK)	Monoclonal Mouse IgG	54 and 60	1:200	Raised against the human N-terminal region of MITF	-
N-cadherin	610920	32/N-Cadherin	78545	BD Transduction Laboratories™	Monoclonal Mouse IgG1	130	1:1000 WB	Raised against amino acids 802-819 of mouse N-cadherin	-

¹ Gift from Dr Nickolai Barlev, University of Leicester, Department of Biochemistry, Leicester, UK

P-cadherin	610228	56/P-Cadherin	7	BD Transduction Laboratories™	Monoclonal Mouse IgG1	120	1:500 WB	Raised against amino acids 72-259 of human P-cadherin	-
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	4377	197G2	6	Cell signalling New England Biolabs (UK) Ltd	Rabbit monoclonal IgG	40	1:1000 WB	Raised against a synthetic phosphopeptide corresponding to residues surrounding Thr202/Tyr204 of human p44 MAP kinase	-
SNAIL2	9585	C19G7	3	Cell signalling New England Biolabs (UK) Ltd	Monoclonal rabbit IgG	30	1:200 WB	Raised against recombinant human Slug protein	-
SNAIL1 ²	-	-	-	In house	Mouse monoclonal	29	1:50 WB	Raised against amino acids 1-82 of the N-terminal domain of SNAIL1	(Franci <i>et al.</i> , 2006)
TWIST1	ab50887	Twist2C1a	GR2224-2	Abcam (Cambridge, UK)	Mouse monoclonal IgG1	21	1:50 WB	Raised against recombinant human Twist	-
Vimentin	550513	RV202	45031	BD Transduction Laboratories™	Mouse monoclonal IgG1	56-60	1:500 WB	Raised against purified cow lens vimentin	-
ZEB1	sc-25388	H-102	G1910	Santa Cruz Biotechnology, INC	Polyclonal rabbit IgG	250	1:2000 WB 1:300 IF	Raised against amino acids 39-140 of human ZEB1	-
ZEB2 (CUK2) ³	-	-	-	In house	Polyclonal rabbit IgG	250	1:5000 WB 1:1500 IF	Raised against the N-terminal 380 amino acids of human ZEB2	(Sayan <i>et al.</i> , 2009; Oztas <i>et al.</i> , 2010)

² Gift from Dr. Antonio Garcia de Herreros, Institut Municipal d'Investigació Mèdica, Parc de Recerca Biomèdica de Barcelona, Spain

³ Available in the laboratory of Dr Eugene Tulchinsky, University of Leicester, Department of Cancer Studies and Molecular Medicine, Leicester, UK

Table 2-4: Secondary antibodies

Antibody	Product number	Lot Number	Company	Type	Dilution
Anti-Mouse Immunoglobulins/HRP	P 0447	00063800	DAKO	Polyclonal goat	1:2000 WB
Anti-Rabbit Immunoglobulins/HRP	P 0448	00072118	DAKO	Polyclonal goat	1:2000 WB
Alexa Fluor® 488 Anti-Mouse IgG Antibodies	A21202	898250	Invitrogen	Polyclonal donkey	1:500 IF
Alexa Fluor® 594 Anti-Rabbit IgG Antibodies	A21207	725861	Invitrogen	Polyclonal donkey	1:500 IF

2.2 CELL CULTURE

2.2.1 ROUTINE CELL MAINTAINENCE

All cell culture procedures were performed in a class II laminar flow cabinet, with cell lines maintained in an incubator at 37 °C, 5% CO₂ and 100% humidity. The range of cell lines used can be found in Table 2-5, including guidance on the type of cell culture media used. All media was stored at 4 °C and warmed to 37 °C prior to use. Tissue culture flasks, T25, T75 and T175 (PAA70075x, PAA70025x, PAA71175x), were purchased from PAA. Cells were maintained in culture and passaged at approximately 70% confluency. Cells were not passaged more than 20 times. Adherent cells were sub-cultured with three washes of phosphate-buffered saline (PBS), prior to trypsinization with 1 X trypsin EDTA. Cells were returned to the incubator at 37 °C for 5 to 10 minutes, after which the flask was gently tapped to detach cells. The 1X trypsin EDTA was neutralised with the addition of cell culture media. Cells were transferred to a 50 ml centrifuge tube (CFT-900-031F) and pelleted via centrifugation at 300 xg for 5 minutes and the pellet re-suspended in 1 ml of media. The cells were seeded at the required cellular density in the correct sized flask and media added. Cells were immediately returned to the incubator. In comparison, for the suspension cell line HL-60, cells were initially pelleted and the pellet resuspended and washed with PBS three times, with pellet formation between each wash step. After the final wash step, cells were re-suspended in 1 ml of media and seeded at the correct density with the required culture media added.

2.2.2 CELL COUNTING

Cells were counted using a Beckman Coulter Z Series Cell and Particle Counter (6605700). Cells were washed, detached, pelleted and re-suspended in 1 ml of media. Ten ml of Isoton® II diluent was added to the Coulter Counter vial from Greiner Bio-Line (668102) (Gloucestershire, UK) and 10 µl of the cellular suspension added. Cells were counted with a lower limit for detection of 8 µm and an upper limit of 20 µm and a dilution factor of 1000. The number of cells required was determined using the

following formula, where V is the volume of cells required in μl , REQ is the required number of cells, DET is the detected cell count and DIL is the dilution factor:-

$$V = (\text{REQ}/\text{DET}) \times \text{DIL}$$

The volume of cells required was then placed a suitable tissue culture flask and re-suspended in tissue culture media.

2.2.3 FREEZING AND THAWING CELL LINES

Cells were washed, trypsinized and pelleted. Cells were subsequently counted, with 2×10^6 cells re-pelleted and re-suspended in 1 mL of freezing media (70% cell culture media with glutamine; 20 % FBS; 10 % DMSO) and aliquoted into Cryo.s™ Freezing Tubes from Greiner Bio-One (123263). These tubes were placed in a Cryo tube freezing container, containing isopropanol, and stored at -80°C overnight. Cells were then transferred into the liquid nitrogen container for long-term storage.

Cells removed from liquid nitrogen were immediately placed in a 37°C water bath to thaw. Cells were then mixed with 10 ml of cell culture media and pelleted by centrifugation. The pellet was re-suspended in 5 mL of complete media, transferred to a T25 tissue culture flask and placed in the incubator.

Table 2-5: Cell lines

Cell line	Culture media	Cellular origin	E-cadherin status	Melanoma associated mutations	References
Human epidermal melanocytes neonatal (HEMN)	Human Epidermal Melanocyte (HEM) basal medium; growth medium supplement; antibiotic	Human neonatal epidermal melanocytes supplied by TCS Cellworks	Positive	None	-
A375 P	RPMI 1640; 10% FBS; 1% P/S	54 year old female; malignant melanoma	Negative	Homozygous B-RAF V600E	(Kozlowski <i>et al.</i> , 1984)
A375 M	RPMI 1640; 10% FBS; 1% P/S	54 year old female; malignant melanoma; derived from the A375P cell line that were injected into the tail vein of nude mice and lung metastases removed	Negative	Homozygous B-RAF V600E	(Kozlowski <i>et al.</i> , 1984)
RPMI-7951	EMEM (MG4655), 1% sodium pyruvate; 1% NEAA; 10% FBS; 1%P/S	18 year old Caucasian female; human malignant melanoma established from lymph node metastasis	Negative	Heterozygous B-RAF V600E	-
WM266-4	DMEM E15-843; 1% NEAA; 10%FBS; 1%P/S	58 year old female; human malignant melanoma derived from the metastatic site	Negative	B-RAF V600D (equivalent to B-RAF V600E in kinase activity)	(Wan <i>et al.</i> , 2004)
SK-MEL-28	DMEM with glutamine; 1% NEAA; 10% FBS; 1% P/S	51 year old male; human melanoma	Positive	Homozygous B-RAF V600E	-
SK-MEL-5	DMEM with glutamine; 1% NEAA; 10% FBS; 1% P/S	24 year old Caucasian female; human cutaneous melanoma	Positive	Heterozygous B-RAF V600E	-
SK-MEL-2	EMEM; 10% FBS; 1% P/S	60 year old Caucasian male; human malignant melanoma obtained from the metastatic site	Positive	Homozygous N-RAS Q61R	
SK-MEL-30	RPMI 1640; 10% FBS; 1% P/S	67 year old Caucasian male; human malignant melanoma established from subcutaneous tumour tissue	Positive	Heterozygous N-RAS Q61K	-

IPC-298	RPMI 1640; 10% FBS; 1% P/S	64 year old female; human cutaneous melanoma established from the primary tumour	Negative	Heterozygous N-RAS Q61L	-
UACC-257	RPMI 1640; 10% FBS; 1%P/S	Human melanoma	Positive	Heterozygous B-RAF V600E	-
MDA-MD-231	DMEM with glutamine; 10% FBS; 1% P/S	51 year old Caucasian female; human breast adenocarcinoma	Negative	N/A	-
SW480	DMEM with glutamine; 10% FBS; 1% P/S	50 year old Caucasian male; human colon adenocarcinoma established from the primary site	Negative	N/A	-
HL-60	RPMI 1640; 10% FBS; 1% P/S	36 year old Caucasian female; leukemia; suspension culture	Negative	N/A	-
A431-ZEB2	DMEM with glutamine; 10% FBS; 1% P/S	85 year old female; human epidermoid carcinoma; transformed to express ZEB2 by addition of 2 µg/µl of doxycycline (DOX) to the culture medium	Positive	N/A	-

2.2.4 TRANSIENT TRANSFECTIONS

Plasmids (Table 2-9) and siRNA (Table 2-7) were transiently transfected into cell lines using the Ingenio® electroporation solution in combination with the GenePulser Xcell electroporator from Bio-Rad (165-2660), which was set at 250 V and 250 μ F.

Cells were trypsinized, pelleted and counted and two million cells aliquoted into a 1.5 ml microcentrifuge safe-lock tube from Fisher Scientific (CFA-112-020P). Cells were again pelleted and re-suspended in 60 μ l of the Ingenio® electroporation solution and subsequently mixed with the appropriate plasmid DNA or siRNA at the required concentration. The sample was transferred to a 4 mm electroporation cuvette from Geneflow (E6-0076) and placed in the electroporator and the cells electroporated. Cells were immediately transferred to a 6 cm² cell culture dish from Greiner Bio-One (628960), containing pre-warmed media and returned to the incubator. For each set of transfections, a positive control for transfection efficiency was included, which consisted of transfection with the pEGFP-C1 plasmid (Table 2-9). Transfection efficiency was determined the following day and cell culture media replaced.

2.2.5 CHEMICAL TREATMENTS

B-RAF and N-RAS mutant cell lines were treated with B-RAF and MEK inhibitors for 24 hours. Stock solutions at 10 mM were produced in DMSO for the B-RAF inhibitor PLX4720 and the MEK inhibitors PD184352 and U0126. The inhibitors were used at a final concentration of 10 μ M dissolved in the appropriate tissue culture media. Additionally, for induction of ZEB2 expression in the A431-ZEB2 cell line, doxycycline at 2 μ g/ml was added to the tissue culture media, from a stock solution of 2 mg/ml in water. Finally, ectopic expression of ZEB1 using the pBI_ZEB1_HA and pUHD172.1 neo vectors (Table 2-9) required transfected cells to be treated with 2 μ g/ml of doxycycline for induction of ZEB1 expression.

2.3 MANIPULATION OF NUCLEIC ACIDS

2.3.1 DNA EXTRACTION

DNA extraction was performed using a phenol-chloroform protocol, a form of liquid-liquid extraction. Cultured cells at the required density were pelleted, as previously described, in a 1.5 ml microcentrifuge tube, and 1 ml of Tris-SDS buffer and 50 μ l of 10 mg/ml proteinase K were added to the pellet. The pellet was incubated over night at 37 °C to efficiently lyse the cells and release the DNA into solution. Samples were then split into two separate 1.5 mL microcentrifuge tubes and 500 μ l of phenol/chloroform/isoamyl alcohol solution (25:24:1) added to separate the DNA from the protein. The solution was vortexed and then centrifuged in an Eppendorf Microcentrifuge 5417 (CFA-112-020P) at 13,000 xg for 3 minutes. The aqueous layer was then removed and retained, which contained the DNA, avoiding the organic phase and white interface, which contained the contaminating protein. This phenol/chloroform/isoamyl alcohol solution wash was repeated if required to obtain a clear aqueous layer. Subsequently, 500 μ l of chloroform/isoamyl alcohol solution was added, vortexed and centrifuged as before, ensuring complete removal of any residual phenol. The aqueous layer was again removed and retained. An ethanol precipitation was then performed, with the addition of 50 μ l of 1 M sodium chloride and 800 μ l of chilled absolute ethanol. Samples were mixed by inverting and stored at -20°C for a minimum of 1 hour. Samples were then centrifuged at 4 °C for 15 minutes, generating a pellet, with the supernatant subsequently removed and discarded. Five hundred μ l of 70% ethanol was added, samples were inverted and again centrifuged at 4 °C for 20 minutes. The ethanol was then removed, avoiding the pellet, which was allowed to air dry. The pellet was re-suspended in 1X trypsin EDTA buffer, the volume of which was dependent upon the size of the pellet. Samples were stored at either 4 °C or -20 °C.

2.3.2 RNA EXTRACTION

RNA extraction was performed by combining the TRI Reagent® method (Chomczynski & Sacchi, 1987) and the RNeasy® Plus Mini Kit. Basically, cells were washed twice with PBS, followed by the addition of 1 ml of TRI Reagent®. TRI Reagent® contains a mix of

guanidine thiocyanate and phenol to dissolve DNA, RNA and protein. Cells were detached by scraping using a cell scraper from VWR (734-1526) (Leicestershire, UK) and transferred to a 1.5 ml microcentrifuge tube. Samples were incubated for 5 minutes, followed by the addition of 200 µl of chloroform. Samples were shaken vigorously for 15 seconds to ensure complete mixing of the TRI Reagent® and chloroform and allowed to incubate for 3 minutes. Samples were centrifuged at 12,000 xg for 15 minutes at 4 °C. This generated a three phase solution; the upper phase containing the RNA, the interface containing the DNA and the lower, organic phase containing the protein. The aqueous phase was removed and retained and the protocol transferred to the RNeasy® Plus Mini Kit, whereby the manufacture's guidelines were followed for the protocol for Purification of Total RNA from Animal Cells from step 5. This involved the addition of ethanol to the RNA sample to ensure RNA binding to the spin column. Multiple wash steps of the column were performed to remove any contaminants and the RNA finally eluted in RNase-free water and stored at -80 °C.

2.3.3 NUCLEIC ACID QUANTIFICATION

Nucleic acid concentration and the total yield were determined using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA). At the beginning and in between samples the pedestal and lid were cleaned with optical instrument cleaner. The program was initialized with ultra-pure water and blanked with the buffer used to dissolve the nucleic acid. Samples were applied onto the pedestal in 1 µl volumes. Samples were measured in duplicate. Sample purity was determined by monitoring the absorbance readings at 260 and 280 nm, with DNA and RNA having an optimal $A_{260/280}$ ratio of 1.8 and 2.1 respectively.

2.3.4 VISUALISATION OF DNA

PCR products and plasmids were separated by horizontal agarose gel electrophoresis with equipment obtained from Bio-Rad (170-4485EDU). DNA in 10 µl volumes was mixed with 2 µl of 6X DNA loading dye. Varying percentages of agarose gels were produced, ranging from 0.8-3%, depending on DNA size. Agarose was dissolved in

either TAE or TB buffers and ethidium bromide added to a final concentration of 2 µg/ml. Samples were loaded, a DNA ladder included, and gels run at 80 volts for 40 minutes. DNA was visualised using an UVP BioDoc-H System UV transilluminator and gel images taken.

2.3.5 VISUALISATION OF RNA

The quality of extracted RNA was determined by visualising the 18S and 28S rRNA subunits. A formaldehyde-agarose gel (1% agarose) was produced by dissolving 1 g of agarose in 74 ml of water by boiling. This was allowed to cool to approximately 55 °C and 16 ml of 37% formaldehyde, 10 ml of 10X MOPS buffer (200 mM MOPS, pH 7.0, 80 mM Sodium Acetate, and 10 mM EDTA, pH 8.0) and ethidium bromide to a final concentration of 2 µg/ml were added, the gel cast into the Rio-Rad gel equipment and allowed to set, all within a fume hood. RNA totalling 2 µg was resuspended to a total of 25 µl with RNA loading buffer. This was heated to 65 °C for 15 minutes and then allowed to cool on ice. The RNA was electrophoresed in 1X MOPS buffer for 40 minutes at 60 volts. The RNA was visualised using the UV transilluminator and gel images taken.

2.3.6 DNA OLIGONUCLEOTIDES

Pelleted DNA primers (Table 2-6) were re-suspended in water to produce a stock solution of 100 µM. A 10 µM working solution was subsequently produced. Additionally, dried siRNA oligonucleotides (Table 2-7) were re-suspended in water to a concentration of 100 µM. Two µl of siRNA were used for each transient transfection.

Table 2-6: DNA oligonucleotides

Primer name	Primer sequence (5'-3')		T _m (°C)	Cycle No.	Product size (bp)	Use	NCBI Accession Number	Annealing position (5'-3')	Exon	Reference
	Forward Primer	Reverse Primer								
E-cad Island 1 meth	TTAGGTTAGAGGGTTATC GCGT	TAATAAAAAATTCACCTACCGA C	57	40	116	MSP	L34545	840-867 960-939	N/A	(Herman <i>et al.</i> , 1996)
E-cad Island 1 unmeth	TAATTTTAGGTTAGAGGG TTATTGT	CACAACCAATCAACAACACA	53	40	96	MSP	L34545	843-867 940-921	N/A	(Herman <i>et al.</i> , 1996)
E-cad Island 2 meth	GTGGGCGGGTCGTTAGT TTC	CTCACAAATACTTTACAAATTCC GACG	57	40	172	MSP	L34545	881-900 1053-1027	N/A	(Graff <i>et al.</i> , 1997)
E-cad Island 2 unmeth	GGTGGGTGGGTGTTAG TTTTGT	AACTCACAAATCTTTACAATTCC AACA	57	40	174	MSP	L34545	881-903 1055-1029	N/A	(Graff <i>et al.</i> , 1997)
E-cad Island 3 meth	GGTGAATTTTGTAGTTAAT TAGCGGTAC	CATAACTAACCGAAAAACGCCG	57	40	204	MSP	L34545	945-971 1149-1129	N/A	(Graff <i>et al.</i> , 1997)
E-cad Island 3 unmeth	GGTAGGTGAATTTTGTAGT TAATTAGTGGTA	ACCCATAACTAACCAAAAACAC CA	57	40	211	MSP	L34545	941-970 1152-1129	N/A	(Graff <i>et al.</i> , 1997)
E-cad Bi	TTTAGATTTTAGTAATTTT AGGTTAGAGGG	ACTCCAAAAACCCATAACTAAC	60	40	333	BSP	L34545	828-857 1160-1139	N/A	N/A
Mi2β	GGCCACGTCCACTACTTG AT	CCCATCTCATCAGCCAAGAT	60	25	356	RT-PCR	NM_001273	2070-2090 2425-2405	13-15	N/A
E-cad	CAGCCACAGACGCGGAC GAT	CAGCCTCCCACGCTGGGGTA	58	25-32	381	RT-PCR	NM_004360	978-997 1358-1339	7-9	N/A
ZEB1	ACAAGCGAGAGGATCAT GGC	TCCTGCTTCATCTGCCTGAG	60	25	126	RT-PCR	NM_001174095	49-68 174-155	1-4b	N/A
GAPDH	TCTTTTGCCTCGCCAGCC GA	GCCAGCATCGCCCCACTTGA	60	25	312	RT-PCR	NM_002046	136-155 447-428	1-6	N/A
BRG1-FLAG	CACCATGGACTACAAGGA CGACGATGACAAGTCCAC TCCAGACCCACCCC	TCAGTCTTCTCGCTGCCAC	62	25	5200	PCR	D26156	61-79 4982-5001	N/A	N/A

Table 2-7: siRNA sequences

Oligo Name	Sense sequence 5'-3'	Anti-sense sequence 5'-3'	References
Control_siRNA	AUGAACGUGAAUUGCUCUAA[dT][dT]	UUGAGCAAUUCACGUUCAU[dT][dT]	(Sayan <i>et al.</i> , 2009)
ZEB1_109651	GGACUCAAGACAUCUCAGU[dT][dG]	ACUGAGAUGUCUUGAGUCC[dT][dG]	(Sayan <i>et al.</i> , 2009)
ZEB1_187234	GCAGACCAGACAGUAUUAC[dT][dG]	GUAAUACUGUCUGGUCUGC[dT][dG]	(Sayan <i>et al.</i> , 2009)
humZEB2	GAACAGACAGGCUUACUUA[dT][dT]	UAAGUAAGCCUGUCUGUUC[dT][dT]	Appendix I
TWIST1	ON-TARGET plus SMARTpool, Thermo Scientific (I-006434-00)		Appendix I
SNAIL2 siRNA	GCAUUUGCAGACAGGUCAA[dT][dT]	UUGACCUGUCUGCAAUUGC[dT][dT]	Appendix I
Brg1A siRNA	GGGUACCCUCAGGACAACA[dT][dT]	UGUUGUCCUGAGGGUACCC[dT][dT]	-
Brg1B siRNA	CGACGUACGAGUACAUCAU[dT][dT]	AUGAUGUACUCGUACGUCG[dT][dT]	-
Mi2βi siRNA	GAUUACGACCUGUUAAGCAG[dC][dT]	CUGCUUGAACAGGUCGUAAUC[dC][dT]	-
Mi2βii siRNA	CAUGAAGGUUAUAAAUACGAA[dT][dT]	UUCGUAAUUUAACCUUCAUG[dT][dT]	-
CtBP siRNA	GGGAGGACCUGGAGAAGUU[dT][dG]	AACUUCUCCAGGUCCUCCC[dT][dG]	-

2.3.7 RESTRICTION ENZYME DIGEST OF PLASMID DNA

To check the quality of the plasmids and to confirm the size of the plasmid insert, restriction digests were performed. Restriction digests were performed as 20 µl reactions, using 1 µg of plasmid, 2 µl of restriction enzyme, 2 µl of the corresponding 10X buffer, with the remaining volume composed of nuclease-free water. The reaction was incubated on a heat block for 1 hour at 37 °C. When a second sequential digestion was required, 1 µl of the first digest was retained to check restriction enzyme activity, with the remaining 19 µl used in the second 40 µl reaction. The additional components of the second digestion include 2 µl of restriction enzyme, 4 µl of the corresponding 10X buffer and 15 µl of nuclease-free water. The reaction was incubated at 37 °C for 2 hours. One µl of the resulting double digest was visualised using a 0.8% TAE agarose gel (Section 2.3.4), along with the uncut plasmid and single digest. Digested plasmids were purified using the Wizard® SV Gel and PCR Clean-Up System (Section 2.3.9).

2.3.8 REVERSE-TRANSCRIPTASE PCR (RT-PCR)

A two-step RT-PCR was performed, the first step generating the cDNA and the second step performing the PCR using sequence specific primers. This meant the cDNA could be used to detect multiple targets.

Generation of cDNA from RNA was performed with the RevertAid H Minus First Strand cDNA Synthesis Kit. All components, including the RNA, were stored on ice throughout. First strand cDNA synthesis was performed using 1 µg of total RNA mixed with 1 µl of an oligo (dT)₁₈ primer and the reaction made up to 12 µl with nuclease free water. The use of the oligo (dT)₁₈ primer ensured that cDNA synthesis commenced at the 3' end of mRNA, due to the presence of a poly(A) tail. Samples were mixed and centrifuged and incubated at 65 °C for 5 minutes, which resulted in RNA denaturation. Subsequently, 4 µl of 5X Reaction Buffer, 1 µl of RiboLock RNase Inhibitor (20 u/µl), 2 µl of 10 mM dNTP Mix and 1 µl of RevertAid H Minus M-MuLV Reverse Transcriptase (200 u/µl) were added. Samples were mixed and centrifuged and incubated at 42 °C for 60 minutes. Samples were then heated to 70 °C for 5 minutes, which terminated the reaction. Samples containing the resulting cDNA were stored at -80 °C prior to further

use. In order to monitor for the presence of genomic DNA contamination and reagent contamination within the cDNA samples, a reverse transcriptase-minus reaction and a no template reaction were performed with each experiment. Additionally, a positive control was performed to assess the efficiency of the reaction, which involved using RNA and primers provided with the kit.

PCR using the cDNA was performed using 1 µl of cDNA, 0.5 µl of 10 µM forward and reverse primers, 10 µl of the BioMix Red MasterMix and 8µl of ultra pure water. The BioMix Red Mastermix contained a red dye, which allowed for direct loading to the agarose gel, without addition of the DNA loading dye. The PCR temperature cycling conditions (Table 2-8) were performed on a GeneAmp PCR System 2400 Thermal Cycler from Perkin-Elmer.

Table 2-8: Temperature cycling conditions for PCR of cDNA

Stage	Temperature (°C)	Duration (sec)	Cycles
DNA denaturation	95	300	1
DNA denaturation	95	30	25-32
Annealing	52-60	30	
Extension	72	30	
Extension	72	300	1
Storage	4	∞	1

2.3.9 DNA PURIFICATION

DNA was purified using the Wizard® SV Gel and PCR Clean-Up System. This product removed contaminants, such as excess nucleotides and primers. The DNA was either purified after excision from an agarose gel or directly from solution. DNA was mixed with a Membrane Binding Solution (4.5M guanidine isothiocyanate, 0.5M potassium acetate (pH 5.0)), which contained guanidine isothiocyanate to denature proteins, so preventing degradation of the DNA. Through a series of centrifugations, the DNA became bound to the silica membrane and washed with a Membrane Wash Solution (10mM potassium acetate (pH 5.0), 80% ethanol, 16.7µM EDTA (pH 8.0)). The DNA was finally eluted in nuclease-free water.

2.3.10 DNA SEQUENCING

Prior to DNA sequencing, PCR products and plasmids were cleaned using the Wizard® SV Gel and PCR Clean-Up System (Section 2.3.9). DNA sequencing was undertaken by GATC Biotech Ltd (London, UK). PCR products at 10-50 ng/μl, plasmids at 30-100 ng/μl and the corresponding sequencing primers at 10 pmol/μl, were sent to the GATC Biotech laboratories. Sequencing data was returned with Phred20 base calling quality and data provided in ABI, SEQ and FAS file formats.

2.4 PREPARATION OF PLASMID DNA

Table 2-9: Plasmids constructs and vectors

Plasmid name	Plasmid	Insert	Use	Provided by
pBI_ZEB1-HA	pBI Tet Vector Clontech 631006	Mouse ZEB1 with N-terminal HA-tag	ZEB1 expression; requires pUHD172.1 neo for expression	In lab
pUHD172.1 neo	pUHD172.1 neo (Gossen <i>et al.</i> , 1995)	N/A	Induction of ZEB1 expression in combination with pBIZEB1	In lab
pcDNA3.1 BRG1	pcDNA3.1 Invitrogen V790-20	Human BRG1 (Accession no. D26156)	Expression of BRG1	Dr Stéphane Ansieau, Centre de Recherche en Cancérologie de Lyon, Lyon, France.
pcDNA3.1 BRG1-FLAG	pcDNA3.1 Invitrogen V790-20	Human BRG1 with N-terminal FLAG-tag	Expression of BRG1 with a N-terminal FLAG-tag	In lab
pcDNA3.1 CtBP-FLAG	pcDNA3.1 Invitrogen V790-20	Human CtBP (isoform 1) with N-terminal FLAG-tag	Expression of CtBP with N-terminal FLAG-tag	Dr G. Browne, Cancer Studies and Molecular Medicine, University of Leicester, Leicester, UK
pGL3 basic	pGL3 basic Promega E1751	N/A	Negative control for luciferase assay- Measures luminometer background	In lab
pGL3 CMVluc	pGL3 basic Promega E1751	CMV promoter	Positive control for luciferase assay	In lab
pGL3 E-cadWT	pGL3 basic Promega E1751	Mouse wild type E-cadherin promoter (-178/+92)	Contains the wildtype mouse E-cadherin promoter used in the luciferase assay	Prof Amparo Cano, Department of Biochemistry, Madrid, Spain (Bolos <i>et al.</i> , 2003; Hennig <i>et al.</i> , 1996)
pGL3 E-cadMUT	pGL3 basic Promega E1751	Mouse E-cadherin promoter mutated at the E-pal site (-178/+92)	Contains the mutated mouse E-cadherin promoter used in the luciferase assay	Prof Amparo Cano, Department of Biochemistry, Madrid, Spain (Bolos <i>et al.</i> , 2003; Hennig <i>et al.</i> , 1996)
pCMV β -gal	pCMV-SPORT1 Invitrogen 10586-014	β -galactosidase (β -gal)	Internal control of transfection efficiency for the luciferase assay and a positive control for gene expression	In lab
pEGFP-C1	pEGFP-C1 Clontech 6084-1	Green fluorescent protein (GFP)-red shifted variant of wildtype GFP	Expression of green fluorescent protein to determine transfection efficiency	In lab

2.4.1 PLASMID MAP

The pcDNA3.1/V5-His-TOPO cloning vector was used with the pcDNA™3.1/V5-His TOPO® TA Expression Kit for protein expression in mammalian cells. This vector was supplied in a linearised form, with single deoxythymidine overhangs at the 3'ends, ready for TA cloning with PCR products amplified by *Taq* polymerase, which provides a single non-template deoxyadenosine addition at the 3'end of PCR products.

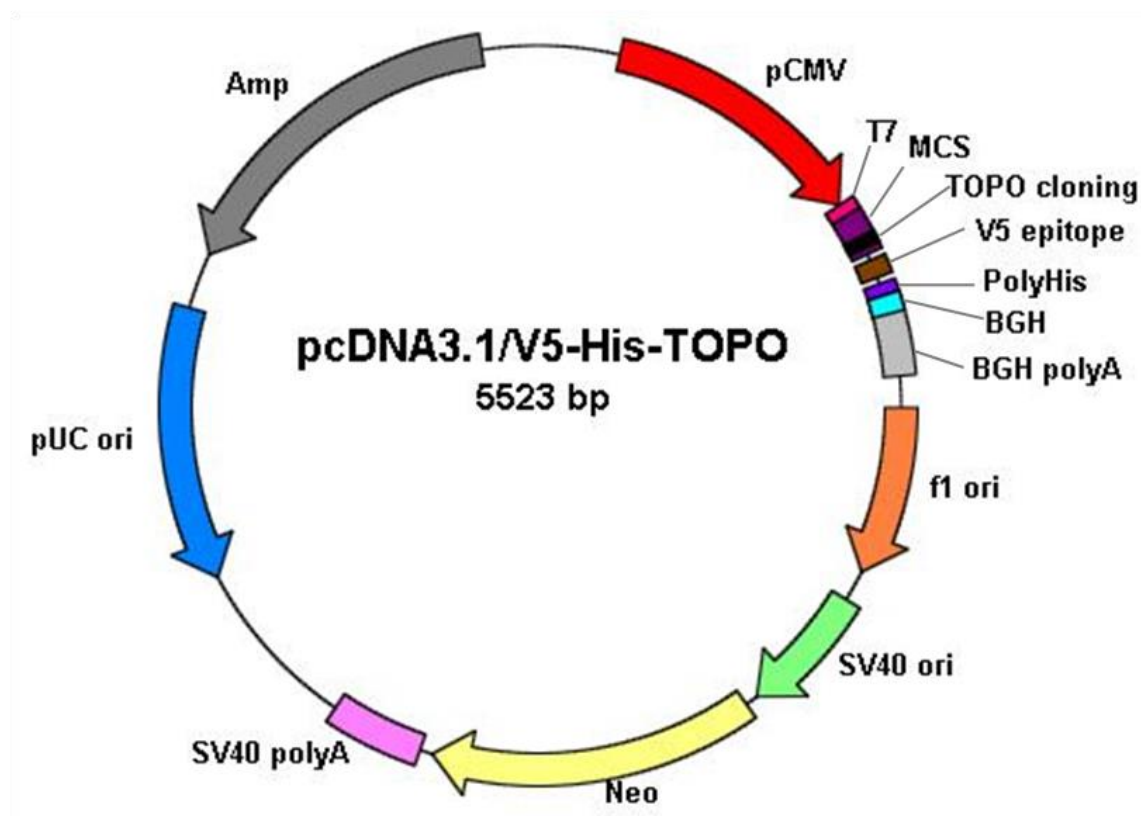


Figure 2-1: Map of the pcDNA3.1/V5-His-TOPO cloning vector

The TOPO cloning site for insertion of the PCR product occurs within the multiple cloning site (MCS), which is under the control of the CMV promoter (pCMV). Selection of successfully transformed *E. coli* cells occurs via the ampicillin (Amp) resistance gene. Plasmid map produced using Plasm Version2.1.5.30.

2.4.2 INTRODUCTION OF FLAG-TAG

A restriction enzyme digest was performed on the original BRG1 expression vector with *Sall* HF in NEBuffer 4 (Section 2.3.7). The resulting digested product was visualised on a 0.8% TAE agarose gel (Section 2.3.4) and compared to the uncut plasmid. Subsequently, a 50 µl PCR was performed consisting of 25 µl of the BioMix Red Taq

Mastermix, 1.5 µl of the 10 µM BRG1-FLAG forward and reverse primers, 1 µl of digested plasmid DNA and 21 µl of ultra-pure water. A PCR-negative reaction was also included. The PCR was performed as in Table 2-8, with an annealing temperature of 62 °C and 25 cycles. Ten µl of the PCR product was visualised on a 0.8 % TAE agarose gel and the remaining product purified (Figure 2.3.9).

The purified PCR product was then cloned into the pcDNA™3.1/V5-His-TOPO® cloning vector using the pcDNA™3.1/V5-His TOPO® TA Expression Kit. This was a one-step cloning reaction whereby the PCR product was mixed with 1 µl of salt solution, 1 µl of the cloning vector, in a final reaction volume of 6 µl. The cloning reaction was incubated at room temperature for 1 hour. The cloning reaction was then placed on ice and 2 µl of the reaction chemically transformed into E-coli (Section 2.4.3.1) and 10 resulting colonies processed for small scale production of plasmid DNA (Section 2.4.3.2.1). The purified plasmid DNA was visualised on a 0.8% agarose gel to identify plasmid with and without insert, and amplified using the BRG1-FLAG primers with PCR composition and conditions as previously performed. Finally, the orientation of the insert was confirmed by sequencing using the T7 forward and BGH reverse sequencing primers provided with the pcDNA™3.1/V5-His TOPO® TA Expression Kit, in accordance with the GATC guidelines (Section 2.3.10). Following confirmation of the correct insert orientation, the plasmid was processed following the protocol for large scale production of plasmid DNA (Section 2.4.3.2.2).

2.4.3 BACTERIAL PRODUCTION OF PLASMID DNA

2.4.3.1 CHEMICAL TRANSFORMATION OF E.COLI

Plasmid DNA was chemically transformed into α-Select Chemically Competent Cells, of either bronze or gold efficiency. Prior to use, cells were stored at -80°C and thawed on ice. Cells were mixed by gentle flicking of the tube and 50 µl of thawed competent cells were aliquoted into pre-chilled 1.5 ml microcentrifuge tubes. 2.5 µl of plasmid DNA was added to the competent cells, gently flicked and incubated on ice for 30 minutes. Cells were then heat shocked in a water bath at 42 °C for 30 seconds.

Samples were returned to ice for 2 minutes. 500 µl of SOC medium (2% (w/v) tryptone; 0.5% (w/v) yeast extract, 8.6 mM NaCl, 2.5 mM KCl, 20 mM MgSO₄, 20 mM glucose) was added to the transformation reaction and orbitally shaken for 1 hour at 37 °C. As a positive control for transformation, pUC19 DNA was provided with the competent cells and used in a separate reaction. Additionally, a negative control for transformation was included, which lacked plasmid DNA and so was antibiotic sensitive.

Meanwhile, previously dissolved and autoclaved LB agar (10g SELECT peptone 140; 5 g SELECT yeast extract; 5 g sodium chloride; 12 g SELECT agar per litre) was melted by heating and allowed to cool prior to the addition of either ampicillin or kanamycin to a final concentration of 100 µg/ml or 50 µg/ml, respectively. This was then poured into culture dishes and allowed to set. 100 µl of the transformation reaction was then spread onto the culture dish and incubated overnight at 37 °C. Single colonies were then picked from the plate using a sterile pipette tip and the bacterial population expanded following the steps for small scale production of plasmid DNA.

2.4.3.2 PURIFICATION OF PLASMID DNA

2.4.3.2.1 SMALL SCALE PRODUCTION OF PLASMID DNA

Colonies were directly picked from the agar plate and added to 3 ml of LB broth (10 g SELECT peptone 140; 5 g SELECT yeast extract; 10 g sodium chloride per litre) containing antibiotic at the previously mentioned concentrations and incubated at 37 °C for approximately 8 hours with orbital shaking. This was classed as the starter culture. The plasmid DNA was then extracted and purified using the NucleoSpin® Plasmid kit, following the protocol for isolation of high-copy plasmid DNA from *E.coli*, which can isolate up to 60 µg of plasmid DNA. The saturated LB broth was initially transferred into a 1.5 ml microcentrifuge tube and the cells pelleted by centrifugation at 11,000 x *g* for 30 seconds. The pellet was resuspended in Buffer A1 containing RNase, followed by the addition of Buffer A2, which contains SDS, resulting in cellular lysis and release of plasmid DNA. Buffer A3 was then added and the tube inverted, ensuring neutralisation of the lysate to allow optimal binding of plasmid DNA to the

silica membrane of the spin columns. Samples were then centrifuged at 11,000 $\times g$ for 5 minutes to pellet cell debris, protein and genomic DNA and the resulting supernatant passed through a spin column by centrifugation at 11,000 $\times g$ for 1 minute, whereby plasmid DNA became bound to the membrane. The membrane was washed by centrifugation in Buffer A4, which contains ethanol to remove contaminants. The DNA was finally eluted in Buffer AE (5 mM Tris/HCl, pH 8.5).

Subsequently, the concentration and quantity of plasmid DNA was determined (Section 2.3.3). Additionally, the integrity of the plasmid DNA was visualised on a 0.8% agarose gel (Section 2.3.4).

In certain situations, the resulting plasmid preparation was further analysed to ensure the presence of the correct insert. This was either performed via a restriction digest (Section 2.3.7), using restriction enzymes that specifically cut the plasmid DNA at known sites to generate identifiable fragments. Alternatively, the plasmid DNA was sent for DNA sequencing (Section 2.3.10).

2.4.3.2.2 LARGE SCALE PRODUCTION OF PLASMID DNA

In order to generate high quantities of purified plasmid DNA, the NucleoBond® Xtra Maxi Plus kit was used, following the protocol for maxi high-copy plasmid purification. This advised recovery of up to 1000 μg of plasmid DNA. A large overnight culture was prepared using 300 ml of LB Broth with antibiotic and diluting the starter culture 1:1000. This culture was grown for 16 hours at 37 °C with orbital shaking. The culture was then pelleted by centrifugation at 6,000 $\times g$ for 20 minutes at 4 °C. The pellet was resuspended in Buffer RES, which contained RNase A. Cells were lysed in Buffer LYS, containing sodium hydroxide and SDS. Samples were mixed by inversion and incubated for 5 minutes. Meanwhile, the NucleoBond® Xtra Column and Filter were equilibrated with Buffer EQU by gravity flow. The cell lysate was then neutralised with Buffer NEU, which contains potassium acetate. The precipitated lysate was then applied to the NucleoBond® Xtra Column and Filter and contaminating protein, chromosomal DNA and cell debris were cleared from the lysate and the plasmid DNA

became bound to the silica resin. The column and filter were then washed with Buffer EQU, after which the column was discarded. The silica resin was washed again and the plasmid DNA eluted under high salt conditions. The protocol was then transferred to the Concentration of NucleoBond® Xtra eluates with the NucleoBond® Finalizers. The eluted DNA was first precipitated by the addition of isopropanol. The sample was then loaded into a syringe with attached NucleoBond® Finalizer, containing another silica membrane, and the sample passed through. The membrane was then washed with the addition of 70% ethanol to the syringe. Excess ethanol was removed from the syringe and the membrane dried. The purified plasmid DNA was subsequently eluted in Buffer Tris (5 mM Tris/HCl, pH 8.5).

The quantity and quality of the purified plasmid DNA was checked and visualised on an agarose gel (Sections 2.3.3 and 2.3.4). The plasmid DNA was then ready for use in downstream process, such as in transient transfections.

2.5 DNA METHYLATION DETECTION METHODS

2.5.1 SODIUM BISULPHITE CONVERSION

The EZ DNA Methylation Gold Kit was used for sodium bisulphite treatment of DNA following the manufacturer's protocol. Briefly, 20 µl of DNA (25 ng/µl) was incubated with 130 µl of resuspended CT Conversion Reagent in a 0.2 ml thin-walled PCR tube. Samples were mixed by inverting the tube, centrifuged and incubated at 98 °C for 10 minutes, followed at 64 °C for 2.5 hours. Six hundred µl of M-Binding Buffer was then added to a Zymo-Spin™ IC Column and the DNA solution added. Samples were inverted and centrifuged at full speed for 30 seconds at room temperature. All subsequent centrifugation steps were performed as such. Flow-through was discarded. One hundred µl of M-Wash Buffer was added to the column and centrifuged. Two hundred µl of M-Desulphonation Buffer was then added, incubated at room temperature for 20 minutes and centrifuged. An additional 200 µl of M-Wash Buffer was added to the column twice and centrifuged. Flow-through was discarded as necessary. The spin column was then placed in a 1.5 ml microcentrifuge tube and

10 µl of M-Elution Buffer added directly to the column matrix. The column was centrifuged and the DNA eluted. The DNA was then ready for PCR or further processing and either stored at 4°C for short term or -20°C for long term storage.

2.5.2 METHYLATION SPECIFIC PCR (MSP)

Methylation-specific PCR (MSP) was performed on sodium bisulphite converted DNA. A 25 µl PCR reaction was produced, including 12.5 µl of ZymoTaq PreMix, 0.8 µl of 10 µM forward and reverse primers and 2 µl of bisulphite converted DNA diluted in 8.9 µl of ultra-pure water. Primers used were specific to the E-cadherin promoter and included methylated and unmethylated version (Table 2-6). PCR conditions were performed as represented in Table 2-10.

Table 2-10: Temperature cycling conditions for MSP

Stage	Temperature (°C)	Duration (seconds)	Cycles
Initial denaturation	95	600	1
Denaturation	95	30	40
Annealing	53/57	30	
Extension	72	30	
Final Extension	72	420	1
Storage	4	∞	1

PCR products were visualised on a 2% TBE agarose gel and variations in the relative intensity of the methylated and unmethylated PCR products were analysed. Samples were analysed in duplicate.

2.5.3 BISULPHITE SPECIFIC PCR (BSP) AND DNA SEQUENCING

Sodium bisulphite treated DNA was amplified in a 50 µL PCR reaction using bisulphite specific primers (Table 2-6). The reaction utilised 25 µl of ZymoTaq PreMix, 1.6 µl of 10 µM forward and reverse primers (E-cad Bi) and 2 µl of bisulphite converted DNA diluted in 19.8 µl of water. PCR conditions were performed as represented in Table 2-11.

Table 2-11: Temperature cycling conditions for BSP

Stage	Temperature (°C)	Duration (seconds)	Cycles
Initial denaturation	95	600	1
Denaturation	95	30	40
Annealing	60	60	
Extension	72	30	
Final Extension	72	420	1
Storage	4	∞	1

Ten µl of PCR product were visualised on a 2% TAE agarose gel. When the band of the required size was present, with minimal primer dimers or unwanted products, the remaining PCR product was purified using the Wizard® SV Gel and PCR Clean-Up System. The eluted DNA was quantified and sent for DNA sequencing, using the reverse PCR primer as the sequencing primer. The presence or absence of methylated cytosine residues was identified within the sequencing data and the relative intensity of the resulting signal compared between sample types. Samples were processed in duplicate.

2.6 MANIPULATION OF PROTEINS

2.6.1 PROTEIN ISOLATION

Cells were obtained from the cell culture incubator and washed three times with PBS. Dependent upon the number of cells present within the flask, an appropriate volume of Laemmli lysis buffer, diluted to 1X, was added. The cells were scraped to promote detachment from the base of the flask. Lysis buffer containing detached cells was pipetted into a 1.5 ml microcentrifuge tube. Samples were then boiled on a heat block at 94°C for 10 minutes. The samples were centrifuged and sonicated on a Soniprep 150 from MSE for 10 seconds to fragment DNA. Protein concentration was then determined or samples were stored at -20°C for future use.

2.6.2 PROTEIN QUANTIFICATION

Protein quantification was performed using the BCA Protein Assay Kit, which is compatible with the SDS detergent present within Laemmli buffer. This was a colorimetric based detection method for total protein quantification, using

bicinchoninic acid (BCA). Quantification occurred in a 96-well format (Greniner, 655180), with 200 μl of BCA reagent required for each sample, consisting of 50 parts BCA Reagent A to 1 part BCA Reagent B. Additionally, a series of protein standards was included, ranging from 2 $\mu\text{g}/\mu\text{l}$ to 25 $\text{ng}/\mu\text{l}$ of bovine serum albumin (BSA). The colorimetric reaction commenced with the addition of 5 μl of pre-boiled protein sample or protein standard. A blank sample was also included, whereby the protein sample was replaced with Laemmli buffer. The 96-well plate was placed on a shaker for 30 seconds, then at 37 °C for 30 minutes. The plate was allowed to cool to room temperature, whereby an absorbance reading at 562 nm was taken on a BioTek ELx808 Absorbance Microplate Reader (BTELX808). The absorbance value obtained for the blank sample was subtracted from the absorbance readings for the protein samples and standards. A standard curve was generated for the protein standards, allowing the concentration of the unknown protein samples to be determined. Protein samples were diluted with the addition of 1X Laemmli buffer to a concentration of 1 $\mu\text{g}/\mu\text{l}$.

In situations when the protein sample to be quantified was suspended in lysis buffer, for example samples to be processed for immunoprecipitation studies, protein quantification occurred via the Coomassie (Bradford) Protein Assay Kit. This technique followed a comparable format as the BCA Protein Assay Kit previously described, except for the replacement of BCA Reagent A and B with 250 μl of Coomassie Reagent and absorbance readings measured at 595nm.

2.6.3 SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

The Mini-PROTEAN Tetra Electrophoresis System from Bio-Rad (165-8006) was used to cast 1.5 mm vertical gels. Varying percentages of resolving gel were produced, comprised of 0.375 M Tris-HCl pH 8.8, 8.0-12% (v/v) 37.5:1 acrylamide:bisacrylamide and 0.1% (w/v) SDS. Polymerisation of the resolving gel was initiated by the addition of 0.1% (w/v) ammonium persulphate and 0.1% TEMED. The gel was poured between two vertical glass plates and water saturated isobutanol applied to the top to remove

air bubbles. The gel was allowed to set for 30 minutes. The isobutanol was subsequently removed and the top of the gel flushed with water and dried. A 5% stacking gel was then applied, consisted of 0.125 M Tris-HCl pH 6.8, 5.0% (v/v) 37.5:1 acrylamide:bisacrylamide and 0.1% (w/v) SDS. Polymerisation of the stacking gel was initiated by the addition of 0.1% (w/v) ammonium persulphate and 0.1% TEMED. The stacking gel was poured between the glass plates and a 16 well comb placed in the top. The gel was allowed to set for an additional 30 minutes.

Meanwhile, proteins samples at 1 µg/µl were combined with protein loading buffer and samples heated to 96 °C for 10 minutes and centrifuged. 20 µl of each sample were loaded into the cast wells, including a protein standard, and electrophoresis performed in protein running buffer at 180 volts for approximately 50 minutes. The bromophenol blue dye front was monitored to check for efficient electrophoresis.

2.6.4 PROTEIN TRANSFER TO NITROCELLULOSE MEMBRANE

After electrophoresis, the gel was immersed in transfer buffer for 5 minutes. Additionally, the Immobilon-P PVDF transfer membrane from Merck Millipore (IPVH00010) (Watford, UK) was immersed in 100% methanol for 5 minutes and then placed in transfer buffer. Wet electrophoresis transfer was performed using the Trans-Blot system from Bio-Rad. The transfer cassette was immersed in transfer buffer and the gel and transfer membrane were sandwiched between filter paper from GE Healthcare (SE1141) and fibre pads (Figure 2-2). Air bubbles were removed by gently rolling over the sandwiched gel and membrane. The transfer cassette was properly orientated within the transfer tank, which was filled with transfer buffer. Protein transfer occurred at 25 volts for 16 hours. After transfer, the membrane was stained with Ponceau S to confirm successful protein transfer and then washed with water to remove the staining.

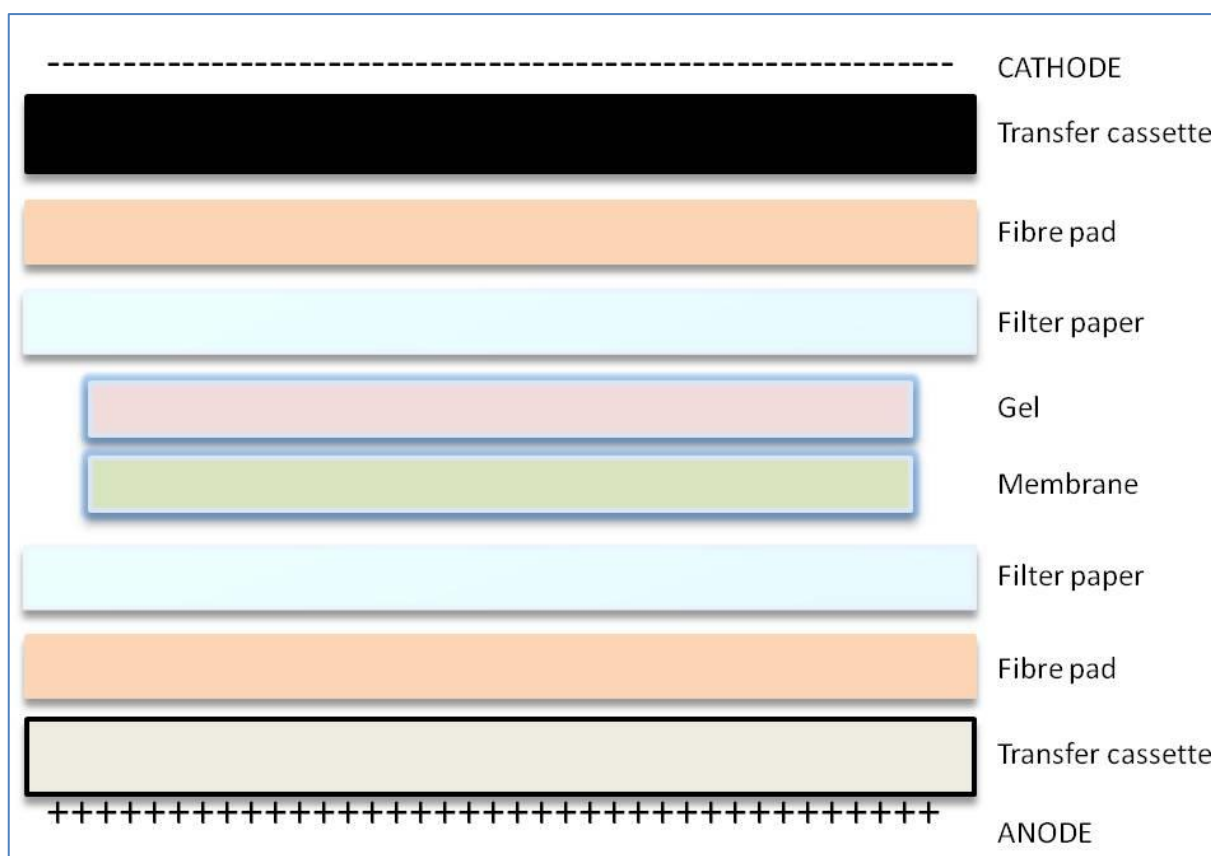


Figure 2-2: Wet protein transfer

The schematic represents the set-up of a wet transfer of proteins from a resolving gel processed via SDS-PAGE onto a PVDF membrane, positioned between two pieces of filter paper and a fibre pad.

2.6.5 IMMUNOLOGICAL DETECTION OF MEMBRANE BOUND PROTEIN

Membranes containing the transferred proteins were incubated in a TBS-T-milk solution for 1 hour at room temperature with shaking. Primary antibody at the appropriate concentration in TBS-T-BSA (Table 2-3) was applied to the membrane and incubated for 1 hour with agitation. Membranes were then washed 3 times for 10 minutes in TBS-T with shaking. The appropriate horseradish peroxidase conjugated secondary antibody (Table 2-4) was then applied to the membrane and incubated for 30 minutes. The membrane wash step was then repeated. The peroxidase activity of the secondary antibody was then detected using the luminal-based Pierce® enhanced chemiluminescence (ECL) Western Blotting Substrate. Equal volumes of Detection Reagents 1 and 2 were combined and 1 ml incubated on the membrane for 1 minute. The presence of chemiluminescence was detected using the CL-XPosure Film from Fisher Scientific (PN34089), in the dark room with the AGFA Curix 60 film developer

(Figure 2-3). The duration of film exposure was specific for each cell line and antibody used. Presented images represent the optimal exposure for the experimental conditions. Samples were processed in duplicate or triplicate.

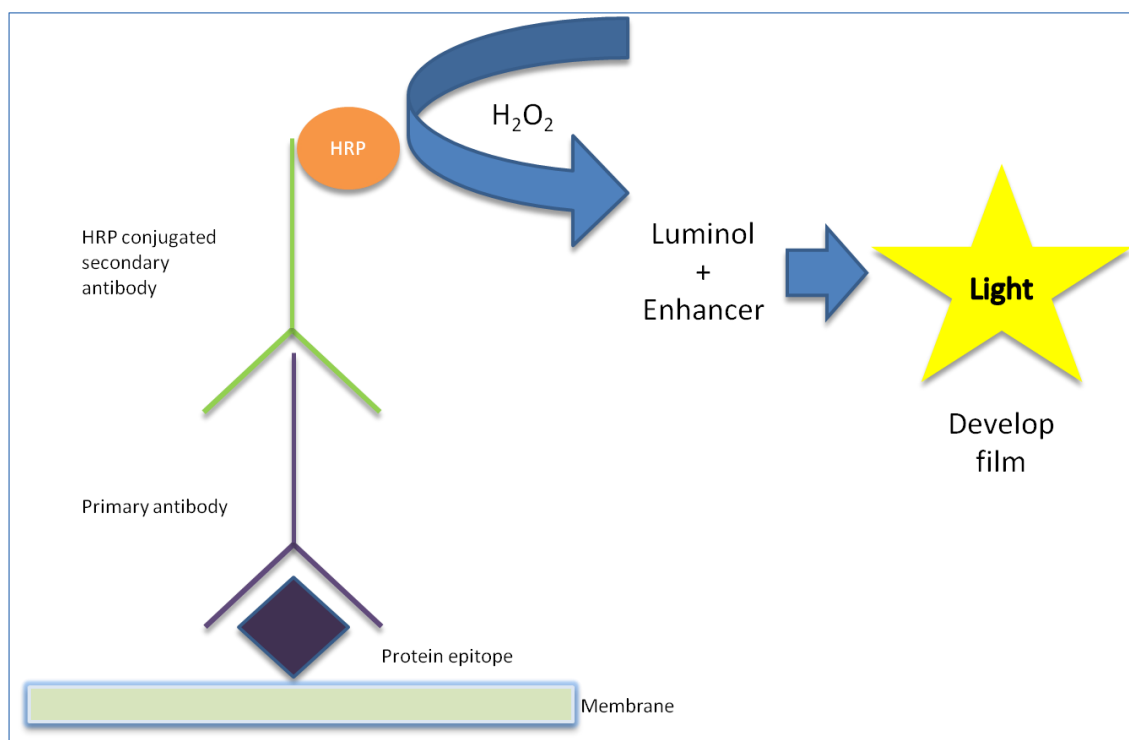


Figure 2-3: Protein detection by enhanced chemiluminescence (ECL)

The schematic represents the luminescence reaction that occurs in the detection of protein following the ECL reaction. The primary antibody, specific to the protein of interest, is bound by a species-specific secondary antibody, which was conjugated to horse radish peroxidase (HRP). HRP is able to catalyze the oxidation of luminol in the presence of H₂O₂, generating light, allowing the immobilised protein to be visualised. This reaction occurs in the presence of enhancers, such as modified phenols, which prolong and increase the intensity of the light emitted (Marquette & Blum, 2006).

The resulting CL-XPosure Film, with visualised protein bands, were scanned using a Canon CanoScan LiDE 35 and the density of the bands determined using the ImageJ software. This allowed the area covered and the intensity of each protein band to be determined and compared to the control sample. When required, a one-way ANOVA (analysis of variance) was performed on the data to test for significant variation between sample types using the GraphPrism 6 for Windows, Version 6.01, with statistical significance identified when the P-value was ≤ 0.05 .

2.7 IMMUNOPRECIPITATION

Prior to the immunoprecipitation (IP), the required cells were transfected (Section 2.2.4) with the appropriate plasmids (Table 2-9).

The initial step of the IP required pre-coupling of the protein-G- Sepharose beads. For each IP, 10 μ l of beads was used, which were washed four times with PBS to remove ethanol required for storage, and finally resuspended in 500 μ l of PBS. For all IPs, an anti-FLAG antibody was used, 1 μ g required for each IP. The anti-FLAG antibody and protein-G-Sepharose beads were mixed by rotation at 4 °C for 1 hour. The antibody coupled protein-G-Sepharose beads were subsequently centrifuged at 16,000 xg for 20 seconds to pellet the beads, the supernatant removed and washed twice with 1 ml of PBS and twice with 1 ml lysis buffer. The beads were finally resuspended in 1X volume of lysis buffer. Pre-coupled beads were stored at 4 °C prior to use.

Transfected cells in tissue culture dishes were placed on top of ice and media removed. The cells were washed twice with ice-cold PBS, PBS removed and 600 μ l of ice-cold lysis buffer added and cells removed by scraping. Cells were transferred into a 1.5 ml microcentrifuge tube and stored on ice. Cell lysates were sonicated for 10 seconds, vortexed and centrifuged at 16,000 xg for 10 minutes at 4 °C to sediment any insoluble material. The protein concentration of the samples was determined by the Coomassie (Bradford) Protein Assay Kit (Section 2.6.2).

For each IP, 1 mg of total protein was added to 20 μ l of the antibody coupled-protein-G-Sepharose beads, with lysis buffer added to a total volume of 500 μ l. The sample was mixed by end-to-end rotation for 1 hour at 4 °C and then centrifuged at 16,000 xg for 20 seconds to sediment the beads. The first supernatant was retained for subsequent analysis, and the beads washed four times with 1 ml of lysis buffer, with the sample gently mixed and centrifuged at 16,000 xg for 20 seconds in between each wash step. Finally, the IP pellet was resuspended in an appropriate volume of 1X Laemmli buffer. The IP sample was mixed and heated to 96 °C for 5 minutes, vortexed and centrifuged at 16,000 xg for 1 minute and stored at -20 °C until analysed by SDS-

PAGE (Section 2.6.3). When IP samples were analysed, the input protein sample, the IP sample and the first supernatant were analysed. Samples were processed in duplicate.

2.8 IMMUNOFLUORESCENCE

Immunofluorescent techniques were originally optimised to determine the protocol for co-visualisation of both nuclear protein, for example ZEB1 and ZEB2, and membranous protein, E-cadherin, staining. This identified the acetone-methanol protocol as the optimal technique for fixation. Cells plated on cover slips from VWR (18×18 mm; thickness no.1; borosilicate glass) (631-0120) were washed twice with PBS and submerged in 1.5 ml of cold acetone-methanol and incubated for 3 minutes. Cover slips were removed and air dried for 10 minutes. The cover slips were then washed with 2 ml PBS prior to the application of 100µL of primary antibodies, at the required dilution (Table 2-3), in complete tissue culture media. The cover slips were incubated for 1 hour. The cover slips were then washed 3 times for 4 minutes each in PBS on a rocker. The secondary antibodies (Table 2-4) were then applied in complete media to the cover slips. These were again incubated for 1 hour. The cover slips were then washed 3 times in PBS, each time for 4 minutes. The second PBS wash included a 1 in 20,000 dilution of DAPI (5 mg/ml in water). The cover slips were then reverse mounted onto Menzel-Gläser microscope slides (25×75×1 mm) (J3800AMNZ), containing a drop of Fluoromount G to ensure attachment. The slides were allowed to dry and the edges sealed with varnish to prevent movement. The cells were then visualised using a light microscope, with representative images recorded once the whole cover slip was analysed. Representative images are shown. Samples were analysed in duplicate.

2.9 LUCIFERASE ASSAY

Cells were initially transfected with the appropriate luciferase reporter construct, the β -galactosidase construct and treated with the appropriate siRNA (Section 2.2.4 and Table 2-7). Following an incubation of 48 hours, cells were obtained from the tissue culture incubator and processed for the luciferase and β -galactosidase assays.

Each 6 cm² tissue culture plate was washed twice with room temperature PBS and cells collected in 1 ml of PBS by scraping into a fresh 1.5 ml microcentrifuge tube. Cells were centrifuged at 70 xg for 5 minutes to form a pellet. The supernatant was removed and discarded and three freeze thaw cycles were performed using a mix of dry ice and ethanol, and then placed in a water bath heated to 37 °C. Cells were then resuspended in 50 µL of diluted lysis buffer and incubated at room temperature for 15 minutes. The sample was then centrifuged at 11,500 xg for 3 minutes, forming a pellet. The pellet remained undisturbed during subsequent analysis.

For the luciferase assay, the lyophilized luciferase assay substrate was re-suspended in luciferase lysis buffer. A Sirius Single Tube Luminometer from Berthold Detection Systems was used to detect the chemiluminescent reaction, following the manufacturer's guidelines. 5 µL of each sample was used to determine luciferase activity, with each experimental condition performed in triplicate, with each sample measured in duplicate. A positive control for luciferase activity was included with each experiment, which included transfection of cells with the pGL3_CMVluc plasmid, which expressed high levels of luciferase under the control of the Cytomegalovirus promoter. In addition, background luminometer readings were determined by transfection of cells with the pGL3_empty plasmid, which did not produce luciferase. Finally, a negative control was performed, which consisted of transfection only with the pGFP plasmid, which also allowed transfection efficiencies to be monitored.

For the β-galactosidase assay, 270 µl of the β-galactosidase mastermix was produced for each sample, combined with 5 µL of sample supernatant and incubated at 37 °C for 20 minutes. Each sample was processed in duplicate. The absorbance reading of each sample was performed on a Sanyo Gallenkamp SPBIO Spectrophotometer at 420 nm.

Following data collection, relative luminescence units (RLU) were calculated by subtracting the background luminometer readings from each test sample and normalising each sample against the corresponding β-galactosidase result. A fold change value was obtained, by dividing the test samples by the control samples.

Finally, a two-way ANOVA was performed on the data to test for significant variation between sample types using the GraphPrism 6 for Windows, Version 6.01, with statistical significance identified when the P-value was ≤ 0.05 .

**CHAPTER 3 : HIERARCHICAL
CONTROL OF THE MR-EMT BY N-
RAS-MEK AND B-RAF-MEK
MELANOMA-DRIVING
PATHWAYS**

3.1 INTRODUCTION

Melanocytes originate from the neural crest cell developmental lineage, a highly migratory cellular population (Sommer, 2011), which express some of the MR-EMT (Gupta *et al.*, 2005). The role of the different MR-EMT in carcinomas is well established (Morel *et al.*, 2012), but is relatively less well studied in neural crest-derived tumours. Here, data is presented that examines the expression of the MR-EMT in malignant melanoma cell lines and investigates how they are regulated in relation to melanoma-initiating mutations within the RAS-RAF MAPK pathway. A parallel study was also undertaken to examine the expression of the MR-EMT in a series of human malignant melanoma specimens (Appendix I).

3.2 AIMS AND OBJECTIVES

Aim: To investigate the expression profile of the MR-EMT and E-cadherin within melanoma cell lines.

Objectives: The expression patterns of the MR-EMT and E-cadherin will be analysed in a panel of human neonatal epidermal melanocytes and melanoma cell lines. The expression of the MR-EMT will then be investigated in relation to melanoma-initiating mutations within the MAPK signalling pathway.

3.3 RESULTS

3.3.1 EXPRESSION OF THE MR-EMT IN MELANOMA

The relative expression of the MR-EMT was examined in malignant melanoma cell lines and compared to neonatal human epidermal melanocytes (NHEMs) (Figure 3-1). NHEMs expressed ZEB2 and SNAIL2 but lacked detectable ZEB1, TWIST1 and SNAIL1. This is consistent with melanocytes in normal skin (Figure 3-2). Variable levels of ZEB2 and SNAIL2 were retained by all the melanoma cell lines. For instance, the N-RAS mutant IPC-298 cell line retained high expression of both ZEB2 and SNAIL2, whilst the B-RAF mutant cell line A375M possessed the lowest expression of both proteins. The expression of ZEB2 and SNAIL2, even though not perfectly correlated, appeared to follow a similar pattern of expression. Additionally, the acquisition of ZEB1 and TWIST1 expression was evident in a subset of the melanoma cell lines. Higher ZEB1 expression

appeared to correlate with reduced ZEB2 expression, exemplified in the A375P (higher ZEB2, lower ZEB1) and A375M (higher ZEB1, lower ZEB2) cells. Finally, SNAIL1 was rarely detected in the melanoma cell lines, which contradicts previous results that identified SNAIL1 mRNA expression in melanoma cell lines (Poser *et al.*, 2001) and at the protein level (Massoumi *et al.*, 2009). However, the reported melanoma cell lines had minimal cross-over with the cell lines analysed here, with only SK-MEL-28 cells co-examined. Interestingly, data presented here agrees with that from human melanoma specimens, whereby only 4% of cases expressed SNAIL1 (Mikesh *et al.*, 2010), therefore SNAIL1 was excluded from further examination.

These initial results are suggestive of a link in expression between ZEB2 and SNAIL2 and between ZEB1 and TWIST1. An interesting point to note is the relative expression of ZEB1 and ZEB2 between the A375P and A375M cell lines. These cell lines represent an important model of melanoma metastasis, with the A375M cell line produced from the lung metastases obtained following intravenous injection of A375P cells. A375M cells were subsequently shown to have a greater metastatic capacity when compared to A375P cells (Kozlowski *et al.*, 1984). A375P cells have higher expression of ZEB2 compared to ZEB1, whilst the reverse expression profile is evident in A375M cells. This may indicate that during the progression of melanoma, from primary to metastatic forms, there is an increase in ZEB1 and down-regulation of ZEB2 expression.

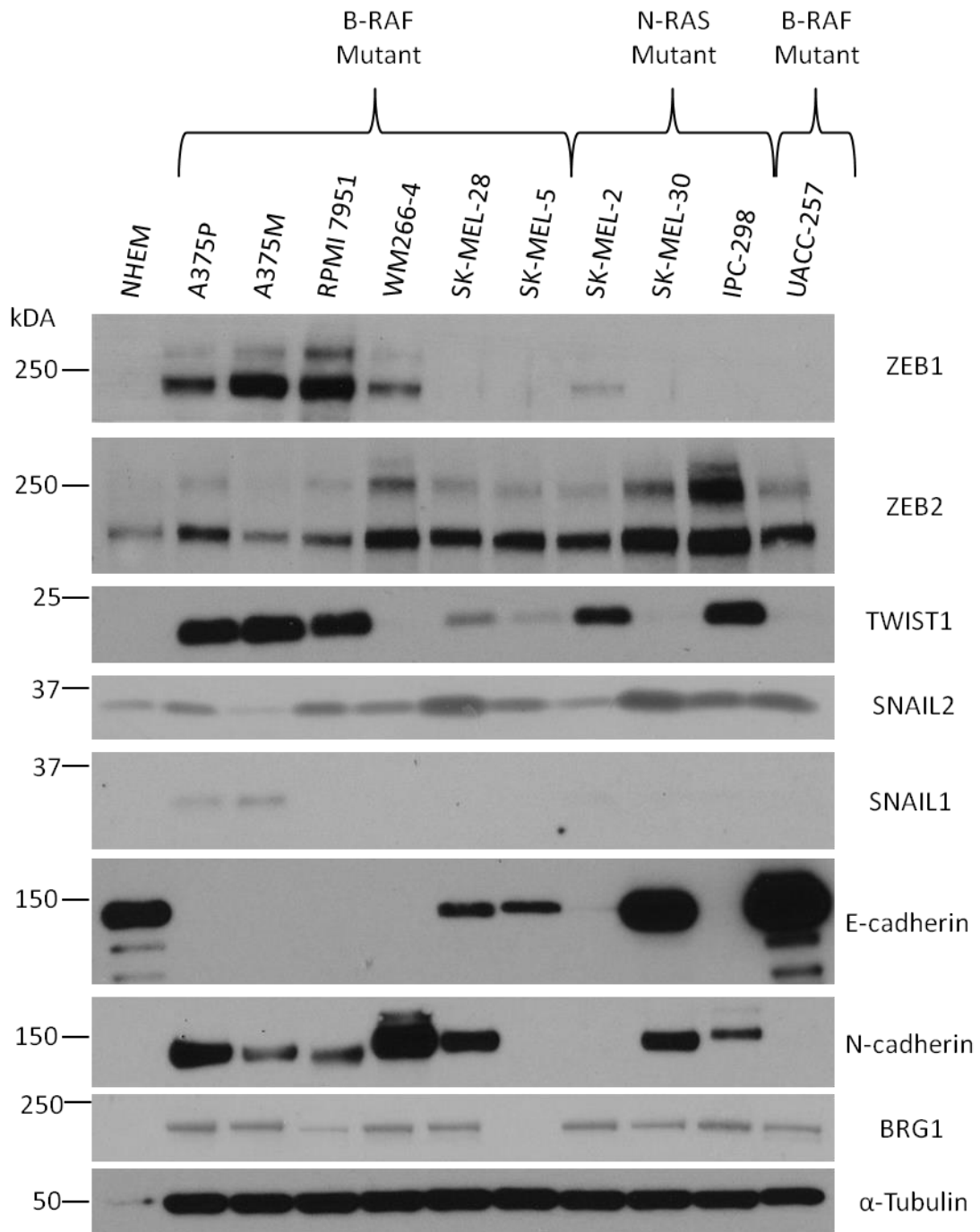


Figure 3-1: ZEB1 and TWIST1 expression is acquired by certain melanoma cell lines. Cells were cultured to 70% confluency and proteins analysed via Western blotting, with α -Tubulin used as a protein loading control. Representative images are presented. ZEB2 and SNAIL2 were expressed in the NHEMs and maintained in the melanoma cell lines. ZEB1 and TWIST1 were absent in the NHEMs but acquired by a sub-set of melanoma cell lines. An inverse relationship between ZEB1 and E-cadherin expression was apparent, whilst ZEB2 and SNAIL2 are co-expressed with E-cadherin.

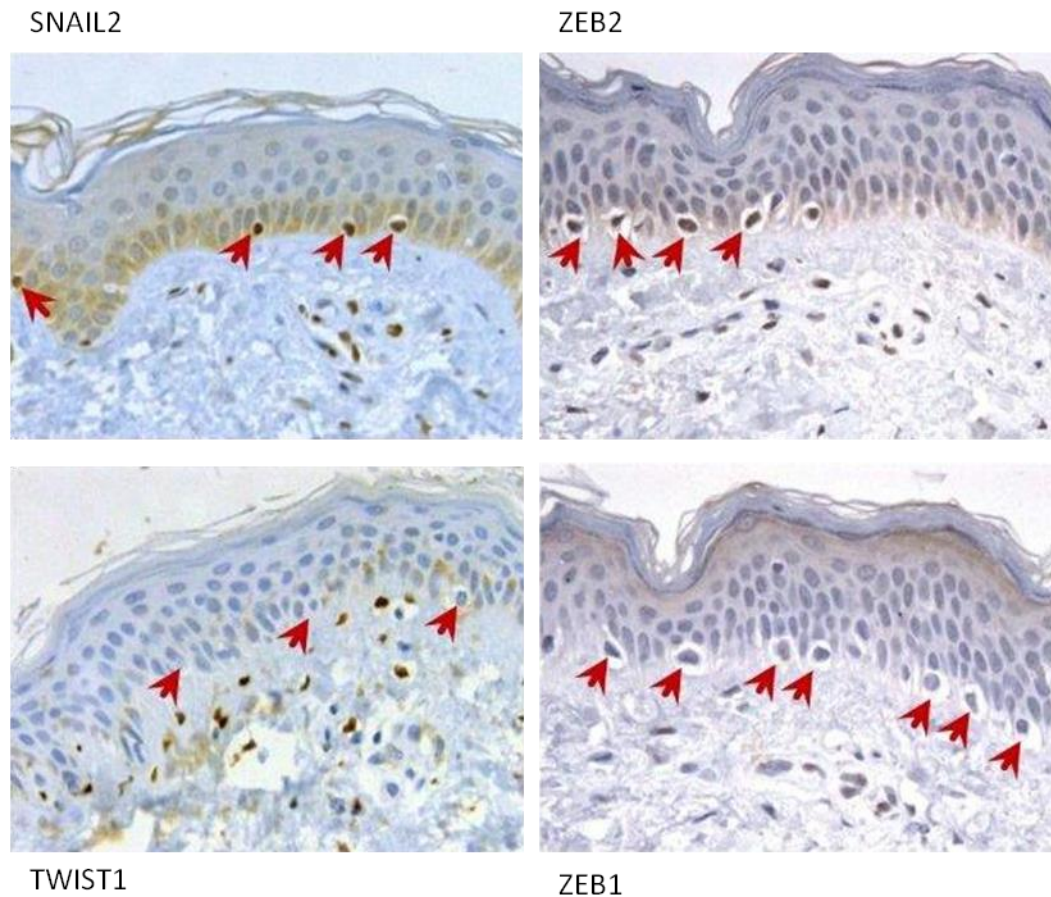


Figure 3-2: Melanocytes in normal skin express ZEB2 and SNAIL2

Immunohistochemical analysis for the MR-EMT was performed on sections of normal skin. Melanocytes are indicated by red arrows. Data provided by Eftychios Papadogeorgakis (Appendix I).

In addition to the MR-EMT, the expression of E-cadherin and N-cadherin was examined (Figure 3-1). The NHEMs expressed high levels of E-cadherin but lacked detectable N-cadherin. Within the melanoma cell lines, the expression of both E-cadherin and N-cadherin was highly heterogeneous. An inverse correlation between the expression of ZEB1 and E-cadherin was evident, with no E-cadherin identified in cells expressing ZEB1. Additionally, high TWIST1 expression was associated with the absence of E-cadherin, for example in IPC-298 cells. Interestingly, high TWIST1 expression occurs within the cell lines SK-MEL-2 and IPC-298, both of which are E-cadherin-negative and have minimal or no ZEB1 expression. This suggests that both ZEB1 and TWIST1 may function as negative regulators of E-cadherin. In comparison, ZEB2 and SNAIL2 were co-expressed with E-cadherin in the NHEMs and malignant melanoma cell lines, indicating that neither functions to repress E-cadherin expression.

It is also evident that a sub-set of the malignant melanoma cell lines have undergone the cadherin switch, with the loss of E-cadherin occurring in conjunction with the acquisition of N-cadherin. Two melanoma cell lines, SK-MEL-28 and SK-MEL-30, co-express E-cadherin and N-cadherin, indicating that they have an intermediary phenotype. The B-RAF mutant UACC-257 cell line has a comparable expression profile to that of the NHEMs, with high expression of ZEB2, SNAIL2 and E-cadherin and no detectable ZEB1, TWIST1 and N-cadherin. This indicates that the UACC-257 cell line remains differentiated, with the least progression towards metastatic melanoma.

Following from the observation that the acquisition of ZEB1 appears to occur in the presence of reduced ZEB2, and that A375P and A375M cells possess opposite expression profiles, ZEB protein expression was analysed at the single cell level (Figure 3-3). The pattern of ZEB1 and ZEB2 expression within both cell lines is heterogeneous, with co-expression of both ZEB proteins. However, it is evident in A375P cells, with the predominance of green (ZEB2) staining, that ZEB2 is the major ZEB protein expressed. In comparison, in A375M cells, the opposite situation was identified, with stronger red (ZEB1) staining, indicating that ZEB1 was present at the expense of ZEB2.

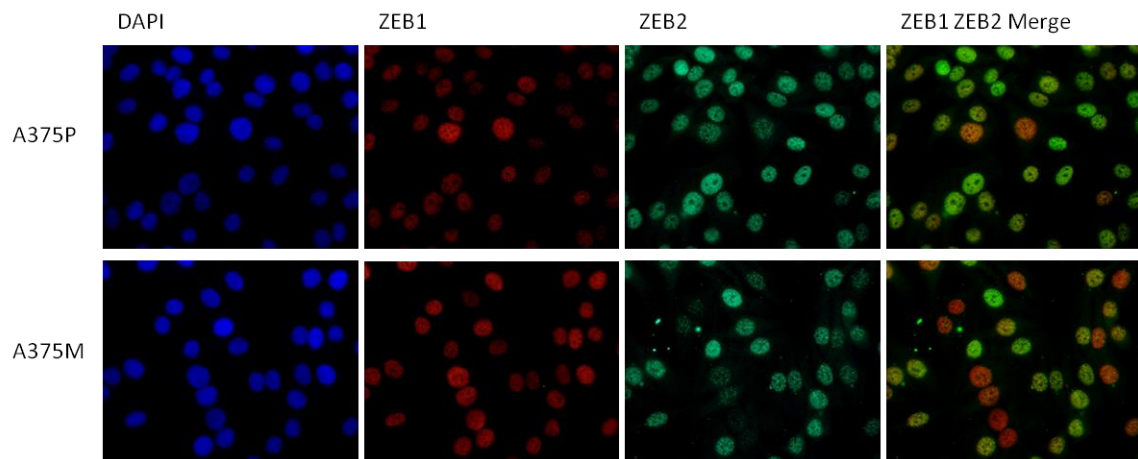


Figure 3-3: Opposing expression of ZEB1 and ZEB2 in the A375 cell lines
Cells were fixed with acetone-methanol and stained with ZEB1 (red), ZEB2 (green) and DAPI (blue). ZEB1 and ZEB2 are expressed by both A375P and A375M cell lines. However, ZEB2 appears to predominant in A375P cells, whilst ZEB1 is more strongly expressed in A375M cells. Representative images are presented (n=2). (60X objective)

3.3.2 MR-EMT AND MELANOMA-INITIATING PATHWAYS

Components of the MAPK signalling pathway are frequently mutated in cases of melanoma, with B-RAF mutations identified in approximately 50% and N-RAS mutations in approximately 15% of cases (Miller & Mihm, 2006). Constitutively activated ERK regulates multiple targets and enhances proliferation, survival and transformation (Hingorani *et al.*, 2003), indicating that these melanoma-initiating mutations have far-reaching effects. With a potential switch in the expression of the MR-EMT identified when the NHEMs and melanoma cell lines were compared (Figure 3-1), the impact of B-RAF and N-RAS mutations were analysed in relation to the expression of the MR-EMT.

The B-RAF inhibitor PLX-4720 and the MEK inhibitors PD184352 and U0126 were used for 24 hours to inhibit the MAPK signalling pathway in the B-RAF mutant cell line A375P and the N-RAS mutant cell line IPC-298 (Figure 3-4). Efficient MAPK pathway inhibition was confirmed by the loss of phosphorylated ERK (pERK). Following B-RAF and MEK inhibition in A375P cells and MEK inhibition in IPC-298 cells, the expression of ZEB2 and SNAIL2 was up-regulated, whilst expression of ZEB1 and TWIST1 was down-regulated. In contrast, use of the B-RAF inhibitor in the N-RAS mutant cell line resulted in up-regulation of pERK, hyper-activation of ZEB1 and loss of ZEB2 and SNAIL2. Interestingly, TWIST1 expression remained constant. These results provide a link between the melanoma-initiating N-RAS and B-RAF mutations and the MR-EMT, and indicate that constitutively active MAPK signalling represses ZEB2 and SNAIL2, whilst activating ZEB1 and TWIST1.

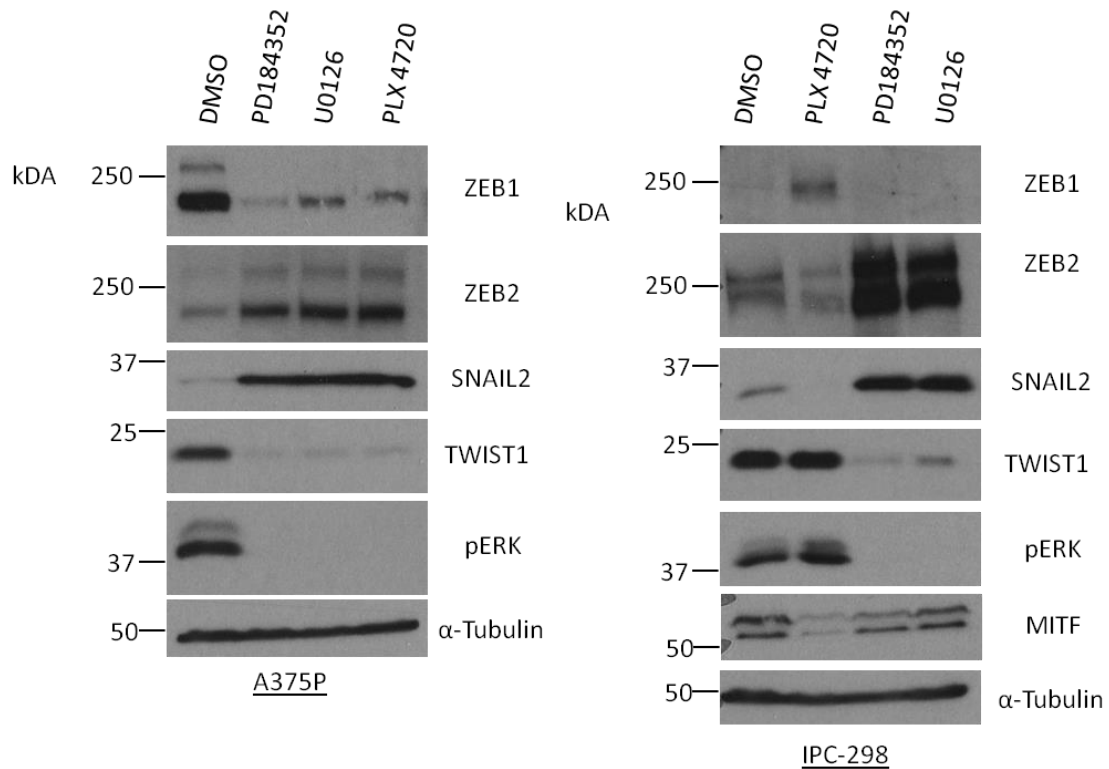


Figure 3-4: Inhibition of oncogenic B-RAF and N-RAS induces a switch in the MR-EMT

The B-RAF mutant A375P cell line and the N-RAS mutant IPC-298 cell line were cultured with 10 μ M of the designated inhibitor or DMSO control for 24 hours. Cells were then lysed and the protein analysed by Western blotting. Following B-RAF and MEK inhibition in the B-RAF mutant cell line A375P, expression of ZEB2 and SNAIL2 were up-regulated whereas ZEB1 and TWIST1 were down-regulated. Comparable results were obtained following MEK inhibition in the N-RAS mutant cell line. In comparison, B-RAF inhibition in IPC-298 cells resulted in up-regulation of ZEB1 and down-regulation of ZEB2 and SNAIL2, with TWIST1 remaining constant. Representative images are presented (n=2). α -Tubulin was used as a protein loading control.

Following the observed influence of MEK inhibition on the expression of the MR-EMT, a kinetics study was undertaken in the A375P cell line, with application of U0126 for varying time intervals over a 24 hour period (Figure 3-5). This confirmed the up-regulation of ZEB2 and SNAIL2 and down-regulation of ZEB1 and TWIST1 but also identified that ZEB2 and SNAIL2 expression increased rapidly following MEK inhibition, with expression elevated after only 4 hours. In comparison, the down-regulation of ZEB1 and TWIST1 expression required prolonged inhibition of MEK signalling, with maximal alterations only observed after 24 hours. This kinetics study identified that U0126-mediated changes in ZEB2 and SNAIL2 precede those observed for ZEB1 and TWIST1.

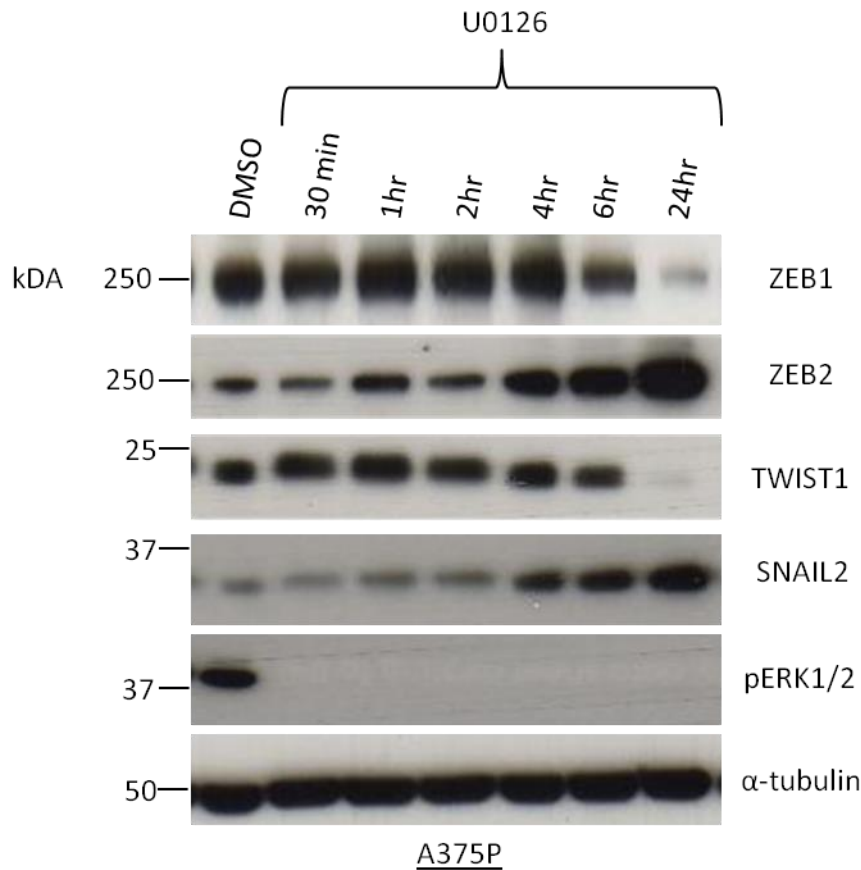


Figure 3-5: Hierarchical organisation of the MR-EMT

A375P cells were incubated with 10 μM of U0126 for varying time intervals, ranging from 30 minutes to 24 hours. Cells were subsequently lysed and the protein analysed by Western blotting. Increased expression of ZEB2 and SNAIL2 occurred after 4 hours of MEK inhibition, whereas down-regulation of ZEB1 and TWIST1 required 24 hours of inhibition for maximal effect. α-Tubulin was used as a protein loading control. Data provided by Eftychios Papadogeorgakis (Appendix I).

3.3.3 ZEB2 REPRESSES ZEB1

The kinetic analysis of the expression of the MR-EMT treated with U0126 suggested that ZEB2 and/or SNAIL2 may function as upstream repressors of ZEB1 and/or TWIST1. It has previously been shown that interactions occur between the ZEB proteins, with activation of ZEB1 in ZEB2^{-/-} mouse embryos, potentially due to the elimination of ZEB2-mediated repression of ZEB1 (Miyoshi *et al.*, 2006). Indeed, in the melanoma cell lines WM266-4 and RPMI-7951, knockdown of ZEB2 resulted in up-regulation of ZEB1 (Figure 3-6). This suggests that ZEB2, at least within melanoma cell lines, has the potential to negatively regulate the expression of ZEB1. Indeed, in the panel of melanoma cell lines (Figure 3-1), it was noted that reduced expression of ZEB2 correlated with increased expression of ZEB1.

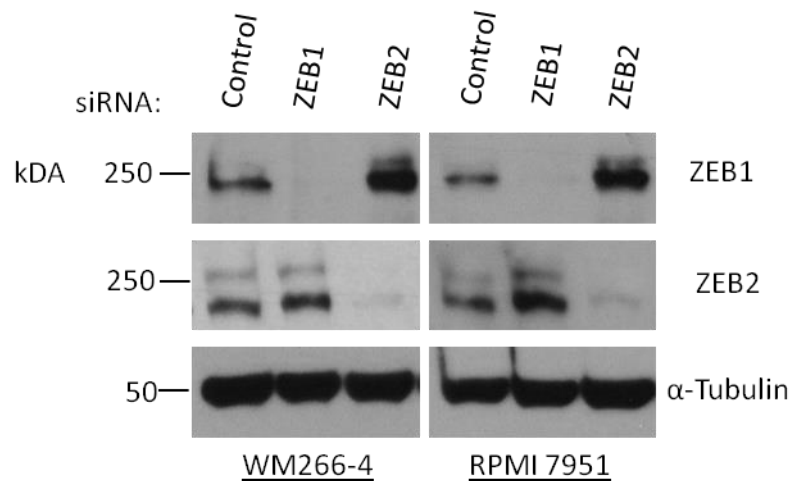


Figure 3-6: ZEB2 is a repressor of ZEB1

WM266-4 and RPMI-7952 cells were transfected with the various siRNAs and incubated for 96 hours. Knockdown of ZEB2 resulted in increased expression of ZEB1. Representative images are presented (n=2). α-Tubulin was used as a protein loading control.

Following MEK inhibition, a ZEB protein switch has been identified, with up-regulation of ZEB2 and down-regulation of ZEB1. To determine whether the MEK-dependent down-regulation of ZEB1 is mediated by increased expression of ZEB2, knockdown of ZEB2 was combined with U0126 treatment in the WM266-4 and A375M cell lines (Figure 3-7). Consistent with previous results, U0126 treatment induced the ZEB protein switch. Additionally, ZEB2 knockdown resulted in an up-regulation of ZEB1. Furthermore, when knockdown of ZEB2 and MEK inhibition were combined, ZEB2 repression attenuated the effect of MEK inhibition on ZEB1 expression, generating an intermediary level of ZEB1 expression. This indicates that induction of ZEB2 expression functions to repress ZEB1.

These data suggest that there is a hierarchical structure in the regulation of the MR-EMT in malignant melanoma cell lines. The expression of the MR-EMT is regulated by N-RAS and B-RAF initiating mutations, which signal through MEK. Inhibition of MEK signalling induces up-regulation of ZEB2, which subsequently functions to repress ZEB1. Importantly, these data were confirmed by immunohistochemical analysis of human melanoma samples (Figure 3-8).

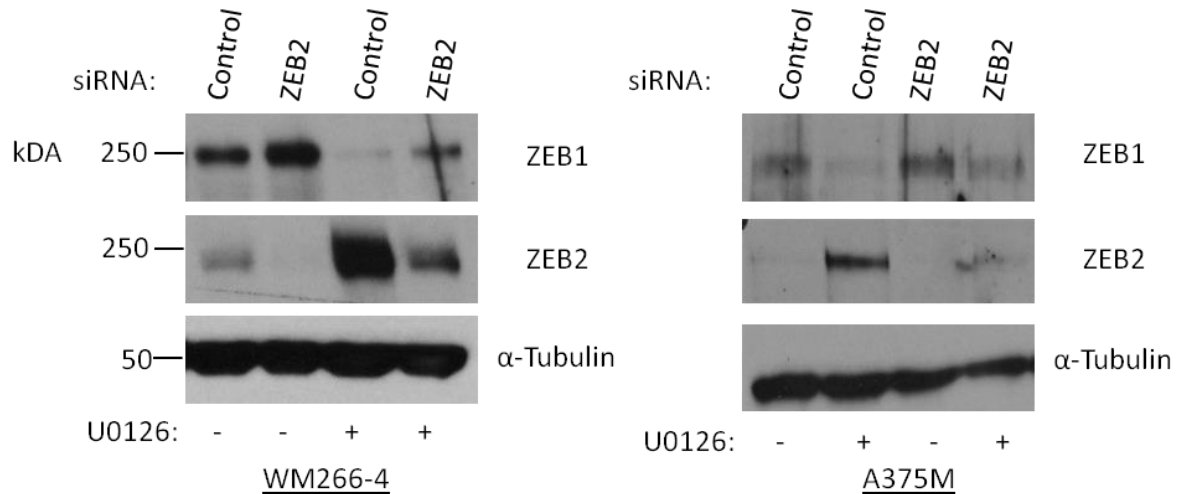


Figure 3-7: Oncogenic B-RAF and N-RAS alleviate ZEB2 repression of ZEB1

Cells were transfected with the siRNAs for a total of 96 hours, with the application of 10 μ M of the MEK inhibitor U0126 for the final 24 hours. Knockdown of ZEB2 with DMSO treatment resulted in upregulation of ZEB1. Application of U0126 resulted in up-regulation of ZEB2, with a corresponding decrease in ZEB1 expression. Interestingly, combined knockdown of ZEB2 and application of U0126 resulted in reduced up-regulation of ZEB2 and an intermediary level of ZEB1 expression. This indicates that MEK-ERK signalling represses ZEB2 and ZEB2 inhibits expression of ZEB1. Representative images are presented (n=2). α -Tubulin was used as a protein loading control.

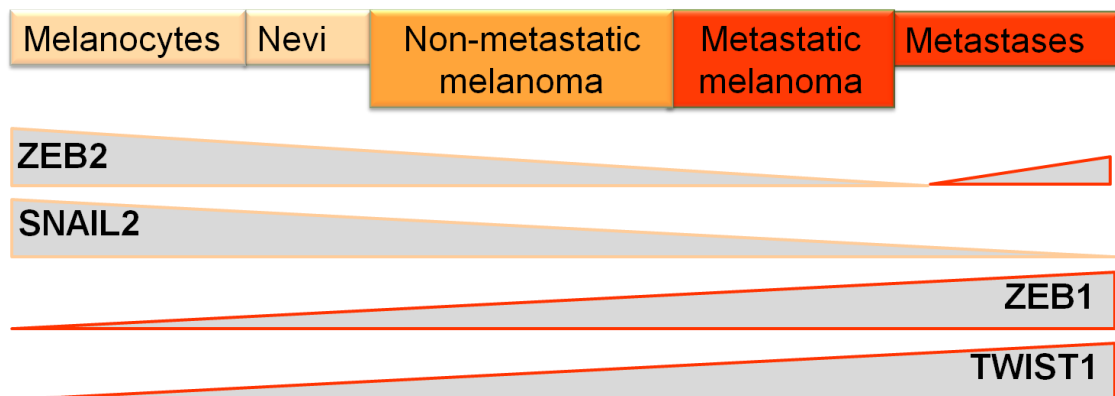


Figure 3-8: A switch in expression of the MR-EMT in melanoma tissue samples

A schematic to describe the changes in the expression of the MR-EMT during the different stages of melanoma progression produced following immunohistochemical analysis of the MR-EMT in human melanoma specimens. Original data provided by Eftychios Papadogeorgakis (Appendix I).

3.4 DISCUSSION

3.4.1 EXPRESSION OF THE MR-EMT IN MELANOMA

The MR-EMT have been widely implicated in the progression of carcinomas through the activation of EMT programs that contribute to tumourigenesis by promoting malignant transformation and tumour progression (Chaffer & Weinberg, 2011). Within this section of the thesis, the expression of the MR-EMT was analysed in NHEMs and melanoma cell lines.

Expression of both ZEB2 and SNAIL2 proteins was identified in NHEMs and melanoma cell lines (Figure 3-1). Previously, SNAIL2 expression was detected in immortalised and transformed melanocytes (Gupta *et al.*, 2005), with higher expression observed in melanocytes when compared to melanoma cell lines (Shirley *et al.*, 2012). However, the data on the expression of SNAIL2 in melanocyte lineage remains contradictory. For example, Poser *et al.* reported SNAIL2 expression in melanoma cell lines but not in melanocytes (Poser *et al.*, 2001). The current results show that SNAIL2 is highly expressed in melanocytes and maintained at varying levels in melanoma cell lines.

Here, a sub-set of the melanoma cell lines have acquired expression of ZEB1, with another, overlapping group gaining expression of TWIST1. Interestingly, the NHEMs did not express ZEB1 and TWIST1 and in the melanoma cell lines reduced expression of ZEB2 correlated with the acquisition of ZEB1. This result suggests that expression of ZEB1 and TWIST1 are acquired during melanoma progression, in conjunction with the down-regulation of ZEB2. A switch in the expression pattern of the MR-EMT was evident in two genetically related cell lines, A375P and A375M. The metastatic A375M cell line has higher expression of ZEB1 and TWIST1 and lower expression of ZEB2 and SNAIL2 when compared to the parental A375P cell line. These observations indicate that there is linked expression of ZEB2 with SNAIL2 and ZEB1 with TWIST1. These data were confirmed in a study performed in a cohort of patients with malignant melanoma and common acquired naevi (Appendix I). While a gradual loss of ZEB2 and SNAIL2 was associated with increased malignancy, expression of ZEB1 and TWIST1 was detected in the most aggressive tumours and predictive of poor survival. SNAIL1 was only

minimally expressed within the melanoma cell lines examined, which is consistent with a study examining melanoma tissue samples, whereby 96% of cases were SNAIL1 negative (Mikesh *et al.*, 2010). Therefore, SNAIL1 was excluded from further investigations.

Expression of E-cadherin was identified in the NHEMs, which is consistent with the fact that melanocytes require E-cadherin for interaction with keratinocytes (Tang *et al.*, 1994). Varying intensities of E-cadherin expression were maintained by a sub-set of melanoma cell lines but absent in half of the cell lines studied. Certain cell lines had undergone the cadherin switch, with the loss of E-cadherin and the acquisition of N-cadherin. This switch is a common feature in melanomagenesis, which promotes interaction of melanoma cells with fibroblasts rather than keratinocytes (Hsu *et al.*, 2000a), resulting in enhanced invasion (Hsu *et al.*, 2000b; Herlyn *et al.*, 2000).

It is worth noting the distinct migratory pattern of both the ZEB1 and ZEB2 proteins. The upper shifted band potentially occurs due to SUMOylation at two conserved lysine residues, which when SUMOylated in ZEB2 does not affect the sub-cellular localisation of the protein, but does reduce the ability of the protein to function as a transcriptional repressor (Long *et al.*, 2005).

3.4.2 THE MR-EMT AND MELANOMA-INITIATING N-RAS AND B-RAF MUTATIONS

Changes in the expression of the MR-EMT were then analysed in relation to mutations within the MAPK signalling pathway (Figure 3-4), which is considered to be a pathway driving melanomagenesis. In the B-RAF mutant cell line A375P, inhibition of B-RAF and MEK resulted in up-regulation of ZEB2 and SNAIL2 and down-regulation of ZEB1 and TWIST1. Additionally, in the N-RAS mutant cell line IPC-298, comparable results were obtained following MEK inhibition. This indicates that B-RAF and N-RAS oncogenic activation results in a switch in the expression of the MR-EMT, promoting expression of ZEB1 and TWIST1, at the expense of ZEB2 and SNAIL2. It appears as though constitutive B-RAF or N-RAS signalling is required to maintain the switch, with short-

term inhibition resulting in a rapid reversal. Interestingly, certain melanoma cell lines have not acquired expression of ZEB1 and TWIST1, even though they contain activating B-RAF and N-RAS mutations. Secondary genetic hits may be required to maintain the MR-EMT expression pattern. These genetic events involve inactivation of inhibitory feedback loops that are known to counteract B-RAF and N-RAS signalling at early stages of melanoma development (Pratilas *et al.*, 2009).

In the N-RAS mutant cell line IPC-298, application of the B-RAF inhibitor resulted in hyper-activation of ZEB1 and repression of ZEB2 and SNAIL2. This result is different from the data obtained in the B-RAF mutant cells and it may be explained by the fact that in the presence of oncogenic N-RAS, B-RAF is usually maintained in an auto-inhibited state. However, following application of a B-RAF inhibitor, a B-RAF/C-RAF complex is formed, which activates C-RAF and enhances downstream MEK-ERK signalling (Heidorn *et al.*, 2010). This hyper-activation of the MAPK pathway correlates with the switch in the MR-EMT, promoting the expression of ZEB1 at the expense of ZEB2 and SNAIL2. It is important to note that expression of TWIST1 does not follow that of ZEB1 after B-RAF inhibition in the N-RAS mutant cell line. This indicates that even though both ZEB1 and TWIST1 are activated by oncogenic B-RAF and N-RAS, their expression is controlled by different mechanisms.

Following the kinetic study of U0126 treatment in A375P cells, up-regulation ZEB2 and SNAIL2 were shown to occur after 4 hours of MEK inhibition, whilst the down-regulation of ZEB1 and TWIST1 required 24 hours (Figure 3-5). This suggested a potential hierarchy in the expression of the MR-EMT, with ZEB2 and SNAIL2 being present upstream of ZEB1 and TWIST1. This was tested with knockdown of ZEB2 in two B-RAF mutant melanoma cell lines, WM266-4 and RPMI-7951, which resulted in increased expression of ZEB1 (Figure 3-6). To link the regulation of ZEB1 by ZEB2 to inhibition of the MAPK signalling pathway, ZEB2 depletion was combined with treatment with U0126 (Figure 3-7). Indeed, knockdown of ZEB2 and MEK inhibition in WM266-4 and A375M cells generated an intermediary level of ZEB1 expression. This indicates that ZEB1 and ZEB2 both function down-stream of MEK within the MAPK

signalling pathway and that ZEB2 is an inhibitor of ZEB1. Taken together, our data suggest that in normal melanocytes, ZEB2 and SNAIL2 are highly expressed, with ZEB2 negatively controlling the expression of ZEB1. However, following oncogenic activation of B-RAF or N-RAS, expression of ZEB2 is down-regulated, allowing increased ZEB1 expression.

The regulation of the ZEB proteins in melanoma appears to be highly divergent from that observed in cells of epithelial origin. The ZEB proteins are co-regulated in epithelial cells, with a double-negative feedback loop involving the ZEB proteins and the miR-200 family of microRNAs. ZEB protein expression induces a mesenchymal phenotype by repression of the miR-200 family, which is counterbalanced by miR-200 expression and the induction of an epithelial phenotype via repression of the ZEB proteins (Hill *et al.*, 2013; Brabletz & Brabletz, 2010). However, regulation of the ZEB proteins must be fundamentally different in melanoma cells, where the expression level of the miR-200 family members is very low (Appendix I). Indeed, ZEB proteins in melanoma appear to belong to a hierarchical pathway, with ZEB2 upstream of, and inhibitory towards, ZEB1.

It is worth noting that even though a linear hierarchy in the expression of the MR-EMT has been described here, with ZEB2 functioning upstream of and repressive towards ZEB1, it is understood that the regulation of the MR-EMT is highly complex rather than simply linear, with the full complement of interactions and levels of regulation still to be investigated. Indeed, intricate interactomes have previously been described to occur between the MR-EMT, whereby inter-regulatory relationships are evident (Taube *et al.*, 2010; Hugo *et al.*, 2011).

3.5 CONCLUSION

NHEMs and melanoma cell lines express ZEB2 and SNAIL2, with a sub-set of the melanoma cell lines gaining expression of ZEB1 and/or TWIST1. ZEB2 and SNAIL2 were co-expressed with E-cadherin, whereas E-cadherin was inversely correlated with ZEB1. Oncogenic B-RAF and N-RAS result in a switch in the expression of the MR-EMT,

promoting the expression of ZEB1 and TWIST1 at the expense of ZEB2 and SNAIL2. ZEB2 acts up-stream of ZEB1 within the MAPK signalling pathway, and is a repressor of ZEB1.

CHAPTER 4 : TRANSCRIPTIONAL REGULATION OF E-CADHERIN IN MELANOMA CELL LINES

4.1 INTRODUCTION

Following from the demonstration of a switch in the repertoire of MR-EMT in melanoma cell lines mediated by melanoma-associated B-RAF and N-RAS mutations, the role of the MR-EMT was addressed in relation to the repression of E-cadherin. E-cadherin is a prototypical target for the MR-EMT (Grooteclaes & Frisch, 2000; Comijn *et al.*, 2001; Cano *et al.*, 2000; Hajra *et al.*, 2002) and loss of E-cadherin is a key feature of the conversion of adenomas to carcinomas (Perl *et al.*, 1998). The cadherin switch within melanocytes, with loss of E-cadherin and up-regulation of N-cadherin, is an important event that disrupts the interaction between melanocytes and keratinocytes (Hsu *et al.*, 2000a). However, the involvement of E-cadherin loss during melanoma progression is controversial, with opposing E-cadherin expression profiles identified at different stages of melanomagenesis (Cowley 1996, Danen 1996, Silye 1998, Sanders 1999, Krengal 2004). The role of the SNAIL1 (Poser *et al.*, 2001; Tsutsumida *et al.*, 2004) and SNAIL2 (Shirley *et al.*, 2012) have previously been studied in relation to the control of E-cadherin expression during melanoma progression. However, due to the contradictory nature of these results, a more comprehensive analysis of the transcriptional control of E-cadherin in malignant melanoma cell lines was undertaken.

The ability of transcription factors, such as the ZEB proteins, to function as repressors or activators depends upon the presence of co-repressors and co-activators. For instance, ZEB1 has been shown to function as a transcriptional activator in the presence of p300-P/CAF (Postigo, 2003). Conversely, both ZEB proteins have been shown to function as E-cadherin transcriptional repressors through interaction with the CtBP co-repressor complex (Shi *et al.*, 2003). Additionally, ZEB1 repressor of E-cadherin has been shown to be partially dependent upon the BRG1 subunit of the SWI/SNF chromatin remodelling complex (Sanchez-Tillo *et al.*, 2010), whilst ZEB2 repression has been shown to function via interaction with the Mi-2 β subunit of the Mi-2/NuRD complex (Verstappen *et al.*, 2008). The presence of such co-repressor complexes could have dramatic consequences on genome-wide expression profiles and could be particularly important during carcinogenesis, with repression of tumour suppressor genes. The differential regulation of E-cadherin expression by the MR-EMT

may be caused by the fact that they have different capacities to interact and co-operate with transcriptional co-repressors. To this end, an investigation into the transcriptional co-repressors(s) involved in the regulation of the E-cadherin gene in melanoma cell lines was undertaken.

In addition to E-cadherin transcriptional repression mediated by the MR-EMT and co-repressors, DNA methylation located at the *CDH1* promoter has been shown to inversely correlate with E-cadherin expression (Reinhold *et al.*, 2007). In order to understand the role of DNA methylation in the transcriptional repression of E-cadherin, DNA methylation at the *CDH1* promoter in melanoma cell lines was analysed.

4.2 AIMS AND OBJECTIVES

Aim: To investigate how E-cadherin is transcriptionally regulated with melanoma cell lines, in relation to the MR-EMT, transcriptional co-repressors and DNA methylation

Objectives: Through the use of transient RNA interference, protein over-expression and reporter assays, the ability of the MR-EMT to control E-cadherin expression will be investigated. Additionally, through transient transfections, reporter assays and co-immunoprecipitation, the involvement of transcriptional co-repressors will be judged. Finally, the level of DNA methylation within the melanoma cell lines will be determined.

4.3 RESULTS

4.3.1 ZEB1 IS A MAJOR E-CADHERIN REPRESSOR IN MELANOMA CELL LINES

Repression of ZEB1 has been shown to result in the re-activation of E-cadherin expression in the E-cadherin negative breast cancer cell line MDA-MB-231 (Eger *et al.*, 2005; Aigner *et al.*, 2007; Sayan *et al.*, 2009) and in the colorectal cancer cell line SW-480 (Sanchez-Tillo *et al.*, 2010). These results were initially replicated to establish a working system of E-cadherin re-expression that could subsequently be transferred to melanoma cell lines.

Two different siRNAs against ZEB1 were tested in MDA-MB-231 cells, siZEB1-10 and siZEB1-18 (Figure 4-1). siZEB1-10 efficiently repressed expression of ZEB1 during an 96 hour incubation resulting in E-cadherin re-expression. In comparison, siZEB1-18 mediated minimal repression of ZEB1, with only minor E-cadherin re-activation. In all subsequent experiments, siZEB1-10 was used. These results were replicated in SW-480 cells, with ZEB1 repression again resulting in re-activation of E-cadherin. This confirmed that ZEB1 depletion triggers *de novo* expression of E-cadherin, correlating with previously published data.

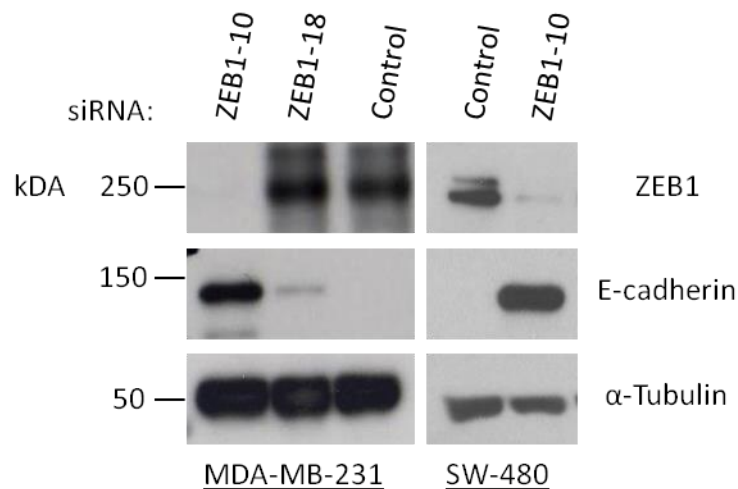


Figure 4-1: ZEB1 represses E-cadherin in carcinoma cell lines
MDA-MB-231 and SW-480 cells were transfected with the various siRNAs and incubated for 96 hours. Western blot analysis identified greater efficiency of ZEB1 protein knockdown using siZEB1-10 when compared to siZEB1-18 in MDA-MB-231 cells, reflected in the strength of E-cadherin re-activation. ZEB1 knockdown resulted in E-cadherin re-expression in MDA-MB-231 and SW-480. Representative images are presented (n=2). α-Tubulin was used as a protein loading control.

As previously stated, a range of melanoma cell lines were examined for expression of E-cadherin (Figure 3-1). Retention of E-cadherin expression was identified in the B-RAF mutant cell lines SK-MEL-28, SK-MEL-5 and UACC-257, and in the N-RAS mutant cell line SK-MEL-30. In comparison, E-cadherin expression was undetectable in the B-RAF mutant cell lines A375P, A375M, WM266-4, RPMI-7951 and in the N-RAS mutant cell lines SK-MEL-2 and IPC-298. The ability of the MR-EMT to repress E-cadherin expression was examined.

The expression of the MR-EMT was inhibited by RNA interference, singularly or in combination, in A375M, RPMI-7951 and WM266-4 cells, and the expression of E-

cadherin examined at the protein level (Figure 4-2a). In the A375M cell line, repression of ZEB1 efficiently up-regulated E-cadherin, which was enhanced with combined knockdown of ZEB1 and ZEB2. However, down-regulation of ZEB2 alone did not result in E-cadherin re-activation. In comparison, in both RPMI-7951 (Figure 4-2a) and WM266-4 cells (Figure 4-8), combined repression of both ZEB1 and ZEB2 was required for re-expression of E-cadherin. In all three cell lines, inhibition of TWIST1 and SNAIL2 did not alter E-cadherin expression, either when repressed singularly or in combination with the ZEB proteins.

The re-activation and subsequent re-localisation of membranous E-cadherin in A375M cells following repression of ZEB1 and ZEB2 was visualised with immunofluorescent staining (Figure 4-2b). Regions of enhanced E-cadherin staining were evident following knockdown of ZEB1, which increased further following combined down-regulation of ZEB1 and ZEB2. However, E-cadherin re-activation only occurred within a sub-set of cells and was not uniform throughout the population. Following knockdown of the ZEB proteins, E-cadherin localised to the membrane, indicating that it was functional in the formation of the adherens junctions.

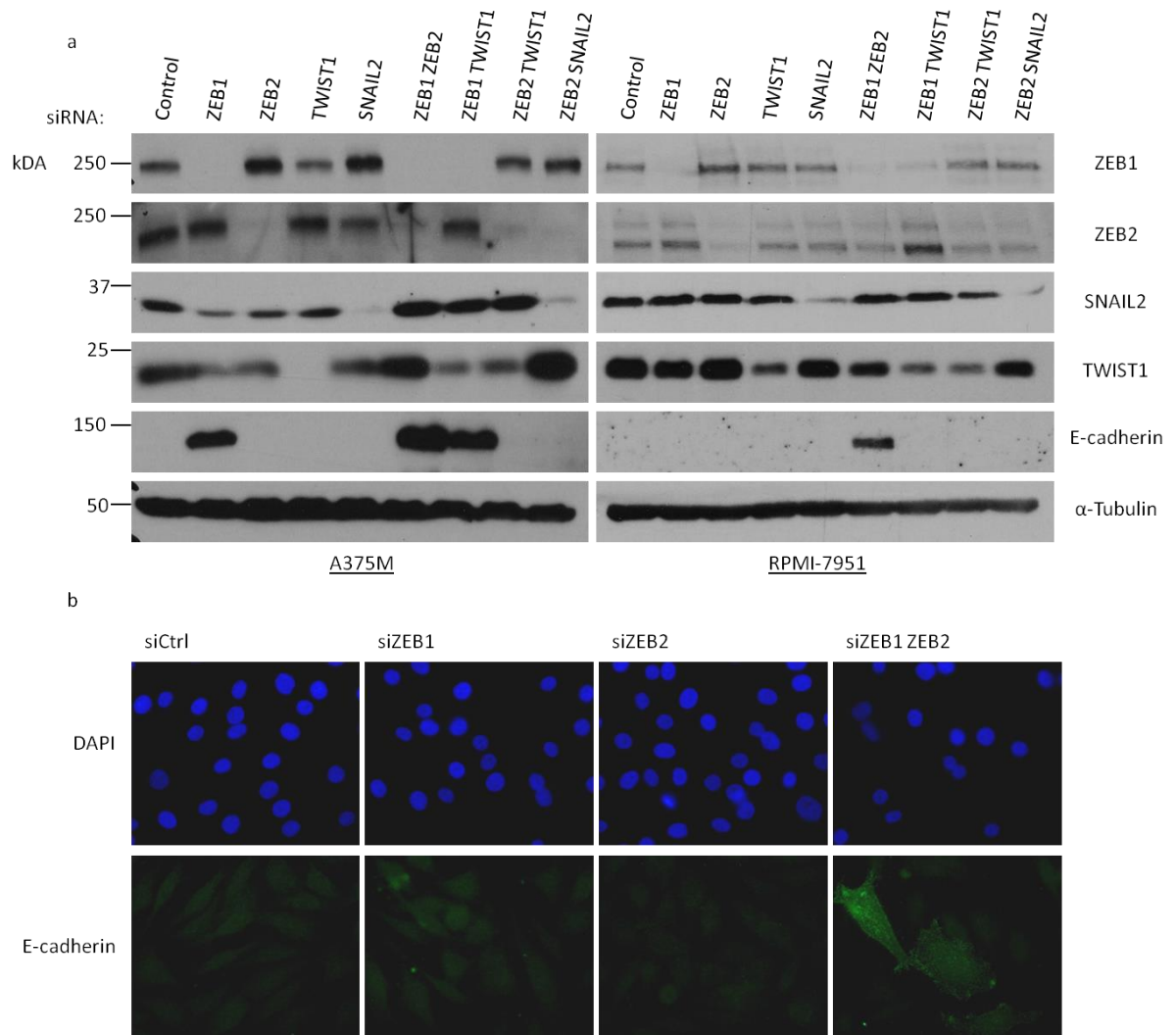


Figure 4-2: Knockdown of ZEB1 and ZEB1-ZEB2 induces E-cadherin re-expression in melanoma cell lines
a) A375M and RPMI-7951 cell lines were transfected with the various siRNA combinations and incubated for 96 hours. Cells were subsequently lysed and the protein expression analysed by Western blotting. Repression of ZEB1 re-activated E-cadherin expression in A375M cells, with combined ZEB1 and ZEB2 down-regulation enhancing the amount of E-cadherin re-expressed. In comparison, in RPMI-7951 cells E-cadherin re-activation required both knockdown of ZEB1 and ZEB2. Representative images are presented (n=2). α-Tubulin was used as a protein loading control.
b) A375M cells were transfected with the various siRNAs and incubated for 96 hours prior to fixation with acetone-methanol and staining for E-cadherin (green) and DAPI (blue). E-cadherin was re-expressed following knockdown of ZEB1, which was increased with combined knockdown of ZEB1 and ZEB2. Representative images are presented (n=2). (60X objective)

The A375 model cell system for melanoma metastasis was then used to assess the degree of E-cadherin silencing in two genetically related melanoma cell lines, which differ in their metastatic potential. Due to the previously established involvement of the ZEB proteins, the ability to re-express E-cadherin was undertaken by inhibition of ZEB1 and ZEB2 singularly and in combination (Figure 4-3a). In A375M, as previously identified, single knockdown of ZEB1 resulted in E-cadherin re-expression, which was undetectable following knockdown of ZEB2. However, combined repression of ZEB1 and ZEB2 had a synergistic effect, with significantly enhanced expression of E-cadherin ($p = \leq 0.01$, $t = 4.36$) (Figure 4-3b). Interestingly, when the parental A375P cell line was exposed to comparable conditions, *de novo* expression of E-cadherin was less efficient, with minimal re-activation identified following knockdown of ZEB1 and re-expression only marginally increased following down-regulation of both ZEB1 and ZEB2.

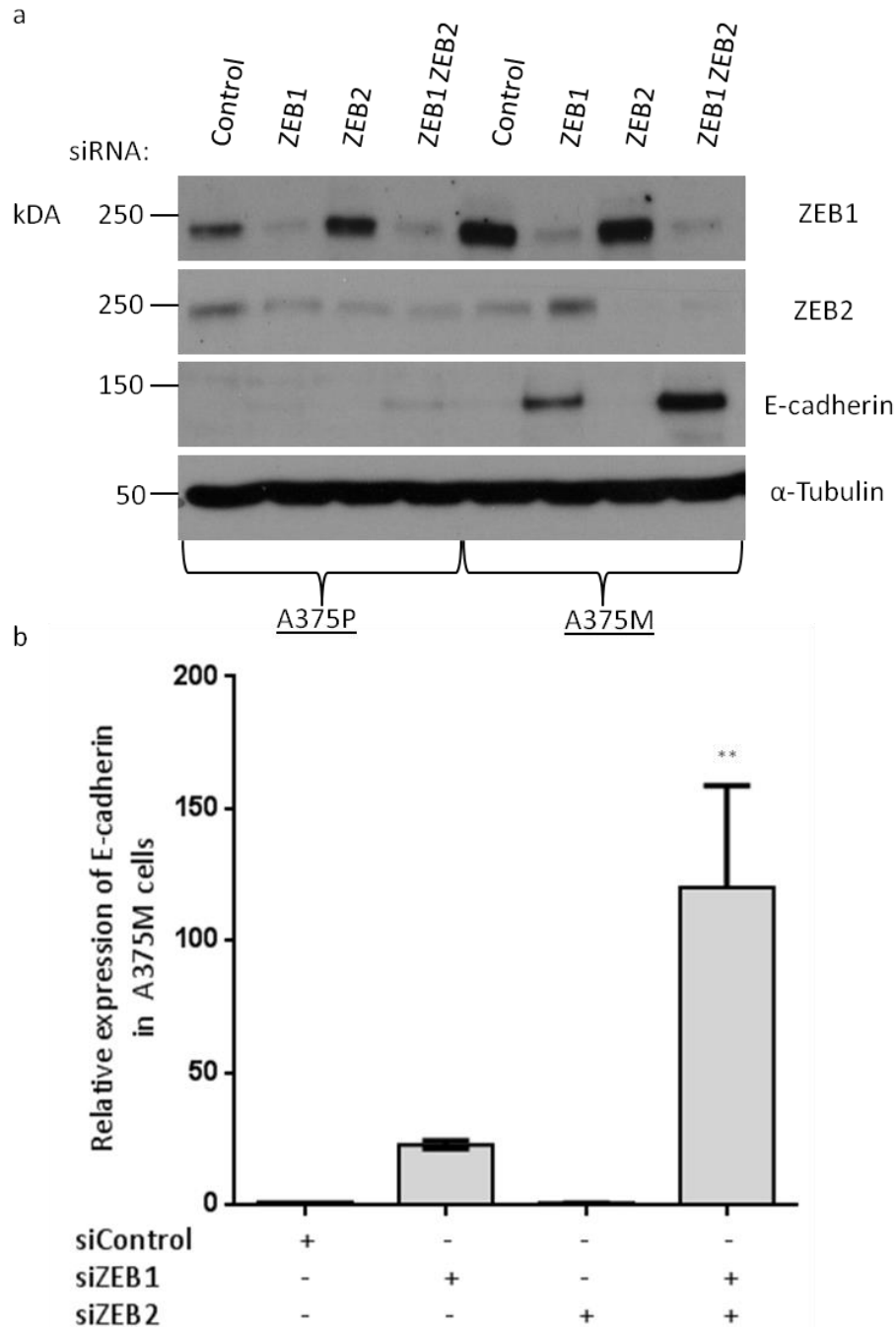


Figure 4-3: ZEB protein repression in the A375 cell lines induces differential E-cadherin re-activation
A375P and A375M cells were transfected with the various siRNAs and incubated for 96 hours.

a) A representative image is presented (n=3). In the metastatic A375M cell line, E-cadherin was re-activated with single knockdown of ZEB1. Additionally, enhanced E-cadherin expression was identified following combined ZEB1 and ZEB2 knockdown. In comparison, in the parental A375P cell line, the strength of E-cadherin re-expression was reduced, with minimal amounts detected after combined ZEB1 and ZEB2 knockdown. α-Tubulin was used as a protein loading control.

b) The relative fold change in expression of E-cadherin in A375M cells following inhibition of the ZEB proteins. Three independent experiments were performed, with the standard error of the mean (SEM) presented for each sample type. The intensity of E-cadherin staining was normalised against α-Tubulin and a fold change in E-cadherin expression generated by comparison to the siControl sample. Significance was tested via a one-way ANOVA (** $p \leq 0.01$).

The MR-EMT repress E-cadherin gene transcription via binding to E-box sequence elements in the E-cadherin promoter (Liu *et al.*, 2005). Wild-type and mutant E-cadherin promoter reporters were analysed to determine whether the repression of E-cadherin transcription in melanoma cell lines occurred via this canonical mechanism (Figure 4-4). The activity of the mutant reporter was significantly higher than that of the wild-type promoter reporter ($p = \leq 0.0001$, $t = 12.57$), indicating that the repression of E-cadherin involves the promoter-based E-boxes. Consistent with the previous conclusion that ZEB1 is a prominent E-cadherin repressor in A375M cells, ZEB1 knockdown also resulted in the significant activation of the wild-type E-cadherin promoter ($p = \leq 0.0001$, $t = 22.13$). In comparison, knockdown of endogenous ZEB2 did not alter the transcriptional activity of the E-cadherin promoter, with the activity remaining at a basal level. This indicates that ZEB2 does not function as a transcriptional repressor of the E-cadherin promoter within melanoma cell lines. This pattern of transcriptional control replicates the previous findings regarding ZEB protein mediated E-cadherin re-activation and confirms that ZEB1-mediated regulation occurs at the transcriptional level.

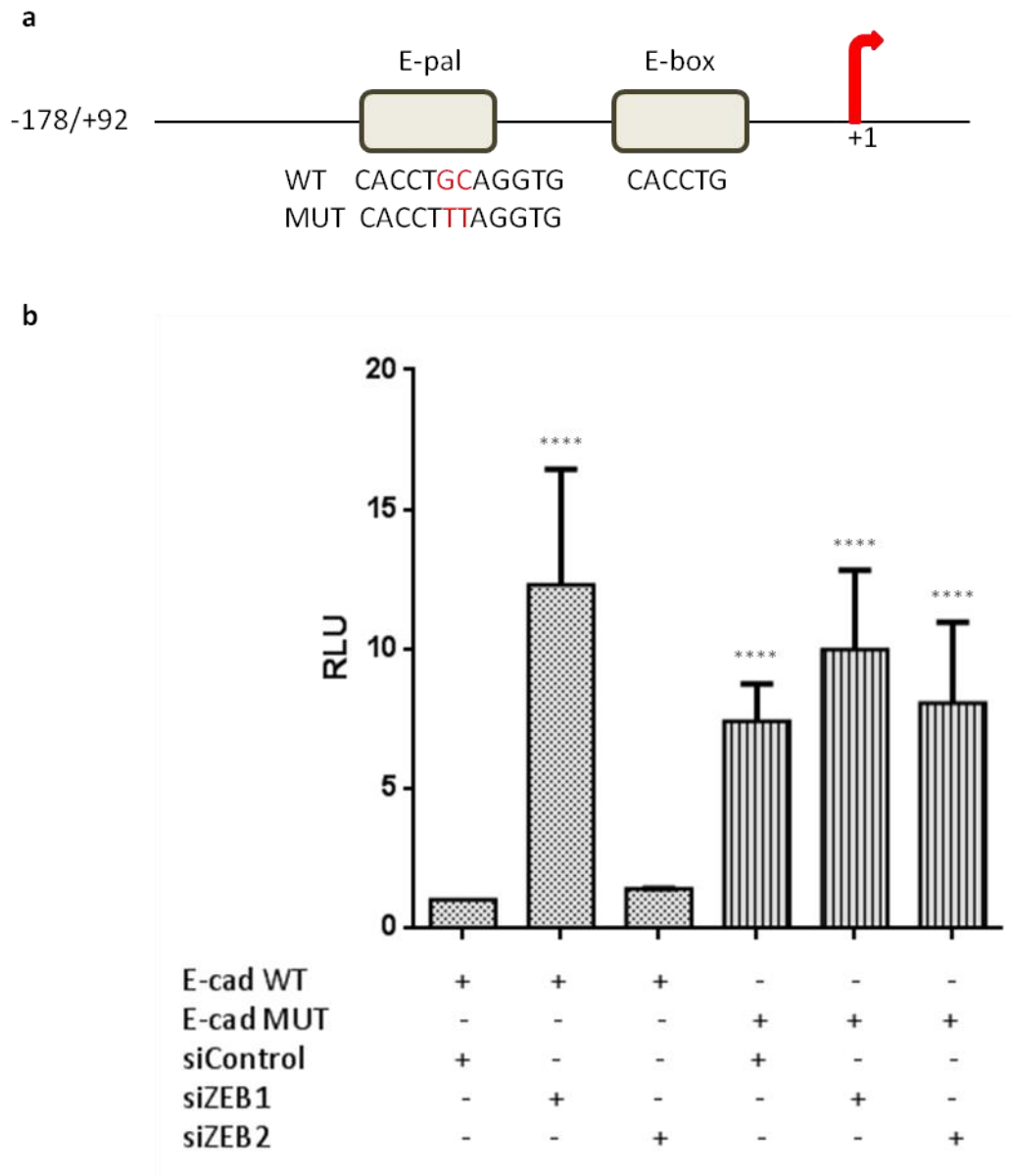


Figure 4-4: ZEB1 transcriptionally represses the E-cadherin promoter

a) A scheme representing the mouse E-cadherin promoter reporter (-178/+92), with +1 marking the transcriptional start site. The E-pal site, consisting of two consecutive E-boxes, was present in a wildtype (WT) and mutated (MUT) form, with the two central GC nucleotides mutated to TT residues, abolishing the E-box binding sites (Bolos *et al.*, 2003; Hennig *et al.*, 1996).

b) A375M cells were co-transfected with 1µg of pGL3 E-cadherin wildtype reporter (E-cad WT) or 1µg of pGL3 E-cadherin mutant reporter (E-cad MUT) along with either 50 nM of scrambled siRNA (siCtrl), ZEB1 siRNA (siZEB1) or ZEB2 siRNA (siZEB2). To normalise transfection efficiency, 1µg of pCMV-β-gal was co-transfected with the E-cadherin reporter and appropriate siRNA. Relative luminescent units (RLU) are expressed as the mean of duplicate readings, with each condition replicated in three independent experiments, with the SEM presented for each sample. ZEB1 was identified as a transcriptional repressor of the E-cadherin promoter, with mutation of the E-boxes allowing transcriptional activation. Significance was tested via a two-way ANOVA, with test samples compared to the control sample containing the wildtype E-cadherin promoter and siControl (**** $p \leq 0.0001$).

Next, a positive approach was used to demonstrate ZEB1-mediated repression of E-cadherin (Figure 4-5). ZEB1 was ectopically expressed for 96 hours in the UACC-257 cell line, which expresses ZEB2 and E-cadherin, but not ZEB1. Via immunofluorescent analysis, ZEB1 expression was shown to be highly nuclear, whereas E-cadherin was localised at the cellular membrane. It was evident that expression of ZEB1 resulted in down-regulation of E-cadherin, with ZEB1-positive cells staining negatively for E-cadherin. This provided further evidence that ZEB1 is a functional repressor of E-cadherin within melanoma cell lines.

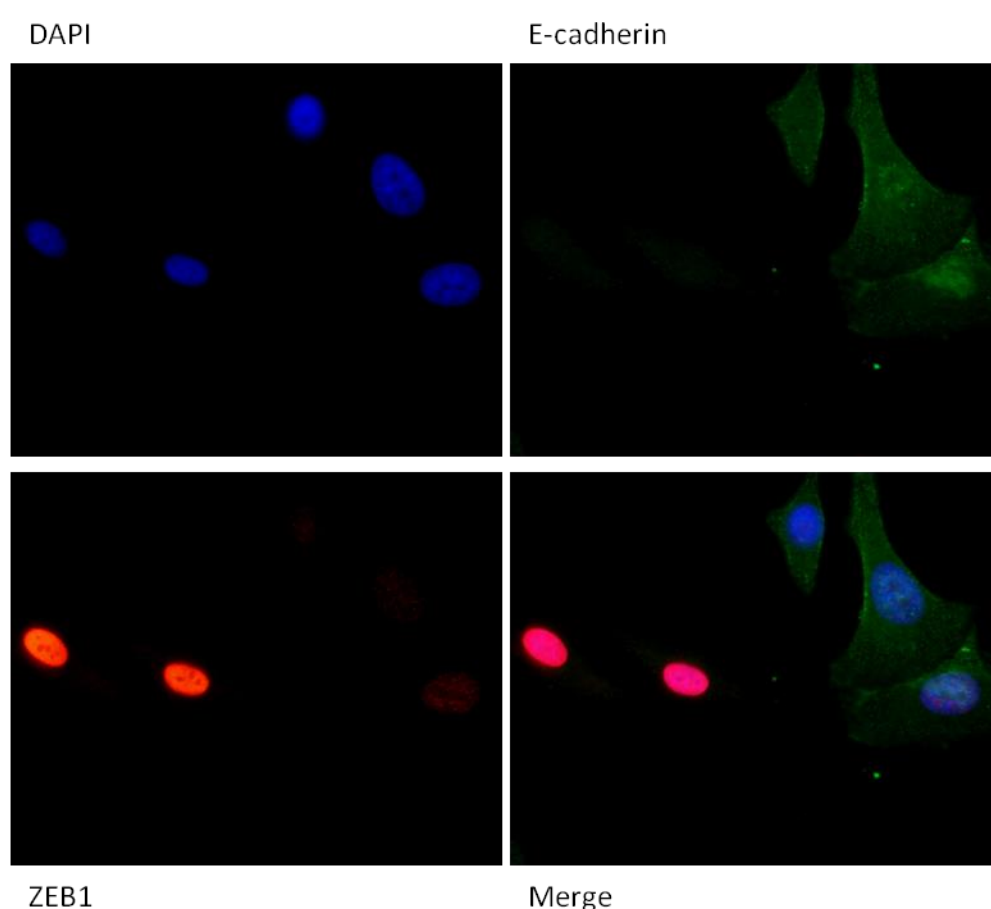


Figure 4-5: ZEB1 represses E-cadherin in the UACC-257 cell line

ZEB1 was transiently expressed in UACC-257 cells for 96 hours by transfection of 4 μg of pBI_ZEB1_HA and 1 μg of pUHD172.1 neo vectors, with ZEB1 expression induced with 2 $\mu\text{g}/\text{ml}$ of doxycycline. Cells were fixed with acetone-methanol and stained for E-cadherin (green), ZEB1 (red) and DAPI (blue). Representative images are presented, with low density cellular clustering required for optimal visualisation of changes in E-cadherin expression ($n=3$). ZEB1 expression resulted in repression of E-cadherin. (60X objective)

4.3.2 ZEB2 AND E-CADHERIN IN MELANOMA CELL LINES

ZEB1-mediated repression of E-cadherin in melanoma cells has been confirmed by several techniques, whereas the relationship between E-cadherin and ZEB2 is more complex. Subsequently, ZEB2 expression was examined in relation to E-cadherin repressor in an epithelial cell system.

ZEB2 functions as a repressor of E-cadherin in epithelial cell lines (Comijn *et al.*, 2001). A model of EMT has been previously established in the squamous epidermoid carcinoma cell line A431, designated A431-ZEB2, which has been engineered with inducible expression of ZEB2 following application of doxycycline. This results in an EMT, with morphological changes, inhibition of epithelial genes and activation of mesenchymal markers (Vandewalle *et al.*, 2005; Mejlvang *et al.*, 2007). Indeed, after induction of ZEB2 expression in A431-ZEB2 cells, there was a decrease in expression of E-cadherin and P-cadherin and a corresponding increase in vimentin and N-cadherin (Figure 4-6a). E-cadherin was lost from the cell boundaries, indicating dissolution of the adherens junctions (Figure 4-6c). This identified ZEB2 as a repressor of E-cadherin and inducer of the cadherin switch. Additionally, ZEB2 expression induced morphological changes, converting the highly compact, clustered and rounded cell line into a scattered, fibroblast-like phenotype, with loss of cell-cell contacts (Figure 4-6b). These features confirmed that ZEB2 was a repressor of E-cadherin and inducer of an EMT in this squamous epidermoid carcinoma cell line.

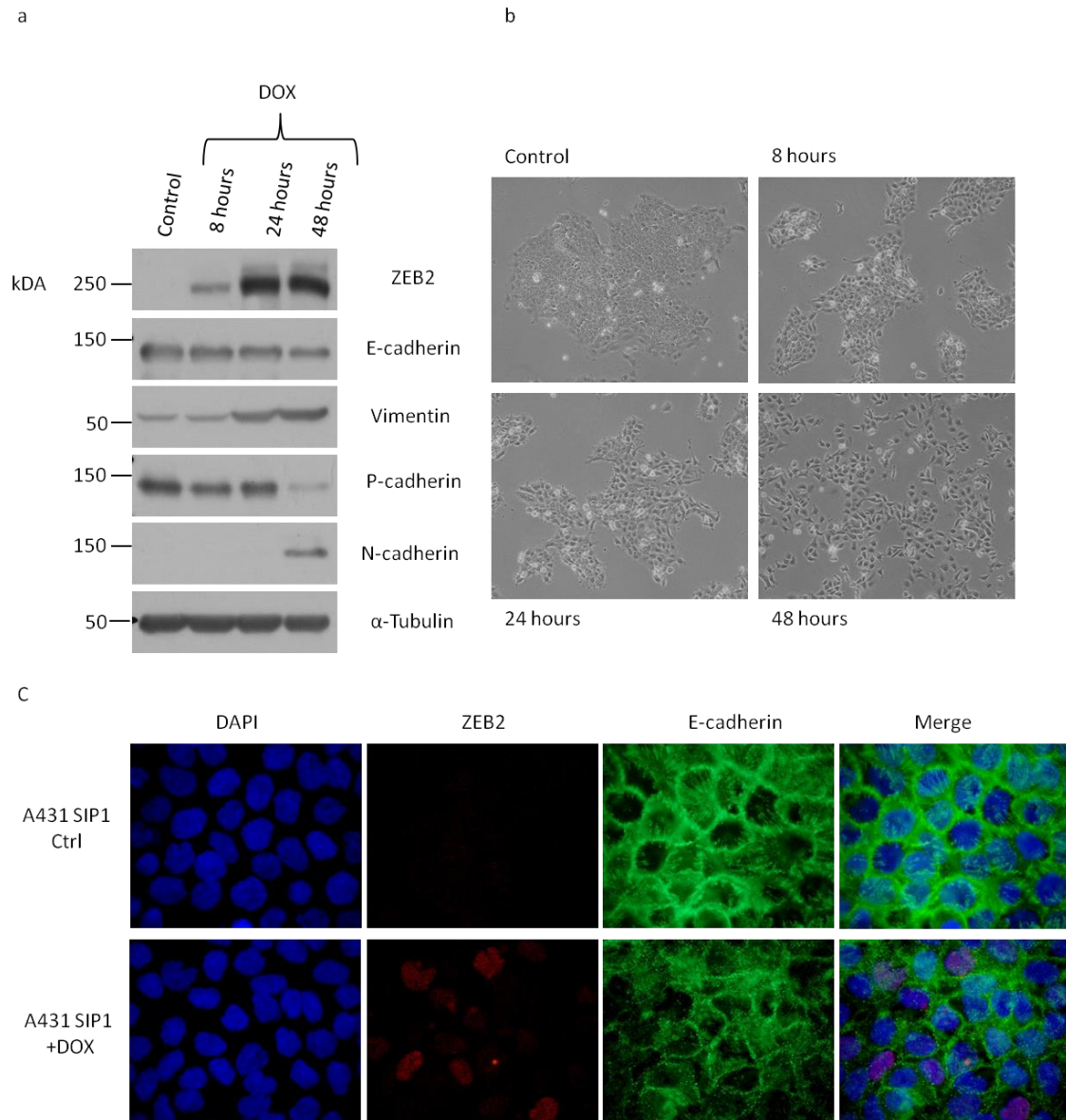


Figure 4-6: ZEB2 represses E-cadherin and induces an EMT in the A431-ZEB2 cell line

A431-ZEB2 cells were treated with 2 μ g/ml of doxycycline over a 48 hour period. Representative images are presented (n=2).

(a) Western blotting analysis of a model of EMT in A431-ZEB2 following induction of ZEB2. Over the time course of ZEB2 induction, E-cadherin and P-cadherin protein levels were reduced, in conjunction with an increase in the expression of vimentin and N-cadherin. These changes correspond to an EMT. α -Tubulin was used as a protein loading control.

(b) Phase contrast images of A431-ZEB2 cells undergoing an EMT due to expression of ZEB2. The epithelial morphology of the cell was lost, with cellular scattering and formation of a mesenchymal phenotype. (10X objective).

(c) ZEB2 expression was induced in A431-ZEB2 cells for 48h hours by treatment with 2 μ g/ml of doxycycline. Cells were then fixed with acetone-methanol and stained for E-cadherin (green), ZEB2 (red) and DAPI (blue). Induction of ZEB2 resulted in a reduction in E-cadherin expression. (60X objective)

However, as previously stated, E-cadherin-positive melanocytes and melanoma cell lines express ZEB2. To confirm co-expression of ZEB2 and E-cadherin, immunofluorescent analysis was performed on UACC-257 cells. ZEB2 was shown to be co-expressed with E-cadherin uniformly within the cell population (Figure 4-7). Staining for both E-cadherin and ZEB2 was strong, with intense localisation of E-cadherin at the cellular membrane, whereas ZEB2 staining was restricted to the nucleus. This indicates that ZEB2 does not function as an E-cadherin transcriptional repressor in this melanoma cell line, an observation that may be expanded to the NHEMs and the other melanoma cell lines. This means that there is a difference in E-cadherin mediated repression between the epithelial A431 and melanoma UACC-257 cell lines.

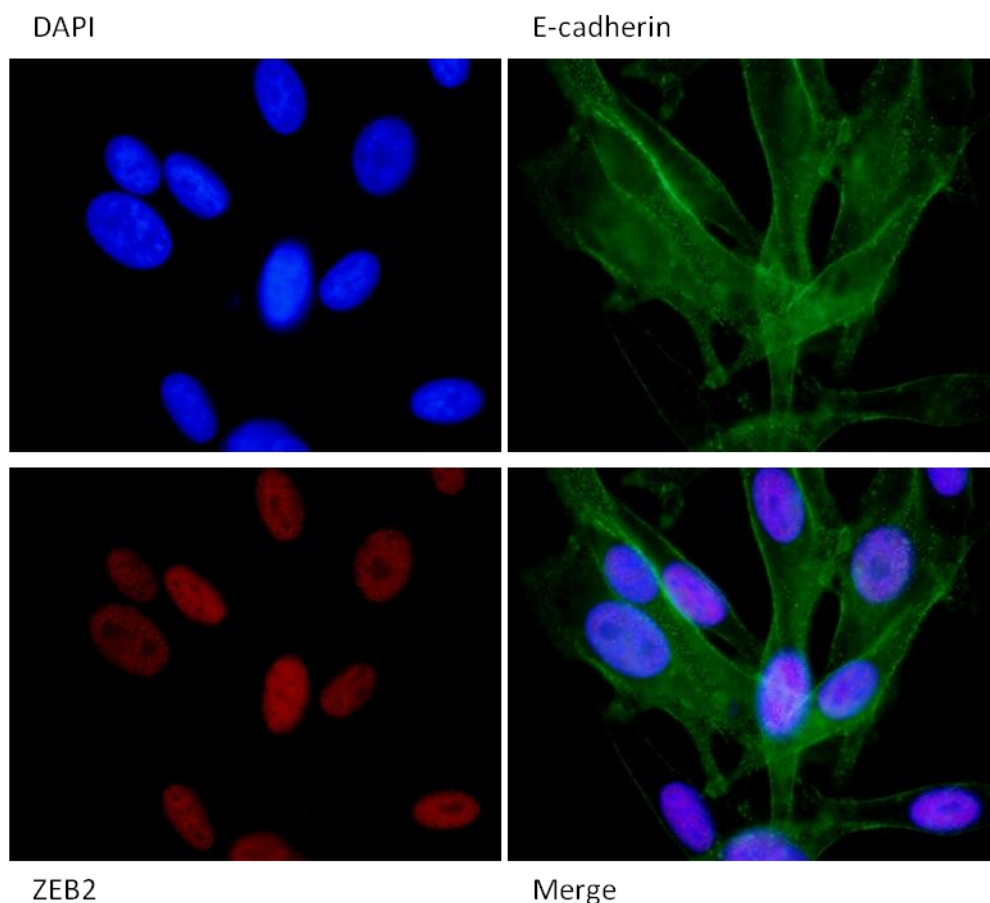


Figure 4-7: ZEB2 and E-cadherin are co-expressed in the UACC-257 cell line
UACC-257 cells were fixed with acetone-methanol and stained for E-cadherin (green), ZEB2 (red) and DAPI (blue). E-cadherin and ZEB2 were uniformly co-expressed within the UACC-257 cells. Representative images are presented (n=2). (60X objective)

Intriguingly, another observation concerning E-cadherin and ZEB2 was that knockdown of ZEB2 reduced, rather than increased, E-cadherin expression in WM266-4 cells, a melanoma cell line with low amounts of endogenous E-cadherin (Figure 4-8a). This may indicate that ZEB2 functions as an activator of E-cadherin expression. Alternatively, this may occur due to the release of ZEB2-mediated repression of ZEB1, which was previously identified within melanoma cell lines, allowing ZEB1-mediated repression of E-cadherin. In contrast to this observation, ZEB2 depletion enhanced activation of E-cadherin when combined with ZEB1 knockdown. In comparison to WM266-4 cells, UACC-257 cells have high endogenous expression of E-cadherin and are ZEB1-negative. Following knockdown of ZEB2 in UACC-257 cells, E-cadherin expression was not altered (Figure 4-8b).

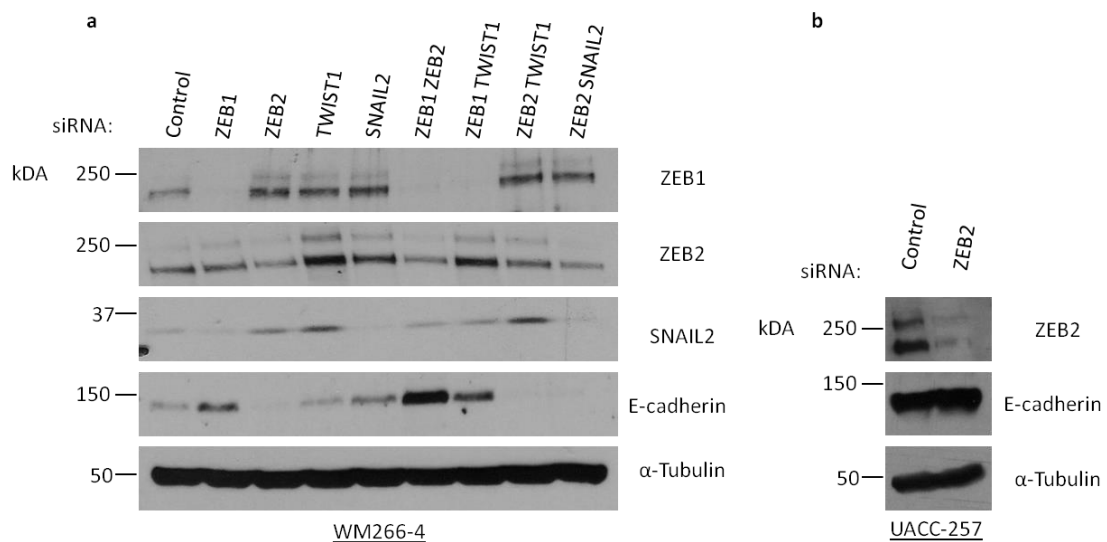


Figure 4-8: Inhibition of ZEB2 induces E-cadherin down-regulation

WM266-4 and UACC-257 cells were transfected with the appropriate siRNAs and incubated for 96 hours. Cells were lysed and the protein analysed by Western blotting.

a) In WM266-4 cells, which have low endogenous E-cadherin expression, knockdown of ZEB2 resulted in reduced E-cadherin expression. SNAIL2 and TWIST1 did not alter E-cadherin expression, with combined knockdown of ZEB1 and ZEB2 enhancing E-cadherin re-expression. Representative images are presented (n=2).

b) In UACC-257 cells, which have a high level of E-cadherin expression, knockdown of ZEB2 did not result in repression of E-cadherin. α -Tubulin was used as a protein loading control.

4.3.3 E-CADHERIN REPRESSION BY ZEB PROTEINS IN MALIGNANT MELANOMA: ROLE OF TRANSCRIPTIONAL CO-REPRESSORS

4.3.3.1 CtBP1/2 AND BRG1

CtBP1 and CtBP2 are canonical co-repressors that act in concert with the ZEB proteins to transcriptionally inactivate the E-cadherin promoter by recruitment of HDACs and HMTs. Importantly, the CtBP1/2 interaction with ZEB1 in epithelial cells is stimulated by a MEK-activated pathway (Shirakihara *et al.*, 2011). The dependence of the ZEB2-CtBP interaction on MEK activity has not been examined. Therefore, activation of MEK by B-RAF or N-RAS mutations may stimulate formation of a ZEB1-CtBP complex at the expense of a ZEB2-CtBP complex.

Knockdown of CtBP using a siRNA targeting both highly homologous genes, *CtBP1* and *CtBP2* (Hildebrand & Soriano, 2002) was undertaken in A375P and A375M cell lines (Figure 4-9). Surprisingly, CtBP depletion produced no effect on E-cadherin expression. Additionally, no additive effect of CtBP and ZEB1 knockdown was observed in A375M cells, in which E-cadherin was induced following use of the siRNA targeting ZEB1. In fact, combining CtBP repression and ZEB1 depletion resulted in reduced E-cadherin expression.

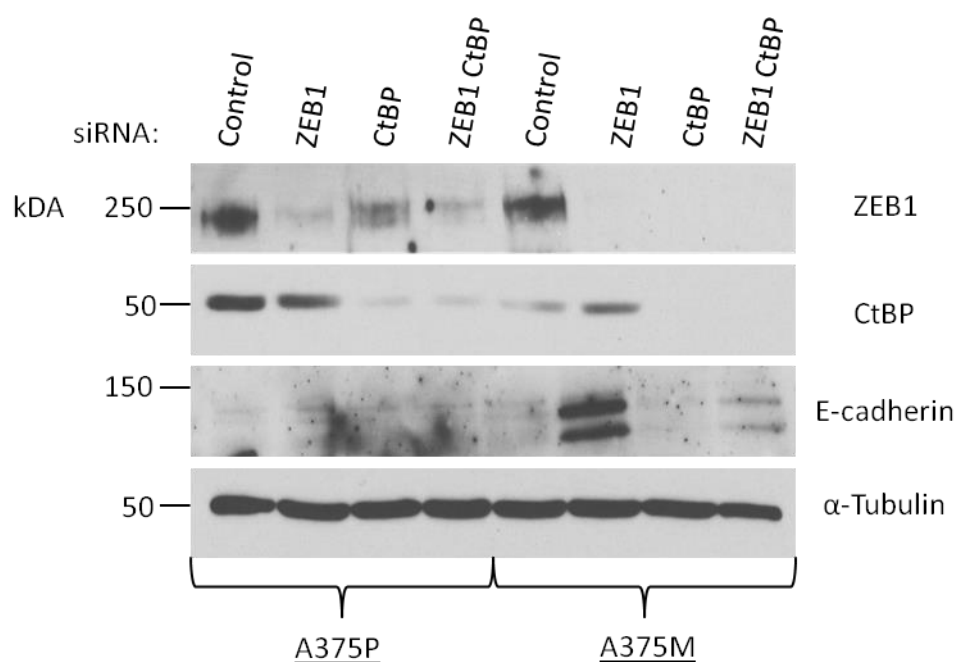


Figure 4-9: CtBP repression does not induce E-cadherin re-activation in melanoma cell lines
A375P and A375M cells were transfected with the siRNAs for 96 hours. E-cadherin re-activation was only apparent in A375M with knockdown of ZEB1. CtBP inhibition did not result in E-cadherin re-expression in either cell line. Representative images are presented (n=2). α -Tubulin was used as a protein loading control.

This lack of cooperation between ZEB1 and CtBP may be a feature unique to melanoma cells. To address this assumption, knockdown of CtBP and ZEB1 was undertaken in a carcinoma cell line, in which cooperation between CtBP and ZEB1 is better established. In a kinetics study in the MDA-MB-231 cell line, 48 hours of ZEB1 repression resulted in *de novo* E-cadherin expression, which was enhanced following 96 hours of ZEB1 depletion (Figure 4-10). Surprisingly, knockdown of CtBP did not result in E-cadherin re-activation and combined repression of ZEB1 and CtBP reduced the strength of E-cadherin re-expression.

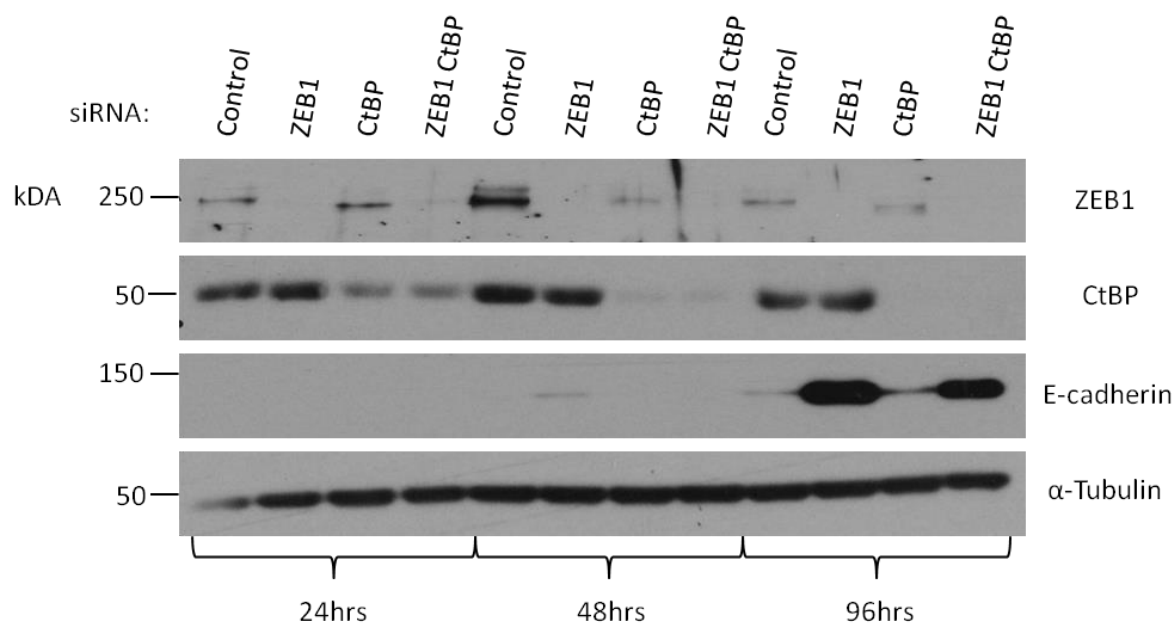


Figure 4-10: CtBP repression does not re-activate E-cadherin expression in a carcinoma cell line. MDA-MB-231 cells were transfected with the various siRNAs and lysed at the designated time points. Knockdown of ZEB1 resulted in re-activation of E-cadherin expression after 48 hours, which was greatly enhanced following 96 hours of ZEB1 repression. CtBP did not appear to act as a transcriptional co-repressor of E-cadherin in this cell line. Representative images are presented (n=2). α-Tubulin was used as a protein loading control.

The observed decrease in E-cadherin expression following combined repression of ZEB1 and CtBP may be the result of cellular toxicity experienced due to depletion of CtBP. Dramatic morphological changes were apparent following knockdown of CtBP, which were enhanced in cells treated with combined repression of ZEB1 and CtBP. The cellular surface area was greatly enlarged, with the formation of cellular protrusions. Also, cellular death was evident (Figure 4-11). Indeed, CtBP has been shown to negatively regulate pro-apoptotic genes (Grooteclaes *et al.*, 2003).

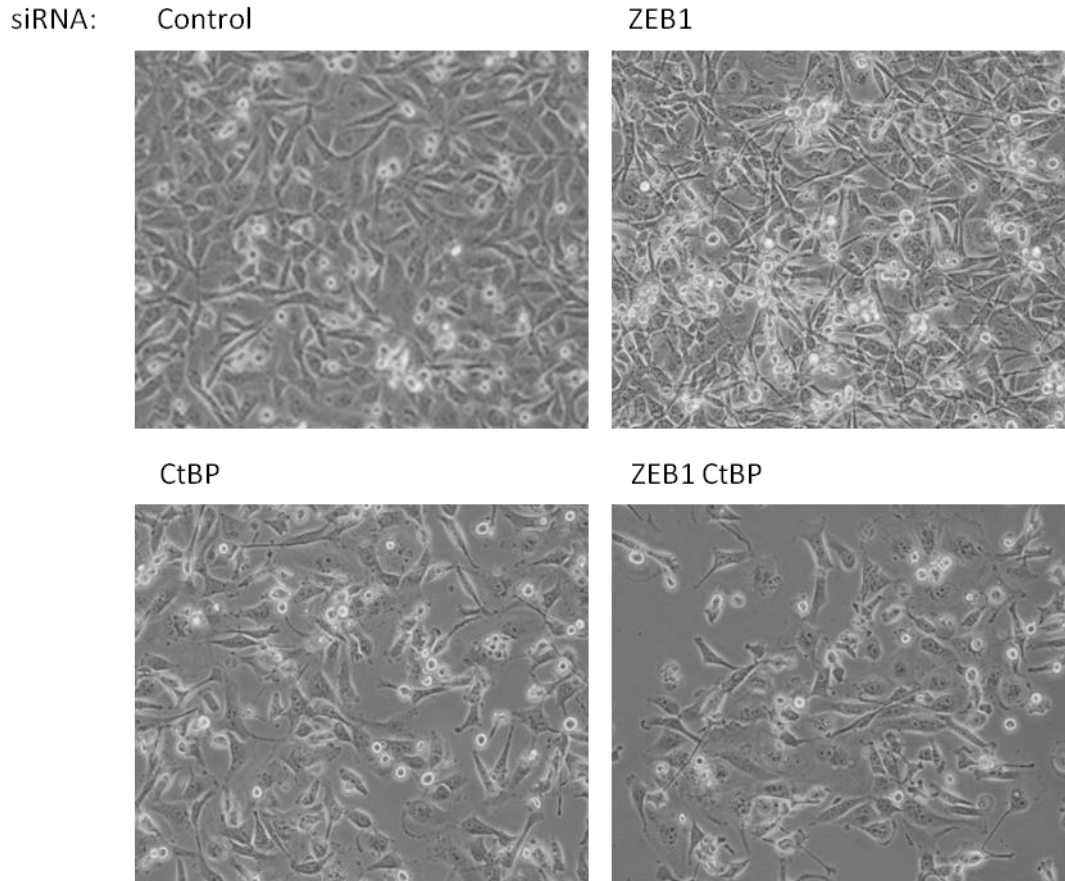


Figure 4-11: Knockdown of CtBP induces cellular toxicity
MDA-MB-231 cells were transfected with the appropriate siRNA and incubated for 96 hours. Morphological changes were evident, especially pronounced with combined ZEB1 and CtBP repression, indicating cellular toxicity was evident. (20X objective)

To avoid complications induced by cellular toxicity on protein expression and stability, a kinetics study of E-cadherin mRNA from MDA-MB-231 cells was undertaken (Figure 4-12). However, matching results were obtained, except that E-cadherin re-activation following ZEB1 repression and combined ZEB1-CtBP repression were comparable, indicating that CtBP does not function cooperatively with ZEB1 to repress E-cadherin.

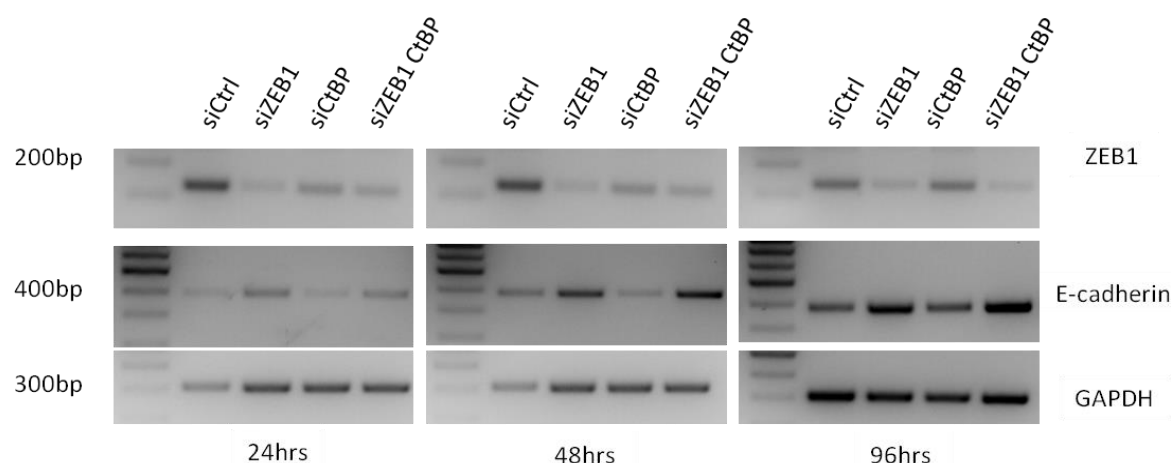


Figure 4-12: CtBP repression does not induce re-expression of E-cadherin mRNA in a carcinoma cell line MDA-MB-231 cells were transfected with the appropriate siRNA and incubated for the indicated time period. RNA was collected and RT-PCR performed. CtBP repression was confirmed by protein analysis (Figure 4-10). ZEB1 knockdown resulted in increased E-cadherin mRNA after only 24 hours of ZEB1 repression. CtBP knockdown did not alter E-cadherin expression. GAPDH was used as a control for mRNA input.

Another candidate co-repressor was BRG1, the ATPase subunit of the SWI/SNF chromatin remodelling complex. BRG1 has been reported to interact with ZEB1 in SW480 cells, contributing to the transcriptional repression of E-cadherin (Sanchez-Tillo *et al.*, 2010). The interaction between ZEB2 and BRG1 has not been assessed in the past.

A screen for BRG1 expression in the melanoma cell lines and NHEMs identified that BRG1 was expressed in all melanoma cell lines, except SK-MEL-5 cells, and absent in the NHEMs (Figure 3-1). In A375M cells, E-cadherin re-expression was identified following repression of ZEB1, with increased re-activation following combined knockdown of ZEB1 and BRG1 (Figure 4-13). Individual knockdown of BRG1 did not alter expression of E-cadherin (Figure 4-18b). It appears as though the repression mediated by BRG1 functions via ZEB1, enhancing the ZEB1-mediated suppression of E-cadherin.

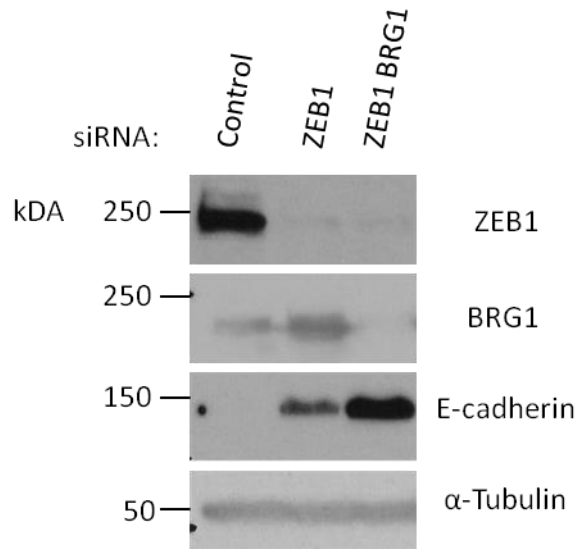


Figure 4-13: Combined ZEB1 and BRG1 repression enhances E-cadherin re-activation

A375M cells were transfected with the various siRNAs and incubated for 96 hours. Protein samples were collected and analysed by Western blotting. Knockdown of ZEB1 re-activated E-cadherin expression, which was enhanced when combined with knockdown of BRG1. Representative images are presented (n=2). α -Tubulin was used as a protein loading control.

Due to the strength of the previously published data linking CtBP and E-cadherin and the current identification that BRG1 repression re-activates E-cadherin in a ZEB1-dependent manner, these transcriptional co-repressors were examined in an E-cadherin promoter reporter assay. This was achieved by co-transfection of the wildtype E-cadherin promoter reporter and siRNA against ZEB1, BRG1 and CtBP in the A375M cell line (Figure 4-14). ZEB1 ($p = 0.0001$, $q = 22.24$), BRG1 ($p = 0.0001$, $q = 11.49$) and CtBP ($p = 0.0001$, $q = 19.30$) all significantly suppressed expression from the E-cadherin promoter in the A375M cell line, with siRNA-mediated repression resulting in transcriptional activation. Repression of ZEB1 resulted in the most significant activation of promoter activity, but an additive effect was observed with combined ZEB1 and CtBP repression ($p = 0.0001$, $q = 28.18$).

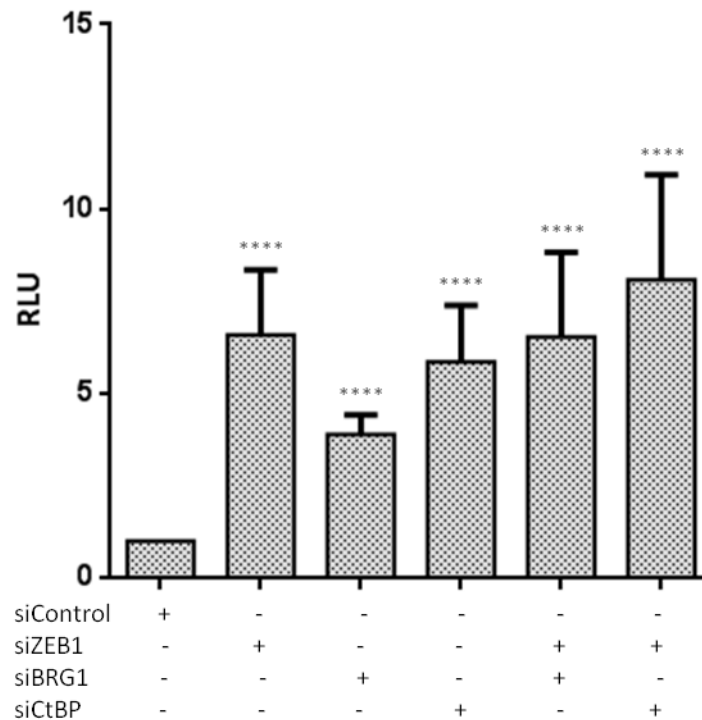


Figure 4-14: BRG1 and CtBP repress the E-cadherin promoter

A375M cells were cotransfected with 1 μ g of pGL3 E-cadherin wildtype promoter reporter, with either 50 nM of control siRNA, siRNA ZEB1, siRNA BRG1 or siRNA CtBP. To normalise transfection efficiency, 1 μ g of pCMV- β -gal was co-transfected with the E-cadherin reporter and appropriate siRNA. Relative luminescent units (RLU) are expressed as the mean of duplicate readings, with each condition replicated in three independent experiments, with the SEM presented for each sample. Both BRG1 and CtBP appear to function as co-repressors of the E-cadherin promoter. Significance was tested via a two-way ANOVA, with test samples compared to the control sample transfected with the siCtrl (**** $p \leq 0.0001$).

The ability of these co-repressors to interact with ZEB1 and ZEB2 was also examined. Due to difficulties with expression of ZEB2 following transient transfection, the A431-ZEB2 cell line was used, which has high ZEB2 expression following doxycycline induction. Additionally, ZEB1 is highly expressed within this cell line following transient transfection. Co-transfection with FLAG-BRG1, FLAG-CtBP or an empty FLAG vector was performed. Following immunoprecipitation with an anti-FLAG antibody, CtBP was shown to strongly interact with both ZEB1 and ZEB2 (Figure 4-15). However, interaction between BRG1 and ZEB1 or ZEB2 was not identified.

Interestingly though, ZEB2 was also shown to interact with CtBP in UACC-257 cells, a cell line that co-expresses ZEB2 and E-cadherin, indicating that the ZEB2-CtBP complex does not function to repress E-cadherin (Figure 4-16).

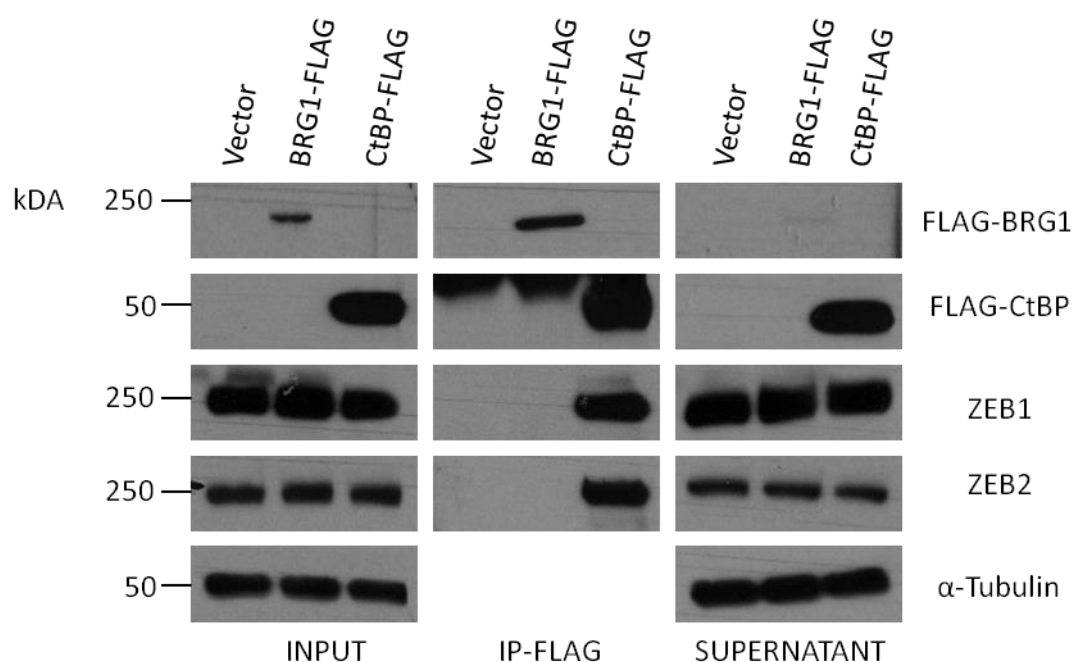


Figure 4-15: ZEB1 and ZEB2 interact with CtBP but not BRG1

A431-ZEB2 cells were induced to express ZEB2 and co-transfected with 4 μ g pBI_ZEB1_HA, 1 μ g of pUHD172.1 neo vectors and either 2 μ g of FLAG-BRG1, 2 μ g of FLAG-CtBP or 2 μ g of empty FLAG vector control and treated with 2 μ g/ml of doxycycline. After 48 hours, cell lysates were processed for immunoprecipitation with an anti-FLAG antibody. Protein pull-down was detected by Western blot. ZEB1 and ZEB2 were shown to interact with CtBP, with no interaction detected with BRG1. α -Tubulin was used as a protein loading control for the input and supernatant samples.

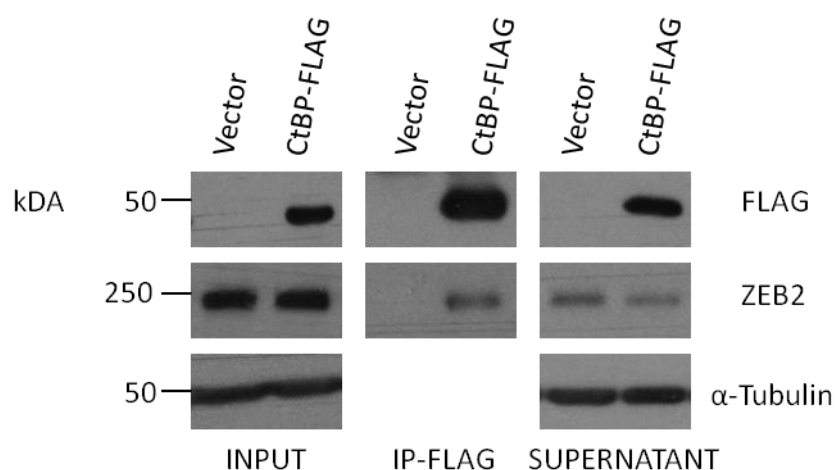


Figure 4-16: ZEB2 interacts with CtBP in an E-cadherin positive melanoma cell line

UACC-257 cells were transfected with 2 μ g of FLAG-CtBP or 2 μ g of an empty FLAG control vector. After 48 hours, lysed cells were processed for immunoprecipitation with an anti-FLAG antibody. Protein pull-down was detected by Western blot. ZEB2 and CtBP were shown to interact in an E-cadherin-positive cell line. α -Tubulin was used as a protein loading control for the input and supernatant samples.

4.3.3.2 Mi-2 β

An interaction with Mi-2 β was previously shown to occur with only one member of the ZEB family, ZEB2 (Verstappen *et al.*, 2008). However, ZEB1 also contains a Mi-2 β binding motif within the N-terminal region (Figure 4-17).

Hs ZEB1, NM_030751	MADGPRCKRRKQANPRRNNVTNYNTVVETNSDSDDEDKLHIVE
Hs ZEB2, NM_001171653	MKQPIMADGPRCKRRKQANPRRKNNVNYDNNVDTGSETDEEDK

Figure 4-17: ZEB1 and ZEB2 proteins contain Mi-2 β binding motifs
The Mi-2 β binding motif (highlighted in yellow) is located within the N-terminal regions of both ZEB1 and ZEB2 (Verstappen *et al.*, 2008).

The ability of ZEB2 and Mi-2 β to regulate E-cadherin expression was tested in WM266-4 cells (Figure 4-18a), which have relatively high expression of ZEB2 and low expression of ZEB1. However, E-cadherin expression was not up-regulated at the protein level following single knockdown of Mi-2 β or knockdown combined with ZEB2. In comparison and as previously mentioned, combined knockdown of ZEB1 and ZEB2 enhanced E-cadherin re-activation, whilst knockdown of ZEB2, without ZEB1, appeared to repress E-cadherin expression.

Likewise, in A375M cells, knockdown of Mi-2 β alone or in combination with ZEB1 depletion demonstrated no effect on E-cadherin (Figure 4-18b). These results suggest that Mi-2 β does not function as a transcriptional co-repressor of E-cadherin, at least in melanoma cell lines. The efficiency of the two siRNAs targeting Mi-2 β was demonstrated at the mRNA level in A375M cells (Figure 4-18c). The interaction of ZEB1 and/or ZEB2 with Mi-2 β was not tested.

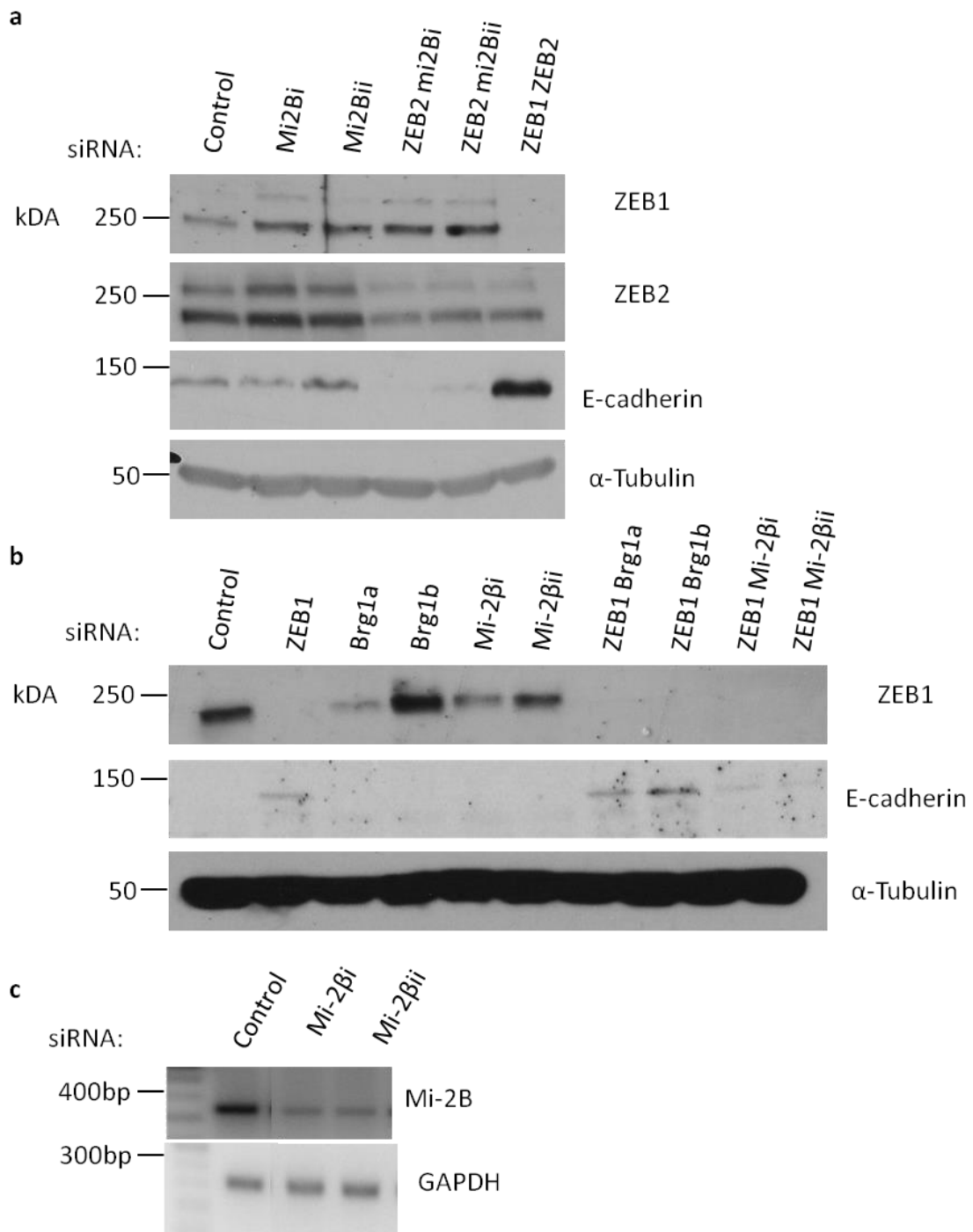


Figure 4-18: Mi-2 β inhibition does not re-activate E-cadherin expression in melanoma cell lines
a) WM266-4 cells and b) A375M cells were transfected with the various siRNAs and incubated for 96 hours. Protein samples were analysed by Western blotting. Mi-2 β did not influence E-cadherin re-expression in either cell line and did not appear to function in conjunction with ZEB2 or ZEB1. α -Tubulin was used as a protein loading control. c) The efficiency of the Mi-2 β siRNA was tested in A375M cells. GAPDH was used as a control for mRNA input.

4.3.4 EPIGENETIC REGULATION OF E-CADHERIN IN MELANOMA CELLS

4.3.4.1 OPTIMISATION OF DNA METHYLATION DETECTION TECHNIQUES

To optimise the DNA methylation detection techniques used, two control cell lines were used. HL-60 is an E-cadherin negative human promyelocytic leukemia cell line, which has a fully methylated E-cadherin promoter. In contrast, A431 is a squamous epidermoid carcinoma cell line, which expresses E-cadherin and possesses an unmethylated *CDH1* promoter. These two cell lines were used to optimise protocols for the detection of DNA methylation using both methylation specific PCR (MSP) and bisulphite specific PCR (BSP) combined with DNA sequencing. Both MSP and BSP require the input DNA to be sodium bisulphite treated, which converts unmethylated cytosines into thymine, whilst retaining methylated cytosines unaltered.

MSP requires primers to specifically amplify sodium bisulphite converted DNA and to selectively amplify either methylated or unmethylated DNA. The primer sets used (Graff *et al.*, 1997) target the E-cadherin proximal promoter around the transcriptional start site (Figure 4-19). Three regions were targeted, designated E-cad CpG island 1, E-cad CpG island 2 and E-cad CpG island 3, each region covered by a methylated and unmethylated primer set. Methylated and unmethylated primers from the same region normally target comparable CpG sites but occasionally due to difficulties with the optimisation of primer length and/or annealing temperatures, different CpG sites are detected by the primers (Table 4-1). The major CpG site discrepancy occurs within the reverse primers targeting CpG island 1, with the methylated primer annealing to CpG site 10, whilst the unmethylated primer anneals to CpG site 8 and 9.

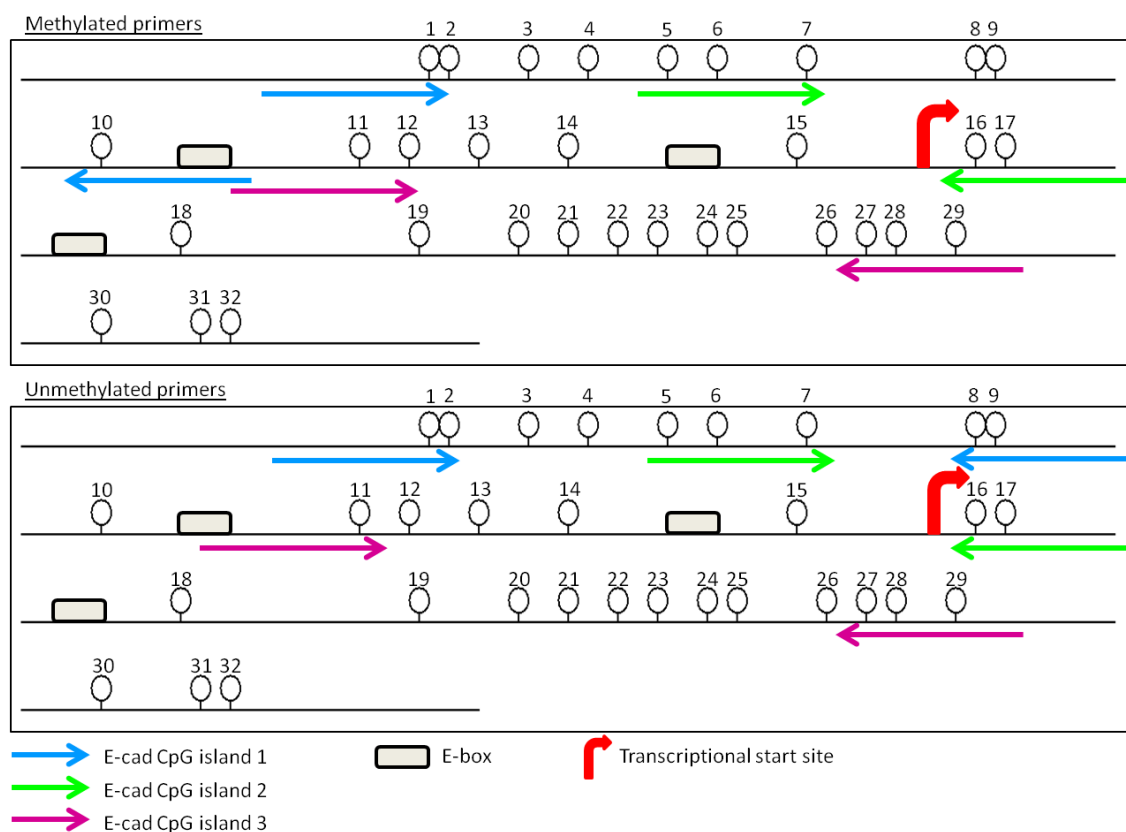


Figure 4-19: MSP primers for the E-cadherin promoter

CpG sites represented in this diagram by lollipops will be referred to throughout the text, commencing with CpG site 1 at the 5' end and terminating with CpG site 32 at the 3' end. Lollipop positioning is reflective of the distance between neighbouring CpG sites. Note the transcriptional start site present upstream of CpG site 16. Forward and reverse methylated and unmethylated primers are represented, positioned according to the CpG sites targeted. The lollipop diagram was generated using BiQ Analyzer software (Bock *et al.*, 2005).

Table 4-1: MSP primers for the E-cadherin promoter

The CpG sites targeted by the methylated and unmethylated MSP primers are presented, with the resulting amplicon size generated following MSP. The CpG sites correspond to the positions in Figure 4-19.

		CpG sites detected		
		Forward Primer	Reverse Primer	Size of amplicon
E-cad CpG island 1	Methylated	1, 2	10	116
	Unmethylated	1, 2	8, 9	96
E-cad CpG island 2	Methylated	5,6,7	16, 17	172
	Unmethylated	5, 6, 7	16, 17	174
E-cad CpG island 3	Methylated	11, 12	27, 28, 29	204
	Unmethylated	11	27, 28, 29	211

Sodium bisulphite converted DNA from HL-60 and A431 cells was amplified by MSP using the methylated and unmethylated primer sets and the resulting amplicons visualised (Figure 4-20). DNA from HL-60 cells was efficiently amplified only by the methylated primers, whilst DNA from A431 cells was amplified by the unmethylated primer sets. This confirmed that the *CDH1* promoter is methylated in HL-60 cells and unmethylated in A431 cells. A faint band was detected with the methylated primers for E-cad island 1 in DNA from A431 cells, suggesting a low level of DNA methylation. These results confirm that the sodium bisulphite conversion, MSP primers and PCR conditions used were optimal to identify and distinguish methylated and unmethylated DNA.

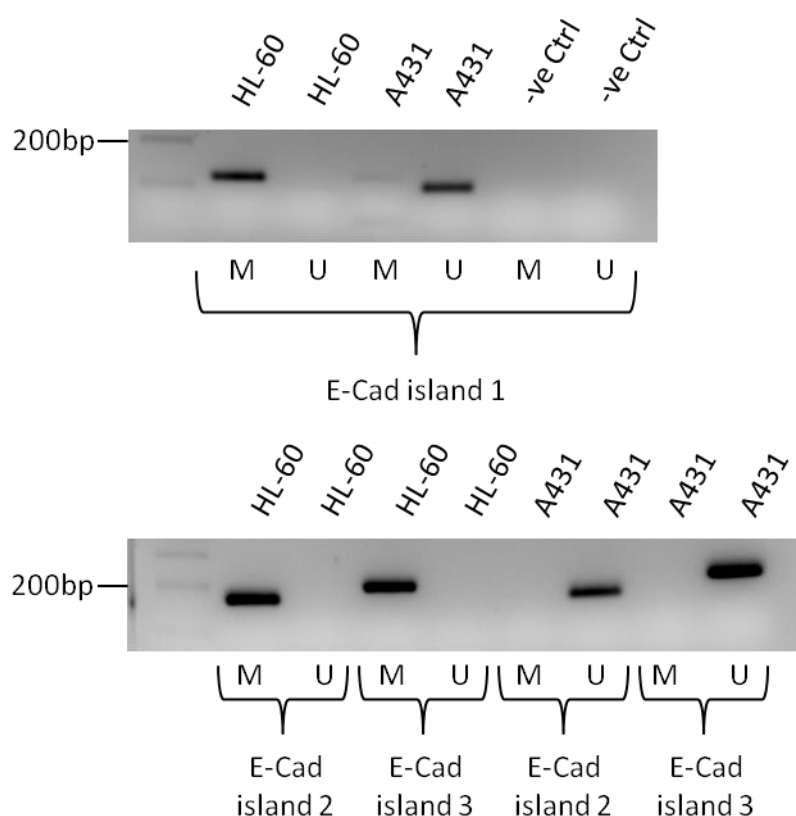


Figure 4-20: Optimisation of MSP in HL-60 and A431 cell lines

DNA from HL-60 and A431 cell lines was extracted, sodium bisulphite converted and amplified using the methylated and unmethylated versions of the E-cad island 1, 2 and 3 primers. Detection of DNA methylation within these regions confirmed the methylated nature of the *CDH1* promoter in HL-60 cells and the unmethylated promoter in A431 cells. Representative images are presented (n=2).

MSP is a rapid technique to identify the overall level of DNA methylation present within a small region of DNA. However, in order to determine the degree of DNA methylation at each CpG site, direct DNA sequencing is required. BSP in combination with DNA sequencing is considered the gold standard in DNA methylation detection techniques. BSP requires the design of primers that target bisulphite converted DNA that anneal outside the CpG island, with no CpG sites present within the primers. This ensures that the primers efficiently amplify both methylated and unmethylated DNA, preventing a bias in the detection of either form. The DNA primers used in BSP for the E-cadherin promoter anneal upstream of CpG site 1 and downstream of CpG site 29 (Figure 4-21). These forward and reverse primers produce an amplicon of 333 bp in length, which once purified, can be cloned, with clones selected for sequencing, resulting in the sequencing of a homogeneous DNA population. Alternatively, the total amplicon product can be directly sequenced using the reverse MSP primer, resulting in a heterogeneous DNA population, meaning that the relative proportion of methylated and unmethylated DNA can be visualised. Within this project, the total amplicon product was sequenced.

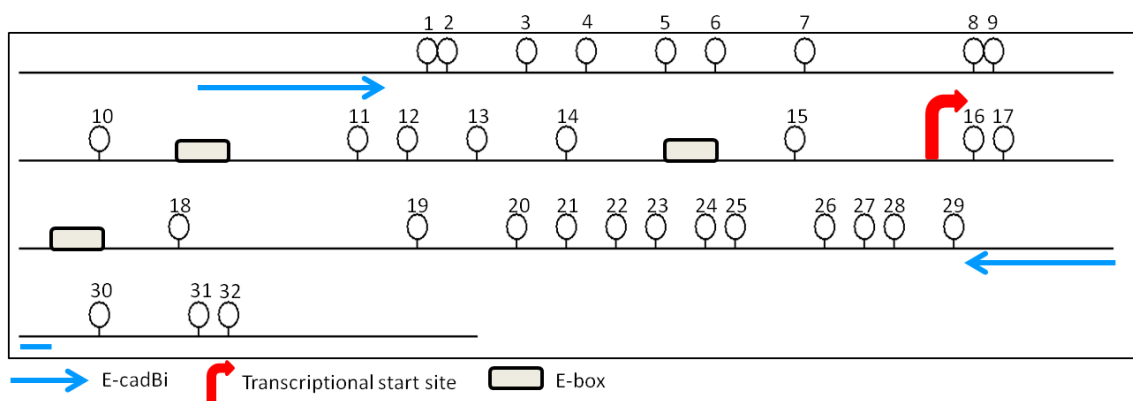


Figure 4-21: BSP primers

Primers for BSP are specific for bisulphite converted DNA, yet target regions outside of the CpG island, containing no CpG sites within either forward or reverse primers. This ensures unbiased amplification of both methylated and unmethylated DNA. This diagram depicts the E-cadBi primers used to amplify sodium bisulphite converted DNA prior to direct sequencing. This primer set amplifies a region containing CpG sites 1 to 29. The lollipop diagram was generated using BiQ Analyzer software (Bock *et al.*, 2005).

The BSP primer sequences and PCR conditions were optimised to ensure the amplification of a single product at the expected size. DNA from HL-60 and A431 cells was efficiently amplified, with ample PCR product produced for direct DNA sequencing (Figure 4-23). The resulting electropherograms enabled single CpG site resolution for the proximal *CDH1* promoter (Figure 4-24). The *CDH1* promoter from the HL-60 cell line was confirmed as fully methylated, with each CpG site retaining a cytosine residue following sodium bisulphite conversion. In comparison, the *CDH1* promoter from A431 cells was fully unmethylated, with thymine residues identified at the CpG sites. Both HL-60 and A431 cells were a homogenous population of cells regarding the DNA methylation status of the E-cadherin promoter. These results validated the sodium bisulphite conversion, BSP and sequencing methods used to study E-cadherin promoter DNA methylation.

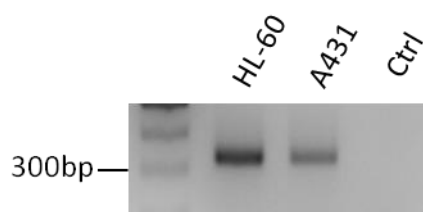


Figure 4-22: Optimisation of BSP amplicon

DNA from HL-60 and A431 cells was sodium bisulphite treated and amplified using the BSP E-cadBi primers. The resulting amplicon was visualised on a 1% TAE gel. A representative image is presented (n=2).

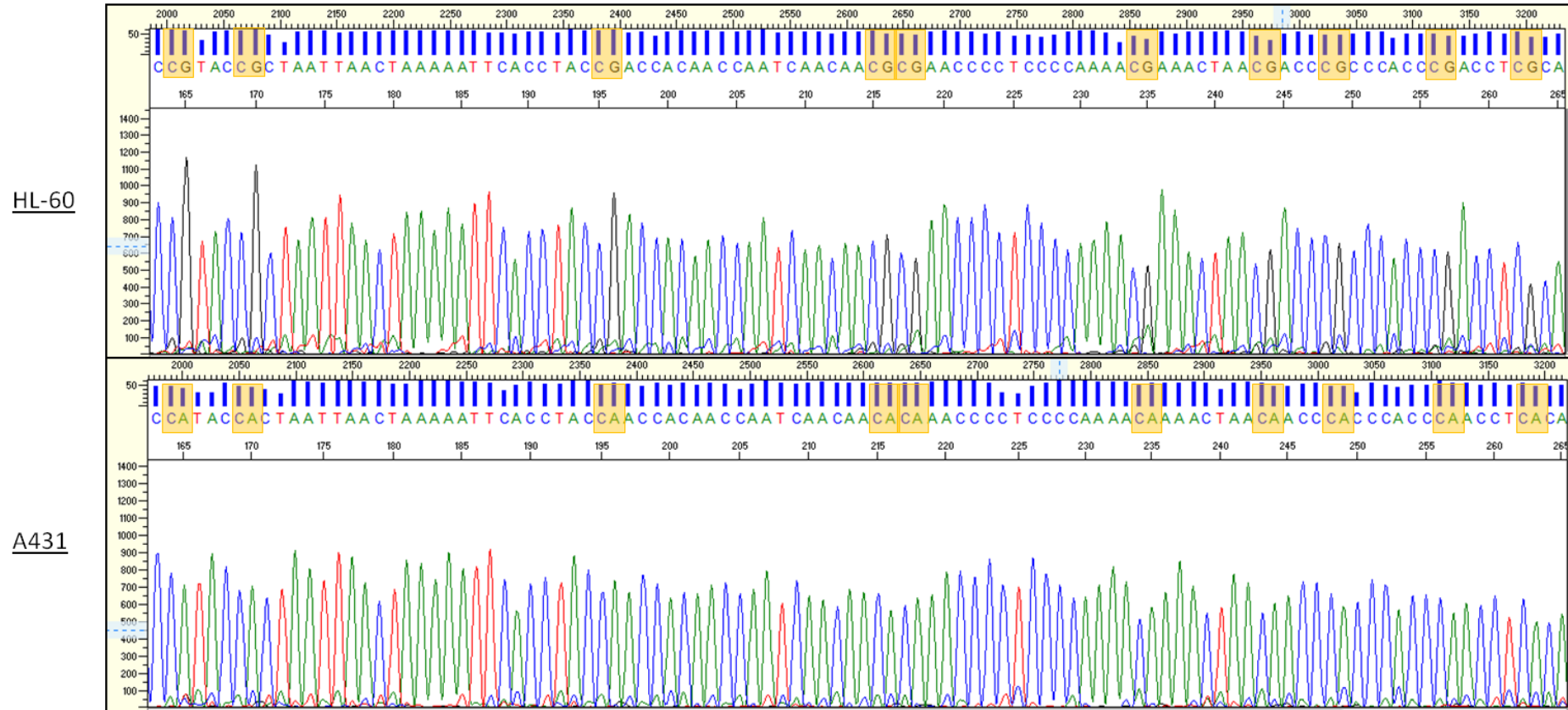


Figure 4-23: DNA methylation in HL-60 and A431 cells

DNA was extracted, sodium bisulphite converted, amplified with E-cadBi sodium bisulphite specific primers and the resulting total amplicon population sent for DNA sequencing. Due to the use of the reverse primer for sequencing, the 5' end of the DNA sequence presented within this image commences with CpG site 12 and the 3' end terminates with CpG site 3. DNA populations from HL-60 and A431 cells are remarkably homogeneous with regard to the methylation status of the E-cadherin promoter, with HL-60 cells possessing a fully methylated *CDH1* promoter, whilst this promoter is completely unmethylated in A431 cells. A representative section of each electropherogram is presented (n=2).

4.3.4.2 DNA methylation and ZEB proteins

Initial DNA methylation studies indicated that expression of ZEB2 in the inducible A431-ZEB2 cell system resulted in increased DNA methylation at the *CDH1* promoter, triggering the repression of E-cadherin. As previously stated, the A431-ZEB2 cell line is a model of EMT, whereby induction of ZEB2 represses E-cadherin (Figure 4-6). A kinetics study of ZEB2 induction was performed and DNA extracted and sodium bisulphite converted. The DNA was subsequently amplified with bisulphite-specific DNA primers, targeted at the *CDH1* promoter. The resulting amplicons were analysed by methylation-specific single strand conformational analysis (MS-SSCA) (Figure 4-24). This is a highly sensitive technique that can distinguish between DNA molecules of the same size due to differences in nucleotide sequence (Bianco *et al.*, 1999). Upon induction of ZEB2, there was an increase in the proportion of methylated allele at the E-cadherin promoter, identified due to a shifted DNA band. This was most pronounced after 96 hours of ZEB2 expression, suggesting that ZEB2 expression triggered *de novo* DNA methylation at the E-cadherin promoter, resulting in transcriptional repression and down-regulation of E-cadherin.

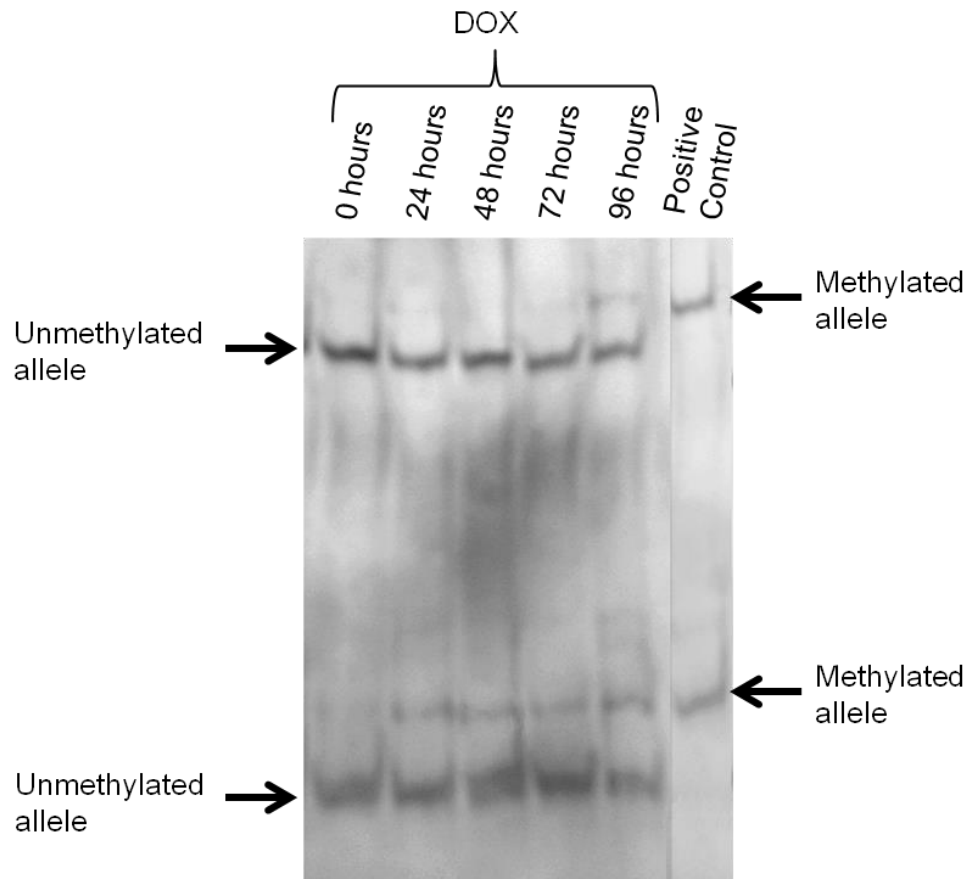


Figure 4-24: ZEB2 induces DNA methylation in A431-ZEB2 cell line

A431-ZEB2 cells were treated with 2 $\mu\text{g/ml}$ doxycycline over the designated time course, inducing the expression of ZEB2. DNA was extracted, sodium bisulphite converted and amplified with E-cadBi primers. The resulting amplicon was analysed by methylation-sensitive single strand conformational analysis (MS-SSCA) and the gel silver stained for visualisation. Following induction of ZEB2 in A431-ZEB2 cells, with the resulting repression of E-cadherin, there was an increase in the amount of methylated DNA at the *CDH1* promoter. Data provided by Dr J Howard Pringle and Dr E Tulchinsky.

Following MS-SSCA, the resulting DNA bands were excised and the DNA purified. The DNA products were sequenced, with the upper shifted band confirmed as originating from methylated DNA, with cytosine residues present at CpG sites. In comparison, DNA from the lower band contained thymine residues at the CpG sites, identifying the DNA as unmethylated (Figure 4-25).

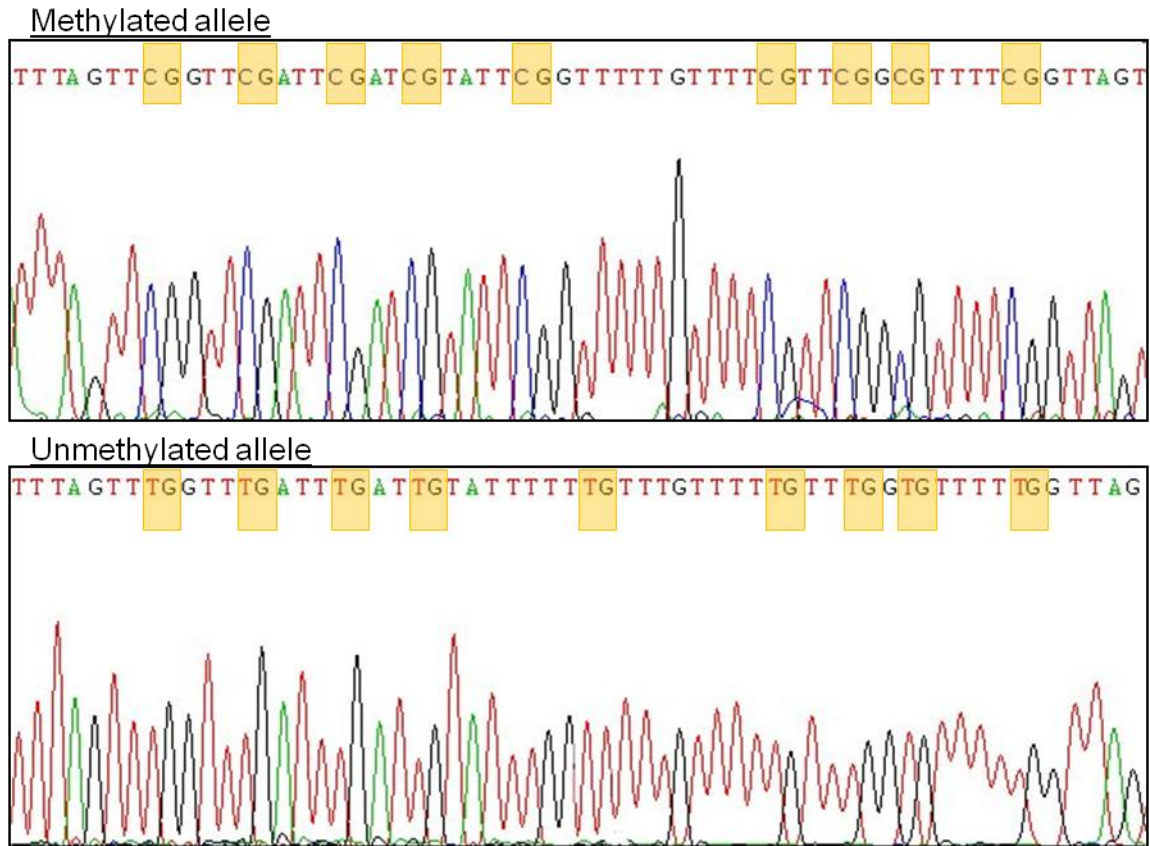


Figure 4-25: ZEB2 induces DNA methylation

Following MS-SSCA (Figure 4-24), the DNA band was excised, purified and the resulting DNA sent for sequencing. The excised methylated band was confirmed to consist of methylated DNA, whereas as the unmethylated band consists of only unmethylated DNA. Data provided by Dr J Howard Pringle and Dr E Tulchinsky.

The results were subsequently repeated in the A431-ZEB2 cell line, with ZEB2 induction for 96 hours, with the resultant EMT, and DNA extracted and processed for BSP and DNA sequencing (Figure 4-26). Control DNA was shown to be unmethylated at the E-cadherin promoter, consistent with the epithelial phenotype of the cell line and the high expression of E-cadherin. However, following expression of ZEB2, no detected increase in DNA methylation was observed, in direct contradiction to the previously described preliminary data. This suggests that ZEB2 does not mediate E-cadherin repression by the induction of DNA methylation.

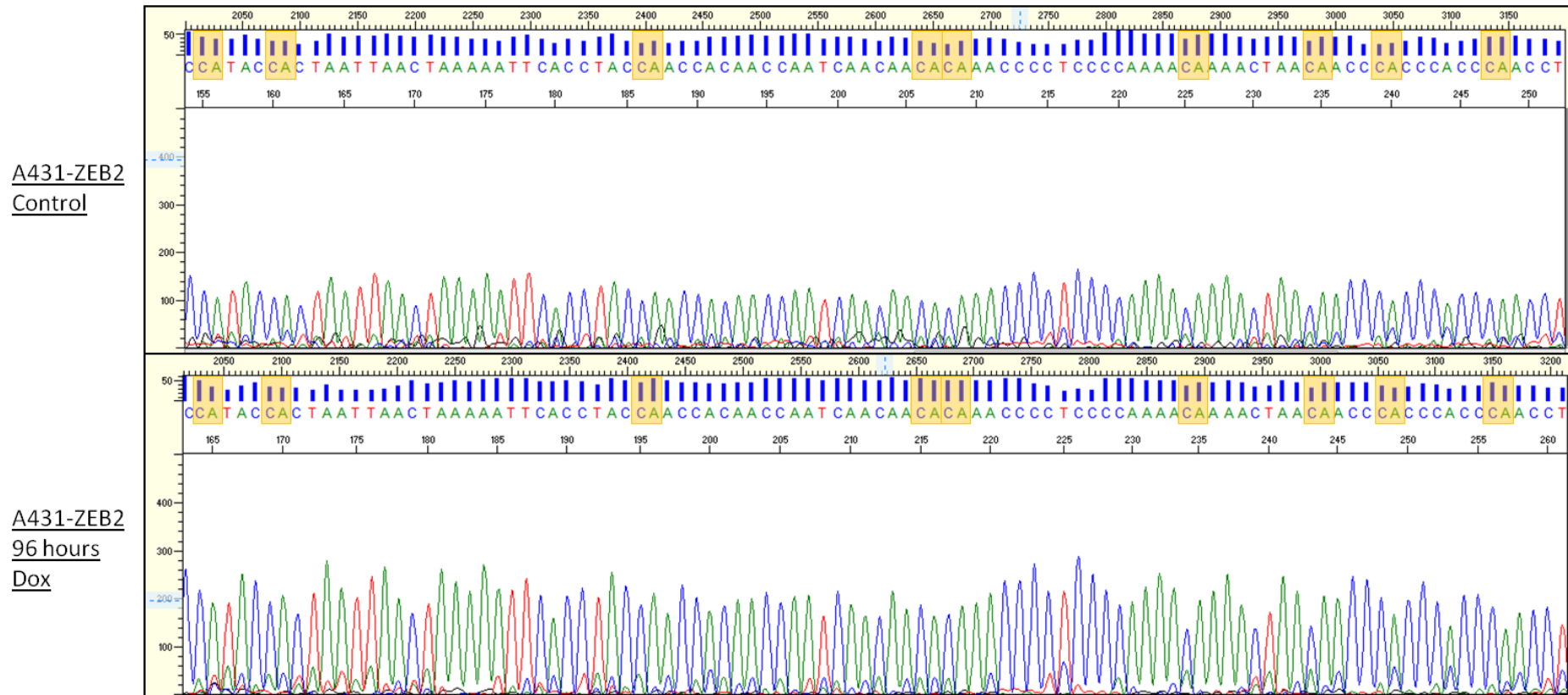


Figure 4-26: ZEB2 expression does not change E-cadherin promoter-based DNA methylation

A431-ZEB2 cells were treated with 2 µg/ml of doxycycline to induce expression of ZEB2 for 96 hours. DNA was extracted, sodium bisulphite converted, amplified with E-cadBi primers and the resulting total amplicon sequenced. Due to the use of the reverse primer for sequencing, the 5' end of the DNA sequence presented within this image commences with CpG site 12 and the 3' end terminates with CpG site 3. Following expression of ZEB2, E-cadherin expression was repressed. However, no additional DNA methylation was detected at the E-cadherin promoter, indicating that ZEB2 does not mediate transcriptional control via induction of *de novo* DNA methylation. A representative section of each electropherogram is presented (n=2).

To further test the role of ZEB proteins in the induction of DNA methylation, the MDA-MB-231 cell line was used, which was previously shown to re-activate E-cadherin expression following repression of the ZEB1 protein. Additionally, the MDA-MB-231 cell line has been extensively used to investigate DNA methylation at the E-cadherin promoter (Graff *et al.*, 1998; Graff *et al.*, 2000).

Following repression of ZEB1 for 96 hours and having confirmed re-expression of E-cadherin, DNA was analysed by MSP using the three methylated and unmethylated primer sets (Table 4-1). MDA-MB-231 cells were heterogeneous for DNA methylation at the *CDH1* promoter, containing both methylated and unmethylated alleles at each position targeted (Figure 4-27). The first region analysed, E-cad CpG island 1, had a higher proportion of DNA methylation compared to the subsequent regions. However, when DNA methylation in control cells and cells with ZEB1 knockdown was compared, there was no difference in the proportion of methylated to unmethylated alleles for island 1 and 3 and only a marginal decrease in the methylated allele following ZEB1 knockdown in island 2. This indicates that no substantial alteration in DNA methylation at the *CDH1* proximal promoter occurs following repression of ZEB1.

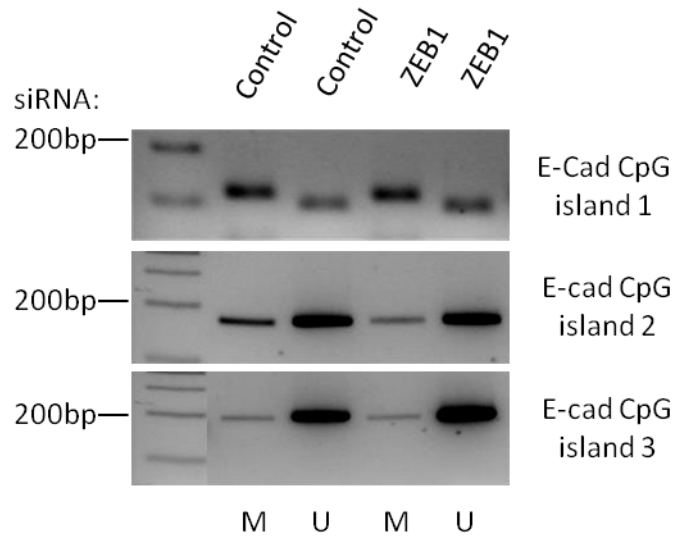


Figure 4-27: ZEB1 repression does not induce DNA demethylation in a carcinoma cell line
MDA-MB-231 cells were transfected with the various siRNAs and incubated for 96 hours. After 48 hours, cells were split so that DNA and protein samples could be collected. Protein was analysed by Western blotting to check for repression of ZEB1 and re-expression of E-cadherin. Subsequently, DNA was extracted, sodium bisulphite converted and amplified with the MSP primers. The resulting amplicons were visualised on a 2% TAE gel. The proportion of methylated to unmethylated alleles at the E-cadherin promoter does not considerably alter following down-regulation of ZEB1 and up-regulation of E-cadherin. E-cadherin re-expression was not dependent upon alterations in DNA methylation. Representative images are presented (n=2).

DNA was also amplified using the BSP primers and DNA sequenced (Figure 4-28). Interestingly, minimal amounts of DNA methylated were detected. The major CpG site to be methylated was CpG site 10, which would have been detected by only the methylated versions of the E-cad CpG island 1 MSP reverse primer, with the unmethylated primer targeting CpG sites 8 and 9. This explains the high proportion of DNA methylation detected within this region. However, the level of DNA methylation did not alter following repression of ZEB1, with comparable cytosine peaks at CpG site 10. This indicates that the overall level of E-cadherin promoter DNA methylation is low and that re-expression of E-cadherin in MDA-MB-231 cells mediated by repression of ZEB1 is not controlled by changes in DNA methylation.

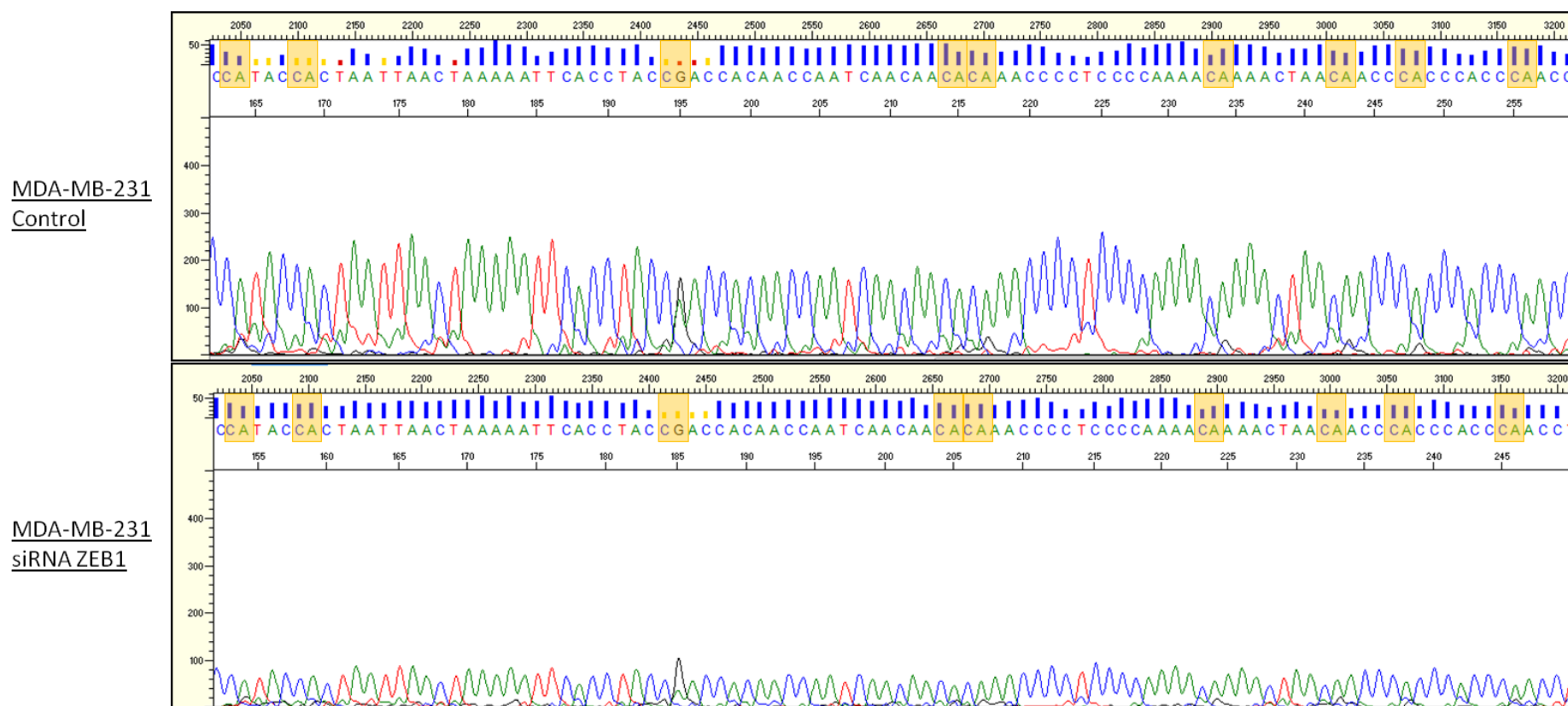


Figure 4-28: Knockdown of ZEB1 does not alter the E-cadherin promoter-based DNA methylation

MDA-MB-231 cells were transfected with the various siRNAs for 96 hours. DNA was extracted, sodium bisulphite converted, amplified with E-cadBi sodium bisulphite specific primers and the resulting total amplicon population sent for DNA sequencing. Due to the use of the reverse primer for sequencing, the 5' end of the DNA sequence presented within this image commences with CpG site 12 and the 3' end terminates with CpG site 3. Following repression of ZEB1 in MDA-MB-231 cells, E-cadherin expression was re-activated. However, when the DNA methylation at the E-cadherin promoter was analysed, no difference was evident between the control cells and cells with ZEB1 repression. This indicates that re-expression of E-cadherin following loss of ZEB1 does not occur due to removal of DNA methylation. A representative section of each electropherogram is presented (n=2).

The MSP and BSP results in A431-ZEB2 and MDA-MB-231 cells did not confirm the preliminary data obtained by MS-SSCA with the A431-ZEB2 cell line, whereby expression of ZEB2 increased the level of DNA methylation at the *CDH1* promoter. DNA methylation is a highly dynamic and changeable feature, with MS-SSCA potentially identifying a minor clone of the A431-ZEB2 cell line that developed increased levels of DNA methylation. The results presented indicate that ZEB proteins do not influence the presence or absence of DNA methylation at the *CDH1* promoter.

4.3.4.3 DNA METHYLATION AND E-CADHERIN REPRESSION IN METASTATIC MELANOMA

In an alternative direction, the role of DNA methylation was investigated in melanoma cell lines, specifically in A375P and A375M cell lines, to determine whether DNA methylation is involved in regulating E-cadherin expression. As previously shown, E-cadherin is more dynamically regulated in A375M cells when compared to A375P cells (Figure 4-3), with enhanced E-cadherin re-expression in A375M cells following inhibition of ZEB1 and combined ZEB1 and ZEB2. Following sodium bisulphite conversion of DNA from A375P and A375M cells, the *CDH1* promoter was analysed to determine the level of DNA methylation by MSP (Figure 4-29) and by BSP, followed by DNA sequencing (Figure 4-30).

When the level of DNA methylation in A375P and A375M cells was compared at the *CDH1* promoter by MSP (Figure 4-29), DNA from A375M cells contained a lower proportion of methylated compared to unmethylated DNA. This was especially evident at E-cad CpG island 3, where no methylated band was present for the A375M sample. In comparison, the DNA from A375P cells was successfully amplified by both the methylated and unmethylated primer sets. Additionally, at CpG island 1, A375M DNA contained lower levels of DNA methylation compared to A375P DNA. Interestingly, there was minimal amplification at E-cad CpG island 2 for A375M DNA. This potentially indicates that the CpG sites targeted by the primers are differentially methylated, unable to be amplified by either primer set.

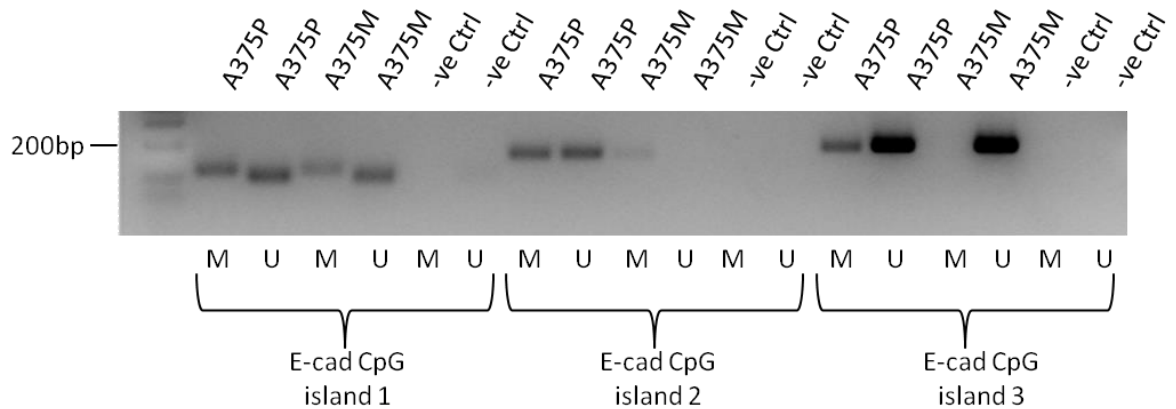


Figure 4-29: Reduced levels of E-cadherin promoter-based DNA methylation in A375M cells compared to A375P cells

DNA was extracted, sodium bisulphite treated and amplified using the MSP primers. The *CDH1* promoter from A375P cells contains higher levels of DNA methylation when compared to A375M cells. The –ve Ctrl was a PCR negative used to check for DNA contamination. A representative image is presented (n=2).

In order to determine the methylation status of individual CpG sites, BSP was also performed and the resulting amplicon sequenced (Figure 4-30). Sequencing confirmed that the *CDH1* promoter of A375P cells contains a greater amount of DNA methylation when compared to the promoter from A375M cells. The electropherogram shown includes CpG sites 12 to 3, with a higher ratio of cytosine to thymine residues identified in the A375P DNA sample when compared to the A375M sample. CpG sites 8 and 9 remain prominently methylated within A375M cells even though the remaining promoter is predominantly unmethylated. A lollipop diagram (Figure 4-31) representing CpG sites 1-18 compares the overall methylation detected at the *CDH1* proximal promoter in A375P and A375M cells. Filled circles represent a methylated cytosine, with clear circles representing an unmethylated cytosine. The level of DNA methylation was much higher at the A375P *CDH1* promoter. Interestingly, DNA from both A375P and A375M cells was unmethylated at the cytosine residues immediately adjacent to the transcriptional start site, which occurs between CpG sites 15 and 16.

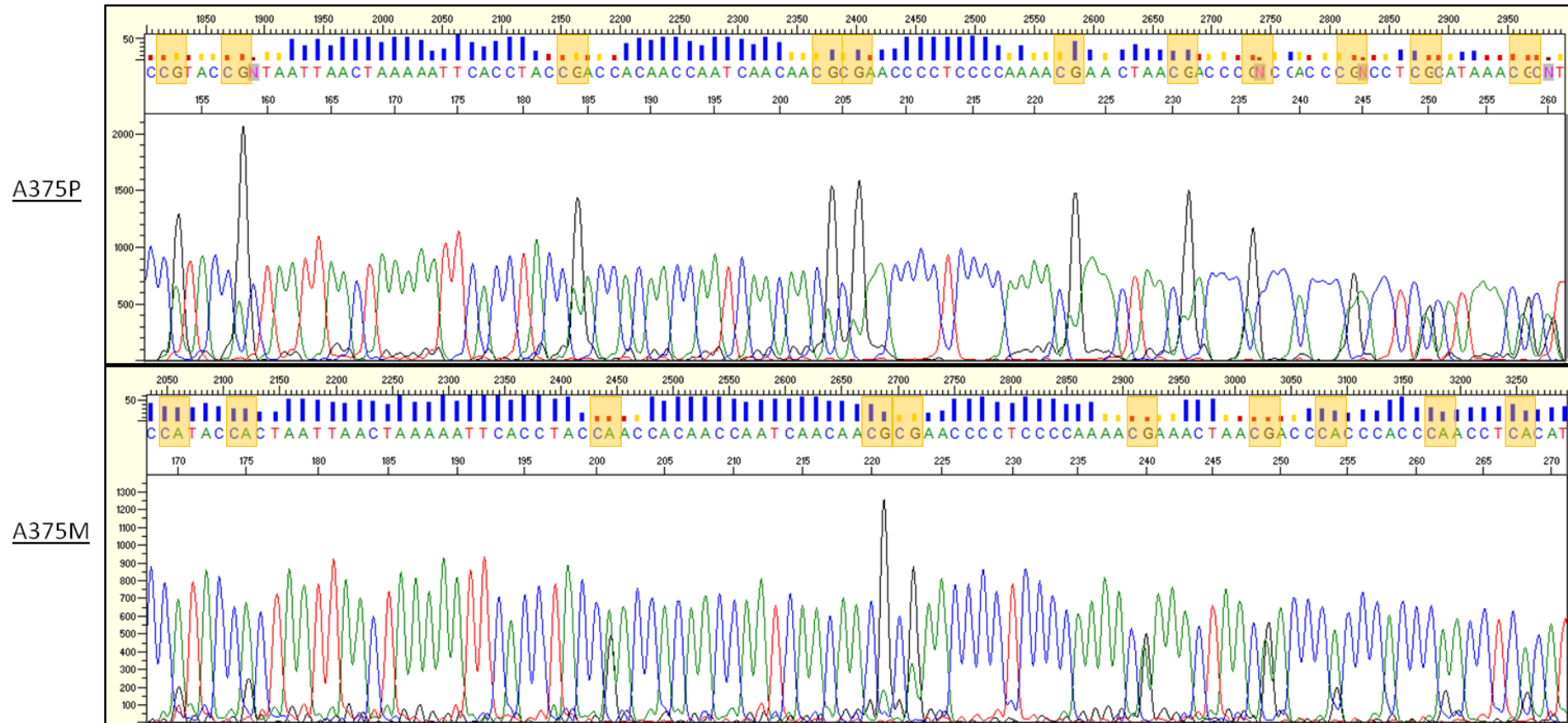


Figure 4-30: Higher levels of E-cadherin promoter-based DNA methylation in A375P cells compared to A375M cells

DNA from untreated A375P and A375M cells was extracted, sodium bisulphite converted, amplified with E-cadBi sodium bisulphite specific primers and the resulting total amplicon population sent for DNA sequencing. Due to the use of the reverse primer for sequencing, the 5' end of the DNA sequence presented within this image commences with CpG site 12 and the 3' end terminates with CpG site 3. Both A375P and A375M cell lines contain a heterogeneous population, whereby both methylated and unmethylated alleles are present at each CpG site. However, A375P cells have a greater level of DNA methylation throughout the *CDH1* promoter. This can be identified by the presence of stronger black peaks within the A375P cell line profile. A representative section of each electropherogram is presented (n=2).

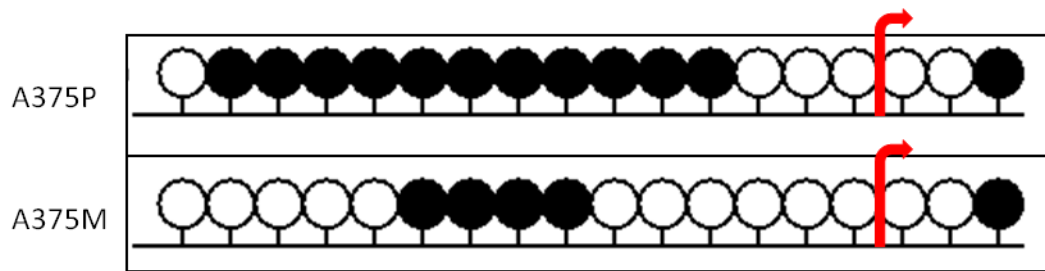


Figure 4-31: E-cadherin promoter-based DNA methylation is more prevalent in A375P cells compared to A375M cells

Within this lollipop diagram, black circles represent methylated CpG sites and white circles represent unmethylated CpG sites. This diagram contains CpG sites 1-18 (5'-3') that were detected following BSP and sequencing using the E-cadBi primers. CpG sites are grouped in a compacted manner for ease of visualisation. Due to the heterogeneous nature of the DNA methylation in A375P and A375M, a CpG site was considered methylated when the methylated peak had a greater height compared to the unmethylated counterpart and vice versa. This diagram shows the higher levels of DNA methylation present at the *CDH1* promoter in A375P compared to A375M cells. The lollipop diagram was generated using BiQ Analyzer software (Bock *et al.*, 2005).

To determine the role of DNA methylation upon E-cadherin re-expression, A375P and A375M cells were treated with the DNA demethylating agent 5-aza-2'-deoxycytidine (5-azaC) for 6 days, in combination with knockdown of ZEB1 and ZEB2 (Figure 4-32). Following treatment with DMSO, knockdown of ZEB1 and ZEB2 resulted in greater E-cadherin re-expression in the A375M cell line compared to A375P. However, following treatment with 5-azaC, re-activation of E-cadherin was comparable following ZEB1 and ZEB2 repression. Interestingly, treatment with 5-azaC and knockdown of ZEB2 did not increase re-activation of E-cadherin above the basal level experienced with only 5-azaC treatment. This indicates that even after the removal of DNA methylation, ZEB2 alone cannot function as a repressor of E-cadherin. These results indicate that the additional repressive factor present within A375P cells is DNA methylation, suggesting that hypermethylation is an important factor in the regulation of melanomagenesis, with the inducibility of E-cadherin correlated with DNA methylation. However, off-target effects of 5-azaC cannot be excluded.

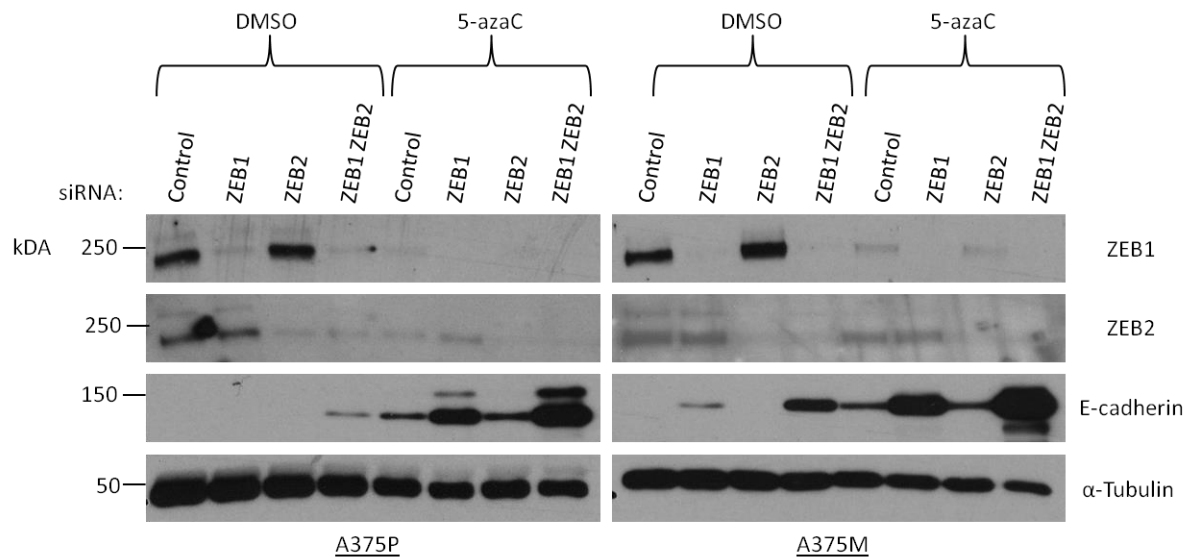


Figure 4-32: DNA demethylation in A375P cells induces E-cadherin re-expression in combination with ZEB1 repression

A375P and A375M cells were incubated with 10 μ M 5-azaC for a total of 6 days, with siRNA transfections occurring with 96 hours remaining. In the DMSO treated controls, only combined ZEB1 and ZEB2 knockdown in A375P resulted in E-cadherin re-expression. However, following application of 5-azaC, single ZEB1 knockdown was sufficient to re-activate E-cadherin. When the double ZEB1 and ZEB2 knockdown were combined with DNA demethylation, the level of E-cadherin re-expression was increased further. Interestingly, DNA demethylation combined with single knockdown of ZEB2 did not re-activate E-cadherin expression beyond that achieved by application of 5-azaC. α -Tubulin was used as a protein loading control. Representative images are presented (n=2).

4.4 DISCUSSION

4.4.1 ZEB1 IS A MAJOR E-CADHERIN REPRESSOR IN MELANOMA CELLS

ZEB1 (Grooteclaes & Frisch, 2000), ZEB2 (Comijn *et al.*, 2001), SNAIL2 (Bolos *et al.*, 2003) and TWIST1 (Yang *et al.*, 2004) have all been shown to function as transcriptional repressors of E-cadherin in various epithelial cell lines. As loss of E-cadherin is also a key feature of melanomagenesis (Tang *et al.*, 1994), the role of the MR-EMT was analysed in relation to the repression of E-cadherin in melanoma cell lines.

Through inhibition of the MR-EMT by RNA interference, the ability to re-activate E-cadherin expression was studied (Figure 4-2 and Figure 4-8). In contrast to results obtained in epithelial cell lines, SNAIL2 and TWIST1 did not appear to have a functional role in the repression of E-cadherin in the E-cadherin-negative melanoma cells A375M, RPMI-7951 and WM266-4. In comparison, E-cadherin re-expression was observed following depletion of ZEB1 in A375M cells, with enhanced re-activation following down-regulation of both ZEB1 and ZEB2. Combined repression of ZEB1 and ZEB2 was required for E-cadherin re-expression in RPMI-7951 and WM266-4 cells, whilst single knockdown of ZEB2 did not induce *de novo* expression of E-cadherin. Interestingly, combined repression of both ZEB proteins was also required for E-cadherin re-expression in the oral squamous cell carcinoma cell line H157, with individual inhibition being ineffective to induce E-cadherin re-activation (Gemmill *et al.*, 2011). The synergistic effect experienced following combined repression of ZEB1 and ZEB2 may be due to ZEB2 playing a compensatory inhibitory role in the absence of ZEB1, the major transcriptional repressor. When ZEB1 is present, ZEB2 is not required for the repression of E-cadherin. However, with down-regulation in ZEB1, ZEB2 is able to function as a repressor of E-cadherin. This compensatory role means that elevated E-cadherin re-expression can be achieved when both ZEB1 and ZEB2 are jointly repressed.

To further investigate the role of ZEB1, a positive approach was employed. After the over-expression of ZEB1 in ZEB2- and E-cadherin-positive UACC-257 cells, E-cadherin

was down-regulated (Figure 4-5). These data suggest that ZEB1 is a predominant repressor of E-cadherin in melanoma cell lines, whilst ZEB2 only has a synergistic role when functioning cooperatively with ZEB1.

The mechanism of E-cadherin repression in melanoma cell lines was also investigated in a *CDH1* promoter reporter-assay (Figure 4-4). E-boxes (CANNTG) within the *CDH1* promoter have been shown to be binding sites for several transcriptional repressors (Liu *et al.*, 2005), including ZEB proteins (Remacle *et al.*, 1999). In the A375M cell line, the *CDH1* promoter was negatively regulated via promoter-based E-boxes, and their mutation resulted in significant transcriptional activation (Figure 4-4). Additionally, the experiments with transient RNA interference have shown that ZEB1, but not ZEB2, had a significant inhibitory effect upon the transcriptional activity of the promoter.

The predicted role of ZEB1 as a transcriptional repressor of E-cadherin appears to be consistent with data obtained in various melanoma cell lines, and involves the binding to E-boxes within the *CDH1* promoter. On the other hand, the involvement of ZEB2 in the regulation of E-cadherin seems to be complex. Though ZEB2 has a strong potential to repress E-cadherin transcription in the epidermoid and colon carcinoma cell lines A431 and DLD-1 (Vandewalle *et al.*, 2005) (Figure 4-6), single knockdown of ZEB2 has no detectable impact upon the level of E-cadherin expression in melanoma cell lines. In contrast, combined depletion of ZEB1 and ZEB2 produced an effect on E-cadherin expression much stronger than that of ZEB1 knockdown alone. Thus, the role of ZEB2 in E-cadherin regulation appears to be fundamentally different between epithelial and melanoma cell lines. Co-expression of ZEB2 and E-cadherin was confirmed to occur uniformly within UACC-257 cells, indicating that ZEB2 does not repress E-cadherin (Figure 4-7). Additionally, and rather intriguingly, ZEB2 appeared to positively regulate E-cadherin expression in the WM266-4 cell line (Figure 4-8), which may be due to the up-regulation of ZEB1 in response to ZEB2 knockdown. Overall, the complexity and divergent roles of the MR-EMT in the regulation of E-cadherin in melanoma cell lines, when compared to epithelial cell systems, may be connected to the hierarchical nature and cross-regulation of the MR-EMT in melanoma cell lines (Chapter 3).

ZEB1 and ZEB2 are structurally similar proteins, both possessing DNA-binding zinc-finger clusters that bind the same DNA sequences, indicating that they may transcriptionally regulate the same set of target genes (Vandewalle *et al.*, 2009). However, ZEB1 and ZEB2 have previously been identified to function in opposing manners in certain situations. For example, differential interaction with transcriptional co-repressors and co-activators can result in ZEB1 and ZEB2 having opposite functions (Postigo *et al.*, 2003). The ability of ZEB1 and ZEB2 to have functionally different roles in transcriptional control may be important in the context of melanocytes and melanoma cell lines. Data suggest that the expression of ZEB1 alters the regulatory function of ZEB2, converting ZEB2 into a transcriptional repressor of E-cadherin. ZEB1 may alter the balance in transcriptional co-activators and co-repressors interacting with ZEB2, which in turn may alter the ability of ZEB2 to interact with and regulate the E-cadherin promoter.

The function of the ZEB proteins is also controlled by post-translational modifications, such as SUMOylation. ZEB2 was shown to be SUMOylated at two sites by the SUMO E3 ligase, Polycomb protein Pc2 (Long *et al.*, 2005). SUMOylation of ZEB2 partially inhibited its ability to repress the E-cadherin promoter, potentially through restricting interactions with CtBP. This indicates that SUMOylation may control conversion of ZEB2 from a transcriptional repressor to an activator. Additionally, prospective SUMOylation sites have been identified in ZEB1 and have been suggested to regulate its activity (Long *et al.*, 2005). Post-translational modifications may provide the ZEB proteins with unique and potentially opposing functions in melanoma cells.

In order to clarify the role of the ZEB proteins in the transcriptional regulation of E-cadherin, it would be advantageous to perform a chromatin immunoprecipitation (ChIP) experiment, whereby interaction of the ZEB1 and ZEB2 proteins with the E-cadherin promoter could be investigated. Analysis could be conducted in E-cadherin-negative melanoma cell lines, E-cadherin and ZEB2-positive melanoma cell lines and E-cadherin-positive epithelial cell lines, in which ZEB2 functionally represses E-cadherin.

This would allow us to determine whether ZEB1 and ZEB2 differentially interact with the E-cadherin promoter within different cellular backgrounds. Additionally, it would be interesting to perform ZEB1 and ZEB2 knockdown experiments within the A375 cell lines and determine whether these alter the ability of the ZEB proteins to interact with the E-cadherin promoter, potentially explaining the enhanced E-cadherin re-activation following combined ZEB protein depletion.

The role of the ZEB proteins in E-cadherin repression was analysed in two A375 cell lines, which represent a model of melanoma metastasis (Figure 4-3). These experiments revealed interesting differences in E-cadherin regulation between these two cell lines, with high levels of *de novo* E-cadherin expression in the metastatic A375M cells and minimal E-cadherin re-activation in the parental A375P cell line. The enhanced capacity for E-cadherin re-expression in the A375M cell line may be an important feature explaining the higher metastatic propensity of these cells. Induction of an MET-like process, with re-expression of E-cadherin, may occur in tumour cells when they interact with cells and soluble factors within the new microenvironment (Yates *et al.*, 2007b). Indeed, re-expression of E-cadherin in colonising tumour cells has been shown to occur in cases of colorectal (Dorudi *et al.*, 1993) and breast carcinomas (Imai *et al.*, 2004; Kowalski *et al.*, 2003).

Phenotypic plasticity does not only relate to the re-expression of E-cadherin in metastatic tumour cells, as the dynamical regulation of the miR-200 family members has also been identified. The presence of a double-negative feedback loop in carcinoma cell lines between the miR-200 family and the ZEB proteins is well established (Hurteau *et al.*, 2006; Christoffersen *et al.*, 2007; Hurteau *et al.*, 2007; Burk *et al.*, 2008; Gregory *et al.*, 2008; Korpál *et al.*, 2008; Park *et al.*, 2008). Repression of the miR-200 family in the primary tumour is advantageous, promoting an EMT by expression of the ZEB proteins, with repression of E-cadherin, and resulting in the formation of migratory and invasive cells. However, re-activation of the miR-200s at the secondary site promotes metastatic colonisation by repression of the ZEB proteins and re-activation of E-cadherin (Korpál *et al.*, 2011). These results indicate that the

expression of miR-200 family can be transiently controlled, allowing for the plastic and dynamic re-expression of E-cadherin when required. Down-regulation of E-cadherin and induction of an EMT-like process at the primary site enhances migration and invasion, ultimately increasing the rate of tumour dissemination. However, at the metastatic site, re-expression of E-cadherin, induced by signals from the local parenchymal cells, may provide a selective advantage, allowing communication with the new microenvironment and enhancing the formation of macro-metastases. The ability to re-activate E-cadherin more efficiently in the A375M cell line may explain the increased rate of metastatic colonisation when compared to A375P cells.

It should be noted that within this study not all E-cadherin repressors have been examined, with additional repressors previously described within malignant melanoma. The T-box transcription factor Tbx3, and potentially Tbx2, have been identified as repressors of E-cadherin in melanoma cell lines, with Tbx3 and E-cadherin inversely correlating in melanoma tissue (Rodriguez *et al.*, 2008). Interestingly, B-RAF^{V600E} induces expression of Tbx3 in melanocytes (Boyd *et al.*, 2012), providing a link between oncogenic B-RAF mutations and repression of E-cadherin. Additionally, expression of GLI2, a transcription factor controlled by the hedgehog signalling pathway, is associated with loss of E-cadherin in melanoma cell lines and human melanoma lesions (Alexaki *et al.*, 2010). Even though the involvement of these additional E-cadherin repressors cannot be excluded, the critical role played by ZEB1 in the transcriptional repression of E-cadherin has been highlighted by the presented data.

Throughout this thesis, small interfering RNA (siRNA)-mediated repression has been extensively employed. Since Elbashir *et al.* in 2001 proved siRNA-based silencing could be achieved in mammalian cells (Elbashir *et al.*, 2001), multiple applications have been developed that utilise the endogenous RNA interference (RNAi) machinery to post-transcriptionally repress gene expression. RNAi utilises short (21-25 nucleotide), double-stranded RNA molecules (siRNAs), which are produced by Dicer, a ribonuclease. These siRNAs are processed by Argonaute, a principle component of the

RNA-induced silencing complex (RISC), which identifies the guide strand of the siRNA, allowing complementary base-pairing with the target mRNA and subsequent specific mRNA degradation. RNAi has become an important means to knockdown target genes in mammalian cells, but also has therapeutic potentials for the treatment of human diseases (Gavrilov & Saltzman, 2012). However, problems associated with the specificity of siRNA targeting have been described (Aagaard & Rossi, 2007). Microarray analysis identified that off-target silencing occurs with the use of siRNA, especially evident in cases of mRNA 3'UTR complementarity to the siRNA seed sequence (Jackson *et al.*, 2003). However, with the use of well-designed and established siRNAs, used at the correct concentration and tested within multiple cell lines, problems associated with siRNA off-target effects can be minimised and overcome.

4.4.2 E-CADHERIN REPRESSION BY ZEB PROTEINS IN MALIGNANT MELANOMA: ROLE OF TRANSCRIPTIONAL CO-REPRESSORS

The ability of CtBP to function as a transcriptional co-repressor of E-cadherin through interaction with the ZEB proteins is well documented (Grooteclaes & Frisch, 2000; Furusawa *et al.*, 1999; Shi *et al.*, 2003; Postigo & Dean, 1999; Grooteclaes *et al.*, 2003). These findings provided the foundation for the analysis of E-cadherin-based transcriptional co-repressors. Additionally, the ZEB proteins have previously been shown to interact differentially with co-repressors and co-activators (Postigo *et al.*, 2003), and it was felt that these opposing interactions may explain the antagonistic function of the ZEB proteins in relation to E-cadherin expression in melanoma cell lines. Moreover, the ATPase chromatin remodelling subunit BRG1 of the SWI/SNF complex and the ATPase chromatin remodelling subunit Mi-2 β of the Mi-2/NuRD complex have been shown to interact with ZEB1 and ZEB2, respectively, contributing to ZEB-mediated repression of E-cadherin (Sanchez-Tillo *et al.*, 2010; Verstappen *et al.*, 2008).

4.4.2.1 TRANSCRIPTIONAL CO-REPRESSORS

Chromatin modifying and remodelling complexes play an intricate role in the regulation of gene expression. Each sub-unit represents only a single component of

much larger, multi-component structures, with several enzymatic activities. Currently, three models have been proposed to explain the interaction of transcriptional co-repressors and co-activators. The classical model involves the direct displacement of co-repressors by co-activators, resulting in a switch from transcriptional repression to activation. The cyclical model describes the continual displacement of co-repressors for co-activators and vice versa. Finally, the combinatorial model envisages both co-repressors and co-activators located together at active, 'primed' or silent promoters, fine-tuning the chromatin structure and histone modifications. The idea of de-repression involves the active removal of co-repressors by co-activators, allowing rapid and dynamic regulation of transcriptional activity (Perissi *et al.*, 2010).

Purification of co-repressor complexes has identified the inter-changeable nature of the components. The NuRD complex is a perfect example, with the Mta subunits, namely Mta1, Mta2 and Mta3, being mutually exclusive of each other and the MBD3 subunit replaced by MBD2. These differences can result in each complex having multiple and potentially opposing functions (McDonel *et al.*, 2009). Within the SWI/SNF complex, such subunit substitutions are also evident for the ATPase subunits, BRG1 and BRM. During osteoblast differentiation, BRG1 may be replaced by BRM, converting the SWI/SNF complex from an activator to a repressor of transcription (Flowers *et al.*, 2009). Additionally, individual components may function as both transcriptional activators and repressors depending upon the presence of specific protein binding partners. Mi-2 β has been shown to contain a C-terminal repressor domain, whilst the N-terminal region functions to activate transcription via interaction with BRG1. Binding between Mi-2 β and BRG1 highlights the potential formation of multi-protein super-complexes (Shimono *et al.*, 2003). The ability to interchange the subunit composition of each repressor complex, and for subunits to have opposing roles, means that transcriptional activity can be finely tuned and dynamically controlled. However, the highly intricate nature of large co-repressor complexes causes difficulties in the analyses and interpretation of the data on the role of individual subunits in transcriptional control.

Post-translational modifications regulate protein-protein interactions in repressor complexes and alter their functional activity. For example, ZEB2 SUMOylation disrupts binding of CtBP, due to the close proximity of the SUMOylation site and CBD (Long *et al.*, 2005). Additionally, CtBP1 is modified by SUMOylation, with SUMOylation being required for its maximal function as a transcriptional co-repressor of E-cadherin in conjunction with ZEB1 (Lin *et al.*, 2003). The presence of post-translational modifications within these co-repressor complexes may profoundly alter the repressive activity of each complex and has the potential to change the complex into an activator of transcription.

4.4.2.2 CtBP, BRG1 and Mi-2 β

Inhibition of CtBP in both A375M and MDA-MB-231 cells did not induce E-cadherin re-expression (Figure 4-9, Figure 4-10, Figure 4-12). In contrast, in E-cadherin promoter reporter assays in A375M cells, repression of CtBP was shown to result in the significant transcriptional activation, characterising CtBP as a co-repressor of E-cadherin. These results indicate that CtBP is able to repress the E-cadherin promoter but removal of CtBP is not sufficient to induce the re-expression of E-cadherin. Interestingly, ZEB1 and ZEB2 have both been previously shown to repress E-cadherin independently of CtBP (van Grunsven *et al.*, 2003) and CtBP was not involved in the ZEB-mediated repression of E-cadherin in NMuMG cells following a TGF- β induced EMT (Shirakihara *et al.*, 2011).

A possible complication with the use of RNA interference-based inhibition of CtBP relates to the anti-apoptotic function of CtBP (Grooteclaes *et al.*, 2003). Following knockdown of CtBP, apoptotic pathways would be activated, resulting in the degradation of membranous E-cadherin and reduced levels of detectable protein. Indeed, cellular toxicity induced by CtBP inhibition can be visualised in MDA-MB-231 cells (Figure 4-11). In these cells, a single ZEB1 knockdown produced greater effect on E-cadherin expression than combined depletion of CtBP and ZEB1 (Figure 4-10). The analysis of mRNA expression has demonstrated equal levels of E-cadherin re-activation in cells treated with siRNA targeting of ZEB1 alone and ZEB1-CtBP in combination

(Figure 4-12). Taken together, these data show that CtBP is not required for down-regulation of E-cadherin by ZEB1.

It is noteworthy that the majority of studies demonstrating a role of CtBP in E-cadherin repression involved the use of promoter-based reporter assays, but not an analysis of E-cadherin re-activation at the mRNA and protein level (Grooteclaes & Frisch, 2000; Furusawa *et al.*, 1999; Postigo & Dean, 1999; Grooteclaes *et al.*, 2003). However, the use of promoter-based reporter assays to study the involvement of co-repressor complexes in transcriptional regulation may not be optimal. The function of these complexes involves chromatin remodelling and enzymatic modifications of histones by HDACs and HMTs, resulting in changes to the chromatin configuration and altering the accessibility of the transcriptional machinery. However, in a promoter reporter assay, the promoter is not present within the normal chromatin context and so inhibition by components of the co-repressor complexes may not generate results that strictly reflect their normal cellular function.

Interestingly, CtBP was shown to strongly interact with both ZEB1 and ZEB2 in A431-ZEB2 cells (Figure 4-15), in which ectopic expression of ZEB proteins induces EMT and down-regulation of E-cadherin (Vandewalle *et al.*, 2005; Mejlvang *et al.*, 2007). However, this interaction was also shown in UACC-257 cells (Figure 4-16), a cell line that co-expresses ZEB2 and E-cadherin. This indicates that the ZEB2-CtBP complex does not result in the transcriptional repression of E-cadherin within melanoma cell lines.

The ability of BRG1 to function as a transcriptional co-repressor of E-cadherin in melanoma cell lines was also examined. In SW-480 cells, ZEB1 recruited BRG1 to the E-cadherin gene promoter to repress transcription. Additionally, BRG1 depletion was sufficient to re-activate E-cadherin expression, and combined knockdown of BRG1 and ZEB1 enhanced the effect (Sanchez-Tillo *et al.*, 2010). Likewise, according to the presented data, BRG1 inhibition in combination with ZEB1 knockdown resulted in enhanced E-cadherin re-expression in A375M cells (Figure 4-13). Furthermore, in E-cadherin promoter reporter assays, knockdown of BRG1 resulted in transcriptional

activation (Figure 4-14). Unexpectedly, the physical interaction between BRG1 and the ZEB proteins was not confirmed in co-immunoprecipitation experiments (Figure 4-15), suggesting that the effect of BRG1 on E-cadherin expression is either indirect or independent of ZEB proteins. Indeed, ZEB1 may interact indirectly with BRG1 by association with an alternative component of the SWI/SNF chromatin remodelling complex, preventing the detection of an interaction by co-immunoprecipitation experiments.

An important feature of co-repressor complexes is the interchangeable nature of the individual sub-units. Indeed, the presence of the BRM and BRG1 subunits has been shown to switch during prostate cancer, with replacement of BRM with BRG1 during disease progression (Sun *et al.*, 2007). A potential role for both ZEB1 and ZEB2 exists in the regulation of the composition of the co-repressor complex, with each preferentially interacting with different components. Indeed, the switch in expression of the MR-EMT that is observed during melanomagenesis may be an important feature in determining the sub-units present within the complex. More detailed analysis of the expression patterns of multiple components of the SWI/SNF complex would be required to validate such a theory, in conjunction with more comprehensive co-immunoprecipitation analysis to confirm definite interactions.

The involvement of Mi-2/NuRD complex in the repression of E-cadherin has been demonstrated to occur via ZEB2 (Verstappen *et al.*, 2008) and also in conjunction with another MR-EMT, TWIST (Fu *et al.*, 2011). However, E-cadherin was not re-expressed in melanoma cells following knockdown of Mi-2 β , irrespective of whether ZEB1 or ZEB2 proteins were present (Figure 4-18). The involvement of Mi-2 β was not investigated further.

4.4.3 EPIGENETIC REGULATION OF E-CADHERIN EXPRESSION IN MELANOMA CELLS

During the reversible progression through EMT-like processes, epigenetic modifications, such as DNA methylation, histone modifications and miRNAs, are

important factors that contribute to the phenotypic conversion of epithelial cells. The classical marker of EMT, E-cadherin, has a central role in the control of epithelial adhesion and restricts the formation of an invasive and metastatic phenotype (Wijnhoven & Pignatelli, 1999). E-cadherin is extensively regulated at the epigenetic level, with inhibitory epigenetic marks resulting in transcriptional repression and induction of an EMT (Wang & Shang, 2012). A dense CpG island has been identified within the promoter and first and second exons of E-cadherin (Berx *et al.*, 1995b). The involvement of DNA methylation in the repression of E-cadherin has been extensively studied, exemplified by the NCI-60 cell lines. Within this panel of cell lines, 29 CpG sites were investigated, which surrounded the transcriptional start site. This identified an inverse correlation between DNA methylation and expression of E-cadherin (Reinhold *et al.*, 2007). In addition to DNA methylation, E-cadherin has been shown to be epigenetically controlled by miRNAs. Repression of E-cadherin was shown to occur via direct targeting of the pro-metastatic miRNA, miR-9, to the E-cadherin 3'UTR, resulting in increased migration and invasiveness (Ma *et al.*, 2010).

4.4.3.1 DNA METHYLATION AND ZEB PROTEINS

Preliminary data provided by Dr J Howard Pringle and Dr Eugene Tulchinsky formed the basis to investigate the role of the ZEB proteins in the regulation of DNA methylation. These results indicated that induction of ZEB2 expression in A431-ZEB2 cells resulted in increased DNA methylation at the *CDH1* promoter, which correlated with repression of E-cadherin expression (Figure 4-25 and Figure 4-26). DNA methylation within mammals is established and maintained by the DNA methyltransferases (DNMTs), DNMT1, DNMT3A and DNMT3B. DNMT1 has a preference for hemi-methylated DNA and so has been linked to the maintenance of DNA methylation following DNA replication. In comparison, DNMT3A and DNMT3B have been assigned the role of *de novo* DNMTs (Bestor, 2000). Indeed, the zinc-fingers and homeoboxes 1 (ZHX1) protein was shown to interact with DNMT3B. ZHX1 contains two zinc-fingers and five homeodomains, with interaction between ZHX1 and DNMT3B occurring via the homeodomains (Kim *et al.*, 2007). The ZEB proteins have a similar protein structure to ZHX1, suggesting that the ZEB proteins may be involved in the

regulation of DNA methylation at the *CDH1* promoter via targeted recruitment of DNMTs. Indeed, a recent paper has identified that SNAIL1 interacts with the G9a methyltransferase and DNMTs, allowing recruitment of these epigenetic modifiers to the *CDH1* promoter during a TGF- β induced EMT. This recruitment resulted in the transcriptional repression of E-cadherin due to enhanced methylation at Lys9 of histone H3 (H3K9me2) and DNA hypermethylation (Dong *et al.*, 2012).

To investigate the role of ZEB proteins in the regulation of DNA methylation, ZEB2 was expressed in the A431-ZEB2 cell line, whilst ZEB1 was inhibited by RNA interference in MDA-MB-231 cells. The DNA methylation at the *CDH1* promoter was subsequently analysed but no alteration in DNA methylation was detected when compared to control samples (Figure 4-27, Figure 4-28, Figure 4-29). In the study by Dong *et al.*, transcriptional repression of E-cadherin appeared to be initiated by H3K9me2, with DNA hypermethylation occurring only after prolonged TGF- β treatment (Dong *et al.*, 2012). This indicates that histone modifications may occur more rapidly than changes in DNA methylation, and may have provided a better marker for the epigenetic regulation of the *CDH1* promoter by the ZEB proteins. Unfortunately due to these negative results, the regulation of DNA methylation by the ZEB proteins was not analysed further.

4.4.3.2 DNA METHYLATION AND METASTATIC MELANOMA

The observation that E-cadherin expression was inducible in A375M cells but not in A375P cells following repression of ZEB1 indicated that A375M cells uniquely lacked an inhibitory factor or possessed an activating factor. DNA methylation was a potential inhibitory factor that could be present within A375P cells to restrict E-cadherin re-expression. Indeed, comparison of the level of DNA methylation, by MSP and BSP (Figure 4-29 and Figure 4-30), at the *CDH1* promoter between A375P and A375M cells identified that the A375P cell line contained higher levels of DNA methylation. The presence of increased DNA methylation at the *CDH1* promoter in A375P cells may explain the resistance to E-cadherin re-activation following repression of ZEB1 and

ZEB2. This may be an important factor in determining the metastatic capacity of the cell lines, with reduced DNA methylation in A375M cells allowing a more dynamic control of E-cadherin expression, increasing rates of metastatic colonisation due to E-cadherin-based interaction with the new microenvironment.

In order to combine the effects of ZEB protein repression and removal of DNA methylation upon the level of E-cadherin re-activation in A375P and A375M cells, the ZEB proteins were repressed in combination with treatment with the demethylating agent 5-azaC (Figure 4-32). Following DNA demethylation, A375P cells responded to ZEB protein repression in a comparable manner to A375M cells. This indicates that the major inhibitory factors controlling E-cadherin re-expression in A375P cells are the ZEB proteins and promoter-based DNA methylation, with the presence of DNA methylation potentially restricting the metastatic capacity of the A375P cell line. The dynamic regulation of DNA methylation during metastatic spread has previously been observed in the regulation of the miR-200 family. The 5' CpG island associated with the miR-200 family is epigenetically regulated by DNA methylation, becoming hypermethylated during an EMT and hypomethylated following a subsequent MET, resulting in transcriptional repression and activation, respectively. This shows that DNA methylation is highly plastic, providing tumour cells with the adaptive ability to successfully metastasis (Davalos *et al.*, 2012). Indeed, a similar pattern of epigenetic plasticity was identified for E-cadherin and $\alpha 6\beta 4$ integrin in NMuMG mouse mammary gland epithelial cells undergoing an EMT and subsequent MET. Gain of DNA methylation and acquisition of repressive histone marks occurred during the EMT, with loss of the corresponding protein. During the MET, protein re-expression occurred, with histone marks modified to an active configuration. Interestingly though, promoters remained hypermethylated (Yang *et al.*, 2009). These studies highlight the flexible nature of epigenetic modifications and their ability to control transcriptional activity. Interestingly, both studies induced the EMT and MET via application and withdrawal of TGF- β , indicating that growth factors present within the microenvironment may be responsible for inducing changes at the epigenetic level.

The level of DNA methylation within both A375 cell lines is heterogeneous at all the CpG sites examined, identified by the presence of both cytosine and thymine residues. This is a potential explanation for the lack of uniform E-cadherin re-expression identified by immunofluorescence in the A375M cell line following repression of the ZEB proteins (Figure 4-2b). The presence of DNA methylation within a sub-set of the A375M cells would prevent E-cadherin re-expression, as experienced in vast majority of the A375P cell population. Additionally, within the A375M cell lines, CpG sites 8 and 9 are strongly methylated, within a region consisting of predominantly unmethylated DNA. These two positions may represent 'seed' CpG sites, which have previously been suggested to prime currently active and unmethylated promoters for *de novo* methylation (Reinhold *et al.*, 2007). The maintenance of DNA methylated at CpG sites 8 and 9 may allow A375M cells to switch between a methylated and unmethylated state depending upon the particular requirements of the tumour cell. Additionally, both cell lines retained unmethylated regions surrounding the E-cadherin transcriptional start site (Figure 4-31). This may indicate that neither cell line is in an 'epigenetically fixed' state but in a reversible 'metastable' state (Thomson *et al.*, 2011), with progression of A375P cells further towards the fixed, fully methylated state. Lack of DNA methylation around the transcriptional start site may allow for a more dynamic regulation of transcription compared to a fully methylated promoter.

Problems associated with the use of 5-azaC should be mentioned. It is a non-specific inhibitor (Stresemann & Lyko, 2008) and is highly mutagenic and cytotoxic, inducing nucleotide transversions at CpG dinucleotides (Jackson-Grusby *et al.*, 1997). Clinical usage of 5-azaC is beneficial in the treatment of myelodysplastic syndrome, a preleukemic bone marrow disorder (Kantarjian *et al.*, 2006), but has also been shown to increase the tumourigenicity of rat chondrosarcoma cells, due to loss of global DNA methylation (Hamm *et al.*, 2009). Interestingly, following application of 5-azaC to the A375 cell lines (Figure 4-32) there was a decrease in the expression of the ZEB proteins, which may result from the off-target effects of 5-azaC. As mentioned, the miR-200 family is regulated by DNA methylation (Davalos *et al.*, 2012), but is normally not expressed in melanoma cell lines (Appendix I). One reason for the reduced

expression of the ZEB proteins may be the activation of the miR-200 family. This would result in repression of the ZEB proteins, impacting upon E-cadherin expression in a manner unconnected to the DNA methylation located at the E-cadherin promoter.

4.5 CONCLUSION

ZEB1 appears to be the primary EMT-related transcriptional repressor of E-cadherin in melanoma cell lines, functioning by interaction with the E-cadherin promoter-based E-boxes. The role of ZEB2 as an E-cadherin transcriptional repressor is highly complex, functioning as a repressor in combination with ZEB1 but independently lacking such ability. Furthermore, the transcriptional co-repressor BRG1 is involved in the regulation of E-cadherin expression, functioning in a ZEB1-dependent manner but with no detectable interaction with either ZEB protein. In comparison, the prototypic E-cadherin transcriptional co-repressor, CtBP, did not functionally control the expression of E-cadherin, even though there is a strong interaction with both ZEB1 and ZEB2. Finally, DNA methylation at the E-cadherin promoter has also been implicated in the negative regulation of E-cadherin expression in melanoma cell lines.

CHAPTER 5 : MR-EMT DIFFERENTIALLY REGULATE MITF EXPRESSION IN MELANOMA CELL LINES

5.1 INTRODUCTION

Here, melanoma-driving pathways, such as oncogenic mutations in N-RAS and B-RAF, have been shown to induce a switch in the MR-EMT. Additionally, E-cadherin has been shown to be co-expressed with ZEB2 and SNAIL2, whilst ZEB1 functions as a repressor of E-cadherin. E-cadherin can be considered as a marker for melanocyte differentiation, which is lost during the dedifferentiation and metastatic progression of malignant melanoma (Hsu *et al.*, 2000a; Hsu *et al.*, 2000b; Herlyn *et al.*, 2000). Therefore, the reprogramming of the MR-EMT was further investigated in relation to melanocyte differentiation. The transcription factor MITF is an important marker of melanocyte differentiation (Sommer, 2011) and was used to study the role of the MR-EMT in the control of differentiation within melanoma cell lines.

5.2 AIMS AND OBJECTIVES

Aim: To investigate how the MR-EMT regulate melanocyte-specific markers of differentiation.

Objectives: MITF will be analysed in relation to the expression of the MR-EMT by the use of RNA interference and ectopic protein expression.

5.3 RESULTS

Within the melanoma cell lines, co-regulation of ZEB2 with SNAIL2 and ZEB1 with TWIST1 has been established (Chapter 3). In WM266-4 and A375P cell lines, knockdown of ZEB2 and SNAIL2 resulted in down-regulation of MITF (Figure 5-1). Additionally, combined inhibition of ZEB2 and SNAIL2 resulted in the synergistic reduction in MITF expression, especially evident in A375P cells. This suggests that ZEB2 and SNAIL2 positively regulated MITF expression. In comparison, combined knockdown of ZEB1 and TWIST1 did not influence MITF expression.

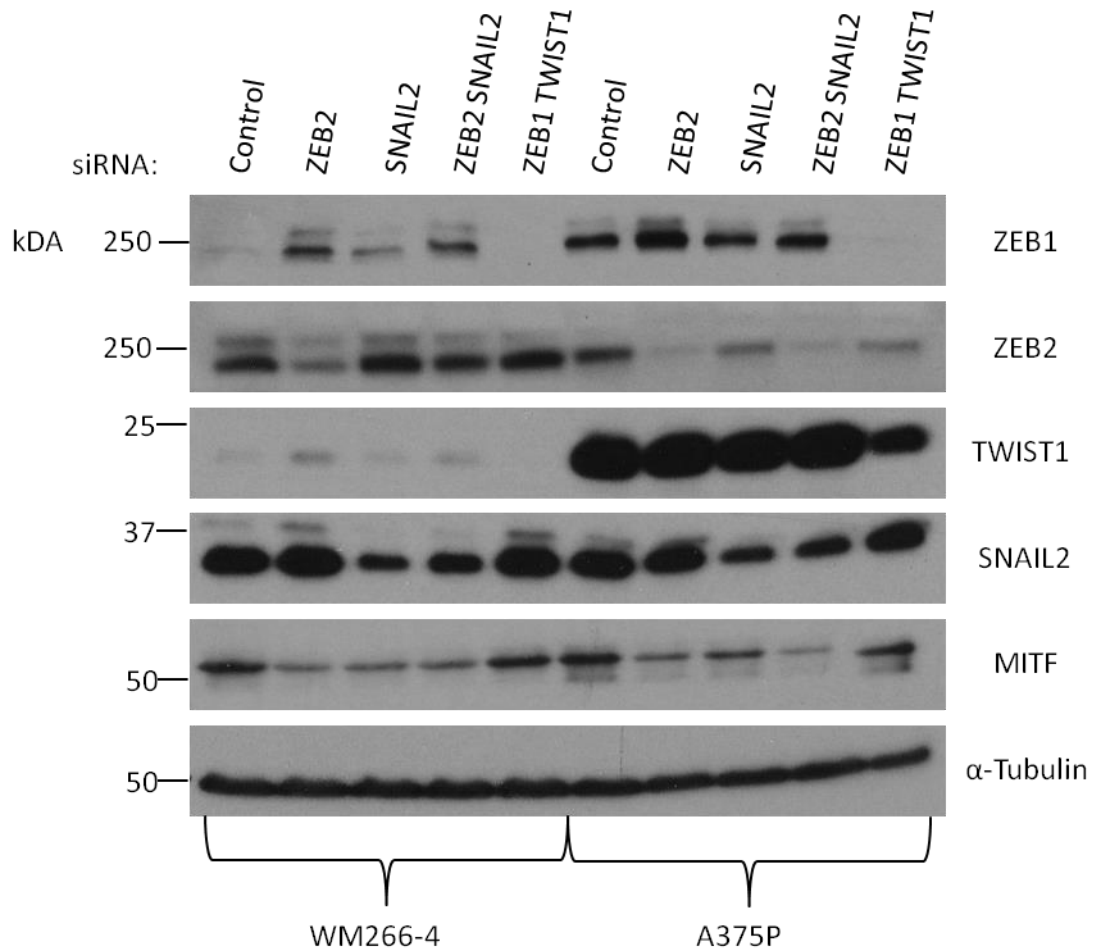


Figure 5-1: ZEB2 and SNAIL2 activate MITF expression

WM266-4 and A375P cells were transfected with the various siRNAs and incubated for 96 hours. Knockdown of ZEB2 and SNAIL2 in both cell lines resulted in reduced MITF expression, with the combined knockdown additively reducing MITF. This indicates the ZEB2 and SNAIL2 function as positive regulators of MITF. α-Tubulin was used as a protein loading control. Representative images are presented (n=2).

To further examine the role of ZEB1 in MITF regulation, ZEB1 was ectopically expressed in ZEB1-negative and MITF-positive melanoma cell lines, SK-MEL-5 and UACC-257 (Figure 5-2a). This resulted in the down-regulation of MITF, indicating that ZEB1 functions as a negative-regulator of MITF. In order to establish the opposing roles of ZEB1 and ZEB2 within a single cell system, ZEB2 was inhibited in UACC-257 cells, resulting in reduced MITF expression (Figure 5-2b). These results suggest that ZEB2 induces MITF expression, whilst ZEB1 inhibits MITF expression.

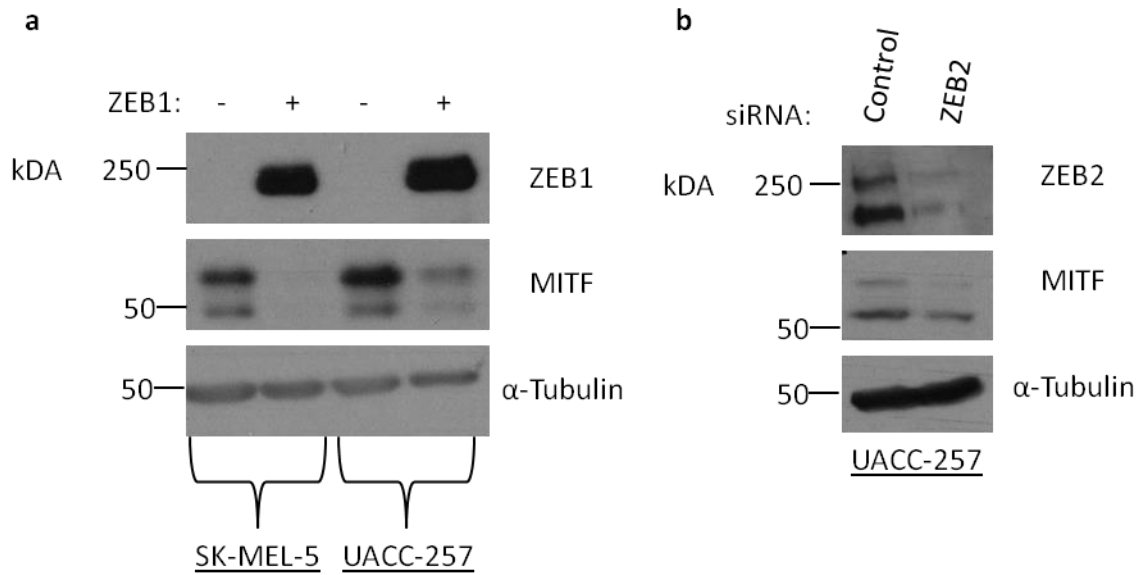


Figure 5-2: ZEB1 represses MITF expression

a) ZEB1 was ectopically expressed in SK-MEL-5 and UACC-257 cells by transient transfection of 4 μ g of pBI_ZEB1_HA and 1 μ g of pUHD172.1 neo vectors for 48 hours in the presence of 2 μ g/ml doxycycline. ZEB1 reduced MITF expression, indicating that ZEB1 is a negative regulator of MITF.

b) Knockdown of ZEB2 in UACC-257 cells resulted in down-regulation of MITF, confirming that ZEB1 and ZEB2 have opposing functions relating to MITF expression within a single cell line. α -Tubulin was used as a protein loading control. Representative images are presented (n=2).

Another interesting observation concerns MITF expression following treatment with the B-RAF inhibitor PLX4720 in the N-RAS mutant cell line IPC-298 (Figure 3-4). B-RAF inhibition for 24 hours resulted in the hyper-activation of the MAPK signalling pathway, up-regulation of ZEB1 and the corresponding down-regulation of MITF expression.

5.4 DISCUSSION

To determine whether the switch in expression of the MR-EMT has a role in regulating features of melanoma differentiation, MITF expression was analysed. A link between the MR-EMT and MITF has previously been identified. MITF was shown to function upstream of *SNAI2*, directly activating *SNAI2* expression by interaction with an E-box sequence located within the *SNAI2* promoter (Sanchez-Martin *et al.*, 2002). Additionally, in retinal pigment cells, ZEB1 was shown to mediate transcriptional repression of MITF-A, by binding to promoter-based E-boxes (Liu *et al.*, 2009).

After single repression of ZEB2 and SNAIL2 in WM266-4 and A375P cells, MITF expression was down-regulated, with a synergistic effect upon MITF following knockdown of both ZEB2 and SNAIL2 in A375P cells (Figure 5-1). This indicates that ZEB2 and SNAIL2 independently function as positive regulators of MITF. With the use of a positive approach, exogenous expression of ZEB1 in SK-MEL-5 and UACC-257 cells resulted in repression of MITF (Figure 5-2). Additionally in UACC-257 cells, inhibition of ZEB2 resulted in down-regulation of MITF. Another observation relating to the regulation of MITF was identified in the initial experiments concerning B-RAF inhibition in the N-RAS mutant cell line IPC-298. In conjunction with increased ZEB1 expression, MITF was down-regulated, after only 24 hours of B-RAF inhibition (Figure 3-4). These results identify that MITF is differentially regulated by the MR-EMT and suggests that they are important in the control of melanoma differentiation.

5.5 CONCLUSION

The MR-EMT are differentially regulated within melanoma cell lines and mediate different functions in relation to melanoma cell differentiation, with ZEB2 and SNAIL2 acting as a positive regulators of MITF, whilst ZEB1 negatively controls MITF expression.

CHAPTER 6 : DISCUSSION

In epithelial tumours, expression of the MR-EMT is regulated by EMT-initiating pathways, resulting in the formation of highly migratory and invasive tumour cells, with increased metastatic potential and stem cell-like properties. Indeed, the MR-EMT have been shown to cooperate with classical oncoproteins and facilitate transformation of immortalised mammary epithelial cells (Morel *et al.*, 2012; Morel *et al.*, 2008). Mechanistically, the MR-EMT (TWIST1 and ZEB1) ensure escape from cellular failsafe programs, senescence and apoptosis. However, it remains unclear which mechanisms coordinate the co-expression of the MR-EMT and the classical oncoproteins. Additionally, it is unclear what the functions of the individual MR-EMT are in neural crest-derived tumours, such as malignant melanoma. By studying the MR-EMT in melanoma cell lines, we identified a novel pathway, whereby the MR-EMT have been shown to be regulated in an opposing manner and to have antagonistic functions (Figure 6-1). In opposition to results obtained within carcinomas, whereby the expression of all the MR-EMT has been shown to be induced within specific epithelial tumour types, with all the MR-EMT involved in the transcriptional repression of E-cadherin, a different system has been identified in melanomagenesis.

With the use of B-RAF and MEK inhibitors, I demonstrated that B-RAF and N-RAS oncogenic pathways cause a switch in the expression of the MR-EMT, with up-regulation of ZEB1 and TWIST1 and down-regulation of ZEB2 and SNAIL2. In addition, my data show that ZEB2 is located up-stream of ZEB1, functioning to negatively regulate ZEB1 expression. Constitutive oncogenic B-RAF or N-RAS signalling was required to maintain the switch in the expression of the MR-EMT, indicating that the switch is reversible. It would be interesting to determine whether this unique pathway is limited to cases of melanoma, or whether it occurs more generally in tumours of neural crest origin, such as glioblastomas, neuroblastomas and schwannomas.

The up-regulation of ZEB1 and TWIST1 in response to B-RAF and N-RAS pathways indicates that they may have an oncogenic role in malignant melanoma. In contrast, ZEB2 and SNAIL2 are down-regulated following oncogenic activation, indicating a possible tumour-suppressive role. This is in agreement with a recent report, in which

ZEB2 has been shown to promote the expression of PTEN in malignant melanoma via a mechanism involving miRNA (Karreth *et al.*, 2011). Our data obtained via collaboration with a group in Lyon confirmed this suggestion, and demonstrated that whereas ZEB1 and TWIST1 facilitate, ZEB2 and SNAIL2 attenuate B-RAF-induced transformation of mouse melanocytes (Appendix I). The opposing activities of these two groups of MR-EMT in malignant melanoma can be linked with their opposing roles in the regulation of MITF, a key transcriptional regulator of melanocyte differentiation. Indeed, ZEB1 was shown to be a negative regulator of MITF, whilst ZEB2 and SNAIL2 were involved in the induction of MITF expression. This is consistent with the correlative expression of SNAIL2 and MITF in naevi and primary melanomas (Shirley *et al.*, 2012). The manner in which the MR-EMT regulate MITF expression has not currently been resolved, and potentially occurs indirectly.

These opposing functions of the MR-EMT may also be linked to the regulation of E-cadherin expression. ZEB1 was shown to be the primary transcriptional repressor of E-cadherin, with ZEB2 appearing to be functionally dependent upon ZEB1. The manner in which ZEB1 and ZEB2 appear to differentially regulate target genes indicates that ZEB2 promotes a differentiated melanocytic phenotype, whilst ZEB1 induces an undifferentiated phenotype, by promoting the down-regulation of E-cadherin and MITF. Indeed, the role of MITF in melanoma cell differentiation is discussed within the framework of the rheostat model of phenotype switching, whereby high expression of MITF in melanoma cells has been linked to a differentiated phenotype, with cells being non-proliferative and non-invasive. Alternatively, the intermediate expression of MITF has been linked to a proliferative state, whilst loss of MITF expression has been correlated with cell cycle arrest and the formation of stem-cell like characteristics, with the formation of an invasive phenotype (Hoek & Goding, 2010). The opposing manner of MITF regulation by the MR-EMT indicates that ZEB2 and SNAIL2 promote the differentiated phenotype, whilst ZEB1 induces the stem-like, invasive phenotype.

The up-regulation of ZEB1 in response to oncogenic B-RAF and N-RAS correlates with the role of ZEB1 as the primary repressor of E-cadherin. Indeed, ZEB1 was the only

MR-EMT to independently regulate E-cadherin expression. ZEB1-mediated repression was also linked to the co-repressor BRG1, an ATPase sub-unit of the SWI/SNF chromatin remodelling complex. This indicates that ZEB1 functions as the DNA-binding partner, allowing the chromatin remodelling complex to interact with the *CDH1* promoter. Additionally, DNA methylation was shown to be a factor that modulates re-expression of E-cadherin in melanoma cell lines and a potential factor in restricting metastatic colonisation.

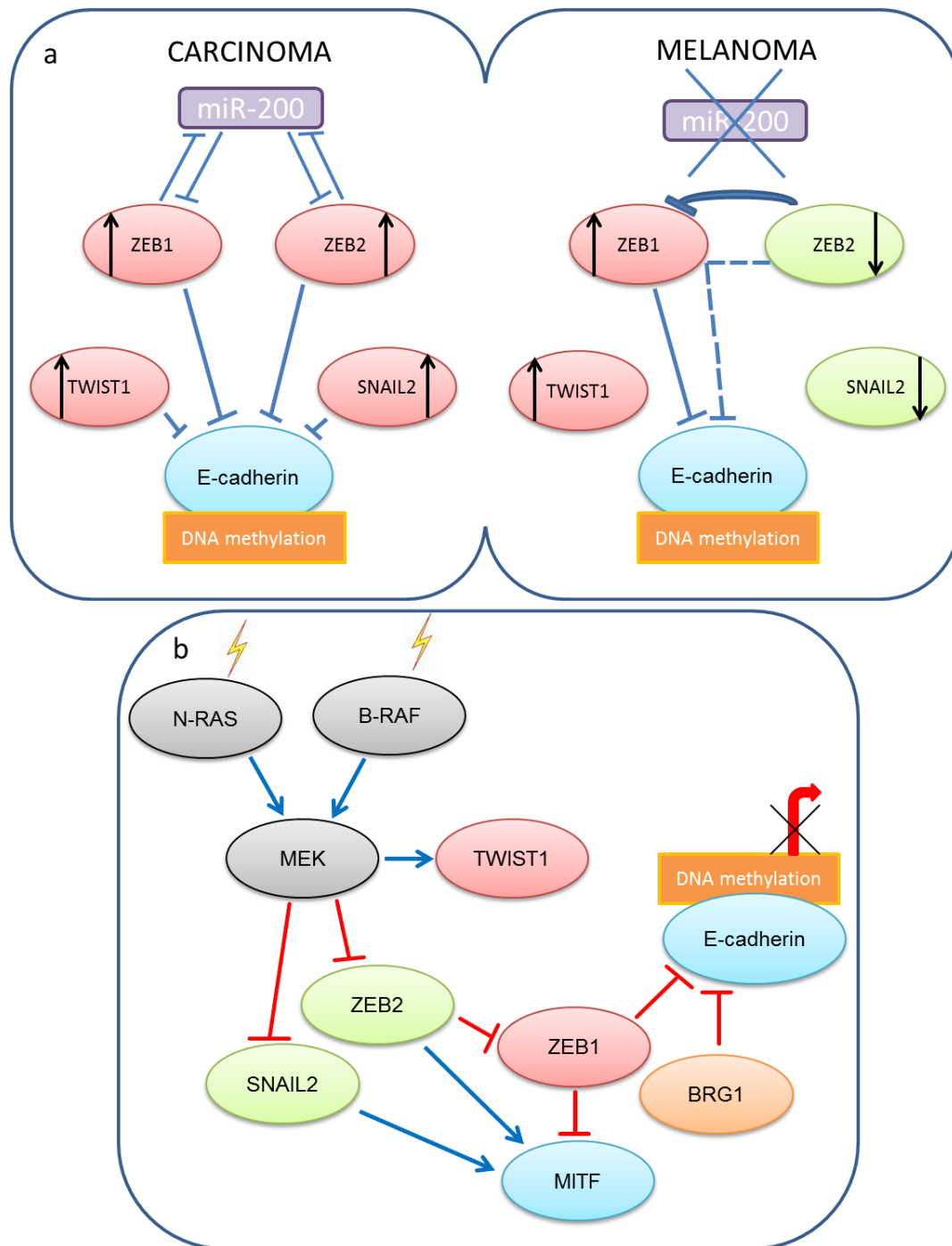


Figure 6-1: Regulation of the MR-EMT in melanoma

(a) The MR-EMT are induced in epithelial tumours and have individually been identified as transcriptional repressors of E-cadherin. In comparison, ZEB2 and SNAIL2 are expressed in normal melanocytes, whilst ZEB1 and TWIST1 are induced during melanomagenesis. ZEB1 is the major transcriptional repressor of E-cadherin, with a synergistic effect identified for ZEB2. TWIST1 and SNAIL2 were unable to function as E-cadherin repressors within melanoma cell lines.

(b) During melanomagenesis, oncogenic B-RAF and N-RAS causes the constitutive activation MAPK signalling pathway, inducing a switch in expression of the MR-EMT, with down-regulation of ZEB2 and SNAIL2 and up-regulation of ZEB1 and TWIST1. ZEB2 and SNAIL2 are positioned up-stream of ZEB1 and TWIST1, with ZEB2 functioning as a repressor of ZEB1. Additionally, ZEB2 and SNAIL2 are positive regulators of MITF, whilst ZEB1 is negative regulator. Furthermore, ZEB1-mediated repression of E-cadherin is increased by the transcriptional co-repressor BRG1. E-cadherin expression was also regulated by the presence of DNA methylation.

6.1 CONCLUSION

During melanoma progression, oncogenic mutations in B-RAF and N-RAS induce a reversible switch in the expression of the MR-EMT, with repression of ZEB2 and SNAIL2, and up-regulation of ZEB1 and TWIST1. ZEB2 and SNAIL2 were shown to be located up-stream of ZEB1 and TWIST1 within the MAPK signalling cascade, with ZEB2 repressing ZEB1. Additionally, ZEB2 and SNAIL2 activate expression of MITF, whilst ZEB1 expression represses MITF. Furthermore, ZEB1 was identified as the primary transcriptional repressor of E-cadherin, with E-cadherin also being regulated by the BRG1 sub-unit of the SWI/SNF chromatin remodelling complex. Finally, E-cadherin expression was also shown to be regulated by DNA methylation.

CHAPTER 7 : APPENDIX I

A Switch in the Expression of Embryonic EMT-Inducers Drives the Development of Malignant Melanoma

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SUMMARY

Aberrant expression of embryonic epithelial-mesenchymal transition-inducing transcription factors (EMT-TFs) in epithelial cells triggers EMT, neoplastic transformation, stemness, and metastatic dissemination. We found that regulation and functions of EMT-TFs are different in malignant melanoma. SNAIL2 and ZEB2 transcription factors are expressed in normal melanocytes and behave as tumor-suppressor proteins by activating an MITF-dependent melanocyte differentiation program. In response to NRAS/BRAF activation, EMT-TF network undergoes a profound reorganization in favor of TWIST1 and ZEB1. This reversible switch cooperates with BRAF in promoting dedifferentiation and neoplastic transformation of melanocytes. We detected EMT-TF reprogramming in late-stage melanoma in association with enhanced phospho-ERK levels. This switch results in E-cadherin loss, enhanced invasion, and constitutes an independent factor of poor prognosis in melanoma patients.

INTRODUCTION

Epithelial-mesenchymal transition (EMT) is a reversible embryonic genetic program, which produces motile cells from polarized epithelia. EMT is driven by a network of embryonic

EMT-inducing transcription factors (EMT-TFs) represented by several protein families, such as SNAIL, TWIST, or ZEB. The aberrant expression of EMT-TFs is frequently observed in various cancer types, particularly in carcinomas, and is often associated with poor prognosis and high risk of metastasis

Significance

In epithelial cells, the aberrant reactivation of transcription factors inducing epithelial-mesenchymal transition (EMT-TFs) is known to facilitate both neoplastic transformation and tumor cell dissemination. We have now shown that during melanomagenesis SNAIL2 and ZEB2 transcription factors drive a melanocytic differentiation program and behave as oncosuppressive proteins, whereas ZEB1 and TWIST1 repress differentiation and possess oncogenic properties. The reversible switch from tumor-suppressive to oncogenic EMT-TF expression pattern is driven by mitogen-activated protein kinase (MAPK)-activating molecular pathways that are critical to melanoma initiation and progression. Our data demonstrate that regulation and function of embryonic EMT-TFs in malignant melanoma and epithelial tumors are dissimilar. Overall, these data provide insight into cooperation between classical oncogenes and embryonic genetic programs during tumor initiation and progression.

(Thiery et al., 2009; Peinado et al., 2007). Their oncogenic potential relies on their ability to promote escape from oncogene-induced fail-safe programs by interfering with critical tumor suppressor pathways including p53, RB, and PP2A (Maestro et al., 1999; Valsesia-Wittmann et al., 2004; Ansieau et al., 2008; Morel et al., 2012). At later stages of carcinoma development, the EMT-resembling processes initiated by EMT-TFs contribute to accelerated tumor progression and metastases (Peinado et al., 2007). Importantly, EMT results in the acquisition of stem cell-like properties including slow proliferation and self-renewal potential (Vega et al., 2004; Meijlvang et al., 2007; Mani et al., 2008; Morel et al., 2008). The flexible and transient nature of EMT pathways determines reversible switches between proliferative and invasive phenotypes and manifests plasticity of tumor cells in aggressive carcinomas (Tsai et al., 2012; Ocaña et al., 2012; Brabletz, 2012a, 2012b). Tumor cell plasticity depends on microenvironmental cues and is responsible for drug resistance and for driving epithelial tumorigenesis through balancing tumor growth and spread (Iliopoulos et al., 2011; Nieto and Cano, 2012).

A high degree of phenotypic plasticity is a hallmark of malignant melanoma (MM), a most aggressive skin cancer originating from neural crest-derived melanocytes. In MM, phenotypic plasticity is controlled by a master regulator of melanocyte development, the microphthalmia-associated transcription factor (MITF) (Cheli et al., 2010; Bell and Levy, 2011). In contrast to the majority of epithelial tissues, normal melanocytes express some EMT-TFs, and this was considered as an intrinsic factor predisposing MM to high metastatic propensity (Gupta et al., 2005). As melanocytes do not belong to epithelial lineage, the term “EMT” cannot be formally attributed to the progression of MM. However, differentiated melanocytes do express E-cadherin, which is required for their contacts with keratinocytes in the basal layer of the epidermis. These communications maintain differentiation state of melanocytes and suppress their proliferation. Loss of E-cadherin that represents a hallmark of EMT in epithelial tumors is also evident in late-stage MM, especially in nodal metastases (Miller and Mihm, 2006; Alexaki et al., 2010).

It is well established that MM is driven by the activation of MEK-ERK signaling, most frequently through the mutations in *BRAF* or *NRAS* oncogenes. However, a molecular link between this pathway, cell plasticity, and EMT-TF network in MM cells remains not studied. Here, we analyze regulation and function of EMT-TFs in MM with the aim to assess a role for EMT-resembling processes in melanomagenesis.

RESULTS

EMT-TF Switching in Human Melanoma Samples

Expression of some EMT-inducers in melanomas has been reported previously (Hoek et al., 2004; Ansieau et al., 2008; Shirley et al., 2012; Mikesch et al., 2010). However, no comprehensive study on the EMT-TF network in MM has yet been performed. We therefore analyzed the expression of SNAIL1, SNAIL2, TWIST1, ZEB1, and ZEB2 proteins in a series of human tissues representing melanoma progression, including normal epidermis, common acquired nevi, primary MM, and metastases. In addition, we evaluated the IHC staining of a selected cohort of primary MM to investigate the clinical significance of the EMT-TF network. In a recent report, SNAIL1 has been shown

to be absent in 96% of MM samples (Mikesch et al., 2010). In agreement with these data, 24 MM samples including 12 primary tumors and matched metastases were SNAIL1-negative; therefore we excluded SNAIL1 from further analyses.

Remarkably, ZEB2 and SNAIL2 showed strong nuclear expression in normal epidermal melanocytes, in which ZEB1 and TWIST1 were absent (Figure S1A available online). The analyses of the melanoma progression series showed two opposing trends within and between lesions. Within lesions, a distinct gradient was observed, where ZEB2 and SNAIL2 were strongly expressed in superficial sites with weaker or absent nuclear staining in the deep sites of nevi ($n = 26$) and primary MMs ($n = 151$) (Wilcoxon matched pairs test, $p < 0.0001$). ZEB1 and TWIST1 showed the opposite gradient with stronger focal staining at the deep sites of primary MMs compared to superficial sites in these lesions ($p < 0.0001$) (Figures 1A and S1). In cortical and medullar parts of metastases, we detected similar gradients of immunopositivity for EMT-TFs ($n = 51$, $p < 0.0001$) (Figures S1B and S1C).

Likewise, a reciprocal pattern of IHC staining was observed between lesions with melanoma progression. We observed a progressive loss of ZEB2 immunopositivity in superficial and deep sites of primary MM and medullar sites of independent metastases ($n = 101$, $p < 0.001$ and $n = 51$, $p < 0.001$ respectively, Jonckheere-Terpstra trend test; Figures 1A and S1B–S1D) and loss of ZEB2 and SNAIL2 staining in matched metastatic lesions ($n = 31$, $p < 0.01$ and $n = 28$, $p < 0.01$ respectively, Page's L trend) (Figure S1E). ZEB1 showed increased immunopositivity with tumor progression. H-score values showed a significant increasing trend from primary MM to medullar sites of independent ($n = 101$, $p < 0.0001$ superficial, $n = 101$, $p < 0.0001$ deep Jonckheere-Terpstra trend test) or matched metastatic lesions ($n = 31$, $p < 0.01$, Page's L trend) (Figures S1D and S1E; Table S1). For TWIST1, the superficial staining showed a significant increasing trend with progression ($n = 101$, $p < 0.014$, Jonckheere-Terpstra trend test) (Table S1).

Overall, we characterized a trend of transition from ZEB2^{high}/SNAIL2^{high}/ZEB1^{low}/TWIST1^{low} toward a ZEB2^{low}/SNAIL2^{low}/ZEB1^{high}/TWIST1^{high} expression pattern. This transition was evident at deeper sites of the lesions and correlated with the level of malignancy.

These findings prompted us to perform survival analyses for all primary melanomas with follow-up data (the Kaplan-Meier method; $n = 98$) for superficial and deep staining of ZEB1, ZEB2, and TWIST1. Significant shorter metastasis-free survival was observed for Breslow depth, high ZEB1, and low ZEB2 levels at deep sites and high TWIST1 at superficial sites (Figure 1B; Table S1). Two-step cluster analysis using IHC H-scores of these EMT-TFs identified three distinct natural groups of expression representing a switch in EMT-TFs from ZEB2/SNAIL2 to ZEB1/TWIST1 with significant differences in clinical outcome (Figure 1B; Table S1). In a multivariate analysis, both superficial and deep staining of ZEB1 and TWIST1 combined with Breslow thickness were all significant independent predictors of time to metastasis. Loss of deep ZEB2 staining showed decreased metastasis-free survival but not independent of tumor depth (Table S1). Together, these data demonstrate that a switch from ZEB2/SNAIL2 to ZEB1/TWIST1 expression constitutes a major risk factor for poor outcome in MM when controlling for other clinico-pathological variables.

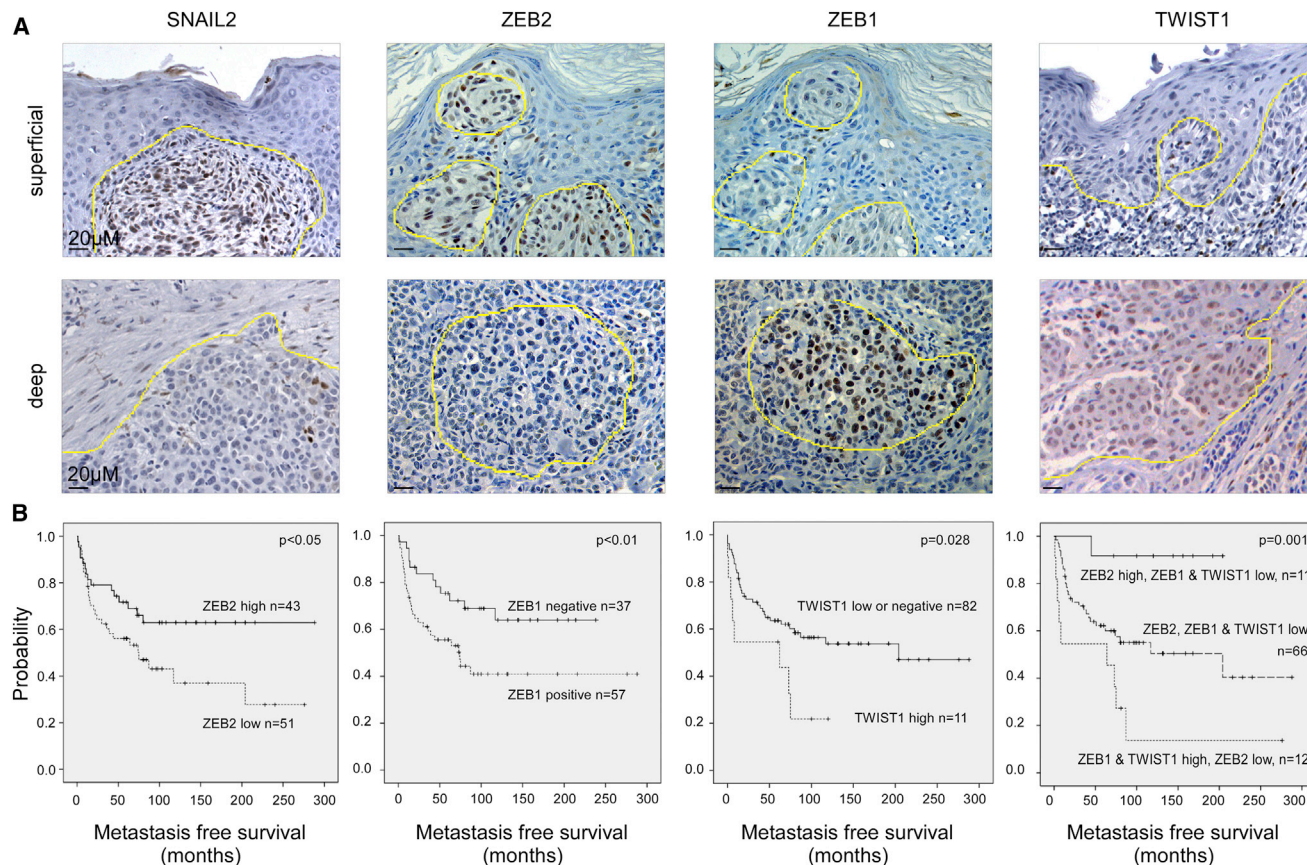


Figure 1. Opposing Trends of EMT-TF Expression in Melanocytic Lesions

(A) Examples of representative IHC analyses of EMT-TF expression at superficial and deep sites of primary melanoma samples. The images exemplify opposing gradients of ZEB2 and SNAIL2 versus ZEB1 and TWIST1 expression within the lesions. Tumor borders are indicated with dashed yellow lines.

(B) Metastasis-free survival of patients with MM according to EMT-TF expression. Note significantly shorter metastasis-free survival of patients with ZEB2-negative tumors expressing ZEB1 and TWIST1.

See also Figure S1 and Table S1.

Activation of the NRAS/BRAF Signaling Pathway Perturbs the Expression of EMT-TFs

Perturbed expression of EMT-TFs in MM samples prompted us to explore a potential interrelationship between this reprogramming and the NRAS or BRAF melanoma-driving pathways. To activate this pathway in melanocytes, we transduced immortalized, but nontransformed murine C57 BL/6J-derived melan-a cells with the tamoxifen-inducible version of a dominant-active truncated variant of BRAF (Δ BRAF-ER). In parallel, human primary melanocytes (NHEM) were infected with a vector expressing a constitutively active mutant BRAF^{V600E}. Consistent with the results of the IHC analyses of normal skin (Figure S1A), nontransformed melanocytes in culture were positive for SNAIL2 and ZEB2, but negative for ZEB1 and TWIST1 (Figure 2A). Activation of the BRAF pathway converted the expression of EMT-TFs to a ZEB2^{low}/SNAIL2^{low}/ZEB1^{high}/TWIST1^{high} pattern consistent with the EMT-TF reprogramming observed by the analyses of deeper lesions of more malignant melanoma samples (Figure 2A).

Next, we addressed whether the constitutive BRAF or NRAS signaling is required to maintain EMT-TF expression pattern in melanoma cell lines. In line with the data obtained in NHEM

and melan-a cells, MEK inhibition upregulated ZEB2 and SNAIL2 and downregulated ZEB1 and TWIST1 in melanoma cell lines with mutations in *BRAF* or *NRAS* (Figure 2B). The effect of a specific BRAF inhibitor was identical with that of MEK inhibitors in *BRAF* mutant cells, such as A375P, but not in an *NRAS* mutant IPC-298 cell line, likely due to the activation of the NRAS-CRAF pathway (Heidorn et al., 2010). Collectively, these data demonstrated that in both murine and human species, BRAF activation drives a rapid and reversible switch in EMT-TF expression, and the activity of this pathway is necessary and sufficient to maintain MM-specific pattern of EMT-TF expression.

Although the absolute majority of melanocytic lesions including nonmalignant nevi contain MAPK-activating mutations, the strength of the signaling is different and modulated by various negative feedback and bypass mechanisms (Wajapeyee et al., 2008; Nikolaev et al., 2012; Li et al., 2012; Martin et al., 2012). Consequently, variable levels of phospho-ERK immunopositivity were reported in the lesions, with higher expression in late-stage melanoma and low or lack of expression in nevi (Saldanha et al., 2004; Uribe et al., 2006; Venesio et al., 2008; Yazdi et al., 2010). To validate the results obtained in vitro, we analyzed phospho-ERK expression in a subset of

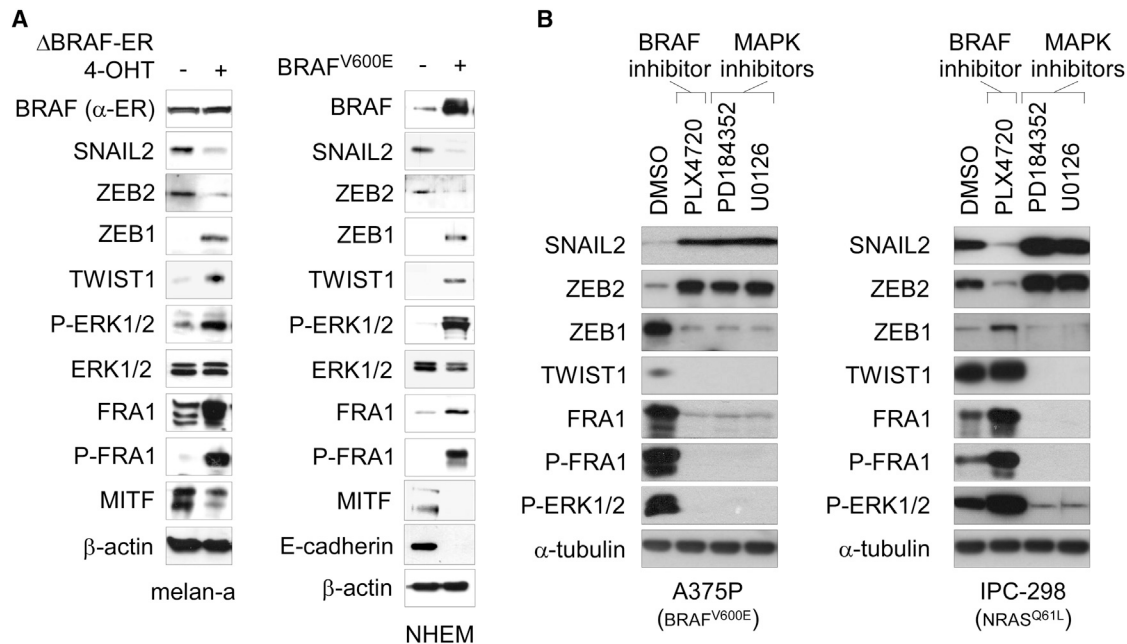


Figure 2. RAS-BRAF Pathway Induces a Reversible Switch in EMT-TF Expression

(A) Activation of BRAF pathway in nontransformed melanocytes upregulates ZEB1, TWIST1, and FRA1, but downregulates ZEB2 and SNAIL2. Expression of an activated form of BRAF in melan-a cells was induced by tamoxifen (4-OHT) treatment of melan-a/ΔBRAF-ER cells for 72 hr. NHEM cells were infected with a lentiviral vector harboring BRAF^{V600E} point mutation. Protein expression was analyzed by immunoblotting.

(B) Chemical inhibitors of BRAF and MEK downregulate ZEB1, TWIST1, and FRA1, but upregulate ZEB2 and SNAIL2. Parental A375 (A375P) and IPC-298 MM cells were treated with indicated inhibitors for 24 hr. Protein expression was analyzed by immunoblotting.

melanocytic lesions with previously characterized expression of EMT-TFs (n = 89). The results showed low phospho-ERK levels in nevi (Figure S2) and elevated expression in cancerous tissues, primary MM (Figure 3A) and metastases (Figures S2B and S2C). Importantly, phospho-ERK immunopositivity in MM samples significantly correlated with elevated ZEB1 and low ZEB2 (Figures 3A and 3B) and represented a prognostic factor for poor survival in MM patients (Figure 3C; Table S1). Therefore, IHC analyses supported the in vitro data and indicated that MEK-ERK pathway regulates EMT-TF reprogramming.

FRA1 Links BRAF Pathway with the Regulatory EMT-TF Network

Zeb2^{-/-} mouse embryos express considerably more ZEB1 in paraxial mesoderm and in neural folds than their wild-type counterparts suggesting that *Zeb1* is ZEB2-regulated (Miyoshi et al., 2006). Likewise, in carcinoma cells, hierarchical reciprocal regulation of EMT-TFs has been reported (Taube et al., 2010; Hugo et al., 2011). These data indicate that regulatory interplay between EMT-TFs may exist also in MM. To address this, we assessed the kinetics of EMT-TF expression in response to MEK inhibition in MM cells. Regulation of all four EMT-TFs was transcriptional, and, importantly, upregulation of *SNAIL2* and *ZEB2* was rapid and apparently preceded repression of *ZEB1* and *TWIST1* (Figure 4A). These data suggested a hierarchy between different EMT-TFs may exist. Indeed, we noted that in some backgrounds depletion of certain individual EMT-TFs affected the expression of the others; i.e., knock-down of *ZEB1* resulted in enhanced expression of *ZEB2* in RPMI-7951

cells (Figure 4B), and depletion of *SNAIL2* upregulated *ZEB1* in A375P and A375M cells (see Figure S6A). Knock-down of *ZEB2* activated *ZEB1* in several MM cell lines including WM-266-4, RPMI-7951, A375P, and A375M (Figures 4B, 4C, and S6A). Moreover, *ZEB2* depletion attenuated downregulation of *ZEB1* by U0126 (Figure 4C). Taken together with our observations that *ZEB2* is replaced with *ZEB1* in melanoma samples (Figures 1 and S1), these data suggest that *ZEB2* acts upstream of *ZEB1* in MEK-EMT-TF pathway.

We next aimed at identifying a molecular link between MEK pathway and EMT-TF network. A double-negative feedback loop involving miR-200 family members and ZEB transcription factors regulates carcinoma cell plasticity. Enhanced expression of the miR-200 family prevails in epithelial cells where it targets *ZEB1* and *ZEB2*, allowing expression of epithelial markers. In mesenchymal cells, an autocrine TGF-β signaling prevents expression of miR-200 and epithelial markers through upregulation of ZEB factors (Brabletz and Brabletz, 2010). Our data indicate that *ZEB1* and *ZEB2* genes are not coregulated in MM, suggesting that the involvement of miR-200 is unlikely. Indeed, miR-200 expression level in melanoma cells was much lower than that in epithelial carcinoma cell lines (Figure 5A). In line with these data, adjunction of miR-200-targeted 3'UTR sequences of *ZEB1* or *ZEB2* to a luciferase reporter impacted on its activity in epithelial, but not in mesenchymal carcinoma or MM cells (Figure S3A).

Given that EMT-TF regulation is transcriptional (Figure 4A), we next evaluated the putative role of FRA1, an AP-1 family member that was required for *ZEB1/2* induction in response

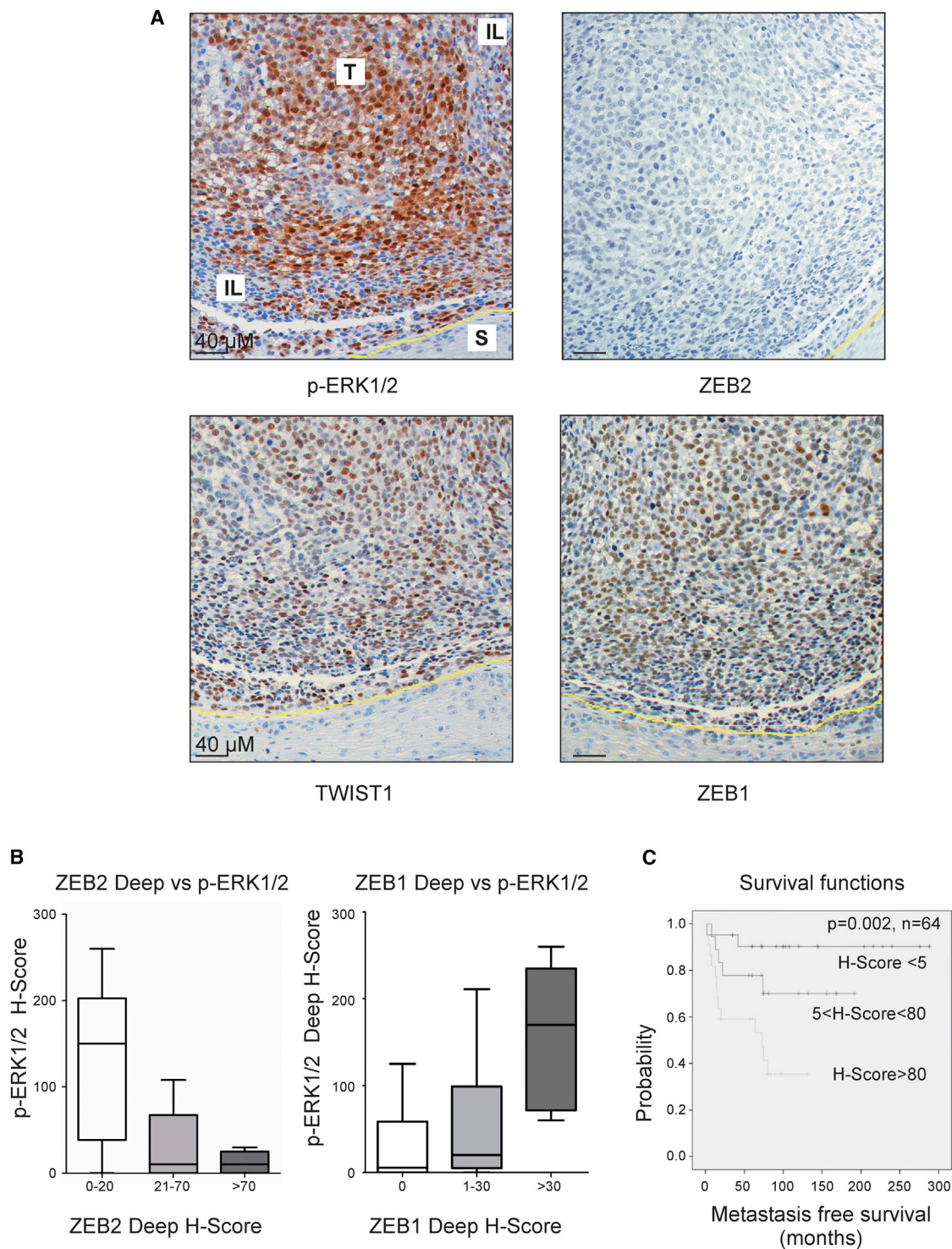


Figure 3. EMT-TF Reprogramming Correlates with ERK Activation

(A) An example illustrating coexpression of phospho-ERK (p-ERK1/2) with ZEB1 and TWIST1 in primary melanoma. T, tumor; IL, infiltrating lymphocytes; S, stroma.

(B) Expression of ZEB2 and ZEB1 correlates with phospho-ERK (p-ERK1/2) in malignant melanomas. Samples (n = 89) were separated in three groups according to the IHC staining for ZEB proteins (H-score) as indicated, and compared with the levels of phospho-ERK. Statistical significance of correlations was determined using Kruskal-Wallis test.

(C) Kaplan-Meier survival analysis of patients with malignant melanoma (n = 64) according to the presence of phospho-ERK in deep areas. Phospho-ERK expression is a highly significant prognostic factor for poor survival.

See also [Figure S2](#).

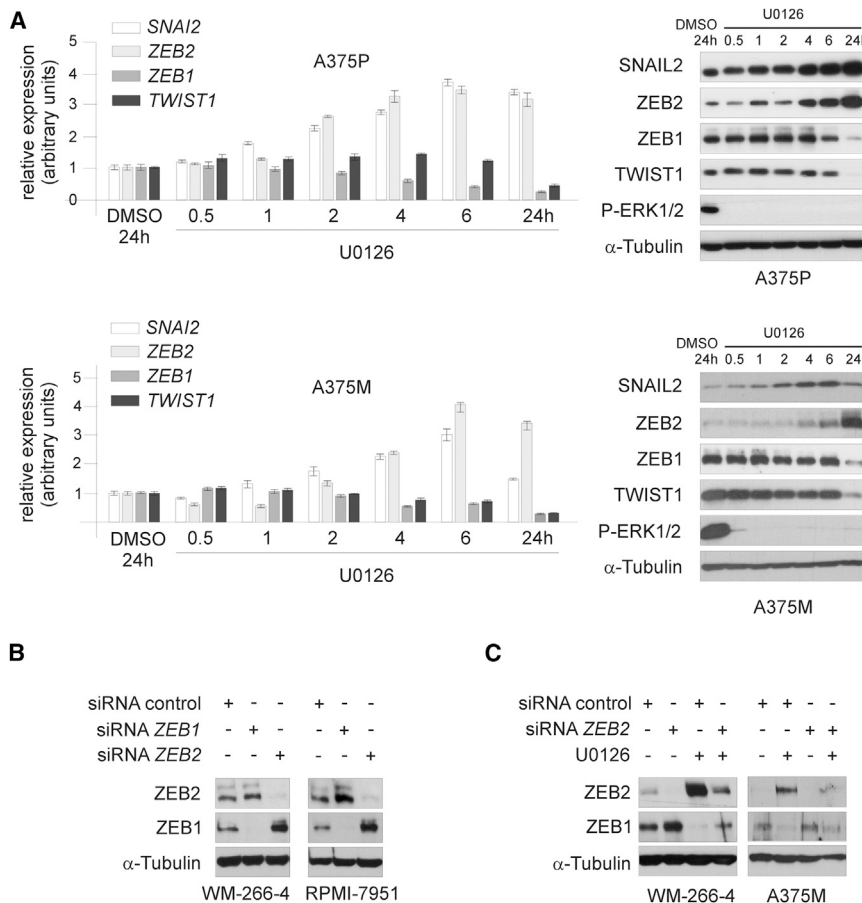


Figure 4. Hierarchical Regulation of EMT-TF Network: ZEB2 Represses ZEB1 in Melanoma Cell Lines

(A) Kinetics of EMT-TF expression was analyzed in A375P and A375M cells treated with 10 μ M U0126 and incubated for different time periods as indicated. EMT-TF expression was analyzed by quantitative PCR (qPCR) (left panels) or immunoblotting (right panels). The results of qPCR analyses are shown as mean $\pm$ SD of triplicate experiments.

(B and C) Melanoma cells were treated with siRNA targeting ZEB1 or ZEB2, left untreated (B), or treated with 10 μ M U0126 or DMSO for 24 hr, as indicated (C) and analyzed by immunoblotting.

2011). Moreover, we characterize a pathway, which operates in melanoma cells and couples *BRAF* and *NRAS* mutations to the EMT-TF network. This pathway is independent of the members of miR-200 family and involves FRA1.

EMT-TF Switch Is a Determinant of BRAF-Induced Transformation

Activation of BRAF or NRAS pathway is a melanoma-initiating event. Therefore, after establishing a link between this pathway and EMT-TF expression, we focused on the role of EMT-TF reprogramming in BRAF-induced transformation. We uncoupled EMT-TF regulation from BRAF mutant in melan-a cells either

to RAS and ERK2 activation in epithelial cells (Shin et al., 2010). In melanocytes and MM cells, FRA1 expression is BRAF-inducible and MEK-dependent (Figure 2), as in other cell types (Casalino et al., 2003). We depleted FRA1 by shRNA in BRAF-transformed melan-a and in A375P MM cells and found that in both cases FRA1 knockdown largely mimicked the effect of MEK inhibition in MM cell lines by upregulating SNAIL2/ZEB2, but downregulating TWIST1 and ZEB1 protein levels (Figure 5B). Next, we aimed to address whether FRA1 has a direct role in transcriptional regulation of EMT-TFs in A375P MM cells. As ZEB1 and ZEB2 genes produce several different transcript variants and utilize alternative promoters (Figure S3B), we employed 5'RACE to identify their transcriptional promoters that are active in MM cells (Figure S3C). Using chromatin immunoprecipitation assay, we detected FRA1 in association with DNA fragments containing a canonical AP-1 binding site localized to the middle part of the ZEB2 transcriptional silencer (Chng et al., 2010) (Figure S3D). Likewise, we identified in vivo FRA1 binding to AP-1 response elements located upstream of transcription start sites of ZEB1, SNAIL2, and TWIST1 genes (Figure S3D). This indicates that FRA1 can directly modulate transcription of EMT-TF-encoding genes downstream of MEK-ERK pathway.

Overall, these data demonstrate mutual regulation of EMT-TFs in MM cells, which resembles complex interactomes reported in EMT models in carcinoma cells (Taube et al., 2010; Hugo et al.,

by ectopic expression of each of four EMT-TFs, or by shRNA-mediated depletion of Zeb1 or Twist1 and examined the transforming activity of BRAF mutant in these conditions. All conditions impacted neither ERK activation nor cell proliferation (data not shown). However, ectopic expression of SNAIL2 or ZEB2, as well as depletion of ZEB1 or TWIST1 reproducibly reduced the oncogenic potential of BRAF as evidenced by the decreased number of colonies formed on soft agar (Figure 6A). In contrast, exogenous ZEB1 or TWIST1 facilitated BRAF-driven transformation of melan-a cells (Figure 6B). In line with these data, in vivo experiments demonstrated that uncoupling BRAF activation from EMT-TF reprogramming either inhibited or delayed tumor development when cells were xenografted in immunocompromised mice (Figure 6C). Data indicate that although SNAIL2 and ZEB2 display tumor suppressor properties, TWIST1 and ZEB1 cooperate with BRAF in melanocyte transformation.

Consistent with the role of FRA1 in orchestrating the EMT-TF switch, its depletion in melan-a/ Δ BRAF-ER cells reduced colony formation, an effect that could be partially rescued by ectopic expression of ZEB1 or TWIST1 (Figure 6D). To corroborate these results we assessed the effect of ZEB2 and SNAIL2 on tumorigenicity of established MM cell lines. In agreement with the data obtained in melan-a cells, simultaneous ectopic expression of ZEB2 and SNAIL2 reduced colony formation potential and in vivo tumorigenicity of both A375P and A375M cell lines (Figure S4).

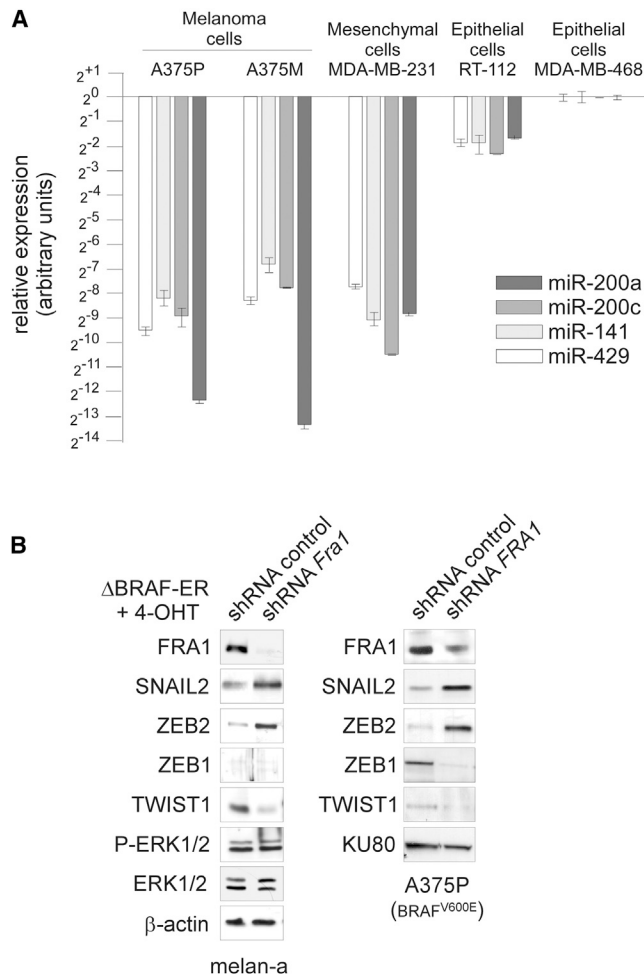


Figure 5. The Switch in EMT-TFs Is miR-200-Independent, but FRA1-Dependent in Melanoma Cells

(A) qPCR analyses of the expression of miR-200 family members in melanoma (A375P and A375M), mesenchymal carcinoma (MDA-MB-231) or epithelial carcinoma (RT112 and MDA-MB-468) cell lines. Data show mean $\pm$ SD of a triplicate experiment.

(B) EMT-TF reprogramming is FRA1-dependent in BRAF-transformed melanocytes and MM cell lines. Tamoxifen (4-OHT)-treated melan-a/ Δ BRAF-ER cells or A375P cells were either infected with a virus expressing shRNA targeting FRA1, or mock infected. Protein expression was analyzed by immunoblotting.

See also Figure S3.

Therefore, SNAIL2 and ZEB2 not only impair BRAF-induced melanocyte transformation, but also have a tumor-suppressive role in established melanoma cell lines.

EMT-TF Switch Affects Melanocyte Differentiation Program

To explore a mechanism by which EMT-TF reprogramming contributes to BRAF-induced transformation we performed gene expression profiling of melan-a-derived cell lines expressing active Δ BRAF-ER alone or in combination with ectopic SNAIL2, ZEB2, TWIST1, or ZEB1. Clustering of the individual EMT-TF gene expression profiles based on their similarity to each other has revealed that ZEB1/TWIST1 and ZEB2/SNAIL2 generated

two pairs of dissimilar gene expression signatures (Figure 7A). Melan-a transformation is associated with the downregulation of *Mitf* and the subsequent inhibition of its target genes, including tyrosinase, an enzyme-controlling melanin production (Wellbrock and Marais, 2005). Beyond its role in cell transformation, subtle regulation of MITF expression is believed to determine the differentiated/proliferative/invasive phenotypic switches occurring in melanomagenesis (Hoek and Goding, 2010). As expected, BRAF activation was associated with the downregulation of *Mitf* expression (Figure 2A) and the repression of genes regulating melanocyte differentiation downstream of MITF (e.g., *Tyr*, *Si*, *Mreg*, *Typr1*, *Trpm1*, *Slc45a2*, *Dct*, *Gpr143*). Enforced expression of SNAIL2 or ZEB2 restored *Mitf* and MITF-target gene expression supporting the assumption that these two EMT-TFs play a role in MITF-driven melanocyte differentiation program. Conversely, ZEB1 and TWIST1 cooperated with the BRAF oncoprotein in downregulating *Mitf* and other differentiation markers (Figures 7A, S5A, and S5B). Consistent with these data, rare soft agar colonies and xenografted tumors generated from melan-a/ Δ BRAF-ER cells ectopically expressing SNAIL2/ZEB2 or depleted of *Zeb1*/*Twist1* were more pigmented than those obtained with the parental cell line (see Figures 6A and 6C). In agreement with the data generated in melan-a cells, siRNA-mediated knockdown of *SNAIL2* or *ZEB2* reduced MITF protein levels in A375 and WM-266-4 melanoma cell lines (Figure 7B). Furthermore, ectopic expression of ZEB1 in ZEB1-negative melanoma cells (SK-MEL-5 and UACC-257) produced a similar effect (Figure 7B). Thus, the repertoire of EMT-TFs in melanoma cells regulates the level of MITF, and EMT-TF reprogramming couples the activation of BRAF-FRA1 and NRAS-FRA1 oncogenic pathways to dedifferentiation and malignant transformation.

The Switch in EMT-TFs Is Associated with the Gain in Invasive Properties and Loss of E-Cadherin

Gene expression analysis of melan-a-derived cells additionally demonstrated that TGF- β -regulated genes (*Bgn*, *Col5A1*, *Fbn1*, *IL6*, *Lox*, *Tagln*, *Thbs1*) (Hoek et al., 2006) and invasion-associated genes (*Mmp1a*, *Mmp3*, *Mmp13*, *Sparc*) were repressed in ZEB2/SNAIL2 group, but activated by TWIST1 and ZEB1 in combination with the BRAF mutant (Figures 7A, S5C, and S5D). Consistent with these data, ectopic expression of TWIST1 or ZEB1 activated, whereas ZEB2 or SNAIL2 repressed, matrigel invasion of melan-a/ Δ BRAF-ER (Figure 8A). Furthermore, although combined knockdown of ZEB1 and TWIST1 in A375P and A375M MM cells decreased their invasiveness, depletion of *SNAIL2* and *ZEB2* had no effect (Figure 8B). Overall, these data point to a role of EMT-TF reprogramming in control of MM cell invasion.

E-cadherin, a prototypical target of SNAIL, TWIST, and ZEB family members, is often downregulated at the invasive front in carcinomas as a result of EMT (Thiery et al., 2009; Brabletz and Brabletz, 2010). Loss of membranous E-cadherin has been documented also in vertical growth-phase melanomas at deep sites in the dermis and in metastatic nodules predominantly in medullar parts (Sanders et al., 1999; Andersen et al., 2004; Alexaki et al., 2010), i.e., in the areas where we identified the EMT-TF switch. To establish a link between EMT-TF reprogramming and loss of E-cadherin in melanoma, we assessed the

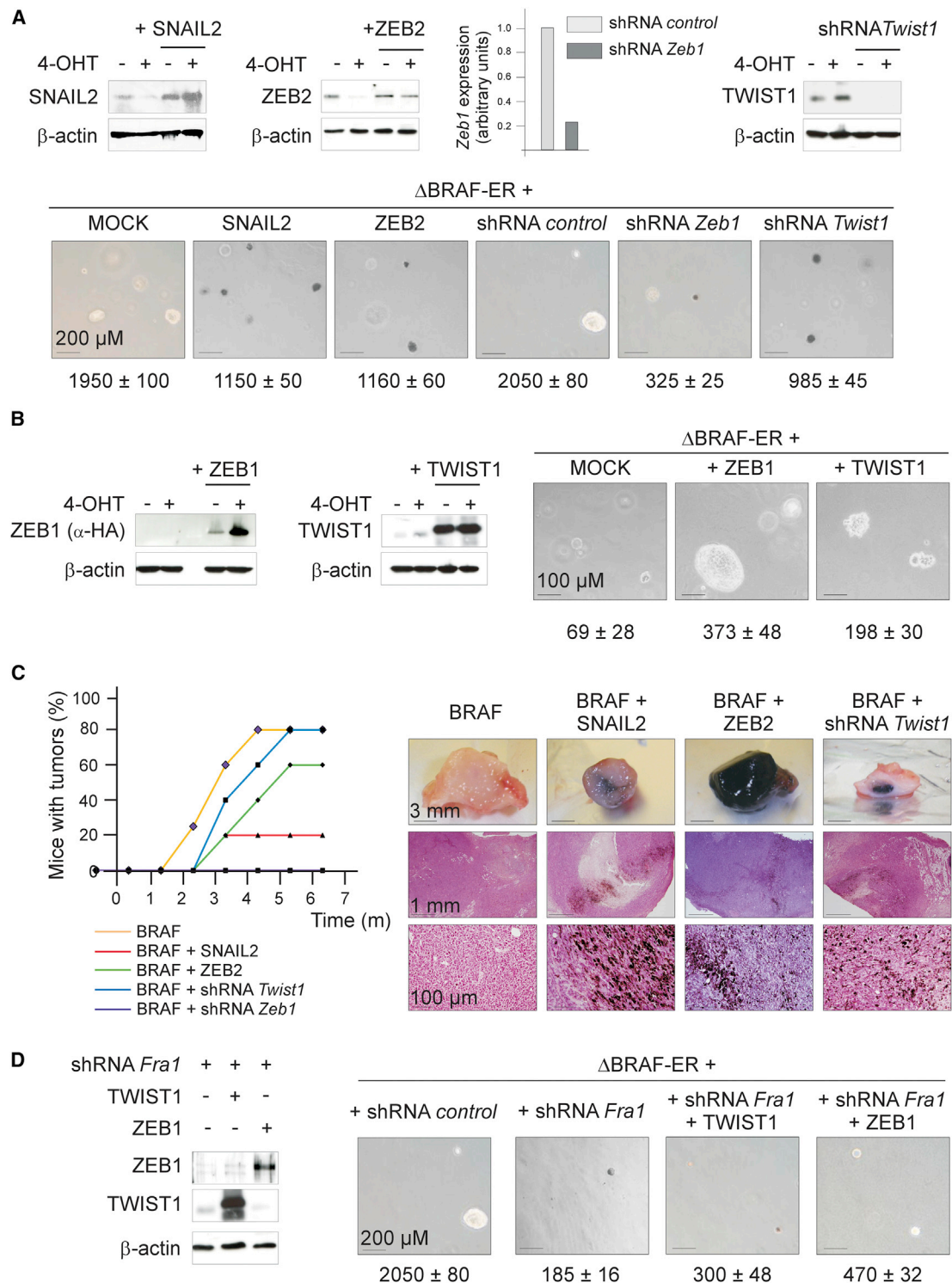


Figure 6. EMT-TF Switch Cooperates with BRAF in Malignant Transformation

(A) EMT-TF expression was modulated by infecting melan-a/ Δ BRAF-ER cells with viral vectors expressing either SNAIL2 or ZEB2. In parallel experiments, *Zeb1* or *Twist1* expression was reduced by shRNA. The BRAF kinase has been activated with 4-OHT 2 weeks before plating. EMT-TF expression was assessed by immunoblotting or qPCR as indicated (upper panels). Colony formation of 4-OHT-treated cells was analyzed, and the results of triplicate experiments ($\pm$ SD) are shown (lower panels).

(B) Melan-a/ Δ BRAF-ER cells were infected with vectors harboring ZEB1 or TWIST1. The BRAF kinase has been activated with 4-OHT immediately before plating. Expression of ZEB1 and TWIST1 was analyzed by immunoblotting (left panels). Colony formation of 4-OHT-treated cells was analyzed, and the results of triplicate experiments ($\pm$ SD) are shown (right panels).

(legend continued on next page)

effect of transient depletion of individual EMT-TFs on E-cadherin in A375P and A375M melanoma cell lines. We found that, among the EMT-inducers, ZEB1 was the only protein required for the repression of the *CDH1* gene in this cellular context (Figure S6A). Consistent with these data, ZEB1, but not ZEB2 depletion-activated wild-type *CDH1* gene promoter, whereas a reporter driven by the promoter with mutagenized E-boxes was insensitive to ZEB1 knockdown (Figure S6B). To substantiate these findings, we transiently expressed ZEB1 in UACC-257 cells expressing E-cadherin, SNAIL2, and ZEB2 (Figure S6C) and observed inhibition of E-cadherin expression in cells positive for ZEB1 (Figure S6D). In agreement with the results of these experiments, no coexpression of E-cadherin and ZEB1 was observed in a panel of melanoma cell lines (Figure S6E).

Next, we assessed whether the expression of E-cadherin and ZEB1 correlated in tumor samples. Given that loss of E-cadherin has been reported in nodal metastases, we analyzed a cohort of ten matched pairs of primary tumors and corresponding metastases by IHC. E-cadherin immunoreactivity revealed a homogeneous staining in superficial areas of metastatic primary melanomas and reduced level in deeper parts in some specimens. Likewise, we observed a gradient of stronger cortical staining for E-cadherin compared to the medullary sites in nodal metastases, where in some areas E-cadherin was entirely lost. Out of ten primary tumors with strong membrane-bound E-cadherin immunoreactivity, eight showed reduced expression in the corresponding metastases. ZEB1 was detected in 20% of primary melanomas (deep areas) and in 40% of paired metastases (medullary metastatic deposits). Strikingly, ZEB1-positive tumor areas showed lack of E-cadherin expression in all cases (Figure 8C). This mutually exclusive pattern of ZEB1 and E-cadherin expression strongly indicates a critical role of ZEB1 in EMT in melanocytic lesions. Expression of ZEB2 was detected in superficial areas of all primary melanomas and in 60% of corresponding metastases. ZEB1-positive tumor cells with absent E-cadherin showed negative ZEB2 protein expression, but in ZEB1-negative melanomas E-cadherin and ZEB2 were coexpressed (Figure 8C). In agreement with these data, downregulation of E-cadherin was concomitant with EMT-TF reprogramming in BRAF^{V600E}-transduced primary human melanocytes (see Figure 2A). Taken together, our data show that in addition to its role in cell transformation, EMT-TF reprogramming contributes to loss of E-cadherin and activation of cell invasion in advanced melanoma.

DISCUSSION

In epithelial backgrounds, EMT-TFs cooperate with mitogenic oncoproteins in malignant transformation, tumor progression, and metastatic dissemination (Ansieau et al., 2008; Thierry

et al., 2009; Rhim et al., 2012). Here, we demonstrate that in a neural crest-derived malignancy, MM, the cross-talk between EMT-TFs and oncogenic pathways has different configuration. We show that normal melanocytes express two EMT-TFs, SNAIL2 and ZEB2, but MM-driving ERK-activating oncogenic pathways induce EMT-TF reprogramming, which involves downregulation of *SNAIL2* and *ZEB2* and upregulation of *TWIST1* and *ZEB1*.

In vitro, we detected EMT-TF reprogramming in untransformed melanocytes in response to the BRAF activation. In melanoma cell lines, MEK inhibition downregulated *ZEB1* and *TWIST1*, but upregulated *ZEB2* and *SNAIL2* indicating that continual MEK-ERK signaling is required to maintain the reconfigured pattern of EMT-TF expression. By the analyses of EMT-TF expression in melanoma samples, we detected EMT-TF switch at late stages of MM progression in deep sites of primary tumors and in medullary metastatic deposits in lymph nodes. High expression of ZEB1 and decreased levels of ZEB2 significantly correlated with high phospho-ERK expression. Remarkably, EMT-TF reprogramming and phospho-ERK immunopositivity represented poor prognostic factors in melanoma patients.

MEK-ERK pathway activation is the central event driving the development of MM. Given high frequencies of mutually exclusive MEK-ERK-activating mutations in *BRAF* (61%), *NRAS* (20%) (Hodis et al., 2012), and a G protein-coupled receptor gene *GRM3* (16.3%) (Prickett et al., 2011), this pathway seems to be activated in most melanocytic lesions including benign nevi. However, there is a documented discrepancy between mutational status of the pathway and phospho-ERK immunopositivity, with MAPK activity being much higher in MM than in common acquired nevi (Saldanha et al., 2004; Uribe et al., 2006; Venesio et al., 2008; Yazdi et al., 2010). This is likely to be caused by the activation of different BRAF- or NRAS-dependent negative feedback loops, such as IGFBP7 or an MEK-inducible dual specificity phosphatases (DUSPs), which repress the MEK-ERK pathway (Wajapeyee et al., 2008; Li et al., 2012; Martin et al., 2012). Diverse mechanisms such as amplification of mutant *BRAF* or *NRAS* alleles, acquired mutations in genes encoding MEK1/2 or activation of EGFR signaling may bypass suppression of MEK-ERK activity at later stages of MM (Udart et al., 2001; Nikolaev et al., 2012). Our data indicate that EMT-TF composition is dependent on activating mutations in *BRAF* or *NRAS* oncogenes, but dynamically regulated thereafter during MM progression mirroring the phospho-ERK expression pattern (Figure 8D).

We also addressed molecular mechanisms linking MEK-ERK pathway with EMT-TF reprogramming. In epithelial cells undergoing EMT, EMT-TFs are upregulated in hierarchical manner. In some EMT models, SNAIL1, SNAIL2, and TWIST1 upregulate

(C) Melan-a/ Δ BRAF-ER cells with the reduced or increased expression of individual EMT-TFs were grafted in immunocompromised mice ($n = 7$). The graph shows the appearance of tumors in each group of mice (left panels). Photographs illustrate high pigmentation of tumors with reduced TWIST1 or increased SNAIL2 and, especially, ZEB2 (right panels).

(D) FRA1 contributes to the oncogenic potential of BRAF. BRAF signaling was induced by 4OH-T treatment in melan-a/ Δ BRAF-ER cells depleted of FRA1 and expressing or not exogenous ZEB1 or TWIST1. As positive control, we used 4-OHT-treated melan-a/ Δ BRAF-ER cells infected with the control vector. Ectopic expression of ZEB1 and TWIST1 was confirmed by immunoblotting (left panels). Oncogenicity was assessed in soft agar colony assay. The data are means $\pm$ SD of triplicate experiments (right panels).

See also Figure S4.

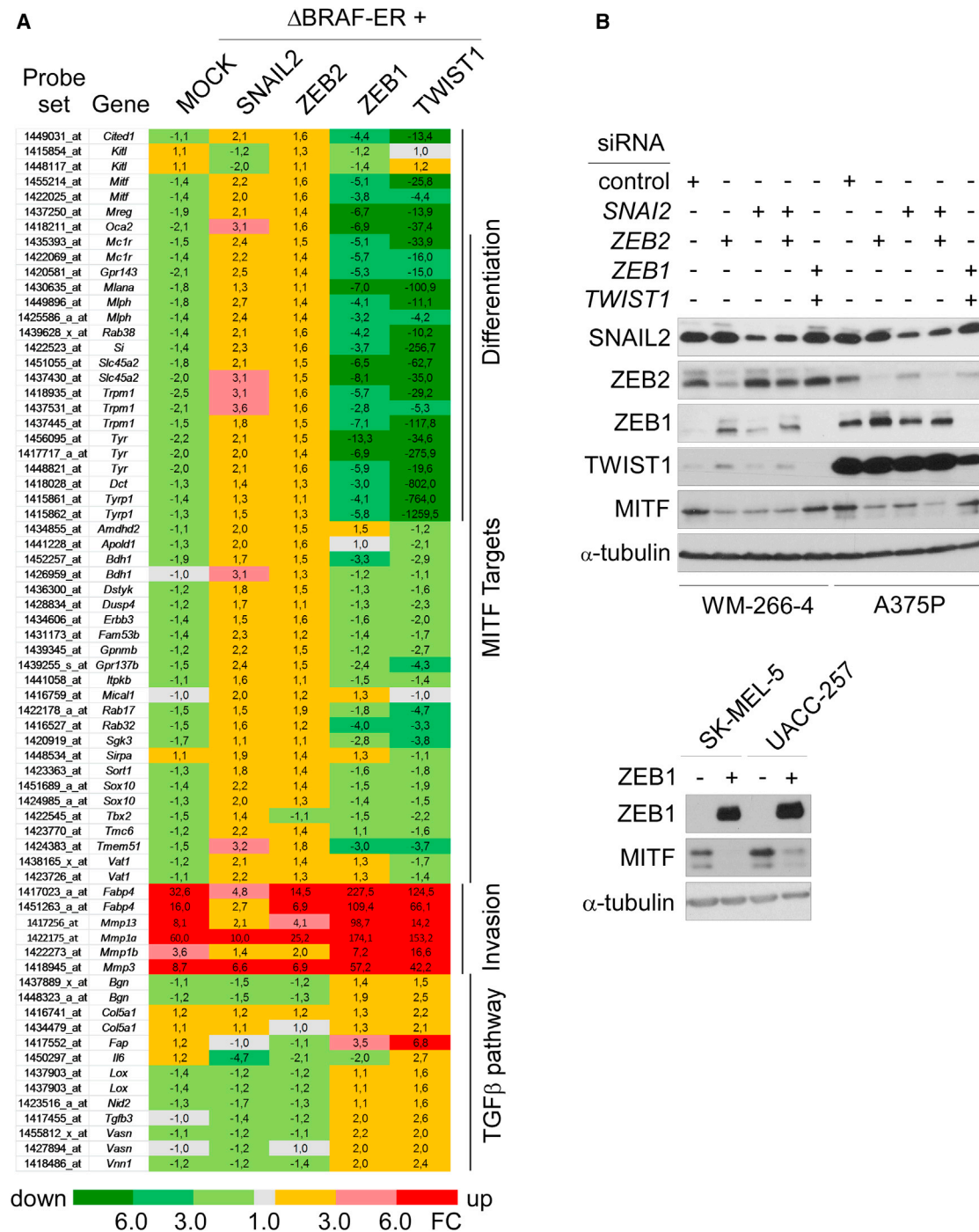


Figure 7. EMT-TF Switch Affects Gene Expression Programs

(A) Genes differentially regulated by ZEB1/TWIST1 and ZEB2/SNAIL2 in melan-a/ Δ BRAF-ER cells. Melan-a/ Δ BRAF-ER cells were infected with either SNAIL2, ZEB2, ZEB1, or TWIST1 retroviral expression vectors, treated with 4-OHT for 1 week, and processed for gene expression profiling. Fold changes are relative to control melan-a/ Δ BRAF-ER cells maintained in the absence of 4-OHT.

(B) The effects of EMT-TFs on MITF expression in MM cell lines. Melanoma cell lines were depleted of EMT-TFs by siRNAs (top panel), or ZEB1 was ectopically expressed in ZEB1-negative cell lines SK-MEL-5 and UACC-257 (bottom panel). EMT-TF expression and the expression of MITF was analyzed by immunoblotting.

See also Figure S5.

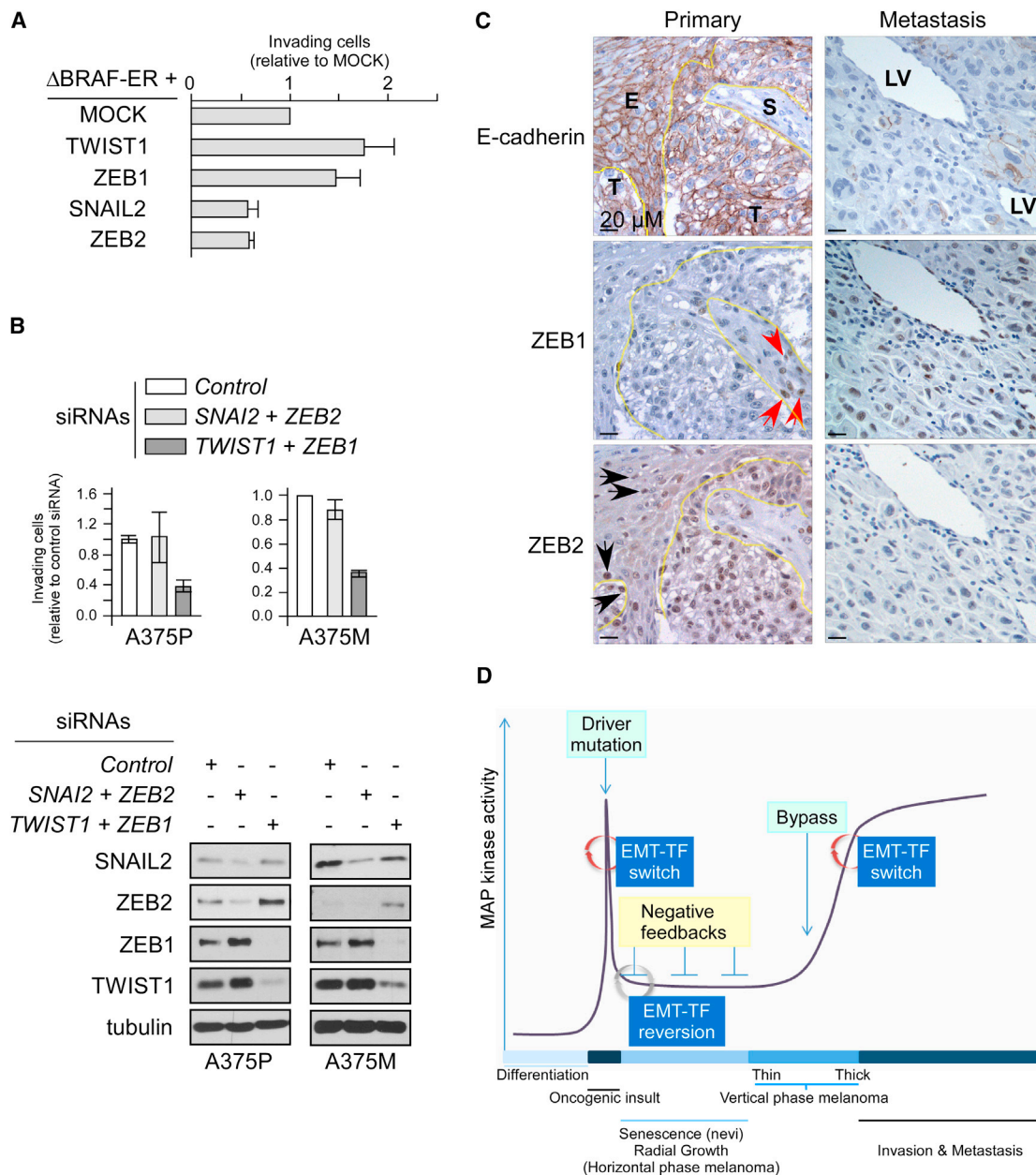


Figure 8. The EMT-TF Switch Is Associated with Increased Invasion and Loss of E-Cadherin

(A) Assessment of invasive potential of melan-a/ Δ BRAF-ER-derived cells in matrigel-coated BD transwells. Melan-a/ Δ BRAF-ER cells were infected with either SNAIL2, ZEB2, ZEB1, or TWIST1 retroviral expression vectors and treated with 4-OHT for 1 week. Fold changes in invasion relative to MOCK-infected melan-a/ Δ BRAF-ER cells in the presence of 4-OHT are shown. Results are shown as the mean $\pm$ SD of triplicate experiments.

(B) Simultaneous knockdown of TWIST1 and ZEB1, but not of SNAIL2 and ZEB2, reduces invasion of A375P or A375M cells into matrigel. The invasion was normalized to that of the cells transfected with control siRNA. Bars represent the mean $\pm$ SD of three independent experiments.

(C) Examples of IHC analyses demonstrating an in vivo correlation between ZEB1, ZEB2, and E-cadherin expression in primary melanoma (left panels) and matched metastases (right panels). T, tumor; S, stroma; E, epidermis; LV, lymphatic vessel. ZEB1-positive cells observed in stromal areas are either invading tumor cells, which underwent EMT-like reprogramming, or stromal components. These cells are marked with red arrows. Black arrows designate melanocytes located in the epidermis.

(D) A hypothetical scheme illustrating modulation of MAPK activity and EMT-TF reprogramming in the time course of tumor progression. Initial activation of MAPK is caused by gain of function MM-driving mutations (BRAFV600E, NRASQ61L, etc.) MAPK activity is suppressed in nevi and in radial growth-phase melanoma through negative feedback loops. The pathway is reactivated in advanced cancer via bypass mechanisms. An early EMT-TF switch is immediately induced by oncogenic mutation, contributes to the neoplastic transformation, and is possibly involved in early dissemination. A late EMT-TF reprogramming is dependent on continual MAPK signaling and leads to loss of E-cadherin and enhanced cell invasion. We hypothesize that the suppression of MAPK activity in nevi or horizontal-phase melanoma causes EMT-TF reversion (suppression of ZEB1 and TWIST1 and reactivation of ZEB2 and SNAIL2).

See also Figure S6.

ZEB1 and *ZEB2* (Taube et al., 2010; Hugo et al., 2011; Dave et al., 2011). In other systems, *TWIST1* is upstream of *SNAIL* family members activating *SNAI2* and *SNAI1* (Casas et al., 2011). The interaction between EMT-TFs is complex also in MM, where we demonstrate their mutual repression. We propose that reciprocal regulation of ZEB proteins in MM mediates dynamic EMT-TF switches in course of melanoma progression (Figure 8D). A regulatory feedback loop involving miR-200 family members and TGF- β is a key mechanism that controls *ZEB1* and *ZEB2* mRNA levels in carcinoma cells (Brabletz and Brabletz, 2010; Hill et al., 2013). However, in MM cells, miR-200 are present at very low levels and not functional. We believe that lack of miR-200 expression (possibly as a result of epigenetic silencing) is in large responsible for the differences in the configurations of EMT-TF networks in MM and epithelial cells. We show that the reorganization of the EMT-TF network in MM is dependent at least in part on *FRA1*, one of the key effectors of the RAS-MEK pathway (Vial et al., 2003). The crosstalk between *FRA1* and EMT-TFs appears to be complex and implies both transcriptional and posttranslational mechanisms (data not shown). However, *FRA1* is associated with regulatory elements within EMT-TFs genes-containing chromatin, and, therefore, it has a potential to directly regulate EMT-TF expression.

We elucidated the functional outcome of EMT-TF reprogramming in MM cells. EMT-TF network plays a more complex role in MM than in epithelial backgrounds, with different EMT-TFs having antagonistic functions. Specifically, although *ZEB1* and *TWIST1* contribute to the malignant transformation, *SNAIL2* and *ZEB2* act as oncosuppressive proteins. Recently, *ZEB2* mRNA was shown to suppress MM growth by enhancing *PTEN* expression in microRNA-dependent manner (Karreth et al., 2011). However, we did not observe any changes in *PTEN* expression levels upon *ZEB2* downregulation in either melan-a or A375M cells (data not shown). This implies that different mechanisms may underlie oncosuppressive properties of *ZEB2*, and our data indicate that antagonistic functions of EMT-TFs in MM correlate with their opposing effects on *MITF* expression and function.

There are several lines of genetic evidence supporting the link between *SNAIL2* and *ZEB2* to *MITF* and melanocyte differentiation (Sánchez-Martín et al., 2002; Van de Putte et al., 2007; Liu et al., 2009). In agreement with our findings, a recent study demonstrated coexpression of *MITF* and *SNAIL2* in MM samples (Shirley et al., 2012). A mechanism by which EMT-TFs regulate *MITF* transcription remains undefined. We failed to detect any effects of EMT-TFs on *MITF* promoter activity in reporter assays (data not shown), suggesting an indirect effect similar to that described for *ATF2* (Shah et al., 2010). The role of *MITF* is not limited to melanoma initiation, and currently its function is being intensively discussed in the context of the mechanisms underlying cell plasticity in MM (Cheli et al., 2010, 2011; Bell and Levy, 2011; Bertolotto et al., 2011). According to the phenotypic plasticity model, the highest *MITF* expression maintains differentiated status of cell-cycle arrested melanocytes. Reduced *MITF* expression results in the transition to proliferative stage, whereas further decrease in the *MITF* level generates invasive and slow-proliferating cells with tumor-initiating properties (Hoek and Goding, 2010). Our data suggest that EMT-TF network operates upstream of *MITF* to control cell plasticity during melanomagenesis. Indeed, *ZEB1* and *TWIST1* induce TGF- β - and invasion-

associated gene signatures in concert with downregulating *MITF*. In line with these findings, *ZEB1* and *TWIST1* stimulate cell invasion; and E-cadherin loss, an attribute of aggressive melanoma, is associated with elevated *ZEB1*.

Of note, nodal metastatic deposits recapitulated the same EMT-TF and phospho-ERK expression gradients that we observed in primary tumors. This may indicate that late-stage MM cells retain the capability to reconstitute “differentiation pattern” of EMT-TF expression (*ZEB2*^{high}/*SNAIL2*^{high}/*ZEB1*^{low}/*TWIST1*^{low}), and MEK-ERK-dependent EMT-TF switches determine tumor cell plasticity during metastatic process via the regulation of *MITF* expression. Alternatively, similar EMT-TF expression patterns in primary and secondary tumors imply that cancer cells may populate lymph nodes prior the feedback mechanisms suppressing MEK-ERK signaling are activated. Then, primary melanomas and lymph node metastases evolve separately, but pass through similar cycles of EMT-TF reprogramming. This explanation is within the framework of a theory suggesting parallel progression of epithelial tumors and metastases (Hüsemann et al., 2008; Ansieau et al., 2008; Stoecklein et al., 2008; Rhim et al., 2012). *ZEB1*^{high}/*TWIST1*^{high} MM cells residing in medullary parts of lymph nodes may represent the source for secondary wave of metastatic dissemination. In line with this suggestion, medullary invasion is predictive of distant metastases and poor survival in patients with MM (Scolyer et al., 2008). On the other hand, our finding that *SNAIL2* and *ZEB2* are highly expressed in cortical areas of lymph node metastases is in agreement with a previous study linking *SNAIL2* with high metastatic propensity of MM (Gupta et al., 2005).

EMT in epithelial tumors is a reversible process, and metastatic carcinoma cells undergo MET in order to colonize distant organs (Korpál et al., 2011; Tsai et al., 2012; Ocaña et al., 2012). It is plausible to speculate that the scenario is similar in MM: melanoma cells cycle between differentiated (*ZEB2*^{high}/*SNAIL2*^{high}/*MITF*^{high}/E-cadherin^{high}) and oncogenic (*ZEB1*^{high}/*TWIST1*^{high}/*MITF*^{low}/E-cadherin^{low}) states to accomplish metastatic process.

Recent clinical trials in patients with BRAF mutant melanoma have shown that the acquired resistance to BRAF-selective inhibitors is an important clinical challenge. In many cases, the mechanism of the resistance was based on the restoration of MEK activity (Emery et al., 2009; Villanueva et al., 2010). Therefore, targeting EMT-TF network downstream of MEK represents an attractive strategy for treatment of MM. Given that *FRA1* plays an important role in EMT-TF reprogramming and is required for melanocyte transformation by BRAF, molecular pathways regulating *FRA1* activity in MM appear as appealing targets for future therapies.

EXPERIMENTAL PROCEDURES

All detailed information on experimental procedures and reagents is provided in the Supplemental Experimental Procedures.

Human Samples and IHC

Melanocytic tissues were obtained from the consenting patients through the Histopathology and Dermatology Departments, University Hospitals of Leicester. Leicestershire Ethics Committee approval was obtained for the tissue analysis (“Molecular pathology of malignant melanoma” REC Ref6791). Histological sections and formalin-fixed paraffin embedded tissue

blocks were retrieved from the archive. The primary tumors (n = 142) were from the lower extremities (21.4%), the trunk (39.8%), the upper extremities (17.3%), the head and neck (16.3%), and acral (4.1%). Of the 51 metastases, 76% were in lymph nodes, and the remaining metastases were in skin or subcutaneous tissue. In addition, 26 common acquired nevi were analyzed. Demographic information on the MM cases included age at primary excision, gender, time to last follow-up visit/clinical metastasis. Stage was determined from the Breslow thickness and ulceration of the primary tumor.

Mouse Injection

Single cell suspensions of melan-a derivatives or A375 MM cell lines ($1-2 \times 10^6$ cells) in PBS/Matrigel (BD Biosciences) (1/1) were injected subcutaneously in the flank of 8-week-old female athymic Swiss nude mice (Charles River Laboratories). Tumor incidence and growth was monitored during different time periods postinjection. Tumors grew up to 1.5 cm in diameter, at which point animals were euthanized. Each tumor was dissected, fixed in formalin, and processed for histopathology examination.

Mice were housed and bred in a specific pathogen-free animal facility “AniCan” at the CRCL, Lyon, France. The experiments were performed in accordance with the animal care guidelines of the European Union and French laws and were validated by the local Animal Ethic Evaluation Committee (CECCAPP).

Statistics

Expression gradients within and between the lesions were analyzed by Wilcoxon matched pairs test and Jonckheere-Terpstra trend test, respectively. Unpaired data were compared by the Mann-Whitney U test or Kruskal-Wallis one-way ANOVA. Paired data were analyzed by the Page L test (Unistat Statistical Package, version 5.0, Unistat) and interrelationships were investigated by linear regression analysis (Stata software package, version 7.0, Stata). Metastasis was investigated by Kaplan-Meier analysis (Unistat) of H-score, which were compared by the log rank Mantel-Haenzel (Peto) test, and by univariate and multivariate Cox regression (Stata).

ACCESSION NUMBERS

The GEO database accession number for the results of microarray experiments reported in this paper is 39030.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.ccr.2013.08.018>.

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Supplemental Information

A Switch in the Expression of Embryonic EMT-Inducers Drives the Development of Malignant Melanoma

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Inventory of Supplemental Information

Supplemental data

Figure S1, related to Figure 1

Table S1, related to Figure 1

Figure S2, related to Figure 3

Figure S3, related to Figure 5

Figure S4, related to Figure 6

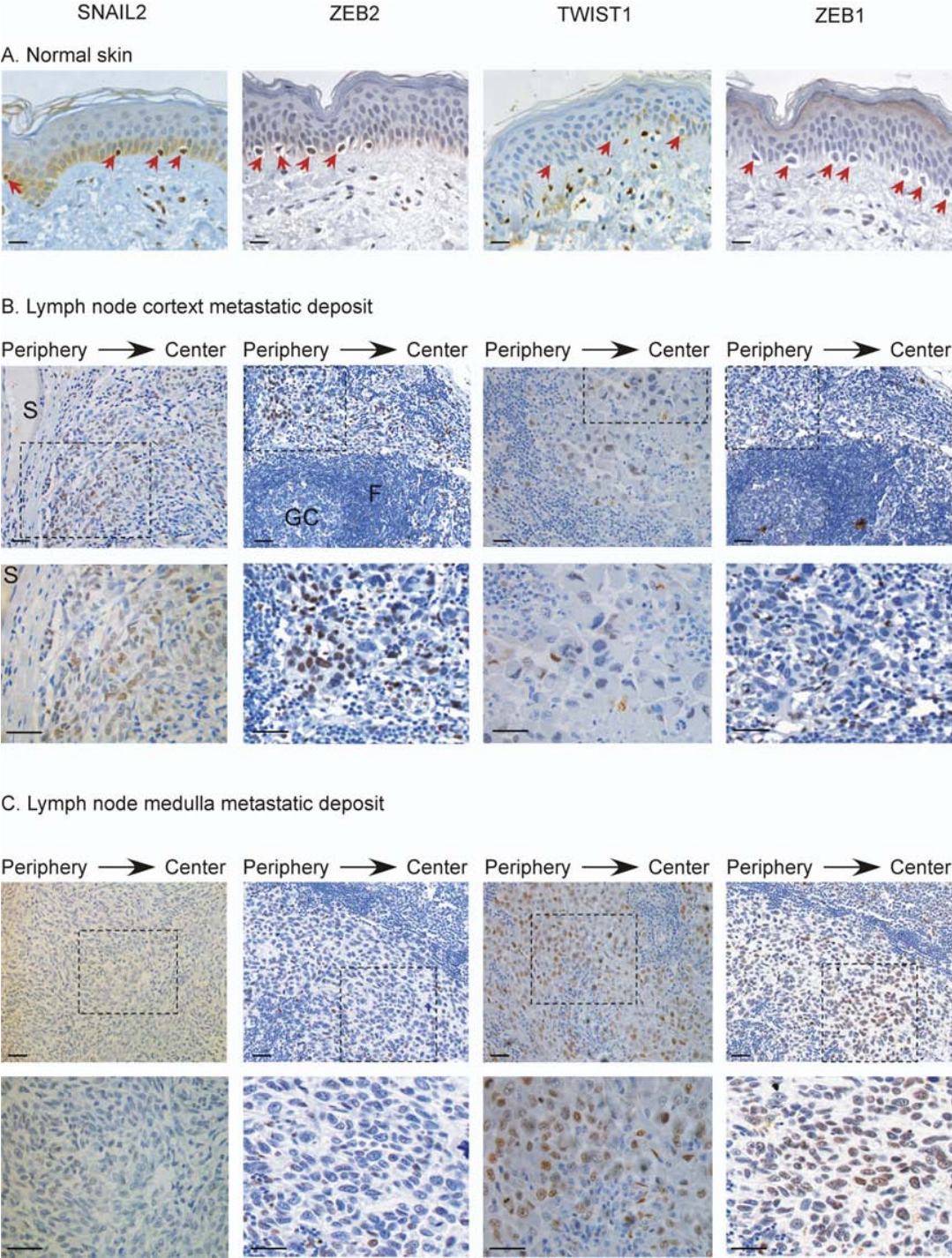
Figure S5, related to Figure 7

Figure S6, related to Figure 8

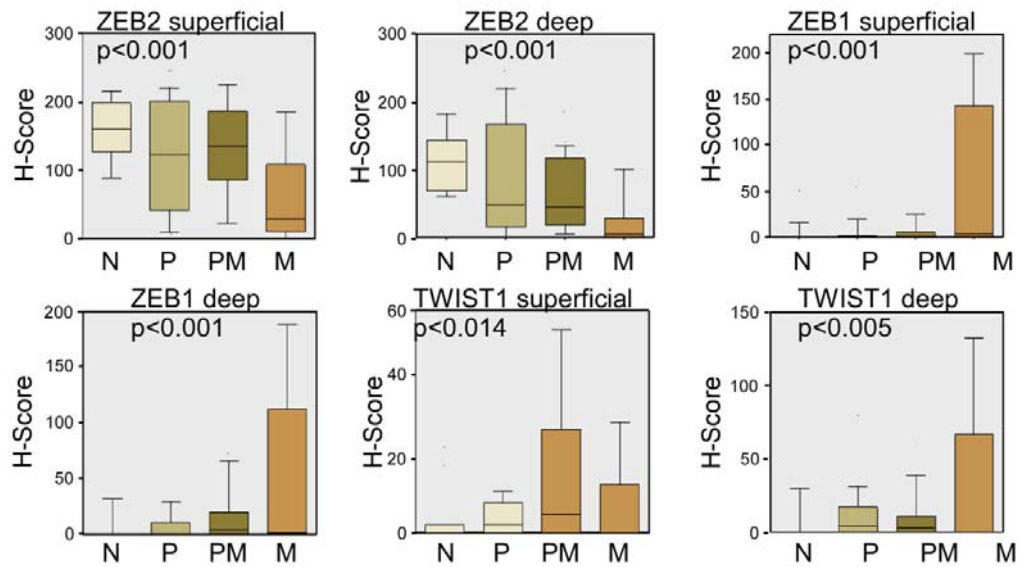
Supplemental experimental procedures

Supplemental references

SUPPLEMENTAL DATA



D



E

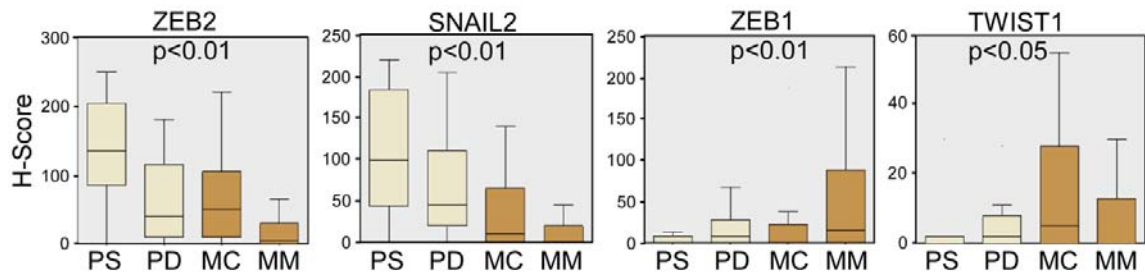


Figure S1, related to Figure 1.

Opposing trends of EMT-TF expression in melanocytic lesions.

(A) IHC analysis of EMT-TF expression in the skin.

Melanocytes embedded in the basal layer of the epidermis are indicated by red arrows. Note that melanocytes are positive for ZEB2 and SNAIL2, but negative for ZEB1 and TWIST1. Keratinocytes do not express EMT-TFs. Scale bar = 40 μ m.

(B, C) Examples of IHC analyses of EMT-TF expression in lymph nodal metastases in patients with advanced MM. Cortical (B) and medullar (C) regions of affected lymph nodes are shown. Images within boxed areas represent predominantly tumor cells and are shown at 40x magnification in adjacent photomicrographs. F, follicles; GC, germinal center; S, subcapsular sinus. Scale bar = 40 μ m.

(D, E) Box and whisker plots of EMT-TF IHC staining (H-Score) for MM independent progression series (D) or matched primary tumor/metastatic lesions series (E). Staining was measured at superficial and deep sites for each melanocytic lesion. Error bars represent the 10th and 90th percentiles. p values represent the significance of a trend test using Jonckheere-Terpstra trend test for the independent series (D) and the Page L trend test for the matched series (E). N, nevi; P, primary melanoma; PM, metastatic primary melanoma; M, metastases. PS, superficial areas in primary melanomas; PD, deep areas in primary melanomas; MC, cortical metastatic deposits in lymph nodes; MM, metastatic deposits in medullary areas. Note opposing gradients of ZEB2 and SNAIL2 versus ZEB1 and TWIST1 expression within the lesions.

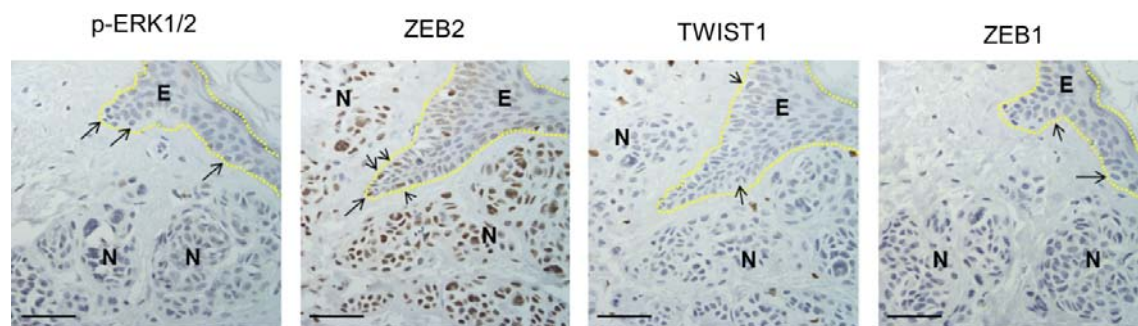
Table S1, related to Figure 1.

Univariate and multivariate Cox proportional hazard analysis for clinical prognostic factors, age, gender, tumor Breslow depth and ulceration with EMT-TF and phospho-ERK immunostaining.

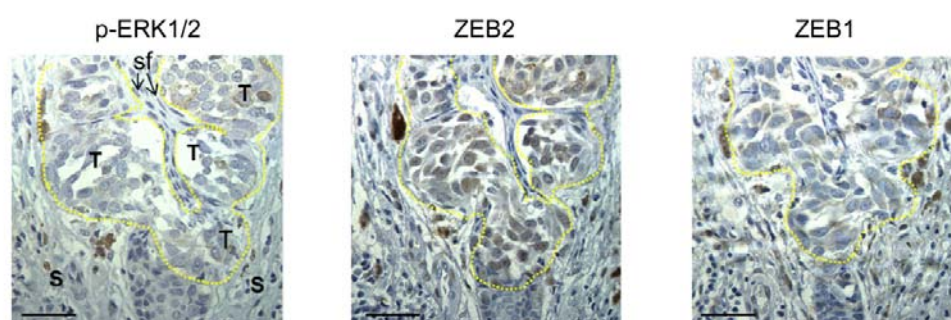
			Univariate Cox Proportional hazard analysis of EMT-TF and p-ERK IHC with metastasis free survival			Multivariate Cox Proportional hazard analysis of EMT-TF or p-ERK IHC and clinical prognostic factors with metastasis free survival		
Variable	Evaluation	N#	HR (95% confidence interval)		P value	HR (95% confidence interval)		P value
Age	<60	46	1			1		
	>60	52	1.64	0.91-2.97	0.01*	0.781	0.40-1.51	0.46
Gender	Female	57	1			1		
	Male	41	1.99	1.12-3.55	0.02*	1.98	1.09-3.62	0.081
Breslow	<1.5 mm	29	1			1		
	>1.5 mm < 4.0mm	43	2.67	1.16-6.17	0.02*	3.09	1.18-8.08	0.022*
	>4.0mm	26	5.10	2.14-12.17	< 0.001***	5.35	1.89-15.12	0.002**
Ulceration	No ulceration	56	1			1		
	ulceration	39	1.81	1.01-3.24	0.049*	1.178	0.58-2.374	0.65
ZEB1 Superficial	H Score (100)	98	6.22	2.19-17.71	<0.001***	5.413	1.74-16.86	0.004**
ZEB2 Superficial	H Score (100)	98	0.89	0.61-1.30	0.557	1.061	0.69-1.63	0.79
TWIST1 Superficial	H Score (100)	92	4.04	1.23-13.29	0.022*	6.452	1.94-21.45	0.002**
p-ERK1/2 Superficial	H Score (100)	63	1.83	1.052-3.19	0.032*	1.40	0.74-2.657	0.302
ZEB1 Deep	H Score (100)	98	4.16	2.02-8.54	<0.001***	3.42	1.53-7.59	0.003**
ZEB2 Deep	H Score (100)	98	0.931	0.35-0.93	0.025*	0.75	0.45-1.25	0.27
TWIST1 Deep	H Score (100)	92	1.73	0.497-6.04	0.389	6.022	1.58-22.94	0.008**
p-ERK1/2 Deep	H Score(100)	63	1.98	1.28-3.07	0.002**	1.72	1.04-2.83	0.034*

Cases were excluded where time-to-event was zero (i.e. 4 cases with pre-existing metastases) P * <0.05 **<0.01 *** < 0.001

A. Expression in nevi



B. Expression in cortical area



C. Expression in medullary area

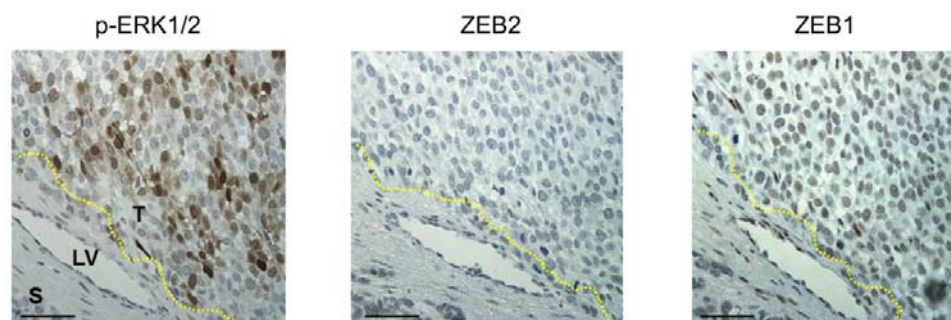
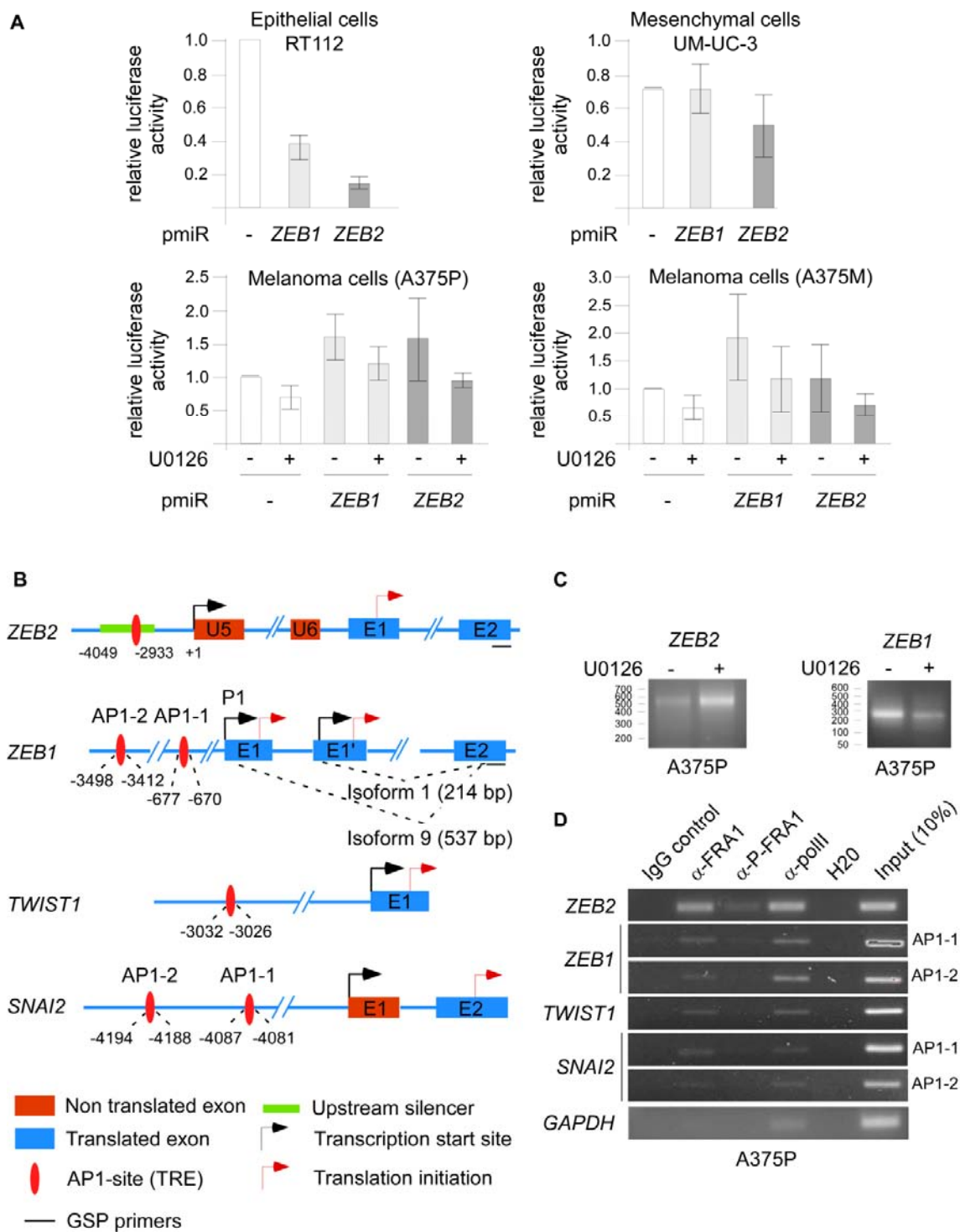


Figure S2, related to Figure 3.

EMT-TF switch correlates with ERK activity. Examples of IHC analyses of phospho-ERK (p-ERK1/2) and EMT-TF expression in a nevus (A) and lymph nodal metastasis (B, C). E, epidermis; N, nevus, melanocytes are indicated by arrows. T, tumor; S, stroma; sf, stromal fibroblasts; LV, lymphatic vessel. The micrographs demonstrate a correlation between EMT-TF reprogramming and phospho-ERK immunopositivity. Scale bar = 40 μ m.



(A) Activity of the firefly luciferase reporters linked to the 3'UTRs of *ZEB1* or *ZEB2* was measured after transient transfection in epithelial (RT112), mesenchymal carcinoma (UM-UC-3) or MM (A375P and A375M) cell lines. U0126 was added to the transfected cells as indicated. The reporter activity was normalized to that of the pMIR-REPORT vector containing no UTRs (-). Data represent mean $\pm$ SD of three independent experiments.

(B) A scheme depicts localization of AP-1 binding sites relative to the *ZEB2*, *ZEB1*, *TWIST1* and *SNAI2* gene structures.

(C) Results of 5'RACE experiments mapping *ZEB2* and *ZEB1* transcription start sites. Note the transcriptional activation of *ZEB2* and repression of *ZEB1* by U0126 in A375P cells.

(D) ChIP using anti-FRA1 and anti-phospho-FRA1 (P-FRA1) antibodies revealed FRA1 interactions with DNA fragments containing AP-1 binding sites upstream of transcription start sites within *ZEB2*, *ZEB1*, *TWIST1* and *SNAI2* genes. ChIP with an anti-PolIII antibody and negative IgG was used as positive and negative controls. Input shows amplification of DNA that was sheared but not subjected to immunoprecipitation.

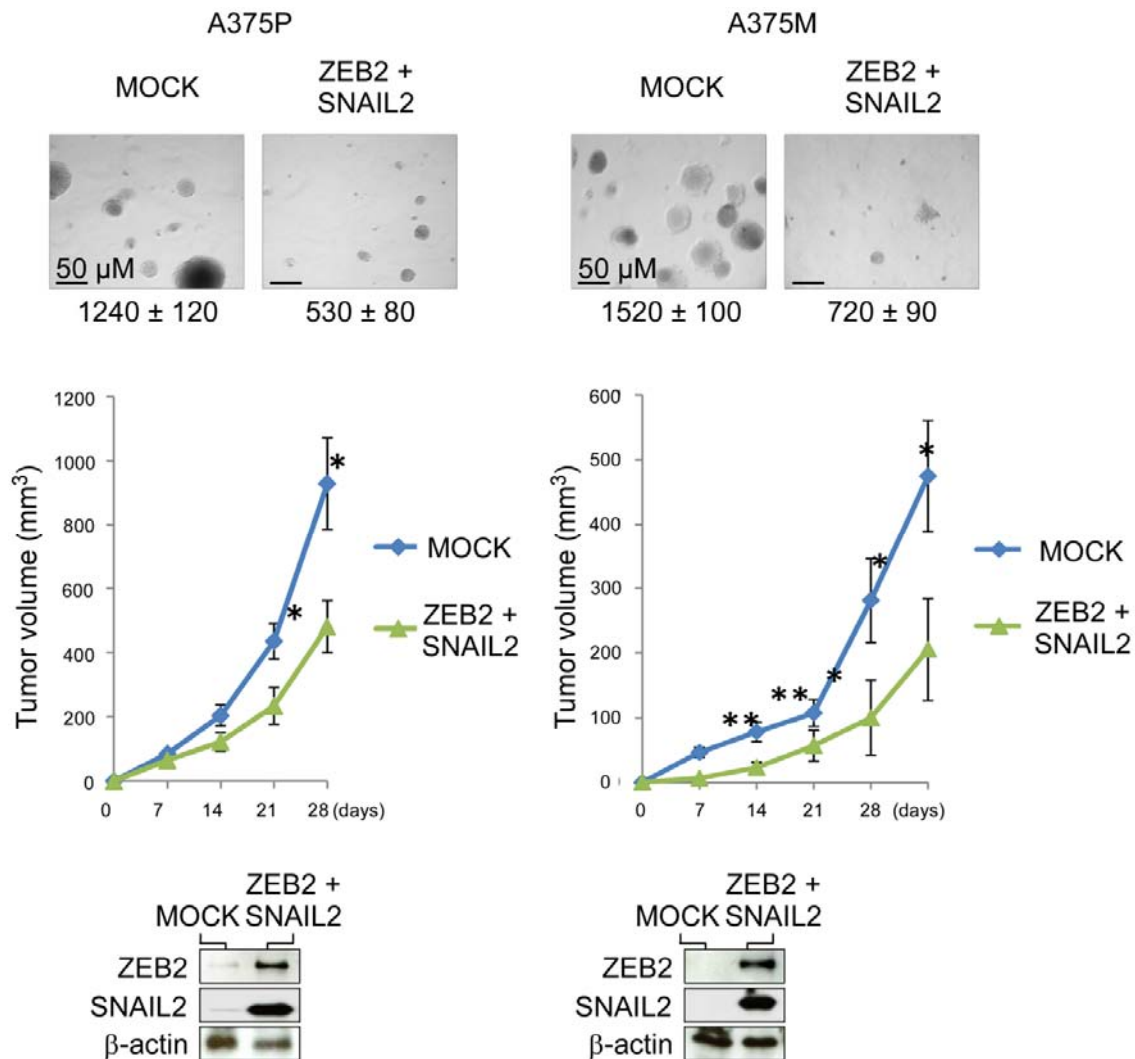
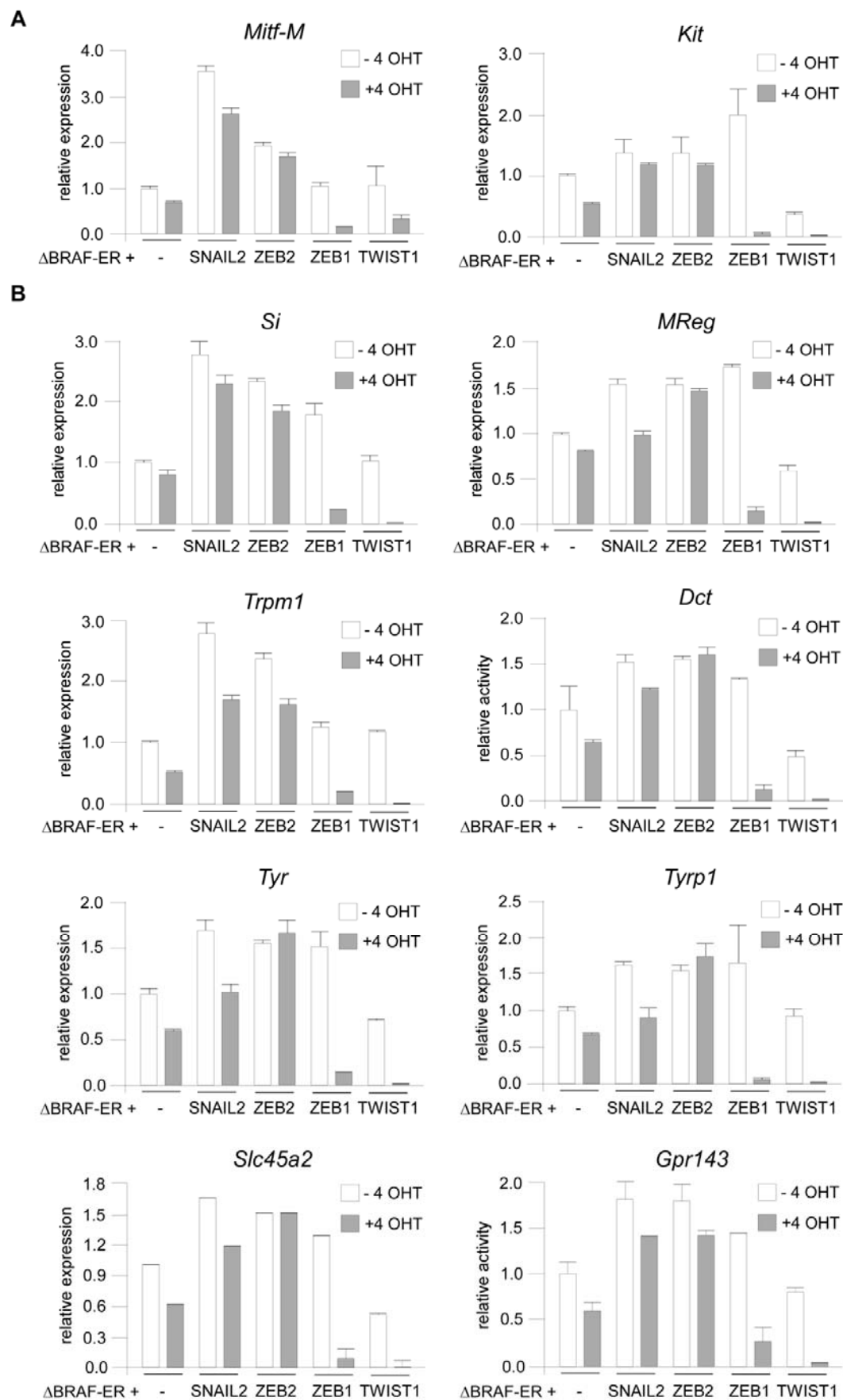


Figure S4, related to Figure 6.

EMT-TF reprogramming impacts on tumorigenicity of melanoma cells.

ZEB2 and SNAIL2 were ectopically expressed in A375P and A375M melanoma cell lines. Tumorigenicity was examined using a soft agar colony assay (top panels) or by xenografting cells in immunocompromised mice. The graphs illustrate the mean tumor size in each group of mice (n=5). Error bars represent standard error of the mean. Significance was analyzed using one tail t test. * p<0.05; ** p<0.01. Note that simultaneous expression of exogenous ZEB2 and SNAIL2 reduces colony formation in soft agar and alleviates tumor growth in xenografted mice.



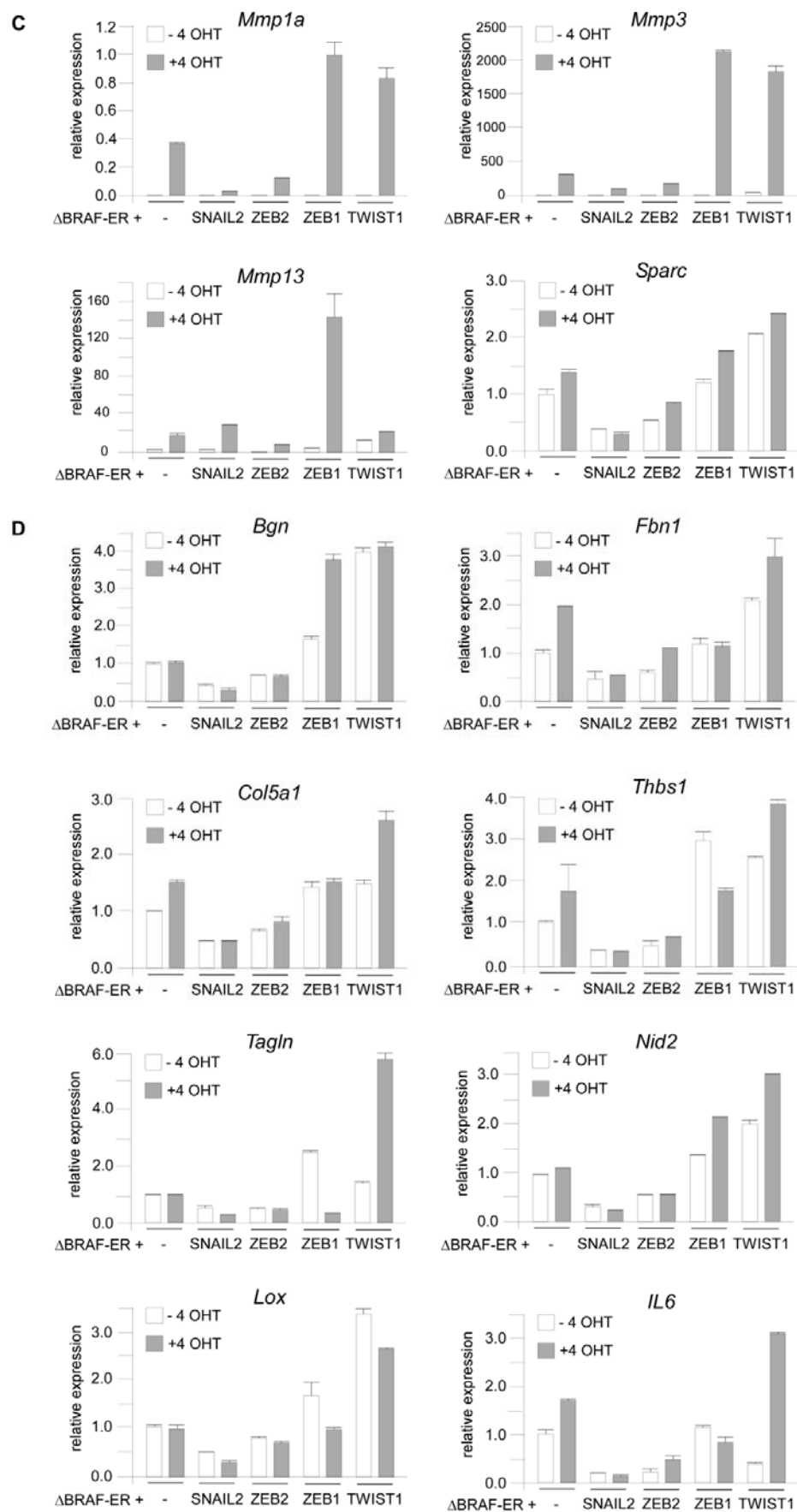


Figure S5, related to Figure 7.

Validation of the array data.

Expression of indicated genes from the following clusters was validated by qPCR.

(A), differentiation; (B), target genes of MITF; (C), invasion; (D), TGF- β pathway.

Data show mean $\pm$ SD of triplicate experiments.

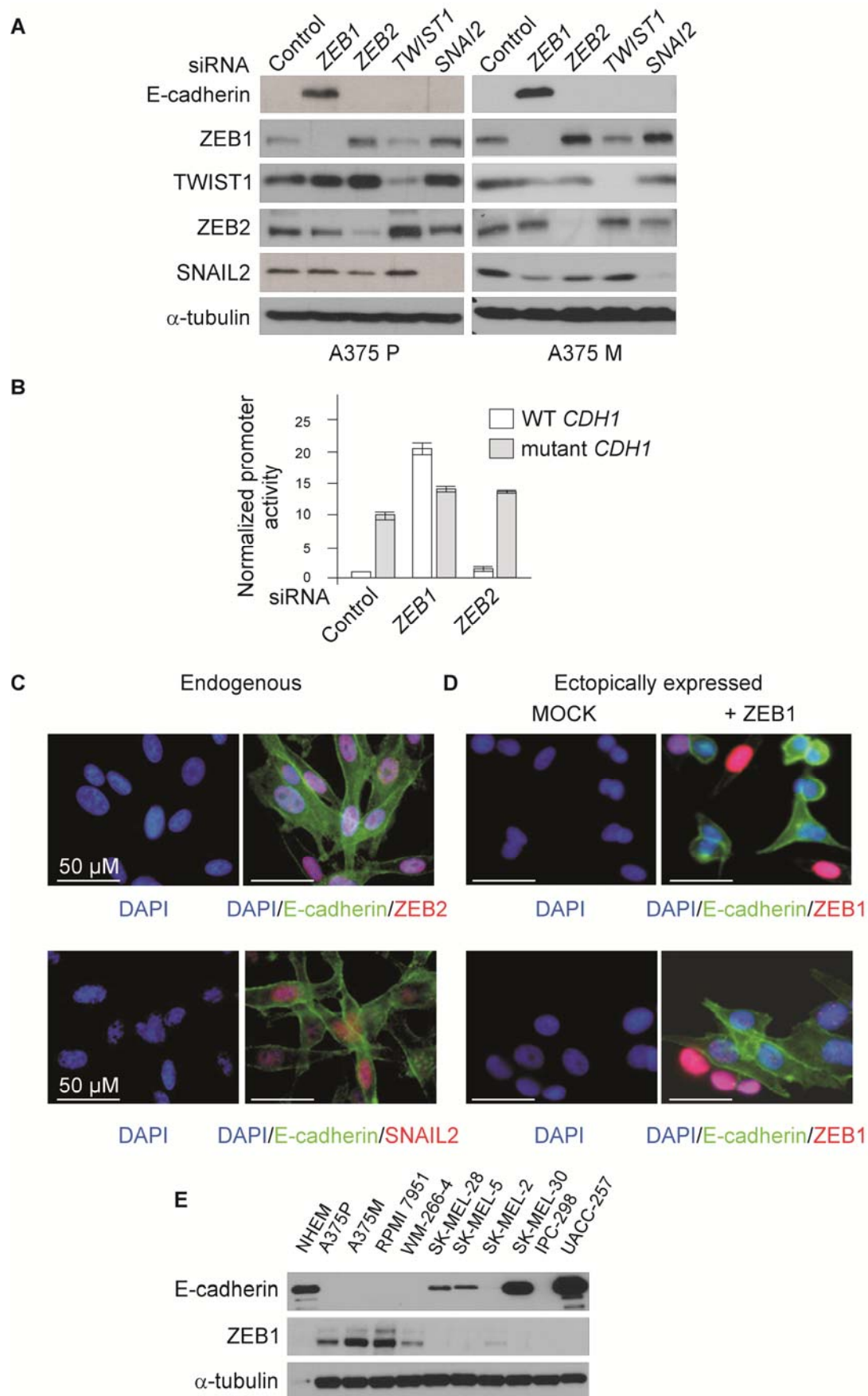


Figure S6, related to Figure 8.

ZEB1 represses *CDH1* expression in melanoma cells.

(A) Analysis of the effect of EMT-TF depletion on the expression of E-cadherin and other EMT-TFs in A375P or A375M cells as assessed by immunoblotting.

(B) Impact of ZEB1 or ZEB2 depletion on *CDH1* promoter activity. Luciferase reporters containing either wild type or mutant E boxes (Bolos et al., 2003) were transiently expressed in A375M cells. Luciferase activity was determined in three independent experiments performed in duplicate with similar results. Results are mean $\pm$ SD of a representative experiment.

(C) E-cadherin is co-expressed with ZEB2 and SNAIL2 in UACC-257 cells as demonstrated by immunofluorescent microscopy.

(D) Ectopic expression of ZEB1 down-regulates E-cadherin in UACC-257 melanoma cells.

(E) Expression levels of E-cadherin and ZEB1 are inversely correlated in a panel of melanoma cell lines.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Constructs and cell lines

Lentiviral vector encoding BRAF^{V600E} (Denoyelle et al., 2006) and retroviral vector encoding a fusion protein consisting of Δ BRAF linked to the T1 form of the human estrogen receptor hormone-binding domain (Pritchard et al., 1995) was generously provided by Dr. Martin McMahon.

Murine HA-ZEB2, murine HA-ZEB1, human SNAIL2, human FLAG-TWIST1, and trans-species shRNA *TWIST1* in pBABE-Puro have been previously described (Ansieau et al., 2008; Morel et al., 2012). The HA-ZEB1 pCMV3.1TOPO expression vector was derived from the HA-ZEB1 pBABE-Puro retroviral construct. To analyze whether miR-200 regulates *ZEB1* and *ZEB2* in MM cells, we generated reporter constructs by cloning 3671-5125 bp and 3618-4935 bp 3'-untranslated sequences respectively of *ZEB1* and *ZEB2* genes (relative to ATG codons) into pMIR-REPORT vector (Invitrogen Carlsbad, CA, USA) vector. To determine *CDH1* promoter activity, we used luciferase reporter vectors containing either a wild-type E-cadherin promoter fragment (-178 - +92 bp) (WT-E-cad), or the same fragment with mutated E-boxes (mutant-E-cad) (Bolos et al., 2003).

Murine shRNA *Zeb1* in pLKO-1 (TRCN0000070819 = NM_011546.1-2785s1c1 and TRCN0000070821 = NM_011546.1-770s1c1) and shRNA *Fra1* (TRCN0000042687 = NM_010235.1-851s1c1 and TRCN0000042683 = NM_010235.1-664s1c1) were purchased from Sigma-Aldrich (St-Louis, MO, USA).

Melan-a cell line (kindly provided by Dorothy Bennett, St. George's Hospital Medical School, London, UK) and melan-a-derivatives were cultured in RPMI medium (Invitrogen) supplemented with 10% FBS (Cambrex), 100 U/ml penicillin-streptomycin (Invitrogen), 2 mM L glutamine (Invitrogen) and 100 nM PMA (Sigma-Aldrich). Δ BRAF-ER was activated by treatment with 50 nM 4-OH-Tamoxifen (Sigma-Aldrich). Primary adult normal human epidermal melanocyte cells (NHEMs) were purchased from Lonza GmbH (Cologne, Germany) and cultured in MBM4 medium supplemented with FBS, CaCl₂, rhFGF-B, PMA, rh-insulin, hydrocortisone, BPE, and endothelin3 (MGM4 bullet kit). WM-266-4, RPMI-7951, SK-MEL-5, and SK-MEL-28 melanoma cell lines were obtained from ATCC. IPC-298 and A375-derived cell lines were obtained from the German Collection of Microorganisms and Cell Cultures

(Leibniz Institut DSMZ, Braunschweig, Germany) and Wellcome Trust Functional Genomics Cell Bank (St. George's, University of London, UK) respectively. Cells were cultured according to the ATCC recommendation and treated with 10 μ M U0126, PD184352 (both purchased from Merck, Darmstadt, Germany), or 10 μ M PLX4720 (SeleckChem, Houston, TX, USA) or with DMSO for the indicated times. UM-UC-3 and RT112 carcinoma cells were cultured in DMEM medium supplemented with 10% FBS (GE Healthcare, Amersham, UK) and non-essential amino acids (Invitrogen).

Retroviral and lentiviral infection

4.10⁶ human embryonic kidney 293T cells were transfected with retroviral or lentiviral expression constructs (10 μ g) in combination with GAG-POL (5 μ g) and ENV expression vectors (10 μ g) using calcium phosphate. Viral stocks were collected 48 h post-transfection, filtered (0.45 μ m) and placed in contact of 2 x 10⁶ melanocytes or melanoma cells for 8 h in the presence of 8 μ g/ml polybrene. 48 h post-infection, cells were selected in presence of puromycin (1.5 μ g/ml), neomycin (100 μ g/ml) or hygromycin (25 μ g/ml) (all from Invitrogen).

siRNA

Transfections of siRNA were performed by electroporation with a single pulse of 250 V and 250 μ Fd by using the Gene Pulser Xcell electroporation system (Bio-Rad, Hercules, CA, USA).

List of siRNAs used for transient transfections:

Target name	siRNA sequence (sense strand)
<i>SNAI2</i>	GGACCACAGUGGCUCAGAA(UU)
<i>ZEB1</i>	GGACUCAAGACAUCUCAGUdTdG
<i>ZEB2</i>	GAACAGACAGGCUUACUUAAdTdT
<i>TWIST1</i>	ON-TARGET plus SMARTpool

siRNA were purchased from Ambion (Austin, TX, USA) or Dharmacon (Lafayette, CO, USA).

Soft-agar colony formation assay

Melan-a cells expressing Δ BRAF-ER or A375 MM cell lines were transduced with cDNA or shRNA retroviral or lentiviral expression vectors and selected with puromycin. Plates were prepared by coating with 0.75% low-melting agarose (Lonza) in growth medium and then overlaid with cell suspension in 0.45% low-melting agarose (2×10^4 cells/well for melan-a or 5×10^3 cells/wells for A375 cells). Melan-a cells were applied in a medium supplemented with 4-OHT (50 nM), PMA (100 nM) and puromycin (1.5 μ g/ml). Plates were incubated for 2-3 weeks at 37°C. Colonies were stained with crystal violet (1 mg/ml Sigma-Aldrich) and counted under microscope.

Transwell invasion assays

Melan-a/ Δ BRAF-ER cells infected with retroviral vectors expressing EMT-TFs, or MM cells with transiently depleted EMT-TFs with siRNAs (48 hours post-transfection) were seeded on matrigel-coated porous membranes (pore size, 8 μ m; BD Biosciences) in transwells. Cells were allowed to invade matrigel towards FBS gradients for 24 hours. Cells that did not migrate were removed using a cotton swab. The membranes were then fixed with methanol and stained with Gurr rapid staining kit (BDH). The number of invaded cells was determined by counting ten random fields using an inverted Nikon TE2000-U microscope.

Immunoblot analysis

Cells were washed twice with phosphate buffered saline (PBS) containing CaCl_2 and then lysed in a 100 mM NaCl, 1% NP40, 0.1% SDS, 50 mM Tris pH 8 RIPA buffer supplemented with a complete protease inhibitor cocktail (Roche, Mannheim, Germany) and phosphatase inhibitors (Sigma-Aldrich). Protein expression was examined by western blot using the anti-HA clone 11 (BabCO), anti-TWIST Twist2C1a (Abcam, Cambridge, MA, USA), anti-P-ERK1/2 (#9106, Cell Signaling

Technology, Danvers, MA, USA), anti-BRAF clone F-7 (sc-5284, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-MITF clone C5 (Thermo Scientific, Huntville, AL, USA), anti-E-cadherin clone 36 (BD Biosciences), anti-ZEB1 H102 (Santa Cruz), anti-ZEB2 (Sayan et al., 2009), anti-ERK1/2 #9102 (Cell Signaling), anti-FRA1 sc-605 (Santa Cruz), anti Phospho-FRA1 #3880 (Cell Signaling), anti-ER HC20 (Santa Cruz) rabbit polyclonal antibodies, and the anti-SNAIL2 G-18 (Santa Cruz) goat polyclonal antibody for primary detection. The anti-SNAIL1 antibody was a gift from Dr Antonio Garcia de Herreros. Loading was controlled using the anti- β -actin clone AC-15 (Sigma-Aldrich), anti-Ku80 clone 7/Ku80 (BD Biosciences) or anti- α -Tubulin (T5168, Sigma-Aldrich) antibodies. Horseradish peroxidase-conjugated rabbit anti-mouse, goat anti-rabbit and donkey anti-goat polyclonal antibodies (Dako, Glostrup, Denmark) were used as secondary antibodies. Western blots were revealed using an ECL detection kit (Amersham) or a western-blotting Luminol reagent (Santa Cruz).

Immunofluorescence analysis

For immunofluorescent staining, cells were transfected with a ZEB1-expressing vector HA-ZEB1 pCMV3.1TOPO or mock-transfected, cultured for 3 days on coverslips and stained according to standard protocols. Briefly, cells were fixed with 4% PFA for 10 min at room temperature, permeabilized with 0.1% Triton X-100 and blocked with 10% FCS in DMEM. Slides were consecutively incubated with the indicated primary and then secondary antibodies. After counter staining with DAPI (Molecular Probes), cells were examined and photographed using a fluorescent Nikon TE2000-U microscope.

Immunohistochemistry

Sections (4 μ m) of formalin-fixed paraffin embedded (FFPE) nevi, melanoma and metastatic melanoma were rehydrated, heated in TE (10 mM Tris, 1 mM EDTA buffer, pH 9.0, or citrate buffer) in a 750W microwave oven at full power for 20 min and allowed to cool down for 20-30 min at room temperature. Sections were incubated in protein blocking solution (Novocastra, Newcastle, UK) and incubated at 4°C overnight in the presence of a primary antibody. Primary antibodies were: rabbit

monoclonal antibodies to human SNAIL2 (clone C19G7 Cell Signaling); rabbit polyclonal antibodies to human ZEB1 sc-H-102 (Santa Cruz Biotech), ZEB2 (Sayan et al., 2009), and TWIST1 sc-R20 (Santa Cruz Biotech); murine monoclonal antibody to human SNAIL1 (Franci et al., 2006), phospho-ERK1/2 #9102 (Cell Signaling) and E-cadherin #610181, (BD Biosciences). Detection was performed using the Novolink™ Polymer System, purchased from Novocastra. Staining was visualized in 3, 3'-diaminobenzidine (DAB) chromogen in Novolink DAB substrate buffer, for 5 min, counterstained in Mayer's Haematoxylin and mounted. Agarose-embedded cell pellets known to be positive or negative for EMT-TFs were used as controls.

Quantitative analysis of immunostaining

Positive staining was scored via the H-score (Kinsel et al., 1989), and three observers agreed the criteria for levels of positive staining. Four representative dermal fields were assessed; two from the superficial tumor close to the epidermal basal lamina in the papillary dermis and two from the deepest tumor sites within the reticular dermis, and a mean H-Score was calculated for both the superficial and deep sites. Superficial and deep sites of the metastatic lesions were also scored using the same criteria. Inter-observer agreement was measured using 12 randomly selected cases and an intra-class correlation coefficient (ICC) indicated high agreement (ICC = 0.92).

5'-RACE (Rapid amplification of cDNA ends)

ZEB2 and *ZEB1* transcription start sites were identified using RACE kit (Roche) according to the manufacturer's protocol. Total RNA was isolated from A375P cells treated or mock treated with 10 μ M U0126 for 24 h and used for the synthesis of the first strand cDNA using the SP2 primer complementary to the exon E2 of *ZEB2* or *ZEB1*. After purification, terminal deoxynucleotidyl transferase was used to add dA tail to 3'-end of cDNA. cDNA was amplified using the oligo-dT anchor primer and *ZEB2*- or *ZEB1*-specific primer pairs (*ZEB2*, TGTTGTGCCAGGGGTGTTCCAC and ACAATTCAGGAAAGATTTTGTTCATGG; *ZEB1*, GTCTGGTCTGTTGGCAGGTCATCC and TCCTCTGGTACACCTTCACAGTCAGC). Amplified DNA was analyzed in agarose gels. For *ZEB2*, DNA was gel-purified, cloned, and the sequence of 20 recombinant plasmid DNAs was determined.

Chromatin immunoprecipitation

Chromatin purification and precipitation was performed following manufacturer's protocols (Active Motif, Rixensart, Belgium). For each chromatin preparation, cells grown in a 70% confluent 15 cm plate were fixed with 1% formalin for 5 min at room temperature. Fixation was stopped by an excess of glycine; nuclei from collected cells were extracted using a Dounce homogenizer and precipitated by brief centrifugation. Chromatin was sheared using sonication with one quarter setting (25 seconds for 4 times on ice). Sheared chromatin was used for each precipitation or input control. Chromatin immunoprecipitation was performed using R-20 anti-FRA1 N-terminal antibody (sc-605, Santa-Cruz Biotech), anti-pospho-FRA1 peptide antibody (New England Biolabs) or negative or positive control antibodies included in the kit. After extensive washing of chromatin-protein G complex, the samples were eluted, reverse cross-linked and treated with Proteinase-K. Following inactivation of Proteinase-K, the sample was used for PCR amplifications using EMT-TF gene-specific or control primer pairs encompassing the AP-1 binding sequence elements. *GAPDH* control primers were from Active Motif).

List of primer pairs used for ChIP

<i>ZEB2</i> , AP-1	F, TGCACGTGAGAAAACCGTTTGGC R, GTGTTGTGACTCAGGAGGTGGGC
<i>ZEB1</i> , AP-1-1	F, ACTCATTCCGCTCTACTAAGGAGGC R, TGACCCGCGCAGCCCGGACTC
<i>ZEB1</i> , AP-1-2	F, CTCCTGGGAGGCTGTATTCTGAAG R, CAGTTCACCGTGGAACAAAGGAG
<i>SNAI2</i> , AP-1-1	F, TCCATCTGAAATTGTGGCAATTG R, GACATCTATTGGTCAGCTTCAGAAGAC
<i>SNAI2</i> , AP-1-2	F, GACGTCATAACATTCCCTTTCTCGC R, GCAATTGCCACAATTTTCAGATGGAC
<i>TWIST1</i> , AP-1	F, TGCAAACATGCCAAGTTTGCAG R, GGGACTACCTTCTTTGGGAATGC

Luciferase reporter assays

Cells were transiently transfected with luciferase reporter vectors along with the β -galactosidase expression vector, pCMV β -gal (Invitrogen). Forty-eight hours post-

transfection, cells were harvested, lysed; and the luciferase activity was measured with a Lumat LB9501 tube luminometer (Berthold). β -galactosidase activity was determined and used for the normalization of luciferase data.

Microarray processing and analysis

Δ BRAF-ER-melan-a cells expressing TWIST1, ZEB1, SNAIL2, or ZEB2 were treated with 4-OHT for 1 week before isolation of total RNA. Microarray processing and data analysis were performed on the ProfileXpert core facility (Bron, France). Total RNA (100 ng) was amplified and biotin-labeled using Kit GeneChip 3' IVT Express and procedures from Affymetrix (Santa Clara, CA, USA, <http://www.affymetrix.com>). Microarrays analyses were performed using high-density oligonucleotide arrays (Mouse Genome 430 2.0 Array, Affymetrix). Fifteen μ g of biotinylated cRNA were fragmented, and hybridization on chip was performed following Affymetrix protocol. Arrays were washed and stained with streptavidin-phycoerythrin using GeneChip Hybridization and Wash stain kit from Affymetrix in a Fluidics Station 450 according to the manufacturer's instructions. The arrays were scanned with a confocal laser (Genechip scanner 3000, Affymetrix).

CEL files were generated using the Affymetrix GeneChip Command Console (AGCC) software 3.0. The complete set of CEL files is available at the GEO database under accession number Geo39030. The obtained data were normalized with Affymetrix Expression Console software using Robust Multiarray Average (RMA) statistical algorithm. Data were analyzed using tools in Partek Genomic Suite 6.4 software (Partek Inc., St. Louis, MO, USA). The retained genes of interest were listed and classified according to their functions using Ingenuity Pathway Analysis and Ingenuity® iReport™ (Mountain View, CA, USA). Probeset intensities were summarized and normalized using RMA, and significant differential expression was determined by a moderated t-test (Limma) using a fold change cutoff of 2.

Transcriptional expression analysis

Total RNA was isolated using Trizol reagent (Sigma-Aldrich) and reverse transcribed using a high cDNA capacity reverse transcription kit (Invitrogen) following the manufacturer's instructions. Real-time PCR intron-spanning assays were designed using the ProbeFinder software (Roche). All reactions, including no-template controls

and RT controls were performed in triplicate on the 96-well StepOnePlus™ Fast Real Time PCR system (Applied Biosystems, Foster City, CA, USA). Human *HPRT1* or mouse *Rplp0* was used for normalization. List of primer pairs and probes used:

Gene	Species	Probe	Primers
<i>ZEB1</i>	human	57	Applied Biosystems (Hs00232783_m1)
<i>ZEB2</i>	human	68	Applied Biosystems (Hs00207691_m1)
<i>TWIST1</i>	human	88	Applied Biosystems (Hs01675818_s1)
<i>SNAI2</i>	human	86	Applied Biosystems (Hs00950344_m1)
<i>HPRT1</i>	human	73	F, TGACCTTGATTTATTTGCATACC
			R, CGAGCAAGACGTTCAATCCT
<i>Zeb1</i>	mouse	57	F, GCCAGCGTCATGATGAAAA
			R, TATCACAATACGGGCAGGTG
<i>Bgn</i>	mouse	40	F, CACTTGGACAACAACAAGCTG
			R, TGATGTTGTTGGAGTGCAGATA
<i>Col5a1</i>	mouse	79	F,GGGCAGAAGGGAAGCAAG
			R, GGCCTTGAGGACCGGTAG
<i>Dct</i>	mouse	6	F, GGCTACAATTACGCCGTTG
			R, CACTGAGAGAGTTGTGGACCAA
<i>Fbn1</i>	mouse	40	F, CAGTGGACCGGGAATGAC
			R, TGGGCAAATATCAGGATCTAATG
<i>Grp143</i>	mouse	18	F, GTATACTTGGTGATCAGGAGATCG
			R, CCTCCACACAGAGCAGCAC
<i>Il6</i>	mouse	55	F, ACGGCCTTCCCTACTTCAC
			R, ACAGGTCTGTTGGGAGTGGT
<i>Kit</i>	mouse	15	F, GATCGCTCTGCGTCCTGTT
			R, CTTGCAGATGGCTGAGACG
<i>Lox</i>	mouse	48	F, CAGGCTGCACAATTCACC
			R, CAAACACCAGGTACGGCTTT

<i>Mitf-M</i>	mouse	27	F, CTAAGTGGTCTGCGGTGTCTC
			R, GGTTTTCCAGGTGGGTCTG
<i>Mmp13</i>	mouse	89	F, GCCAGAACTTCCCAACCAT
			R, TCAGAGCCCAGAATTTTCTCC
<i>Mmp1A</i>	mouse	94	F, TGTGTTTCACAACGGAGACC
			R, GCCCAAGTTGTAGTAGTTTTCCA
<i>Mmp3</i>	mouse	7	F, TTGTTCTTTGATGCAGTCAGC
			R, GATTTGCGCCAAAAGTGC
<i>Mreg</i>	mouse	6	F, TTCGTAATCAGCAGACCAAAGA
			R, CTGCCGCAGGGTGTAGATA
<i>Nid2</i>	mouse	10	F, ATGACCAGCACACTTGTATCTTG
			R, AGGTGTGACTGCCATCGAG
<i>Silver</i>	mouse	76	F, AGCTTCCTTCCCGTGCTT
			R, CACCAAGCCAGTCCTGATTC
<i>Slc45a2</i>	mouse	29	F, GAGATGCGGTCGTATCAGC
			R, TGATGCTTATGGCCCAGAT
<i>Sparc</i>	mouse	73	F, GCCCCTCAGCAGACTGAA
			R, GTCTCCTCCACCACGGTTT
<i>Tagln</i>	mouse	13	F, CCTTCCAGTCCACAAACGAC
			R, GTAGGATGGACCCTTGTTGG
<i>Thbs1</i>	mouse	22	F, CACCTCTCCGGGTTACTGAG
			R, GCAACAGGAACAGGACACCTA
<i>Trp1</i>	mouse	67	F, GGTGCGGGATGTAAAGAAGA
			R, TGTAGTTGCAGCGGTAGGC
<i>Tyr</i>	mouse	26	F, TCGTCACCCTGAAAATCCTAA
			R, CTGATCTGCTACAAATGATCTGC
<i>Tyrp1</i>	mouse	104	F, ATGGATATGGGTGCACGAG
			R, CCTTGTAAGTGAAGCCCTCCT
<i>Rplpo</i>	mouse	9	F, ACTGGTCTAGGACCCGAGAAG

			R, TCCCACCTTGTCTCCAGTCT
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To analyze the expression of miRs, quantitative real-time PCR Taqman® MicroRNA assay (Applied Biosystems) was used. MiR-132, miR-345 and miR-191 were identified as the most appropriate endogenous control genes for miR expression. Selection of stable endogenous control genes was carried out using geNorm algorithm-based software.

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