ALTERED NUCLEAR TRANSPORT IN TRANSFORMED CELLS

A thesis submitted for the degree of Doctor of Philosophy at

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Copyright Notices

Notice 1

Notice 2

ADDENDUM

p8, at the end of Fig. 1.1 legend: add “Original figure”.

p23, at the end of Fig. 1.2c legend: add “Original figure. See text for citations”.

p50 line 21: should read:

“as the ratio of the signal for nuclear transport factor relative to that for α/β tubulin *(signals were obtained from the same blot)*...”

p52, line 4: should read:

“...all of the Impα/β1- and Impβ1-recognised proteins *with the exception of TRF-1***,** localised more strongly....”

p60, Fig. 2.4 legend, line 2: should read:

“Endogenous nuclear transport factors *Impα1, Impα3 and Impα4 (all c. 58 kDa), Impβ1 (97 kDa), Exp-1 (116 kDa), CAS (100 kDa), RCC1 (45 kDa), Ran (27 kDa)* in whole cell extracts of...”

p60, Fig. 2.4 legend, line 3: should read:

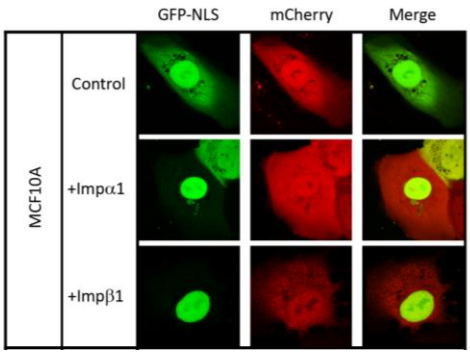
“...with α/β tubulin *(55 kDa; the blots shown are the corresponding tubulin for Exp-1)* as a loading control, and SV40 T-ag *(97 kDa)* control denoting...”

p60, Fig. 2.4 legend, line 5: After “...from a single typical experiment from a series of ≥ 3 separate experiments.” add:

“Representative blot images for MCF10A/CA1h cells were cropped and merged from blots originally comprised of the MCF10 series presented in Chapter 3.”

p60, Fig. 2.4 legend, line 8: should read:

“...signal obtained for the nuclear transport factor protein normalised to that of *the corresponding signal from the same blot for* α/β tubulin in transformed cells...”



p62, Fig. 2.5a:

A new image containing a more representative depiction of Impβ1 overexpressing MCF10A cells has been added (right).

p84, Fig. 3.2a, line 2: should read:

“(**a**) Western analysis of Importin (Imp) expression levels and control protein α/β tubulin (*the blot shown is the corresponding tubulin for Impα3)* in MCF10 breast cancer cell series.”

p87, Fig. 3.3 legend, line 4: should read:

"... transfected 48 h post siRNA treatment to express the Impα/β1-recognised GFP-T-ag (114-135) fusion protein *(two representative images are shown in the top and bottom panels)* and (**d**)..."

p88, line 6: After: "...Impβ1 using actin as an internal control protein." add:

"Signals for Impα1, Impβ1 and actin were quantified at set exposure times for titrated amounts of protein from IDC3 and DCIS extracts during Western blotting experiments to ensure all signals were in a linear range (**Supp. Fig. 3.5**)."

p90, Fig. 3.5 legend, line 3: should read:

"Densitometric analysis of Impα1 and Impβ1 signals relative to the corresponding signal for the loading control protein actin in human breast tumour tissues", instead of "Digitised images such as those shown in (**a**) were subjected to densitometric analysis."

p92, paragraph 2, line 12:

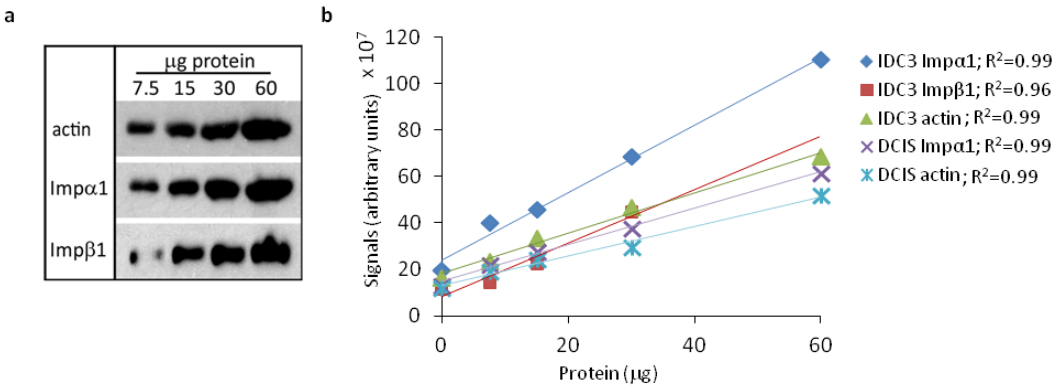
After "...net efficiency of Impβ1-dependent import in cells that are overexpressing Impα1, such as cancer cells." add:

"We presume that the reason the result for MCF10A/CA1h cells here in Chapter 3 contradicts the findings of Chapter 2 where Impβ1-recognised NLS-containing proteins localised more strongly in the nucleus of transformed MCF10CA1h compared to non-transformed MCF10A cells may be due to a change in transfection reagent. **Figure S2.1** shows transfection reagents may differentially affect Imp/Exp levels; Fugene HD (used in Chapter 3) did not affect levels of key modulators of nuclear import efficiency such as Impα1, Impβ1 or CAS, whilst Lipofectamine 2000 (used in Chapter 2) caused c. 2-fold reduction in CAS levels compared to untreated cells."

p110, Fig. 4.1, line 6: should read:

"...signal obtained for Impβ1 normalised to that for α/β tubulin control protein (*the blot shown is the corresponding tubulin for Impβ1)* in transformed relative to non-transformed..."

Chapter 3, p100: add Supplementary Figure S3.5:



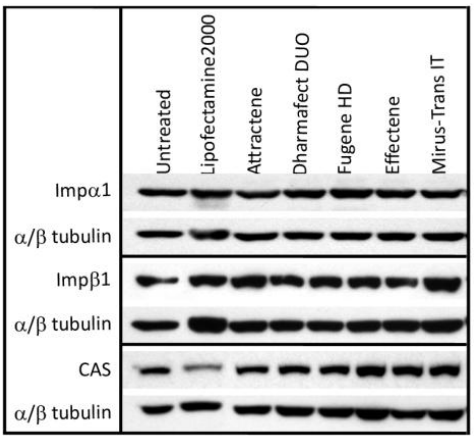
ERRATA

pXII, Table of Contents, lines 7-11:

4.4.1.1, 4.4.1.2, 4.4.1.3, 4.4.1.4 and 4.4.1.5 should read: *4.4.1, 4.4.2, 4.4.3, 4.4.4 and 4.4.5*

p67, Fig. S2.1c:

The lane containing the ladder has been cropped from the middle and bottom panels so that the bands between the rows align (right).



p107: "4.4.1.1 Mammalian cell culture and siRNA treatment" should read: *"4.4.1* Mammalian cell culture and siRNA treatment"

p108: *"*4.4.1.2 Quantitative real-time reverse transcription-PCR*"* and "*4.4.1.3* Preparation of cell extracts and Western blotting*"* should read: "*4.4.2* Quantitative real-time reverse transcription-PCR*" and* "*4.4.3* Preparation of cell extracts and Western blotting*",* respectively.

p109: "4.4.1.4 XTT assays" and "4.4.1.5 Statistical significance" should read: "*4.4.4* XTT assays" and "*4.4.5* Statistical significance"

Supplementary Figure S3.5 Verification of Western blot signal linearity for Impα1 and actin in ductal tumour tissues. (**a**) Titrated amounts of protein from representative IDC3 and DCIS (not shown) samples were subjected to Western analysis for Impα1 and actin signals 3 s and 90 s exposure times respectively. (**b**) Densitometric analysis of blots such as those given in (**a**) are given as signal intensity (arbitrary units) for μg protein. The line of best fit and goodness of fit (R^2) are given to indicate the linearity of signals.

DECLARATION FOR THESIS BASED OR PARTIALLY BASED ON CONJOINTLY PUBLISHED OR UNPUBLISHED WORK

General Declaration

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and Research Master's regulations the following declarations are made:

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

This thesis includes 1 original paper published in a peer reviewed journal and 2 unpublished publications. The core theme of the thesis is investigating altered nuclear transport properties of transformed and malignant cell types. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Department of Biochemistry and Molecular Biology under the supervision of Prof. David Jans.

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

In the case of Chapters 2, 3 and 4 my contribution to the work involved the following:

Thesis chapter	**Publication title**	**Publication status***	**Nature and extent of candidate's contribution**
2	Global enhancement of nuclear localisation signal-dependent nuclear transport in transformed cells	Published	Designed the study, performed all experimental work/analysis and prepared the manuscript for publication.
3	Enhanced Importin α/β1-dependent nuclear import efficiency in a model of breast tumour progression	Manuscript in preparation	Designed the study, performed all experimental work/analysis (but did not clone the pIRES-Impα3-mCherry plasmid), and prepared the manuscript for publication.
4	Hypersensitivity of malignant breast cancer cell types to knockdown of the nuclear transporter Importin β1	Submitted	Designed the study, performed all experimental work/analysis (but did not but did not perform the XTT assay in Fig. 4.2c), and prepared the manuscript for publication.

I have / have not (circle that which applies) renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: ……

Date: ……

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Prof. David Jans, for believing and trusting in me, supporting my passions and goals, and for the excellent advice and guidance he has given me over the past years. His approach in teaching has made me independent, confident and prepared for the real world of science; I was never made to feel like "just a student", but a valued member of the team, whose thoughts and opinions about the research were listened to and debated, if necessary. He has given me incredible freedom and responsibility to conduct my studies, but was always available if I needed advice. For all these reasons, I am so grateful.

I would also like to acknowledge Katharine Adcroft and Cassandra David, whose support, humour and company during this PhD were greatly valued. If laughter is known to prolong life then I would be another year younger thanks to my dear friend Steven Heaton and my running buddy Linda Wiltzer, who lightened up the mood when things were not going so great. It is always stimulating bouncing back ideas about science and life; Steve and Linda were able to provide many a great conversations, and for that I would like to personally thank them.

Although unusual, I would also like to acknowledge my dogs who are like family to me, (the late) Uncle Harry, Jamie and Agent. Even if they can not read this thesis and if they could, would probably opt to rip it up instead of reading it (the essence of incredi naughty), they have played a central role in my life by giving me a healthy work-life balance and taught me so much about myself during the PhD years.

The last thank you goes to my mother Sirkka. Without her I would not have been able to follow my passion in terms of doing a PhD. We do not possess many grand belongings, but we do have each other's friendship and support. Without her, the completion of this PhD would not have been possible, and I really mean that, financially and otherwise.

ABSTRACT

Fundamental to eukaryotic cell function, trafficking into and out of the nucleus is conventionally mediated by the Importin (Imp) and Exportin (Exp) transporters respectively, of which there are multiple Impα, Impβ and Exp forms expressed in humans. Nuclear transport can be regulated at many levels, including through modulation of the Imp/Exp nuclear transport machinery itself. Interestingly, altered Imp/Exp levels are associated with cellular malignancy and tumour disease progression in terms of tumour grade, stage, aggressiveness and patient prognosis, but the functional relevance with respect to changes in Imp/Exp activity and nuclear transport has not been investigated.

Through rigorous quantitative live cell imaging of a variety of isogenic non-transformed/transformed cell systems from various origins, including the MCF10A human breast tumour progression series, this thesis shows that nuclear accumulation of Impα/β1-recognised nuclear localisation signal (NLS)-containing proteins, but not their NLS-mutated derivatives, is up to 7-fold increased in malignant compared to non-transformed cell types. This is associated with a significantly faster rate of nuclear import in transformed cells, as revealed by analysis of an Impα/β1-recognised cargo using fluorescence recovery after photobleaching. Nuclear accumulation of NLS/nuclear export signal-containing (shuttling) proteins was also enhanced in transformed cell types, experiments using the nuclear export inhibitor leptomycin B demonstrating that efficient Exp-1-mediated nuclear export is not impaired in transformed compared to non-transformed cells. Enhanced nuclear import and export efficiencies were found to correlate with 2- to-4-fold higher expression of Impα1, β1 and Exp-1 in the various transformed cell types, as indicated by quantitative Western analysis.

Trafficking dependent on Impα/β1 was found to be selectively enhanced during the benign to malignant switch in the MCF10 model of human breast tumour progression (basal triple negative type, invasive ductal carcinoma), with the degree of enhancement correlating to advancing tumour disease state of the cells. In contrast, the nuclear accumulation of Impβ1-recognised cargoes was c. 2-fold decreased in malignant breast cells. Quantitative RT-qPCR and/or Western analysis indicate this is specifically associated with progressively increased expression of Impα1, α3, β1 and the Impα re-exporter CAS (Exp-2) in malignant compared to benign/non-transformed breast cell types, with RNAi/overexpression approaches establishing Impα1 levels to be the primary basis of elevated Impα/β1-dependent nuclear import efficiency and decreased Impβ1-dependent nuclear import

efficiency. Underlining the physiological relevance of these observations, the degree of Impα1 expression was found to correlate with increasing tumour grade in clinical ductal carcinoma samples.

Excitingly, the studies in this thesis show that targeting Impβ1 activity through RNAi is up to 33-fold more efficient in decreasing the viability of transformed/ductal carcinoma cell types compared to isogenic non-transformed counterparts, and is highly potent with tumour selective activity shown at subnanomolar siRNA concentrations. Flow cytometric analysis in malignant cells treated with Impβ1 siRNAs implied this effect was due to increased cell death. Importantly, the tumour selective killing activity was found to be specific to Impβ1 siRNA, and was not observed for siRNAs targeting other Imps such as Impα1, α3, CAS or Exp-1 that were also found to be overexpressed in malignant cell types.

The results here established for the first time that enhanced Impα/β1-dependent nuclear import efficiency at a global scale is associated with malignant transformation and that transformed/tumour cell types display hypersensitivity to inhibition of Impβ1 through siRNA-mediated knockdown. The findings raise the exciting possibility that the enhanced Impα/β1-dependent nuclear import of transformed cells may be exploited to facilitate tumour cell-specific drug delivery, and have implications for development of tumour-selective anti cancer strategies through RNAi/drugs targeted specifically at Impβ1.

PUBLICATIONS

Kuusisto H. V., Wagstaff K. M., Alvisi G., Roth D. M. and Jans D. A. (2012) Global enhancement of nuclear localisation signal-dependent nuclear transport in transformed cells. *FASEB Journal,* 26, 1181-1193.

Kuusisto H. V. and Jans D. A. (2014) Enhanced Importin α/β1-dependent nuclear import efficiency in a model of breast tumour progression. In preparation for submission.

Kuusisto H. V. and Jans D. A. (2014) Hypersensitivity of malignant breast cancer cell types to knockdown of the nuclear transporter Importin β1. Submitted.

Wiltzer L., Okada K., Yamaoka S., Larrous F., **Kuusisto H. V**., Sugiyama M., Blondel D., Bourhy H., Jans D. A., Ito N., and Moseley G. W. (2014) Interaction of Rabies Virus P-Protein With STAT Proteins is Critical to Lethal Rabies Disease. *Journal of Infectious Diseases.* doi:10.1093/infdis/jit829.

CONFERENCE ATTENDANCE

7th Biannual Australasian Gene Therapy Society Meeting

4th – 6th May (2011), Bio21 Institute, Parkville, Victoria, Australia.

Oral presentation. "Altered nuclear transport in transformed cells; a prospective tool for anti-cancer gene therapy?"

Winner, best student oral presentation.

Australian Society for Biochemistry and Molecular Biology (ASBMB) Combio Conference

25th-29th September (2011), Cairns Convention Centre, Queensland, Australia.

Poster presentation. "Altered nuclear transport in transformed cells; a prospective tool for anti-cancer therapy?"

Winner, best student poster presentation.

Department of Biochemistry and Molecular Biology Annual PhD Conference

10th-11th November (2011), Monash University, Clayton, Australia.

Oral presentation. "Altered nuclear transport in transformed cell types".

Australian Society for Biochemistry and Molecular Biology (ASBMB) Combio Conference

29th September – 3rd October (2013), Perth Convention Centre, Western Australia, Australia.

Oral presentation. "Increased nuclear import efficiency in a breast tumour progression model; potential for targeted cancer therapies."

Recipient of student bursary.

Monash Comprehensive Cancer Consortium (MCCC) Monthly Symposia

24th October (2013), Monash University, Clayton, Australia.

Oral presentation. "Altered nuclear transport in tumour cells; potential for targeted cancer therapies."

Table of Contents

LIST OF ABBREVIATIONS

AP-1	Activator protein-1
ARM	Armadillo repeat
BRE	Elsten's modification of the Bloom & Richardson grading system
CAS	Cellular apoptosis susceptibility protein
CK2	Protein kinase casein kinase 2
CLSM	Confocal laser scanning microscopy
CREB	cAMP-response element binding protein
CT	Cellular targeting (domain)
Cy3	Cyanine 3
DCIS	Ductal carcinoma *in situ*
DEM	Diethyl maleate
DMSO	Dimethyl sulfoxide
E2F	Elongation 2 factor initiator
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
ES cells	Embryonic stem cells
Exp	Exportin
FACS	Fluorescence-activated cell sorting
FRAP	Fluorescence recovery after photobleaching
GDP	Guanosine 5'-diphosphate
GFP	Green fluorescent protein
GST	Glutathione S-transferase
GTP	Guanosine-5'-triphosphate
H_2O_2	Hydrogen peroxide
Hα-Ras	Harvey rat sarcoma viral oncogene homologue
Her2Neu	Erb-B2 growth factor receptor
HIV-1	Human immunodeficiency virus-1
HPV	Human papilloma virus
IBB	Importin beta binding domain
IC_{50}	Half maximal inhibitory concentration
IDC	Invasive ductal carcinoma
Iκ-B	Inhibitor kappa-B
Imp	Importin
IRES	Internal ribosomal entry site
kDa	Kilo Dalton
LD_{50}	Half maximal lethal dose
LMB	Leptomycin B
MAPK	Mitogen-activated protein kinase
MDa	Mega Dalton
miRNA	Micro RNA
MRT	Modular recombinant transporter
NE	Nuclear envelope
NES	Nuclear export signal
NF-κB	Nuclear factor-kappa B
NFY	Nuclear factor Y

NLS	Nuclear localisation signal
NPC	Nuclear pore complex
NT	Nuclear targeting (domain)
NT siRNA	Non-targeting siRNA
NTF2	Nuclear transport factor 2
Nup	Nucleoporin
PKA	Protein kinase A
PMA	Phorbol myristate acetate
PR	Progesterone receptor
pRb	Retinoblastoma protein
PTHrP	Parathyroid hormone-related protein
Ran	Ras-related nuclear protein
RanBP1	Ran binding protein 1
RanBP3	Ran binding protein 3
RanGAP1	RanGTPase activating protein 1
RanGEF	Ran guanine nucleotide exchange factor
RCC1	Regulator of chromosome condensation 1
Rev	Human immunodeficiency virus regulator of virion expression
RNAi	RNA interference
RT-PCR	Reverse transcription-polymerase chain reaction
SD	Standard deviation
SE	Standard error
SINE	Selective inhibitor of nuclear export
siRNA	Silencing RNA
SREBP-1a	Sterol regulatory element-binding protein-1a
SV40	Simian virus 40
T-ag	Large tumour antigen
TNBC	Triple negative breast cancer
tNTS	Tumour cell-specific nuclear targeting signal (from Chicken anaemia virus VP3)
TRF-1	Telomeric repeat-binding factor-1
UT	Untreated
UTR	Untranslated region
VP3	(Chicken anaemia virus) Viral protein 3
UL54	Human cytomegalovirus DNA polymerase catalytic subunit UL54

PREFACE

This thesis is presented as a 'thesis by publication' due to the fact that a number of publications have arisen from this study. Chapters 2, 3 and 4 are presented as individual manuscripts, irrespective of their publication status. To maintain continuity, the formatting and numerical referencing system is kept consistent throughout, irrespective of whether the work is published or unpublished. The manuscripts are presented in their published/submitted format within the Appendix section. Chapters that include submitted/published manuscripts contain the mandatory declaration of authorship information at the beginning of the chapter. When required, a preface/explanatory paragraph is included within a Chapter to illustrate the publication status of the work and to ensure continuity between the chapters. A general discussion of all the results shown within this thesis is presented in Chapter 5.

CHAPTER 1

Literature Review

1 Literature Review

1.1 INTRODUCTION

Signal-dependent bidirectional trafficking between the nucleus and cytoplasm is conventionally mediated by the Importin (Imp) superfamily of nuclear transporters and controls the activity of many signalling factors (such as those involved in gene transcription), which modulate cell growth, differentiation, proliferation, apoptosis, response to DNA damage, migration, inflammation and angiogenesis. Regulation of nuclear transport, central to changes in cellular phenotype, can occur by modulation of the cargo itself, or through alteration of Imps. Interestingly, altered Imp levels are associated with cellular malignancy and tumour disease progression in terms of tumour grade, stage, aggressiveness and patient prognosis, but the functional relevance with respect to changes in Imp activity and nuclear transport has not been fully elucidated. Increased Imp levels may contribute substantially to more efficient nuclear localisation of signalling factors important for maintenance of increased replication, migration, DNA repair, inflammation or angiogenesis, and decreased differentiation or apoptosis. How protein translocation into the nucleus is mediated in Imp-dependent or –independent fashion is discussed below.

1.2 NUCLEOCYTOPLASMIC TRANSPORT

1.2.1 Nuclear pore complexes

The eukaryotic cell nucleus is enveloped by phospholipid membranes collectively called the nuclear envelope, which acts as a barrier between the cell's genetic material and cytoplasmic components. Translocation of molecules between the nucleus and cytoplasm occurs exclusively through large (c. > 60 MDa) dynamic sieve-like structures called nuclear pore complexes (NPCs), comprised of c. 30-40 different nucleoporin (Nup) proteins, which can accommodate rapid bidirectional transport of < 1000 molecules/sec (1). Nups are responsible for maintaining the structural integrity and permissiveness of the pores, and play a critical role in nuclear transport, as they offer transient docking sites for nuclear transporters (2). The NPC also allows passive diffusion of molecules c. 60 kDa (3) or < 9 nm in diameter, although on a physiological scale this is an exceedingly slow process (4). Most small and large molecules (such as proteins and signalling

factors) utilise specialised transport machinery to gain access into and out of the nucleus in a signal-mediated, energy-dependent process.

1.2.2 Nuclear protein import

1.2.2.1 Nuclear localisation signals

Conventional nuclear import is dependent on the presence of nuclear localisation signals (NLSs) on cargo proteins, which are recognised by members of the Importin (Imp) superfamily of transporters. Two broad classes of NLSs were initially described, monopartite NLSs that consist of a simple 3-5 basic amino acid (aa.) sequence comparable to that of the well-characterised Simian virus 40 (SV40) large tumour antigen (T-ag) ($PKKKRKV^{132}$) (5), and bipartite NLSs that consist of 2 clusters of basic residues separated by a spacer region of 10-12 basic aa. such as the NLS of *Xenopus laevis* histone chaperone protein nucleoplasmin (KR-spacer-$KKKKL^{171}$) (6, 7). Other more varied NLSs have been described, such as the non-basic, glycine- and asparagine-rich M9 sequence on the A1 protein of the heterogeneous nuclear ribonucleoprotein participle hnRNP A1 (8), the proline- and aspartic acid residue rich NLS form proto-oncogene c-myc (9), and conformational NLSs such as the one found in the C-terminal region of the Rabies Virus Phosphoprotein (10) or the central double stranded RNA binding domain of the RNA-editing enzyme ADAR1 (11)).

1.2.2.2 Importins

NLSs are generally recognised by the α and β Imps subtypes, of which multiple isoforms are expressed in humans (12, 13), either through Impβ alone or through an Impα adapter protein heterodimerised with Impβ1 (see following Sections). Based on sequence homology, there are 3 subfamilies of Impαs; P (Impα1/α8), Q (Impα3/α4) and S (Impα5/α6/α7) (14), where members of different subfamilies have about 50% sequence identity at the amino acid level, whereas within a subfamily the identity is at least 80% (15, 16). Impα8 is a recently discovered, highly divergent member of the Impα family that most closely resembles Impα1 in terms of structure, but its cellular localisation, cargo recognition and sequence identity differs greatly to that of Impα1 (16), therefore its placement into the α1 subfamily is highly tentative. In contrast to Impαs, the Impβs are quite distinct from one another (12).

Imps appear to have a specific set of cargoes with which they interact, although there is some redundancy in cargo recognition between the Imp isoforms (see **Table 1.1**). For example Impα1 recognises pluripotency factor Oct4, cell cycle modulators CDK2 and E2F1, and proteins involved in DNA repair such as CHK2 and NBS1, whereas Impα3 recognises components of the Ran system RCC1 and RanBP3 important for the nuclear transport cycle, as well as the Signal transducer and activator of transcription (STAT) 2 protein, whilst Impα5 imports STAT1 and 3, and differentiation inducing factors Brn2 and Oct6. However the above Imps can all corporate in the import of the proto-oncogene c-myc, whilst Impα3 and α5, but not α1 both mediate nuclear import of cyclin dependent kinase inhibitor $p27^{kip1}$. Another example is that Impβ1 recognises constitutive transcription factors activator protein-1 (AP-1) and cAMP-response element binding protein (CREB), whilst Impβ2 recognises the mRNA export factor TAP, but both can import ribosomal proteins and histones. Cargoes recognised by Impαs include those involved in initiation, maintenance or inhibition of differentiation, immune and inflammatory responses, DNA repair, cell cycle regulation, apoptosis and migration (**Table 1.1**). This and the fact that the expression of Impα isoforms becomes altered during cellular changes in phenotype (see Sections 1.3.1.1.2.2 and 1.5.1.1) has led to the idea that Impαs may be important for co-operating both housekeeping, as well as "higher order" responses, being central to diseases (17, 18) and development (14). The fact that many constitutively imported proteins and DNA/RNA modulating factors important for gene expression machineries are recognised by ubiquitously expressed Impβs (**Table 1.1**), and that most Impβ isoforms are evolutionarily conserved in lower eukaryotes, amphibians, insects and yeast, suggests a potential housekeeping nuclear transport role (12).

1.2.2.3 Nuclear import by the Importin α/β1-heterodimer

Nuclear import mediated by the Impα/β1-heterodimer is carefully controlled at a number steps. During productive import, Impα is recognised by Impβ1 through its flexible N-terminal Importin beta binding domain (IBB) resulting in the dislodgement of the overlapping auto-inhibitory loop bound to the major and minor binding grooves located in middle (Armadillo (ARM) repeats 3/4) and C-terminal (ARM repeats 7/8) portions of Impα respectively (**Fig. 1.1**), to allow binding to NLSs on cargo proteins (19).

Table 1.1: Selected examples of cellular proteins recognised and imported into the nucleus by Importins.

Importin	Protein	Function	References
Impα1 (KPNA2 / Rch-1)	c-myc	Oncogenic transcription factor	(20)
	NBS1	DNA repair	(21)
	CHK2	DNA repair	(22)
	Sp-1	Transcription factor (multiple targets)	(23)
	Sept9_i1	Co-factor for HIF1α mediated signalling	(24)
	PLAG1	Oncogenic transcription factor	(25)
	IGFBP-2	Transcription factor, glucose metabolism	(26)
	Taspase	Protease, pluripotency maintenance	(27)
	CREBBP (CBP/p300)	Transcriptional co-activator	(28)
	CBC	mRNA processing factor	(29)
	PABPC	mRNA processing factor	
	E2F1	Cell cycle progression	(30)
	CDK2	Cell cycle progression	(31)
	TAFs	Transcriptional co-activator (RNA pol II)	(32)
	ADAR3	RNA editing	(33)
	Oct4	Transcription factor, pluripotency	(34)
	Rac1	Signalling protein, cell motility/adhesion and MAPKKK activation	(35)
Impα3 (KPNA4 / Qip1)	p53	DNA repair, cell cycle inhibition, apoptosis	(36)
	c-myc	Oncogenic transcription factor	(37)
	NAC-1	Transcription factor, pluripotency	(38)
	Sp-1	Transcription factor (multiple targets)	(23)
	RanBP3	RanGTP generating co-factor	(39)
	RCC1	RanGTP generating factor	(40)
	Daxx	Transcriptional regulation, cell death	(41)
	HDAC1	Gene expression, cell proliferation and differentiation	(42)
	RNA Helicase A	RNA processing factor, gene expression	(43)
	IRF9/STAT2	Transcription factor, inflammatory responses	(44)
	NF-κB	Transcription factor, inflammatory responses	(45)
	$p27^{kip1}$	Cell cycle regulator	(46)
Impα5 (KPNA1/ Srp-1)	Brn2	Transcription factor, differentiation	(34)
	Oct6	Transcription factor, differentiation	(34)
	c-myc	Oncogenic transcription factor	(47)
	ADAR2	RNA editing	(33)
	STAT1	Transcription factor, inflammatory responses	(48)
	STAT3	Transcription factor, inflammatory responses	(49)
	HDAC1	Gene expression, cell proliferation and differentiation	(42)
	$p27^{kip1}$	Cell cycle regulator	(46)
Impβ1 (KPNB1/ NTF97)	rPL23a, rPS7, rPL5, rPL6, rPL18a	Ribosomal proteins	(50, 51)
	Histones	Gene expression	(52, 53)
	SMAD3	Transcription factor, TGF-β induced cytokine signalling	(54)
	CREB	Transcription factor, cAMP-induced signalling	(55)
	AP-1 (c-Jun)	Oncogenic transcription factor, steroid genes	(55)
	PTHrP	Polyhormone signalling factor	(56)
	TRF-1	DNA maintenance	(57)
	SREBP-1a	Transcription factor, fatty acid and cholesterol metabolism	(58)
Impβ2 (Tpn)	hnRNPs (eg. hnRNP A1)	mRNA binding proteins	(59)
	ADAR1	RNA processing factor	(60)
	TAP	RNA export factor	(61)
	HuR	mRNA stability	(62)
	rPL23a, rPL5, rPS7	Ribosomal proteins	(50)
	Histones	Gene expression, chromatin stability	(52, 53)
	PABP2	RNA processing factor	

Importin	Protein	Function	References
Imp5 (RanBP5)	Histones	Gene expression	(52, 53)
	rPL23a, rPL5, rPS3a, rPS7	Ribosomal proteins	(50)
	Rag-2	DNA recombinase	
Imp7	Histones	Gene expression	(52, 53)
(Impβ7/RanBP7)	rPL23a, rPL5, rPS7 (rPL4, rPL6, rPS3a)*	Ribosomal proteins, translation	(50, 51)
	ERK2, MEK1	Transcription factor – apoptosis and cell cycle	(63)
	PRH	Transcriptional repressor, haematopoietic differentiation	(64)
	AR	Transcription factor ^	(65)
	GR	Transcription factor	(66)
Imp9	rPS7, rPS3, rPS7, rPS9, rL19, rPL18a	Ribosomal proteins, translation	(51)
(RanBP9)	Histones	Gene expression	(52)
	Hsp27	Chaperone, stress resistance and actin re-organisation	(51)

*In a heterodimer with Impβ1.
^ Non-functional import complex / retention factor resulting in repression of AR activity.

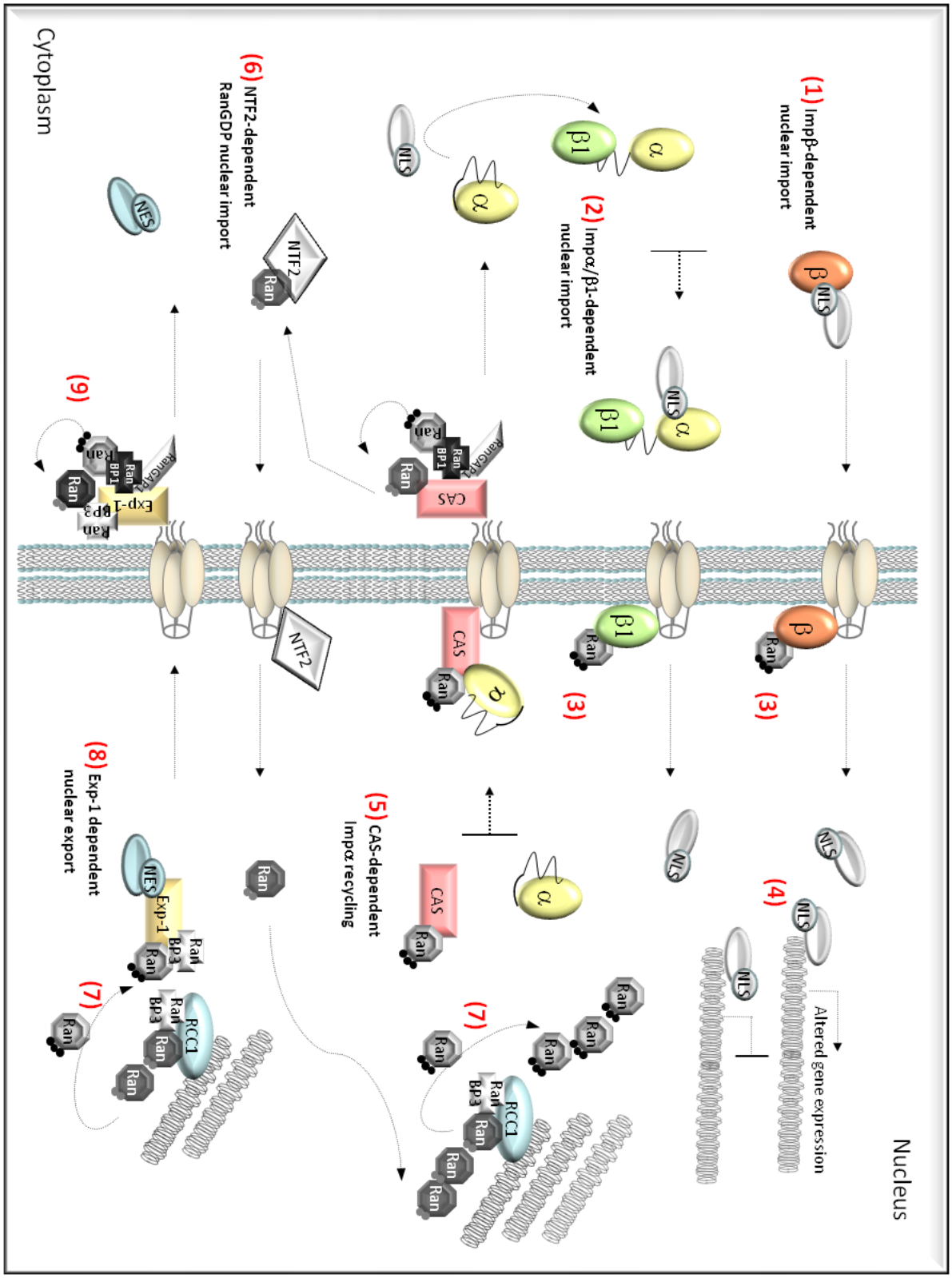
Abbreviations: ADAR, Adenosine deaminase acting on RNA; AP-1, activator protein-1; AR, androgen receptor; CBC, cap-binding complex; CDK2, cyclin dependent kinase 2; CHK2, checkpoint kinase 2; CREB, cAMP response element binding protein; CREBBP/CBP, CREB binding protein; E2F1, elongation 2 factor initiator 1; ERK2 (MAPK1), extracellular signal-regulated kinase 2 / mitogen-activated protein kinase 1; GR, glucocorticoid receptor; HDAC1, histone deacetylase 1; HIF1α, hypoxia inducible factor 1α; Hsp27, heat shock protein 27; IGFBP-2, insulin-like growth factor binding protein 2; MAPKKK, mitogen-activated protein kinase kinase kinase; MEK (MAP2K1), mitogen-activated protein kinase kinase 1; NAC1, nucleus accumbens-associated protein 1 ; NBS1, nijmegen breakage syndrome 1; Oct4, octamer binding factor 4; PABP2, poly(A) binding protein 2; PABPC, poly(A) binding protein, cytoplasmic; PLAG1, pleiomorphic adenoma gene 1; PRH, proline-rich homeodomain protein; PTHrP, parathyroid hormone-related protein; Rac-1, ras-related C3 botulinum toxin substrate 1; rPL, L ribosomal protein (large subunit); rPS, S ribosomal protein (small subunit); Sept9_i1, septin 9 isoform 1; SMAD3, mothers against decapentaplegic homolog 3; SREBP-1a, sterol regulatory element-binding protein-1a; STAT, signal transducer of activated transcription; TAP, transporter associated with antigen processing ; TAFs, transcription associated factors; Tpn, transportin; TRF-1, telomeric repeat-binding factor-1.

Translocation through the NPC of the trimeric Impα/β1:cargo complex is mediated by binding of Impβ1 onto hydrophobic FXFG repeat-containing Nups such as Nup358, Nup214 and Nup153 (19). In the nucleus, Nup50 appears to bind to both Impβ1 and Impα to stimulate Impα/β1-dependent import (67); docking at the Nup153-Nup50 interface facilitates the disassembly of the Imp:cargo complex by dislodging certain cargoes from Impα (68), in part by pushing the equilibrium towards prevention of NLS re-binding (69). Binding to Impβ1 of the monomeric guanine nucleotide binding protein Ran, in activated GTP-bound form, effects the release of Impβ1 from Impα enabling closure of the Impα autoinhibitory loop (containing the IBB domain) preventing both NLS and Impβ1 re-binding to Impα, ensuring the import complex does not re-assemble (68, 70, 71) (**Fig. 1.1**). Displacement of cargo-free Impα from Nup50 is triggered by competitive binding of the specialised export transporter (Exportin) cellular apoptosis susceptibility protein (CAS), in its RanGTP-bound form to the Nup50 binding site of Impα, allowing re-export of Impα by CAS to the cytoplasm, to ensure that Impα is ready for another round of nuclear import (68) (**Fig. 1.1**). High levels of nuclear

RanGTP are essential to nuclear import, providing directionality and cycling of import receptors during the transport process, and are provided by the exclusively nuclear chromatin-bound Ran guanine exchange factor (RanGEF) RCC1, and its co-factor RanBP3 (39) (**Fig. 1.1**).

Nuclear import through multiple Impα adaptors forming the Impα/β1-mediated pathway present in yeast and multicellular organisms promotes complexity and dynamic range due to improved flexibility in cargo recognition. There is one Impα in yeast, whilst three Impα isoforms have been identified in the worm (72) and fruitfly (73), and up to seven isoforms in mammals (14), this expansion of the number of Impα isoforms expressed implying important diversification of functional role in complex organisms. Other IBB domain containing proteins apart from Impα that can also serve as adaptors for Impβ1-mediated trafficking through the NPC include XRIPα , a specific adaptor of the replication protein A involved in DNA metabolism (74) and snurportin, which is the import adaptor of ribosomal U snRNPs, a large class of ribonucleoproteins complexes critical for RNA splicing (75).

Figure 1.1 Facilitated nuclear protein transport. Nuclear import can be mediated by the Importin (Imp) superfamily of transporters of which there are α and β types, whereby **(1)** Impβ alone or **(2)** an Impβ1 bound Impα adapter recognise nuclear localisation signals (NLSs) on cargo proteins. Translocation of the cargo:Imp complex into the nucleus through the nuclear pore complex (NPC) is mediated by Impβ binding to FXFG repeat Nucleoporins (Nups) in NPCs. **(3)** In the nucleus, binding of Ran in the activated GTP-bound form to Impβ promotes the disassembly of the import complex resulting in the release of the cargo protein into the nucleoplasm, **(4)** ensuing its nuclear functions such as transcription factor-mediated changes in gene expression. **(5)** The Exportin (Exp) CAS (Exp-2), when bound to RanGTP is responsible for the cytoplasmic recycling of Impαs subsequent to a round of protein nuclear import. Exps such as CAS and Impβs are thought to mediate their own recycling subsequent to a round of nuclear export and import respectively (not shown). **(6)** The nuclear import of hydrolysed Ran (RanGDP) by the non-Imp transporter NTF2 ensures RanGTP production in the nucleus, **(7)** where the nuclear chromatin-associated Ran guanine nucleotide exchanger factor RCC1, facilitated by RanBP3, generates RanGTP from RanGDP. **(8)** Exps are Impβ homologues that mediate nuclear protein export. Given by way of example is Exp-1 that recognises nuclear export signals (NESs) on cargo proteins when bound to RanGTP, which is further stimulated by binding of co-factor RanBP3. The nuclear export complex then traverses through the NPC into the cytoplasm, where **(9)** unloading of the cargo from Exp-1 is affected by hydrolysis of GTP-bound Ran by cytoplasmic RanGAP1 facilitated by RanBP1.


Nucleus
Cytoplasm
(1) Impβ-dependent nuclear import
(2) Impα/β1-dependent nuclear import
(3)
(4)
Altered gene expression
(5) CAS-dependent Impα recycling
(6) NTF2-dependent RanGDP nuclear import
(7)
(8) Exp-1 dependent nuclear export
(9)
NLS
NES
Ran
RanBP1
RanGAP1
RanBP3
RCC1
CAS
NTF2
Exp-1

1.2.2.4 Nuclear import by Impβs

Nuclear translocation mediated by Impβ is a simple and rapid process (76), through the fact that the cytoplasmic concentration of RanGTP is low, enabling free Impβ to bind to NLSs, initiating nuclear import through the NPC. As for Impα/β1-type import, Impβ family members mediate nuclear translocation of their cargoes by direct binding to Nups (12). Once in the nucleus the transport complex dissociates upon binding of RanGTP and the cargo is released into the nucleoplasm (**Fig. 1.1**). Impβs may also form heterodimeric complexes with one another, such as Impβ1/Imp7 (77), which are thought to give flexibility and amplify the cargo recognition ability of Impβ1 (12). Unlike Impαs, RanGTP-bound Impβs are thought to mediate their own recycling back into the cytoplasm, where the Nup358, RanGTPase RanGAP and co-factor RanBP1 activate the hydrolysis of RanGTP to trigger the release of Impβ from RanGDP (78). Intriguingly, Impβs can serve a dual function as transporters and/or cytoplasmic chaperones for basic type DNA/RNA binding proteins such as histones and ribosomal proteins (51).

1.2.2.5 Imp-independent nuclear import

Nuclear translocation can also occur in Imp-independent fashion, an obvious example being nuclear import of RanGDP by the non-Imp protein NTF2 (79) (**Fig. 1.1**). Other examples include nuclear transport of the heat shock protein factor Hsp70 mediated by the non-Imp-related transport protein Hikeshi (80), and of the high-mobility group (HMG)-box-containing chromatin-remodelling factors SRY (sex-determining region on the Y chromosome *SRY*) (81) and SOX9 by the calcium binding protein calmodulin (82). Nuclear translocation may also occur by direct binding to Nups within the NPC, such as for the tissue growth factor (TGF)-β pathway associated transcription factors SMAD3/4 (83), and signalling molecule β-catenin (84). Certain factors are able to use dual Imp/Imp-independent pathways for nuclear translocation, to ensure nuclear access under diverse conditions; Impβ1-recognised SRY/SOX9 proteins are one example, where increased calcium levels inhibit conventional Impβ1-mediated nuclear import (85) to favour calmodulin-dependent trafficking (86, 87). Calcium pulses are associated with fertilisation and embryo development (88) thus a dual import mechanism may be critical for SRY/SOX9 nuclear roles in initiation of male sex differentiation.

1.2.3 Nuclear protein export

1.2.3.1 Nuclear export signals

As for nuclear import, nuclear export of proteins requires a targeting signal, called a nuclear export signal (NES) on the cargo protein that is recognised by Impβ homologues called Exportins (Exps). Although NESs are generally short, hydrophobic, often leucine-rich patches within cargo proteins (the prototypical NES sequence is LxxxLxxLxL (89)), where isoleucine, valine or phenylalanine residues may substitute for leucine, other NESs (such as the NES in transcription factor NFAT (SAIVAAINALTT^{321}) (90)) appear to be quite distinct.

1.2.3.2 Exportins

Seven Exps (1, 2, t, 4-7), homologues of Impβ, are expressed in humans. The best characterised is Exp-1 (Crm-1), which recognises leucine-rich NESs (91) and mediates nuclear export of a variety of functionally distinct endogenous factors such as tumour suppressor and oncoproteins, proteins involved in control of cell cycle progression and apoptosis, and contributes to nuclear export of diverse RNAs such as rRNA (92-94). Many different cargos have been described for Exp-1 to date, probably owing due to the availability of a specific inhibitor for Exp-1 function, leptomycin B (LMB) (95). Apart from Exp-1 and unlike their Impβ1 counterparts, Exps appear to have evolved with specialised functions, although only a limited number of cargoes are currently known for Exps other than Exp-1 (96). Exps with specialised roles include CAS (Exp-2) that facilitates the recycling of the Impα isoforms to the cytoplasm, to enable subsequent rounds of nuclear import thereby being a central component of Impα/β1-dependent import (97) (**Fig. 1.1**) and Exp-t and Exp-5, which are responsible for nuclear export of tRNAs and miRNAs respectively (94).

1.2.3.3 Exp-dependent nuclear protein export

In the nucleus only RanGTP-bound Exps can recognise NESs on a cargo proteins (89), resulting in complex translocation through the NPC via Exp-mediated binding of Nups, converging with Impβ-mediated import pathways (98). Exp-1 utilises an additional co-factor RanBP3 that enhances the interaction between leucine-rich NESs and Exp-1 stimulating nuclear export (99, 100), possibly through bringing the export complexes into close proximity of RanGTP-generating RCC1 (101) (**Fig. 1.1**). Once in the cytoplasm, Nup88 and Nup214 act as docking sites for the export complexes and the complex is dissociated in close proximity of Nup358, which facilitates by the hydrolysis of

RanGTP by the cytoplasmic factors Ran guanine hydrolysing factor RanGAP1 and co-factor RanBP1 (102), resulting in cargo expulsion from the complex (**Fig. 1.1**). It is presumed that like Impβs, Exps mediate their own recycling back into the nucleus subsequent to a round of nuclear export, but do so in the absence of Ran binding (103).

1.3 REGULATION OF NUCLEAR TRANSPORT

Effective signal transduction relies on efficient nuclear targeting of signalling factors/effectors to induce phenotypic changes, and with myriads of signalling pathways converging at the level of nuclear trafficking, regulation of nuclear transport is essential. Regulation is a multi-tiered process that occurs through a number of different mechanisms that involve modulation of either individual cargoes, or nuclear transport components such as Nups or Imps/Exps, thereby affecting the nuclear transport of multiple cargoes. Specific focus in the following Sections is devoted to transport regulation through effects on the nuclear transport apparatus itself.

1.3.1 Modulation of components of the nuclear transport machinery

Although the general principles of the mechanisms for Imp-dependent nuclear protein transport have been largely elucidated, the extent to which the components of the nuclear transport machinery represent a dynamic, potentially interdependent system and the degree to which this may be a means to modulate nuclear transport efficiency, is not clear. Current advances in this area are discussed below.

1.3.1.1 Alterations in levels

1.3.1.1.1 Nucleoporins

Studies in the fruitfly present direct evidence that NPC components can regulate cargo shuttling. During embryogenesis, dorsal-ventral polarity of the fruitfly embryo is regulated by the transcription factor Dorsal, the orthologue of NF-κB, in response to Toll receptor signalling, which inhibits the fly orthologue of I-κB, the cytoplasmic retention factor Cactus, to trigger Dorsal nuclear import. To ensure Dorsal nuclear activity, Exp-1 activity is inhibited through increased expression of Nup88 and Nup214 in ventral, but not dorsal cells, where Exp-1 becomes sequestered by the Nup88-Nup214 complex at the cytoplasmic rim of the NPC leading to inhibition of Dorsal nuclear export and

altered transcription of its gene targets (**Fig. 1.2a**) (73, 104, 105). It is interesting that a number of Nups such as Nup50, important for Impα/β1 mediated nuclear import (see Section 1.2.2.3) and GP210, responsible for anchoring the NPC to the nuclear membrane among others, exhibit cell type-specific expression in the mouse and the fruitfly (106). The relevance of such alterations has not yet been fully characterised but has been predicted to alter the NPC composition/transport capacity and/or modulate gene expression directly (106, 107).

Further examples of altered nuclear transport efficiency arising due to changes in Nup levels include polio-, rhino- and influenza-virus induced degradation of distinct Nups to inhibit nuclear transport of host proteins or mRNAs, which contributes to efficient viral replication and evasion from host immunity (108-110).

1.3.1.1.2 Importins / Exportins and Ran regulators

1.3.1.1.2.1 Proof of principle experiments in model cell systems

The first studies to investigate the effects of nuclear transport machinery levels on transport efficiency in model cell systems concentrated solely on whether components of the Ran system (such as RCC1, Ran import carrier NTF2, RanGAP, RanBP1 and Ran itself) could alter RanGDP:RanGTP distribution and nuclear import efficiency. The Ran system appears to be robustly buffered to resist effects from depletion of its components in computational transport system analysis and experimental microinjection studies (111, 112). Microinjection studies performed by Riddick et al. in intact HeLa cells indicate that addition of exogenous Ran, NTF2 or RanBP1, but not RCC1 or RanGAP appear to stimulate the initial rate of NLS-dependent nuclear accumulation of an Impα-recognised GST-GFP-NLS (113) by up to 2-fold compared to controls, presumably through increasing nuclear RanGTP levels; overall capacity (efficiency) of NLS nuclear import at 30 min post-injection was similarly affected (76). Imp levels were also found to alter both nuclear import rate and efficiency,

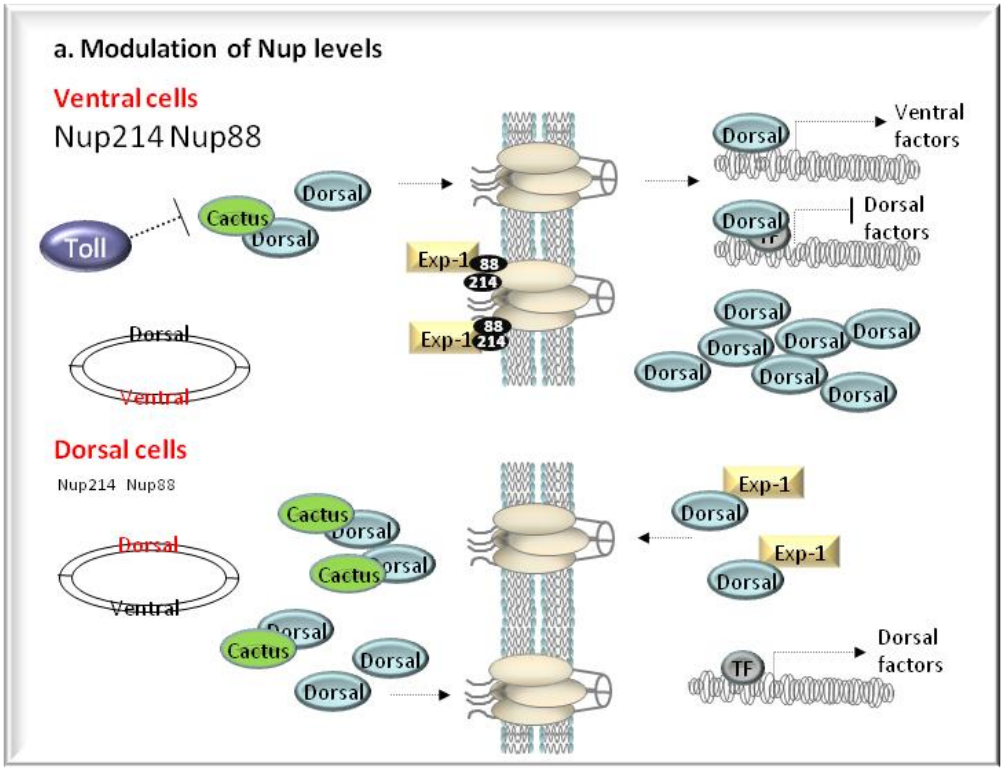


Figure 1.2 Regulation of nuclear protein transport (a) **By modulation of Nup levels.** Regulation of spatial organisation in developing fruitfly embryos occurs through Nf-κB (Dorsal) signalling. Dorsal is expressed in both dorsal and ventral cells, where it is retained in the cytoplasm by the Iκ-B like retention factor Cactus. (Top) In ventral cells the activation of Toll receptor signalling results in inhibition of Cactus-mediated retention resulting Dorsal nuclear import, where high levels of Nup214 and Nup88 contribute to the nuclear trapping of Dorsal by sequestering Exp-1 at the nuclear rim inhibiting Dorsal nuclear export leading to expression of ventral promoting cell factors and inhibition of dorsal promoting factors. (Bottom) In dorsal cells the levels of Nup214/88 are decreased compared to ventral cell types; Dorsal that has escaped Cactus-mediated cytoplasmic retention to localise in the nucleus of dorsal cells is rapidly exported by Exp-1 due to lack of Nup-mediated Exp-1 sequestration allowing expression of dorsal promoting factors. TF, transcription factor. Adapted from (73).

with microinjection of Impα1 stimulating GST-GFP-NLS import rate by c. 2-fold and GST-NES-GFP-NLS nuclear accumulation at steady-state by up to 5-fold, indicating that under the conditions tested Impα1 is a major limiting factor for nuclear import (76, 113). In contrast, microinjection of Impβ1 suppressed efficiency of nuclear translocation of Impα-recognised fusion proteins GST-GFP-NLS and GST-NES-GFP-NLS. Computational models predicted increased Impβ1 concentrations deplete nuclear RanGTP, which was verified by experimental models in which co-injection of Ran with Impβ1 and GST-GFP-NLS was shown to reverse the suppression of cargo import by Impβ1 (113). It is

plausible that under the test conditions excess Impβ1 may compete with Impα/β1:cargo complexes for free RanGTP in the nucleus, decreasing cargo release from and arresting Impα/β1:cargo complexes at the NPC; surprisingly the nuclear import efficiency and rate of an Impβ1-recognised cargo in cells injected with Impβ1 was not tested by the investigators. Conversely, Yang and Musser (114) reported increased Impβ1 concentration decreased interaction time and improved cargo nuclear import efficiency of an Impα-recognised NLS from Simian virus 40 (SV40) large tumour antigen (T-ag NLS), but the bulk of these studies were performed in non-intact as opposed to intact cells. Although the concentrations of endogenous Imp and NPC components are not readily measurable in intact as compared to non-intact systems that are reconstituted in an exogenous buffer with specified amounts of transport components, intact systems are more physiologically relevant in terms of presence of competing cargoes and endogenous components.

Clearly the complex nature of the nuclear transport machinery, and the difficulty of finding malleable systems to probe it, makes definitive interpretations based on data from *in vitro*/microinjected cells systems challenging at best.

1.3.1.1.2.2 Altered Importin levels during cell development

That Impαs levels may play distinct roles in cells is implied by descriptive studies showing that levels or activity of Imps are different in various tissue types (115, 116), cell types (20, 115) and phenotypes such as SV40-transformed versus non-transformed cells (117), quiescent versus proliferating cells (118), senescing (aged) compared to young fibroblasts (119), immature vascular smooth muscle cells and embryonic stem cells undergoing differentiation into fully differentiated contractile smooth muscle cells (120) and cardiomyocytes respectively (121), and blood cells induced to differentiate *in vitro* into macrophage and neutrophil lineages compared to undifferentiated cells (122). Impα and Impβ expression patterns/localisation also change during developmental stages in the worm (72), fruitfly (73) and during murine spermatogenesis (123-129) to favour the nuclear translocation of factors that control cellular development at specific time points.

Interestingly, Impα expression switches from α1 to α5 upon stimulation of differentiation by phorbol myristate acetate (PMA) or DMSO treatment of human promyelocytic leukaemia HL-60 cells into macrophage or granulocyte lineages respectively (122). A subsequent landmark study by

Yasuhara et al. demonstrated how switching of Impα1 to α5 expression could determine a neuronal cell fate in mouse embryonic stem (ES) cells. In pluripotent ES cells, the levels of Impα1 are high, resulting in nuclear accumulation of the Impα1-recognised pluripotency factor Oct3/4 and upregulation of its gene targets; in contrast Impα5 is barely detectable (**Fig. 1.2b**) (130). During retinoic acid-induced differentiation, levels of Impα1 decrease with a concomitant increase in Impα5 levels leading to inhibition of Oct3/4 nuclear import and protein levels, and increased nuclear import and levels of the Impα5-recognised differentiation inducing factors Tuj1, Nestin and Brn2 (130). The transient knockdown of Impα1 expression in pluripotent ES cells was sufficient to decrease the pluripotency state, and trigger the expression of differentiation-inducing factors and Impα5 itself (130). Further, high levels of Impα1 in undifferentiated ES cells appear to effect cytoplasmic retention of differentiation inducing factor Oct6, expressed in undifferentiated cells. This inhibition is thought to occur by the binding of the Impα1 C-terminal region (rather than the major NLS binding site of Impα1 that recognises Oct3/4) to Oct6 resulting in a transport incompetent Impα1/β1:Oct6 complex, and inhibition of Oct6 nuclear translocation and differentiation-inducing activities (**Fig. 1.2b**). The binding of NLSs to the conventional major and minor NLS binding sites of Impα (ARM repeats 3/4 and 7/8, respectively) leads to productive nuclear import (34). Importantly, ectopic expression of Impα1 in ES cells induced to differentiate by retinoic acid treatment is sufficient to inhibit progression of differentiation to a neuronal cell type by induction of cell death, meaning Impα1 activity must be inhibited (in addition to Impα5 activity being increased) to allow neural differentiation to occur (34).

Impα3 levels are also known to increase in differentiating mouse ES cells compared to pluripotent cells (130, 131). In ES cells undergoing embryonic body differentiation, this is associated with re-localisation of Impα3 from the nucleus to the cytoplasm and reduced Oct3/4 expression (131). That Impα3 may negatively regulate Oct3/4 expression is indicated by the fact that ectopic expression of Impα3 in ES cells decreases levels of endogenous Oct3/4 by 50% compared to non-transfected controls, speculated to occur due to increased Impα3-mediated import of differentiation-inducing transcription factors (131).

Other examples where Impα levels may influence development/differentiation include in keratinocytes induced to differentiate by interferon (IFN)-γ-treatment, during which Impα1

expression was found to be essential for the nuclear translocation of IFN-response factor-1 (IRF-1) and expression of targets known to be involved in epidermal differentiation (132), and during induction of muscle cell development (myogenesis) *in vitro* where myotube size and myocyte migration both depend on increased levels of Impα1 (15).

The clear implication from the above is that altered Impα levels may modulate and/or are integrally linked to cell development and differentiation.

1.3.1.2 Post-translational modifications

1.3.1.2.3 Nucleoporins

A possible link between post-translational modifications on nuclear transport capacity has been suggested by several reports that show broad spectrum kinase or phosphatase inhibitors affect nuclear transport capacity. Studies in digitonin permebialised cells indicate that pre-incubation of cells with alkaline phosphatase can inhibit nuclear import of albumin-conjugated nucleoplasmin and T-ag NLS containing peptides, which is relieved by the addition of exogenous protein kinase A (PKA) (133). Consistent with this, treatment of cells with a peptide inhibitor of the PKA kinase inhibits nuclear import of T-ag NLS, serum response factor and cyclin A cargoes (134). The fact that all tested cargo peptides in the above studies lacked phosphorylation sites indicates that phosphorylation-regulation of nuclear transport components. Interestingly, the Impβ1-binding regions of Nup50, Nup153 and Nup214 have been shown to be phosphorylated by the mitogen activated protein kinase (MAPK) extracellular signal-regulated kinases (ERK) (a downstream target antagonised by PKA-mediated cell growth inhibition (135)), resulting in reduced Impβ1 affinity with these NPC components, inhibiting docking and release of cargoes from Imp:cargo complexes, without altering the structural integrity of the NPC itself (136). ERK-dependent phosphorylation of Nups and their altered distribution/functionality may play a role in impaired Impα/β1- and Exp-1- dependent nuclear transport capacity during oxidative stress both in cells treated with nonlethal doses of the oxidant diethyl maleate (DEM) (137, 138) or high doses of hydrogen peroxide (H_2O_2) (139, 140), or regulate transport-independent roles of Nups during mitosis (see Section 1.4) (136, 141).

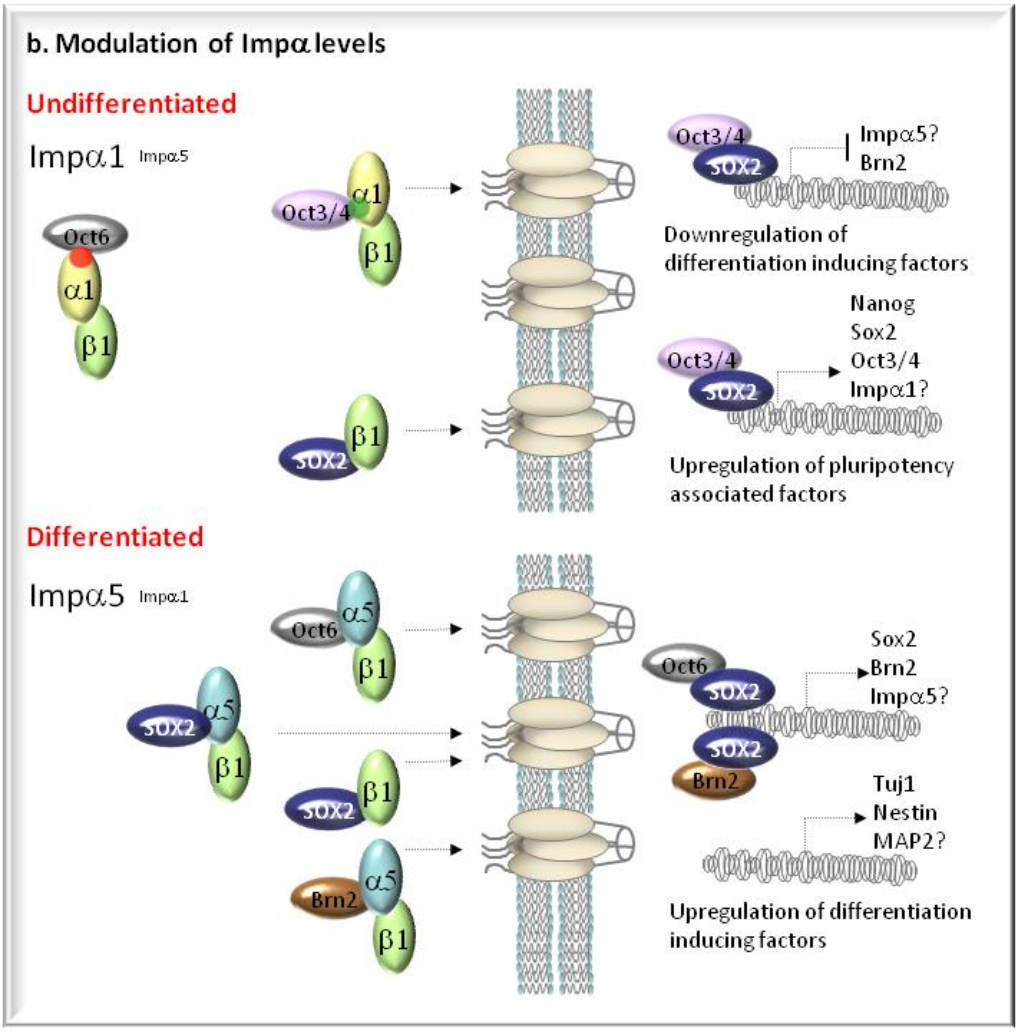


Figure 1.2 Regulation of nuclear protein transport (b) By modulation of Impα levels. Impα subtype switching modulates neural differentiation in mouse embryonic stem cells. (Top) In undifferentiated cells high levels of Impα1 maintain pluripotency. The N-terminal NLS binding site (green shading) of Impα1 recognises the pluripotency factor Oct3/4, resulting in Oct3/4 nuclear import through the Impα1/β1 heterodimer and altered expression of Oct3/4 targets involved in maintenance of pluripotency. Upregulated targets may include Impα1 itself providing a positive feedback loop, whereas downregulated targets include differentiation inducing factors such as Brn2 and Impα5. In undifferentiated cells the C-terminus of Impα1 (red shading) when bound to Impβ1 retains differentiation inducing factors expressed in undifferentiated cells, such as Oct6, in a transport incompetent complex preventing Oct6 nuclear translocation. (Bottom) Neural differentiation can be triggered by RNAi-induced Impα1 knockdown or by loss of Oct3/4 and Impα1 expression induced by retinoic acid treatment resulting in inhibition of Oct3/4 activity and Oct6 cytoplasmic retention, and increased Impα5 levels leading to nuclear import of Impα5-recognised differentiation inducing factors such as Brn2 and Oct6. SOX2 co-regulates Oct3/4, Oct6 and Brn2 targets during both pluripotency and differentiation and is not subject to regulation. Adapted from (14, 34).

1.3.1.2.4 Importins and Ran regulators

Imps can undergo post-translational modifications that may regulate their activity. Both Impα1 and Impα7, but not Impα3 can be acetylated *in vitro*/*in vivo* by the histone acetyl transferase (HAT) CREB binding protein (CBP)/p300, due to a HAT-recognised consensus sequence (G/SK) in N-terminal region of Impα1 (GK^{22}) and Impα7 (GK^{9}) that is absent from Impα3 (28, 142). The residue K^{22} is located within the linker region of the IBB domain of Impα1, and although it is non-essential for Impβ1 binding (142), its side chains contribute to Van der Waals interactions with W^{472} on Impβ1; acetylation at K^{22} neutralises the positive charge of the residue possibly facilitating an amide NH interaction with the hydrophobic W (tryptophan) ring, which may improve binding between Impα1 and Impβ1 (143). Strikingly, although a number of studies have reported acetylation of Impαs, the studies have not directly addressed whether this may modulate Impα affinity for Impβ1 and in turn, Impα/β1-dependent nuclear import efficiency.

The AMP-activated protein kinase (AMPK), an enzyme that responds to cellular metabolic stress, has been shown to phosphorylate Impα1 at S^{105} (143). Significantly, the S^{105} residue is located in the NLS-binding site of Impα1, where negative charge at the site normally supplied by phosphorylation has been suggested to increase binding for basic type NLSs (143). It appears that Impα/β1-dependent nuclear import rates of a GFP-T-ag NLS fusion protein increase during the cell cycle without a change in the overall steady-state level of accumulation, peaking at late G1 and S phases due to increased Impα1:Impβ1 heterodimerisation (144), presumably to accommodate the cells need for efficient and timely import through specific pathways promoting DNA replication and transcriptional regulation. Interestingly, both Impα1 and Impβ1 are found phosphorylated only during G2/M, but this does not appear to relate to altered nuclear import, but rather may play a role mitotic progression (see Section 1.4) (144).

CAS, the exportin for Impαs is also believed to be subject to post-translational modifications. CAS is phosphorylated by MEK1 *in vitro* (145), either indirectly or directly by components in the MAPK and Akt signalling pathways; inhibition of the MAPK MEK1 or Akt results in CAS nuclear or cytoplasmic re-distribution respectively (145, 146). The extent to which this may regulate CAS localisation and activity in untreated cells is unknown, while the effects of cytoplasmic CAS mislocalisation on Impα/β1-dependent nuclear import efficiency have not been elucidated.

The RCC1 cofactor RanBP3 is another nuclear transport component whose activity may undergo regulation by MAPK/ribosomal S6 kinase 1(RSK1)- and Akt-dependent phosphorylation depending on growth stimuli (39). In PMA-stimulated human embryonic kidney cells, MAPK/RSK1-dependent signalling leads to phosphorylation of RanBP3 at S^{58}. Although this does not influence RanBP3s ability to interact with Exp-1, the S^{58} site appears to be important for RanGDP to GTP conversion by RCC1-RanBP3 complexes *in vitro*, possibly through increasing Ran affinity for RCC1:RanBP3 complexes. Interestingly, treatment of cells with growth factors can also lead to RanBP3 phosphorylation at S^{58} by Akt (39). That this may have implications for global nuclear import efficiency is suggested by the fact that RanBP3 silencing in cells transfected to express a non-phosphorylatable S^{58} RanBP3 mutant increases cytoplasmic localisation of Ran and inhibits nuclear import of microinjected T-ag NLS and ribosomal protein L12 through Impα/β1- and Imp11-dependent import pathways respectively, compared to cells transfected with wild-type RanBP3 (39).

Other components of the nuclear transport machinery able to be phosphorylated include the RanGEF RCC1 (147) and Exportin Exp-1 (148) that relate to their specific non-transport roles in mitosis (see Section 1.4). The precise effect of post-translational modification of other nuclear transport components remains to be fully elucidated.

1.3.1.3 Negative regulators of nuclear import

A number of factors have been reported to inhibit Imp-dependent nuclear import of cargo proteins. These include BRAP2, a BRCA1-binding protein that binds phosphorylated NLSs of several cellular (and viral) proteins to inhibit their nuclear import (149, 150), the putative tumour suppressor protein EI24 (PIG8), a p53-induced gene shown to inhibit Impα/β1 and Impβ1-dependent import through direct Impβ1 sequestration by its IBB-like domain (151); ARHI (DIRAS 3), a tumour suppressor protein that appears to function in a similar manner to EI24, in that it binds to a number of Imps (such as Impβ1, Imp7, Imp9 and Impαs) repressing the nuclear import of proteins such as the growth-inducing and anti-apoptotic transcription factor STAT3 in semi-intact cells (152); and CC3, a proapoptotic protein that binds Impβ isoforms such as Impβ1 and β2 to inhibit nuclear import of their cargoes, which is associated with the induction of cell death (153).

1.3.2 Modulation of cargoes

Nuclear transport can be precisely controlled by a number of different mechanisms at the level of individual cargoes leading to transport changes, which only affect the particular cargo in question. This process has already been extensively studied by others and is reviewed in detail elsewhere (154). Briefly, regulation by post-translational modifications in close proximity of nuclear targeting signals (such as phosphorylation) is the most common mechanism, acting to modulate target signal affinity for Imps/Exps in an enhancing or inhibitory fashion, by initiating structural changes that expose nuclear targeting signal/s on the cargo protein. This is shown by way of example in **Fig. 1.2c** for the inducible tumour suppressor transcription factor p53, which translocates into the nucleus in response to stress signals conditional on deubiquitinylation of the p53 NLS by the ubiquitin hydrolase Herpesvirus-associated ubiquitin-specific protease (HAUSP) in parallel with degradation of the E3 ubiquitin protein ligase double minute 2 protein (MDM2), to enable p53 recognition by the Impα3/β1 heterodimer (36). Modulation of target signal affinity can also occur by intra- or intermolecular masking, where nuclear target signal recognition by Imps is prevented by masking by domains within the cargo protein itself, or by heterologous factors. An example is the tetramerisation of p53 protein in the nucleus, which results in masking of the p53 NES and inhibition of Exp-1-mediated nuclear export essential to p53-dependent transcriptional function (155) (**Fig. 1.2c**). Finally, cargoes may also undergo nuclear or cytoplasmic retention through binding to nuclear or cytoplasmic anchoring proteins respectively. In the case of p53, its nuclear import can be inhibited by cytoplasmic sequestration to the p53-associated Parkin-like protein Parc (156) or Hsp70 family member mortalin (mot-2) (157). During cell stress mot-2 is displaced from p53 by upregulation and binding of the ubiquitin ligase like domain containing Ubxn2a to mot-2 (157), whilst Parc levels are downregulated by the calcium-dependent cysteine protease calpain through a proteosomal and caspase independent mechanism, which is associated with p53 nuclear translocation (158) (**Fig. 1.2c**).

Interestingly, Imps themselves can inhibit nuclear import of particular cargoes. For example, the Impβ homologue Imp7 binds the Impα/β1-recognised NLS of the androgen receptor (AR) retaining AR in the cytoplasm, until hormone stimulation with androgen displaces Imp7 to allow binding of Impα/β1 enabling AR nuclear import/transcriptional activity (65). Another example is Impα1 that acts as a retention protein to inhibit the nuclear import of differentiation inducing factors Brn2 and

Oct6 to maintain pluripotency in mouse embryonic stem cells, via its C-terminal domain that prevents productive nuclear import although the mechanism is currently incompletely understood (159). Impαs such as Impα1, have been reported to inhibit the nuclear translocation of distinct Impβ1-recognised cargoes such as chromatin associated factors telomeric repeat factor (TRF)-1 (57) and histone H1, hormone-related signalling factors parathyroid hormone related protein (PTHrP) (56) and sterol regulatory-element binding protein (SREBP)-2 (58, 160), DNA replication associated protein Cdc7 (161), and Snail, a transcriptional repressor regulating epithelial to mesenchymal phenotype switching and embryonic development (160), through a number of different mechanisms shown *in vitro* using reconstituted cell systems and binding assays. For example, Impαs can compete with Impβ1 to bind the Snail NLS to inhibit Snail nuclear import (160), whereas for proteins imported into the nucleus by Impβ1 alone such as Cdc7 (161), SREBP-2 (58, 160), CREB (55), TRF-1 (57) and PTHrP (56), the addition of recombinant Impα decreases their nuclear import efficiency even though the protein themselves do not bind Impα, most likely due to competition of Impα for Impβ1, decreasing the amount of available Impβ1 that is free to bind cargoes.

It is apparent that there are multiple distinct mechanisms that can regulate protein nuclear transport, either through direct modulation of individual cargoes, or of nuclear transport components themselves. In addition, the modulation of nuclear transport components may potentially bear relevance to non-nuclear transport roles of the nuclear transport components, which are discussed in the following Section.

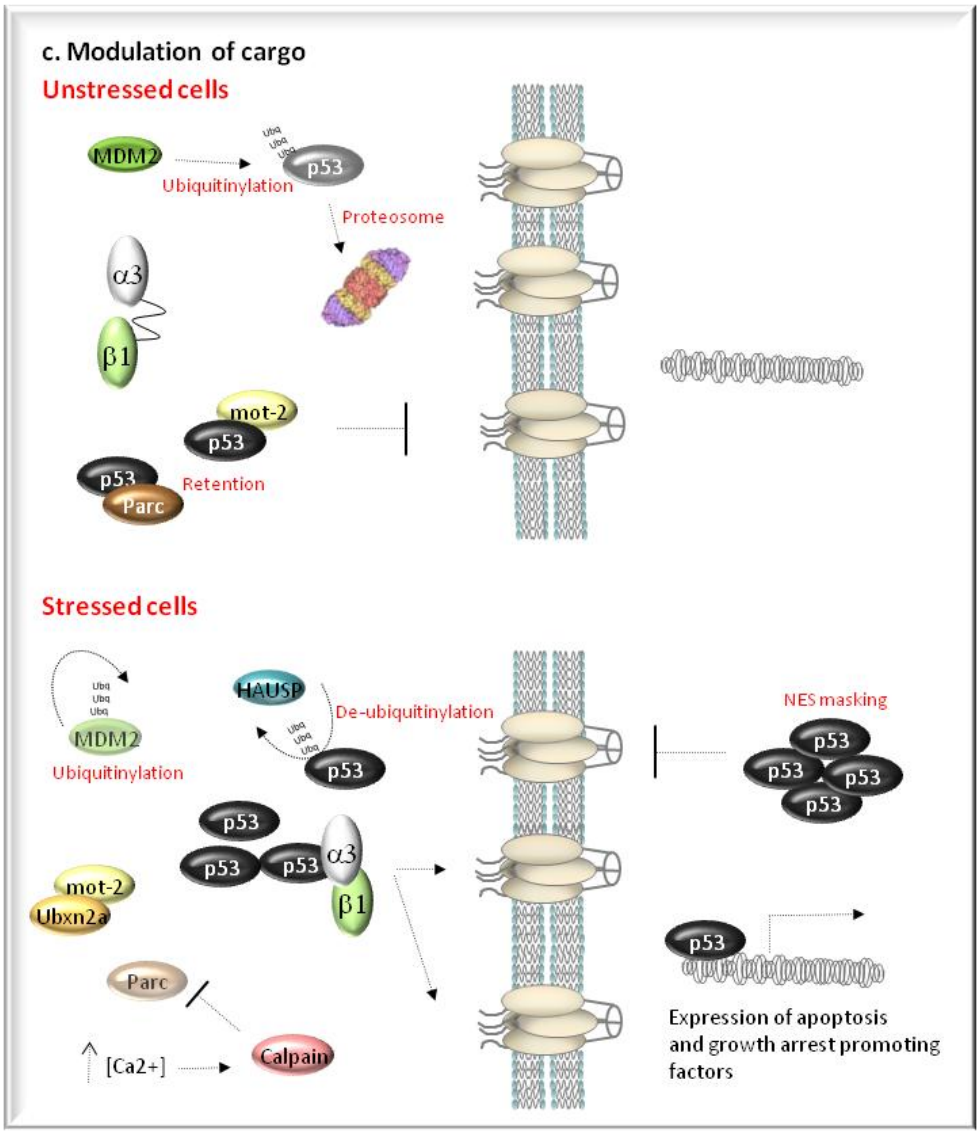


Figure 1.2 Regulation of nuclear protein transport (c) By modulation of cargo. Nuclear localisation of the inducible tumour suppressor transcription factor p53 is regulated by a variety of mechanisms in response to stress signals. (Top) In resting/unstressed cells the p53 NLS is ubiquitinylated by the E3 ubiquitin ligase MDM2 inhibiting Impα3/β1 recognition, leading to p53 cytoplasmic trapping and degradation in the proteasome. Other factors that prevent p53 NLS – Imp recognition are the p53-associated Parkin-like protein Parc and the Hsp70 family member mot-2, which bind and retain p53 in the cytoplasm. (Bottom) DNA damage/stress upregulates/activates the ubiquitin-like domain containing factor Ubxn2a and calcium-dependent cysteine protease calpain, relieving p53 cytoplasmic retention by sequestering mot-2 and degrading Parc respectively. MDM2 – p53 association is inhibited and MDM2 degraded by self-ubiquitinylation leading to HAUSP-dependent deubiquitinylation p53, recognition of the p53 NLS by the Impα3/β1 heterodimer and p53 nuclear import. In the nucleus, p53 forms tetramers masking the NES (155) leading to inhibition of Exp-1-dependent p53 nuclear export and the expression of growth arrest promoting and pro-apoptotic p53 targets. Other interacting factors of the MDM2/HAUSP/p53 complexes are not shown.

1.4 NON-TRANSPORT FUNCTIONS OF THE COMPONENTS OF THE NUCLEAR TRANSPORT MACHINERY

In addition to their well-established roles in nuclear transport during interphase, components of the nuclear transport machinery have transport-independent roles during cell cycle progression, namely through regulation of DNA replication at S-phase, mitotic spindle formation, kinetochore-microtubule attachment during prometaphase and nuclear envelope break down/reassembly during mitosis. In particular, the small GTPase Ran has emerged as a key regulator in this, signalling multiple chromatin-mediated events during the phases of mitosis (162). Not surprisingly, an essential role for numerous Ran-binding proteins such as Imps, CAS, Exp-1, RCC1, RanBP1, RanGAP1 and Nup358 has been implicated.

During S phase, dependent on high levels/activity of RanGTP and Cyclin-CDK2, Exp-1 binding to the DNA replication initiation factor MCM helicase in the nucleus inhibits its activity and restricts DNA replication to a single round; this inhibition is not relieved until after nuclear envelope breakdown (163, 164). During mitotic progression into prophase, phosphorylation of Nups such as Nup98 by CDK1 is important for NPC disassembly and nuclear membrane breakdown (141, 165), during which Impα and Impβ1 localise at the spindle poles most likely through microtubule-dependent transport mechanisms, where they are found in association with the NLSs of a number of spindle assembly factors (SAFs) such as NUMA, lamin B, TPX and the kinesin XCTK2, resulting in inactivation of SAF activity (164). The primary role of Ran at these localised centres is to release SAFs from an inhibitory complex with Imps, and relies on the conversion of RanGDP to RanGTP by the RCC1:RanBP3 complex, which are closely associated with chromatin and therefore mitotic chromosomes during mitosis, thereby providing the necessary spatiotemporal regulation of spindle formation (166).

One of the most important roles for Ran is its capability to regulate the mitotic spindle checkpoint. Ran regulates the attachment of microtubules to the kinetochores of mitotic chromosomes, the formation of stable κ-fibres (kinetochore fibres) as well as the segregation of sister chromatids during anaphase and telophase respectively, by regulating Exp-1-dependent recruitment of kinetochore regulatory factors, RanBP1 and Nup358-RanGAP1 complexes to kinetochores (148, 167). The timely release of factors from Exp-1 is ensured through RanGTP hydrolysis by the localised activity of kinetochore-binding RanGAP1, RanBP1 and Nup358 allowing progression to telophase (166, 168). Interestingly, increased ectopic expression of Impβ1 can relocalise RanGAP1-Nup358

complexes through Nup358 binding to inhibit Exp-1-mediated delivery of kinetochore regulatory factors through lack of RanGTP hydrolysis, resulting in G2/M arrest; an effect that can be overcome by increasing levels of Exp-1 or RanGAP1 (169) indicating nuclear transport component levels are in fine balance to regulate cell cycle progression. RCC1 is phosphorylated by cyclin B-CDK1 during chromosomal congression at the metaphase plate to prevent its binding to Impα/β1 (147), most likely to ensue RanGTP production within close vicinity of chromatin and kinetochore associated factors such as Exp-1. The exportin CAS is also known to play a role in the mitotic spindle checkpoint in yeast (170) and in mammalian cells where it is found associated with the mitotic spindle (171). The silencing of CAS results in the inability to degrade cyclin B, an important event to trigger the progression through the mitotic spindle checkpoint leading to separation of sister chromatids during anaphase, leading to cell cycle arrest at the G2/M checkpoint (172). Distinct nuclear transport factors such as Impβ1, Ran and RCC1 regulate the assembly of the nuclear envelope and NPC formation during telophase through Impβ1-mediated binding of FG-repeat Nups that is inhibited by RanGTP found in high concentrations localised near chromatin-bound RCC1 (166).

Other roles for CAS have been described in tumour necrosis factor (TNF)- and toxin-induced apoptosis (173) (hence the name cellular apoptosis susceptibility protein), tumour cell migration (174), and in the regulation of transcription of p53-target genes (175). It is still unclear whether the roles of CAS in apoptosis, migration and proliferation are linked to CASs function as a nuclear export receptor for Impαs, or whether CAS can mediate these activities through domains that are separate to its function as a nuclear transport factor, but CASs ability to localise at the plasma membrane and microvesicles (174, 176, 177) would suggest the latter is likely to be the case.

Nups have non-transport related functions, in that they can modify chromatin and regulate gene transcription both in a positive and negative manner (178). Studies in the fruitfly and worm show that NPCs/distinct Nups can dynamically associate with promoter regions and regulate transcription of developmental or stress-induced genes, either in the interior or periphery of the nucleus respectively (178). In mammalian cells, Nup98 is known to exist in a dynamic intranuclear form, or a tethered NPC-localised form (179). Whereas the tethered form of Nup98 is essential for multiple pathways of RNA export (180), the dynamic form of Nup98 is known to associate with the 3'UTR of the mRNA encoding the p53-induced protein $p21^{cip1}$, preventing its degradation by the exosome,

enhancing its expression (181), and facilitates transcriptional reactivation of recently expressed IFN-γ-induced genes (178).

Impα1 may also regulate transcription during certain circumstances. Cargo- and Impβ1-free Impα1 is able to translocate into the nucleus using an import mechanism that does not require ATP or ATP hydrolysis, and that does not overlap with that of Impβ1 (182) upon stress-induced Ran gradient collapse in cells treated with high doses of the oxidant H_2O_2 (140). This is associated with depletion of cellular ATP levels that are normally required for nuclear transport and apoptotic progression, Impα1-DNA interaction and downregulation of histones, stress-responsive and apoptosis-related genes, as well as upregulation of caspase-independent serine/threonine kinase (STK)35 expression at the mRNA level, thought to occur through Impα1-mediated inhibition of an STK35 transcriptional repressor (159), although protein levels of these targets were not measured over time due to rapid cell death induced by the H_2O_2 treatment. Ectopic expression of STK35 enhances caspase-independent cell death during H_2O_2 treatment, suggesting a potential role for Impα1 in facilitating non-apoptotic cell death in response to lethal H_2O_2 levels (159, 183).

It is clear from the above examples that nuclear transport components can serve roles outside of nuclear transport, especially during cell cycle/mitotic progression and/or transcriptional regulation. The following Section discusses how nuclear transport components become altered during malignant transformation and how this may relate to tumorigenesis.

1.5 ALTERED NUCLEAR TRANSPORT COMPONENTS IN CANCER CELLS

That altered nuclear transport component activity may be linked to diseases is an emerging concept (184-186). Elucidating how this can contribute to and promote malignant transformation has become of significant interest due to the fact that nuclear transport components become altered in tumour cells as well as in patient tumour tissues when compared to their non-transformed, matched counterparts. The types of alterations commonly associated with tumorigenesis are outlined below.

1.5.1 Altered expression levels

1.5.1.1 Importins

There has been a large increase in recent publications reporting altered (usually elevated) levels of Imps and/or Exps in malignant cell types. The best studied is Impα1, with overexpression of transcript and/or protein reported in a surprisingly varied and large number of different cancers such as melanoma, invasive ductal breast carcinoma (IDC), carcinomas of the bladder, liver, lung (squamous and non-small cell types), stomach, oesophagus, in prostate, ovarian and cervical cancers and in infiltrative astrocytomas (see **Table 1.2**). The degree of Impα1 overexpression (including redistribution to the nucleus) and/or mRNA expression is associated to a significant ($p < 0.05$) extent with tumour progression and aggressiveness in IDC, infiltrative astrocytoma, colon, head and neck carcinomas, gastric adenocarcinoma and in ovarian (germ and epithelial type), oesophageal and hepatocellular carcinoma patients, where levels correlate positively with tumour grade and/or stage, and inversely with patient outcomes such as disease-free and overall survival, and tumour relapse post-treatment (**Table 1.2**). As a consequence of these studies, the suitability of Impα1 levels as a prognostic marker for cancer is currently under examination. The precise mechanisms underlying increased levels of Impα1 in cancer are not well understood, although increased KPNA2 (the gene encoding Impα1) promoter activity and cDNA levels have been shown to occur in oesophageal carcinoma (187) and IDC (188), respectively. This may be through increased activity of the transcription factors Sp-1 or E2F that appear to upregulate Impα1 expression in normal fibroblast (189) and cervical cancer cell lines (190) respectively; indeed the activity of these factors is often found upregulated in cancers (191-193) and the fact that both transcription factors utilise Impα1 for their own nuclear translocation (23, 30), may represent a positive feedback loop to enhance Impα1 expression/their own nuclear localisation.

Impα1 expression has been shown to promote proliferation, tumorigenicity and the invasive potential of luminal type MCF-7 breast cancer cells (194), epithelial ovarian carcinoma (195) and non-small cell lung cancer cells (30). Whilst Impα1 silencing inhibits the proliferation of various types of cancer cell lines such as luminal and basal type MCF-7 and MDA-MB-231 breast cancer (194, 195), non-small cell lung cancer (30), CLI-5 lung adenocarcinoma (195), gastric carcinoma (196), and types of ovarian epithelial cells (HeLa, EF-O21, Sk-OV3), where it can be associated with G2/M phase arrest (195), Impα1 silencing does not alter the growth properties of CLI-0 lung

adenocarcinoma (195), or other ovarian epithelial carcinoma cell lines (CaSki, SiHa, C33A, MS751) (197), meaning that Impα1 activity may modulate growth/oncogenic potential in different cancer types and lines in different ways.

Fewer studies to date have reported overexpression of Impβ1, which occurs in cervical, gastric and bladder carcinomas, whilst Impβ2 and Imp7 mRNA levels appear to be upregulated in colorectal carcinoma patients and Impα7 mRNA levels are found elevated in chronic lymphoid leukaemia (CLL) patients (**Table 1.2**). It is clear that increased levels in malignant cells of Imps other than Impα1 have not been consistently reported, but whether this is because the mRNA levels of these Imps is not upregulated in cancers, or simply due to low coverage, is unclear. Most studies have attempted to characterise the gene expression profiles of cancer tissues by way of mRNA array analysis, but this obviously does not assess protein expression levels; clearly, investigation of the Imp protein levels in specific subsets of cancers are urgently needed to give the full picture of the nuclear transport machinery in malignant cells.

Table 1.2: Components of the nuclear transport machinery found altered in tumour disease and cellular transformation.

Protein	Tumour/transformed cell type (cell line/tissue)	Alteration/significance*	Reference/s (and proposed mechanism)
Impα1 / KPNA2 / Rch-1	Invasive ductal carcinoma (breast)	Overexpression (protein) is associated with high tumour grade, negative hormone receptor status, basal-like and Her-2Neu breast tumour types as well as event free and overall patient survival especially in Grade 2 but not Grade 3 tumours from high-risk breast cancer patients regardless of dosage of chemotherapy	(198)
		Overexpression (mRNA and protein) is an independent negative prognostic factor for overall survival especially in node positive patients One of the top 20 overexpressed (mRNA^) genes in Grade 3 *vs.* Grade 1 tumours	cDNA^^ increased in 32% of tested tumours suggesting CNV (188) (199)
	Ductal carcinoma *in situ* (breast)	Overexpression (protein) correlates positively with tumour grade, stage, lymph node and ER status and decreased disease-free survival in patients with invasive carcinoma and with high nuclear grade in patients with carcinoma *in situ*	(200)
	Lymph node negative breast cancer	Included in a 76-gene expression signature (mRNA^) for prediction of unfavourable patient prognosis (distant recurrence) within 5 years	(201)

Protein	Tumour/transformed cell type (cell line/tissue)	Alteration/significance*	Reference/s (and proposed mechanism)
Impα1 / KPNA2 contd.	Breast cancer cell line (ZR-75-1)	Mutation resulting in a C-terminally deleted truncation variant (comprising of the IBB domain) able to bind Impβ1 but unable to bind NLSs Expression of the IBB domain variant in cells results in increased cytoplasmic p53 mislocalisation	Nonsense mutation (202)
	Ovarian cancer tissues and cell lines (OVSAHO, OV56, OV90, COV644, CaO3, COVAR4, SKOV3, and EFO21)	Overexpression (mRNA^ and protein) is associated with tumour de-differentiation, high stage and poor patient prognosis (decreased disease free survival/overall survival) and increased tumour volumes in mice	(195)
	Ovarian germ cell carcinoma	Overexpression (protein) is associated with high tumour grade, tumour relapse, increased resistance to therapy, and is an independent prognostic factor for 5 year overall survival	(203)
	Epithelial ovarian cancer	Overexpression (mRNA, protein) is associated with high tumour grade and stage, histologic type, tumour relapse, and poor 5-year overall survival rate	(204)
	Prostate cancer	High nuclear Impα1 expression (protein) is a strong and independent predictor of biochemical relapse§ in patients treated by radical prostatectomy	(205)
	Colon cancer, head and neck cancer, lung, gastric, breast, oral, and pancreatic cancer	Overexpression (mRNA^/protein) is associated with high tumour grade and shorter overall survival in colon cancer and head and neck cancer patients	(206)
	Squamous oesophageal carcinoma, lung cancer	Positive Impα1 staining (protein) occurs in c. 50% of tested carcinoma samples correlating with poor differentiation, increased tumour depth, stage and invasiveness and poor patient prognosis Increased Impα1 expression (mRNA^) correlates with nuclear mislocalisation	(187, 207, 208)
	Gastric carcinoma	Nuclear Impα1 expression (mRNA^/protein) in primary lesions and metastatic lymph nodes is associated with increased tumour stage and depth, venous invasion and poor patient survival	(196)
	Gastric adenocarcinoma	Overexpression (mRNA^/protein) is associated with increased tumour size and grade, lymph node involvement and tumour node metastasis and is an independent prognostic indicator of patient survival	(209)
	Bladder carcinoma	Overexpression (protein) is associated with tumour progression from non-invasive to muscle-invasive carcinoma and significantly shorter progression-free survival independent of tumour grade/stage in patients treated with transurethral resection of the bladder	(210)

Protein	Tumour/transformed cell type (cell line/tissue)	Alteration/significance*	Reference/s (and proposed mechanism)
Impα1 / KPNA2 contd.	Hepatocellular carcinoma	Overexpression (protein) occurs in c. 36% of tested carcinoma tissues correlating with increased tumour stage, invasiveness, early recurrence after resection and poor patient survival	(211)
	Infiltrative astrocytoma	High (nuclear) Impα1 expression (protein) is associated with high tumour grade and decreased overall and progression-free survival	(212)
	Melanoma	Overexpression (mRNA, protein) is associated with poor patient outcome	(213)
	Cervical cancer (HPV-16 infected) cell lines (HeLa, SiHa, CaSki, ME180, MS751); SV40-transformed lung fibroblasts (SVW138)	Overexpression (mRNA, protein)	Increased E2F regulated KPNA2 promoter activity (190, 197)
Impα7 / KPNA6	Chronic lymphocytic leukaemia	Overexpression (mRNA)	(214)
Impα8 / KPNA7	Pancreatic cancer cell lines (AsPC-1, BxPC-3, Capan-1, Capan-2, CFPAC-1, HPAC, HPAF-II, Hs700T, Hs766T, MIA PaCa-2, PANC-1, Su.86.86, SW 1990)	Overexpression (mRNA)	Amplification of 7q22 amplicon (215)
Impβ1 / KPNB1 / NTF97	Cervical cancer (HPV-infected) cell lines (HeLa, SiHa, CaSki, ME180,MS751); SV40-transformed lung fibroblasts (SVW138)	Overexpression (mRNA, protein) is important for tumour cell viability	(197)
	Gastric carcinoma, bladder carcinoma	Overexpression (mRNA) ^	(216, 217)
Impβ2 / Tpn	Colorectal carcinoma	Overexpression (mRNA)^ is an indicator of poor patient prognosis and survival	(218)
Imp7 / RanBP7	Colorectal carcinoma	Overexpression (mRNA) is associated with increased cell proliferation (PCNA stain)	(219)
Exp-1 / Crm-1	Ovarian cancer and ovarian cancer cell lines (OVCAR-3, SKOV-3, CAOV-3, ES-2, A2780, Mdah2744, OAW42, EFO21, EFO27, FU-OV-1, PA-1)	Overexpression (protein) is associated with high tumour stage and mitotic index and dedifferentiation Cytoplasmic Exp-1 mislocalisation correlates with high tumour stage and grade	(220)

Protein	Tumour/transformed cell type (cell line/tissue)	Alteration/significance*	Reference/s (and proposed mechanism)
Exp-1 / Crm-1 contd.	Ovarian cancer	Overexpression (mRNA, protein) is associated with cytoplasmic Topo IIα localisation	(221)
	Cervical cancer (HPV-infected) cell lines (HeLa, SiHa, CaSki, ME180, MS751), SV40-transformed lung fibroblasts (SVW138)	Overexpression (mRNA, protein) is important for tumour cell viability	Increased HPV-associated Sp-1/NFY activity (197, 222)
	Pancreatic adenocarcinoma	Overexpression (protein) is associated with lymphadenopathy, increased tumour size, and decreased overall and progression-free patient survival	(223)
	Lung adenocarcinoma	Part of a 4 gene signature (mRNA) associated with poor overall survival in Stage 1 and 2 cancer patients from multiple cohorts	(224)
	Glioma	Overexpression (protein) is associated with high grade of malignancy and decreased overall patient survival	(225)
	Osteosarcoma	Overexpression (protein) [#] is associated with high tumour grade, and decreased overall and progression-free survival	(226)
Exp-4	Hepatocellular carcinoma and associated cell lines (not specified), breast carcinoma	Gene deletion results in loss of Exp-4-mediated nuclear export activity results in EIF5A and SMAD3 nuclear retention and transcription of TGF-β gene targets leading to tumour growth in mice Associated with poor patient survival in breast carcinoma	XPO4 (Exp-4 gene) homozygous deletion (227)
	Hepatocellular carcinoma (primarily HBV-associated)	Downregulation (mRNA and protein) is associated with increased tumour size, de-differentiation and is an independent prognostic factor for patient survival	(228)
Exp-5	Urothelial carcinoma, malignant pleural mesothelioma	Overexpression (mRNA) [^#]	(229, 230)
Exp-T	Malignant pleural mesothelioma, hepatocellular carcinoma	Overexpression (mRNA/protein) [^#]	(230, 231)
CAS / Cse1l / Exp-2	Breast cancer	Overexpression (protein) is associated with increased tumour grade and aggressiveness Nuclear CAS expression is associated with increased tumour grade	Amplification of 20q13 locus (232, 233)
	Ovarian cancer	Overexpression (protein) is associated with high tumour grade and FIGO classified tumour stage and adverse patient outcome in terms of decreased overall survival	Amplification of 20q13 locus (234, 235)
	Serous ovarian cancer	Overexpression (protein) is associated with high tumour grade	(236)

Protein	Tumour/transformed cell type (cell line/tissue)	Alteration/significance*	Reference/s (and proposed mechanism)
CAS / Cse1l / Exp-2 contd.	Endometrial carcinoma	Overexpression (protein) is associated with transition from simple and complex hyperplasia to carcinoma and decreased overall patient survival	(237)
	Liver neoplasms**	Overexpression (protein) is associated with tumour dedifferentiation	(238)
	Melanoma	Overexpression (protein) is associated with high UICC classified tumour stage	(239)
	Bladder carcinoma	High nuclear CAS expression level (protein) correlates with poor overall survival but not stage, tumour penetration or lymph node metastasis	(240, 241)
	Colorectal carcinoma	High cytoplasmic CAS expression (protein) correlates with increased tumour stage and depth of penetration, lymph node metastasis and overall patient survival CAS overexpression induces lung and liver metastasis of B16F10 melanoma and HT-29 colon cancer cells in mice, respectively	(242)
	Prostate tumour xenograft, colon cancer, lymphoid neoplasms	Overexpression (mRNA/protein) ^	Amplification of 20q13 locus (233, 243-247)
Nup88	Breast carcinoma	Overexpression (mRNA) & is associated with axillary node invasion, altered oncogene and tumour suppressor expression and DNA aneuploidy but not grade or stage	(248)
	Cervical carcinoma	Overexpression (protein) is associated with increased tumour grade	(249)
	Endometrial carcinoma	Overexpression (mRNA) is associated with myometrial invasion but not grade or stage	(250)
	Liver neoplasms	Overexpression (protein) is associated with high tumour grade and dedifferentiation	(251)
	Colon adenocarcinoma	Overexpression (protein) is associated with high tumour grade and dedifferentiation	(252)
	Colorectal carcinoma	Overexpression (protein) is associated with metastasis &	(253)
	Colorectal carcinoma	Increased secretion is associated with increased tumour depth and stage	(254)
	Melanoma	Overexpression (protein) is associated with transition of primary to metastatic melanoma	(255)

Protein	Tumour/transformed cell type (cell line/tissue)	Alteration/significance*	Reference/s (and proposed mechanism)
Nup88 contd.	Invasive ovarian carcinoma, cervical carcinoma, stomach and colon adenocarcinomas, carcinomas of the breast, lung and liver, prostate and endometrial cancer, HBV-positive hepatocellular carcinoma cell lines	Overexpression (mRNA/protein)	(251, 256)
	Breast carcinoma, bladder and prostate carcinomas, melanoma, several sarcomas and glioma	Overexpression (protein)/cytoplasmic mislocalisation	
Nup98	Leukaemia	Fusion to homeodomain (ie. HOXA9) or non-homeodomain (ie. Topo I and IIβ, NSD1) genes In the case of HOXA9 fusions, this induces aberrant HOX target gene transcription through deregulated HOX-Nup98 nuclear import and transcription enhancing activities	Chromosomal translocations (257)
Nup214	Leukaemia	Fusion to SET and DEK chromatin binding protein genes; fusion to ABL1 In the case of SET/DEK fusions colocalisation of Exp-1 with SET-Nup214 may result in reduced nuclear protein export; fusion to ABL1 results in increased ABL1 tyrosine kinase activity	Chromosomal translocations 9q32:9q34 (SET) (258) 6p23:9q34 (DEK) 9q34 amplification and circularisation (ABL1)(257)

*All stated findings are statistically significant ($p < 0.05$)
^ Based on cDNA microarray data
^^Based on dot plot and RNA *in situ* hybridisation data
§ Biochemical recurrence is a rise in the concentration of blood prostate specific antigen (PSA) after treatment with surgery or radiation and may mean that the cancer has relapsed
# Sample number (*n*) for normal compared to tumour tissue group is low or *n* is not stated
** Includes both viral hepatitis and hepatocellular carcinomas
& Data (or primary data) not shown

Abbreviations: ABL1, abelson murine leukaemia viral oncogene homologue 1; BRCA1, breast and ovarian cancer susceptibility protein 1; CAS, cellular apoptosis susceptibility protein; CNV, copy number variation; EIF5A, eukaryotic translation initiation factor 5A; Exp, exportin; FIGO, the international federation of gynaecology and obstetrics**;** HBV, hepatitis B virus; HOX, homeobox transcription factor; HPV, human papilloma virus; Imp, importin; KPN, karyopherin; Nup, nucleoporin; NTF97, nuclear transport factor 97; PCNA, proliferating cell nuclear antigen; SV40, simian Virus 40; TGF-β, tumour growth factor β; Tpn, transportin; Topo I and Topo IIβ, topoisomerase I and topoisomerase II β; UICC, the international union against cancer.

1.5.1.2 Exportins

In terms of Exps, Exp-1 is commonly overexpressed in tumours with the degree of overexpression correlating with overall survival in ovarian, cervical, pancreatic and lung cancer patients, and in osteosarcoma and glioma (**Table 1.2**). Although the basis of Exp-1 overexpression is not understood, NFY and Sp-1 transcription factors have been implicated in this context in cervical carcinoma cells and SV40-transformed fibroblasts (222). The high correlation between Exp-1 overexpression and cancer, and the availability of Exp-1 inhibiting leptomycin B (LMB)-derivative compounds has led to Exp-1 being the focus of anti-cancer strategies, with specific small molecular inhibitors currently under Phase I and II clinical investigation (259). A number of tumour suppressors (such as p53, pRb, FOXO, $p21^{cip1}$ and $p27^{kip1}$) and chemotherapy drug targets (such as topoisomerases) use Exp-1 for nuclear export, meaning that elevated Exp-1 levels may result in cytoplasmic redistribution of these proteins, promoting tumour progression and drug resistance (260).

CAS (Exp-2) is another Exp showing increased levels in numerous cancers including colon, bladder, liver, breast and endometrial carcinomas and melanoma; CAS overexpression is implicated in tumour progression (**Table 1.2**). In many instances higher levels of CAS expression occur due to chromosomal amplification of the 20q13 locus, a region which is commonly found altered in cancers of the colon, breast and ovarian carcinomas and is associated with increased metastatic and invasive potential (234, 238, 242, 246). CAS is associated with matrix metalloprotease-2 (MMP-2)-dependent metastasis, as CAS and MMP-2 co-localise in microvesicles at the plasma membrane and are secreted more readily from invasive/metastatic colon cancers and melanomas than from non-invasive controls (176, 261, 262). CAS is found secreted in the urine of patients with bladder carcinoma or atypical urothelial growth but not in healthy controls (241). This has raised considerable interest in terms of CAS as a potential prognostic marker for cancer.

Ectopic overexpression of CAS and its RNAi-mediated silencing increases and decreases migration and invasion respectively, of melanoma cells *in vitro* and lung metastasis in mice (174). Given that CAS can translocate to the extracellular membrane where it is secreted in vesicles to increase MMP secretion, which are key players in metastatic tumour progression (263), and induces tubulin dephosphorylation and polymerisation associated with increased migration (264), it seems likely that CASs role in metastasis may not relate directly to its nuclear transport function. Interestingly

high expression of CAS can lead to cell death, possibly through a p53-dependent mechanism as CAS is known to act as a co-factor in p53-mediated transcription through its ability to bind to chromatin (175), although induction of cell death by CAS is also known to occur in p53 null cells (265). Cancer cells that overexpress CAS appear to be able to circumvent apoptosis, presumably by altering the activity of apoptotic pathways that are regulated by CAS.

1.5.1.3 Nucleoporins

The non-FG repeat Nup88 appears to be the only NPC component thus far found to be overexpressed in multiple tumour types including tumours of the colon, breast, cervix, endometrium and liver (**Table 1.2**); since its overexpression is associated with tumour grade, depth, invasion potential and dedifferentiation, it has been proposed as a tumour biomarker. Although Nup88 expression is associated with tumour progression, its relevance to tumour biology and the mechanisms by which Nup88 expression is enhanced in tumour cells has not been fully elucidated. Interestingly, Nup88 overexpression is not associated with a concomitant increase in levels of its binding partner Nup214 or other Nups such as Nup153, suggesting no general upregulation in NPC numbers/functionality (257). Nup88, important for structural integrity of NPCs, does not directly participate in nuclear transport of Imp-recognised cargoes, but together with Nup214 participates in the Exp-1-dependent export of pre-ribosomal complexes (266), although its importance to the general Exp-1-dependent export pathway in different cell system is currently unclear (257). Overexpressed Nup88 is found mislocalised in the cytoplasm of cancer cells (257); whether this can mislocalise Nup88-Nup214 complexes/their binding partner Nup358 to the cytoplasm affecting protein nuclear trafficking efficiency in tumours is not yet known. Interestingly, Nup88 is found complexed with Nup214 throughout the cell cycle and is found localised to spindles and spindle poles after nuclear envelope breakdown (267). Increased levels of Nup88 enhance the occurrence of multi nucleated cells, which may contribute to genetic instability through aneuploidy and polyploidy that is often associated with cancer cells, meaning Nup88 overexpression may influence cancer through a non-nuclear transport related mechanism (267).

1.5.2 Other alterations

1.5.2.1 Importins / Exportins

Other less common alterations to Imps in cancer include redistribution in subcellular localisation, homozygous gene loss and expression of truncation variants (**Table 1.2**). Re-distribution of both CAS and Impα1 occurs in breast cancer, both showing a change from a predominantly cytoplasmic to nuclear pattern in hyperplasic compared to invasive carcinoma respectively, with strong nuclear Impα1 expression being an independent adverse risk factor for overall survival and recurrence-free survival in primary and node positive invasive breast cancers (198, 232). An example of transport components affected by protein truncations in cancer include an Impα1 C-terminal truncation variant comprising only the IBB domain (also known as CAN/Rch1) derived from a breast cancer cell line, its expression resulting in nuclear import inhibition of Impα/β1-recognised proteins such as p53 to promote tumorigenesis (151, 202). Exp-5 is another example of a transport component that is found mutated in a number of cancers (gastric, colon and endometrial) due to microsatellite instability in the XPO5 gene, resulting in the expression of a C-terminally truncated protein devoid of miRNA binding activity (268). A critical function of Exp-5 is to export pre-miRNAs into the cytoplasm for further processing into mature (active) miRNA; XPO5 mutations trap pre-miRNAs (such as tumour suppressive *let*7 family members, miR-138 and miR-145) inside the nucleus preventing miR-dependent suppression of oncoprotein factors such as c-myc and *k*-Ras promoting tumour formation in mice (268).

Another Exportin, Exp-4 (XPO4) is found deleted in breast and liver cancer (227, 228). XPO4 deletion results in increased nuclear trapping and constitutive activity of the Exp-4-recognised transcription factors SMAD3 and EIF5A that drive the expression of TGF-β target genes, causing the initiation of tumours in mice (227). The fact that EIF5A and SMAD3 expression are vital for full induction of tumorigenesis in XPO4 null cells, which is inhibited by the ectopic restoration of Exp-4 levels, strongly implies that the tumour suppressive activity of Exp-4 relates specifically to its role as an Exportin for its specific cargoes.

1.5.2.2 Nucleoporins

Apart from Nup88, which is found mislocalised in breast carcinoma cells, where its redistribution from the NPC into the cytoplasm is most likely affected by its increased expression

levels (269), several cancer-associated fusion proteins involving chromosomal rearrangement of Nups with transcription factors have been identified (**Table 1.2**) and implicated in playing a role in leukaemogenesis. These Nup chromosomal translocations are varied in type and have already been reviewed in detail elsewhere (257, 270). Briefly, certain chromosomal translocations in acute myeloid leukaemia, for example, result in the expression of fusion proteins comprising the hydrophobic FG/GLFG repeat domain of Nup98 and the DNA-binding and transcriptional domains of homeodomain transcription factors (most commonly HoxA9); through the Nup98 portion, nuclear translocation is enhanced, leading to constitutive Hox-induced transcription of dedifferentiation inducing factors (257). Another example is fusion of Nup214 to the nuclear phosphoproteins Dek and Set in AML patients, resulting in partial loss of the Nup88 binding domain on Nup214 required for Nup214 tethering to the NPC leading to its nuclear relocalisation and possibly increased Dek/Set-mediated chromatin modulation (257). The fact that Nup214-fusion proteins retain the ability to associate with Exp-1 may mean nuclear export dependent activity may be altered by the fusion protein (257).

As discussed above, Imps/Exps or NPC components can become altered in cellular transformation in a number of different ways. Overexpression of Impα1, CAS, Exp-1 or Nup88 correlates with tumour formation, progression and aggressiveness in many different types of cancers from different tissue origins, which could be stemming from a generalised mechanism; increased Imp/Exp levels, for example, may result in altered nuclear transport activity that in itself is a fundamental aspect of tumour disease progression. Misregulation of nuclear trafficking at the level of individual cargoes is already known to contribute to diseases such as cancer (see (184, 185)); strikingly the extent to which altered Imp/Exp activities can influence nuclear transport efficiency in tumour/transformed cell types and how this in turn relates to tumour progression is not clear.

1.6 CONCLUDING REMARKS

That aberrant nuclear transport of individual proteins can contribute to cancer has been well documented (154, 186, 271-273). However, limited knowledge exists regarding the extent to which mechanisms of nuclear transport regulation might relate to more global effects on nuclear transport components themselves, and how this may influence the localisation of particular proteins

in tumorigenesis. As summarised here, a number of specific Imps and Exps are overexpressed in so many different types of cancers, implying that there may be some sort of selection in tumour progression for enhanced Imp activity, through its fundamental importance to the cancer cell biology. Although studies have begun to attempt to demonstrate altered Imp/Exp expression in various types of cancer cells, the effect of altered Imp/Exp levels on global nuclear transport efficiency in cancer has never been examined, with essentially nothing known about whether altered nuclear trafficking pathways may be associated with particular types of tumours or tumour origins, what types of cargoes mislocalise as a result, and how this relates, potentially mechanistically, to tumour progression.

A significant limitation in terms of model systems is the scarcity of isogenic human normal/tumour cell systems, which means that deciphering tumour-specific nuclear transport alterations in a panel of tumour types is difficult. Understanding the mechanistic basis of changes to the nuclear transport machinery may be paramount to understanding of much of tumour cell biology itself; simply pinpointing the contribution of specifically altered Imps/Exps to cargo re-distribution in cancer, although integral to understanding the impact of altered Imp/Exp levels, is an enormous challenge. Thus, a top down approach looking at the overall effects that altered nuclear transport components can have on the common features of tumour cells, would appear to be an important first step in furthering our understanding in this context.

1.7 HYPOTHESIS AND AIMS OF THIS THESIS

The present study sets out to utilise a variety of isogenic non transformed/transformed cell line pairs and an *in vitro* model for breast tumour progression to examine in detail the comparative nucleocytoplasmic transport efficiencies in transformed compared to non-transformed cells, the underlying mechanism(s) for these alterations and tests the effects of Imp/Exp inhibition on viability of non-transformed compared to malignant cells.

The hypothesis to be investigated is ***that, through increased levels of Imps/Exps, transformed/ tumour cells display enhanced nuclear trafficking efficiency correlating with tumorigenic disease progression state***.

More specifically this study aimed:

1. To compare in detail the efficiency of nucleocytoplasmic transport in tumour/transformed and non-transformed cell pairs of isogenic origin
2. To identify components of the nuclear transport machinery, which show altered expression in tumour/transformed cells compared to non-transformed counterparts, as well as in breast tumour tissues
3. To use overexpression/RNAi approaches to modulate the levels of selected Imps/Exps identified in Aim 2, to monitor the extent to which this modifies nuclear import efficiency and cell viability in transformed cell type.

The nuclear import and export properties of various types of transformed/non-transformed cell pairs are compared in Chapter 2. These studies are extended to a breast tumour progression model in Chapter 3, in which nuclear transport component expression levels are modulated to identify the mechanistic basis for altered nuclear transport efficiency and inferences made to patient tumour tissues. The effects of RNAi-mediated Imp/Exp knockdown on viability of non-transformed compared to transformed cell types are documented in Chapter 4. Importantly, the results of this study are relevant to understanding of fundamental aspects in tumour disease biology and the development of novel tumour-targeted therapies, which is discussed in Chapter 5.

CHAPTER 2

Global enhancement of nuclear localisation signal-dependent nuclear transport in transformed cells

DECLARATION FOR THESIS CHAPTER 2

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
Contributed to the conception and design of the study. Performed and was involved in all aspects of the experiments and prepared the manuscript for publication.	90%

The following co-authors contributed to the work:

Name	**Nature of contribution**
Kylie M. Wagstaff	Assistance with fluorescence recovery after photobleaching studies and cell culture. Critical review of the manuscript.
Daniela M. Roth	Assistance with fluorescence recovery after photobleaching studies. Critical review of the manuscript.
Gualtiero Alvisi	Contributed to the concept and design of the study. Critical review of the manuscript.
David A. Jans	Contributed to the concept and design of the study. Critical review of the manuscript.

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date**

Main Supervisor's Signature		**Date**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

2 Global enhancement of nuclear localisation signal-dependent nuclear transport in transformed cells

2.1 PREFACE

Chapter 2 of this thesis is composed of a manuscript published in *The F*ASEB *Journal* entitled "Global enhancement of nuclear localisation signal-dependent nuclear transport in transformed cells".

2.2 ABSTRACT

Fundamental to eukaryotic cell function, nucleocytoplasmic transport can be regulated at many levels, including through modulation of the Importin/Exportin (Imp/Exp) nuclear transport machinery itself. Although Imps/Exps are overexpressed in a number of transformed cell lines/patient tumour tissues, the efficiency of nucleocytoplasmic transport in transformed cell types compared to non-transformed cells has not been investigated. Here we use quantitative live cell imaging of three isogenic non-transformed/transformed cell pairs to show that nuclear accumulation of nuclear localisation signal (NLS)-containing proteins, but not their NLS-mutated derivatives, is increased up to 7-fold in MCF10CA1h human epithelial breast carcinoma cells and in Simian Virus 40 (SV40)-transformed fibroblasts of human and monkey origin, compared to their non-transformed counterparts. The basis for this appears to be a significantly faster rate of nuclear import in transformed cell types, as revealed by analysis using fluorescence recovery after photobleaching for the human MCF10A/MCF10CA1h cell pair. Nuclear accumulation of NLS/nuclear export signal-containing (shuttling) proteins was also enhanced in transformed cell types, experiments using the nuclear export inhibitor leptomycin B demonstrating that efficient Exp-1-mediated nuclear export was not impaired in transformed compared to non-transformed cells. Enhanced nuclear import and export efficiencies were found to correlate with 2- to-4-fold higher expression of specific Imps/Exps in transformed cells, as indicated by quantitative Western analysis, with ectopic expression of Imps able to enhance NLS nuclear accumulation levels up to 5-fold in non-transformed MCF10A cells. The findings indicate that transformed cells possess altered nuclear transport properties most likely due to the overexpression of Imps/Exps. The findings have

important implications for the development of tumour-specific drug nanocarriers in anti-cancer therapy.

2.3 INTRODUCTION

Tumour cells are characterised by a plethora of changes in signal transduction, which affect tumour cell metabolism, as well as proliferative potential and resistance to apoptosis. Ultimately, these cellular responses to extra- and intracellular signalling pathways converge at the level of the nucleus to effect phenotypic changes in transcription/chromatin structure/cell cycle control/DNA repair etc. Protein transport into the nucleus through the nuclear envelope-embedded nuclear pore complexes (NPCs) is dependent on nuclear localisation signals (NLSs), which are recognised by members of the Importin (Imp) superfamily of cellular transport receptors, of which there are multiple α and β types (13, 274). A well-characterised example is Impβ1 which, either directly or through an Impα adapter, recognises basic-type NLSs, and mediates translocation of the Imp:cargo complex through the NPC into the nucleus, where the complex dissociates upon binding to Impβ1 of the monomeric guanine nucleotide binding protein Ran in activated GTP-bound form (275). Nuclear protein export occurs analogously, whereby generally hydrophobic nuclear export signals (NESs) are recognised by Exportins (Exps), homologues of Impβ1 in combination with RanGTP (275). The best characterised Exps include Exp-1 (Crm-1), which recognises leucine-rich NESs (91), and CAS (Cse1l), which facilitates recycling of Impαs to the cytoplasm to enable subsequent rounds of nuclear import (97).

Nucleocytoplasmic transport can be regulated by a number of distinct mechanisms (see (47)), which are central to cellular processes such as differentiation and oncogenesis (154). One mechanism appears to be through alterations in the levels of expression of specific Imps. For example, the levels of different Impα isoforms alter during sperm development in the mouse testis (123, 125), embryonic stem (ES) cell differentiation (130), maintenance of pluripotency in ES cells (131), and in the central nervous system in the early postnatal and adult stages (276). Interestingly, recent reports indicate alterations in the expression levels of Imps/Exps in patient tumour tissue and in transformed cell lines, with elevation of Imp expression in breast (188, 198-201), bladder (210, 217), oesophageal (187, 207), cervical (197), hepatocellular (211), colon (277) and ovarian carcinomas

(204), and also in Simian Virus 40 (SV40) or Human Papilloma Virus-16 transformed cell lines (197), whilst Exps are also found overexpressed in carcinomas of the breast (232, 233), liver (238) and ovaries (220, 221, 234) and in numerous virally-transformed and cancer cell lines (197, 220, 232, 233, 238). To date, however, no study has analysed the effect of altered Imp/Exp expression on nuclear transport efficiency in transformed cells, with essentially nothing known regarding the comparative transport efficiency in tumour *versus* normal cells.

Here we examine nuclear transport efficiency quantitatively in three isogenic pairs of transformed/non-transformed cells for the first time, and relate this to levels of Imp/Exp expression. We show that Impα/β1- and Impβ1-dependent nuclear transport efficiencies and rates are increased in transformed cells compared to non-transformed counterparts, and that this correlates with increased levels of Impα1, Impβ1 and the Ran guanine nucleotide exchange factor RCC1 in transformed cells. Exp-1 levels were also elevated in the transformed cell types, which was associated with an increased responsiveness to the Exp-1 specific inhibitor leptomycin B (LMB) of NLS-/NES-containing cargo proteins, implying higher Exp-1 activity in transformed compared to non-transformed cells. The results overall have important relevance to the development of tumour-specific drug nanocarriers in anti-cancer therapy (278-280).

2.4 MATERIALS AND METHODS

2.4.1 Mammalian expression constructs

Plasmid pEGFP (Clontech) encoding GFP alone was used as a control for transfection studies. The pEGFP-T-ag(114-135), pEGFP-T-ag(111-135), pEGFP-T-ag(111-135)NLSm (containing a $K^{128}T$ mutation), pEPI-UL54(1145-1161), pDEST53-UL54(1145-1161)NLSm (containing $R^{1157}A$ and $R^{1159}A$ mutations), pEPI-VP3(74-121), pEPI-VP3(74-121)NLS1/2m (containing $KK^{86-87}NN$ and $RR^{117-118}NN$ mutations), pEGFP-PTHrP(1-141) and pEPI-Rev(2-116) expression constructs have all been described previously (279, 281-286). The TRF-1 (337-440) fragment (57) was introduced into the Gateway™ system (Invitrogen, Carlsbad, CA, USA) (281) by PCR using *att*B site-containing primers, and subsequent BP and LR recombination reactions to generate to yield plasmid pEPI-TRF-1(337-440). The bicistronic pIRES-mCherry expression plasmid was generated using a modified version of the pIRES-GFP plasmid (a kind gift from KA Steiner, Children's Medical Research Institute, New South

Wales, Australia), in which the GFP fragment was first removed by XbaI/SmaI digestion before ligation with PCR amplified mCherry cDNA. pIRES-Imp-mCherry was generated by ligation of a PCR amplified Impα1 or Impβ1 cDNA fragment using the NotI/BamHI sites. The integrity of all plasmid constructs was verified by DNA sequencing.

2.4.2 Mammalian cell culture, transfection and Leptomycin B treatment

The three isogenic non-transformed/transformed cell pairs used in this study are described in **Table 2.1**. In the case of the 1BR3/1BR3Neo (1BR3N) human skin fibroblast (287) and the CV-1/COS-7 African Green Monkey kidney fibroblast (288) cell pairs, the transformed derivative was generated by transfection with plasmids containing SV40 genomic sequences for either the early region (1BR3N, pre-crisis stage) or a replication defective (-ori) mutant (COS-7, post-crisis stage). In the case of the MCF10A/MCF10CA1h human breast epithelial cell pair, the non-transformed MCF10A cell line was isolated from a female patient suffering from fibrocystic disease, whereas the tumorigenic MCF10CA1h line was generated from *Ha*-Ras transformed MCF10A cells, passaged serially *in vivo,* in mice (289).

1BR3 and 1BR3N cells (both at passages 6-12) were cultured in DMEM supplemented with 15% foetal calf serum and 2 mM L-glutamine (all from Invitrogen). CV-1 and COS-7 cells were cultured in DMEM supplemented with 10% FCS and 2 mM L-Glutamine (282, 290), whilst MCF10A (passage < 72) and MCF10CA1h (passage < 62) cells were cultured in DMEM/F12 media supplemented with 5% horse serum (Invitrogen), 1 mM sodium pyruvate, 10 mM HEPES, 2 mM L-glutamine, 0.5 μg/ml hydrocortisone, 10 μg/ml insulin, 20 ng/ml human recombinant EGF and 100 ng/ml cholera toxin (all from Sigma-Aldrich, St. Louis, MO, USA), as previously (289). All of the cell line pairs were transfected using Lipofectamine 2000 Transfection Reagent (Invitrogen) as previously (282). Where appropriate, transfected cells were incubated for 3-4 h in the presence 2.8 μg/ml of the Exp-1 specific nuclear export inhibitor LMB, prior to microscopic imaging.

2.4.3 Confocal laser scanning microscopy and image analysis

Cells were imaged live (X100 oil immersion objective, zoom at 1.0, 60 μm pinhole, 4 and 15% laser power for 488nm and 568nm lasers respectively, heated stage) 16-24 h post-transfection using a Nikon TSI 100 confocal laser scanning microscope (CLSM; Nikon, Tokyo, Japan) using a Kalman setting of 2. Image analysis was performed using the ImageJ v1.41 public domain software

(U.S. National Institutes of Health, Bethesda, MD, USA) to determine the nuclear (Fn), cytoplasmic (Fc) and background (Fb) fluorescence. Briefly, a mean density measurement of pixel numbers was made on a non-saturated region of interest (ROI) of equal size (ROI = 30 arbitrary units) in the nuclear and cytoplasmic compartments, respectively, whilst Fb measurements were made by placing the ROI on a non-transfected, autofluorescent region near the cells outer perimeter. The ratio of nuclear to cytoplasmic fluorescence (Fn/c) was then determined according to the formula: Fn/c = (Fn - Fb)/(Fc - Fb) (290). Fold-differences in nuclear accumulation between non-transformed and transformed cell lines were calculated according to the formula: [T Fn/c]/[NT Fn/c], where T Fn/c is transformed Fn/c, and NT Fn/c is non-transformed Fn/c.

2.4.4 Fluorescence recovery after photobleaching

Fluorescence recovery after photobleaching (FRAP) experiments were performed using a modified method from (285). MCF10A/CA1h cells were transfected as above to express GFP-T-ag(114-135) and imaged live using an Olympus Fluoview 1000 CLSM (100x oil immersion objective; Olympus, Tokyo, Japan) 16-24 h later. Prior to bleaching, two images using 3% laser power were taken at 8 μs/pixel and a zoom of 2-fold. By zooming 120-fold in the nuclear region and increasing the laser power to 100%, an area of c. 5% of the nucleus was photobleached (1 scan, 100 μs/pixel, 2500 and 4000 ms for MCF10A and MCF10CA1h cells, respectively) and the cells then immediately scanned at 3% laser power (8 μs/pixel and zoom of 2-fold) and images subsequently captured at 5 s intervals for up to 280 s to monitor the recovery of fluorescence in the nucleus, and the decay of fluorescence in the cytoplasm. The level of specific nuclear (Fn - Fb) and cytoplasmic (Fc - Fb) fluorescence at each time interval was determined by image analysis as above, and values expressed in terms of fractional recovery of fluorescence (values at each time interval divided by the maximal post-bleach value), with the data for fractional recovery of specific nuclear fluorescence fitted exponentially according to the formula ($y= (1-a)\ e^{-bx}$) to determine the recovery half-times ($t_{1/2}$), as previously (285). The rate of exponential decay of cytoplasmic fluorescence was also determined for Fc-b values at 0-280 s post bleach using the equation ($y=y0+ae^{-bx}$).

2.4.5 Preparation of cell extracts and Western blotting

Equal numbers of living non-transformed and transformed cells, as determined by haemocytometer/trypan blue staining were harvested by trypsinisation and centrifugation, and washed once with PBS prior to freezing at -20°C for 30 min. Cell pellets were resuspended in ice-cold

lysis buffer (150 mM NaCl; 50 mM Tris-HCl pH 7.3; 1% Triton X-100 with complete, EDTA-free protease inhibitor cocktail; Roche, Indianapolis, IN, USA), lysed at 4°C for 30 min and centrifuged at 14,200 rpm for 30 min at 4°C (Hereaus Instruments, New Plainfield, SJ, USA) to pellet insoluble material. The total protein concentration in each extract was estimated using the Bradford Dye Reagent (Bio-RAD, Hercules, CA, USA). Equal amounts (25 μg) of protein from each cell extract was then separated by SDS-PAGE (12% reducing gel), transferred onto nitrocellulose membrane and probed with a pre-determined (optimised) concentration of anti-Impα1 (1:2000 dilution), Impβ1 (1:500 dilution), Exp-1 (1:500 dilution), CAS (1:500 dilution), Ran (1:2000 dilution), RCC1 (1:250 dilution) monoclonal (all from BD Biosciences, San Jose, CA, USA) or anti-Impα3 (1:250 dilution) and Impα4 (1:500 dilution) polyclonal (Abcam, Cambridge, MA, USA) primary antibodies, followed by the appropriate host IgG-HRP secondary antibody (Chemicon, Temecula, CA, USA) according to manufacturer's recommendations and protein visualised using the Chemiluminescence Reagent (Perkin-Elmer, Wellesley, MA, USA or Millipore, Bedford, MA, USA). Where necessary, the membranes were then stripped of antibody using Western strip buffer (25 mM glycine, 1% SDS, pH 2) at 60°C, blocked and re-probed with anti-α/β tubulin (Cell Signaling, Danvers, MA, USA) or anti-T-ag (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) followed by the appropriate host-IgG-HRP secondary antibodies and visualised as previously. The intensity of the resulting bands for all proteins was estimated by densitometry using an Alpha Imager (Alpha Innotec, Santa Clara, CA, USA) for image capture, and the 1D electrophoresis gel analysis module from ImageQuant TL software (Amersham Biosciences, Piscataway, NJ, USA) for analysis of the resulting images; results are given as the ratio of the signal for nuclear transport factor relative to that for α/β tubulin in transformed cells, relative to the respective value for the non-transformed cell line of the isogenic cell pair.

2.4.6 Statistical analysis

The significance of differences in results between transformed/non-transformed cells was determined using the Student's (or Welch corrected) *t* test for unpaired data (two-tailed *p* value) or Mann-Whitney test for non-parametric data, as appropriate.

2.5 RESULTS

To compare the efficiency of nuclear transport in transformed and non-transformed cells we utilised three distinct non-transformed/transformed cell pairs, in which the transformed and non-transformed cell lines both share the same genetic ('isogenic') background (see **Table 2.1**); 1) the primary human skin fibroblast cell line 1BR3, and its SV40-transformed counterpart 1BR3N (287), 2) the non-transformed monkey kidney fibroblast CV-1 cell line together with its SV40-transformed counterpart COS-7 (288), and 3) the non-transformed, spontaneously immortalised MCF10A breast epithelial cell line (291), and its fully malignant counterpart, MCF10CA1h, generated by *in vivo* passaging through serial implantation of a *Ha*-Ras transfected MCF10A cell line in mice (289).

Table 2.1: Isogenic cell pairs used in this study.

	Transformed status	***Origin***	***Derivation***
1BR3	Non-transformed	Human skin	Fibroblast cells derived from a skin biopsy (287).
1BR3N	Transformed	1BR3 cell line	1BR3 cells stably transfected with plasmid pSV3neo, which contains the early region of SV40 encoding for small, middle and large tumour antigens (287).
CV-1	Non-transformed	Monkey kidney	Fibroblast cells derived from the African Green monkey (292).
COS-7	Transformed	CV-1 cell line	CV-1 cells stably transfected with an origin defective mutant of SV40 plasmid (-ori) which encodes for SV40 proteins, including the tumour antigens (288).
MCF10A	Non-transformed	Human breast	Spontaneously immortalised line from non-malignant human breast epithelium (291).
MCF10CA1h	Transformed	MCF10A transfected with *Ha*-Ras	Derived from serial mouse xenograft implantations of MCF10A cells transfected with oncogenic *Ha*-Ras which progressed to a carcinoma. Majority of MCF10CA1h cells form well-differentiated carcinomas with low metastatic potential in mice (289).

The cell lines were transfected to express either GFP alone or various NLS-containing GFP-fusion proteins (see **Table 2.2**), which are transported into the nucleus by either Impα/β1 (281, 293) or Impβ1 (57, 294), and subcellular localisation analysed by quantitative live cell confocal laser scanning microscopy (CLSM) 16-24 h later.

Table 2.2: Nuclear targeting sequences within the fusion proteins used in this study.

GFP-fusion protein	*NLSs (basic residues in bold)*	*NESs (hydrophobic residues underlined)*
Impα/β1-recognised		
T-ag(114–135)	P**KKKRK**V[132] (5, 293, 295)	*N/A*
T-ag(111–135)	As above	*N/A*
T-ag(111-135) NLSm	P**K**T**KRK**V[132] (282)	*N/A*
UL54(1145-1161)	PA**KKRA**R[1159] (281)	*N/A*
UL54(1145-1161) NLSm	PA**KK**AAA[1159] (281)	*N/A*
Impβ1-recognised		
TRF-1(337-440)	**KKKK**ES**RR**[356] (57)	*N/A*
Impβ1- and Exp-1-recognised		
VP3(74-121)	**K**PPS**KKR**–12 aa spacer-**R**P**R**TA**KRR**I**K**L[121] (290, 294)	VSKLKESLI[105] (279, 290)
VP3(74-121) NLS1/2m	**K**PPSNN**R**–12 aa spacer-**R**P**R**TA**K**NNI**K**L[121] (279)	As above
PTHrP(1-141)	RYLTQETNKVETYKEQPLKTPG**KKKK**G**K**P[94] (56, 296)	LSDTSTTSLELL[136] (56, 296)
Rev(2-116)*	**R**QA**RR**N**RRRR**W**R**E**R**Q**R**Q[51] (297)	LPPLERLTL[83] (298, 299)

* The HIV-1 Rev NLS is also recognised by Imp7, Impβ3, Imp9 and Impβ2 transporters (300, 301).

Abbreviations: GFP, green fluorescent protein; Imp, importin; N/A, not applicable; NES, nuclear export signal; NLS, nuclear localisation signal; NLSm, nuclear localisation signal mutant; PTHrP, parathyroid hormone-related protein; Rev, human immunodeficiency virus regulator of virion expression; T-ag, simian virus 40 large tumour antigen; TRF-1, telomeric repeat-binding factor-1; UL54, human cytomegalovirus DNA polymerase catalytic subunit UL54; VP3, chicken anaemia virus viral protein 3.

As expected, GFP localised uniformly throughout the nucleus and cytoplasm in all cell lines (**Fig. 2.1a**) due to its lack of nuclear targeting signals and small size (27 kDa) which is well below the molecular weight cut-off for passive-type transport through the NPC (c. 50-70 kDa) (3). In contrast, all of the Impα/β1- and Impβ1-recognised NLS-containing proteins localised more strongly in the nucleus of the transformed 1BR3N, COS-7 and MCF10CA1h cells, compared to their non-transformed counterparts (**Fig. 2.1a**). Image analysis was performed to determine at the single cell level the mean ratio of nuclear-to-cytoplasmic fluorescence (Fn/c, see *Materials and Methods*), statistical (*t*-test) analysis confirming significantly ($p < 0.0001$) higher levels of nuclear accumulation in the transformed cells compared to their non-transformed counterparts for all of the NLS-containing proteins (**Fig. 2.1b**), with the most pronounced differences in the levels of nuclear accumulation apparent for Impα/β1-recognised NLSs in 1BR3N (c. 7-fold) and MCF10CA1h (c. 6-fold) cells compared to non-transformed counterparts (**Fig. 2.1b**). The extent of nuclear accumulation was similarly assessed for GFP-fusion constructs in which the NLSs carry inactivating mutations (see

Table 2.2); in all cases, the nuclear accumulation of the NLS-mutated fusion proteins as well as that of GFP alone was completely comparable between the transformed and non-transformed lines in each cell pair (**Fig. 2.1c**, MCF10A/CA1h cells not shown), indicating that the large-scale differences in nuclear accumulation efficiency were specific to NLS- (and thus Imp-) dependent transport and not due to altered passive diffusion properties of the NPC in transformed cells.

Western analysis (not shown) indicated essentially identical levels of expression of the various GFP fusion proteins in the transformed and non-transformed lines, but to assess formally at the single cell level whether the results for increased nuclear accumulation in transformed compared to non-transformed cells may be attributable to increased expression levels of ectopically expressed proteins in the transformed compared to the non-transformed cells, we compared the Fn/c values for several NLS-containing GFP fusion proteins in the MCF10A/CA1h cells, expressing the proteins at either high (Fn-b > 1500 arbitrary fluorescence units), medium (Fn-b = 500-1500) or low (Fn-b < 500) levels (see **Fig. 2.1d** and *Materials and Methods*). Completely consistent with the whole population analysis (**Fig. 2.1b**)*,* 3- to-6-fold higher levels of nuclear accumulation were observed in transformed cells regardless of the levels of expression, clearly indicating that the extent of nuclear accumulation is not related to the concentration of nuclear import cargo expressed.

To test whether the increased nuclear accumulation in transformed cells is associated with an increase in the rate of nuclear import, we performed fluorescence recovery after photobleaching (FRAP) experiments as previously (285) on the MCF10A/CA1h cell pair expressing GFP-T-ag(114-135) whereby the nucleus of a single cell was bleached, and the return of non-bleached fluorescence from the cytoplasm to the nucleus monitored by CLSM for up to 280 s (**Figs. 2.2a**, **2.2b***,* also see *Materials and Methods*). The fractional recovery for specific nuclear fluorescence (Fn-b), calculated from the initial (pre-bleach) fluorescence and maximal (post-bleach) fluorescence recovery value, was then plotted for each time point to enable the half-time ($t_{1/2}$) for 50% maximal recovery to be determined. Whilst the mean maximal fractional recoveries of GFP-T-ag(114-135) did not differ significantly between MCF10A/CA1h cells (**Table 2.3**), the $t_{1/2}$ was significantly ($p < 0.05$) reduced by about 2-fold in transformed MCF10CA1h cells ($t_{1/2}$ = 16.0 ± 1.9 s) compared to the non-transformed MCF10A cells ($t_{1/2}$ = 27.4 ± 4.9 s) (see **Fig. 2.2c***;* **Table 2.3**) .

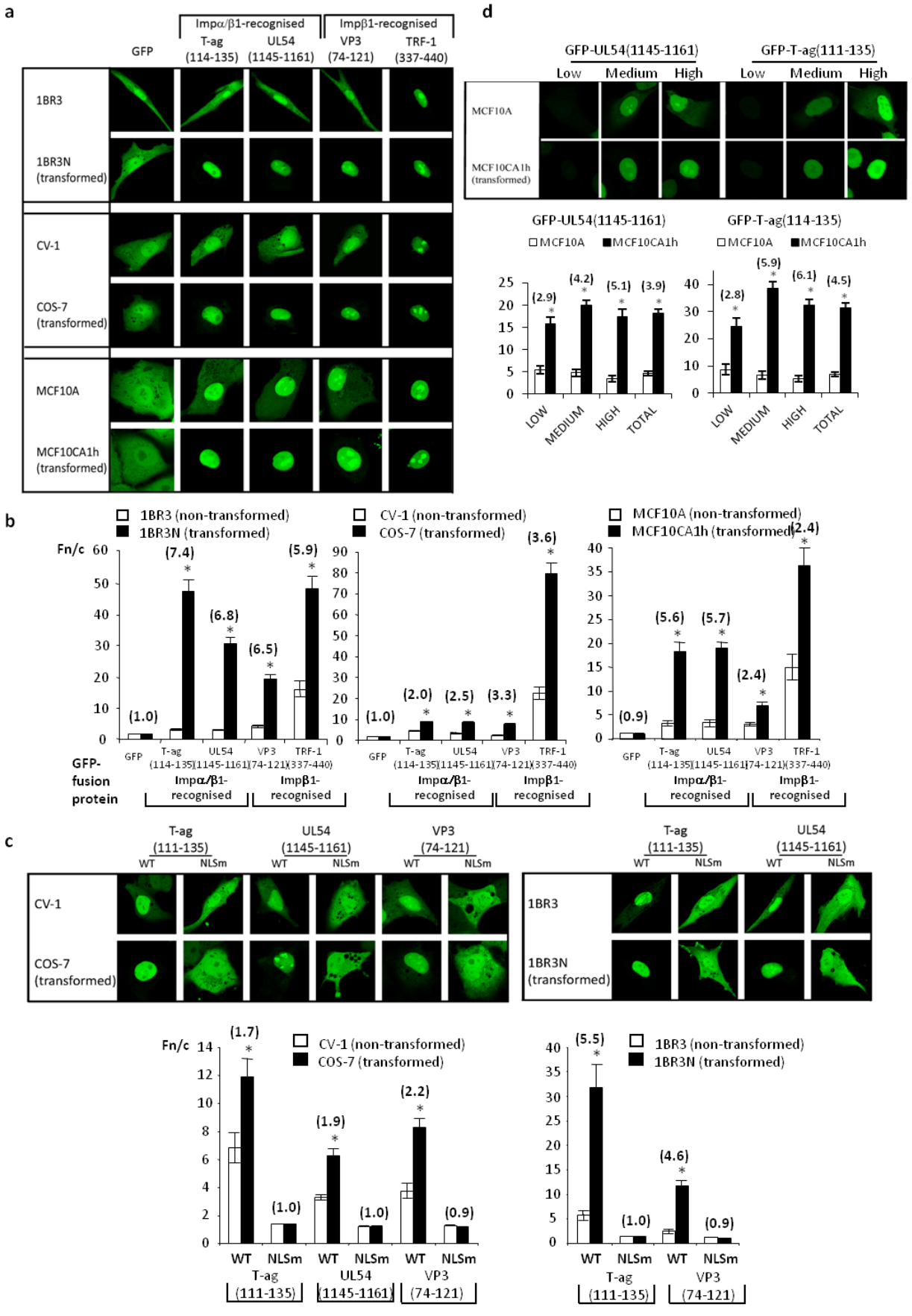

a
Impα/β1-recognised
Impβ1-recognised
GFP
T-ag (114-135)
UL54 (1145-1161)
VP3 (74-121)
TRF-1 (337-440)
1BR3
1BR3N (transformed)
CV-1
COS-7 (transformed)
MCF10A
MCF10CA1h (transformed)
d
GFP-UL54(1145-1161)
GFP-T-ag(111-135)
Low
Medium
High
MCF10A
MCF10CA1h (transformed)
GFP-UL54(1145-1161)
GFP-T-ag(114-135)
MCF10A
MCF10CA1h
LOW
MEDIUM
HIGH
TOTAL
b
1BR3 (non-transformed)
1BR3N (transformed)
CV-1 (non-transformed)
COS-7 (transformed)
MCF10A (non-transformed)
MCF10CA1h (transformed)
Fn/c
GFP-fusion protein
Impα/β1-recognised
Impβ1-recognised
c
T-ag (111-135)
UL54 (1145-1161)
VP3 (74-121)
WT
NLSm
CV-1
COS-7 (transformed)
1BR3
1BR3N (transformed)
Fn/c
CV-1 (non-transformed)
COS-7 (transformed)
1BR3 (non-transformed)
1BR3N (transformed)

Figure 2.1 Impα/β1- and Impβ1-dependent NLS-nuclear protein import is more efficient in transformed than in non-transformed cells. (**a**) Typical CLSM images of 1BR3/1BR3N (top), CV-1/COS-7 (middle) and MCF10A/CA1h (bottom) isogenic cell line pairs (see **Table 2.1**), transfected to express GFP alone or the indicated NLS-containing GFP-fusion proteins (see **Table 2.2**) analysed live 16-24 h post-transfection (X100 oil immersion objective). (**b**) Quantitative analysis of the level of nuclear accumulation (Fn/c) of fusion proteins in digitised images such as those in (**a**) in 1BR3/1BR3N (left), CV-1/COS-7 (centre) and MCF10A/CA1h (right) isogenic cell lines. Results represent the mean ± SE (n > 33, from a series of ≥ 3 different experiments); the fold-change in Fn/c ratio (indicated in parenthesis) represents the ratio of the Fn/c values in transformed *vs.* non-transformed cells. * p < 0.0001 *vs.* non-transformed cells. (**c**) Quantitative analysis of the relative levels of nuclear accumulation (Fn/c) of digitised images of CV-1/COS-7 (left) and 1BR3/1BR3N (right) cells transfected to express the indicated NLS-containing wild-type (WT) or NLS-mutated (NLSm) GFP-fusion proteins and analysed live 24 h post-transfection (X100 oil immersion objective). Results represent the mean ± SE (n > 22); the fold-change in Fn/c ratio for each cell pair is indicated in parentheses. * p < 0.0001 *vs.* non-transformed cells. (**d**) Quantitative analysis of the relative levels of nuclear accumulation (Fn/c) of digitised images of MCF10A/CA1h cells transfected to express the indicated NLS-containing GFP-fusion proteins in high (Fn-b > 1500), medium (Fn-b = 1500-500) and low (Fn-b < 500) expressing cell populations. Results represent the mean ± SE (n > 14). * p < 0.009 *vs.* MCF10A cells.

Consistent with this, the rate of loss of specific cytoplasmic fluorescence (Fc-b) was c. 3–fold faster in the transformed ($t_{1/2}$ = 28.2 ± 3.7 s) than in the non-transformed ($t_{1/2}$ = 101 ± 24.8 s) cells, indicating significantly (p < 0.0154) faster transport from the cytoplasm to the nucleus in transformed cells (**Fig. 2.2b***;* **Table 2.3**). The results indicate that the enhancement of NLS-dependent nuclear import efficiency observed in transformed cells is due to a faster nuclear import rate.

We also tested the subcellular localisation of a number of different NLS/NES-containing GFP-fusion proteins (**Table 2.2**; **Fig. 2.3a**), able to shuttle between the nucleus and cytoplasm dependent on Impβ1 and Exp-1 (56, 290, 294, 296-298) in the transformed/non-transformed cell pairs, in the absence and presence of the Exp-1-specific inhibitor leptomycin B (LMB), where the extent of the increase in protein nuclear accumulation (Fn/c) in the presence compared to absence of LMB was used as an indicator of the strength of nuclear export by Exp-1 in each cell line (see **Table 2.4**).

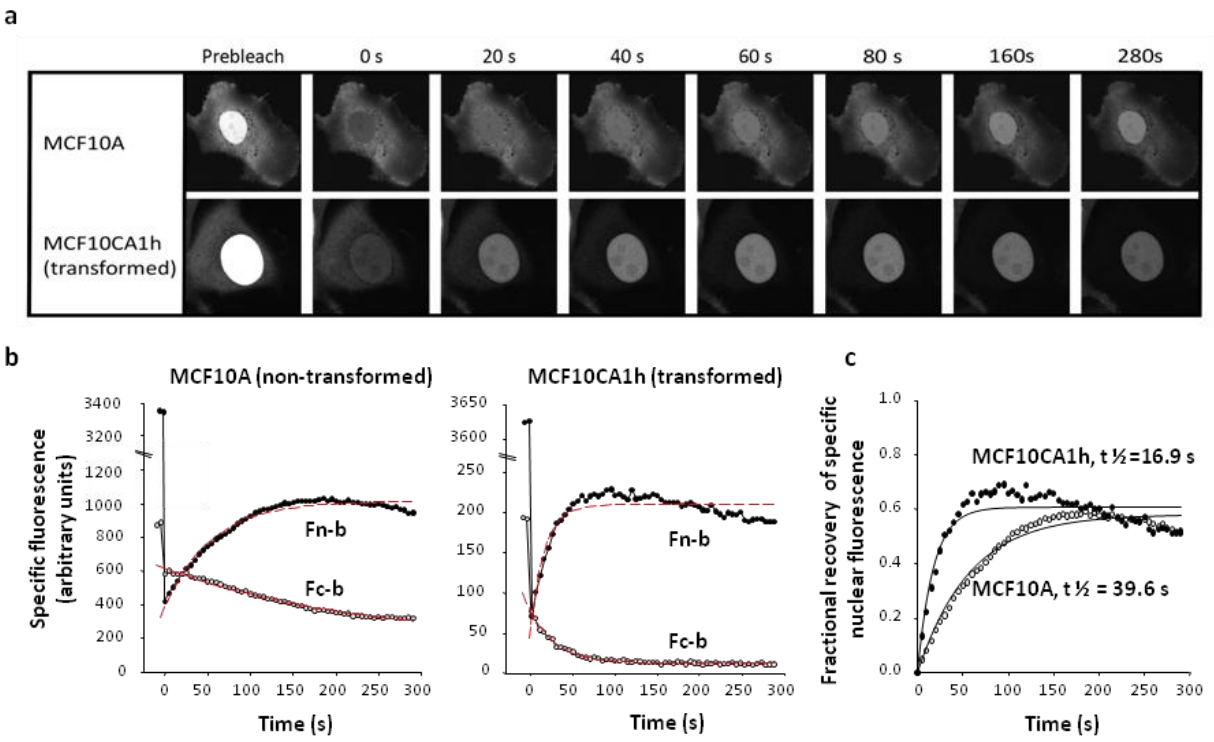


Table 2.3 Pooled data from a single representative fluorescence recovery after photobleaching experiment for GFP-T-ag(114-135) in populations of non-transformed MCF10A and transformed MCF10CA1h breast epithelial cells.

	MCF10A	MCF10CA1h	Normal / tumour
Half-time (t ½) for:			
Nuclear recovery (Fn-b)	27.4 ± 4.9 s	16.0 ± 1.9 s*	1.7
Cytoplasmic decay (Fc-b)	101 ± 24.8 s	28.2 ± 3.7 s*	3.6
Maximal fractional recovery	0.82 ± 0.06	0.91 ± 0.05	0.9
Initial Fn/c	4.3 ± 0.60	16.7 ± 1.9*	*N/A*
Initial fluorescence (arbitrary units):			
Fn-b	3218 ± 69	3466 ± 46*	0.93
Fc-b	964 ± 136	246 ± 31*	3.9

Results are for the mean +/- *SE* (n = 13). * $p < 0.05$ *vs.* MCF10A cells, Student's *t* test, unpaired data, 2-tailed.

Figure 2.2 The rate of nuclear import is increased in transformed cells compared to non-transformed cells. (**a**) Representative CLSM images (X100 oil immersion objective) of photobleaching experiments in MCF10A/CA1h cells transfected to express GFP-T-ag(114-135). At 16-24 h post-transfection a small (zoom 120-fold) area of the nucleus was photobleached (2500-4000 ms, 100% laser power) and monitored for the return of fluorescence by CLSM at 5 s intervals for a total of 280 s. (**b**) Quantitative representation of the specific nuclear (Fn-b) and cytoplasmic (Fc-b) fluorescence values in pre-bleach (-10 to -5 s), bleach (0 s) and post-bleach (5 to 280 s) images from (**a**) in single typical MCF10A and MCF10CA1h cells. The percentage loss of specific fluorescence in the nuclear and cytoplasmic compartments (Fn-b and Fc-b at 0 s time-point, compared to initial Fn-b and Fc-b pre-bleach values) due to photobleaching was comparable between the two lines (data not shown). (**c**) Quantitative representation of the fractional recovery of specific nuclear fluorescence, where values for Fn-b at respective time points were divided by the post-bleach maximal value and normalised to the 0 s post-bleach time point at each time interval from digitised images such as those in (**a**), is shown for single typical cells from data sets obtained for MCF10A/CA1h cell pairs. The half-maximal time ($t_{1/2}$) of recovery of specific nuclear fluorescence recovery is shown for MCF10A (r^2 = 0.84) and MCF10CA1h (r^2 = 0.97) cells.

Table 2.4: Quantitative analysis of nuclear accumulation of NLS/NES-containing GFP-fusion proteins in the absence as compared to the presence of LMB treatment in non-transformed and transformed cell lines; increased sensitivity to LMB treatment responsiveness in transformed cells.

	Non-transformed			Transformed			T [+LMB /-LMB] / NT [+LMB /-LMB]
GFP-fusion protein	**- LMB**	**+ LMB**	**+ LMB / - LMB**	**- LMB**	**+ LMB**	**+ LMB / - LMB**	
	1BR3			**1BR3N**			
GFP alone	1.52 ± 0.04 (135)	1.57 ± 0.06 (58)	1.00	1.42 ± 0.02 (156)	1.70 ± 0.06 (41)	1.20	1.2
VP3(74-121)	2.89 ± 0.23 (114)	4.02 ± 0.54 (91)*	1.40	21.9 ± 1.94 (142)	24.8 ± 3.36 (110)	1.10	0.8
PTHrP(1-141)	2.85 ± 0.23 (112)	4.22 ± 0.58 (67)*	1.50	32.7 ± 5.73 (50)	91.6 ± 15.10 (21)*	2.80	1.9
Rev(2-116)	0.37 ± 0.04 (37)	18.2 ± 3.51 (50)*	50.00	0.16 ± 0.03 (67)	42.2 ± 5.00 (43)*	256.00	5.2
	CV-1			**COS-7**			
GFP alone	1.45 ± 0.062 (65)	1.38 ± 0.033 (51)	1.00	1.60 ± 0.05 (25)	1.68 ± 0.037 (41)	1.00	1.0
VP3(74-121)	2.34 ± 0.059 (205)	2.80 ± 0.123 (159)*	1.20	8.20 ± 0.40 (162)	7.52 ± 0.28 (80)	0.92	0.8
PTHrP(1-141)	4.78 ± 0.35 (44)	7.46 ± 1.22 (30)*	1.56	11.8 ± 0.60 (77)	21.9 ± 1.24 (77)*	1.85	1.2
Rev(2-116)	0.40 ± 0.013 (122)	26.8 ± 2.04 (102)*	67.30	0.28 ± 0.021 (75)	26.7 ± 2.23 (196)*	96.00	1.4
	MCF10A			**MCF10CA1h**			
GFP alone	1.24 ± 0.03 (41)	1.21 ± 0.02 (38)	1.00	1.14 ± 0.02 (61)	1.15 ± 0.02 (54)	1.00	1.0
VP3(74-121)	4.09 ± 0.43 (58)	3.73 ± 0.50 (63)	0.90	8.06 ± 0.61 (72)	9.71 ± 0.75 (85)	1.20	1.3
PTHrP(1-141)	3.67 ± 0.38 (75)	7.90 ± 0.56 (58)*	2.15	6.42 ± 0.47 (67)	20.14 ± 1.52 (60)*	3.14	1.5
Rev(2-116)	1.56 ± 1.79 (75)	9.17 ± 1.28 (59)*	5.90	0.50 ± 0.05 (69)	15.6 ± 1.37 (91)*	31.10	5.3

Values for - LMB and + LMB represent mean ± *SE Fn/c;* numbers in parentheses indicate *n*. T [+LMB/-LMB], transformed +LMB/-LMB; NT [+LMB/-LMB], non-transformed +LMB/-LMB. *$p < 0.0001$ *vs.* - LMB.

We found that the NLS/NES-containing proteins generally showed significantly ($p \leq 0.003$) higher levels of nuclear accumulation in the transformed cell lines compared to their non-transformed counterparts in both the absence and presence of LMB treatment (**Fig. 2.3b**), consistent with the idea that nuclear import efficiency is enhanced in transformed compared to non-transformed cells. GFP-Rev(2-116) was an exception, failing to show significantly enhanced nuclear accumulation in the presence of LMB in transformed COS-7 compared to non-transformed CV-1 cells, probably as a result of the fact that Rev nuclear import can be modulated by a number of different Imps, including Impβ1 (297, 302), 7, 3, 9 and transportin (Impβ2) (300, 301). Most of the fusion proteins showed significantly ($p \leq 0.03$) increased nuclear accumulation in the presence of LMB in both non-transformed and transformed cell lines, indicating robust nuclear export activity in its absence (**Table 2.4**). GFP-VP3(74-121) was the only exception to this, exhibiting nuclear accumulation that was not affected by LMB in any of the transformed cell lines, consistent with the fact that VP3-mediated nuclear export is inhibited in transformed cells as a result of tumour cell-specific phosphorylation near the VP3 NES (279, 290).

Importantly, we found that the extent of the increase in nuclear accumulation of NLS/NES-containing proteins apart from VP3 in the presence as compared to the absence of LMB ([Fn/c + LMB] / [Fn/c - LMB]) was much greater in the transformed cell types compared to non-transformed cells (**Table 2.4**), implying enhanced Exp-1 activity in the transformed cell lines under normal conditions in the absence of LMB. Reduced nuclear export is clearly not the basis of higher nuclear accumulation of NLS-containing cargoes in the transformed compared to the non-transformed cell lines. Consistent with this, GFP-Rev(2-116), which preferentially localises in the cell cytoplasm due to the presence of a dominant, Exp-1-recognised NES, showed significantly ($p < 0.0001$) lower levels of nuclear accumulation in the absence of LMB treatment in transformed as compared to non-transformed cells, but either equivalent or significantly higher nuclear accumulation in its presence (**Fig. 2.3b**). The clear implication is that the various transformed cells show higher intrinsic levels of Exp-1-dependent export activity, in addition to the observed increased nuclear import efficiency (see above).

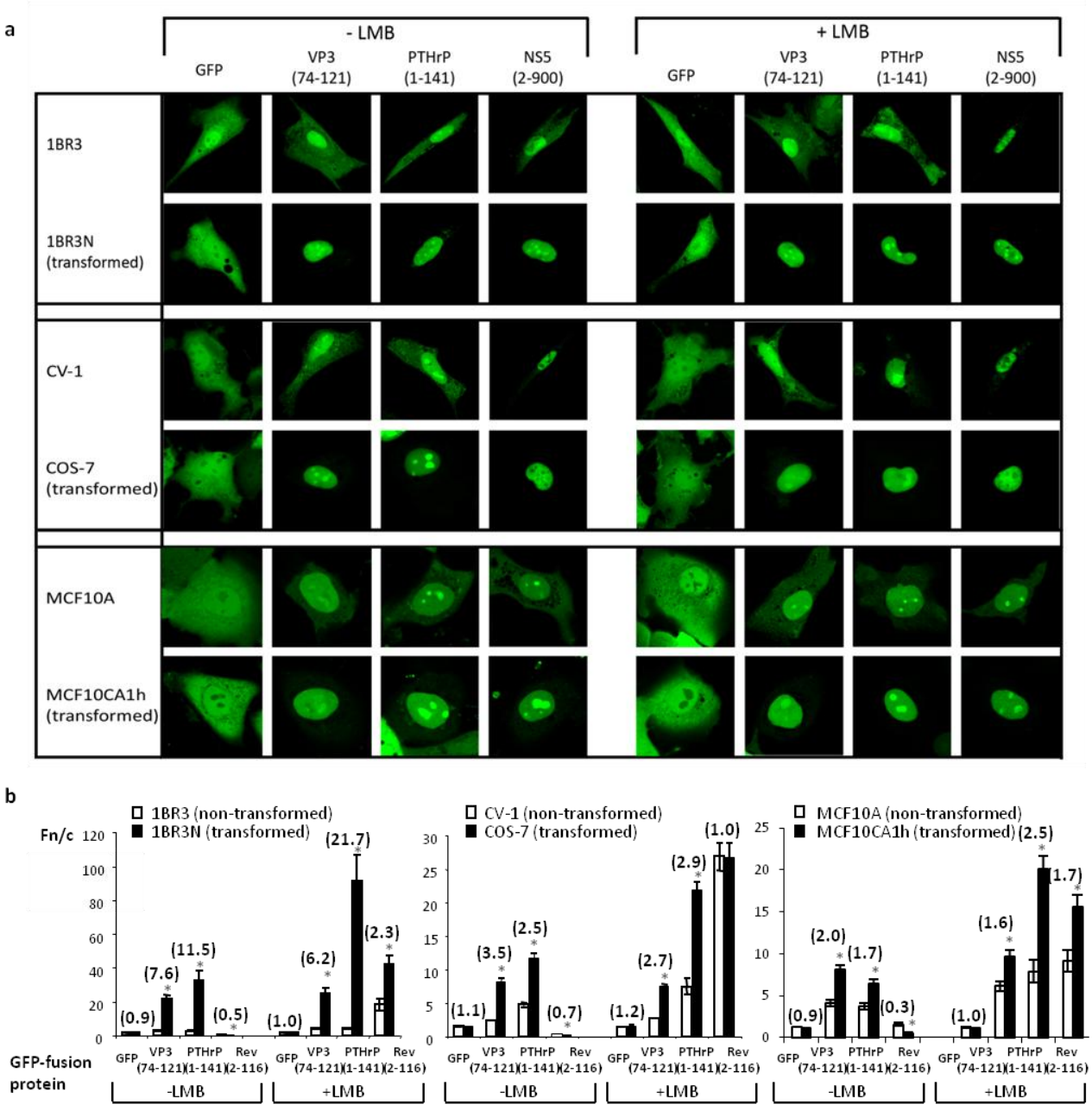
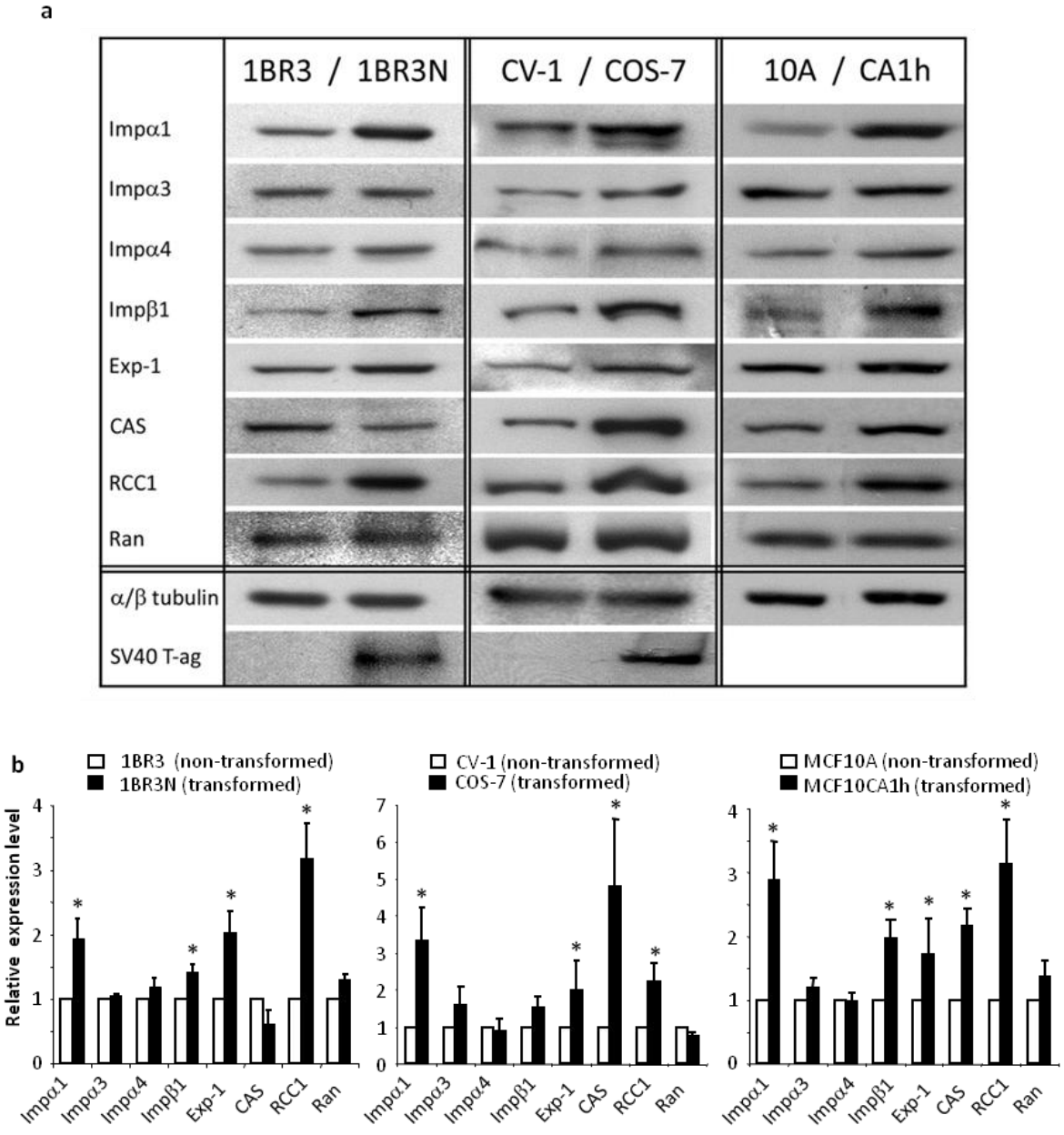


Figure 2.3 Reduced nuclear export is not the basis of enhanced nuclear accumulation of NLS/NES-containing proteins in transformed compared to non-transformed cells. (**a**) Typical CLSM images of 1BR3/1BR3N (top), CV-1/COS-7 (middle) and MCF10A/CA1h (bottom) cells, transfected to express GFP alone or the indicated NLS/NES-containing GFP-fusion proteins (see **Table 2.2**), in the absence (-) or presence (+) of 2.8 μg/ml of the specific Exp-1 inhibitor leptomycin B (LMB) 3-4 h prior to imaging and analysed live 16-24 h post-transfection (X100 oil immersion objective). (**b**) Quantitative analysis of the relative levels of nuclear accumulation (Fn/c) between transformed *vs.* non-transformed cells from digitised images such as those shown in (**a**) in 1BR3/1BR3N (left), CV-1/COS-7 (centre) and MCF10A/CA1h (right) isogenic cell lines. Results represent the mean ± SE (n > 20, typical results from a series of ≥ 2 separate experiments); the fold-change in Fn/c ratio for each cell pair is indicated in parentheses. * $p \leq 0.003$ *vs.* non-transformed cells.

Since the globally enhanced nuclear transport efficiencies could relate to differences in the levels of components of the nuclear transport machinery, we assessed the levels of Imps/Exps/Ran/Ran regulatory proteins in our non-transformed/transformed cell pairs by Western analysis/densitometry. After optimisation, analysis was performed for a range of Imps (Impα1, Impα3, Impα4 and Impβ1), Exps (Exp-1 and CAS), Ran and RCC1 in whole cell extracts of 1BR3/1BR3N, CV-1/COS-7 and MCF10A/CA1h cells. We found Impα1, Impβ1, Exp-1, CAS and RCC1 proteins were all expressed at markedly higher levels in the transformed cells compared to their non-transformed counterparts (**Fig. 2.4a**), with densitometric analysis (values expressed relative to the respective levels of the control protein α/β tubulin) revealing c. 2- to-4-fold higher levels of these components in transformed compared to non-transformed cells ($p < 0.05$, $n \geq 3$, for Impα1, Exp-1 and RCC1; see **Fig. 2.4b**).

To test whether enhanced Imp levels can enhance nuclear accumulation of proteins we co-transfected non-transformed MCF10A cells to express a bicistronic pIRES-mCherry Impα1 or Impβ1-encoding plasmid and GFP-T-ag(114-135) and assessed nuclear accumulation in live cells 40 h post-transfection (**Fig. 2.5a**). Expression of Impα1 and Impβ1 significantly ($p < 0.04$) increased GFP-NLS nuclear accumulation levels by c. 5- and 2.5-fold, respectively, compared to the control plasmid in MCF10A cells (**Fig. 2.5b**), demonstrating formally that the overexpression of either Impα1 or Impβ1 can enhance nuclear import efficiency in non-transformed cells.

Figure 2.4 Impα1, Impβ1, Exp-1, CAS and RCC1 are overexpressed in transformed compared to non-transformed cells. (**a**) Endogenous nuclear transport factors in whole cell extracts of 1BR3/1BR3N (left panel), CV-1/COS-7 (middle panel) and MCF10A/CA1h (right panel) cell lines were detected by Western analysis using the indicated antibodies, with α/β tubulin as a loading control, and SV40 T-ag control denoting transformed/non-transformed cell status as appropriate. Results are from a single typical experiment from a series of ≥ 3 separate experiments. (**b**) Quantitative analysis of the relative expression levels of nuclear transport factors based on Western analysis as per (**a**) for 1BR3/1BR3N (left), CV-1/COS-7 (centre) and MCF10A/CA1h (right) cell pairs, as determined by densitometry is given as the ratio of the intensity of the signal obtained for the nuclear transport factor protein normalised to that for α/β tubulin in transformed cells, relative to non-transformed cells. Results represent the mean ± SE ($n \geq 3$), * $p < 0.05$ *vs.* non-transformed cells.


a
1BR3 / 1BR3N
CV-1 / COS-7
10A / CA1h
Impα1
Impα3
Impα4
Impβ1
Exp-1
CAS
RCC1
Ran
α/β tubulin
SV40 T-ag
b
1BR3 (non-transformed)
1BR3N (transformed)
CV-1 (non-transformed)
COS-7 (transformed)
MCF10A (non-transformed)
MCF10CA1h (transformed)
Relative expression level
Impα1
Impα3
Impα4
Impβ1
Exp-1
CAS
RCC1
Ran

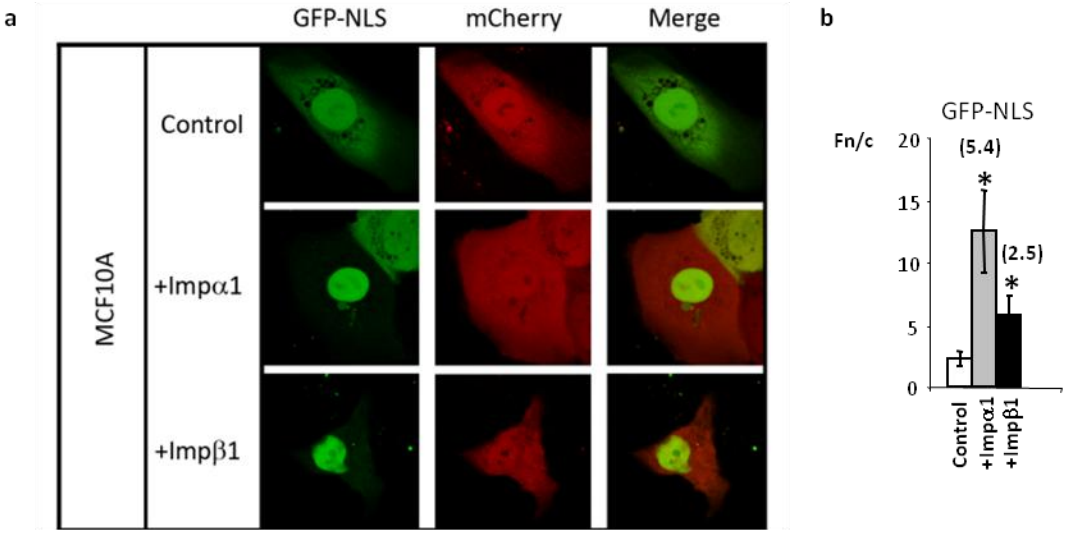


Figure 2.5 Overexpression of Impα1 or Impβ1 in non-transformed cells enhances protein nuclear accumulation. (**a**) CLSM images of normal MCF10A cells co-transfected to express GFP-T-ag(114-135) and Impα1, Impβ1 or control (empty vector) imaged 40 h post-transfection. mCherry expression from the pIRES-Imp-mCherry bicistronic plasmid was used to identify transfected cells. (**b**) Quantitative analysis of the relative levels of nuclear accumulation (Fn/c) of control (empty vector-transfected) or Imp-expressing MCF10A cells. Results represent the mean +/- SE (n > 41, results are from a single typical experiment in a series of three separate experiments). * p < 0.05 *vs.* MCF10A control.

2.6 DISCUSSION

This study uses transformed/non-transformed cell pairs from various origins and high resolution quantitative approaches to demonstrate for the first time that conventional, NLS-/Imp-dependent nuclear protein import is globally enhanced in transformed cells compared to their non-transformed counterparts, correlating with elevated levels of specific components of the cellular nuclear transport machinery. Although elevated Imp/Exp levels have been previously reported in tumour/transformed cells and tissues (211, 220, 303, 304), this is the first study to document quantitative differences in non-transformed/transformed cell lines of isogenic origin, and hence enables conclusions to be drawn with respect to the nuclear transport machinery and transformation status without the complications of confounding factors associated with the use of cells of different types/origins/patients (20, 116, 122). Further, it is the first study to explore the

kinetic parameters of nuclear transport in living transformed and non-transformed cells, establishing through fluorescence-based approaches and FRAP studies that the efficiency and rate of nuclear import of various NLS-containing cargoes utilising conventional, Imp-dependent pathways is enhanced in three different transformed cell lines compared to their non-transformed counterparts. The analysis of control molecules (GFP alone and NLS-mutated derivatives) established that the passive diffusion properties of the nuclear pore are not altered in the various lines, and that the enhanced transport in the transformed lines is NLS-dependent, with analysis of cell sub-populations with varying expression levels clearly indicating that differences between the transformed/non-transformed lines are not attributable to differing expression levels of the respective heterologous transport cargoes (305, 306). Rather, we propose that the basis of enhanced nuclear transport relates directly to increased levels of a subset of Imp/Exp molecules (Impα1, Impβ1, CAS and Exp-1) and the Ran nucleotide exchange factor RCC1, wherein proof-of-principle experiments involving overexpression of specific Imps in non-transformed cells show increased nuclear transport efficiency. The clear implication is that increased nuclear transport efficiency may be a key feature of transformed cells.

Significantly increased nuclear import efficiencies and rates were observed for various different Impα/β1 and Impβ1-recognised NLS-containing proteins in three different transformed cell lines compared to their non-transformed counterparts; two of the lines were SV40-transformed (1BR3N and COS-7) and the other (MCF10CA1h), representative of tumorigenic breast carcinoma cells. The observations with respect to the latter cell line represent the first report of enhanced nuclear import in non-virally transformed/tumour cell lines/tissues, but, consistent with our results here, enhanced nuclear import efficiency has previously been reported for SV40-transformed mouse fibroblasts (117, 307); enhanced nuclear import through conventional Impα/β1 and Impβ1-dependent pathways may be a key feature of transformation by DNA tumour viruses such as SV40 to ensure the maximal nuclear concentration and activity of key host and viral gene products (see(308)). We also analysed the efficiency of nuclear protein export for the first time in our three transformed/non-transformed cell pairs, enhanced responsiveness to the Exp-1 inhibitor LMB in the transformed cells suggesting higher intrinsic Exp-1 activity than in non-transformed cells. This was consistent with significantly increased Exp-1 protein levels in all of the transformed lines compared to their non-transformed counterparts.

Several studies in model cell types have shown that nuclear import efficiency can be influenced by Imp concentration (113, 114, 306). Here we observed significantly higher levels of both Impα1 and Impβ1 in transformed as opposed to non-transformed cells, in parallel with increased levels of the Ran guanine exchange factor, RCC1 which is important for unloading of import cargoes from Imps in the nucleus, as well as the Exportins CAS, which is important for recycling Impα back to the cytoplasm after a round of nuclear import, and Exp-1. Since enhanced nuclear import efficiency in the transformed cell lines is dependent on a functional NLS, and that ectopically expressed Imps clearly enhance nuclear transport efficiency in non-transformed MCF10A breast cells, it does not seem unreasonable to propose that the enhanced nuclear transport efficiency in the transformed cells observed here is a direct result of the increased levels of specific nuclear transport components, which are observed in many transformed cell lines and tumour samples (197, 200, 207, 216, 217, 220, 221, 309). Enhanced nuclear trafficking through higher levels of Imps/Exps could potentially impact on the nuclear targeting of many growth and proliferation regulating signalling molecules/transcription factors such as mitogen activated protein kinase (304), or cytoplasmic localisation of growth inhibitory factors such as $p27^{kip}$ or $p21^{cip}$, and thereby promote the transformed cell phenotype. The extent to which enhanced (amplified) signalling between the nucleus and cytoplasm may influence tumour biology/cancer pathology remains to be determined, but it is an intriguing possibility that enhanced nuclear transport may be a critical factor in tumour cell growth/proliferation/progression, and hence a potential target for therapeutic intervention to treat cancer (eg. see (271, 310, 311)).

Importantly, the results here raise the exciting possibility that the enhanced nuclear transport properties of transformed cells may be exploited to facilitate tumour cell-specific drug delivery. It is already known that the addition of NLSs to modular recombinant transporters (MRTs, comprised of assemblies of independently functioning protein domains capable of mediating cellular entry, endosomal escape and efficient nuclear targeting of photosensitising drugs (278, 280, 312)) can improve cell killing over 50-fold by inducing the localised generation of DNA-damaging reactive oxygen species (ROS) in the cell nucleus, the most sensitive site for ROS-induced damage (313, 314). Since the efficacy of cell killing through MRTs relies to a large extent on the efficiency of drug delivery to the nucleus, the fact that certain transformed cells possess intrinsically more efficient nuclear import implies that MRT killing action should be greater in transformed than non-transformed cells, supporting the idea of the utility of nuclear drug delivery in anti-cancer therapy.

Finally, the findings here have strong relevance to studies where SV40-induced transformation is used to increase cellular life span and growth potential to generate apparently 'normal' model cell types ie. from primary cells/mouse embryonic fibroblasts for a whole range of different applications (eg. studies of development). Based on the results here, it would seem judicious to be cautious in interpreting results using such cells, since SV40-transformed cells will almost certainly have significantly enhanced nuclear transport properties, which in turn may alter cell phenotype in various, yet-to-be-identified ways. In short, it is important to note that the use of SV40 in cell lines may well not be suitable for many studies for which they are currently used as accepted practice.

In summary, the results show that several transformed cell types from different origins possess enhanced Imp- and also Exp-dependent nuclear transport, correlated with increased levels of Imp/Exp proteins. The results are important for future anti-cancer therapies relying on nuclear drug delivery, as well as cell biological studies relying on transformation by SV40. Our future work will focus on how altered nucleocytoplasmic transport efficiency can be exploited optimally to achieve tumour-selective nuclear drug delivery using non-viral delivery approaches.

2.7 ACKNOWLEDGEMENTS

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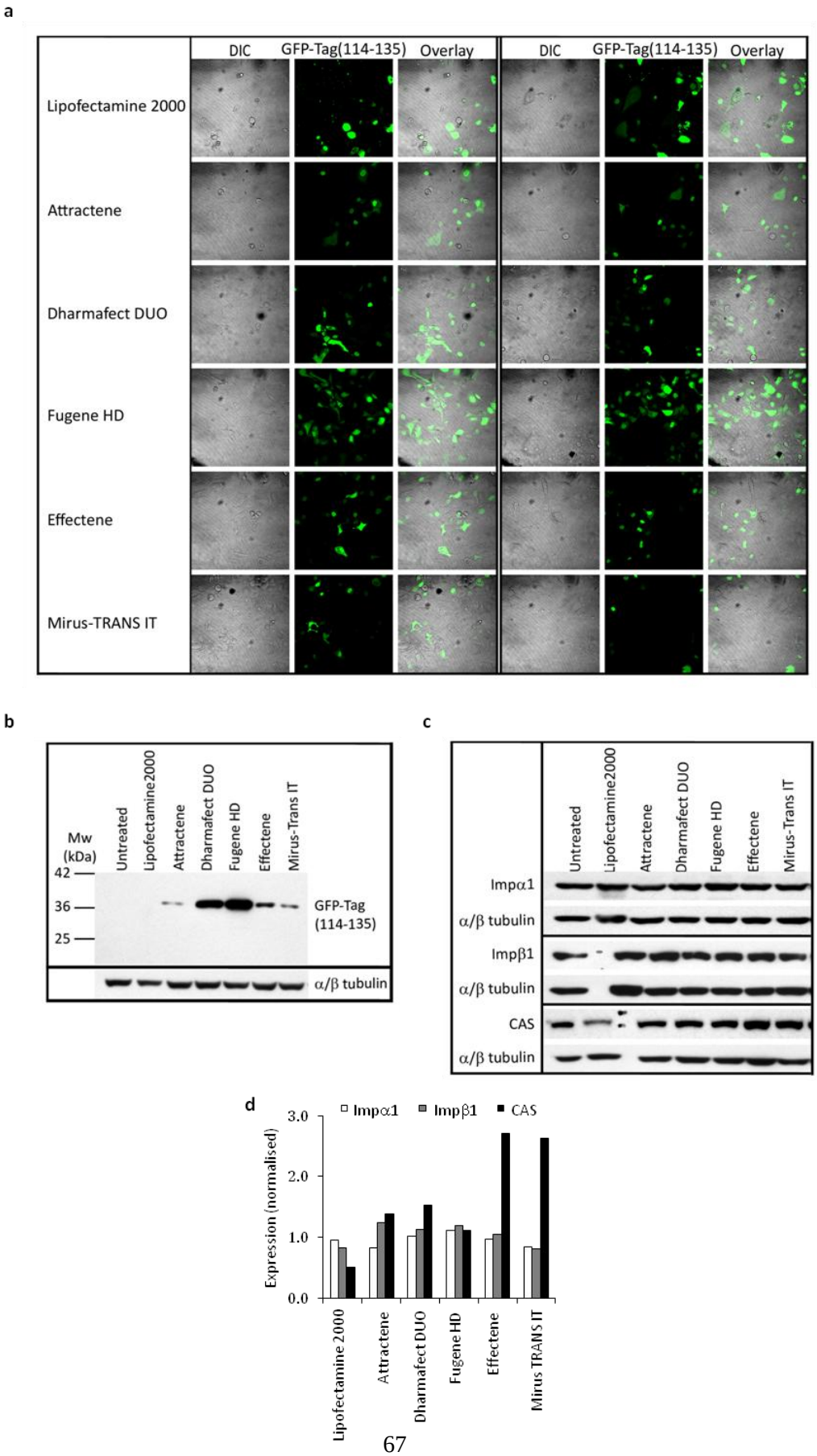
ADDITIONAL INFORMATION NOT SUBMITTED WITH KUUSISTO ET AL. 2012

2.8 SUPPLEMENTARY FIGURES/DATA

2.8.1 Comparison of transgene expression efficiency and effect on Imp/CAS levels of commercially available transfection reagents in MCF10CA1h breast cells

Prior to performing the studies described in Chapter 3, the effects of a number of commercially available transfection reagents on 1) transgene expression levels and 2) Imp/Exp levels, were tested in the malignant MCF10CA1h cell line. The basis for these experiments was to select a reagent that was capable of achieving the highest transgene expression levels from an ectopically expressed plasmid, with minimal effects of endogenous Imp/Exp levels and cell viability. MCF10CA1h cells were transfected with a GFP-Tag(114-135)-encoding plasmid using either Lipofectamine 2000 (Invitrogen), Dharmafect DUO (Dharmacon), Fugene HD (Promega), Effectene, Attractene (both from Qiagen) or Mirus TRANS-IT (Mirus Bio) according to manufacturer's instructions, and transgene expression detected 24 h pt by live cell CLSM (X20 oil immersion lens, heated stage, Nikon; **Supp. Fig. 2.1a**) or Western analysis (**Supp. Fig. 2.1b**) using an anti-GFP antibody (1:1000, Roche). Highest levels of transgene expression were obtained using Fugene HD and Dharmafect DUO, whilst transgene expression in the Lipofectamine2000-transfected sample was barely detectable by Western analysis most likely due to high cell death in the latter (not shown).

Supplementary Figure 2.1 Comparison of transgene expression efficiency and effect on Imp/CAS levels of commercially available transfection reagents in malignant breast cells. (**a**) MCF10CA1h cells were transfected to express GFP-T-ag NLS (114-135) using the indicated reagents and imaged live 24 h later by sequential DIC and CLSM. (**b**) Cell samples from (**a**) were lysed and whole cell extracts were subjected to Western analysis using an anti-GFP antibody (1:1000 dilution, Roche), followed by blotting for the endogenous control protein α/β tubulin. The molecular weight (Mw) marker (numbers represent kDa) is given on the left and shows no degredation of GFP-T-ag(114-135) in the samples. (**c**) Western and (**d**) densitometric analysis showing levels of Imps/CAS in transfected samples from (**a**/**b**) normalised to the tubulin control protein and to corresponding values in untreated MCF10CA1h cells. Results are from a single representative experiment in a set of two separate experiments.


a
DIC
GFP-Tag(114-135)
Overlay
DIC
GFP-Tag(114-135)
Overlay
Lipofectamine 2000
Attractene
Dharmafect DUO
Fugene HD
Effectene
Mirus-TRANS IT
b
Mw (kDa)
42
36
25
Untreated
Lipofectamine2000
Attractene
Dharmafect DUO
Fugene HD
Effectene
Mirus-Trans IT
GFP-Tag (114-135)
α/β tubulin
c
Untreated
Lipofectamine2000
Attractene
Dharmafect DUO
Fugene HD
Effectene
Mirus-Trans IT
Impα1
α/β tubulin
Impβ1
α/β tubulin
CAS
α/β tubulin
d
Impα1
Impβ1
CAS
Expression (normalised)
3.0
2.0
1.0
0.0
Lipofectamine 2000
Attractene
Dharmafect DUO
Fugene HD
Effectene
Mirus TRANS IT

Impα1, β1 and CAS levels were detected in the samples by Western and densitometric analysis (**Supp. Fig. 2.1cd**); levels of the nuclear transport components were minimally affected by Fugene HD, whereas all other reagents either increased (Effectene, Attractene, Dharmafect DUO, Mirus TRANS-IT) or decreased (Lipofectamine 2000) endogenous Imp/Exp levels. Reagents that induced the least cell death upon transfection as assessed by visual inspection were Attractene, Effectine and Mirus TRANS-IT followed by Dharmafect DUO and Fugene HD, whilst Lipofectamine 2000 induced the highest levels of cell death (not shown). Given that Fugene HD satisfied the requirements of high transgene expression with minimal effects on Imp/CAS levels where appropriate, all subsequent transfection studies in Chapter 3 were performed using Fugene HD.

CHAPTER 3

Enhanced Importin $\alpha/\beta 1$-dependent nuclear import efficiency in a model of breast tumour progression

DECLARATION FOR THESIS CHAPTER 3

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
Contributed to the conception and design of the study. Performed and was involved in all aspects of the experiments and prepared the manuscript for publication.	95%

The following co-authors contributed to the work:

Name	**Nature of contribution**
David A. Jans	Contributed to the conception and design of the study. Critical review of the manuscript.

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date**

Main Supervisor's Signature		**Date**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

3 Enhanced Importin α/β1-dependent nuclear import efficiency in a model of breast tumour progression

3.1 PREFACE

Chapter 3 of this thesis is composed of a manuscript prepared for submission in *Molecular Oncology* entitled "Enhanced Importin α/β1-dependent nuclear import efficiency in a model of breast tumour progression".

3.2 ABSTRACT

Trafficking into and out of the nucleus is conventionally mediated by the Importin (Imp) family of transporters, which are overexpressed in a variety of tumour cell types. Using the MCF10 cell model of human breast tumour progression, we show here that trafficking dependent on Impα/β1 but not Impβ1 is selectively enhanced in malignant breast (basal type invasive ductal carcinoma) cell types compared to isogenic non-transformed and hyperplasic (benign) counterparts, with the degree of enhancement correlating to advancing tumour disease state of the cells. Quantitative Western and RT-qPCR analysis indicate that this is associated with progressively increased expression of Impα1, α3, β1 and CAS in malignant cell types, with RNAi/overexpression approaches establishing Impα1 levels to be the primary basis of elevated Impα/β1-dependent nuclear import efficiency. Underlining the physiological relevance of these observations, the degree of Impα1 expression was found to relate with increasing tumour grade, in clinical ductal carcinoma samples. This is the first study to describe altered Impα/β1-dependent nuclear import as a novel feature of tumour disease progression, with implications for the development of tumour-selective therapeutic approaches.

3.3 INTRODUCTION

Signal-dependent nuclear protein import is central to cellular signal transduction events including the regulation of altered gene expression and phenotype change. Protein translocation into the nucleus is dependent on the presence of nuclear localisation signals (NLSs), which are

recognised by the Importin (Imp) family of nuclear transporters, of which there are multiple α and β forms (Impα1, α3-7 and Impβ1-5, β7-9, β11-13) in humans (12, 13). NLSs can be recognised by Impβs such as Impβ1 or the Impα/β1 heterodimer, after which the Imp:cargo complex translocates through the nuclear envelope-embedded nuclear pore complexes (NPCs) into the nucleoplasm, where the complex dissociates upon binding to Impβ1 of the monomeric guanine nucleotide binding protein, Ran, in activated GTP-bound form to enable disassembly of the import complex (89). Nuclear protein export occurs analogously, whereby nuclear export signals (NESs) are recognised by Exportins (Exps), homologues of Impβ1, in combination with RanGTP (89). Of multiple Exps expressed in humans, the best characterised are Exp-1 (Crm-1), which recognises leucine-rich NESs (91) and whose activity is inhibited by the specific inhibitor leptomycin B (95), and CAS (cellular apoptosis susceptibility protein, Cse1l), which facilitates recycling of Impα to the cytoplasm for subsequent rounds of nuclear import, thereby playing a key role in Impα/β1-dependent import (97).

An increasing body of evidence implicates altered Imp expression as a key driver and/or contributor to cellular processes, including male germ cell differentiation (spermatogenesis) (123, 125, 315), embryonic stem cell differentiation (34, 130) and pluripotency (131), induction of senescence in keratinocytes (119), responses to oxidative stress (316, 317) and cancer-related changes in breast (194) and ovarian cells (195). Tumorigenesis is a multi-step process that can be characterised by the accumulation of genotypical and phenotypical alterations to a cell, which allow cancer initiation, maintenance and progression to more aggressive phenotypes associated with metastasis. We previously showed that overexpression of Impα1 and β1 occurs in a variety of transformed cell lines, correlating with increased nuclear protein import efficiencies (318). In breast carcinomas (invasive ductal carcinoma and ductal carcinoma *in situ*), the degree of Impα1 (nuclear) overexpression has been positively linked to tumour grade, stage, aggressiveness and inversely to patient prognosis such as disease free and overall survival (188, 199, 200), as well as to poor responses to chemotherapy in advanced (high stage) breast carcinoma patients (198). Surprisingly, the effects of Imp overexpression on the nuclear transport efficiencies during breast tumour progression have not been examined, with essentially nothing known about the comparative nuclear import efficiencies of cell arising from differing tumour progression states from isogenic origins.

Here we use a model of human breast tumour progression (289) to show for the first time that Impα/β1- but not Impβ-dependent nuclear import becomes selectively and progressively enhanced

with increasing cellular tumorigenicity, and is associated with a benign to malignant switch in phenotype. We demonstrate that the level of expression of Impα1, and to a lesser extent of Impβ1 and CAS, is a key driver of improved Impα/β1-dependent import efficiency, and formally show that Impα1 expression can modulate the efficiency of nuclear import in cells. Results for altered Imp expression in patient samples of ductal carcinomas from different disease grades correlate with our findings in the model system, underlining the key importance of Imp expression in tumour progression, with implications for anti-cancer therapeutic development in the future.

3.4 MATERIALS AND METHODS

3.4.1 Mammalian expression constructs

Plasmid pEGFP (Clontech, Mountain View, CA, USA) encoding GFP alone was used as a control for transfection studies. The mammalian cell GFP fusion protein expression vectors encoding pEGFP-T-ag (114-135), pEGFP-T-ag (111-135), pEGFP-T-ag (111-135) NLSm (containing a $K^{128}T$ mutation), pEPI-pUL54 (1145-1161), pDEST53-pUL54 (1145-1161) NLSm (containing $R^{1157}A$ and $R^{1159}A$ mutations), pEPI-p53 (2-394), pEGFP-pRb (766-928), pEPI-VP3 tNTS (74-121) NESm, pEGFP-PTHrP (66-108), pEPI-TRF-1 (337-441), pEPI-aF10 (696-794) and pEPI-Rev (2-74), as well as pIRES-Imp-mCherry expression constructs, such as those encoding Impα1 and Impβ1 in a polycistronic gene with an mCherry marker have all been described previously (279, 281-285, 318, 319). In the case of the pIRES-CAS-mCherry and pIRES-Impα3-mCherry constructs, the CAS and Impα3 cDNAs were a kind gift from Dr. Carol Prives (Columbia University, New York, USA) and Dr. Yoichi Miyamoto (Monash University, Victoria, Australia) respectively, and were ligated into the pIRES plasmid using NotI and BamHI sites.

3.4.2 Mammalian cell culture, transfection and siRNA treatment

The properties of the MCF10 isogenic breast tumour progression model are summarised in **Table 3.1** (289). MCF10A (passage < 72), MCF10AT (passage < 75), MCF10CA1h (passage < 62) and MCF10CA1a (passage < 106) cells were cultured in DMEM/F12 Ham's media supplemented with 5% horse serum (Invitrogen, Carlsbad, CA, USA), 1 mM sodium pyruvate, 10 mM HEPES, 2 mM L-glutamine, 0.5 μg/ml hydrocortisone, 10 μg/ml bovine insulin, 20 ng/ml human recombinant EGF and 100 ng/ml cholera toxin (all from Sigma-Aldrich, St. Louis, MO, USA), as previously (289).

Depending on the assay 2.5-4.0 x 10^5 cells were seeded in 6 well tissue culture plates and transfected 24 h later using Fugene HD transfection reagent (Promega, Madison, WI, USA) according to manufacturer's instructions. Where appropriate, transfected cells were incubated for 3-4 h in the presence 2.8 μg/ml of the Exp-1 specific nuclear export inhibitor leptomycin B, prior to microscopic imaging.

For RNAi assays, 1.15 x 10^5 cells were seeded into 6 well tissue culture plates, and treated 24 h later with 10 nM commercially available non-targeting (NT) siRNA control or Impα1, Impα3, CAS (Cse1l) or Impβ1 ONTarget SMARTPool siRNAs (Dharmacon, GE Healthcare, Little Chalfont, Buckinghamshire, UK) where appropriate, using RiboCellIn siRNA transfection reagent (BioCellChallenge, Toulon-Cedex, France) according to the manufacturer's instructions. Cells were incubated for 48 h before re-transfecting with a plasmid encoding pEGFP-T-ag (114-135), and subjected to live cell CLSM and Western analysis 24 h later.

3.4.3 Confocal laser scanning microscopy (CLSM) and image analysis

Cells were imaged live (X100 oil immersion objective, zoom at 1.0, 60 μm pinhole, 4 and 15% laser power for 488nm and 568nm lasers respectively, heated stage) 16-24 h (single transfection) or 41-48 h (dual transfection) pt using a Nikon TSI 100 confocal laser scanning microscope (Nikon, Tokyo, Japan). Image analysis was performed using the ImageJ v1.41 public domain software (U.S. National Institutes of Health, Bethesda, MD, USA) to determine the nuclear (Fn) cytoplasmic (Fc) and background (Fb) fluorescence. Briefly, a mean density measurement of pixel numbers was made on a region of interest (ROI, within linear range) of equal size (ROI = 30 arbitrary units) in the nuclear and cytoplasmic compartments, respectively, whilst Fb measurements were made by placing the ROI on a non-transfected, autofluorescent region near the cells outer perimeter. The ratio of nuclear to cytoplasmic fluorescence (Fn/c) was then determined according to the formula: Fn/c = (Fn-Fb)/(Fc-Fb) (318). Fold-differences in nuclear accumulation between non-transformed and transformed cell lines were calculated according to the formula: [transformed Fn/c] / [non-transformed Fn/c].

3.4.4 Preparation of cell extracts and extracts from tissue samples for Western blotting/ densitometric analysis

Cells at log growth phase were detached, counted and washed twice in PBS prior to centrifugation and freezing at -80°C. Thawed pellets were washed twice with ice cold PBS prior to

resuspension (100 μl/10^6 cells) in ice-cold RIPA lysis buffer (50 mM Tris HCl pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS and 1 mM EDTA) with fresh EDTA-free complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA), homogenised through a fine 26G needle and lysed at on ice for 15 min followed by centrifugation at 10,000*g* for 30 min at 4°C to pellet insoluble material.

The human breast tumour tissue samples used in this project were provided by the Victorian Cancer Biobank (supported by the Victorian State Government) with appropriate ethics approval (HREC project #CF12/1949-2012001064). Samples comprised 12 DCIS (high grade), 15 IDC (grade 1), 19 IDC (grade 2) and 17 IDC (grade 3) samples (see **Supp. Table S3.1**). 15 mg of human breast tissue from each sample was homogenised using a TissueRuptor and extracts prepared using the AllPrep DNA/RNA/protein extraction kit (both from Qiagen, Venlo, Limburg, Netherlands) according to the manufacturer's instructions. Protein pellets from tissues were solubilised in Urea buffer (8 M Urea, 65 mM DTT, 2% CHAPS, 25 mM Tris-HCl pH 6.8) at 95°C for 5 mins.

The total protein concentration in each cell extract and tissue sample was estimated using the Bradford Dye Reagent (Bio-RAD, Hercules, CA, USA). 20 μg of protein from each cell extract (or 30 μg of protein from tissues) was then separated by SDS-PAGE (10 or 12% reducing gel), transferred onto nitrocellulose membrane (PALL Corporation, Port Washington, NY, USA) and probed with anti-Impα1, Impβ1, Impβ2, Imp9 monoclonal (BD Biosciences, San Jose, CA, USA), anti-Impα3, Impα4 (Abcam, Cambridge, MA, USA), Impβ1, Impβ3 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or Imp7 (GeneTex Inc., Irvine, CA, USA) polyclonal primary antibodies as required, followed by the appropriate host IgG-HRP secondary antibody (Chemicon, Temecula, CA, USA) according to the manufacturer's recommendations and the proteins visualised using Western Lightning Chemiluminescence Reagents (from Perkin-Elmer, Wellesley, MA, USA or Millipore, Bedford, MA, USA). The nitrocellulose membranes were stripped of antibody using Western strip buffer (25 mM glycine, 1% SDS, pH 2), blocked and then re-probed either with anti-α/β tubulin monoclonal (Cell Signaling, Danvers, MA, USA) or actin polyclonal (Santa Cruz Biotechnology Inc.) antibodies followed by the appropriate host-IgG-HRP secondary antibodies and visualised as previously.

In the case of the cell extracts, a 4-20% gradient gel was loaded in parallel for Coomassie staining to show total protein in each sample. The resulting bands for all HRP-visualised proteins for both

tumour tissues and cell extracts including Coomassie stained gels were scanned using an Alpha Imager (Alpha Innotec, Santa Clara, CA, USA), and the intensity of the bands was calculated using the Image Quant TL software (GE Healthcare, Little Chalfont, UK). For cell lines, the results are given as expression in terms of the ratio of the signal for Imp or Exp relative to that for α/β tubulin in transformed/malignant cells, relative to the respective value for the non-transformed cell line of the isogenic cell set.

3.4.5 Immunofluorescence assays

0.4 x 10^5 MCF10A and MCF10CA1h cells were seeded onto coverslips in a 12 well tissue culture plate, then fixed and permebialised with 4% paraformaldehyde and 0.1% Triton-X-100 (both from Sigma-Aldrich) respectively 48 h later. The samples were blocked using 1% BSA and immunostained using monoclonal anti-Impα1 (BD Biosciences), Impβ1 (NTF97, Abcam), CAS (Cse1l, Sigma-Aldrich), and polyclonal anti-Impα3 (Abcam) antibodies followed by the appropriate host FITC-conjugated IgG secondary antibodies (Molecular Probes, Life Technologies). The samples were then mounted onto microscopic slides using Prolong Gold anti-fade mounting media containing DAPI (Molecular Probes) to allow counterstaining of nuclear DNA. Proteins were visualised using the TCS SP5 confocal microscope (Leica Microsystems, X60 oil immersion objective) and the Fn/c ratio calculated using the Image J software (NIH).

3.4.6 Statistical significance

The significance ($p < 0.05$) of differences in results between transformed and non-transformed cells/tissues was determined using the Student's (or Welch corrected) t test for unpaired data (2-tailed p value), as appropriate, using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA).

3.5 RESULTS

3.5.1 Impα/β1-dependent nuclear import efficiency is enhanced in fully malignant breast tumour cells but not in benign or non-transformed counterparts

We set out to examine changes in nuclear import during tumour disease progression utilising the well-characterised MCF10 *in vitro* model of human ductal breast tumour progression (**Table 3.1**) (289, 320) comprising of non-transformed, spontaneously immortalised MCF10A cells

(291, 321), their mutant *Ha-Ras* (oncogenic $G^{12}V$ variant) stably transfected MCF10AT counterpart, and two fully malignant counterparts MCF10CA1h and MCF10CA1a, generated by serial trocar implantation of the MCF10AT line in mice. The tumorigenicity of these cell types has been previously graded based on colony forming potential on soft agar, immunocytochemical expression and karyotypic profiles, hormone/growth receptor (ER/PR/HER/EGFR) status, tumour forming ability in mice and the histological appearance of these ensuing growths (289, 322). When injected into the flanks of mice, MCF10AT cells produce palpable growths representative of benign hyperplasic tissue of myoepithelial origin that can give rise to various different breast tumour types (323), whereas MCF10CA1h cells form generally well-differentiated glandular adenocarcinomas and MCF10CA1a cells form undifferentiated carcinomas with minimal ductal and squamous components that are fully metastatic to the lungs (289). As expected, non-transformed MCF10A cells do not form palpable growths. Apart from mimicking increasingly advanced tumour disease stages, the MCF10 series has the advantage that all of the lines are derived from the same progenitor thus sharing an identical genetic background, meaning that any differences in cell properties including those relating to nuclear import are likely to be attributable to transformation status, rather than confounding factors such as differences in cell type, tissue or patient origin.

Table 3.1: The MCF10 breast tumour progression model.

	Transformed status	***Origin***	***Derivation***
MCF10A	Non-transformed	Human breast	Spontaneously immortalised line from non-malignant human breast epithelium (291).
MCF10AT	Benign, hyperplasic	MCF10A	Derived from MCF10A stably transfected with mutant ($G^{12}V$) *Ha*-Ras (under neomycin resistance), which were implanted into mice to form benign growths. When serially passaged in mice, gives rise to multiple different types of breast carcinomas (324).
MCF10CA1h	Fully malignant, non-metastatic	MCF10AT	Derived from serial mouse xenograft implantations of MCF10AT cells, which progressed to a carcinoma. MCF10CA1h cells form moderately-differentiated adenocarcinomas with low metastatic potential (289).
MCF10CA1a	Fully malignant, metastatic	MCF10AT	Derived from serial mouse xenograft implantations of MCF10AT cells, which progressed to a carcinoma. MCF10CA1a cells form poorly-differentiated carcinomas with high metastatic potential (289).

The various lines were transfected to express a number of well-characterised NLS-containing GFP fusion proteins recognised either by either the Impα/β1 heterodimer (T-ag, pUL54, pRb and p53) (5, 281, 282, 295, 325-327), Impβ1 alone (VP3 tNTS, PTHrP, SREBP-1a and TRF-1) (56, 57, 290, 294, 296), or multiple Impβs (HIV-1 Rev) (300, 301) (see **Table 3.2; Fig. 3.1a**). None of the fusion proteins contain a functional nuclear export sequence, with the exception of p53, whose nuclear export can be inhibited by the Exp-1-specific inhibitor leptomycin B (LMB). Transfected cells were subjected to live cell confocal scanning laser microscopy (CLSM; **Fig. 3.1a**), and the extent of nuclear accumulation (the nuclear to cytoplasmic fluorescence ratio - Fn/c) determined by image analysis as previously (318) (**Fig. 3.1b**). Intriguingly, the levels of nuclear accumulation for all proteins recognised by the Impα/β1 heterodimer were significantly increased ($p < 0.002$) in both fully malignant cell types, MCF10CA1h and MCF10CA1a (> 2- and 4-fold respectively) compared to their non-transformed and benign counterparts (**Fig. 3.1b**). Surprisingly, this was not the case for the nuclear accumulation of Impβ1/Impβ-recognised cargoes, some of which were significantly ($p < 0.05$) decreased compared to non-transformed counterparts (**Fig. 3.1b**). The non-Imp-recognised GFP-aF10 and GFP alone control proteins did not show altered nuclear accumulation in the transformed cell types (**Fig. 3.1b**), implying that the observed enhanced nuclear localisation efficiency was NLS/Imp-dependent. To test this more formally non-transformed MCF10A and malignant MCF10CA1h cells were transfected to express NLS mutated (NLSm) derivatives of Impα/β1 recognised T-ag and pUL54 proteins, neither showed differences in the extent of nuclear accumulation in malignant cells compared to non-transformed counterparts (**Fig. 3.1c**), implying that the results are not attributable to a difference in the passive diffusion properties of the NPC between the cell lines.

Complementing the fusion protein studies the nuclear localisation of several endogenous factors (the proto oncogene c-myc, the cell cycle protein cyclin D1 and the DNA repair factor Rad50) whose nuclear translocation is known to depend on the Impα/β1 pathway (30, 195, 328, 329) was also found to be significantly ($p < 0.0001$) up to almost 4-fold increased in malignant MCF10CA1h cells compared to non-transformed MCF10A counterparts (**Supp. Fig. 3.1ab**). This was in contrast to the Impβ2-recognised mRNA-binding factor hnRNP A1 (59, 330) that localised to similar levels in the nucleus of both cell types.

Table 3.2: Nuclear targeting sequences within the fusion proteins used in this study.

	NLS/s (basic residues in bold)
Impα/β1-recognised	
T-ag (114–135)	P**KKKRK**V[132] (5, 293, 295)
T-ag (111–135)	As above
T-ag (111-135) NLS mutant	P**K**T**KRK**V[132] (282)
pUL54 (1145-1161)	PA**KKRA**R[1159] (281)
pUL54 (1145-1161) NLS mutant	PA**KK**AAA[1159] (281)
pRb (766-928)	**KR**SAEGSNPP**K**PL**KKLR**[877] (325, 327)
p53 (2-394)^	**KR**ALPNNTSSSPQP**KKK**P[322]; L**KSKK**GQ[375]; **R**H**KK**LM[384] (326)
Impβ1-recognised	
VP3 (74-121)	**K**PPS**KKR**–12 aa. spacer-**R**P**R**TA**KRRIK**L[121] (290, 294)
PTHrP (66-108)	RYLTQETNKVETYKEQPLKTPG**KKKK**G**K**P[94] (56, 296)
TRF-1 (337-441)	**KKKK**ES**RR**[356] (57)
SREBP-1a (2-487)	Within the HLH-Zip domain (between aa. 343-460) (58)
Impβ-recognised*	
HIV-1 Rev (2-74)	**R**QA**RR**N**RRRR**W**R**E**R**Q**R**Q[51] (300, 301).
Non Imp-recognised	
aF10 (696-794)	N/A (319)

^ p53 contains Exp-1-recognised NESs.

* Apart from Impβ1, the HIV-1 Rev NLS is also recognised by Imp7, Impβ1, Impβ3, Imp9 and Impβ2.

Abbreviations: aa. amino acid; HIV-1 Rev, human immunodeficiency virus regulator of virion expression; Imp, importin; NES, nuclear export signal; NLS, nuclear localisation signal; p53, tumour suppressor protein 53; PTHrP, parathyroid hormone-related protein; pRb, retinoblastoma protein; SREBP-1a, sterol regulatory element-binding protein-1a; T-ag, simian virus 40 large tumour antigen; TRF-1, telomeric repeat-binding factor-1; pUL54, human cytomegalovirus DNA polymerase catalytic subunit UL54; VP3, chicken anaemia virus viral protein 3.

To exclude the possibility that the observed enhanced nuclear import may be the result of a faster cell proliferation in the malignant lines, we compared the growth properties of all of the lines in the MCF10 cell system (**Supp. Fig. S3.2**). Significantly, although the MCF10A line proliferated more slowly than the malignant cell lines at log growth phase (doubling time of 10 compared to 5-6 h, respectively), the benign MCF10AT cells proliferated as rapidly as the malignant MCF10CA1h and MCF10CA1a lines; since the latter both show significantly enhanced Impα/β1-dependent nuclear import activity compared to MCF10AT, but all 3 lines proliferate at the same rate, the enhanced nuclear import efficiency must relate to the malignant transformation status of the cells, rather than proliferation rate *per se*. The results overall thus indicate a selective enhancement in Impα/β1- but not Impβ1/Impβ-dependent nuclear import in the malignant lines of the MCF10 breast tumour progression series, relating strongly to the tumour progression state of the cells.

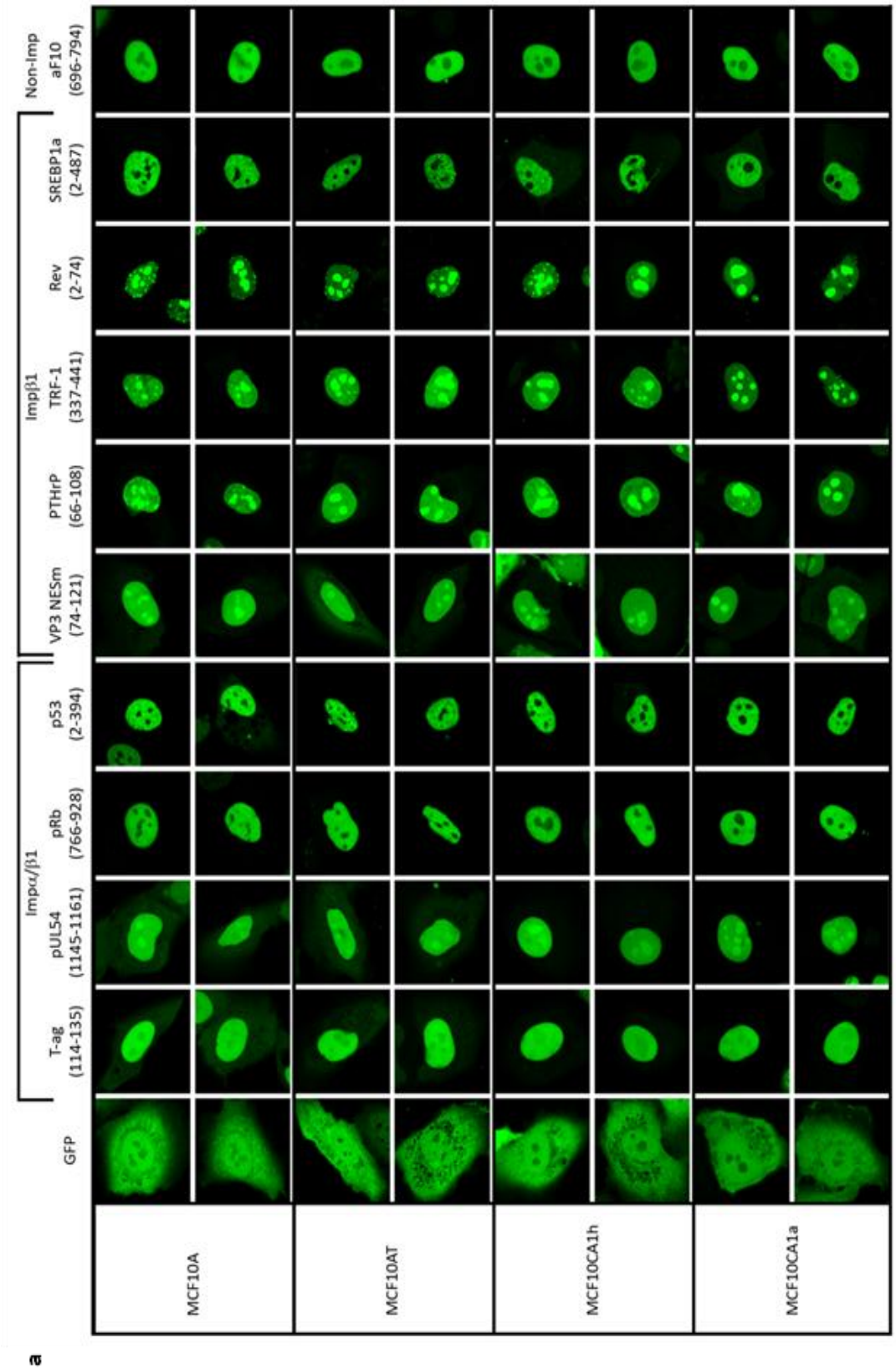
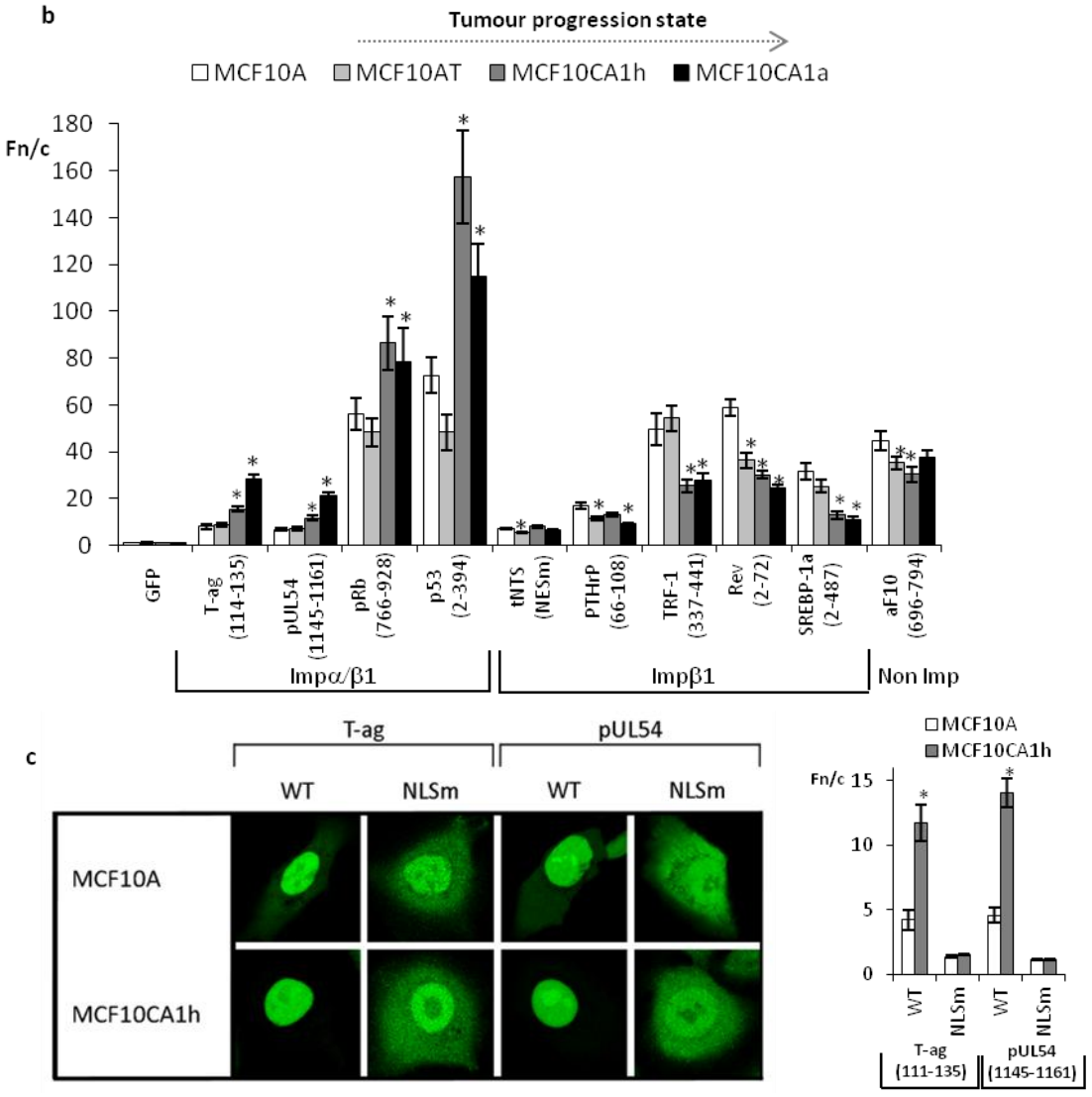

a
MCF10A
MCF10AT
MCF10CA1h
MCF10CA1a
GFP
Impα/β1
T-ag (114-135)
pUL54 (1145-1161)
pRb (766-928)
p53 (2-394)
Impβ1
VP3 NESm (74-121)
PTHrP (66-108)
TRF-1 (337-441)
Rev (2-74)
SREBP1a (2-487)
Non-imp
aF10 (696-794)

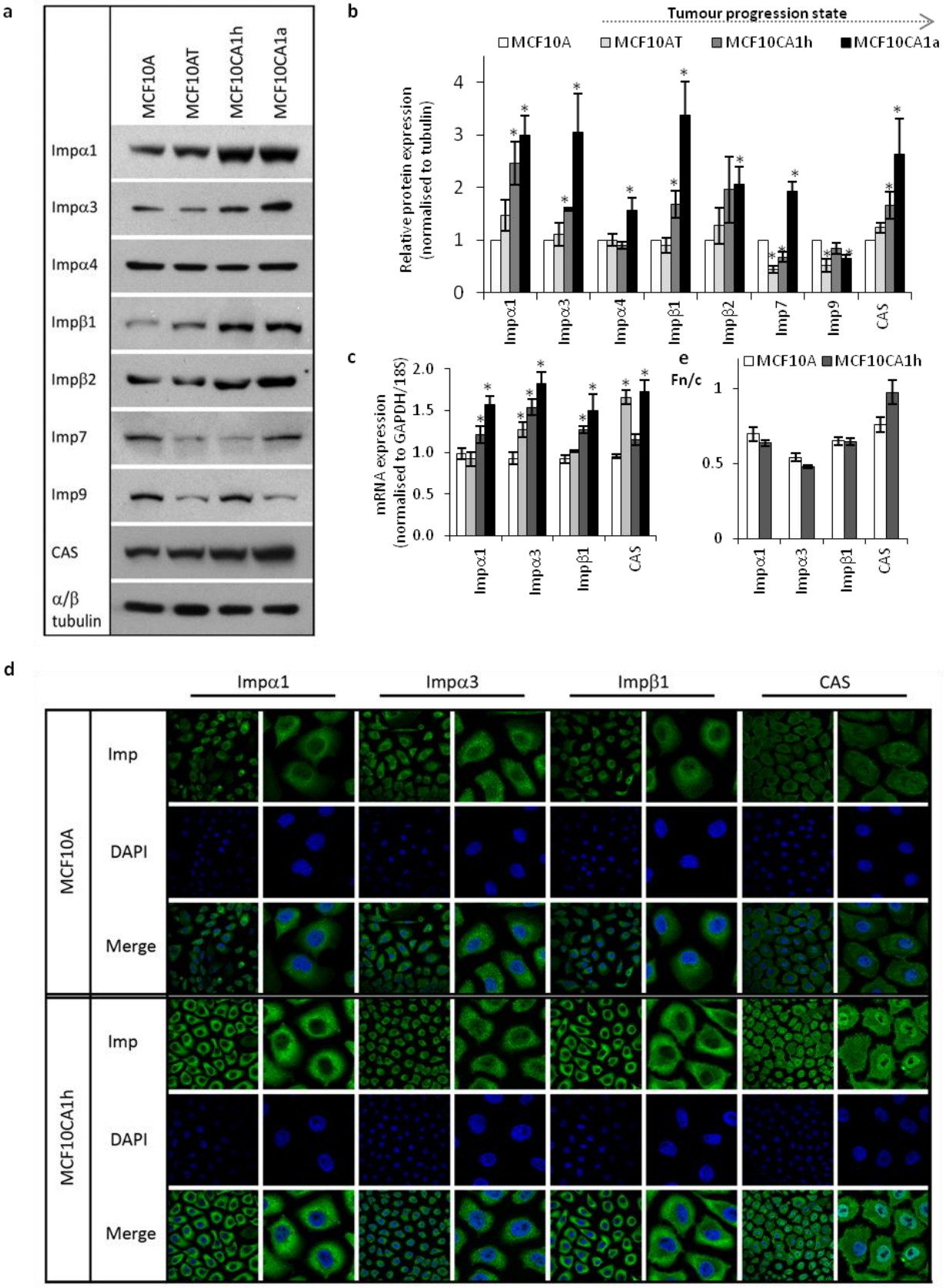
Figure 3.1 Impα/β1-dependent nuclear import efficiency is enhanced in malignant compared to benign/non-transformed breast cells. (**a**) The MCF10 series of cell lines was transiently transfected to express the indicated NLS-containing GFP fusion proteins recognised either by Impα/β1 or Impβ1 alone or in the case of HIV-1 Rev, multiple Impβs, and imaged live 16-24 h post transfection (pt) by confocal laser scanning microscopy (CLSM). GFP alone or a non-Imp recognised nuclear localising construct (GFP-aF10) were used as controls. (**b**) Image analysis of images such as those shown in (**a**) was used to determine the nuclear to cytoplasmic fluorescence (Fn/c) ratio for each construct in each cell line (non-transformed, white bars; transformed, grey/black bars); results represent the mean +/- SE from a single typical experiment (n > 37) in a set of three separate experiments. * p < 0.05 *vs.* MCF10A cells. (**c**) NLS-dependence of enhanced import efficiency. Non-transformed MCF10A (white bars) and malignant MCF10CA1h cells (grey bars) were transfected to express the indicated Impα/β1-recognised WT and NLS-mutated (NLSm) proteins and the extent of nuclear accumulation determined by image analysis. Results are from a single typical experiment (n > 27) in a set of three separate experiments. * p < 0.05 *vs.* MCF10A cells.



3.5.2 Increased expression of specific Imps with tumour progression

Our previous findings documented overexpression of specific Imps in a variety of tumour cell types (see Chapter 2) (318), but no formal analysis has been performed to examine Imp expression during stages of tumour progression. We determined the expression levels of Impα 1, α3 and α4, Impβ1, β2, β7 (Imp7) and β9 (Imp9), and CAS in the MCF10 breast tumour progression series by Western/densitometric analysis, normalising results to the α/β tubulin loading control (**Fig. 3.2a**); that α/β tubulin was an internal control representative of total protein levels in each cell type, was confirmed by Coomassie staining of total protein, and Western/densitometric analysis for actin (**Supp. Fig. S3.3**). Strikingly, although we found all of the Imps except Imp9 to be overexpressed to some extent in the metastatic high grade MCF10CA1a cells compared to non-transformed counterparts (**Fig. 3.2a**), quantitative analysis revealed significantly ($p < 0.035$) higher expression of Impα1, α3, β1 and CAS in both fully malignant cell types (MCF10CA1h and MCF10CA1a; c. 2- and 3.5-fold increased respectively) compared to their benign and non-transformed counterparts (**Fig. 3.2b**).

Figure 3.2 Progressively elevated expression of Importins in malignant breast cells compared to benign and non-transformed counterparts. (**a**) Western analysis of Importin (Imp) expression levels and control protein α/β tubulin in MCF10 breast cancer cell series. (**b**) Digitised images such as those shown in (**a**) were subjected to densitometric analysis. Results represent the mean +/- SE (n = 8) expression level normalised to the tubulin control in transformed/malignant cells (grey/black bars) relative to the normalised expression level in non-transformed MCF10A cells (white bars). * $p < 0.05$ *vs.* MCF10A cells. (**c**) Endogenous Impα1, α3, β1 and CAS transcript levels in the MCF10 tumour progression series were detected by real-time qRT-PCR. Results represent the mean +/- SE (n = 4) mRNA expression normalised to the geometric mean of GAPDH and 18S internal reference targets in transformed/malignant (grey or black bars) and non-transformed cells (white bars). (**d**) Endogenous Impα1, α3, β1 and CAS proteins in non-transformed MCF10A (top) and malignant MCF10CA1h (bottom) cells were detected by immunostaining and CLSM (X20 objective, left panels; zoomed-in images of single cells shown in the right panels). (**e**) Digitised images such as those shown in (**d**) were analysed for the mean +/- SE Fn/c ($n > 31$) as per the legend to **Fig. 3.1b**. Results are from a single experiment in a series of two experiments. * $p < 0.05$ *vs.* MCF10A cells.


a
MCF10A
MCF10AT
MCF10CA1h
MCF10CA1a
Impα1
Impα3
Impα4
Impβ1
Impβ2
Imp7
Imp9
CAS
α/β tubulin
b
Tumour progression state
MCF10A
MCF10AT
MCF10CA1h
MCF10CA1a
Relative protein expression (normalised to tubulin)
Impα1
Impα3
Impα4
Impβ1
Impβ2
Imp7
Imp9
CAS
c
mRNA expression (normalised to GAPDH/18S)
Impα1
Impα3
Impβ1
CAS
e
MCF10A
MCF10CA1h
Fn/c
Impα1
Impα3
Impβ1
CAS
d
Impα1
Impα3
Impβ1
CAS
MCF10A
MCF10CA1h
Imp
DAPI
Merge

Importantly, the extent of overexpression in the malignant cell types compared to the normal cells correlated with the extent of increased Impα/β1 dependent nuclear import efficiency. Real time RT-qPCR confirmed significantly ($p < 0.03$) increased Impα1, α3 and β1 transcript abundance in the malignant cell types (**Fig. 3.2c**), whilst immunostaining analysis of non-transformed MCF10A and malignant MCF10CA1h cells indicated the localisation of the endogenous proteins was similar between the cell types for Impα1, α3 and β1, although CAS appeared to localise to a greater extent in cytoplasmic structures in non-transformed cells (**Fig. 3.2de**). Interestingly, we found protein levels of other transport components such as Imp9 not increased in the malignant cell types, whilst Impα4, Impβ2 and Imp7 were all significantly ($p < 0.05$) upregulated in the highly metastatic MCF10CA1a, but not in non-metastatic MCF10CA1h cells compared to non-transformed counterparts (**Fig. 3.2ab**). The data implicates Impα1, α3, β1 and CAS levels as potential drivers of increased Impα/β1-dependent nuclear import efficiency occurring during a benign to malignant phenotype switch, and implies that alterations to the level of expression of other Imps may also occur during tumour progression to highly malignant phenotypes.

3.5.3 Impα1 levels affect nuclear import efficiency in the MCF10 tumour progression model

To formally test whether Impα1, α3, Impβ1 or CAS levels can modulate nuclear import efficiency we knocked down their expression in malignant MCF10CA1h cells using RNAi (**Fig. 3.3ab**), and assessed the effect of this on the nuclear translocation efficiency of the Impα/β1-recognised, NLS-containing GFP-T-ag (114-135) fusion protein (GFP-NLS) by live cell CLSM and image analysis. Interestingly, the silencing of Impα1, Impβ1 and CAS, but not Impα3 expression significantly ($p < 0.05$) decreased nuclear accumulation to levels similar to those observed in non-transformed MCF10A cells (**Fig. 3.3cd**). That the extent of the reduction in nuclear accumulation of GFP-NLS in response to Impα1 knockdown was not greater than 30% compared to non-targeting (NT) siRNA-treated cells (**Fig. 3.3d**) is attributable to the fact that the GFP-NLS can be recognised by other Impα isoforms (115, 331-333). In contrast, silencing of Impβ1 and CAS had much more pronounced effects in that they reduced nuclear accumulation of the fusion protein by up to 70% compared to NT siRNA-treated cells (**Fig. 3.3d**), most likely as the activities of these proteins are central to the Impα/β1-dependent import pathway.

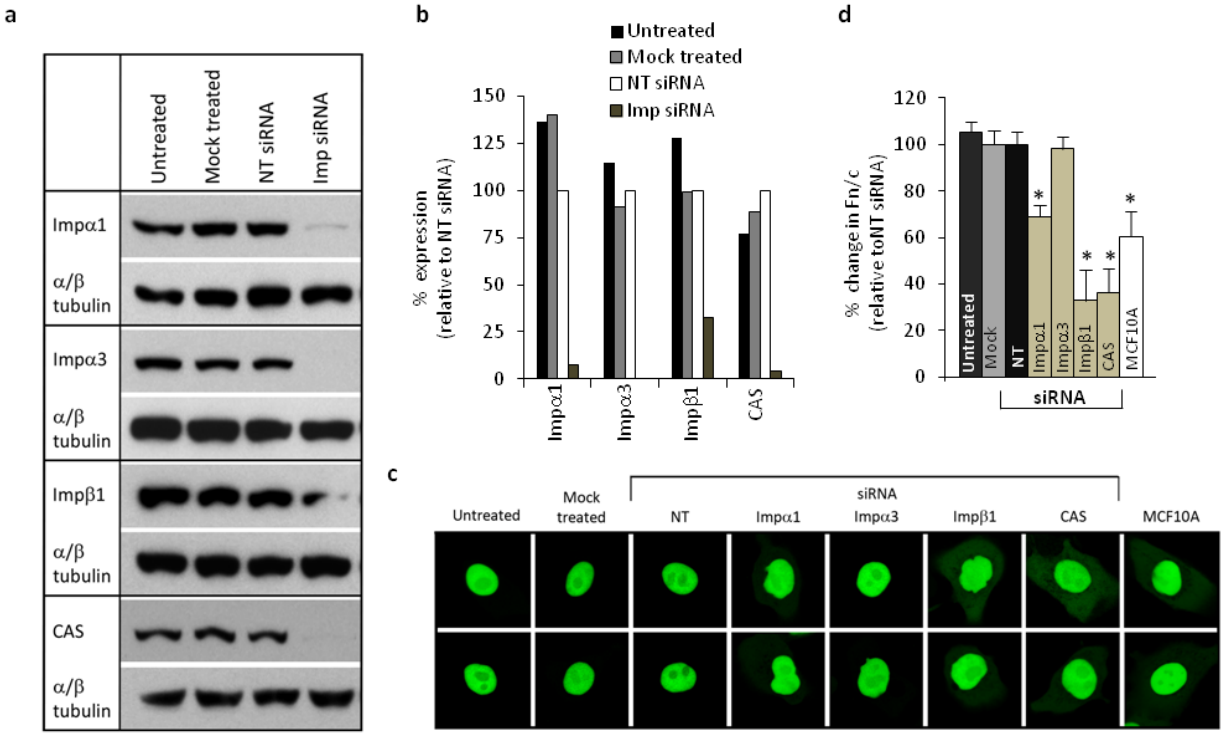


Figure 3.3 Cancer-associated Imps modulate nuclear import efficiency. (**a**) Malignant MCF10CA1h cells were treated with siRNA for Impα1, α3, β1, CAS or non-targeting (NT) controls, and knockdown assessed at 72 h pt by Western and (**b**) densitometric analysis as per the legend to **Fig. 3.2b**. Results represent the relative expression of Imps compared to NT siRNA-treated controls from a single typical experiment in a series of three separate experiments. (**c**) Cells were transfected 48 h post siRNA treatment to express the Impα/β1-recognised GFP-T-ag (114-135) fusion protein and (**d**) analysed as per the legend to **Fig. 3.1b**. Results represent the mean % change in Fn/c +/- SE (n > 146) of NT siRNA-treated controls in a series of three experiments. * p < 0.05 *vs.* NT siRNA-treated cells.

We complemented these studies by ectopically expressing these proteins in non-transformed MCF10A cells using a bicistronic pIRES expression plasmid capable of expressing two separate proteins from a single transcript through the presence of an internal ribosomal entry (IRES) site, encoding both the protein of interest and an mCherry reporter protein (**Fig. 3.4a**). Imp overexpression was confirmed by FACS (sorting for mCherry positive events) and Western/densitometric analysis (**Supp. Fig. S3.4**); a comparable level of overexpression was achieved in non-transformed MCF10A cells to that in malignant MCF10CA1h and MCF10CA1a cells. The nuclear accumulation of the GFP-NLS protein was significantly (p < 0.05) increased by

overexpression of Impα1, Impβ1 or CAS compared to control (empty plasmid transfected) MCF10A cells by c. 3- and 1.5-fold respectively, but consistent with our previous findings GFP-NLS nuclear accumulation was not affected by overexpression of Impα3 (**Fig. 3.4b**). Although comparable levels of overexpression of Impα1 and Impβ1 proteins were observed in MCF10CA1h and MCF10CA1a cells, Impα/β1-dependent nuclear import appeared to be more sensitive to changes in Impα1 than Impβ1 levels. This, together with the fact that Impα1 becomes highly overexpressed early in malignant transformation, implicates Impα1 as being primarily responsible for the enhanced Impα/β1-dependent nuclear import efficiency in MCF10CA1h and MCF10CA1a cells.

To test the effect of altered Impα1 or Impβ1 levels on Impβ1-dependent import, we overexpressed each protein in non-transformed MCF10A cells and tested the nuclear accumulation ability of Impβ1-recognised NLS-containing proteins GFP-tNTS (NESm), -PTHrP (66-108) and -TRF-1 (337-441). In contrast to the Impα/β1-recognised cargo, increased expression of Impα1 significantly ($p < 0.05$) decreased (c. 1.25 to 2.0-fold) nuclear accumulation of all of the Impβ1-recognised NLS-containing proteins compared to empty vector transfected controls (**Fig. 3.4c**). Unexpectedly and in contrast to the Impα/β1-recognised cargoes, overexpression of Impβ1 did not significantly increase nuclear accumulation of the Impβ1-recognised cargoes (**Fig. 3.4c**). Overall, these results suggest that although increasing Impα/β1-dependent import, the overall effect of overexpressing Impα1 appeared to be to inhibit Impβ1-dependent nuclear import.

3.5.4 Impα1 overexpression is associated with increasing tumour grade in ductal carcinomas

To put the above results in a clinically relevant context, we investigated the expression of Impα1 and Impβ1 in patient samples of invasive ductal carcinoma (IDC) and ductal carcinoma *in situ* (DCIS). Protein was extracted from tissues from patient groups ($n \geq 12$) which were selected according to tumour grade (BRE grading system 1-3, DCIS nuclear grading system, **Supp. Table 3.1**), and then subjected to Western (**Fig. 3.5a**) and densitometric (**Fig. 3.5b**) analysis for Impα1 and Impβ1 using actin as an internal control protein.

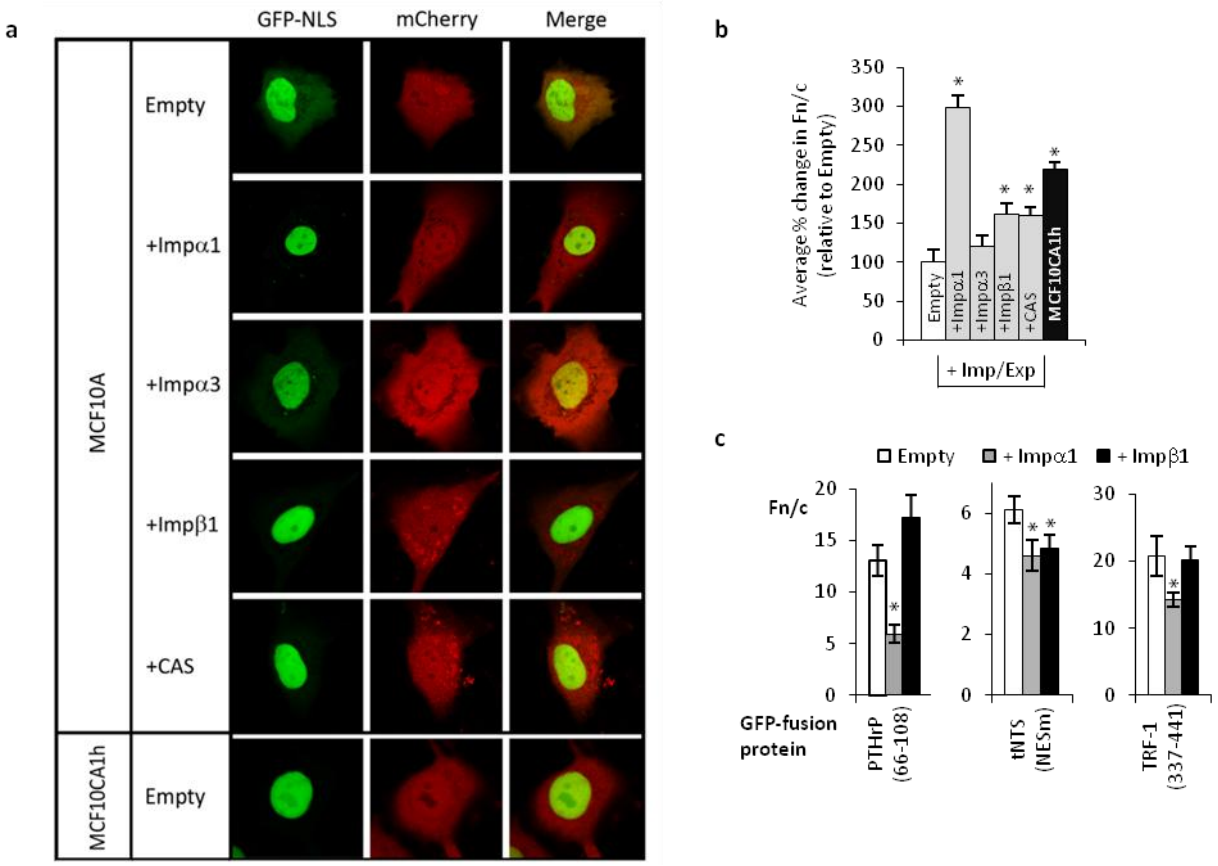


Figure 3.4 Impα1 levels drive enhanced nuclear import efficiency in the MCF10 cell model. (**a**) Non-transformed MCF10A cells were transiently co-transfected to express Impα1, α3, β1, CAS or a non-Imp-containing plasmid control (empty vector) all encoding the mCherry fluorescent marker, together with the Impα/β1–recognised NLS-containing fusion protein GFP-T-ag (114-135), and imaged 41-48 h pt as per **Fig. 3.1a**. (**b**) The Fn/c ratio in the mCherry expressing population was then determined from the digitised images as per the legend to **Fig. 3.1b**; results are for the mean % change in Fn/c +/- SE (n > 256) in Imp overexpressing cells in relation to empty vector control in a series of five experiments. * p < 0.05 *vs.* MCF10A control. (**c**) Non-transformed MCF10A cells were co-transfected to express Impα1 or Impβ1 and the indicated NLS-containing GFP fusion proteins recognised by Impβ1, and subjected to image analysis as per **Fig. 3.1b**. * p < 0.05 *vs.* MCF10A control.

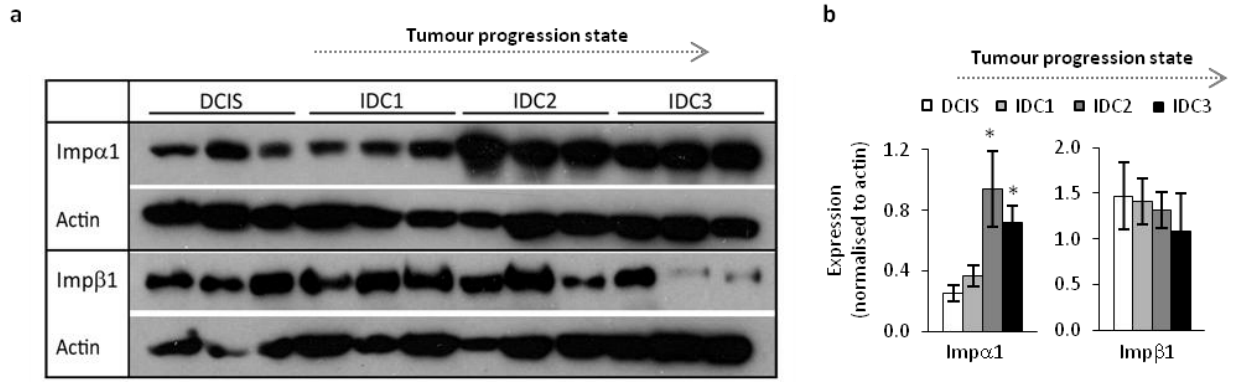


Figure 3.5 Increased Impα1 levels in invasive ductal carcinomas of the breast correlates with high tumour grade. (**a**) Western analysis of Impα1 or Impβ1 levels in representative mammary samples taken from ductal carcinoma *in situ* (DCIS, high grade) or invasive ductal carcinomas (IDC1-3 representing BRE grades 1-3). (**b**) Digitised images such as those shown in (**a**) were subjected to densitometric analysis. Results are given for the mean +/- SE (n > 12) Imp levels normalised to that of the actin internal control. * p < 0.05 *vs.* IDC1 sample.

The tumour tissues were chosen based on grade and hormone receptor status to mimic our *in vitro* model as closely as possible. We found significantly (p < 0.05) increased Impα1 expression associated with high tumour grades (2.6- and 2-fold increased in grade 2 and grade 3 IDC respectively) compared to grade 1 IDC and DCIS controls (**Fig. 3.5b**). This was not the case for Impβ1, whose expression remained constant throughout the tumour samples (**Fig. 3.5b**). Our data thus indicates that increased Impα1, but not Impβ1 occurs in high compared to low grade IDC and DCIS.

3.6 DISCUSSION

The MCF10 model is an ideal test system for fundamental questions in cancer biology, as the study of clinical samples is complicated by cellular, genetic, environmental and treatment heterogeneities, whilst the commonplace use of non-isogenic *in vitro* cell models makes it difficult to determine whether changes observed were associated with cancer or due to variations in genetic backgrounds. We show here for the first time that Impα/β1- dependent but not Impβ1-dependent nuclear import pathways become selectively enhanced during the benign to malignant switch

phenotype in the isogenic MCF10 model of breast tumour progression. Interestingly, this is not a simple by-product of increased cellular proliferation as benign MCF10AT cells, which have acquired the ability to grow as fast as their fully malignant counterparts, did not exhibit altered nuclear import properties, implying that the changes in Impα/β1 activity were specifically associated with malignant transformation. We found that overexpression primarily of Impα1, but also to a lesser degree Impβ1 and CAS were associated with improved nuclear import efficiency in malignant cells and identified Impα1 as the key driver of Imp-dependent nuclear import efficiency changes in tumour progression.

To our surprise, overexpression or silencing of Impα3 did not affect GFP-NLS nuclear import, even though the NLS of T-antigen is known to be recognised by Impα3 (115, 331-333). Preferential binding of Impαs to certain NLSs is known to occur in cells and may differ in cell types due to the differential expression levels of competing cargo NLSs (20), so that it is possible that high expression of endogenous Impα3 recognised cargoes may saturate Impα3 dependent import in MCF10A/CA1h cells and/or that the T-ag NLS is simply recognised by Impαs such as Impα1 with higher affinity *in vivo* than Impα3. Although Impα3 is reported to be important for nuclear import of Ran guanine exchange factor RCC1, essential for maintenance of high RanGTP levels in the nucleus, we did not detect any alterations to RCC1 protein nuclear localisation nor nuclear RanGTP accumulation when Impα3 expression was inhibited by siRNA (not shown). It is likely that multiple import pathways exist for important nuclear transport components such as RCC1, either through other Imps such as Impα4 or Imp-independent mechanisms (334), suggesting that cells have evolved multiple, redundant systems to maintain the integrity of the RanGTP gradient, which is so important to nucleocytoplasmic transport itself.

It is interesting to note that the Imp which had the most pronounced effects on GFP-NLS nuclear accumulation through Impα/β1 pathway when overexpressed in non-transformed MCF10A cells was Impα1, with Impβ1 and CAS levels playing secondary "housekeeping" roles by way of accommodating nuclear import (through NPC binding) and re-export of Impαs respectively. This is consistent with the findings of Riddick and Macara who found Impα to be a limiting factor for nuclear import in model systems (76, 113); in contrast to Riddick and Macara we did not, however, observe inhibition of nuclear import due to increased Impβ1 or CAS expression in non-transformed cells (76, 113). It has been proposed from studies in intact cells that as the total cellular

concentration of Karyopherins (Imps/Exps) exceeds 15 μM, the system becomes saturated resulting in reduced nuclear import kinetics/efficiency (306). This is especially true for Imps/Exps that bind to NPCs and RanGTP, such as Impβ1 and contributes to "futile cycling"; a non-productive import event where excess Impβ1 travels through the NPCs devoid of cargo, to bind RanGTP and Nups at the nuclear rim of the NPC before returning to the cytoplasm (76). Impβ1 must react with RanGTP prior to being released from the Nups inside the nuclear rim of the NPC, thus continuous cycling depletes the availability of RanGTP for disassembly of cargo:Imp complexes resulting in their arrest at the pore. As the concentration of Imps/Exps has not been measured in the MCF10 model we can only assume that total Imp/Exp levels are within a range (see (114)) that increased Impβ1/CAS concentrations act to increase Impα/β1-dependent nuclear import efficiency, rather than inhibit it.

We also noted that Impβ1 expression is increased in malignant cells, but without a significant increase in Impβ1-dependent nuclear import. Rather, Impβ1-dependent nuclear import efficiency was decreased up to 2-fold in malignant cells expressing high levels of Impα1, implying that Impβ1 is preferentially directed to the Impα/β1 pathway, presumably at the expense of Impβ1-dependent import. Consistent with this, we found that overexpression of Impα1 decreased whilst overexpression of Impβ1 failed to effect nuclear accumulation of Impβ1-recognised NLS-containing proteins such as TRF-1 and PTHrP, and both were able to increase nuclear accumulation of an Impα/β1-recognised protein in non-transformed cells. Given that Impα1 does not directly bind to the aforementioned Impβ1-recognised factors but can competitively inhibit their nuclear translocation (57, 335) and itself has a high affinity for Impβ1 (c. 10nM) (115), it is conceivable that Impα1 levels may compete for Impβ1, reducing Impβ1 availability for its cargoes thereby decreasing the net efficiency of Impβ1-dependent import in cells that are overexpressing Impα1, such as cancer cells.

Our work shows overexpression of Impα1 in patient tumour tissues of ductal breast carcinomas with increasing disease severity and strongly suggests Impα/β1- but not Impβ1-dependent nuclear import efficiency is upregulated *in vivo* in ductal carcinoma patients. Impα1 overexpression has been previously described in tumour tissues from invasive ductal carcinoma, where it is linked with tumour aggressiveness and poor patient prognosis (188, 198-200), presumably through enhanced nuclear localisation of numerous Impα1 recognised cargoes (such as transcription factors and proteins controlling cell cycle, invasion, apoptosis, proliferation and differentiation) that contribute

to tumour progression. Indeed, we showed increased nuclear localisation of cancer-associated endogenous proteins such as cyclin D1, c-myc and Rad50 is evident in malignant cells compared to non-transformed counterparts. Impα1 has been shown to promote oncogenic potential in both ovarian and luminal type breast carcinoma cells (194, 195), potentially through the upregulation of the transcriptional activity of c-myc targets, whilst Impα1 is implicated in inducing E2F1 transcription factor related proliferation/cell cycle progression in non-small cell lung carcinoma cells through regulating E2F1 nuclear import and expression of its downstream targets (30).

The fact that silencing of Impα1 expression is anti-proliferative in certain tumour cells such as luminal type breast carcinoma, CL1-5 non small cell lung cancer or epithelial ovarian carcinoma (30, 194, 195) but not in others (cervical, CL1-0 non small cell lung cancer (30) and MCF10 basal carcinoma breast (not shown) cells) indicates Impα1 may promote differential pathological functions in different types of cancers. Clearly, this may in part be related to the specialised cargoes that Impα1 is importing in the different cell types as well as the cargo abundance in these cells; the effect of Impα1 overexpression on cargo re-distribution of both Impα1 and β1 recognised factors in cancer needs to be determined and compared in different types of malignancies before the broader impacts of elevated Impα1 activity on tumour formation, maintenance and progression can be properly assessed. Intriguingly, this study suggests that increased Impα1 levels can, apart from enhancing Impα/β1-dependent import, decrease the nuclear localising ability of Impβ1-recognised cargoes in cells overexpressing it, such as malignant cells. Whether decreased efficiency in nuclear localisation of Impβ1-recognised factors is relevant to promoting tumorigenesis remains to be elucidated.

The fact that Impα/β1 dependent nuclear import activity is enhanced during breast tumour progression and is specifically associated with malignant transformation *in vitro* and *in vivo* has implications for anti-cancer approaches. The study suggests improved Impα1 activity could be exploited to target drugs into the nucleus of malignant cells more efficiently, than their non-transformed or benign/rapidly proliferating counterparts. This could be achieved through the use of an Impα1-recognised NLS in either viral (336) or non-viral multi-modular delivery systems (278), which contain modules to target other tumour enhanced pathways (such as enhanced EGFR expression) on the cell surface, that are loaded with toxic nuclear-acting genes (337) or drugs (313).

In summary, this study represents the first comprehensive analysis of changes to nuclear trafficking properties during tumour progression, demonstrating that Impα/β1-dependent import activity is selectively and progressively upregulated during tumour progression due to an increasing degree of Impα1 overexpression, and associated with a concomitant decrease in Impβ1-dependent import efficiency. The fact that overexpression of Impα1 is also observed in grade 2 and 3 compared to grade 1 invasive ductal carcinomas, correlating with the benign to malignant switch in the MCF10 model of ductal tumour progression, underlines the physiological importance and potential impact of the observations.

3.7 ACKNOWLEDGEMENTS

The authors would like to thank Katharine Adcroft for skilled technical assistance with this study. This work was supported by the National Health and Medical Research Council (Australia; grant APP1032143 and Senior Principal Research Fellowship 384109/APP1002486). All microscopic imaging was performed at the Monash Micro Imaging facility and flow cytometry conducted using the cytometers provided by the Flowcore facility (both located at Monash University, Clayton, VIC, Australia).

3.8 SUPPLEMENTARY METHODS

3.8.1 Immunofluorescence assays

0.4 x 10^5 MCF10A and MCF10CA1h cells were seeded onto coverslips in a 12 well tissue culture plate, then fixed and permebialised as per *Materials and Methods* and immunostained using monoclonal anti-hnRNP A1 (Sigma Aldrich), cyclin D1, c-myc (both from Santa Cruz Biotechnology) and Rad50 (Abcam) antibodies followed by the appropriate host FITC-conjugated IgG secondary antibodies (Molecular Probes, Life Technologies). The samples were mounted onto microscopic slides using Prolong Gold anti-fade mounting media containing DAPI (Molecular Probes) to counterstain nuclear DNA. Proteins were visualised using the TCS SP5 confocal microscope (Leica Microsystems; X60 oil immersion objective) and the Fn/c ratio calculated using the Image J software (NIH) as per *Materials and Methods*.

3.8.2 Cell proliferation assays

0.4 x 10^5 cells were seeded in triplicate wells onto a 12 well tissue culture plate and cell proliferation monitored over 6 days post-seeding. Adherent cells were detached by trypsinisation at 24 h intervals, centrifuged at 600*g*, and pellets resuspended in DMEM/F12 Ham's media without serum, containing 1 mM EDTA to prevent cell clumping prior to counting using a haemocytometer. Population doubling time was calculated at log growth phase using the formula: pdt = (0.693**t*)/LN(Xe/Xb) where *t* is the incubation time, and Xe and Xb represent the number of cells at the end and beginning of the incubation time, respectively.

3.8.3 Quantitative real-time RT-PCR

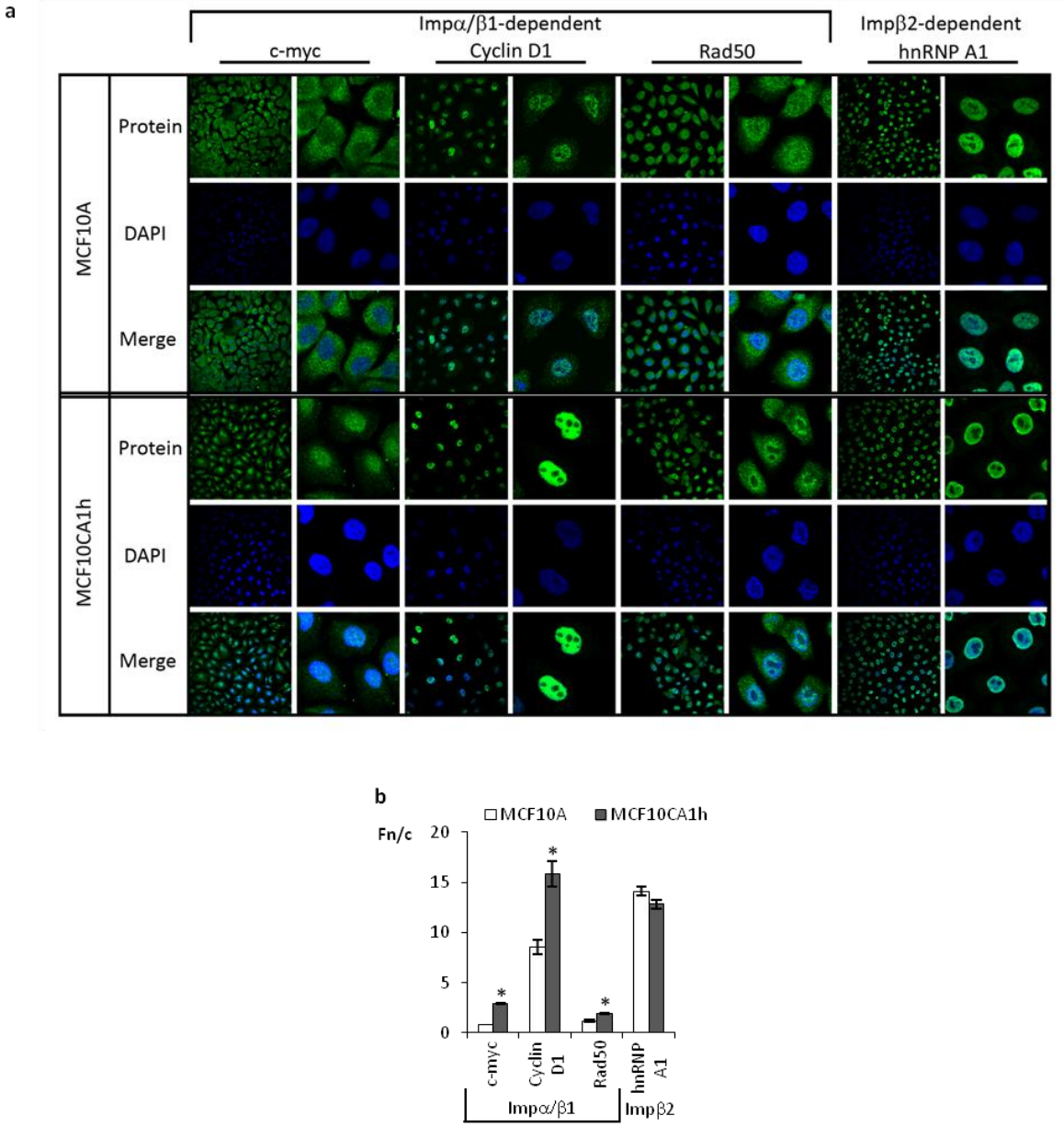
RNA was isolated from cells at 48 h post cell re-seeding using the RNeasy mini kit (Qiagen) according to manufacturer's instructions, DNAse-digested using RNAse-free DNAse (Ambion) and the purity and yield of the RNA was measured using the Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The RNA was reverse-transcribed by first strand cDNA synthesis using 500 ng of total RNA and Superscript III Reverse Transcriptase/random hexamer mix (Invitrogen), and 20 ng cDNA product was then amplified using the SensiMix SYBR Master Mix (Bioline, Alexandria, NSW, Australia) and 1 μM forward and reverse primer mix for: Impα1 (sense: 5'-ACCAAGGCTGTGGTAGATGG-3'; anti-sense: 5'-GAACTGCAAGGAGAGCCAAC-3'), Impα3 (sense: 5'-ACTGATGCTGGCAATGAACA-3'; anti-sense: 5'-GGGATGTGTCAGGAGTGCTG-3', detects both

transcript variants 1 and 2), Impβ1 (sense: 5'-AAGCCGCAGATTCTGTCAGT-3'; anti-sense: 5'-TTCCAAGCAGCTTTCCCTTA-3'), CAS (sense: 5'-AACAGAATGTCCCCCAATGA-3'; anti-sense: 5'-AAGTGACTGTGCCAGGTGAA-3', detects both transcript variants 1 and 2) and 18S (sense: 5'-TCCCCCAACTTCTTAGAGG-3'; anti-sense: 5'-CTTATGACCCGCACTTACTG-3'). The additional primers used for determination of the appropriate reference targets (GAPDH, YWHAZ, UBC, HPRT1 and ACTB; not shown) have all been previously described (338). Quantitative real-time RT-PCR was performed using the Applied Biosystems 7900HT Fast Real-Time PCR system (Applied Biosystems, Life Technologies, Mulgrave, Victoria, Australia)) for triplicate reactions. The comparative threshold cycle (C_t) method for relative quantification from a calibration curve ($r^2 \geq 0.99$, efficiency = 90-110%) was used for the calculation of mean amplicon quantity in non-transformed and transformed cells, and was normalised to the geometric means of both GAPDH and 18S internal reference controls as determined from a set of tested genes/products by the *geNorm* function on qBase (not shown) (338), where the *geNormM* value represents the average expression stability of each target, where a target with the lowest *M* value has the highest stability. The specificity of the primers was tested *in silico* using a BLASTn search and was further verified by gel electrophoresis, melt curve analysis and sequencing.

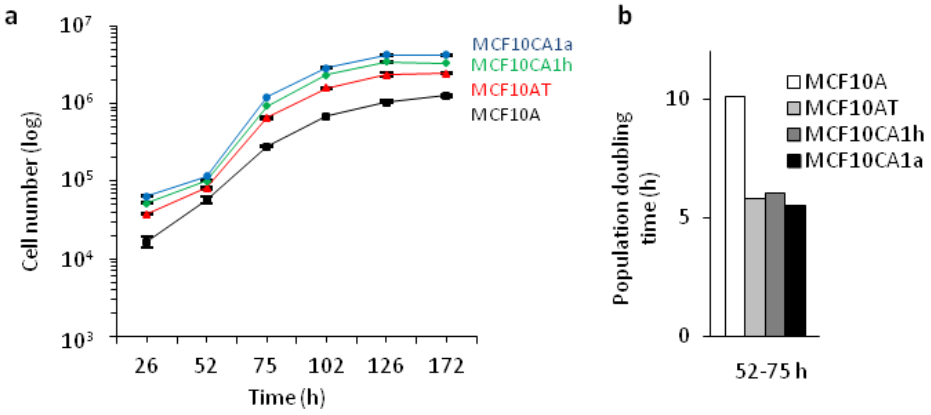
3.8.4 Fluorescence-Activated Cell Sorting

6×10^5 MCF10A cells were seeded onto 10 cm dishes and transfected to express pEGFP-T-ag (114-135) and pIRES-mCherry plasmids encoding Imps using Fugene HD (Promega) according to manufacturer's instructions. The cells were incubated for 41-48 h detached by trypsinisation and resuspended in FACS buffer (0.5% FCS, 2 mM EDTA and 10 mM HEPES in PBS, all from Invitrogen) and subjected to 2-way sorting into mCherry and non-mCherry-expressing populations using the BD Influx cell sorter (BD Biosciences). Cells were pelleted post sort at 600*g* and washed once in PBS at 4°C prior to lysis and Western/densitometric analysis.

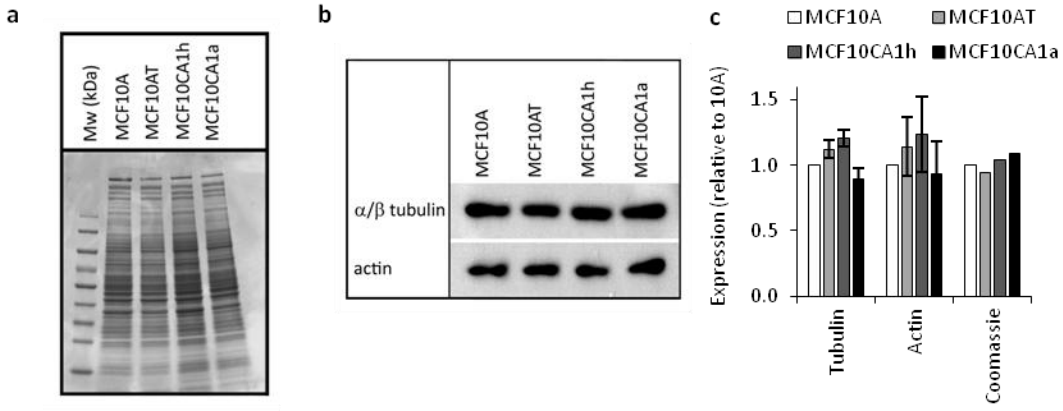
3.9 SUPPLEMENTARY FIGURES



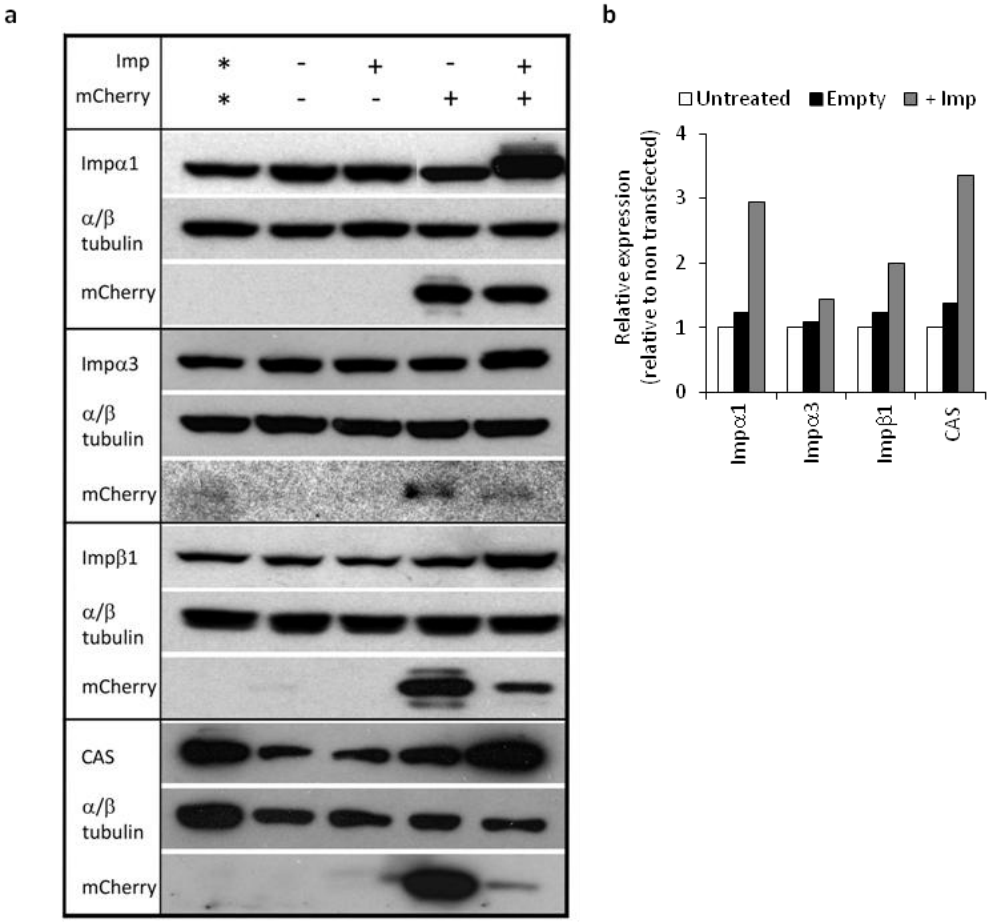
Supplementary Figure S3.1 Nuclear localisation of endogenous proteins dependent on Impα/β1-mediated trafficking is enhanced in malignant compared to non-transformed breast cells. (**a**) CLSM images of non-transformed MCF10A and malignant MCF10CA1h cells immunostained for the indicated proteins that depend on the Impα/β1 heterodimer for their nuclear localisation. The Impβ2-recognised mRNA binding protein hnRNP A1 was used as a control. (**b**) Digitised images such as those shown in (**a**) were analysed for the Fn/c ratio as per **Fig. 3.1b**. Results are given as mean +/- SE (n > 32), from a single experiment in a series of two experiments. * p < 0.0001 *vs.* MCF10A cells.



Supplementary Figure S3.2 Cell growth and population doubling times of the MCF10 cell series. (**a**) Cell growth in the MCF10 cell series was measured by way of counting Trypan blue excluding cells. Results are given as log cell number over 172 h (7 days) in 24 h increments. (**b**) Population doubling time (h) at log growth-phase (52-72 h). Results are from a single experiment in a series of two experiments.



Supplementary Figure S3.3 Verification of the loading control for Western blotting in the MCF10 cell series. (**a**) Total protein levels assessed by way of Coomassie Stain in the MCF10 cell series from a representative assay in two separate experiments. (**b**) Western and (**c**) densitometric analysis representing the mean +/- *SE* (n = 7) for expression of α/β tubulin or actin as per **Fig. 3.2b**, or total protein from (**a**).



Supplementary Figure S3.4 Ectopic expression of Imps/CAS in non-transformed MCF10A breast cells. Imp overexpressing and empty vector transfected cell populations were sorted by FACS at 41-48 h pt using a 2-way sort and levels of Impα1, α3, β1 and CAS expression determined by (**a**) Western and (**b**) densitometric analysis in mCherry expressing (+) and non-expressing (-) populations. * represents untreated cells whilst (–) Imp indicates empty vector-transfected samples. Densitometric analysis represent the intensity of the signal obtained for Imps normalised to that for α/β tubulin relative to empty vector transfected controls in mCherry expressing (+) populations and are from a single experiment in a series of two experiments.

3.10 SUPPLEMENTARY TABLE

Supplementary Table S3.1: Human breast tumour tissues used in the study.

Group	*Grade*	*N*	*ER/PR/Her2 status*
DCIS	High (nuclear)^	12	Mixed types
IDC	1 (BRE)* Well differentiated	15	+/+ / -
IDC	2 (BRE)* Moderately differentiated	19	+/+ / -
IDC	3 (BRE)* Poorly differentiated	17	- / - / -

^ Nuclear grade refers to the number of mitotic nuclei representing amount of proliferation.

* BRE is Elsten's modification of the Bloom & Richardson grading system (339). The BRE score is represented by the number of tubules (ducts), nuclear pleiomorphisms and mitotic nuclei present in a tissue sample as assessed by way of histology/staining, and is used as a predictor of invasion/metastatic spread.

Abbreviations: DCIS, ductal carcinoma *in situ*; ER, estrogen receptor; Her2, Erb-B2 growth factor receptor; IDC, invasive ductal carcinoma; PR, progesterone receptor.

CHAPTER 4

Hypersensitivity of malignant breast cancer cell types to knockdown of the nuclear transporter Importin β1

DECLARATION FOR THESIS CHAPTER 4

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
Contributed to the conception and design of the study. Performed and was involved in all aspects of the experiments and prepared the manuscript for publication.	95%

The following co-authors contributed to the work:

Name	**Nature of contribution**
David A. Jans	Contributed to the conception and design of the study. Critical review of the manuscript.

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date**

Main Supervisor's Signature		**Date**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

4 Hypersensitivity of malignant breast cancer cell types to knockdown of the nuclear transporter Importin β1

4.1 PREFACE

Chapter 4 of this thesis is composed of a manuscript prepared for submission in *International Journal of Cancer* entitled "Hypersensitivity of malignant breast cancer cell types to knockdown of the nuclear transporter Importin β1".

4.2 ABSTRACT

We and others have previously reported that Impβ1, a member of the Importin family of nuclear transporters, is overexpressed in a number of tumour cell lines and in patient tumour tissues. Here we show for the first time that the degree of Impβ1 overexpression correlates with disease progression state in the MCF10 human breast tumour progression system. Excitingly, we find that targeting Impβ1 activity through RNAi is up to 33-fold more efficient in decreasing the viability of malignant ductal carcinoma cells compared to isogenic non-transformed counterparts, and is highly potent with tumour selective activity at subnanomolar siRNA concentrations. Flow cytometric analysis in malignant cells treated with Impβ1 siRNAs implied this effect was due to increased cell death. Importantly, the tumour selective killing activity was found to be specific to Impβ1 siRNA, and was not observed for siRNAs targeting other Imps. Our study raises the exciting possibility of anti-cancer therapies targeted specifically at Impβ1.

4.3 INTRODUCTION

Nuclear transport mediated by the Importin (Imp) superfamily of transport receptors is central to eukaryotic cell function, with regulated nuclear import and export of signalling molecules integral to processes such as transcription, translation, cell cycle progression and apoptosis. Imp expression is known to be altered in various types of cancers (such as cervical, breast, ovarian and lung (17)), but that this may impact on nuclear transport efficiency has only been demonstrated recently (318). Importin β1 (Impβ1) is an important member of the Importin family of nuclear

transporters that is highly expressed in transformed cells such as in Simian Virus 40 (SV40) large T-antigen-mediated transformation, Human Papillomavirus-16 (HPV-16) E6/E7-transformed epithelial cells and in gastric, bladder, breast and cervical cancer cell lines (197, 216, 217, 318). Highly conserved in mammals, and ubiquitously expressed in human tissues and cells, Impβ1 is critical for early embryonic development in the mouse, worm and fly (340, 341) because of key roles in both interphase and mitosis (342). Through its ability to mediate interaction of transport complexes with the nucleoporin (Nup) proteins of the nuclear pore complex (NPC), Impβ1 mediates nuclear import during interphase of a range of different proteins, including of cargoes bound directly to Impβ1 such as the signalling molecule parathyroid hormone-related protein PTHrP (56), cyclin B1 (12), chromatin remodelling factors such as SOX9 (82) and SRY (81) and basic loop helix factors such as activator protein-1 AP-1 and cAMP-response element-binding protein CREB (55). Impβ1 can also mediate nuclear translocation of cargoes through the action of Impα adapters that directly recognise other cargoes, such as the tumour suppressor proteins pRb (325, 343) and p53 (326), inducible transcription factors such as the signal transducers and activators of transcription (STATs) (344) and NF-κB family members (45). Once in the nucleus, dissociation of the transport complexes is effected by binding to Impβ1 of the guanine binding protein Ran in activated GTP-bound form.

Impβ1 also plays a role after nuclear membrane breakdown during entry into mitosis, where either it alone or together with Impα regulates static spindle formation in a Ran-dependent manner by mediating the delivery of spindle assembly factors (such as the nuclear mitotic apparatus NuMA and microtubule associated TPX2 proteins) to spindle poles (274), and has indirect effects on dynamic microtubule attachment at kinetochores (169). Impβ1 is critical to the formation of the NPC/nuclear envelope architecture during telophase through its ability to recruit Nups and other nuclear envelope components (166).

In this study we use an isogenic breast tumour progression model to show for the first time that treatment of cells with Impβ1 siRNA is up to 33-fold more efficient in killing benign and malignant basal type ductal breast cells compared to non-transformed cell counterparts, with potent tumour-selective activity even at subnanomolar doses. Silencing RNA to other Imps did not show this effect. Impβ1 looms as an exciting target for selective and potent tumour cell killing approaches.

4.4 MATERIALS AND METHODS

4.4.1.1 Mammalian cell culture and siRNA treatment

The MCF10 breast tumour progression series was obtained from Prof. Fred Miller/Dr. Steven Santner at the Karmanos Cancer Institute, Detroit, USA. It comprises the non-transformed MCF10A ductal breast epithelial cell line, its *Ha-Ras* ($G^{12}V$ mutated) transformed benign counterpart MCF10AT, and two fully malignant counterparts, the non-metastatic MCF10CA1h and metastatic MCF10CA1a cell lines generated by serial trocar implantation (passaging) in mice (289). In the case of the 1BR3/1BR3.N human skin fibroblast (287) cell pair, the transformed derivative was generated by transfection with a plasmid containing SV40 genomic sequences for the early region comprising of the transforming small and large T-antigen products.

MCF10A (passage < 75), MCF10AT (passage < 71), MCF10CA1h (passage < 60) and MCF10CA1a (passage < 105) cells were cultured in DMEM/F12 Ham's media supplemented with 5% horse serum (Invitrogen, Carlsbad, CA, USA), 1 mM sodium pyruvate, 10 mM HEPES, 2 mM L-glutamine, 0.5 μg/ml hydrocortisone, 10 μg/ml bovine insulin, 20 ng/ml human recombinant EGF and 100 ng/ml cholera toxin (all from Sigma-Aldrich, St. Louis, MO, USA), as previously, whilst 1BR3 and 1BR3.N cells (both at passages < 18) were cultured in DMEM supplemented with 15% FCS and 2 mM L-glutamine (Invitrogen), as previously (318).

For Impβ1 titration assays, comparative Imp silencing efficiency assays and high dose siRNA assays in the MCF10 cell system, 2.3 x 10^5 cells were seeded into 6 cm dishes, treated 24 h later with ONTARGETplus SMARTPool siRNAs (Dharmacon, GE Healthcare, Little Chalfont, UK) specific for Impα1, Impα3, Exp-1, CAS, Impβ1 or non-targeting control siRNA, where appropriate, at the indicated doses using RiboCellIn siRNA transfection reagent (BioCellChallenge, Toulon-Cedex, France) according to manufacturer's instructions, and incubated for 48 h before re-seeding onto 96- (3.3 x 10^3 cells) and 6- (1.0 x 10^5 cells) well culture plates for XTT and protein analysis respectively. 24 h later, cells were re-treated with another dose of siRNA, and incubated for the indicated time points prior to Western and/or XTT analysis.

4.4.1.2 Quantitative real-time reverse transcription-PCR

RNA was isolated from cells at 48 h post cell re-seeding using the RNeasy mini kit (Qiagen, Venlo, Limburg, Netherlands) according to the manufacturer's instructions, prior to DNAse digestion using RNAase-free DNAse (Ambion) and reverse-transcription by first strand cDNA synthesis using 500 ng of total RNA and Superscript III Reverse Transcriptase/random hexamers (Invitrogen). 20 ng cDNA was then amplified using the SensiMix SYBR Master Mix (Bioline, Alexandria, NSW, Australia) and 1 μM Impβ1 forward and reverse primer mix (sense: 5'-AAGCCGCAGATTCTGTCAGT-3'; anti-sense: 5'-TTCCAAGCAGCTTTCCCTTA-3'). Quantitative RT-PCR was performed using the Applied Biosystems 7900HT Fast Real-Time PCR system (Applied Biosystems, Life Technologies, Mulgrave, Victoria, Australia) for triplicate reactions. The comparative threshold cycle (C_T) method was used for the calculation of expression fold-change between normal and transformed cells, and normalised to GAPDH (sense: 5'-TGCACCACCAACTGCTTAGC-3'; anti-sense: 5'-GGCATGGACTGTGGTCATGAG-3') and 18S (sense: 5'-TCCCCCAACTTCTTAGAGG-3'; anti-sense: 5'-CTTATGACCCGCACTTACTG-3') internal reference targets as determined from a set of tested genes by the *geNorm* function on qBase (not shown) (338).

4.4.1.3 Preparation of cell extracts and Western blotting

Cells were washed twice with ice-cold PBS prior to incubation in ice-cold RIPA lysis buffer (50 mM Tris HCl pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS and 1 mM EDTA) with fresh 5 x EDTA-free complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA), scraped and lysed at 4°C for 30 min and centrifuged at 10,000*g* for 30 min at 4°C to pellet insoluble material. The total protein concentration in each extract was estimated using the Bradford Dye Reagent (Bio-RAD, Hercules, CA, USA). 20 μg of protein from each cell extract was then separated by SDS-PAGE (10% reducing gel), transferred onto nitrocellulose membrane (PALL Corporation, Port Washington, NY, USA) and probed with anti-Impα1, Impβ1, Exp-1, CAS monoclonal (all from BD Biosciences, San Jose, CA, USA) or anti-Impα3 polyclonal (Abcam, Cambridge, MA, USA) primary antibodies, followed by the appropriate host IgG-HRP secondary antibody (Chemicon, Temecula, CA, USA) according to the manufacturer's recommendations, and protein visualised using the Western Chemiluminescence Reagent (from Perkin-Elmer, Wellesley, MA, USA or Millipore, Bedford, MA, USA). The membranes were then stripped of antibody using Western strip buffer (25 mM glycine, 1% SDS, pH 2), blocked and re-probed with anti-α/β tubulin

(Cell Signaling, Danvers, MA, USA) antibody followed by the appropriate host-IgG-HRP secondary antibodies and visualised as previously. The intensity of the resulting bands for all proteins was estimated by densitometry using an Alpha Imager (Alpha Innotec, Santa Clara, CA, USA) for image capture and the 1D electrophoresis gel analysis module from Image Quant TL software (GE Healthcare, Little Chalfont, Buckinghamshire, UK); results are expressed in terms of the ratio of the signal for Imp or Exp relative to that for α/β tubulin in transformed cells, relative to the respective value for the non-transformed cell line of the isogenic cell pair/set.

4.4.1.4 XTT assays

The effect of Impβ1 siRNA on cell proliferation was determined using the XTT assay as per the manufacturer's instructions. Briefly, siRNA-treated cell samples were re-seeded onto 96-well assay plates, re-treated with siRNA and washed at the indicated time points, and the XTT/PMS reagents (Sigma-Aldrich) incubated in phenol-free DMEM/F12 Ham's media for 6 h before reading the change in absorbance at 690 and 450nm using the FluoSTAR Optima plate reader (BMG LabTech). Specific absorbance was calculated by the following equation: (OD_{450} (sample) –OD_{450} (blank))-(OD_{690} (sample) - OD_{690} (blank)) and given as the mean (+/- SD) absorbance calculated from 5 repeat wells/sample. The mean specific absorbance was normalised at each time point to that of the non-targeting siRNA-treated control in each cell line, and the value used as an indicator of cell viability.

4.4.1.5 Statistical significance

The significance ($p < 0.05$) of differences in results between transformed and non-transformed cells was determined using the Student's (or Welch corrected) *t*-test for unpaired data (2-tailed p value), as appropriate using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA).

4.5 RESULTS

4.5.1.1 Impβ1 overexpression in malignant breast cells

Real-time RT-qPCR and Western analysis was applied to the MCF10 tumour progression model of invasive ductal carcinoma, which comprises the non-transformed ductal epithelial cell line MCF10A, its mutant ($G^{12}V$) *Ha-Ras*-transfected benign counterpart MCF10AT and two fully malignant counterparts, the non-metastatic MCF10CA1h and metastatic MCF10CA1a lines generated from serial trocar implantation of MCF10AT cells in mice (289). Both malignant lines showed significantly ($p < 0.05$) higher expression of Impβ1 at both the transcript (**Fig. 4.1a**) and protein (**Fig. 4.1b**) levels compared to non-transformed and benign counterparts, with the degree of overexpression correlating with the tumour progression state of the cells.

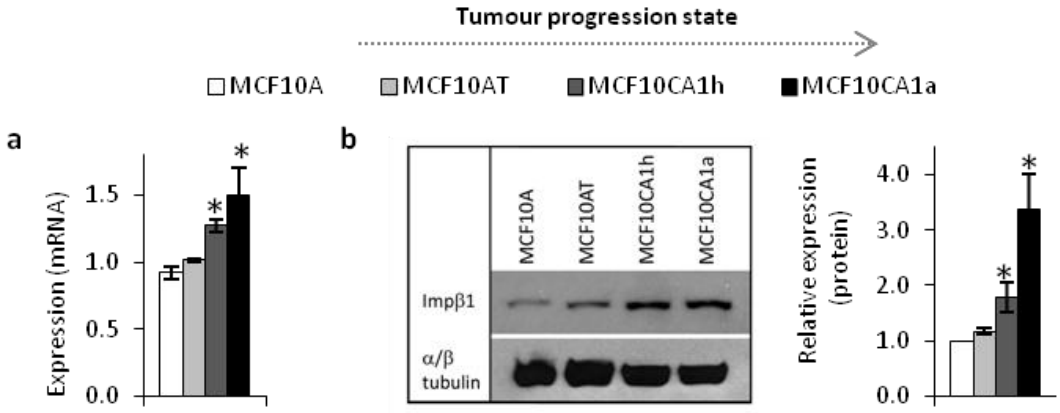


Figure 4.1 Elevated expression of Impβ1 in malignant breast cancer cells. (**a**) Levels for Impβ1 mRNA in the MCF10 tumour progression series as detected by real-time qRT-PCR. Results represent the mean +/- *SE* (n = 4), for expression normalised to the geometric mean of GAPDH and 18S internal reference targets in transformed (grey or black bars) and non-transformed cells (white bar). * $p < 0.05$ *vs.* non-transformed cells. (**b**) Western (left) and densitometric (right) analysis of Impβ1 levels in the MCF10 cell series. Results represent the mean +/- *SE* (n = 6) for the ratio of the intensity of the signal obtained for Impβ1 normalised to that for α/β tubulin control protein in transformed, relative to non-transformed cells. * $p < 0.05$ *vs.* MCF10A cells.

4.5.1.2 Impβ1 siRNA inhibits the proliferation of transformed but not non-transformed breast cell types

To test the effects of Impβ1 knockdown on cell proliferation and viability, we transiently treated the cells of the MCF10 series with 10 nM siRNA specific to Impβ1, followed by detachment at 48 h post treatment, and retreatment with siRNAs, prior to assessment of cell proliferation using the XTT assay (**Fig. 4.2a**) for up to 6 days post retreatment; direct cell counts (**Supp. Fig. S4.1**) yielded very similar results.

Although treatment with Impβ1 siRNA decreased the viability of both transformed and non-transformed cells compared to untreated and non-targeting siRNA-treated controls (**Fig. 4.2a**), this effect was significantly ($p < 0.05$) more pronounced (c. 6-fold) in the transformed/malignant cell types compared to non-transformed counterparts (**Fig. 4.2b**). Strikingly, low doses (0.5 nM) of siRNA failed to affect the viability of non-transformed cells, but decreased tumour cell viability by 50% relative to non-targeting siRNA-treated controls (**Fig. 4.2c**). Dose-response experiments as per **Fig. 4.2a** revealed that while nM doses of Impβ1 siRNA decreased the number of viable cells for both non-transformed and malignant lines, doses at, or below 1 nM resulted in selective effects on malignant but not non-transformed cells (**Fig. 4.2d**), with c. 33-fold higher siRNA potency in tumour compared to non-transformed cells (estimated absolute LD_{50} of 0.15 and 5 nM, respectively).

To confirm the findings in the MCF10 cell system, we used an additional isogenic transformed cell system; the primary human fibroblast cell line 1BR3 together with its SV40 large T-antigen-transformed counterpart 1BR3.N. Cells were treated with 1 and 10 nM Impβ1 siRNAs and viability measured at day 4 (3 days post siRNA re-treatment) using the XTT assay. Consistent with the findings above, 1 nM Impβ1 siRNA decreased viability of the transformed 1BR3.N, but not primary 1BR3 cells (**Supp. Fig. S4.2a**); Western/densitometric analysis confirming efficient silencing of Impβ1 expression in both primary and transformed cells (**Supp. Fig. S4.2bc**).

Testing the silencing efficiency of increasing concentrations of Impβ1 siRNA in the MCF10 cell series (**Fig. 4.3a**) revealed that Impβ1 silencing was c. 20-fold more efficient in malignant MCF10CA1h cells than non-transformed MCF10A cells at day 3 (72 h, log growth-phase; **Fig. 4.3b**, absolute IC_{50} of 0.066 compared to 1.3 nM, respectively), with greater silencing efficiency observed also in benign

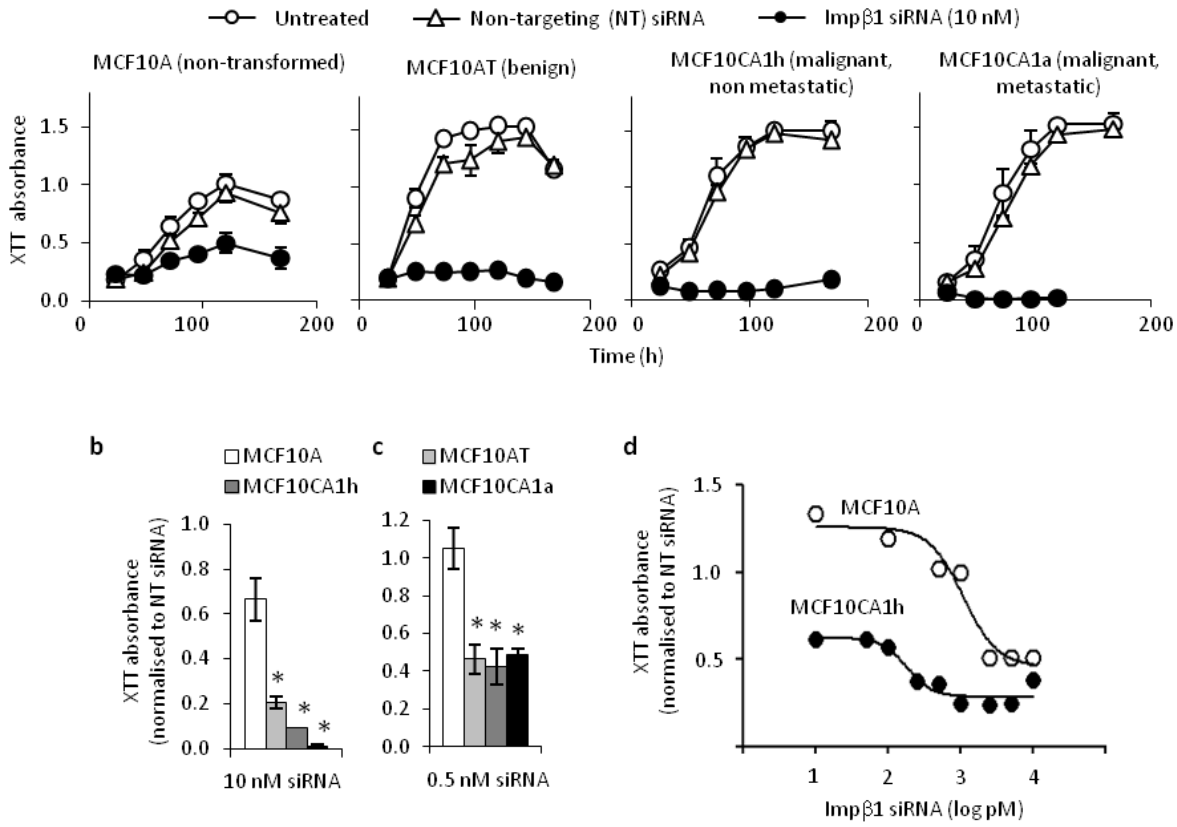


Figure 4.2 Transformed but not non-transformed breast cell types are hypersensitive to Impβ1 knockdown. (**a**) XTT assays for cells of the MCF10 cell series treated twice with 10 nM Impβ1 or non-targeting (NT) siRNA, or for untreated (UT) controls at the indicated time points after replating. Results for XTT absorbance are for the mean +/- *SD* from a single representative experiment in a series of three experiments. (**b**) Results for cell viability measured on day 3 (72 h) from (**a**) in non-transformed (white bars) and transformed cells (grey/black bars) and (**c**) in cells treated with 0.5 nM siRNA. Results represent the ratio of the mean +/- *SD* absorbance relative to NT siRNA-treated cells. * $p < 0.0001$ *vs.* MCF10A cells. (**d**) LD_{50} curves on day 3 (72 h) for non-transformed (white spheres) and malignant (black spheres) cells treated twice with the indicated concentrations of Impβ1 siRNA. Results are from a single typical experiment in a set of two independent experiments.

MCF10AT and malignant MCF10CA1a lines (**Fig. 4.3cd**). Efficiency of uptake of labelled siRNAs appeared to be identical in all of the lines of the MCF10 series (**Supp. Fig. S4.3**). To investigate whether the decreased viability in Impβ1 siRNA-treated cells may be due to a block in cell cycle progression and/or induction of cell death, we performed propridium iodide staining of malignant MCF10CA1h cells treated twice with 10 nM Impβ1 siRNA on day 4 (96 h) followed by flow cytometry

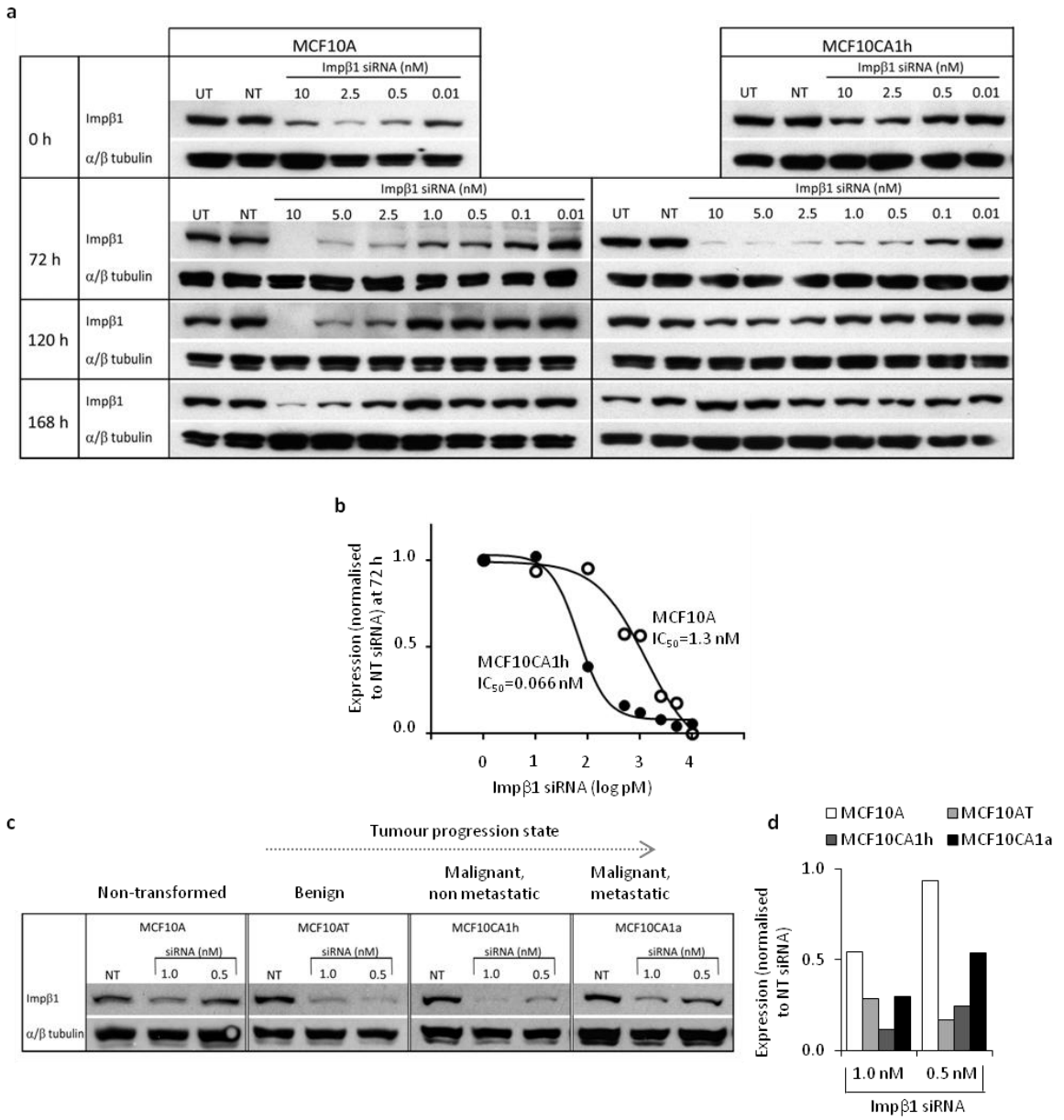
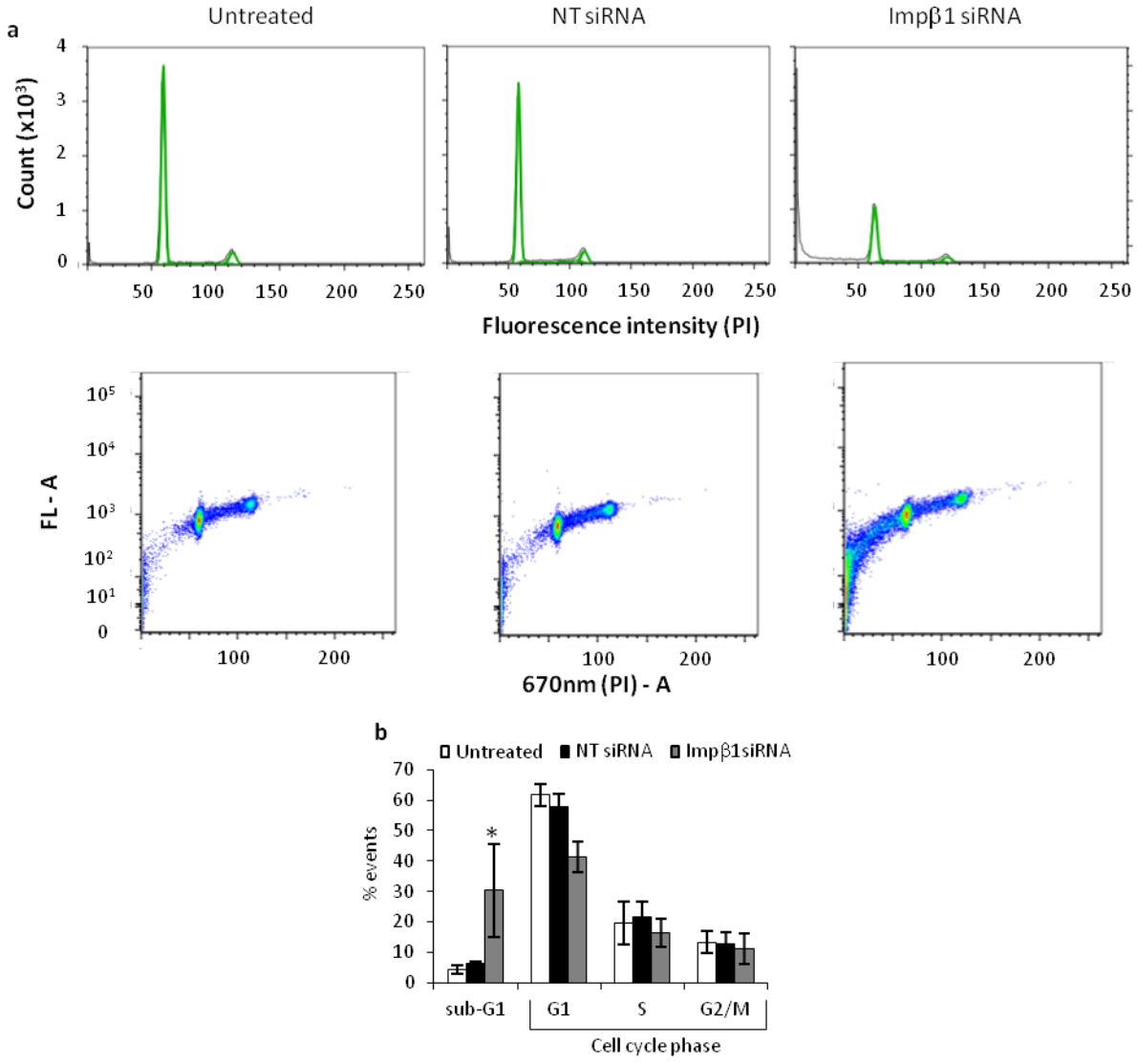


Figure 4.3 Increased silencing of Impβ1 in transformed breast cells compared to isogenic non-transformed cells. Analysis of Impβ1 expression in the MCF10 series treated with Impβ1 siRNA. (**a**) Results for Western analysis for Impβ1 expression in non-transformed MCF10A (left panel) and malignant MCF10CA1h (right panel) cells treated twice with increasing concentrations of Impβ1 siRNA or a non-targeting (NT) siRNA, and untreated (UT) controls at the indicated time points post cell replating (compare to **Fig. 4.2a** for corresponding XTT time course values). Results are from a single typical experiment in a set of two independent experiments. (**b**) Densitometric analysis from images such as those shown in (**a**). Results represent the mean for the level of protein expression in Impβ1 siRNA-treated samples, normalised to NT siRNA-treated controls at day 3 (72 h) in non-transformed (white spheres) and malignant (black spheres) cells. The IC_{50} is given for both cell types. (**c**) Western and (**d**) densitometric analysis for effect of 0.5 or 1 nM Impβ1 siRNA on Impβ1 levels in the MCF10 cell series. Results are from a single typical experiment in a set of two independent experiments.

and DNA content analysis (**Fig. 4.4a**). Impβ1 siRNA significantly (p = 0.0433) increased the sub-G1 population compared to untreated and non-targeting siRNA-treated controls, with a corresponding but non-significant decrease in the G1 phase population (**Fig. 4.4b**). No significant differences were observed in the G2/M and S phase populations compared to non-targeting siRNA-treated controls. These data indicate Impβ1 siRNA's anti-proliferative effect occurs through the induction of cell death in malignant cells.

Overall, the results imply that low doses of Impβ1 siRNA selectively decrease viability of different transformed and malignant cell types but not primary/non-transformed counterparts.



4.5.1.3 Tumour-selective targeting activity is specific to Impβ1 siRNA

To test whether the tumour selectivity of Impβ1 siRNA induced killing activity reflected a general hypersensitivity of tumour cells to knockdown/inhibition of components of the cellular nuclear transport machinery, we treated non-transformed MCF10A and malignant MCF10CA1h cells twice with siRNAs targeting Impα1, α3, β1, CAS (Cse1l) or Exp-1 (Crm-1) at 1 or 10 nM as required to induce efficient gene silencing, and monitored viability (**Fig. 4.5a**). Although silencing of Exp-1 and to some extent CAS resulted in significantly decreased viability ($p < 0.05$) compared to non-targeting siRNA-treated controls, none of the Imp/Exp siRNAs other than that for Impβ1 ($p < 0.05$) affected the malignant cells selectively (**Fig. 4.5a**). Impβ1 was efficiently silenced in malignant (c. 50%) but not in MCF10A cells (0%) as expected (1 nM siRNA), whilst all of the other Imp and Exp proteins showed similar silencing efficiency across both cell types (**Fig. 4.5b**). Based on these data, malignant cells appear to be uniquely hypersensitive to knockdown of Impβ1, and not affected specifically by siRNA to other Imps/Exps.

4.6 DISCUSSION

We show here for the first time that Impβ1 siRNA is highly potent in inhibiting the proliferation of basal type tumour cells arising from ductal epithelia and transformed primary cells, but not their non-transformed isogenic counterparts, although at higher (≥ 10 nM) doses we observed partial decreases in viability even in normal/non-transformed cell types. Intriguingly, this hypersensitivity is evident even though malignant cells express higher levels of Impβ1 transcript/protein (197, 216, 217, 318).

Figure 4.4 Silencing of Impβ1 induces cell death. (**a**) Flow cytometric analysis of the DNA content in malignant MCF10CA1h cells treated twice with 10 nM Impβ1 or non-targeting (NT) siRNAs at day 3 from a single representative assay in a series of three experiments. (**b**) Cell cycle analysis of samples such as those shown in (**a**). Results are for the % of total events in sub-G1/the indicated cell cycle phases, representing the mean +/- SE (n = 3). * $p < 0.05$ *vs.* NT siRNA-treated cells.

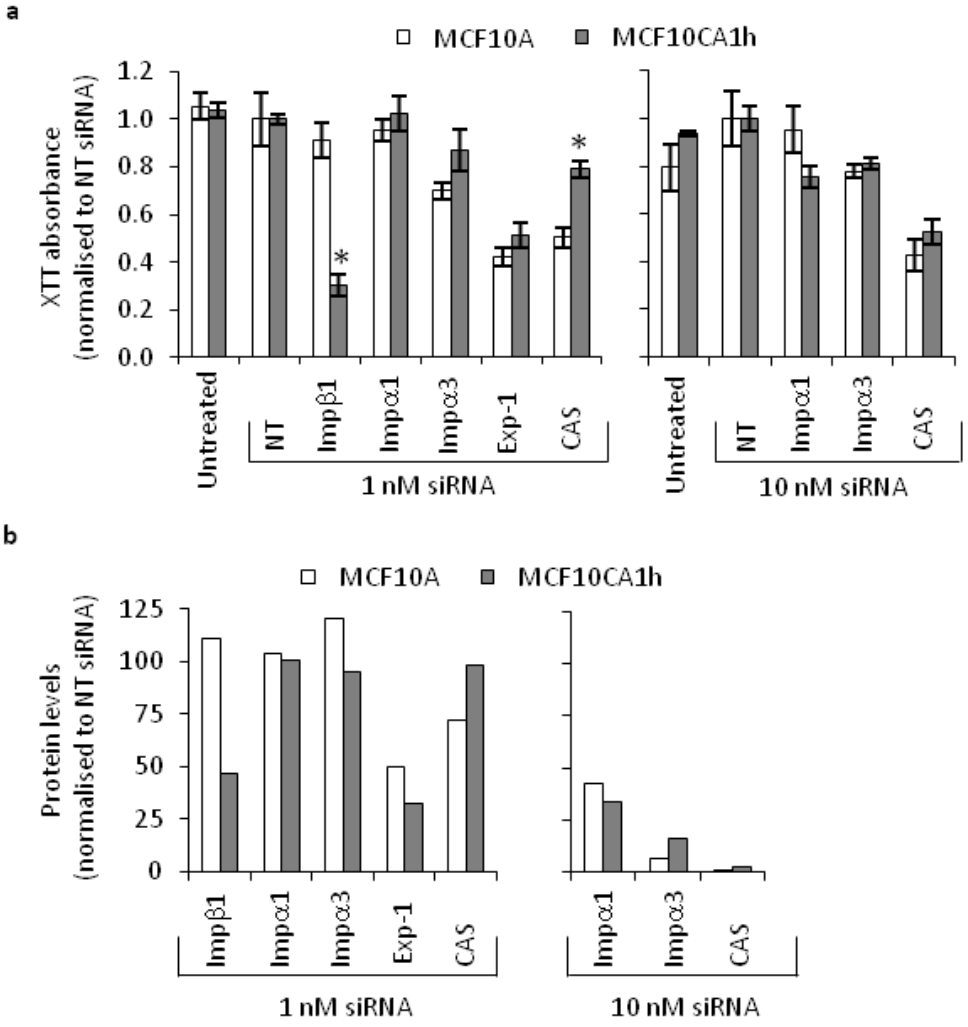


Figure 4.5 Tumour enhanced silencing and cell killing activity is specific to Impβ1 siRNA. (**a**) Proliferation of non-transformed MCF10A and malignant MCF10CA1h cells treated twice with 1 (left) or 10 (right) nM siRNA for the indicated Imp/Exp or non-targeting (NT) control was measured using the XTT assay on day 3 as per **Fig. 4.2b**. * $p < 0.05$ *vs.* MCF10A cells. (**b**) Western (not shown)/densitometric analysis of Imp expression in samples from (**a**) in cells treated with 1 (left) or 10 (right) nM siRNAs as per **Fig. 4.3c**. Results are from a single representative experiment in a series of ≥ 4 separate experiments.

Impβ1 plays a central role in nuclear trafficking of important signalling molecules such as transcription factors through action alone, or as a heterodimer with adaptors such as Impα, snurportin (75) and even Imp7. It is also a key driver of mitotic exit and G1 phase progression. The precise mechanism of tumour cell hypersensitivity to reduced levels of Impβ1 is not clear, but presumably relates to these key cellular roles of Impβ1 as opposed to those of other Imps/Exps, and the fact that faster growing tumour cells, although expressing higher levels of Impβ1, are more dependent on ("addicted to") Impβ1 than non-transformed cells. Comparable tumour cell "addiction" has been observed for other cellular factors, oncogenic or otherwise, such as cyclin D1 (345), c-myc (346), DNA damage response kinases ATM and CHK1 (347), and the reactive oxygen species (ROS) inhibiting pyruvate dehydrogenase kinase (347), whereby targeting these factors by RNAi or small molecular inhibitors can result in selective cancer cell death. As for these factors, Impβ1 levels may be critical to support oncogenic tumour promoting functions driving cell cycle progression and proliferation in transformed cells, whilst being less critical in non-transformed cell types. The increased sensitivity to Impβ1 knockdown of the tumour cells at low siRNA concentrations is not clear, especially as the breast tumour cell types in this study replicate faster than normal MCF10A cells (not shown) which can result in the dilution of siRNA, and have a higher abundance of Impβ1 transcript and protein in untreated cells, both of which can negatively impact RNAi efficiency (348, 349). Significantly, this is clearly not a generalised mechanism, as we found silencing of other Imp and Exp proteins was similar in MCF10CA1h tumour compared to non-transformed cells thus improved silencing may relate to inherent differences in target recognition/individual siRNA potencies etc.

Targeting the components of the nuclear transport system through inhibition of nuclear transporter activity is already underway in preclinical and clinical trials for SINEs (selective inhibitors of nuclear export), which are small molecular inhibitors that target the activity of the Impβ1-homologue Exp-1 (Crm-1) (260), although the realistic utility of these novel leptomycin B (95) like molecules in a clinical context remains to be established, predominantly of their high toxicity (259). Our studies in transformed breast and skin cells indicate that targeting of Impβ1-dependent activity using siRNA is highly toxic to tumour but not non-transformed cell types, which raises the question of whether Impβ1 siRNAs may prove useful in other cancer cell and animal models, and whether targeting of Impβ1 may also be used in therapy. Indeed, inhibition of Impβ1 activity by way of RNAi is a potent

killer of lung carcinoma, head and neck carcinoma cells (350) and cervical cancer lines (40, 197). Impβ1 has been suggested as a novel candidate for next generation mitotic exit inhibitors for cancer therapy (351), presumably through effects on mitotic exit. Whether small molecule inhibitors targeting Impβ1 transport activity may have comparable effects to siRNAs remains to be established in the future.

4.7 CONCLUSION

In conclusion, this is the first comprehensive demonstration of hypersensitivity of isogenic tumour compared to normal cell types to Impβ1 inhibition in a disease progression model of human basal type breast carcinoma. Future work in this laboratory is aimed at pursuing the observations here to assess their potential application in tumour selective therapies.

4.8 ACKNOWLEDGEMENTS

The authors would like to thank Katharine Adcroft for skilled technical assistance with this study. This work was supported by the National Health and Medical Research Council (Australia; grant APP1032143 and Senior Principal Research Fellowship 384109/APP1002486). Microscopic imaging was performed at the Monash Micro Imaging Facility and flow cytometry conducted using the cytometers provided by the Flowcore Facility (both located at Monash University, Clayton, VIC, Australia).

4.9 SUPPLEMENTARY METHODS

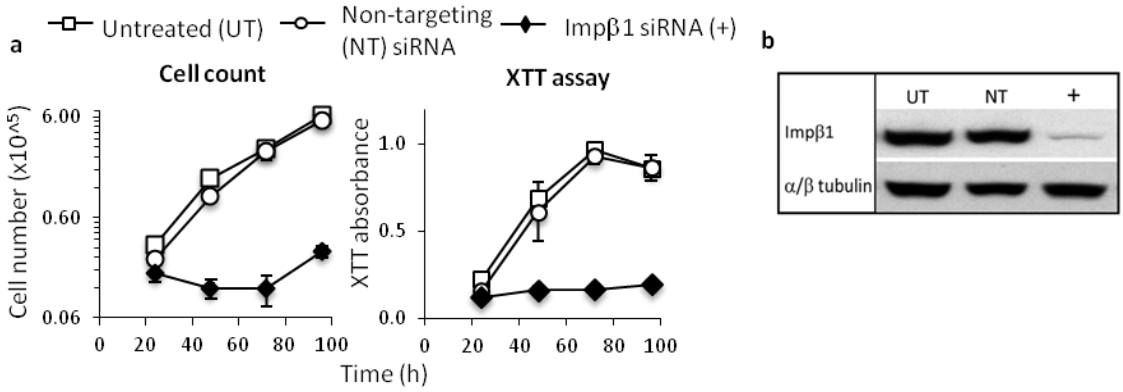
4.9.1 Cell proliferation assays

MCF10CA1h cells were treated once with siRNAs and 48 h later 0.4 x 10^5 cells were replated onto 12 well tissue culture plates in triplicate wells/sample and retreated 24 h later using 10 nM non-targeting or Impβ1 siRNAs. At 24 h intervals, the cells were detached by trypsinisation, and counted using a haemocytometer for a total of four days.

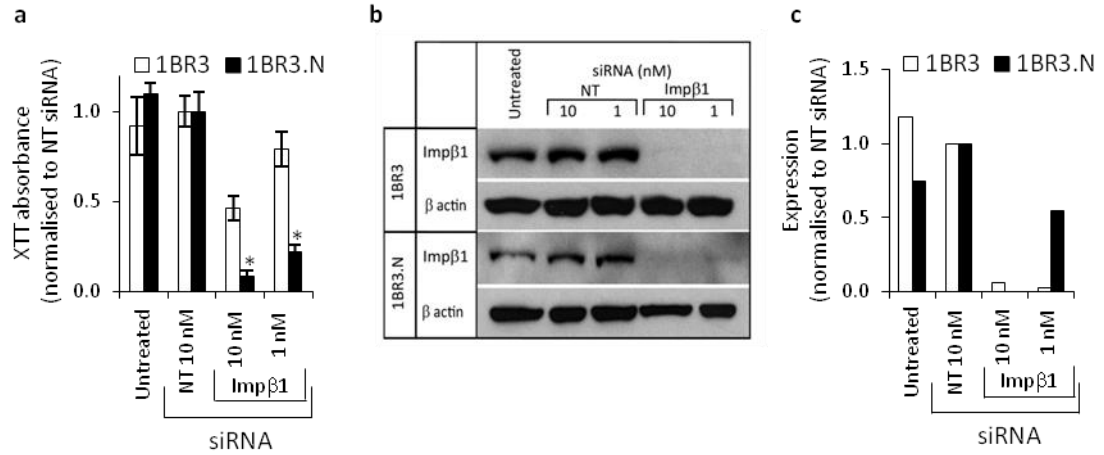
4.9.2 Flow cytometric analysis and siRNA uptake assay

For analysis of the effect of the siRNAs on cell death and the cell cycle, MCF10CA1h cells were plated at 2.3 x 10^5 cells in 6 cm dishes, treated with 10 nM Impβ1 siRNA and 48 h later 6 x 10^5 cells re-seeded onto 10 cm dishes. Cells were re-treated with siRNA and fixed in 80% ethanol 3 days post-treatment. Subsequent to fixing and RNase A treatment, cells were stained using propidium iodide to indicate the DNA content and the cell cycle/death profiles were analysed using the LSR II flow cytometer (BD Biosciences). Quantification of the percentage of singlet cells at different stages of the cell cycle was performed using FlowJo software (TreeStar Inc., Ashland, OR, USA) and Dean Jett Fox analysis. For analysis of siRNA uptake in MCF10 cell panel, 2.3 x 10^5 cells were seeded onto 6 cm dishes and transfected with 1 or 10 nM Cy3-labelled ONTARGETplus SMARTPool siRNAs (Dharmacon) using RiboCellIn siRNA transfection reagent (BioCellChallenge) according to the manufacturer's instructions, and incubated for 24 h before subjecting the cells to flow cytometric analysis using a BD Biosciences LSR II cytometer (Flowcore, Monash University, Clayton, Victoria, Australia). Silencing RNA localisation was analysed in parallel by fluorescence and bright field microscopy using 10x and 40x objectives (Olympus IX71 invert microscope, dry lens; Olympus, Tokyo, Japan).

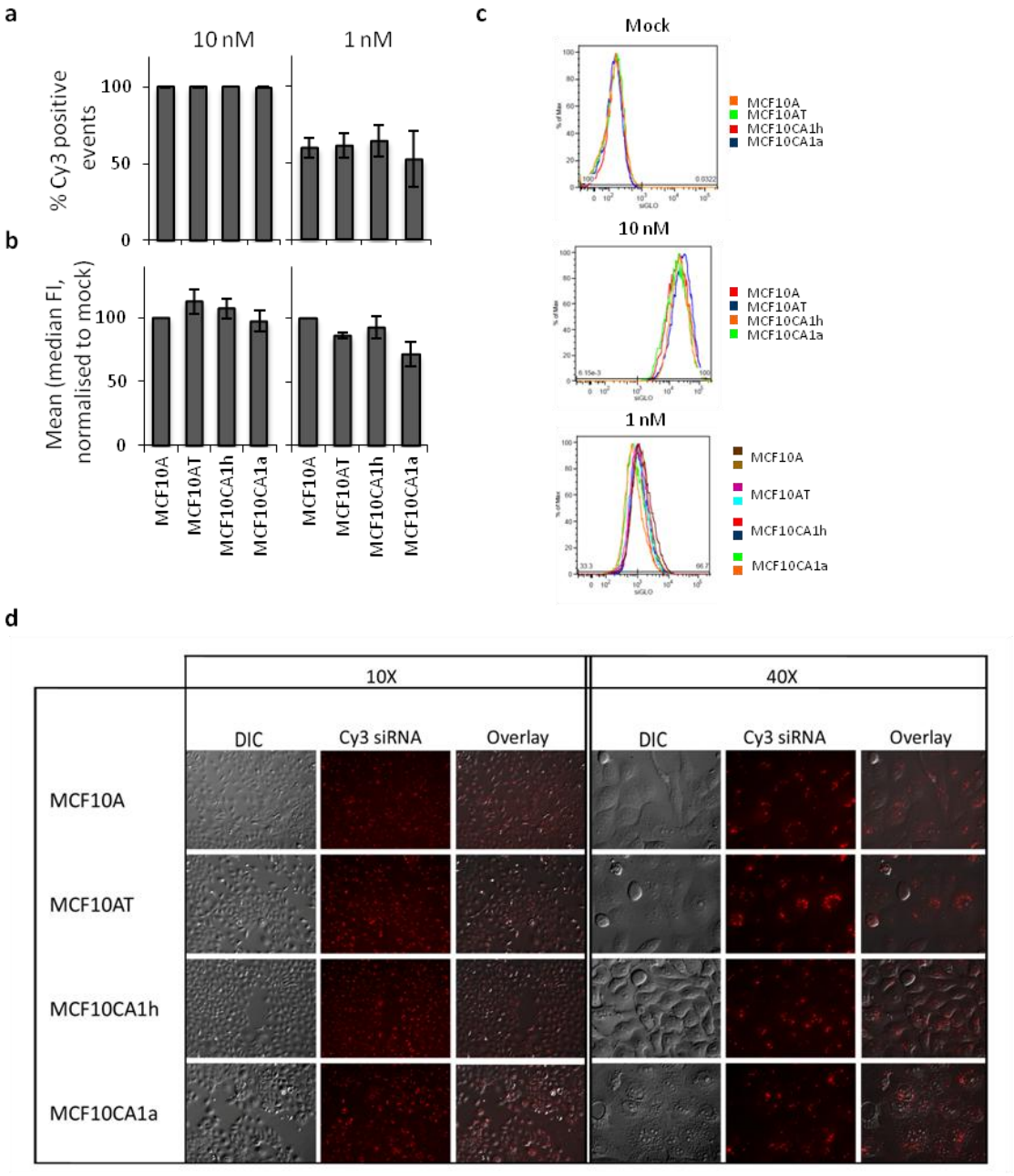
4.10 SUPPLEMENTARY FIGURES



Supplementary Figure S4.1 Comparison of XTT and cell count assays for the effect of Impβ1 siRNA. (**a**) Malignant MCF10CA1h cells were treated twice with 10 nM non-targeting (NT) or Impβ1 siRNAs and cell numbers monitored over four days post cell replating by cell counting (left) or an XTT assay (right). Results represent the mean +/- *SE* cell number (left) or mean +/- *SD* specific XTT absorbance (right), from a single representative experiment in two separate experiments. (**b**) Western analysis for Impβ1 expression in siRNA-treated samples from (**a**). + refers to Impβ1 siRNA-treated cells. UT, untreated.



Supplementary Figure S4.2 Silencing of Impβ1 decreases viability in transformed fibroblasts but not in their normal primary counterparts. (**a**) Cell proliferation measured on day 4 (96 h) using the XTT assay as per **Fig. 4.2** in normal 1BR3 (white bars) and transformed 1BR3.N cells (black bars) treated with 1 or 10 nM Impβ1 or non-targeting (NT) siRNAs. Results are for the mean +/- *SD* absorbance normalised to the NT siRNA-treated controls (n = 3). * p < 0.0001 *vs.* 1BR3 cells. (**b**) Western and (**c**) densitometric analysis for Impβ1 expression in samples from (**a**). Results are from a single typical experiment in a series of three separate experiments.



Supplementary Figure S4.3 siRNA uptake in the MCF10 cell series. (**a**) Uptake of Cy3-labelled siRNA in cell lines of the MCF10 series treated with the indicated concentrations of Cy3-labelled siRNA. Results for the % Cy3 positive events represent the mean +/- *SE* (n = 4). (**b**) Median fluorescence intensities (FI) in the MCF10 cell panel of samples from (**a**) are presented as the mean /- *SE*, relative to MCF10A cells. (**c**) Overlayed curves of Cy3 signals in MCF10 cell series in mock- and Cy3-transfected cells from a single typical experiment. (**d**) Fluorescence microscopic images of Cy3-labelled siRNAs in the MCF10 series.

CHAPTER 5

General Discussion

5 General Discussion

5.1 INTRODUCTION

Cancer is one of the most common causes of morbidity and mortality in developed countries (352, 353), because there remains a dire need for therapies that selectively kill tumour but not normal healthy bystander cells, and that can overcome the phenomenon of tumour-mediated drug resistance. In order to discover novel ways in which to target tumours selectively, a basic understanding of the types of cellular changes that are fundamental to tumour diseases is required. Nuclear trafficking of proteins through Imps is central to cell function. Altered nuclear protein localisation through changes in Imp levels is known to occur during phenotypic changes associated with cell differentiation, maintenance of pluripotency, tissue development (14, 131) while aberrations in this process are associated with diseases such as atherosclerosis (18), viral infection, neurodegenerative and inflammatory-related disease (184, 354).

At the commencement of the work described in this thesis, there were several reports based on qualitative approaches of overexpression of CAS (232, 234, 238, 246) or Impα1 (188, 198, 199, 213) in patient tumour tissues associated with high tumour grade and poor patient prognosis. Subsequent studies have largely focussed on these proteins, seeking to establish their potential as disease progression biomarkers in cancer patients (17, 177). Surprisingly, no studies have addressed the functional consequences of Imp overexpression in cancer, with essentially nothing known about comparative nuclear transport efficiencies between transformed/tumour and non-transformed cells.

5.2 INVESTIGATING NUCLEAR TRANSPORT PROPERTIES OF TRANSFORMED CELL TYPES USING ISOGENIC CELL SYSTEMS

The aim of this thesis was to investigate the types of alterations to nuclear transport properties occurring in tumour compared to non-transformed cell types using sets of isogenic non-transformed/transformed cell systems, to determine whether altered Imp activity is a common feature of transformed cell types, which transport pathways may become altered as a consequence, what might be the mechanisms thereof, and whether inhibiting the activity of overexpressed

nuclear transport components could selectively kill tumour cells. The use of isogenic cell systems is a powerful way to address questions relating to fundamental tumour biology, by avoiding the confounding variables associated with the use of non-isogenic cell types from different patients, tissue sources or cell types. The studies here were aimed at examining nuclear import and export efficiencies through conventional Impα/β1, Impβ1 and Exp-1-dependent pathways in a variety of cell types (discussed in Chapters 2 and 3) using quantitative methods at the single cell level.

This study shows for the first time that Impα/β1-dependent and to a lesser degree Impβ1-dependent import efficiency and rate is enhanced in a range of tumour/transformed cell types (**Table 5.1**), and that this correlates with overexpression of Impα1, Impβ1, and the Ran guanine nucleotide exchange factor RCC1 in all of the transformed cell types, with proof-of-principle type experiments showing Impα1 and Impβ1 levels as the key contributions for increased nuclear import activity through the Impα/β1-mediated pathway (**Fig. 2.5**, **Fig. 3.3** and **Fig. 3.4**). The fact that a number of transformed cell types display similar characteristics with respect to increased nuclear transport properties, and that Impα1 and to a lesser degree Impβ1, are all found overexpressed in numerous types of cancers (**Table 1.2**), indicate that enhanced nuclear import may be an as yet to be identified hallmark feature (355) of cellular transformation.

5.3 ENHANCED NUCLEAR EXPORT IN TRANSFORMED CELLS

This study apart from investigating nuclear import, was also the first to test Exp-1-dependent nuclear export efficiencies in transformed cell types, to document enhanced nuclear protein export activity occurs in a variety of transformed cell types, and that this relates with increased Exp-1 expression in transformed compared to non-transformed counterparts. Exp-1 has been reported to be overexpressed in many tumour types (**Table 1.2**), making this finding highly relevant to human cancer. Exp-1 mediates the nuclear export of multiple tumour suppressor factors (including p53, pRb, FOXO, $p21^{cip}$ and $p27^{kip}$). Nuclear localisation of tumour suppressors is vital for their proliferation inhibiting and/or apoptosis promoting activities; elevated Exp-1 activity may

Table 5.1: Summary of investigations testing nuclear import efficiency of NLS-containing fusion proteins in transformed/tumour *versus* isogenic non-transformed cell types.

a) SV40/T-ag-transformed systems

GFP-fusion protein	*Import pathway*	*Efficiency (fold change) vs. non-transformed*	
		1BR3.N	***COS-7***
T-ag (114-135)	Impα/β1	Increased (7.4)*	Increased (2.0)*
pUL54 (1145-1161)	Impα/β1	Increased (6.8)*	Increased (2.5)*
VP3 (74-121)[#]	Impβ1	Increased (6.5)*	Increased (3.3)*
TRF-1 (337-441)	Impβ1	Increased (5.9)*	Increased (3.6)*

b) Breast tumour progression series[%]

GFP-fusion protein	*Import pathway*	*Efficiency (fold change) vs. non-transformed*		
		MCF10AT[1]	***MCF10CA1h***[2]	***MCF10CA1a***[3]
T-ag (114-135)	Impα/β1	No change (1.1)	Increased (1.9)*	Increased (3.6)*
pUL54 (1145-1161)	Impα/β1	No change (1.0)	Increased (1.7)*	Increased (3.1)*
pRb (766-928)	Impα/β1	No change (0.9)	Increased (1.5)*	Increased (1.4)*
p53 (2-394)[^]	Impα/β1	Decreased (0.7)*	Increased (2.2)*	Increased (1.6)*
pUL44 (425-433)	Impα/β1 (v. weak NLS)	No change (0.9)	Increased (1.2)*	Increased (1.1)*
VP3 (74-121) NESm	Impβ1	Decreased (0.8)*	No change (1.1)	No change (0.9)
PTHRP (66-108)	Impβ1	Decreased (0.7)*	Decreased (0.8)*	Decreased (0.5)*
	Inhibition by Impα			
TRF-1 (337-441)	Impβ1	No change (1.1)	Decreased (0.5)*	Decreased (0.6)*
	Inhibition by Impα			
Rev (2-74)	Impβ1	Decreased (0.6)*	Decreased (0.5)*	Decreased (0.4)*
	Impβ2, Impβ3, Imp7, Imp9			
SREBP-1a (2-487)	Impβ1	Decreased (0.5)*	Decreased (0.4)*	Decreased (0.5)*
aF10 (696-794)	Non Imp	Decreased (0.8)*	Decreased (0.7)*	No change (0.9)

*$p < 0.05$ *vs*. non-transformed cells, Student's *t* test, 2 tailed.
[#] VP3 (74-121) contains the tumour specific nuclear targeting signal from chicken anaemia virus.
[%]Change of transfection reagent as per **Supp. Fig. S2.1**.
[1]Benign, hyperplasic; [2]Malignant, moderately to well differentiated, non-metastatic; [3]Malignant, poorly differentiated, metastatic.
[^]In leptomycin B treated samples (+LMB) as the p53 (2-394) construct contains Exp-1-recognised NESs.

Abbreviations: GFP, green fluorescent protein; Imp, importin; NESm, nuclear export signal mutant; NLS, nuclear localisation signal; PTHrP, parathyroid hormone-related protein; pRb, retinoblastoma protein; SREBP-1a, sterol regulatory element-binding protein-1a; SV40, simian virus 40; T-ag, large tumour antigen; TRF-1, telomeric repeat-binding factor-1; VP3, viral protein 3.

favour increased nuclear export of these factors where cytoplasmic redistribution renders them inactive, promoting tumour progression and resistance to therapies (93, 260) (**Fig. 5.1**). Enhanced Exp-1 activity can also influence the subcellular distribution of Exp-1-recognised chemotherapeutic targets such as topoisomerases in myeloma cells, where inhibition of Exp-1 sensitises the cells to chemotherapy-induced killing (356) (**Fig. 5.1**). Inhibiting Exp-1 activity through selective inhibitors of nuclear export (SINEs) are currently under investigation as anti-cancer therapies (see below) (260).

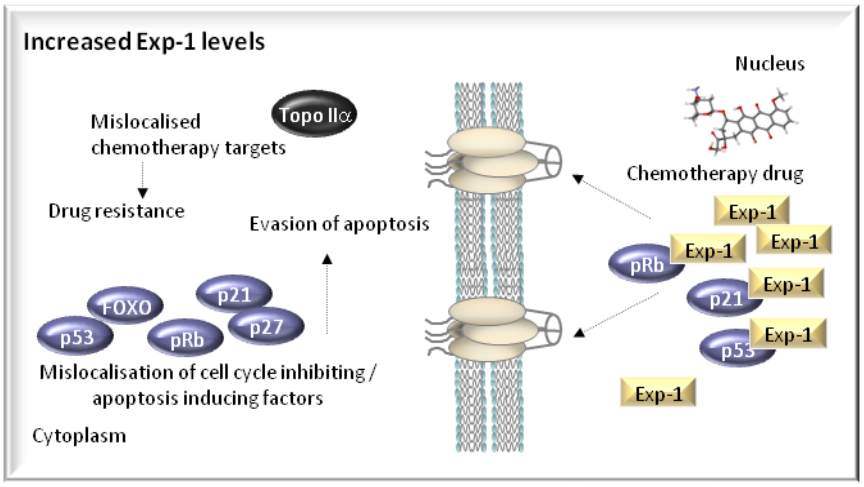


Figure 5.1 Potential effects of increased Exp levels on tumour progression. Increased Exp-1-dependent nuclear export of factors (such as p53 and FOXO) important for apoptosis induction may be a contributing factor to therapy resistance in Exp-1 overexpressing tumour cells. Increased Exp-1 levels can lead to increased nuclear export of chemotherapy targets such as Topoisomerase Iiα (Topo Iiα) resulting in acquired drug resistance of Topo IIα targeting chemotherapeutic agents such as doxorubicin and VP-16.

5.3.1 Targeting Exp-1 activity through small molecular inhibitors

SINEs are leptomycin B (LMB)-related compounds, in that they bind and inhibit the reactive site C^{528} in the NES binding groove of Exp-1 resulting in proteosomal degradation of Exp-1, but unlike LMB they exhibit higher potency, reversible (transient) inhibition and oral bioavailability (357). SINEs can induce nuclear re-distribution of tumour suppressors (ie. FOXO, p53, pRb) leading to amplified growth suppressing activity and initiation of death in many types of tumour cells (260, 358-360), although it is still unclear whether this occurs in a tumour selective fashion. KPT-335, KPT-251 and

Selinexor (KPT-330) are SINEs that are currently being tested in pre-clinical and Phase 1 clinical trials respectively, for blood and other types of cancers (259). Selinexor and KPT-251, due to their transient, reversible nature are claimed to possess tumour selective killing activity *in vivo* thus circumventing some of the toxic side-effects commonly associated with first generation Exp-1 inhibitors (such as LMB and analogues thereof) that led to their withdrawal from clinical trials in the first place. The basis of this tumour selectivity, however, is not clear. Significantly, mice bearing acute myeloid leukaemia (AML) treated with either agent do not survive past 35 days and have a significantly elevated haematocrit, platelet count, and decreased reticulocyte and lymphocyte counts compared to vehicle treated controls (361, 362). Furthermore, Phase 1 trials in dogs bearing non-Hodgkin's lymphoma (NHL) resulted in numerous gastrointestinal side effects during oral treatment with KPT-335 (259), suggesting that development of selective tumour therapies through Exp-1 targeting drugs may not be achievable. Supporting this idea, the studies presented in this thesis showed there was no selectivity of treatment when Exp-1 activity was transiently inhibited using Exp-1 siRNAs, in that the viability of both non-transformed and malignant breast cells was similarly affected (**Fig. 4.5**).

5.4 ENHANCEMENT OF IMPα/β1-DEPENDENT NUCLEAR IMPORT DURING BREAST TUMOUR PROGRESSION

Breast cancer is the most common cancer in women. About 15% of diagnosed tumours are of the triple negative breast cancer (TNBC) subtype meaning the tumours do not express progesterone, or estrogen receptors and lack genetic amplification of the HER-2/Neu (Erb-B2) growth factor receptor. TNBC can be early onset, is the most severe and aggressive form of breast cancer (363). In contrast to non-TNBC tumours that are responsive to highly selective hormonal (ie. Tamoxifen) and/or antibody conjugated therapies (ie. Herceptin), current therapies for TNBC are limited to use of conventional approaches such as chemotherapy, and mastectomy (364, 365), although TNBC is also known to have high recurrence rates post tumour resection (363). Our study aimed to investigate nuclear transport properties in this particular subset of breast tumours using the basal TNBC type MCF10 invasive ductal breast tumour progression model (289, 366) (discussed in Chapter 3), and to test whether targeting nuclear transport components overexpressed in transformed cells by way of siRNA would enable tumour selective killing.

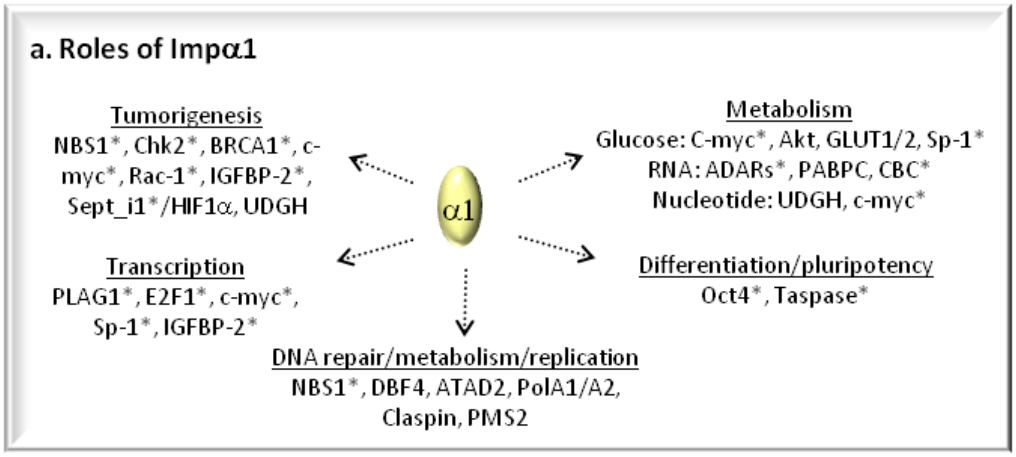
This study showed Impα/β1-dependent nuclear import efficiency to be greatly enhanced in malignant MCF10 breast cell types but not in benign or non-transformed counterparts, with some notable decreases in Impβ1-dependent import efficiency especially for cargoes that are known to be strongly nuclear such as TRF-1, PTHrP and SREBP-1a (**Table 5.1b**). Interestingly, the fact that nuclear import efficiency through the Impα/β1 heterodimer was found unaltered in the benign cell type that proliferates at the same rate as its malignant counterparts (**Supp. Fig. S3.2**) indicates altered nuclear transport properties may not be a simple byproduct of a faster doubling rate, but associated specifically with changes related to malignant transformation. Consistent with the findings presented in Chapter 2 (318), enhanced nuclear import efficiency was associated with progressively increased levels of Impα1 and Impβ1 (among others) with Impα1 identified as a key component modulating altered nuclear import efficiencies in the MCF10 series (**Fig. 3.4**). Increased Impα1 levels were also detected in grade 2 and 3 patient samples of invasive ductal carcinoma (IDC), compared to benign IDC grade 1 counterparts (**Fig. 3.5**) implying that the findings presented in this study are clinically relevant. Impα1 overexpression has been recently described in IDC (see **Table 1.2**), where the severity of the disease (grade, stage, recurrence post therapy) is known to correlate with degree of Impα1 overexpression (188, 198). This is true for several different types of cancers (**Table 1.2**), suggesting that Impα1 driven enhancement of Impα1/β1 dependent import efficiency may be present in many tumour types. Our findings in the MCF10 tumour progression model are significant in terms of examining the importance of altered Impα1 activity to tumorigenesis (see below), and are highly relevant to the design of tumour selective agents (see Section 5.5).

5.4.1 Relevance of enhanced Impα/β1-dependent protein nuclear import efficiency to tumorigenesis

To date, seven Impα isoforms (Impα1, α3-8) have been identified in human cells, which are divided into 3 subfamilies comprising of Impα1/8, Impα3/4 and Impα5/6/7 (see Section 1.2.2.2). The findings presented in this study indicate activity of Impα1, but not of other Impα isoforms tested is enhanced during the benign to malignant transformation switch, and modulates enhanced import capacity during tumour progression. Impα1 has very specific nuclear transport (see **Table 1.1**, Section 1.3.1.1.2.2 and **Fig. 5.2a**) and non-transport roles in cells (see Section 1.4). Impα1 maintains pluripotency in mouse embryonic stem cells through nuclear import of pluripotency-associated transcription factors (130, 131), while its C-terminal domain acts as a retention factor for

differentiation inducing factors (34). During oxidative stress Impα1 appears to be able to translocate into the nucleus without Impβ1, where it facilitates the transcription of stress-related genes and induction of non-apoptotic cell death (159). Impα1 is also involved in maintaining cellular homeostasis through supporting DNA, RNA, and nucleotide metabolism. For instance, Impα1 binds to capped polyAAA mRNAs through the cap binding complex (CBC) to facilitate CBC-mediated mRNA export and translation (29) through Impβ1, where Impβ1 binding to the Impα1:CBC:capped mRNA complex in the cytoplasm displaces capped mRNA from the CBC allowing EIF4-dependent mRNA translation and CBC nuclear import. In the nucleus the Impα1/β1:CBC complex dissociates upon RanGTP binding to Impβ1 allowing CBC to re-bind capped RNAs for re-export (29). Impα1 supports DNA repair through importing the NBS1/Mre11/Rad50 DNA repair factor complex (21) and transcription factors E2F1 (30) and c-myc (195), resulting in altered transcriptional programming to support increased metabolism and replication. Growth during oxidative stress, altered DNA replication, repair and metabolic processes, and the degree of cellular (de)differentiation all relate to tumour disease progression (355, 367, 368) and may be linked to altered Impα1 activity (**Fig. 5.2a**).

In terms of cargo nuclear re-distribution, our studies in transformed systems show enhancement of nuclear import and export mediated by the Impα1/β1 heterodimer and Exp-1 respectively can occur simultaneously, implying that endogenous proteins carrying an Impα1-recognised NLS, and an Exp-1-recognised NES would shuttle between the nucleus and the cytoplasm more efficiently, while proteins carrying one or the other sequence only (eg. where one targeting signal has become inactive), would be predominantly enhanced in nuclear or cytoplasmic accumulation respectively. Such redistribution presumably influences tumour progression, formation and/or maintenance by increasing the nuclear translocation efficiency of Impα1-recognised factors that are involved in differentiation/pluripotency, motility, metabolism, DNA repair/replication and tumorigenesis such as Impα1-recognised transcription factors whose downstream targets themselves are involved in these processes (see **Table 1.1** and **Fig. 5.2a**).


a. Roles of Impα1
Tumorigenesis
NBS1*, Chk2*, BRCA1*, c-myc*, Rac-1*, IGFBP-2*, Sept_i1*/HIF1α, UDGH
Metabolism
Glucose: C-myc*, Akt, GLUT1/2, Sp-1*
RNA: ADARs*, PABPC, CBC*
Nucleotide: UDGH, c-myc*
α1
Transcription
PLAG1*, E2F1*, c-myc*, Sp-1*, IGFBP-2*
Differentiation/pluripotency
Oct4*, Taspase*
DNA repair/metabolism/replication
NBS1*, DBF4, ATAD2, PolA1/A2, Claspin, PMS2

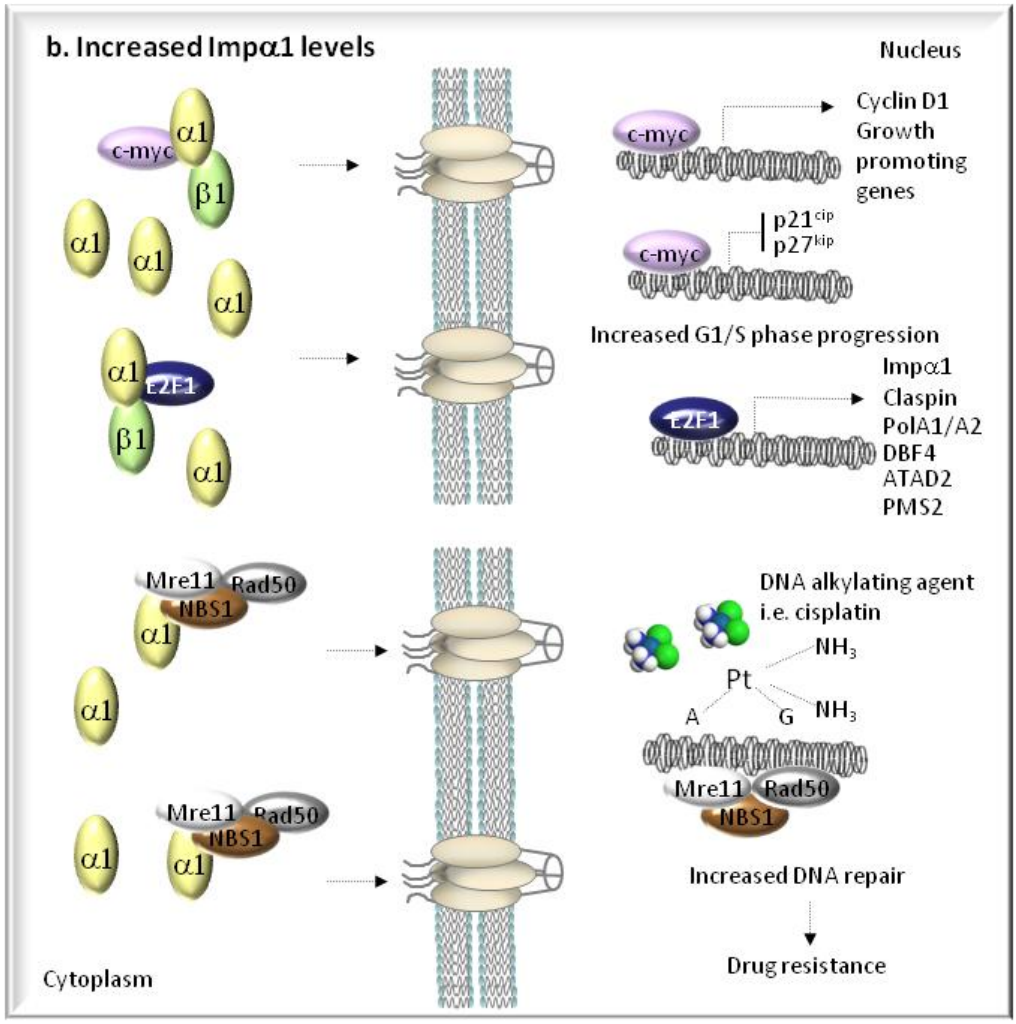

b. Increased Impα1 levels
Nucleus
c-myc
α1
β1
E2F1
Cyclin D1
Growth promoting genes
p21cip
p27kip
Increased G1/S phase progression
Impα1
Claspin
PolA1/A2
DBF4
ATAD2
PMS2
Mre11
Rad50
NBS1
DNA alkylating agent
i.e. cisplatin
NH3
Pt
A
G
Increased DNA repair
Drug resistance
Cytoplasm

Figure 5.2 Potential effects of increased Impα1 levels on tumour progression. (**a**) Impα1 increases the expression levels/activity of numerous signalling factors involved in metabolism, transcription, differentiation, and DNA repair and replication. * denotes factors that translocate into the nucleus through Impα1 (see **Table 1.1**). (**b**) Increased levels of Impα1 in tumour cells may promote tumorigenesis indirectly by enhancing the nuclear import and transcriptional activity of tumour-associated proteins such as c-myc and E2F1 resulting in altered expression of their downstream targets known to be involved in DNA replication (Claspin, Pol1, DBF4, ATAD2, PMS2) and cell cycle regulation ($p21^{cip}$, $p27^{kip}$, cyclin D1), to promote increased G1/S phase transition. Increased activity of E2F1 can further promote increased expression of Impα1 indicating a positive feedback loop may exist between the two proteins. Impα1 levels may increase the severity of tumours directly by playing a role in acquired cisplatin drug resistance, due to enhanced nuclear import of DNA repair factors such as the NBS1/Mre11/Rad50 complex, resulting in improved repair of purine crosslinking adducts in cisplatin-treated cells.

Consistent with this idea, increased Impα1 levels by ectopic expression have been shown to promote proliferation, tumorigenicity and invasive potential of luminal type MCF-7 breast cancer cells *in vitro* where increased Impα1 levels are associated with upregulation of its recognised cargoes involved in tumour progression such as the oncogenic pluripotency factor Oct4, NF-κB family member RelA (p65) and Rho-GTPase Rac1 (194). In ovarian cells, Impα1 upregulation can act in an oncogenic manner by increasing the levels, nuclear localisation and transcriptional activity of c-myc with multiple changes in signal pathways such as increased Akt and FOXO3a activity and decreased expression of cell cycle inhibitors $p21^{cip}$ and $p27^{kip}$ through c-myc-mediated transcriptional repression, to promote formation of epithelial ovarian carcinoma *in vitro* and *in vivo* (195) (**Fig. 5.2b**). In non-small cell lung carcinoma cells, Impα1 levels are critical for nuclear translocation and activity of E2F1 resulting in transcription of its downstream targets involved in DNA metabolism, replication, repair and/or cell cycle control that are required for initiation and progression through the S-phase (such as Claspin, PolA1 and PolA2, DBF4 and ATAD2), which are known to be involved in lung tumour progression (30). Interestingly, enhanced E2F1 activity itself can upregulate Impα1 expression, which may result in a positive feedback loop, further amplifying the effects of Impα1-mediated E2F1 nuclear import (30, 190) (**Fig. 5.2b**).

It should be noted that the exact outcomes of increased Impα1 expression may vary according to different cancer cell types, as the Imp binding affinity of targeting signals can be influenced indirectly by competing factors other than those governed by the targeting signal itself. It is now

evident that different Impα isoforms display distinct NLS binding properties (7, 369), which appears to be influenced strongly by both the nature of the NLS and protein context (370). For instance the nuclear transcription factor STAT1 is preferentially recognised by Impα5, but not Impα1 (48), whilst RCC1, the nuclear RanGTP nucleotide exchange factor utilises Impα3 far more efficiently than Impα5 or any other Impα isoform for its nuclear entry (40) (**Table 1.1**). More reflective of *in vivo* circumstances where multiple cargoes would compete for Impαs, when two known substrates of Impα3/4 are added together in competition binding assays (RCC1 and the nucleoplasmin (NPM)) one (RCC1) will preferentially bind to Impα3/4, whilst the other (NPM) will bind the remaining Impα isoforms (Impα1, α5 and α7) despite the fact that both are known to bind Impα3/4. Since studies here (and see (76)) implicate Impα1 as a limiting component, the cellular concentration of NLS containing proteins, their Impα binding affinities and the levels of other Impα isoforms may all influence the activity of Impα1, and in turn the nuclear transport efficiency of its recognised cargoes, meaning that Impα1 levels alone may not be the only factor determining the extent of altered cargo nuclear translocation and activity in cancer cells.

To add to this complexity, the levels of negative regulators of nuclear import (NRNI) such as EI24, BRAP2 or ARHI (discussed in Section 1.3.1.3) may also influence Impα1 activity in cells. For example, increased levels of EI24 (151) or ARHI (152) in tumour cells may prevent enhanced Impα/β1-dependent nuclear import activity even in the presence of increased levels of Impα1, through their ability to inhibit Impα/β1-dependent import. Although increased/decreased Impα1 levels were sufficient to modulate the nuclear import efficiency through the Impα/β1 pathway, indicating a simple relationship between Imp expression and nuclear import efficiency in this study, NRNI-mediated regulation cannot be excluded as an added layer of complexity that may be present in other cell types.

5.4.2 Relevance of decreased Impβ1-dependent protein nuclear import efficiency to tumorigenesis

That Impα1 overexpression may inhibit the nuclear import of certain Impβ1-recognised cargoes such as TRF-1 and PTHrP (**Table 5.1b** and **Fig. 3.4c**) in the MCF10 breast tumour progression model is intriguing. An interesting question arises from the findings of this study; could the negative regulation of Impβ1-dependent nuclear import by Impα1 be of significance to tumour disease progression or are the tumour associated functions of Impα1 primarily due to increased Impα1/β1-

dependent nuclear import efficiency? Nuclear PTHrP plays a role in mammary cells by inhibiting growth and enhancing differentiation (335), whilst TRF-1 protects telomere stability and length, and mice deficient in TRF-1 develop preneoplastic skin lesions as early as 1-6 days after birth (371); reduced nuclear localisation of PTHrP and TRF-1 may act to promote growth and dedifferentiation associated with increasingly malignant cell types and telomeric damage associated with neoplastic lesions, respectively. Whether the nuclear import of other Impβ1-recognised proteins may be affected by Impα1 overexpression was not examined here. Since neither PTHrP nor TRF-1 bind Impα and yet both show inhibition of nuclear import by addition of recombinant Impα in *in vitro* reconstituted transport systems (56, 57), is consistent with the idea that Impα levels may compete with Impβ1 to increase Impα/β1-dependent import at the expense of Impβ1-dependent import. Consistent with this, we found nuclear localisation of other Impβ1-recognised fusion proteins such as SREBP-1a, significantly decreased in Impα overexpressing malignant MCF10CA1h and MCF10CA1a cells (**Table 5.1b**). Whether inhibition of Impβ1-dependent import occurs in tumour cell types other than in the MCF10 system has not been extensively examined here, although the Impβ1-recognised TRF-1 appeared to localise to a much stronger extent in the nucleus of Impα1 overexpressing SV40-transformed cell types compared to non-transformed counterparts (**Table 5.1b** and **Fig. 2.1**). Clearly, further studies are required to test the nuclear import efficiency of Impβ1-recognised factors in various normal/transformed cell systems overexpressing Impα1 to determine whether Impα1 modulates Impβ1-dependent trafficking of specific proteins (see Section 1.3.2), or on a global scale.

5.4.3 Targeting Impα1 in anti-cancer therapy

Increased Impα1 activity in tumours has been proposed as a novel target for anti-cancer therapy after several studies concluded that silencing of Impα1 expression by way of RNAi inhibits the proliferation of several types of cancer cells (see Section 1.5.1.1). Molecular inhibitors of the Impα/β1-mediated pathway currently comprise of supraphysiological NLSs that bind many Impα isoforms such as bimax1 and 2 (372) and the broad spectrum anti-parasite agent Ivermectin (373). However none of these agents are likely to be targeted specifically at Impα1 but rather, inhibit the activity of multiple Impα isoforms. Whether specific inhibition of Impα1 or in fact any of the Impα isoforms could induce selective death of tumour but not normal cells is not yet clear. In fact the studies here in the MCF10 breast tumour progression model show that silencing Impα1 expression had very little to no effect on the growth of malignant cell types (**Fig. 4.5**), which is entirely

consistent with findings in CL1-0 lung adenocarcinoma (195) and ovarian epithelial carcinoma cell lines (CaSki, SiHa, C33A, MS751) (197). This suggests that cancer types and lines from different origins have differential sensitivities to Impα1 inhibition.

5.5 EXPLOITATION OF ENHANCED NUCLEAR IMPORT EFFICIENCY FOR TUMOUR SELECTIVE DRUG DELIVERY

There remains an urgent need for discovery and development of novel tumour-selective agents. Nanocarriers termed modular recombinant transporters (MRTs) (314) and other variations thereof (374) have great potential in achieving targeted delivery of cytotoxic agents to tumour cells. The premise behind this tumour selective action is through the inclusion of multiple tumour enhanced targeting domains, to produce a polypeptide/vehicle in which each domain mediates the progressive amplification of delivery of nuclear acting agents into tumour, but not to non-tumour cells. These domains include, but are not restricted to the use of a cellular targeting domain in the form of a receptor binding ligand, an endosomal escape domain for pH-dependent release from endosomes into the cytoplasm, a drug binding domain, which facilitates the attachment of nuclear-acting drugs and finally, a nuclear targeting domain allowing for efficient drug nuclear translocation (314, 375). Nuclear-acting drugs suitable for delivery include photosensitising agents such as chlorin e6, suicide genes such as the herpes simplex virus thymidine kinase (337), and chemotherapies such as topoisomerase inhibitors and DNA damaging agents. This study indicates a previously unrecognised potential that upregulation of Impα1/β1-dependent import may have, in terms of improving selective drug delivery into tumours to aid tumour selective killing by MRTs (**Fig. 5.3a**). The inclusion specifically of Impα1-recognised NLSs as a part of the multimodular complex, may provide an extra layer of selectivity in targeted drug delivery and is relevant to therapies utilising both non-virally (376), virus-like (377) and oncolytic virus-derived (378) vectors, which inherently rely on Imps for efficient drug delivery or virus production (**Fig. 5.3a**). Furthermore, as upregulation of Impα1/β1-dependent nuclear import activity appears to be a general feature of cellular transformation (Chapter 2) and is likely to be associated with high grade malignancies (Chapter 3), the findings presented here are highly relevant to improving tumour selective drug delivery approaches especially aimed at advanced forms of the disease, for which there is often very little in the way of therapies.

5.6 TUMOUR SELECTIVE HYPERSENSITIVITY TO IMPβ1 SILENCING

Apart from exploitation of enhanced Impα1 activity in tumour cells described above, inhibition of nuclear transport component activity has also been proposed as a potential target for anti-cancer therapy (185, 260, 271, 272). As discussed earlier (see Sections 5.3.1 and 5.4.3), Exp-1 inhibition by SINEs (Karyopharm), the most advanced agents in terms of the drug development pipeline results in potent tumour cell killing activity across a multitude of cancer types, whilst Impα1 has been recently proposed as a novel anti-cancer target, although whether Impα1 inhibition can induce killing across a broad range of tumours remains questionable. Significantly, it is unclear whether approaches targeting either Exp-1 or Impα1 activity may result in targeted killing of tumour cells leaving non-tumour cells unharmed, limiting their usability.

Non-transformed and malignant cell models were compared here for the first time to show that targeting Impβ1, in contrast to a panel of other nuclear transport components (including Exp-1 or Impα1) by way of RNAi is 33-fold more potent in killing transformed/malignant breast cell types compared to non-transformed counterparts, and at nanomolar/subnanomolar siRNA concentrations induced selective death of transformed cell types whilst leaving non-transformed cells unaffected (**Fig. 4.2**). Whereas the transformed MCF10 breast cells displayed more efficient Impβ1-dependent silencing compared to its non-transformed counterpart after treatment, the transformed non-breast 1BR3.N line, despite showing less efficient silencing compared to its normal counterpart, still showed increased sensitivity to treatment (**Fig. 4.3** and **Supp. Fig. S4.2**). It is quite possible that the tumour selective action of Impβ1 siRNA may be due to increased sensitivity to changes in either Impα/β1- or Impβ1-dependent nuclear import efficiencies, and/or mitotic progression in transformed cells, as Impβ1 is known to play a role in both (379) (see Sections 1.2.2 and 1.4). Irrespective of the mechanism/s (which were beyond the scope of this thesis) and consistent with the findings shown here, treatment with Impβ1 siRNA has been shown to inhibit the proliferation of various types of cervical carcinoma cell lines (40, 197, 350) and nerve sheath tumour cells (380), although none of the aforementioned studies addressed the anti-proliferative effects and silencing efficiencies of Impβ1 siRNA in non-transformed cell types, especially those from isogenic origins.

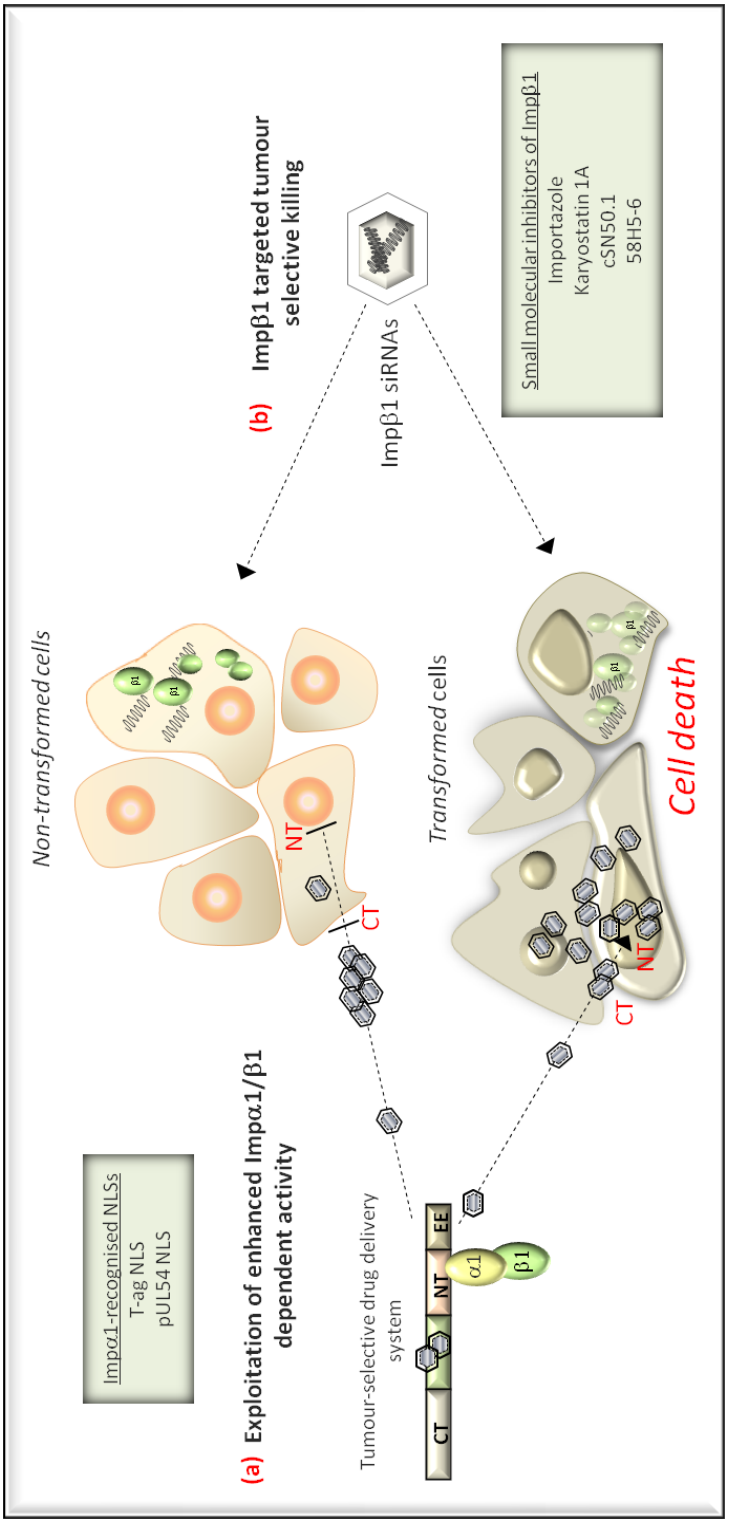

Non-transformed cells
Transformed cells
(a) Exploitation of enhanced Impα1/β1 dependent activity
(b) Impβ1 targeted tumour selective killing
Impβ1 siRNAs
Impα1-recognised NLSs
T-ag NLS
pUL54 NLS
Small molecular inhibitors of Impβ1
Importazole
Karyostatin 1A
cSN50.1
58H5-6
Tumour-selective drug delivery system
CT
NT
EE
α1
β1
Cell death

Figure 5.3. Exploitation of altered nuclear import properties in tumour cells; potential for targeted cancer therapies? (**a**) Enhanced Impα1/β1-dependent import activity occurring during the benign to malignant switch may be exploited through the inclusion of Impα1-recognised NLSs (ie. Tag or pUL54 NLS) in targeted drug delivery systems to further drive tumour selective nuclear drug delivery. Shown by way of example are modular recombinant transporters, non-viral nanocarriers comprised of domains/ligands that confer cell targeting (CT; through receptor mediated endocytosis), endosomal escape (EE), drug attachment of nuclear acting drugs such as photosensitisers (hexagons), suicide genes or DNA targeting agents, and nuclear targeting (NT; through NLS-dependent Imp binding), where selective delivery could be progressively amplified through the combined activity of tumour targeting CT and NT domains. (**b**) Targeting of Impβ1 activity through siRNA-mediated silencing can result in enhanced killing of transformed cell types compared to non-transformed counterparts through tumour hypersensitivity to Impβ1 knockdown. The potential for small molecular inhibitors (in particular cSN50.1 and 58H5-6) to induce selective killing of transformed cell types is not yet known.

Silencing of Impβ1 in malignant breast cells in the study here was associated with a significant increase in sub-G1 cell population and a concomitant decrease in G1-phase cells, indicative of cell death without cell cycle arrest (**Fig. 4.4**). This is entirely consistent with the findings in cervical carcinoma cells, where cell death induced by Impβ1 silencing was associated with PARP cleavage, caspase 3/7 activation, elevated p53/p21 expression and cytochrome c release from the mitochondria suggestive of apoptosis (197, 379). Using highly sensitive real-time imaging methods at the single cell level, silencing of Impβ1 has been shown to result in a delay from post-mitotic exit (G2/M progression) (351), which may be attributable to Impβ1's role in nuclear envelope assembly during mitosis (381). Mitotic delay was not able to be detected in this study as the DNA content analysis showing cell cycle progression was conducted using end point measurements from cell populations.

Impβ1 is an exciting target for novel tumour-selective therapies (**Fig. 5.3b**). The preliminary findings in this study need to be extended to include larger panels of non-transformed/malignant cell lines and a mouse tumour xenograft model derived from the MCF10 series (289), in order to assess the feasibility of Impβ1 inhibiting agents (siRNA or other) for cancer therapy. The efficacy of siRNA in cancer therapy has been limited due to poor intracellular delivery and is dependent on the development of nanocarrier delivery systems. An exciting application would be to utilise Impβ1 siRNAs in delivery systems comprising of tumour-selective components that target factors overexpressed at the tumour cell membrane. Examples of these approaches include siRNA encapsulated with liposomes displaying growth receptor ligands (ie. vascular endothelial growth

factor (VEGF)) at the exterior (382), antibody-mediated cell targeting of siRNAs through Erb-B2 binding and internalisation (383) or DNA-siRNA aptamer chimeras (384). The latter is a particularly attractive approach for targeted siRNA delivery as DNA is highly stable, elicits very little immunogenicity, and is easy to produce. In aptamer-form, DNA is comprised of short strands of nucleic acids assembled in conformations that can bind factors present at the cell surface such as the prostate specific membrane antigen overexpressed in prostate cancer cells, mediating tumour selective targeting as well as modifications allowing for siRNA attachment and processing in cells (384).

5.6.1 Targeting Impβ1 through small molecular inhibitors

Small molecular inhibitors are an attractive choice for anti-cancer therapies as they are easily produced, readily cross the plasma membrane, have defined mechanisms of action and are orally active. The concept and applicability of Imp-targeting small molecular inhibitors for disease therapy has already been demonstrated in a mouse model of atherosclerosis where the intraperitoneal delivery of a peptide comprising of a 10 aa. NLS from NF-κB p50, with flanking cysteines for circularisation attached to a hydrophobic cell penetrating peptide (cSN50.1), resulted in inhibition of Impα/β1- and Impβ1-dependent nuclear import of proteins involved in inflammatory and lipid metabolism, and lowered cholesterol and atherosclerosis in treated compared to control animals (18). However, lack of selectivity is by far the biggest challenge that is hindering the development of novel Impβ1-targeting compounds. Novel inhibitors of Impβ1 include the pyrrole compound karyostatin 1A (385), the 2,4-diaminoquinazoline Importazole (386) and the alpha helix mimetic 58H5-6 (387) (**Fig. 5.3b**). Both karyostatin 1A and Importazole inhibit the binding of RanGTP to Impβ1 thus preventing cargo release in the nucleus, and have the potential to arrest the activity of import pathways other than those mediated by Impβ1 through clogging of Impβ1 transport complexes at the NPC through preventing cargo release by RanGTP (385, 386). The alpha helix mimetic 58H5-6 has a different mechanism of action in that it appears to bind the FxFG-repeat binding region of Impβ1, preventing Impβ1 translocation through the NPC (387); the fact that treatment of cells with 58H5-6 does not alter the nuclear import of Impβ2-recognised M9 protein whilst efficiently inhibiting nuclear import of an Impα/β1-recognised cargo (an Impβ1-recognised cargo was not tested) indicates it may be a more suitable candidate for future studies, in terms of maximising tumour cell selectivity and minimising side effects, compared to karyostatin 1A and

Importazole. Whether or not these compounds can be utilised instead of Impβ1 siRNAs to kill cancer cells in a selective fashion is unknown, but would be of interest to determine in future studies (**Fig. 5.3b**).

5.7 MECHANISM/S FOR NUCLEAR TRANSPORT COMPONENT OVEREXPRESSION IN TRANSFORMED CELLS

The study here using transformed cell types from a number of different origins indicate that overexpression of several nuclear transport components (namely Impα1 and possibly Impα3, Impβ1, Exp-1, CAS and the Ran component RCC1) are generally associated with malignant transformation. The fact that the expression of multiple components increases concurrently both at the protein and transcript levels suggests altered activity of a shared factor, such as a transcription factor or miRNA, both of which are known to be deregulated in cancer may be responsible (388, 389). Transcription factors known to target Imps are E2F/Dp1 (Impα1 and Impβ1) (190), Sp-1 and the NFY transcriptional complex (Exp-1) (222), whilst miR-30, miR-137, miR-26 and miR-181b have been proposed to target Impβ1 (380), CAS (390), Impα1 (391) and Impα3 (392) respectively. miRNA deregulation in particular provides an attractive explanation by which multiple Imps might become upregulated at any one time during a switch from benign to malignant transformation. Interestingly, Imps such as Impα3, α5, and β1 are known to possess identical miRNA binding sites in their 3' UTR that become affected by single nucleotide polymorphisms in human diseases such as breast cancer (393), although this isn't surprising considering Imps possess long (ie. 1900bp, 5100bp and 1200bp for Impα3, α5, and β1 respectively) 3'UTR regions. Future studies need to concentrate on identifying miRNAs whose expression becomes altered in malignant cells compared to non-transformed counterparts by microarray and qPCR verification, analysing their Imp targeting potential by TargetScan software and whether altering the expression of these miRNAs can influence levels of Impα1, α3, β1, Exp-1 or CAS contributing to their altered activities in malignant cells. Enhanced transcription factor activity may also be a consequence of altered miRNA expression/activity. Analysis of transcription factor binding sites on 5' UTR regions of Imp genes to identify shared transcription factors may be useful, followed by molecular studies measuring promoter activity of predicted sites in non-transformed compared to malignant cell types.

5.8 CONCLUDING REMARKS

A number of recent studies show that nuclear import is not only essential for cell function and constitutive housekeeping roles, but rather that the activity of Imp isoforms is tightly regulated during specific developmental stages, which in turn can modulate nuclear import efficiencies critical to phenotypic change. Cancer appears to be similar, in that deregulation of Imp/Exp expression and activity may represent a key contribution to tumour disease progression. The studies presented here suggest that enhanced Impα1/β1-dependent nuclear import efficiency is associated with a switch from benign to malignant transformation due to overexpression of Impα1 in malignant but not in non-transformed/benign cells and correlates these findings to those in tissues of increasing tumour grade from patient samples. Questions that remain include the identity of the key endogenous proteins that become mislocalised as a result of altered Imp activity, the effects on the pathways they regulate and how this impacts on tumour progression to highly malignant cell types. As increased levels of Impα1 are known to correlate with increased tumour grade, stage, metastasis and decreased overall patient survival, future studies should primarily focus on determining whether Impα1 contributes to the most significant features of disease progression, such as metastasis and invasive potential, which have the most profound impact on patient survival/prognosis.

That altered nuclear trafficking may be a hallmark of cell transformation, raises the exciting possibility of exploring this as a novel way to amplify selective killing of tumour cells. Enhanced Impα/β1-dependent import activity could be exploited to improve tumour selective targeting of nuclear-acting drugs by the inclusion of Impα1-recognised NLSs in a broad range of delivery systems, regardless of whether they are viral, non-viral or virus-like in origin. Alternatively, inhibiting the activity of nuclear transport components that tumour cells have become addicted to, such as Impβ1, by way of siRNA or other agents may yet be another way to selectively eradicate tumours. Future studies are required to determine the feasibility of either approach across a panel of cancers from various origins starting with those from TNBC, and in mouse xenografts representing aggressive/higher grade tumour types, where the ultimate goal would be to provide viable treatment options for the most vulnerable patients. Exploiting altered Imp activities in cancers may be a significant advancement towards achieving tumour selective killing and importantly towards safer and more effective anti-cancer strategies.

References

References

1. Suntharalingam, M., and Wente, S. R. (2003) Peering through the pore: nuclear pore complex structure, assembly, and function. *Dev Cell* **4**, 775-789
2. Tran, E. J., and Wente, S. R. (2006) Dynamic nuclear pore complexes: life on the edge. *Cell* **125**, 1041-1053
3. Cardarelli, F., Serresi, M., Bizzarri, R., Giacca, M., and Beltram, F. (2007) In vivo study of HIV-1 Tat arginine-rich motif unveils its transport properties. *Mol Ther* **15**, 1313-1322
4. Gorlich, D., and Kutay, U. (1999) Transport between the cell nucleus and the cytoplasm. *Annu Rev Cell Dev Biol* **15**, 607-660
5. Kalderon, D., Richardson, W. D., Markham, A. F., and Smith, A. E. (1984) Sequence requirements for nuclear location of simian virus 40 large-T antigen. *Nature* **311**, 33-38
6. Robbins, J., Dilworth, S. M., Laskey, R. A., and Dingwall, C. (1991) Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. *Cell* **64**, 615-623
7. Jans, D. A., Forwood J.K. (2005) Nuclear protein import: distinct intracellular import receptors for different types of import substrates. In *Nuclear import and export in plants and animals* (V.Cytovsky, T. T. a., ed) pp. 136-159, Bioscience Publishers, Austin, Texas
8. Siomi, H., and Dreyfuss, G. (1995) A nuclear localization domain in the hnRNP A1 protein. *J Cell Biol* **129**, 551-560
9. Conti, E., and Kuriyan, J. Crystallographic analysis of the specific yet versatile recognition of distinct nuclear localization signals by karyopherin α. *Structure* **8**, 329-338
10. Moseley, G. W., Filmer, R. P., DeJesus, M. A., and Jans, D. A. (2007) Nucleocytoplasmic distribution of rabies virus P-protein is regulated by phosphorylation adjacent to C-terminal nuclear import and export signals. *Biochemistry* **46**, 12053-12061
11. Barraud, P., Banerjee, S., Mohamed, W. I., Jantsch, M. F., and Allain, F. H. (2014) A bimodular nuclear localization signal assembled via an extended double-stranded RNA-binding domain acts as an RNA-sensing signal for transportin 1. *Proc Natl Acad Sci U S A*
12. Chook, Y. M., and Suel, K. E. (2011) Nuclear import by karyopherin-s: recognition and inhibition. *Biochim Biophys Acta* **1813**, 1593-1606
13. Goldfarb, D. S., Corbett, A. H., Mason, D. A., Harreman, M. T., and Adam, S. A. (2004) Importin alpha: a multipurpose nuclear-transport receptor. *Trends Cell Biol* **14**, 505-514
14. Yasuhara, N., Oka, M., and Yoneda, Y. (2009) The role of the nuclear transport system in cell differentiation. *Semin Cell Dev Biol* **20**, 590-599
15. Hall, M. N., Griffin, C. A., Simionescu, A., Corbett, A. H., and Pavlath, G. K. (2011) Distinct roles for classical nuclear import receptors in the growth of multinucleated muscle cells. *Dev Biol* **357**, 248-258
16. Kelley, J. B., Talley, A. M., Spencer, A., Gioeli, D., and Paschal, B. M. (2010) Karyopherin alpha7 (KPNA7), a divergent member of the importin alpha family of nuclear import receptors. *BMC Cell Biol* **11**, 63
17. Christiansen, A., and Dyrskjot, L. (2013) The functional role of the novel biomarker karyopherin 2 (KPNA2) in cancer. *Cancer Lett* **331**, 18-23
18. Liu, Y., Major, A. S., Zienkiewicz, J., Gabriel, C. L., Veach, R. A., Moore, D. J., Collins, R. D., and Hawiger, J. (2013) Nuclear transport modulation reduces hypercholesterolemia, atherosclerosis, and fatty liver. *J Am Heart Assoc* **2**, e000093

19. Stewart, M. (2007) Molecular mechanism of the nuclear protein import cycle. *Nat Rev Mol Cell Biol* **8**, 195-208
20. Nadler, S. G., Tritschler, D., Haffar, O. K., Blake, J., Bruce, A. G., and Cleaveland, J. S. (1997) Differential expression and sequence-specific interaction of karyopherin alpha with nuclear localization sequences. *J Biol Chem* **272**, 4310-4315
21. Tseng, S. F., Chang, C. Y., Wu, K. J., and Teng, S. C. (2005) Importin KPNA2 is required for proper nuclear localization and multiple functions of NBS1. *J Biol Chem* **280**, 39594-39600
22. Zannini, L., Lecis, D., Lisanti, S., Benetti, R., Buscemi, G., Schneider, C., and Delia, D. (2003) Karyopherin-alpha2 protein interacts with Chk2 and contributes to its nuclear import. *J Biol Chem* **278**, 42346-42351
23. Ito, T., Kitamura, H., Uwatoko, C., Azumano, M., Itoh, K., and Kuwahara, J. (2010) Interaction of Sp1 zinc finger with transport factor in the nuclear localization of transcription factor Sp1. *Biochem Biophys Res Commun* **403**, 161-166
24. Golan, M., and Mabjeesh, N. J. (2013) SEPT9_i1 is required for the association between HIF-1alpha and importin-alpha to promote efficient nuclear translocation. *Cell Cycle* **12**, 2297-2308
25. Braem, C. V., Kas, K., Meyen, E., Debiec-Rychter, M., Van De Ven, W. J., and Voz, M. L. (2002) Identification of a karyopherin alpha 2 recognition site in PLAG1, which functions as a nuclear localization signal. *J Biol Chem* **277**, 19673-19678
26. Azar, W. J., Zivkovic, S., Werther, G. A., and Russo, V. C. (2014) IGFBP-2 nuclear translocation is mediated by a functional NLS sequence and is essential for its pro-tumorigenic actions in cancer cells. *Oncogene* **33**, 578-588
27. Bier, C., Knauer, S. K., Docter, D., Schneider, G., Kramer, O. H., and Stauber, R. H. (2011) The importin-alpha/nucleophosmin switch controls taspase1 protease function. *Traffic* **12**, 703-714
28. Bannister, A. J., Miska, E. A., Gorlich, D., and Kouzarides, T. (2000) Acetylation of importin-alpha nuclear import factors by CBP/p300. *Curr Biol* **10**, 467-470
29. Dias, S. M., Wilson, K. F., Rojas, K. S., Ambrosio, A. L., and Cerione, R. A. (2009) The molecular basis for the regulation of the cap-binding complex by the importins. *Nat Struct Mol Biol* **16**, 930-937
30. Wang, C. I., Chien, K. Y., Wang, C. L., Liu, H. P., Cheng, C. C., Chang, Y. S., Yu, J. S., and Yu, C. J. (2012) Quantitative proteomics reveals regulation of karyopherin subunit alpha-2 (KPNA2) and its potential novel cargo proteins in nonsmall cell lung cancer. *Mol Cell Proteomics* **11**, 1105-1122
31. Neganova, I., Vilella, F., Atkinson, S. P., Lloret, M., Passos, J. F., von Zglinicki, T., O'Connor, J. E., Burks, D., Jones, R., Armstrong, L., and Lako, M. (2011) An important role for CDK2 in G1 to S checkpoint activation and DNA damage response in human embryonic stem cells. *Stem Cells* **29**, 651-659
32. Soutoglou, E., Demeny, M. A., Scheer, E., Fienga, G., Sassone-Corsi, P., and Tora, L. (2005) The nuclear import of TAF10 is regulated by one of its three histone fold domain-containing interaction partners. *Mol Cell Biol* **25**, 4092-4104
33. Maas, S., and Gommans, W. M. (2009) Identification of a selective nuclear import signal in adenosine deaminases acting on RNA. *Nucleic Acids Res* **37**, 5822-5829
34. Yasuhara, N., Yamagishi, R., Arai, Y., Mehmood, R., Kimoto, C., Fujita, T., Touma, K., Kaneko, A., Kamikawa, Y., Moriyama, T., Yanagida, T., Kaneko, H., and Yoneda, Y. (2013) Importin

alpha subtypes determine differential transcription factor localization in embryonic stem cells maintenance. *Dev Cell* **26**, 123-135

35. Sandrock, K., Bielek, H., Schradi, K., Schmidt, G., and Klugbauer, N. (2010) The Nuclear Import of the Small GTPase Rac1 is Mediated by the Direct Interaction with Karyopherin α2. *Traffic* **11**, 198-209
36. Marchenko, N. D., Hanel, W., Li, D., Becker, K., Reich, N., and Moll, U. M. (2010) Stress-mediated nuclear stabilization of p53 is regulated by ubiquitination and importin-alpha3 binding. *Cell Death Differ* **17**, 255-267
37. Koch, H. B., Zhang, R., Verdoodt, B., Bailey, A., Zhang, C. D., Yates, J. R., 3rd, Menssen, A., and Hermeking, H. (2007) Large-scale identification of c-MYC-associated proteins using a combined TAP/MudPIT approach. *Cell Cycle* **6**, 205-217
38. Okazaki, K., Nakayama, N., Nariai, Y., Nakayama, K., Miyazaki, K., Maruyama, R., Kato, H., Kosugi, S., Urano, T., and Sakashita, G. (2012) Nuclear localization signal in a cancer-related transcriptional regulator protein NAC1. *Carcinogenesis* **33**, 1854-1862
39. Yoon, S.-O., Shin, S., Liu, Y., Ballif, B. A., Woo, M. S., Gygi, S. P., and Blenis, J. (2008) Ran-binding protein 3 phosphorylation links the Ras and PI3-kinase pathways to nucleocytoplasmic transport. *Mol Cell* **29**, 362-375
40. Quensel, C., Friedrich, B., Sommer, T., Hartmann, E., and Kohler, M. (2004) In vivo analysis of importin alpha proteins reveals cellular proliferation inhibition and substrate specificity. *Mol Cell Biol* **24**, 10246-10255
41. Yeung, P. L., Chen, L. Y., Tsai, S. C., Zhang, A., and Chen, J. D. (2008) Daxx contains two nuclear localization signals and interacts with importin alpha3. *J Cell Biochem* **103**, 456-470
42. Joshi, P., Greco, T. M., Guise, A. J., Luo, Y., Yu, F., Nesvizhskii, A. I., and Cristea, I. M. (2013) The functional interactome landscape of the human histone deacetylase family. *Mol Syst Biol* **9**, 672
43. Aratani, S., Oishi, T., Fujita, H., Nakazawa, M., Fujii, R., Imamoto, N., Yoneda, Y., Fukamizu, A., and Nakajima, T. (2006) The nuclear import of RNA helicase A is mediated by importin-alpha3. *Biochem Biophys Res Commun* **340**, 125-133
44. Banninger, G., and Reich, N. C. (2004) STAT2 nuclear trafficking. *J Biol Chem* **279**, 39199-39206
45. Fagerlund, R., Kinnunen, L., Kohler, M., Julkunen, I., and Melen, K. (2005) NF-{kappa}B is transported into the nucleus by importin {alpha}3 and importin {alpha}4. *J Biol Chem* **280**, 15942-15951
46. Sekimoto, T., Fukumoto, M., and Yoneda, Y. (2004) 14-3-3 suppresses the nuclear localization of threonine 157-phosphorylated p27(Kip1). *Embo J* **23**, 1934-1942
47. Jans, D. A., Xiao, C. Y., and Lam, M. H. (2000) Nuclear targeting signal recognition: a key control point in nuclear transport? *Bioessays* **22**, 532-544
48. Sekimoto, T., Imamoto, N., Nakajima, K., Hirano, T., and Yoneda, Y. (1997) Extracellular signal-dependent nuclear import of Stat1 is mediated by nuclear pore-targeting complex formation with NPI-1, but not Rch1. *Embo J* **16**, 7067-7077
49. Ma, J., and Cao, X. (2006) Regulation of Stat3 nuclear import by importin α5 and importin α7 via two different functional sequence elements. *Cellular Signalling* **18**, 1117-1126
50. Jakel, S., and Gorlich, D. (1998) Importin beta, transportin, RanBP5 and RanBP7 mediate nuclear import of ribosomal proteins in mammalian cells. *Embo J* **17**, 4491-4502

51. Jakel, S., Mingot, J.-M., Schwarzmaier, P., Hartmann, E., and Gorlich, D. (2002) Importins fulfil a dual function as nuclear import receptors and cytoplasmic chaperones for exposed basic domains. *Embo J* **21**, 377-386
52. Muhlhausser, P., Muller, E. C., Otto, A., and Kutay, U. (2001) Multiple pathways contribute to nuclear import of core histones. *EMBO Rep* **2**, 690-696
53. Baake, M., Bauerle, M., Doenecke, D., and Albig, W. (2001) Core histones and linker histones are imported into the nucleus by different pathways. *Eur J Cell Biol* **80**, 669-677
54. Xiao, Z., Liu, X., and Lodish, H. F. (2000) Importin beta mediates nuclear translocation of Smad 3. *J Biol Chem* **275**, 23425-23428
55. Forwood, J. K., Lam, M. H., and Jans, D. A. (2001) Nuclear import of Creb and AP-1 transcription factors requires importin-beta 1 and Ran but is independent of importin-alpha. *Biochemistry* **40**, 5208-5217
56. Lam, M. H., Briggs, L. J., Hu, W., Martin, T. J., Gillespie, M. T., and Jans, D. A. (1999) Importin beta recognizes parathyroid hormone-related protein with high affinity and mediates its nuclear import in the absence of importin alpha. *J Biol Chem* **274**, 7391-7398
57. Forwood, J. K., and Jans, D. A. (2002) Nuclear import pathway of the telomere elongation suppressor TRF1: inhibition by importin alpha. *Biochemistry* **41**, 9333-9340
58. Nagoshi, E., Imamoto, N., Sato, R., and Yoneda, Y. (1999) Nuclear import of sterol regulatory element-binding protein-2, a basic helix-loop-helix-leucine zipper (bHLH-Zip)-containing transcription factor, occurs through the direct interaction of importin beta with HLH-Zip. *Mol Biol Cell* **10**, 2221-2233
59. Bonifaci, N., Moroianu, J., Radu, A., and Blobel, G. (1997) Karyopherin beta2 mediates nuclear import of a mRNA binding protein. *Proc Natl Acad Sci U S A* **94**, 5055-5060
60. Fritz, J., Strehblow, A., Taschner, A., Schopoff, S., Pasierbek, P., and Jantsch, M. F. (2009) RNA-regulated interaction of transportin-1 and exportin-5 with the double-stranded RNA-binding domain regulates nucleocytoplasmic shuttling of ADAR1. *Mol Cell Biol* **29**, 1487-1497
61. Truant, R., Kang, Y., and Cullen, B. R. (1999) The human tap nuclear RNA export factor contains a novel transportin-dependent nuclear localization signal that lacks nuclear export signal function. *J Biol Chem* **274**, 32167-32171
62. Güttinger, S., Mühlhäusser, P., Koller-Eichhorn, R., Brennecke, J., and Kutay, U. (2004) Transportin2 functions as importin and mediates nuclear import of HuR. *Proc Natl Acad Sci U S A* **101**, 2918-2923
63. Lorenzen, J. A., Baker, S. E., Denhez, F., Melnick, M. B., Brower, D. L., and Perkins, L. A. (2001) Nuclear import of activated D-ERK by DIM-7, an importin family member encoded by the gene moleskin. *Development* **128**, 1403-1414
64. Ploski, J. E., Topisirovic, I., Park, K. W., Borden, K. L., and Radu, A. (2009) A mechanism of nucleocytoplasmic trafficking for the homeodomain protein PRH. *Mol Cell Biochem* **332**, 173-181
65. Ni, L., Llewellyn, R., Kesler, C. T., Kelley, J. B., Spencer, A., Snow, C. J., Shank, L., and Paschal, B. M. (2013) Androgen induces a switch from cytoplasmic retention to nuclear import of the androgen receptor. *Mol Cell Biol* **33**, 4766-4778
66. Hakim, A., Barnes, P. J., Adcock, I. M., and Usmani, O. S. (2013) Importin-7 mediates glucocorticoid receptor nuclear import and is impaired by oxidative stress, leading to glucocorticoid insensitivity. *The FASEB Journal*

67. Lindsay, M. E., Plafker, K., Smith, A. E., Clurman, B. E., and Macara, I. G. (2002) Npap60/Nup50 is a tri-stable switch that stimulates importin-alpha:beta-mediated nuclear protein import. *Cell* **110**, 349-360
68. Matsuura, Y., and Stewart, M. (2005) Nup50/Npap60 function in nuclear protein import complex disassembly and importin recycling. *Embo J* **24**, 3681-3689
69. Pumroy, R. A., Nardozzi, J. D., Hart, D. J., Root, M. J., and Cingolani, G. (2012) Nucleoporin Nup50 stabilizes closed conformation of armadillo repeat 10 in importin alpha5. *J Biol Chem* **287**, 2022-2031
70. Makise, M., Mackay, D. R., Elgort, S., Shankaran, S. S., Adam, S. A., and Ullman, K. S. (2012) The Nup153-Nup50 protein interface and its role in nuclear import. *J Biol Chem* **287**, 38515-38522
71. Ogawa, Y., Miyamoto, Y., Oka, M., and Yoneda, Y. (2012) The interaction between importin-alpha and Nup153 promotes importin-alpha/ beta-mediated nuclear import. *Traffic*
72. Adam, S. A. (2009) The nuclear transport machinery in Caenorhabditis elegans: A central role in morphogenesis. *Semin Cell Dev Biol* **20**, 576-581
73. Mason, D. A., and Goldfarb, D. S. (2009) The nuclear transport machinery as a regulator of Drosophila development. *Semin Cell Dev Biol* **20**, 582-589
74. Jullien, D., Gorlich, D., Laemmli, U. K., and Adachi, Y. (1999) Nuclear import of RPA in Xenopus egg extracts requires a novel protein XRIPalpha but not importin alpha. *Embo J* **18**, 4348-4358
75. Huber, J., Cronshagen, U., Kadokura, M., Marshallsay, C., Wada, T., Sekine, M., and Luhrmann, R. (1998) Snurportin1, an m3G-cap-specific nuclear import receptor with a novel domain structure. *Embo J* **17**, 4114-4126
76. Riddick, G., and Macara, I. G. (2007) The adapter importin-alpha provides flexible control of nuclear import at the expense of efficiency. *Mol Syst Biol* **3**, 118
77. Jakel, S., Albig, W., Kutay, U., Bischoff, F. R., Schwamborn, K., Doenecke, D., and Gorlich, D. (1999) The importin beta/importin 7 heterodimer is a functional nuclear import receptor for histone H1. *Embo J* **18**, 2411-2423
78. Hamada, M., Haeger, A., Jeganathan, K. B., van Ree, J. H., Malureanu, L., Walde, S., Joseph, J., Kehlenbach, R. H., and van Deursen, J. M. (2011) Ran-dependent docking of importin-beta to RanBP2/Nup358 filaments is essential for protein import and cell viability. *J Cell Biol* **194**, 597-612
79. Ribbeck, K., Lipowsky, G., Kent, H. M., Stewart, M., and Gorlich, D. (1998) NTF2 mediates nuclear import of Ran. *Embo J* **17**, 6587-6598
80. Kose, S., Furuta, M., and Imamoto, N. (2012) Hikeshi, a nuclear import carrier for Hsp70s, protects cells from heat shock-induced nuclear damage. *Cell* **149**, 578-589
81. Harley, V. R., Layfield, S., Mitchell, C. L., Forwood, J. K., John, A. P., Briggs, L. J., McDowall, S. G., and Jans, D. A. (2003) Defective importin beta recognition and nuclear import of the sex-determining factor SRY are associated with XY sex-reversing mutations. *Proc Natl Acad Sci U S A* **100**, 7045-7050
82. Malki, S., Nef, S., Notarnicola, C., Thevenet, L., Gasca, S., Mejean, C., Berta, P., Poulat, F., and Boizet-Bonhoure, B. (2005) Prostaglandin D2 induces nuclear import of the sex-determining factor SOX9 via its cAMP-PKA phosphorylation. *Embo J* **24**, 1798-1809
83. Xu, L., Alarcon, C., Col, S., and Massague, J. (2003) Distinct domain utilization by Smad3 and Smad4 for nucleoporin interaction and nuclear import. *J Biol Chem* **278**, 42569-42577

84. Sharma, M., Jamieson, C., Johnson, M., Molloy, M. P., and Henderson, B. R. (2012) Specific armadillo repeat sequences facilitate beta-catenin nuclear transport in live cells via direct binding to nucleoporins Nup62, Nup153, and RanBP2/Nup358. *J Biol Chem* **287**, 819-831
85. Sweitzer, T. D., and Hanover, J. A. (1996) Calmodulin activates nuclear protein import: a link between signal transduction and nuclear transport. *Proc Natl Acad Sci U S A* **93**, 14574-14579
86. Kaur, G., and Jans, D. A. (2011) Dual nuclear import mechanisms of sex determining factor SRY: intracellular Ca2+ as a switch. *Faseb J* **25**, 665-675
87. Argentaro, A., Sim, H., Kelly, S., Preiss, S., Clayton, A., Jans, D. A., and Harley, V. R. (2003) A SOX9 defect of calmodulin-dependent nuclear import in campomelic dysplasia/autosomal sex reversal. *J Biol Chem* **278**, 33839-33847
88. Berridge, M. J., Bootman, M. D., and Lipp, P. (1998) Calcium--a life and death signal. *Nature* **395**, 645-648
89. Macara, I. G. (2001) Transport into and out of the nucleus. *Microbiol Mol Biol Rev* **65**, 570-594
90. Klemm, J. D., Beals, C. R., and Crabtree, G. R. (1997) Rapid targeting of nuclear proteins to the cytoplasm. *Curr Biol* **7**, 638-644
91. Fukuda, M., Asano, S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M., and Nishida, E. (1997) CRM1 is responsible for intracellular transport mediated by the nuclear export signal. *Nature* **390**, 308-311
92. Turner, J. G., and Sullivan, D. M. (2008) CRM1-mediated nuclear export of proteins and drug resistance in cancer. *Curr Med Chem* **15**, 2648-2655
93. Nguyen, K. T., Holloway, M. P., and Altura, R. A. (2012) The CRM1 nuclear export protein in normal development and disease. *Int J Biochem Mol Biol* **3**, 137-151
94. Kohler, A., and Hurt, E. (2007) Exporting RNA from the nucleus to the cytoplasm. *Nat Rev Mol Cell Biol* **8**, 761-773
95. Kudo, N., Matsumori, N., Taoka, H., Fujiwara, D., Schreiner, E. P., Wolff, B., Yoshida, M., and Horinouchi, S. (1999) Leptomycin B inactivates CRM1/exportin 1 by covalent modification at a cysteine residue in the central conserved region. *Proc Natl Acad Sci U S A* **96**, 9112-9117
96. Guttler, T., and Gorlich, D. (2011) Ran-dependent nuclear export mediators: a structural perspective. *Embo J* **30**, 3457-3474
97. Kutay, U., Bischoff, F. R., Kostka, S., Kraft, R., and Gorlich, D. (1997) Export of importin alpha from the nucleus is mediated by a specific nuclear transport factor.[see comment]. *Cell* **90**, 1061-1071
98. Kutay, U., Izaurralde, E., Bischoff, F. R., Mattaj, I. W., and Gorlich, D. (1997) Dominant-negative mutants of importin-[beta] block multiple pathways of import and export through the nuclear pore complex. *Embo J* **16**, 1153-1163
99. Englmeier, L., Fornerod, M., Bischoff, F. R., Petosa, C., Mattaj, I. W., and Kutay, U. (2001) RanBP3 influences interactions between CRM1 and its nuclear protein export substrates. *EMBO Rep* **2**, 926-932
100. Lindsay, M. E., Holaska, J. M., Welch, K., Paschal, B. M., and Macara, I. G. (2001) Ran-binding protein 3 is a cofactor for Crm1-mediated nuclear protein export. *J Cell Biol* **153**, 1391-1402
101. Nemergut, M. E., Lindsay, M. E., Brownawell, A. M., and Macara, I. G. (2002) Ran-binding protein 3 links Crm1 to the Ran guanine nucleotide exchange factor. *J Biol Chem* **277**, 17385-17388

102. Hutten, S., and Kehlenbach, R. H. (2007) CRM1-mediated nuclear export: to the pore and beyond. *Trends Cell Biol* **17**, 193-201
103. Kutay, U., and Guttinger, S. (2005) Leucine-rich nuclear-export signals: born to be weak. *Trends Cell Biol* **15**, 121-124
104. Roth, P., Xylourgidis, N., Sabri, N., Uv, A., Fornerod, M., and Samakovlis, C. (2003) The Drosophila nucleoporin DNup88 localizes DNup214 and CRM1 on the nuclear envelope and attenuates NES-mediated nuclear export. *J Cell Biol* **163**, 701-706
105. Xylourgidis, N., Roth, P., Sabri, N., Tsarouhas, V., and Samakovlis, C. (2006) The nucleoporin Nup214 sequesters CRM1 at the nuclear rim and modulates NFkappaB activation in Drosophila. *J Cell Sci* **119**, 4409-4419
106. Capelson, M., and Hetzer, M. W. (2009) The role of nuclear pores in gene regulation, development and disease. *EMBO Rep* **10**, 697-705
107. D'Angelo, M. A., Gomez-Cavazos, J. S., Mei, A., Lackner, D. H., and Hetzer, M. W. (2012) A change in nuclear pore complex composition regulates cell differentiation. *Dev Cell* **22**, 446-458
108. Ghildyal, R., Jordan, B., Li, D., Dagher, H., Bardin, P. G., Gern, J. E., and Jans, D. A. (2009) Rhinovirus 3C protease can localize in the nucleus and alter active and passive nucleocytoplasmic transport. *J Virol* **83**, 7349-7352
109. Park, N., Katikaneni, P., Skern, T., and Gustin, K. E. (2008) Differential targeting of nuclear pore complex proteins in poliovirus-infected cells. *J Virol* **82**, 1647-1655
110. Satterly, N., Tsai, P.-L., van Deursen, J., Nussenzveig, D. R., Wang, Y., Faria, P. A., Levay, A., Levy, D. E., and Fontoura, B. M. A. (2007) Influenza virus targets the mRNA export machinery and the nuclear pore complex. *Proc Natl Acad Sci U S A* **104**, 1853-1858
111. Gorlich, D., Seewald, M. J., and Ribbeck, K. (2003) Characterization of Ran-driven cargo transport and the RanGTPase system by kinetic measurements and computer simulation. *Embo J* **22**, 1088-1100
112. Smith, A. E., Slepchenko, B. M., Schaff, J. C., Loew, L. M., and Macara, I. G. (2002) Systems analysis of Ran transport. *Science* **295**, 488-491
113. Riddick, G., and Macara, I. G. (2005) A systems analysis of importin-{alpha}-{beta} mediated nuclear protein import. *J Cell Biol* **168**, 1027-1038
114. Yang, W., and Musser, S. M. (2006) Nuclear import time and transport efficiency depend on importin beta concentration. *J Cell Biol* **174**, 951-961
115. Kohler, M., Speck, C., Christiansen, M., Bischoff, F. R., Prehn, S., Haller, H., Gorlich, D., and Hartmann, E. (1999) Evidence for distinct substrate specificities of importin alpha family members in nuclear protein import. *Mol Cell Biol* **19**, 7782-7791
116. Kohler, M., Ansieau, S., Prehn, S., Leutz, A., Haller, H., and Hartmann, E. (1997) Cloning of two novel human importin-alpha subunits and analysis of the expression pattern of the importin-alpha protein family. *FEBS Lett* **417**, 104-108
117. Feldherr, C. M., Lanford, R. E., and Akin, D. (1992) Signal-mediated nuclear transport in simian virus 40-transformed cells is regulated by large tumor antigen. *Proc Natl Acad Sci U S A* **89**, 11002-11005
118. Feldherr, C. M., and Akin, D. (1991) Signal-mediated nuclear transport in proliferating and growth-arrested BALB/c 3T3 cells. *J Cell Biol* **115**, 933-939
119. Kim, S. Y., Ryu, S. J., Ahn, H. J., Choi, H. R., Kang, H. T., and Park, S. C. Senescence-related functional nuclear barrier by down-regulation of nucleo-cytoplasmic trafficking gene expression. *Biochem Biophys Res Commun* **391**, 28-32

120. Nakamura, S., Hayashi, K. i., Iwasaki, K., Fujioka, T., Egusa, H., Yatani, H., and Sobue, K. Nuclear import mechanism for myocardin family members and their correlation with vascular smooth muscle cell phenotype. *J Biol Chem* **285**, 37314-37323
121. Perez-Terzic, C., Faustino, R. S., Boorsma, B. J., Arrell, D. K., Niederlander, N. J., Behfar, A., and Terzic, A. (2007) Stem cells transform into a cardiac phenotype with remodeling of the nuclear transport machinery. *Nat Clin Pract Cardiovasc Med* **4 Suppl 1**, S68-76
122. Kohler, M., Fiebeler, A., Hartwig, M., Thiel, S., Prehn, S., Kettritz, R., Luft, F. C., and Hartmann, E. (2002) Differential expression of classical nuclear transport factors during cellular proliferation and differentiation. *Cell Physiol Biochem* **12**, 335-344
123. Hogarth, C. A., Calanni, S., Jans, D. A., and Loveland, K. L. (2006) Importin alpha mRNAs have distinct expression profiles during spermatogenesis. *Dev Dyn* **235**, 253-262
124. Hogarth, C. A., Jans, D. A., and Loveland, K. L. (2007) Subcellular distribution of importins correlates with germ cell maturation. *Dev Dyn* **236**, 2311-2320
125. Loveland, K. L., Hogarth, C., Szczepny, A., Prabhu, S. M., and Jans, D. A. (2006) Expression of nuclear transport importins beta 1 and beta 3 is regulated during rodent spermatogenesis. *Biol Reprod* **74**, 67-74
126. Arjomand, A., Baker, M. A., Li, C., Buckle, A. M., Jans, D. A., Loveland, K. L., and Miyamoto, Y. (2014) The alpha-importome of mammalian germ cell maturation provides novel insights for importin biology. *Faseb J* **28**, 3480-3493
127. Ly-Huynh, J. D., Lieu, K. G., Major, A. T., Whiley, P. A., Holt, J. E., Loveland, K. L., and Jans, D. A. (2011) Importin alpha2-interacting proteins with nuclear roles during mammalian spermatogenesis. *Biol Reprod* **85**, 1191-1202
128. Whiley, P. A., Miyamoto, Y., McLachlan, R. I., Jans, D. A., and Loveland, K. L. (2012) Changing subcellular localization of nuclear transport factors during human spermatogenesis. *Int J Androl* **35**, 158-169
129. Miyamoto, Y., Baker, M. A., Whiley, P. A., Arjomand, A., Ludeman, J., Wong, C., Jans, D. A., and Loveland, K. L. (2013) Towards delineation of a developmental α-importome in the mammalian male germline. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **1833**, 731-742
130. Yasuhara, N., Shibazaki, N., Tanaka, S., Nagai, M., Kamikawa, Y., Oe, S., Asally, M., Kamachi, Y., Kondoh, H., and Yoneda, Y. (2007) Triggering neural differentiation of ES cells by subtype switching of importin-alpha.[Erratum appears in Nat Cell Biol. 2007 Apr;9(4):479]. *Nat Cell Biol* **9**, 72-79
131. Young, J. C., Major, A. T., Miyamoto, Y., Loveland, K. L., and Jans, D. A. (2011) Distinct effects of importin {alpha}2 and {alpha}4 on Oct3/4 localization and expression in mouse embryonic stem cells. *Faseb J*
132. Umegaki, N., Tamai, K., Nakano, H., Moritsugu, R., Yamazaki, T., Hanada, K., Katayama, I., and Kaneda, Y. (2007) Differential regulation of karyopherin alpha 2 expression by TGF-beta1 and IFN-gamma in normal human epidermal keratinocytes: evident contribution of KPNA2 for nuclear translocation of IRF-1. *J Invest Dermatol* **127**, 1456-1464
133. Mishra, K., and Parnaik, V. K. (1995) Essential role of protein phosphorylation in nuclear transport. *Exp Cell Res* **216**, 124-134
134. Gauthier-Rouviere, C., Vandromme, M., Lautredou, N., Cai, Q. Q., Girard, F., Fernandez, A., and Lamb, N. (1995) The serum response factor nuclear localization signal: general implications for cyclic AMP-dependent protein kinase activity in control of nuclear translocation. *Mol Cell Biol* **15**, 433-444

135. Schmitt, J. M., and Stork, P. J. S. (2002) PKA Phosphorylation of Src Mediates cAMP's Inhibition of Cell Growth via Rap1. *Mol Cell* **9**, 85-94
136. Kosako, H., Yamaguchi, N., Aranami, C., Ushiyama, M., Kose, S., Imamoto, N., Taniguchi, H., Nishida, E., and Hattori, S. (2009) Phosphoproteomics reveals new ERK MAP kinase targets and links ERK to nucleoporin-mediated nuclear transport. *Nat Struct Mol Biol* **16**, 1026-1035
137. Crampton, N., Kodiha, M., Shrivastava, S., Umar, R., and Stochaj, U. (2009) Oxidative stress inhibits nuclear protein export by multiple mechanisms that target FG nucleoporins and Crm1. *Mol Biol Cell* **20**, 5106-5116
138. Kodiha, M., Tran, D., Qian, C., Morogan, A., Presley, J. F., Brown, C. M., and Stochaj, U. (2008) Oxidative stress mislocalizes and retains transport factor importin-alpha and nucleoporins Nup153 and Nup88 in nuclei where they generate high molecular mass complexes. *Biochim Biophys Acta* **1783**, 405-418
139. Czubryt, M. P., Austria, J. A., and Pierce, G. N. (2000) Hydrogen peroxide inhibition of nuclear protein import is mediated by the mitogen-activated protein kinase, ERK2. *J Cell Biol* **148**, 7-16
140. Miyamoto, Y., Saiwaki, T., Yamashita, J., Yasuda, Y., Kotera, I., Shibata, S., Shigeta, M., Hiraoka, Y., Haraguchi, T., and Yoneda, Y. (2004) Cellular stresses induce the nuclear accumulation of importin alpha and cause a conventional nuclear import block. *J Cell Biol* **165**, 617-623
141. Kosako, H., and Imamoto, N. (2010) Phosphorylation of nucleoporins: signal transduction-mediated regulation of their interaction with nuclear transport receptors. *Nucleus* **1**, 309-313
142. Lott, K., and Cingolani, G. (2011) The importin beta binding domain as a master regulator of nucleocytoplasmic transport. *Biochim Biophys Acta* **1813**, 1578-1592
143. Wang, W., Yang, X., Kawai, T., Lopez de Silanes, I., Mazan-Mamczarz, K., Chen, P., Chook, Y. M., Quensel, C., Kohler, M., and Gorospe, M. (2004) AMP-activated protein kinase-regulated phosphorylation and acetylation of importin alpha1: involvement in the nuclear import of RNA-binding protein HuR. *J Biol Chem* **279**, 48376-48388
144. Yasuhara, N., Takeda, E., Inoue, H., Kotera, I., and Yoneda, Y. (2004) Importin alpha/beta-mediated nuclear protein import is regulated in a cell cycle-dependent manner. *Exp Cell Res* **297**, 285-293
145. Scherf, U., Kalab, P., Dasso, M., Pastan, I., and Brinkmann, U. (1998) The hCSE1/CAS protein is phosphorylated by HeLa extracts and MEK-1: MEK-1 phosphorylation may modulate the intracellular localization of CAS. *Biochem Biophys Res Commun* **250**, 623-628
146. Lorenzato, A., Biolatti, M., Delogu, G., Capobianco, G., Farace, C., Dessole, S., Cossu, A., Tanda, F., Madeddu, R., Olivero, M., and Di Renzo, M. F. (2013) AKT activation drives the nuclear localization of CSE1L and a pro-oncogenic transcriptional activation in ovarian cancer cells. *Exp Cell Res* **319**, 2627-2636
147. Hutchins, J. R. A., Moore, W. J., Hood, F. E., Wilson, J. S. J., Andrews, P. D., Swedlow, J. R., and Clarke, P. R. Phosphorylation Regulates the Dynamic Interaction of RCC1 with Chromosomes during Mitosis. *Curr Biol* **14**, 1099-1104
148. Wu, Z., Jiang, Q., Clarke, P. R., and Zhang, C. (2013) Phosphorylation of Crm1 by CDK1-cyclin-B promotes Ran-dependent mitotic spindle assembly. *J Cell Sci* **126**, 3417-3428
149. Fulcher, A. J., Roth, D. M., Fatima, S., Alvisi, G., and Jans, D. A. (2010) The BRCA-1 binding protein BRAP2 is a novel, negative regulator of nuclear import of viral proteins, dependent on phosphorylation flanking the nuclear localization signal. *Faseb J* **24**, 1454-1466

150. Asada, M., Ohmi, K., Delia, D., Enosawa, S., Suzuki, S., Yuo, A., Suzuki, H., and Mizutani, S. (2004) Brap2 functions as a cytoplasmic retention protein for p21 during monocyte differentiation. *Mol Cell Biol* **24**, 8236-8243
151. Lieu, K. G., Shim, E. H., Wang, J., Lokareddy, R. K., Tao, T., Cingolani, G., Zambetti, G. P., and Jans, D. A. (2014) The p53-induced factor Ei24 inhibits nuclear import through an importin beta-binding-like domain. *J Cell Biol* **205**, 301-312
152. Huang, S., Chang, I. S., Lin, W., Ye, W., Luo, R. Z., Lu, Z., Lu, Y., Zhang, K., Liao, W. S. L., Tao, T., Bast, R. C., Jr., Chen, X., and Yu, Y. ARHI (DIRAS3), an imprinted tumour suppressor gene, binds to importins and blocks nuclear import of cargo proteins. *Biosci Rep* **30**, 159-168
153. King, F. W., and Shtivelman, E. (2004) Inhibition of nuclear import by the proapoptotic protein CC3. *Mol Cell Biol* **24**, 7091-7101
154. Poon, I. K. H., and Jans, D. A. (2005) Regulation of nuclear transport: central role in development and transformation? *Traffic* **6**, 173-186
155. Stommel, J. M., Marchenko, N. D., Jimenez, G. S., Moll, U. M., Hope, T. J., and Wahl, G. M. (1999) A leucine-rich nuclear export signal in the p53 tetramerization domain: regulation of subcellular localization and p53 activity by NES masking. *Embo J* **18**, 1660-1672
156. Nikolaev, A. Y., Li, M., Puskas, N., Qin, J., and Gu, W. (2003) Parc: a cytoplasmic anchor for p53. *Cell* **112**, 29-40
157. Sane, S., Abdullah, A., Boudreau, D. A., Autenried, R. K., Gupta, B. K., Wang, X., Wang, H., Schlenker, E. H., Zhang, D., Telleria, C., Huang, L., Chauhan, S. C., and Rezvani, K. (2014) Ubiquitin-like (UBX)-domain-containing protein, UBXN2A, promotes cell death by interfering with the p53-Mortalin interactions in colon cancer cells. *Cell Death Dis* **5**, e1118
158. Woo, M. G., Xue, K., Liu, J., McBride, H., and Tsang, B. K. (2012) Calpain-mediated processing of p53-associated parkin-like cytoplasmic protein (PARC) affects chemosensitivity of human ovarian cancer cells by promoting p53 subcellular trafficking. *J Biol Chem* **287**, 3963-3975
159. Yasuda, Y., Miyamoto, Y., Yamashiro, T., Asally, M., Masui, A., Wong, C., Loveland, K. L., and Yoneda, Y. (2012) Nuclear retention of importin alpha coordinates cell fate through changes in gene expression. *Embo J* **31**, 83-94
160. Sekimoto, T., Miyamoto, Y., Arai, S., and Yoneda, Y. (2011) Importin alpha protein acts as a negative regulator for Snail protein nuclear import. *J Biol Chem* **286**, 15126-15131
161. Kim, B. J., and Lee, H. (2006) Importin-β Mediates Cdc7 Nuclear Import by Binding to the Kinase Insert II Domain, Which Can Be Antagonized by Importin-α. *J Biol Chem* **281**, 12041-12049
162. Quimby, B. B., Wilson, C. A., and Corbett, A. H. (2000) The interaction between Ran and NTF2 is required for cell cycle progression. *Mol Biol Cell* **11**, 2617-2629
163. Yamaguchi, R., and Newport, J. (2003) A role for Ran-GTP and Crm1 in blocking re-replication. *Cell* **113**, 115-125
164. Mosammaparast, N., and Pemberton, L. F. (2004) Karyopherins: from nuclear-transport mediators to nuclear-function regulators. *Trends Cell Biol* **14**, 547-556
165. Laurell, E., Beck, K., Krupina, K., Theerthagiri, G., Bodenmiller, B., Horvath, P., Aebersold, R., Antonin, W., and Kutay, U. (2011) Phosphorylation of Nup98 by multiple kinases is crucial for NPC disassembly during mitotic entry. *Cell* **144**, 539-550
166. Clarke, P. R., and Zhang, C. (2008) Spatial and temporal coordination of mitosis by Ran GTPase. *Nat Rev Mol Cell Biol* **9**, 464-477
167. Rensen, W. M., Mangiacasale, R., Ciciarello, M., and Lavia, P. (2008) The GTPase Ran: regulation of cell life and potential roles in cell transformation. *Front Biosci* **13**, 4097-4121

168. Budhu, A. S., and Wang, X. W. (2005) Loading and unloading: orchestrating centrosome duplication and spindle assembly by Ran/Crm1. *Cell Cycle* **4**, 1510-1514
169. Roscioli, E., Di Francesco, L., Bolognesi, A., Giubettini, M., Orlando, S., Harel, A., Schinina, M. E., and Lavia, P. (2012) Importin-beta negatively regulates multiple aspects of mitosis including RANGAP1 recruitment to kinetochores. *J Cell Biol*
170. Behrens, P., Brinkmann, U., and Wellmann, A. (2003) CSE1L/CAS: its role in proliferation and apoptosis. *Apoptosis* **8**, 39-44
171. Scherf, U., Pastan, I., Willingham, M. C., and Brinkmann, U. (1996) The human CAS protein which is homologous to the CSE1 yeast chromosome segregation gene product is associated with microtubules and mitotic spindle. *Proc Natl Acad Sci U S A* **93**, 2670-2674
172. Ogryzko, V. V., Brinkmann, E., Howard, B. H., Pastan, I., and Brinkmann, U. (1997) Antisense inhibition of CAS, the human homologue of the yeast chromosome segregation gene CSE1, interferes with mitosis in HeLa cells. *Biochemistry* **36**, 9493-9500
173. Brinkmann, U., Brinkmann, E., Gallo, M., Scherf, U., and Pastan, I. (1996) Role of CAS, a human homologue to the yeast chromosome segregation gene CSE1, in toxin and tumor necrosis factor mediated apoptosis. *Biochemistry* **35**, 6891-6899
174. Liao, C.-F., Luo, S.-F., Li, L.-T., Lin, C.-Y., Chen, Y.-C., and Jiang, M.-C. (2008) CSE1L/CAS, the cellular apoptosis susceptibility protein, enhances invasion and metastasis but not proliferation of cancer cells. *J Exp Clin Cancer Res* **27**, 15
175. Tanaka, T., Ohkubo, S., Tatsuno, I., and Prives, C. (2007) hCAS/CSE1L associates with chromatin and regulates expression of select p53 target genes.[see comment]. *Cell* **130**, 638-650
176. Liao, C. F., Lin, S. H., Chen, H. C., Tai, C. J., Chang, C. C., Li, L. T., Yeh, C. M., Yeh, K. T., Chen, Y. C., Hsu, T. H., Shen, S. C., Lee, W. R., Chiou, J. F., Luo, S. F., and Jiang, M. C. (2012) CSE1L, a novel microvesicle membrane protein, mediates Ras-triggered microvesicle generation and metastasis of tumor cells. *Mol Med* **18**, 1269-1280
177. Tai, C. J., Hsu, C. H., Shen, S. C., Lee, W. R., and Jiang, M. C. (2010) Cellular apoptosis susceptibility (CSE1L/CAS) protein in cancer metastasis and chemotherapeutic drug-induced apoptosis. *J Exp Clin Cancer Res* **29**, 110
178. Burns, L. T., and Wente, S. R. (2014) From Hypothesis to Mechanism: Uncovering Nuclear Pore Complex Links to Gene Expression. *Mol Cell Biol* **34**, 2114-2120
179. Griffis, E. R., Altan, N., Lippincott-Schwartz, J., and Powers, M. A. (2002) Nup98 is a mobile nucleoporin with transcription-dependent dynamics. *Mol Biol Cell* **13**, 1282-1297
180. Powers, M. A., Forbes, D. J., Dahlberg, J. E., and Lund, E. (1997) The vertebrate GLFG nucleoporin, Nup98, is an essential component of multiple RNA export pathways. *J Cell Biol* **136**, 241-250
181. Singer, S., Zhao, R., Barsotti, A. M., Ouwehand, A., Fazollahi, M., Coutavas, E., Breuhahn, K., Neumann, O., Longerich, T., Pusterla, T., Powers, M. A., Giles, K. M., Leedman, P. J., Hess, J., Grunwald, D., Bussemaker, H. J., Singer, R. H., Schirmacher, P., and Prives, C. (2012) Nuclear pore component Nup98 is a potential tumor suppressor and regulates posttranscriptional expression of select p53 target genes. *Mol Cell* **48**, 799-810
182. Miyamoto, Y., Hieda, M., Harreman, M. T., Fukumoto, M., Saiwaki, T., Hodel, A. E., Corbett, A. H., and Yoneda, Y. (2002) Importin [alpha] can migrate into the nucleus in an importin [beta]- and Ran-independent manner. *Embo J* **21**, 5833-5842
183. Miyamoto, Y., Loveland, K. L., and Yoneda, Y. (2012) Nuclear importin alpha and its physiological importance. *Commun Integr Biol* **5**, 220-222

184. Faustino, R. S., Nelson, T. J., Terzic, A., and Perez-Terzic, C. (2007) Nuclear transport: target for therapy. *Clin Pharmacol Ther* **81**, 880-886
185. Hill, R., Cautain, B., de Pedro, N., and Link, W. (2013) *Targeting nucleocytoplasmic transport in cancer therapy* Vol. 5
186. Hung, M. C., and Link, W. (2011) Protein localization in disease and therapy. *J Cell Sci* **124**, 3381-3392
187. Sato, F., Abraham, J. M., Yin, J., Kan, T., Ito, T., Mori, Y., Hamilton, J. P., Jin, Z., Cheng, Y., Paun, B., Berki, A. T., Wang, S., Shimada, Y., and Meltzer, S. J. (2006) Polo-like kinase and survivin are esophageal tumor-specific promoters. *Biochem Biophys Res Commun* **342**, 465-471
188. Dahl, E., Kristiansen, G., Gottlob, K., Klaman, I., Ebner, E., Hinzmann, B., Hermann, K., Pilarsky, C., x00Fc, rst, M., Klinkhammer-Schalke, M., Blaszyk, H., Knuechel, R., Hartmann, A., Rosenthal, A., x00E, and Wild, P. J. (2006) Molecular profiling of laser-microdissected matched tumor and normal breast tissue identifies karyopherin alpha2 as a potential novel prognostic marker in breast cancer. *Clin Cancer Res* **12**, 3950-3960
189. Kim, S. Y., Kang, H. T., Han, J. A., and Park, S. C. (2012) The transcription factor Sp1 is responsible for aging-dependent altered nucleocytoplasmic trafficking. *Aging Cell* **11**, 1102-1109
190. van der Watt, P. J., Ngarande, E., and Leaner, V. D. (2011) Overexpression of Kpnbeta1 and Kpnalpha2 importin proteins in cancer derives from deregulated E2F activity. *PLoS ONE* **6**, e27723
191. Deacon, K., Onion, D., Kumari, R., Watson, S. A., and Knox, A. J. (2012) Elevated SP-1 transcription factor expression and activity drives basal and hypoxia-induced vascular endothelial growth factor (VEGF) expression in non-small cell lung cancer. *J Biol Chem* **287**, 39967-39981
192. Li, L., and Davie, J. R. (2010) The role of Sp1 and Sp3 in normal and cancer cell biology. *Ann Anat* **192**, 275-283
193. Nevins, J. R. (2001) The Rb/E2F pathway and cancer. *Hum Mol Genet* **10**, 699-703
194. Noetzel, E., Rose, M., Bornemann, J., Gajewski, M., Knuchel, R., and Dahl, E. (2012) Nuclear transport receptor karyopherin-alpha2 promotes malignant breast cancer phenotypes in vitro. *Oncogene* **31**, 2101-2114
195. Huang, L., Wang, H. Y., Li, J. D., Wang, J. H., Zhou, Y., Luo, R. Z., Yun, J. P., Zhang, Y., Jia, W. H., and Zheng, M. (2013) KPNA2 promotes cell proliferation and tumorigenicity in epithelial ovarian carcinoma through upregulation of c-Myc and downregulation of FOXO3a. *Cell Death Dis* **4**, e745
196. Altan, B., Yokobori, T., Mochiki, E., Ohno, T., Ogata, K., Ogawa, A., Yanai, M., Kobayashi, T., Luvsandagva, B., Asao, T., and Kuwano, H. (2013) Nuclear karyopherin-alpha2 expression in primary lesions and metastatic lymph nodes was associated with poor prognosis and progression in gastric cancer. *Carcinogenesis* **34**, 2314-2321
197. van der Watt, P. J., Maske, C. P., Hendricks, D. T., Parker, M. I., Denny, L., Govender, D., Birrer, M. J., and Leaner, V. D. (2009) The Karyopherin proteins, Crm1 and Karyopherin beta1, are overexpressed in cervical cancer and are critical for cancer cell survival and proliferation. *Int J Cancer* **124**, 1829-1840
198. Gluz, O., Wild, P., Meiler, R., Diallo-Danebrock, R., Ting, E., Mohrmann, S., Schuett, G., Dahl, E., Fuchs, T., Herr, A., Gaumann, A., Frick, M., Poremba, C., Nitz, U. A., and Hartmann, A.

(2008) Nuclear karyopherin alpha2 expression predicts poor survival in patients with advanced breast cancer irrespective of treatment intensity. *Int J Cancer* **123**, 1433-1438

199. Sotiriou, C., Wirapati, P., Loi, S., Harris, A., Fox, S., Smeds, J., Nordgren, H., Farmer, P., Praz, V., Haibe-Kains, B., Desmedt, C., Larsimont, D., Cardoso, F., Peterse, H., Nuyten, D., Buyse, M., Van de Vijver, M. J., Bergh, J., Piccart, M., and Delorenzi, M. (2006) Gene expression profiling in breast cancer: understanding the molecular basis of histologic grade to improve prognosis. *J Natl Cancer Inst* **98**, 262-272
200. Dankof, A., Fritzsche, F. R., Dahl, E., Pahl, S., Wild, P., Dietel, M., Hartmann, A., and Kristiansen, G. (2007) KPNA2 protein expression in invasive breast carcinoma and matched peritumoral ductal carcinoma in situ. *Virchows Arch* **451**, 877-881
201. Wang, Y., Klijn, J. G. M., Zhang, Y., Sieuwerts, A. M., Look, M. P., Yang, F., Talantov, D., Timmermans, M., Meijer-van Gelder, M. E., Yu, J., Jatkoe, T., Berns, E. M. J. J., Atkins, D., and Foekens, J. A. (2005) Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. *Lancet* **365**, 671-679
202. Kim, I. S., Kim, D. H., Han, S. M., Chin, M. U., Nam, H. J., Cho, H. P., Choi, S. Y., Song, B. J., Kim, E. R., Bae, Y. S., and Moon, Y. H. (2000) Truncated form of importin alpha identified in breast cancer cell inhibits nuclear import of p53. *J Biol Chem* **275**, 23139-23145
203. He, L., Ding, H., Wang, J. H., Zhou, Y., Li, L., Yu, Y. H., Huang, L., Jia, W. H., Zeng, M., Yun, J. P., Luo, R. Z., and Zheng, M. (2012) Overexpression of karyopherin 2 in human ovarian malignant germ cell tumor correlates with poor prognosis. *PLoS ONE* **7**, e42992
204. Zheng, M., Tang, L., Huang, L., Ding, H., Liao, W.-T., Zeng, M.-S., and Wang, H.-Y. (2010) Overexpression of karyopherin-2 in epithelial ovarian cancer and correlation with poor prognosis. *Obstet Gynecol* **116**, 884-891
205. Grupp, K., Habermann, M., Sirma, H., Simon, R., Steurer, S., Hube-Magg, C., Prien, K., Burkhardt, L., Jedrzejewska, K., Salomon, G., Heinzer, H., Wilczak, W., Kluth, M., Izbicki, J. R., Sauter, G., Minner, S., Schlomm, T., and Tsourlakis, M. C. (2013) High nuclear karyopherin alpha 2 expression is a strong and independent predictor of biochemical recurrence in prostate cancer patients treated by radical prostatectomy. *Mod Pathol*
206. Rachidi, S. M., Qin, T., Sun, S., Zheng, W. J., and Li, Z. (2013) Molecular Profiling of Multiple Human Cancers Defines an Inflammatory Cancer-Associated Molecular Pattern and Uncovers KPNA2 as a Uniform Poor Prognostic Cancer Marker. *PLoS ONE* **8**, e57911
207. Sakai, M., Sohda, M., Miyazaki, T., Suzuki, S., Sano, A., Tanaka, N., Inose, T., Nakajima, M., Kato, H., and Kuwano, H. (2010) Significance of karyopherin-{alpha} 2 (KPNA2) expression in esophageal squamous cell carcinoma. *Anticancer Res* **30**, 851-856
208. Troyanskaya, O. G., Garber, M. E., Brown, P. O., Botstein, D., and Altman, R. B. (2002) Nonparametric methods for identifying differentially expressed genes in microarray data. *Bioinformatics* **18**, 1454-1461
209. Li, C., Ji, L., Ding, Z. Y., Zhang, Q. D., and Huang, G. R. (2013) Overexpression of KPNA2 correlates with poor prognosis in patients with gastric adenocarcinoma. *Tumour Biol* **34**, 1021-1026
210. Jensen, J. B., Munksgaard, P. P., Sorensen, C. M., Fristrup, N., Birkenkamp-Demtroder, K., Ulhoi, B. P., Jensen, K. M.-E., Orntoft, T. F., and Dyrskjot, L. (2011) High expression of karyopherin-alpha2 defines poor prognosis in non-muscle-invasive bladder cancer and in patients with invasive bladder cancer undergoing radical cystectomy. *Eur Urol* **59**, 841-848
211. Yoshitake, K., Tanaka, S., Mogushi, K., Aihara, A., Murakata, A., Matsumura, S., Mitsunori, Y., Yasen, M., Ban, D., Noguchi, N., Irie, T., Kudo, A., Nakamura, N., Tanaka, H., and Arii, S.

(2011) Importin-α1 as a Novel Prognostic Target for Hepatocellular Carcinoma. *Annals of Surgical Oncology*, 1-11

212. Gousias, K., Becker, A. J., Simon, M., and Niehusmann, P. (2012) Nuclear karyopherin a2: a novel biomarker for infiltrative astrocytomas. *J Neurooncol* **109**, 545-553
213. Winnepenninckx, V., x00E, ronique, Lazar, V., Michiels, S., Dessen, P., Stas, M., Alonso, S. R., Avril, M.-F., x00E, oise, Ortiz Romero, P. L., Robert, T., Balacescu, O., Eggermont, A. M. M., Lenoir, G., Sarasin, A., Tursz, T., van den Oord, J. J., Spatz, A., and Melanoma Group of the European Organization for Research and Treatment of, C. (2006) Gene expression profiling of primary cutaneous melanoma and clinical outcome. *J Natl Cancer Inst* **98**, 472-482
214. Mascarenhas, C. D., Ferreira da Cunha, A., Brugnerotto, A. F., Gambero, S., de Almeida, M. H., Carazzolle, M. F., Pagnano, K. B., Traina, F., Costa, F. F., and de Souza, C. A. (2013) Identification of Target Genes Using Gene Expression Profile of Granulocytes From Chronic Myeloid Leukemia (Cml) Patients Treated With Tyrosine Kinase Inhibitors. *Leuk Lymphoma*
215. Laurila, E., Vuorinen, E., Savinainen, K., Rauhala, H., and Kallioniemi, A. (2013) KPNA7, a nuclear transport receptor, promotes malignant properties of pancreatic cancer cells in vitro. *Exp Cell Res*
216. Wu, M.-S., Lin, Y.-S., Chang, Y.-T., Shun, C.-T., Lin, M.-T., and Lin, J.-T. (2005) Gene expression profiling of gastric cancer by microarray combined with laser capture microdissection. *World J Gastroenterol* **11**, 7405-7412
217. Dyrskjot, L., Kruhoffer, M., Thykjaer, T., Marcussen, N., Jensen, J. L., Moller, K., and Orntoft, T. F. (2004) Gene expression in the urinary bladder: a common carcinoma in situ gene expression signature exists disregarding histopathological classification. *Cancer Res* **64**, 4040-4048
218. Eschrich, S., Yang, I., Bloom, G., Kwong, K. Y., Boulware, D., Cantor, A., Coppola, D., Kruh, x00F, ffer, M., Aaltonen, L., Orntoft, T. F., Quackenbush, J., and Yeatman, T. J. (2005) Molecular staging for survival prediction of colorectal cancer patients. *J Clin Oncol* **23**, 3526-3535
219. Li, S. R., Gyselman, V. G., Dorudi, S., and Bustin, S. A. (2000) Elevated levels of RanBP7 mRNA in colorectal carcinoma are associated with increased proliferation and are similar to the transcription pattern of the proto-oncogene c-myc. *Biochem Biophys Res Commun* **271**, 537-543
220. Noske, A., Weichert, W., Niesporek, S., x00F, ske, A., Buckendahl, A.-C., Koch, I., Sehouli, J., Dietel, M., and Denkert, C. (2008) Expression of the nuclear export protein chromosomal region maintenance/exportin 1/Xpo1 is a prognostic factor in human ovarian cancer. *Cancer* **112**, 1733-1743
221. Faggad, A., Darb-Esfahani, S., Wirtz, R., Sinn, B., Sehouli, J., x00F, nsgen, D., Lage, H., Weichert, W., Noske, A., Budczies, J., x00Fc, ller, B. M., Buckendahl, A.-C., x00F, ske, A., Eldin Elwali, N., Dietel, M., and Denkert, C. (2009) Topoisomerase IIalpha mRNA and protein expression in ovarian carcinoma: correlation with clinicopathological factors and prognosis. *Mod Pathol* **22**, 579-588
222. van der Watt, P. J., and Leaner, V. D. The nuclear exporter, Crm1, is regulated by NFY and Sp1 in cancer cells and repressed by p53 in response to DNA damage. *Biochim Biophys Acta* **1809**, 316-326
223. Huang, W.-y., Yue, L., Qiu, W.-s., Wang, L.-W., Zhou, X.-h., and Sun, Y.-j. (2009) Prognostic value of CRM1 in pancreas cancer. *Clin Invest Med* **32**, E315

224. Akagi, I., Okayama, H., Schetter, A. J., Robles, A. I., Kohno, T., Bowman, E. D., Kazandjian, D., Welsh, J. A., Oue, N., Saito, M., Miyashita, M., Uchida, E., Takizawa, T., Takenoshita, S., Skaug, V., Mollerup, S., Haugen, A., Yokota, J., and Harris, C. C. (2013) Combination of protein coding and noncoding gene expression as a robust prognostic classifier in stage I lung adenocarcinoma. *Cancer Res* **73**, 3821-3832
225. Shen, A., Wang, Y., Zhao, Y., Zou, L., Sun, L., and Cheng, C. (2009) Expression of CRM1 in human gliomas and its significance in p27 expression and clinical prognosis. *Neurosurgery* **65**, 153-159; discussion 159-160
226. Yao, Y., Dong, Y., Lin, F., Zhao, H., Shen, Z., Chen, P., Sun, Y.-J., Tang, L.-N., and Zheng, S.-E. (2009) The expression of CRM1 is associated with prognosis in human osteosarcoma. *Oncol Rep* **21**, 229-235
227. Zender, L., Xue, W., Zuber, J., Semighini, C. P., Krasnitz, A., Ma, B., Zender, P., Kubicka, S., Luk, J. M., Schirmacher, P., McCombie, W. R., Wigler, M., Hicks, J., Hannon, G. J., Powers, S., and Lowe, S. W. (2008) An oncogenomics-based in vivo RNAi screen identifies tumor suppressors in liver cancer. *Cell* **135**, 852-864
228. Liang, X. T., Pan, K., Chen, M. S., Li, J. J., Wang, H., Zhao, J. J., Sun, J. C., Chen, Y. B., Ma, H. Q., Wang, Q. J., and Xia, J. C. (2011) Decreased expression of XPO4 is associated with poor prognosis in hepatocellular carcinoma. *J Gastroenterol Hepatol* **26**, 544-549
229. Catto, J. W. F., Miah, S., Owen, H. C., Bryant, H., Myers, K., Dudziec, E., Larr, x00E, St, x00E, phane, Milo, M., Rehman, I., Rosario, D. J., Di Martino, E., Knowles, M. A., Meuth, M., Harris, A. L., and Hamdy, F. C. (2009) Distinct microRNA alterations characterize high- and low-grade bladder cancer. *Cancer Res* **69**, 8472-8481
230. Roe, O. D., Anderssen, E., Sandeck, H., Christensen, T., Larsson, E., and Lundgren, S. (2010) Malignant pleural mesothelioma: genome-wide expression patterns reflecting general resistance mechanisms and a proposal of novel targets. *Lung Cancer* **67**, 57-68
231. Shiraki, K., Fujikawa, K., Sugimoto, K., Ito, T., Yamanaka, T., Suzuki, M., Yoneda, K., Takase, K., and Nakano, T. (2006) Cellular apoptosis susceptibility protein and proliferation in human hepatocellular carcinoma. *Int J Mol Med* **18**, 77-81
232. Behrens, P., Brinkmann, U., Fogt, F., Wernert, N., and Wellmann, A. (2001) Implication of the proliferation and apoptosis associated CSE1L/CAS gene for breast cancer development. *Anticancer Res* **21**, 2413-2417
233. Brinkmann, U., Gallo, M., Polymeropoulos, M. H., and Pastan, I. (1996) The human CAS (cellular apoptosis susceptibility) gene mapping on chromosome 20q13 is amplified in BT474 breast cancer cells and part of aberrant chromosomes in breast and colon cancer cell lines. *Genome Res* **6**, 187-194
234. Brustmann, H. (2004) Expression of cellular apoptosis susceptibility protein in serous ovarian carcinoma: a clinicopathologic and immunohistochemical study. *Gynecol Oncol* **92**, 268-276
235. Ouellet, V., x00E, ronique, Guyot, M.-C., Le Page, C., x00E, cile, Filali-Mouhim, A., Lussier, C., Tonin, P. N., Provencher, D. M., and Mes-Masson, A.-M. (2006) Tissue array analysis of expression microarray candidates identifies markers associated with tumor grade and outcome in serous epithelial ovarian cancer. *Int J Cancer* **119**, 599-607
236. Stawerski, P., Wagrowska-Danilewicz, M., Stasikowska, O., and Danilewicz, M. (2010) Immunoexpression of CAS protein is augmented in high grade serous ovarian tumors. *Pol J Pathol* **61**, 219-223

237. Peiro, G., Diebold, J., Baretton, G. B., Kimmig, R., x00F, and hrs, U. (2001) Cellular apoptosis susceptibility gene expression in endometrial carcinoma: correlation with Bcl-2, Bax, and caspase-3 expression and outcome. *Int J Gynecol Pathol* **20**, 359-367
238. Wellmann, A., Flemming, P., Behrens, P., Wuppermann, K., Lang, H., Oldhafer, K., Pastan, I., and Brinkmann, U. (2001) High expression of the proliferation and apoptosis associated CSE1L/CAS gene in hepatitis and liver neoplasms: correlation with tumor progression. *Int J Mol Med* **7**, 489-494
239. Böni, R., Wellmann, A., Man, Y. G., Hofbauer, G., and Brinkmann, U. (1999) Expression of the proliferation and apoptosis-associated CAS protein in benign and malignant cutaneous melanocytic lesions. *Am J Dermatopathol* **21**, 125-128
240. Chang, C.-C., Tai, C.-J., Su, T.-C., Shen, K.-H., Lin, S.-H., Yeh, C.-M., Yeh, K.-T., Lin, Y.-M., and Jiang, M.-C. (2012) The prognostic significance of nuclear CSE1L in urinary bladder urothelial carcinomas. *Ann Diagn Pathol* **16**, 362-368
241. Tai, C. J., Liao, C. F., Su, T. C., Shen, K. H., Chang, C. C., Lin, S. H., Yeh, C. M., Shen, S. C., Lee, W. R., Chiou, J. F., Lin, C. H., Chen, Y. C., Shih, H. Y., Yeh, K. T., and Jiang, M. C. (2012) Presence of CSE1L protein in urine of patients with urinary bladder urothelial carcinomas. *Int J Biol Markers* **27**, e280-284
242. Tai, C.-J., Su, T.-C., Jiang, M.-C., Chen, H.-C., Shen, S.-C., Lee, W.-R., Liao, C.-F., Chen, Y.-C., Lin, S.-H., Li, L.-T., Shen, K.-H., Yeh, C.-M., Yeh, K.-T., Lee, C.-H., Shih, H.-Y., and Chang, C.-C. (2013) Correlations between cytoplasmic CSE1L in neoplastic colorectal glands and depth of tumor penetration and cancer stage. *Journal of Translational Medicine* **11**, 29
243. Bar-Shira, A., Pinthus, J. H., Rozovsky, U., Goldstein, M., Sellers, W. R., Yaron, Y., Eshhar, Z., and Orr-Urtreger, A. (2002) Multiple genes in human 20q13 chromosomal region are involved in an advanced prostate cancer xenograft. *Cancer Res* **62**, 6803-6807
244. Bertucci, F., x00E, ois, Salas, S., x00E, bastien, Eysteries, S., x00E, verine, Nasser, V., x00E, ry, Finetti, P., Ginestier, C., Charafe-Jauffret, E., Loriod, B., x00E, atrice, Bachelart, L., x00Ef, Montfort, J., x00E, x00F, me, Victorero, G., x00E, ve, Viret, F., x00E, x00E, ric, Ollendorff, V., Fert, V., Giovaninni, M., Delpero, J.-R., Nguyen, C., Viens, P., Monges, G., x00E, ve, Birnbaum, D., Houlgatte, R., x00E, and mi (2004) Gene expression profiling of colon cancer by DNA microarrays and correlation with histoclinical parameters. *Oncogene* **23**, 1377-1391
245. Brinkmann, U., Brinkmann, E., Gallo, M., and Pastan, I. (1995) Cloning and characterization of a cellular apoptosis susceptibility gene, the human homologue to the yeast chromosome segregation gene CSE1. *Proc Natl Acad Sci U S A* **92**, 10427-10431
246. Brinkmann, U. (1998) CAS, the human homologue of the yeast chromosome-segregation gene CSE1, in proliferation, apoptosis, and cancer. *Am J Hum Genet* **62**, 509-513
247. Wellmann, A., Krenacs, L., Fest, T., Scherf, U., Pastan, I., Raffeld, M., and Brinkmann, U. (1997) Localization of the cell proliferation and apoptosis-associated CAS protein in lymphoid neoplasms. *Am J Pathol* **150**, 25-30
248. Agudo, D., Gomez-Esquer, F., Martinez-Arribas, F., Nunez-Villar, M. J., Pollan, M., and Schneider, J. (2004) Nup88 mRNA overexpression is associated with high aggressiveness of breast cancer. *Int J Cancer* **109**, 717-720
249. Brustmann, H., and Hager, M. (2009) Nucleoporin 88 expression in normal and neoplastic squamous epithelia of the uterine cervix. *Ann Diagn Pathol* **13**, 303-307
250. Schneider, J., Martinez-Arribas, F., and Torrejon, R. (2010) Nup88 expression is associated with myometrial invasion in endometrial carcinoma. *Int J Gynecol Cancer* **20**, 804-808

251. Knoess, M., Kurz, A. K., Goreva, O., Bektas, N., Breuhahn, K., Odenthal, M., Schirmacher, P., Dienes, H. P., Bock, C. T., Zentgraf, H., and zur Hausen, A. (2006) Nucleoporin 88 expression in hepatitis B and C virus-related liver diseases. *World J Gastroenterol* **12**, 5870-5874
252. Zhang, Z.-Y., Zhao, Z.-R., Jiang, L., Li, J.-C., Gao, Y.-M., Cui, D.-S., Wang, C.-J., Schneider, J., Wang, M.-W., and Sun, X.-F. (2007) Nup88 expression in normal mucosa, adenoma, primary adenocarcinoma and lymph node metastasis in the colorectum. *Tumour Biol* **28**, 93-99
253. Emterling, A., Skoglund, J., Arbman, G., Schneider, J., Evertsson, S., Carstensen, J., Zhang, H., and Sun, X.-F. (2003) Clinicopathological significance of Nup88 expression in patients with colorectal cancer. *Oncology* **64**, 361-369
254. Zhao, Z. R., Zhang, L. J., Wang, Y. Y., Li, F., Wang, M. W., and Sun, X. F. (2012) Increased serum level of Nup88 protein is associated with the development of colorectal cancer. *Med Oncol* **29**, 1789-1795
255. Zhang, H., Schneider, J., and Rosdahl, I. (2002) Expression of p16, p27, p53, p73 and Nup88 proteins in matched primary and metastatic melanoma cells. *Int J Oncol* **21**, 43-48
256. Martinez, N., Alonso, A., Moragues, M. D., Ponton, J., and Schneider, J. (1999) The nuclear pore complex protein Nup88 is overexpressed in tumor cells. *Cancer Res* **59**, 5408-5411
257. Xu, S., and Powers, M. A. (2009) Nuclear pore proteins and cancer. *Semin Cell Dev Biol* **20**, 620-630
258. Saito, S., Miyaji-Yamaguchi, M., and Nagata, K. (2004) Aberrant intracellular localization of SET-CAN fusion protein, associated with a leukemia, disorganizes nuclear export. *Int J Cancer* **111**, 501-507
259. London, C. A., Bernabe, L. F., Barnard, S., Kisseberth, W. C., Borgatti, A., Henson, M., Wilson, H., Jensen, K., Ito, D., Modiano, J. F., Bear, M. D., Pennell, M. L., Saint-Martin, J.-R., McCauley, D., Kauffman, M., and Shacham, S. (2014) Preclinical Evaluation of the Novel, Orally Bioavailable Selective Inhibitor of Nuclear Export (SINE) KPT-335 in Spontaneous Canine Cancer: Results of a Phase I Study. *PLoS ONE* **9**, e87585
260. Turner, J. G., Dawson, J., and Sullivan, D. M. (2012) Nuclear export of proteins and drug resistance in cancer. *Biochem Pharmacol* **83**, 1021-1032
261. Stella Tsai, C.-S., Chen, H.-C., Tung, J.-N., Tsou, S.-S., Tsao, T.-Y., Liao, C.-F., Chen, Y.-C., Yeh, C.-Y., Yeh, K.-T., and Jiang, M.-C. (2010) Serum cellular apoptosis susceptibility protein is a potential prognostic marker for metastatic colorectal cancer. *Am J Pathol* **176**, 1619-1628
262. Tung, M.-C., Tsai, C.-S. S., Tung, J.-N., Tsao, T.-Y., Chen, H.-C., Yeh, K.-T., Liao, C.-F., and Jiang, M.-C. (2009) Higher prevalence of secretory CSE1L/CAS in sera of patients with metastatic cancer. *Cancer Epidemiol Biomarkers Prev* **18**, 1570-1577
263. Deryugina, E. I., and Quigley, J. P. (2006) Matrix metalloproteinases and tumor metastasis. *Cancer Metastasis Rev* **25**, 9-34
264. Tai, C. J., Shen, S. C., Lee, W. R., Liao, C. F., Deng, W. P., Chiou, H. Y., Hsieh, C. I., Tung, J. N., Chen, C. S., Chiou, J. F., Li, L. T., Lin, C. Y., Hsu, C. H., and Jiang, M. C. (2010) Increased cellular apoptosis susceptibility (CSE1L/CAS) protein expression promotes protrusion extension and enhances migration of MCF-7 breast cancer cells. *Exp Cell Res* **316**, 2969-2981
265. Jiang, M.-C., Yeh, C.-M., Tai, C.-J., Chen, H.-C., Lin, S.-H., Su, T.-C., Shen, S.-C., Lee, W.-R., Liao, C.-F., Li, L.-T., Lee, C.-H., Chen, Y.-C., Yeh, K.-T., and Chang, C.-C. (2013) CSE1L modulates Ras-induced cancer cell invasion: correlation of K-Ras mutation and CSE1L expression in colorectal cancer progression. *Am J Surg* **206**, 418-427

266. Bernad, R., Engelsma, D., Sanderson, H., Pickersgill, H., and Fornerod, M. (2006) Nup214-Nup88 nucleoporin subcomplex is required for CRM1-mediated 60 S preribosomal nuclear export. *J Biol Chem* **281**, 19378-19386
267. Hashizume, C., Nakano, H., Yoshida, K., and Wong, R. W. (2010) Characterization of the role of the tumor marker Nup88 in mitosis. *Mol Cancer* **9**, 119
268. Melo, S. A., Moutinho, C., Ropero, S., Calin, G. A., Rossi, S., Spizzo, R., Fernandez, A. F., Davalos, V., Villanueva, A., Montoya, G., Yamamoto, H., Schwartz, S., Jr., and Esteller, M. (2010) A genetic defect in exportin-5 traps precursor microRNAs in the nucleus of cancer cells. *Cancer Cell* **18**, 303-315
269. Gould, V. E., Orucevic, A., Zentgraf, H., Gattuso, P., Martinez, N., and Alonso, A. (2002) Nup88 (karyoporin) in human malignant neoplasms and dysplasias: correlations of immunostaining of tissue sections, cytologic smears, and immunoblot analysis. *Hum Pathol* **33**, 536-544
270. Simon, D. N., and Rout, M. P. (2014) Cancer and the nuclear pore complex. *Adv Exp Med Biol* **773**, 285-307
271. Chahine, M. N., and Pierce, G. N. (2009) Therapeutic targeting of nuclear protein import in pathological cell conditions. *Pharmacol Rev* **61**, 358-372
272. Kau, T. R., Way, J. C., and Silver, P. A. (2004) Nuclear transport and cancer: from mechanism to intervention. *Nat Rev Cancer* **4**, 106-117
273. Wang, S.-C., and Hung, M.-C. (2005) Cytoplasmic/nuclear shuttling and tumor progression. *Ann N Y Acad Sci* **1059**, 11-15
274. Harel, A., and Forbes, D. J. (2004) Importin beta: conducting a much larger cellular symphony. *Mol Cell* **16**, 319-330
275. Pemberton, L. F., and Paschal, B. M. (2005) Mechanisms of receptor-mediated nuclear import and nuclear export. *Traffic* **6**, 187-198
276. Hosokawa, K., Nishi, M., Sakamoto, H., Tanaka, Y., and Kawata, M. (2008) Regional distribution of importin subtype mRNA expression in the nervous system: Study of early postnatal and adult mouse. *Neuroscience* **157**, 864-877
277. Kirschbaum-Slager, N., Parmigiani, R. B., Camargo, A. A., de Souza, S. J., and x00E (2005) Identification of human exons overexpressed in tumors through the use of genome and expressed sequence data. *Physiol Genomics* **21**, 423-432
278. Glover, D. J., Lipps, H. J., and Jans, D. A. (2005) Towards safe, non-viral therapeutic gene expression in humans. *Nat Rev Genet* **6**, 299-310
279. Kuusisto, H. V., Wagstaff, K. M., Alvisi, G., and Jans, D. A. (2008) The C-terminus of apoptin represents a unique tumor cell-enhanced nuclear targeting module. *Int J Cancer* **123**, 2965-2969
280. Wagstaff, K. M., and Jans, D. A. (2009) Nuclear drug delivery to target tumour cells. *Eur J Pharmacol* **625**, 174-180
281. Alvisi, G., Ripalti, A., Ngankeu, A., Giannandrea, M., Caraffi, S. G., Dias, M. M., and Jans, D. A. (2006) Human cytomegalovirus DNA polymerase catalytic subunit pUL54 possesses independently acting nuclear localization and ppUL44 binding motifs. *Traffic* **7**, 1322-1332
282. Moseley, G. W., Roth, D. M., DeJesus, M. A., Leyton, D. L., Filmer, R. P., Pouton, C. W., and Jans, D. A. (2007) Dynein light chain association sequences can facilitate nuclear protein import. *Mol Biol Cell* **18**, 3204-3213

283. Poon, I. K. H., Oro, C., Dias, M. M., Zhang, J. P., and Jans, D. A. (2005) A tumor cell-specific nuclear targeting signal within chicken anemia virus VP3/apoptin.[comment]. *J Virol* **79**, 1339-1341
284. Pryor, M. J., Rawlinson, S. M., Butcher, R. E., Barton, C. L., Waterhouse, T. A., Vasudevan, S. G., Bardin, P. G., Wright, P. J., Jans, D. A., and Davidson, A. D. (2007) Nuclear localization of dengue virus nonstructural protein 5 through its importin alpha/beta-recognized nuclear localization sequences is integral to viral infection. *Traffic* **8**, 795-807
285. Roth, D. M., Moseley, G. W., Glover, D., Pouton, C. W., and Jans, D. A. (2007) A microtubule-facilitated nuclear import pathway for cancer regulatory proteins. *Traffic* **8**, 673-686
286. Ghildyal, R., Ho, A., Dias, M., Soegiyono, L., Bardin, P. G., Tran, K. C., Teng, M. N., and Jans, D. A. (2009) The respiratory syncytial virus matrix protein possesses a Crm1-mediated nuclear export mechanism. *J Virol* **83**, 5353-5362
287. Arlett, C. F., Green, M. H., Priestley, A., Harcourt, S. A., and Mayne, L. V. (1988) Comparative human cellular radiosensitivity: I. The effect of SV40 transformation and immortalisation on the gamma-irradiation survival of skin derived fibroblasts from normal individuals and from ataxia-telangiectasia patients and heterozygotes. *Int J Radiat Biol* **54**, 911-928
288. Gluzman, Y. (1981) SV40-transformed simian cells support the replication of early SV40 mutants. *Cell* **23**, 175-182
289. Santner, S. J., Dawson, P. J., Tait, L., Soule, H. D., Eliason, J., Mohamed, A. N., Wolman, S. R., Heppner, G. H., and Miller, F. R. (2001) Malignant MCF10CA1 cell lines derived from premalignant human breast epithelial MCF10AT cells. *Breast Cancer Res Treat* **65**, 101-110
290. Poon, I. K. H., Oro, C., Dias, M. M., Zhang, J., and Jans, D. A. (2005) Apoptin nuclear accumulation is modulated by a CRM1-recognized nuclear export signal that is active in normal but not in tumor cells. *Cancer Res* **65**, 7059-7064
291. Soule, H. D., Maloney, T. M., Wolman, S. R., Peterson, W. D., Jr., Brenz, R., McGrath, C. M., Russo, J., Pauley, R. J., Jones, R. F., and Brooks, S. C. (1990) Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. *Cancer Res* **50**, 6075-6086
292. Jensen, F. C., Girardi, A. J., Gilden, R. V., and Koprowski, H. (1964) INFECTION OF HUMAN AND SIMIAN TISSUE CULTURES WITH ROUS SARCOMA VIRUS. *Proc Natl Acad Sci U S A* **52**, 53-59
293. Hubner, S., Xiao, C. Y., and Jans, D. A. (1997) The protein kinase CK2 site (Ser111/112) enhances recognition of the simian virus 40 large T-antigen nuclear localization sequence by importin. *J Biol Chem* **272**, 17191-17195
294. Wagstaff, K. M., and Jans, D. A. (2006) Intramolecular masking of nuclear localization signals: analysis of importin binding using a novel AlphaScreen-based method. *Anal Biochem* **348**, 49-56
295. Kalderon, D., Roberts, B. L., Richardson, W. D., and Smith, A. E. (1984) A short amino acid sequence able to specify nuclear location. *Cell* **39**, 499-509
296. Pache, J. C., Burton, D. W., Deftos, L. J., and Hastings, R. H. (2006) A carboxyl leucine-rich region of parathyroid hormone-related protein is critical for nuclear export. *Endocrinology* **147**, 990-998
297. Henderson, B. R., and Percipalle, P. (1997) Interactions between HIV Rev and nuclear import and export factors: the Rev nuclear localisation signal mediates specific binding to human importin-beta. *J Mol Biol* **274**, 693-707

298. Askjaer, P., Jensen, T. H., Nilsson, J., Englmeier, L., and Kjems, J. (1998) The specificity of the CRM1-Rev nuclear export signal interaction is mediated by RanGTP. *J Biol Chem* **273**, 33414-33422
299. Hakata, Y., Yamada, M., Mabuchi, N., and Shida, H. (2002) The carboxy-terminal region of the human immunodeficiency virus type 1 protein Rev has multiple roles in mediating CRM1-related Rev functions. *J Virol* **76**, 8079-8089
300. Arnold, M., Nath, A., Hauber, J., and Kehlenbach, R. H. (2006) Multiple importins function as nuclear transport receptors for the Rev protein of human immunodeficiency virus type 1. *J Biol Chem* **281**, 20883-20890
301. Gu, L., Tsuji, T., Jarboui, M. A., Yeo, G. P., Sheehy, N., Hall, W. W., and Gautier, V. W. (2011) Intermolecular masking of the HIV-1 Rev NLS by the cellular protein HIC: novel insights into the regulation of Rev nuclear import. *Retrovirology* **8**, 17
302. Truant, R., and Cullen, B. R. (1999) The arginine-rich domains present in human immunodeficiency virus type 1 Tat and Rev function as direct importin beta-dependent nuclear localization signals. *Mol Cell Biol* **19**, 1210-1217
303. Nakashima, T., Masuda, A., Sekiguchi, T., Nishimoto, T., and Uemura, T. (1994) Preliminary findings of chromosomal alterations and expression of cell cycle genes in head an neck tumors. *European Archives of Oto-Rhino-Laryngology* **251 Suppl 1**, S87-90
304. Smith, E. R., Cai, K. Q., Smedberg, J. L., Ribeiro, M. M., Rula, M. E., Slater, C., Godwin, A. K., and Xu, X.-X. Nuclear entry of activated MAPK is restricted in primary ovarian and mammary epithelial cells. *PLoS ONE* **5**, e9295
305. Tachibana, T., Hieda, M., and Yoneda, Y. (1999) Up-regulation of nuclear protein import by nuclear localization signal sequences in living cells. *FEBS Lett* **442**, 235-240
306. Timney, B. L., Tetenbaum-Novatt, J., Agate, D. S., Williams, R., Zhang, W., Chait, B. T., and Rout, M. P. (2006) Simple kinetic relationships and nonspecific competition govern nuclear import rates in vivo. *J Cell Biol* **175**, 579-593
307. Feldherr, C., and Akin, D. (1995) Stimulation of nuclear import by simian virus 40-transformed cell extracts is dependent on protein kinase activity. *Mol Cell Biol* **15**, 7043-7049
308. Fulcher, A. J., and Jans, D. A. (2011) Regulation of nucleocytoplasmic trafficking of viral proteins: An integral role in pathogenesis? *Biochim Biophys Acta*
309. Mortezavi, A., Hermanns, T., Seifert, H.-H., Baumgartner, M. K., Provenzano, M., Sulser, T., Burger, M., Montani, M., Ikenberg, K., Hofstadter, F., Hartmann, A., Jaggi, R., Moch, H., Kristiansen, G., and Wild, P. J. KPNA2 Expression Is an Independent Adverse Predictor of Biochemical Recurrence after Radical Prostatectomy. *Clin Cancer Res* **17**, 1111-1121
310. Mutka, S. C., Yang, W. Q., Dong, S. D., Ward, S. L., Craig, D. A., Timmermans, P. B., and Murli, S. (2009) Identification of nuclear export inhibitors with potent anticancer activity in vivo. *Cancer Res* **69**, 510-517
311. Lane, D. P., Cheok, C. F., and Lain, S. (2010) p53-based cancer therapy. *Cold Spring Harb Perspect Biol* **2**, a001222
312. Sobolev, A. S. (2009) Novel modular transporters delivering anticancer drugs and foreign DNA to the nuclei of target cancer cells. *J BUON* **14 Suppl 1**, S33-42
313. Akhlynina, T. V., Jans, D. A., Rosenkranz, A. A., Statsyuk, N. V., Balashova, I. Y., Toth, G., Pavo, I., Rubin, A. B., and Sobolev, A. S. (1997) Nuclear targeting of chlorin e6 enhances its photosensitizing activity. *J Biol Chem* **272**, 20328-20331

314. Rosenkranz, A. A., Lunin, V. G., Gulak, P. V., Sergienko, O. V., Shumiantseva, M. A., Voronina, O. L., Gilyazova, D. G., John, A. P., Kofner, A. A., Mironov, A. F., Jans, D. A., and Sobolev, A. S. (2003) Recombinant modular transporters for cell-specific nuclear delivery of locally acting drugs enhance photosensitizer activity. *Faseb J* **17**, 1121-1123
315. Itman, C., Miyamoto, Y., Young, J., Jans, D. A., and Loveland, K. L. (2009) Nucleocytoplasmic transport as a driver of mammalian gametogenesis. *Semin Cell Dev Biol* **20**, 607-619
316. Stochaj, M. K. a. U. (2012) Nuclear Transport: A Switch for the Oxidative Stress-Signaling Circuit? *Journal of Signal Transduction* **2012**, 18 pages
317. Young, J. C., Ly-Huynh, J. D., Lescesen, H., Miyamoto, Y., Browne, C., Yoneda, Y., Koopman, P., Loveland, K. L., and Jans, D. A. (2013) The nuclear import factor importin alpha4 can protect against oxidative stress. *Biochim Biophys Acta* **1833**, 2348-2356
318. Kuusisto, H. V., Wagstaff, K. M., Alvisi, G., Roth, D. M., and Jans, D. A. (2012) Global enhancement of nuclear localization-dependent nuclear transport in transformed cells. *Faseb J* **26**, 1181-1193
319. Cai, Y., Gao, Y., Sheng, Q., Miao, S., Cui, X., Wang, L., Zong, S., and Koide, S. S. (2002) Characterization and potential function of a novel testis-specific nucleoporin BS-63.[Erratum appears in Mol Reprod Dev 2002 Feb;61(2):279]. *Mol Reprod Dev* **61**, 126-134
320. Neve, R. M., Chin, K., Fridlyand, J., Yeh, J., Baehner, F. L., Fevr, T., Clark, L., Bayani, N., Coppe, J.-P., Tong, F., Speed, T., Spellman, P. T., DeVries, S., Lapuk, A., Wang, N. J., Kuo, W.-L., Stilwell, J. L., Pinkel, D., Albertson, D. G., Waldman, F. M., McCormick, F., Dickson, R. B., Johnson, M. D., Lippman, M., Ethier, S., Gazdar, A., and Gray, J. W. (2006) A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell* **10**, 515-527
321. Cowell, J. K., LaDuca, J., Rossi, M. R., Burkhardt, T., Nowak, N. J., and Matsui, S. (2005) Molecular characterization of the t(3;9) associated with immortalization in the MCF10A cell line. *Cancer Genet Cytogenet* **163**, 23-29
322. Strickland, L. B., Dawson, P. J., Santner, S. J., and Miller, F. R. (2000) Progression of premalignant MCF10AT generates heterogeneous malignant variants with characteristic histologic types and immunohistochemical markers. *Breast Cancer Res Treat* **64**, 235-240
323. Miller, F. R. (2000) Xenograft models of premalignant breast disease. *J Mammary Gland Biol Neoplasia* **5**, 379-391
324. Miller, F. R., Soule, H. D., Tait, L., Pauley, R. J., Wolman, S. R., Dawson, P. J., and Heppner, G. H. (1993) Xenograft model of progressive human proliferative breast disease. *J Natl Cancer Inst* **85**, 1725-1732
325. Hu, W., Kemp, B. E., and Jans, D. A. (2005) Kinetic properties of nuclear transport conferred by the retinoblastoma (Rb) NLS. *J Cell Biochem* **95**, 782-793
326. Liang, S. H., and Clarke, M. F. (1999) A bipartite nuclear localization signal is required for p53 nuclear import regulated by a carboxyl-terminal domain. *J Biol Chem* **274**, 32699-32703
327. Zacksenhaus, E., Bremner, R., Phillips, R. A., and Gallie, B. L. (1993) A bipartite nuclear localization signal in the retinoblastoma gene product and its importance for biological activity. *Mol Cell Biol* **13**, 4588-4599
328. Alao, J. P. (2007) The regulation of cyclin D1 degradation: roles in cancer development and the potential for therapeutic invention. *Mol Cancer* **6**, 24
329. Teng, S. C., Wu, K. J., Tseng, S. F., Wong, C. W., and Kao, L. (2006) Importin KPNA2, NBS1, DNA repair and tumorigenesis. *J Mol Histol* **37**, 293-299

330. Fridell, R. A., Truant, R., Thorne, L., Benson, R. E., and Cullen, B. R. (1997) Nuclear import of hnRNP A1 is mediated by a novel cellular cofactor related to karyopherin-beta. *J Cell Sci* **110 (Pt 11)**, 1325-1331
331. Seki, T., Tada, S., Katada, T., and Enomoto, T. (1997) Cloning of a cDNA encoding a novel importin-alpha homologue, Qip1: discrimination of Qip1 and Rch1 from hSrp1 by their ability to interact with DNA helicase Q1/RecQL. *Biochem Biophys Res Commun* **234**, 48-53
332. Bian, X.-L., Rosas-Acosta, G., Wu, Y.-C., and Wilson, V. G. (2007) Nuclear import of bovine papillomavirus type 1 E1 protein is mediated by multiple alpha importins and is negatively regulated by phosphorylation near a nuclear localization signal. *J Virol* **81**, 2899-2908
333. Melen, K., Fagerlund, R., Franke, J., Kohler, M., Kinnunen, L., and Julkunen, I. (2003) Importin alpha nuclear localization signal binding sites for STAT1, STAT2, and influenza A virus nucleoprotein. *J Biol Chem* **278**, 28193-28200
334. Nemergut, M. E., and Macara, I. G. (2000) Nuclear import of the ran exchange factor, RCC1, is mediated by at least two distinct mechanisms. *J Cell Biol* **149**, 835-850
335. Lam, M. H. C., Thomas, R. J., Martin, T. J., Gillespie, M. T., and Jans, D. A. (2000) Nuclear and nucleolar localization of parathyroid hormone-related protein. *Immunol Cell Biol* **78**, 395-402
336. Akhlynina, T. V., Jans, D. A., Statsyuk, N. V., Balashova, I. Y., Toth, G., Pavo, I., Rosenkranz, A. A., Naroditsky, B. S., and Sobolev, A. S. (1999) Adenoviruses synergize with nuclear localization signals to enhance nuclear delivery and photodynamic action of internalizable conjugates containing chlorin e6. *Int J Cancer* **81**, 734-740
337. Alvisi, G., Poon, I. K. H., and Jans, D. A. (2006) Tumor-specific nuclear targeting: promises for anti-cancer therapy? *Drug Resist Updat* **9**, 40-50
338. Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3**, RESEARCH0034
339. Elston, C. W., and Ellis, I. O. (2002) Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. C. W. Elston & I. O. Ellis. Histopathology 1991; 19; 403-410. *Histopathology* **41**, 151-152, discussion 152-153
340. Miura, K., Yoshinobu, K., Imaizumi, T., Haruna, K., Miyamoto, Y., Yoneda, Y., Nakagata, N., Araki, M., Miyakawa, T., Yamamura, K.-i., and Araki, K. (2006) Impaired expression of importin/karyopherin beta1 leads to post-implantation lethality. *Biochem Biophys Res Commun* **341**, 132-138
341. Tirian, L., Puro, J., Erdelyi, M., Boros, I., Papp, B., Lippai, M., and Szabad, J. (2000) The Ketel(D) dominant-negative mutations identify maternal function of the Drosophila importin-beta gene required for cleavage nuclei formation. *Genetics* **156**, 1901-1912
342. Quan, Y., Ji, Z.-L., Wang, X., Tartakoff, A. M., and Tao, T. (2008) Evolutionary and transcriptional analysis of karyopherin beta superfamily proteins. *Mol Cell Proteomics* **7**, 1254-1269
343. Zacksenhaus, E., Jiang, Z., Hei, Y. J., Phillips, R. A., and Gallie, B. L. (1999) Nuclear localization conferred by the pocket domain of the retinoblastoma gene product. *Biochim Biophys Acta* **1451**, 288-296
344. Reich, N. C., and Liu, L. (2006) Tracking STAT nuclear traffic. *Nat Rev Immunol* **6**, 602-612

345. Choi, Y. J., Li, X., Hydbring, P., Sanda, T., Stefano, J., Christie, A. L., Signoretti, S., Look, A. T., Kung, A. L., von Boehmer, H., and Sicinski, P. (2012) The requirement for cyclin D function in tumor maintenance. *Cancer Cell* **22**, 438-451
346. Soucek, L., Whitfield, J., Martins, C. P., Finch, A. J., Murphy, D. J., Sodir, N. M., Karnezis, A. N., Swigart, L. B., Nasi, S., and Evan, G. I. (2008) Modelling Myc inhibition as a cancer therapy. *Nature* **455**, 679-683
347. Luo, J., Solimini, N. L., and Elledge, S. J. (2009) Principles of cancer therapy: oncogene and non-oncogene addiction. *Cell* **136**, 823-837
348. Arvey, A., Larsson, E., Sander, C., Leslie, C. S., and Marks, D. S. (2010) Target mRNA abundance dilutes microRNA and siRNA activity. *Mol Syst Biol* **6**, 363
349. Sandy, P., Ventura, A., and Jacks, T. (2005) Mammalian RNAi: a practical guide. *Biotechniques* **39**, 215-224
350. Martens-de Kemp, S. R., Nagel, R., Stigter-van Walsum, M., van der Meulen, I. H., van Beusechem, V. W., Braakhuis, B. J. M., and Brakenhoff, R. H. (2013) Functional genetic screens identify genes essential for tumor cell survival in head and neck and lung cancer. *Clin Cancer Res* **19**, 1994-2003
351. Schmitz, M. H., Held, M., Janssens, V., Hutchins, J. R., Hudecz, O., Ivanova, E., Goris, J., Trinkle-Mulcahy, L., Lamond, A. I., Poser, I., Hyman, A. A., Mechtler, K., Peters, J. M., and Gerlich, D. W. (2010) Live-cell imaging RNAi screen identifies PP2A-B55alpha and importin-beta1 as key mitotic exit regulators in human cells. *Nat Cell Biol* **12**, 886-893
352. AACR, A. (2012) Cancer in Australia: an overview 2012. Vol. Cancer series no. 74. Cat. no. CAN 70. , AIHW, Canberra
353. Ferlay J, S. I., Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray, F. *Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11*
354. Aggarwal, A., and Agrawal, D. K. (2014) Importins and Exportins Regulating Allergic Immune Responses. *Mediators of Inflammation* **2014**, 14
355. Hanahan, D., and Weinberg, Robert A. Hallmarks of Cancer: The Next Generation. *Cell* **144**, 646-674
356. Turner, J. G., Marchion, D. C., Dawson, J. L., Emmons, M. F., Hazlehurst, L. A., Washausen, P., and Sullivan, D. M. (2009) Human multiple myeloma cells are sensitized to topoisomerase II inhibitors by CRM1 inhibition. *Cancer Res* **69**, 6899-6905
357. Tai, Y. T., Landesman, Y., Acharya, C., Calle, Y., Zhong, M. Y., Cea, M., Tannenbaum, D., Cagnetta, A., Reagan, M., Munshi, A. A., Senapedis, W., Saint-Martin, J. R., Kashyap, T., Shacham, S., Kauffman, M., Gu, Y., Wu, L., Ghobrial, I., Zhan, F., Kung, A. L., Schey, S. A., Richardson, P., Munshi, N. C., and Anderson, K. C. (2014) CRM1 inhibition induces tumor cell cytotoxicity and impairs osteoclastogenesis in multiple myeloma: molecular mechanisms and therapeutic implications. *Leukemia* **28**, 155-165
358. Sakakibara, K., Saito, N., Sato, T., Suzuki, A., Hasegawa, Y., Friedman, J. M., Kufe, D. W., Vonhoff, D. D., Iwami, T., and Kawabe, T. (2011) CBS9106 is a novel reversible oral CRM1 inhibitor with CRM1 degrading activity. *Blood* **118**, 3922-3931
359. Turner, J. G., Dawson, J., Emmons, M. F., Cubitt, C. L., Kauffman, M., Shacham, S., Hazlehurst, L. A., and Sullivan, D. M. (2013) CRM1 Inhibition Sensitizes Drug Resistant Human Myeloma Cells to Topoisomerase II and Proteasome Inhibitors both In Vitro and Ex Vivo. *J Cancer* **4**, 614-625

360. Mendonca, J., Sharma, A., Kim, H. S., Hammers, H., Meeker, A., De Marzo, A., Carducci, M., Kauffman, M., Shacham, S., and Kachhap, S. (2014) Selective inhibitors of nuclear export (SINE) as novel therapeutics for prostate cancer. *Oncotarget*
361. Etchin, J., Sanda, T., Mansour, M. R., Kentsis, A., Montero, J., Le, B. T., Christie, A. L., McCauley, D., Rodig, S. J., Kauffman, M., Shacham, S., Stone, R., Letai, A., Kung, A. L., and Thomas Look, A. (2013) KPT-330 inhibitor of CRM1 (XPO1)-mediated nuclear export has selective anti-leukaemic activity in preclinical models of T-cell acute lymphoblastic leukaemia and acute myeloid leukaemia. *Br J Haematol* **161**, 117-127
362. Etchin, J., Sun, Q., Kentsis, A., Farmer, A., Zhang, Z. C., Sanda, T., Mansour, M. R., Barcelo, C., McCauley, D., Kauffman, M., Shacham, S., Christie, A. L., Kung, A. L., Rodig, S. J., Chook, Y. M., and Look, A. T. (2013) Antileukemic activity of nuclear export inhibitors that spare normal hematopoietic cells. *Leukemia* **27**, 66-74
363. Brouckaert, O., Wildiers, H., Floris, G., and Neven, P. (2012) Update on triple-negative breast cancer: prognosis and management strategies. *Int J Womens Health* **4**, 511-520
364. Engebraaten, O., Vollan, H. K., and Borresen-Dale, A. L. (2013) Triple-negative breast cancer and the need for new therapeutic targets. *Am J Pathol* **183**, 1064-1074
365. Hudis, C. A., and Gianni, L. (2011) Triple-negative breast cancer: an unmet medical need. *Oncologist* **16 Suppl 1**, 1-11
366. Chavez, K. J., Garimella, S. V., and Lipkowitz, S. (2010) Triple negative breast cancer cell lines: one tool in the search for better treatment of triple negative breast cancer. *Breast Dis* **32**, 35-48
367. Hanahan, D., and Weinberg, R. A. (2000) The hallmarks of cancer. *Cell* **100**, 57-70
368. Sosa, V., Moline, T., Somoza, R., Paciucci, R., Kondoh, H., and ME, L. L. (2013) Oxidative stress and cancer: an overview. *Ageing Res Rev* **12**, 376-390
369. Miyamoto, Y., Imamoto, N., Sekimoto, T., Tachibana, T., Seki, T., Tada, S., Enomoto, T., and Yoneda, Y. (1997) Differential modes of nuclear localization signal (NLS) recognition by three distinct classes of NLS receptors. *J Biol Chem* **272**, 26375-26381
370. Friedrich, B., Quensel, C., Sommer, T., Hartmann, E., and Kohler, M. (2006) Nuclear localization signal and protein context both mediate importin alpha specificity of nuclear import substrates. *Mol Cell Biol* **26**, 8697-8709
371. Martinez, P., Thanasoula, M., Munoz, P., Liao, C., Tejera, A., McNees, C., Flores, J. M., Fernandez-Capetillo, O., Tarsounas, M., and Blasco, M. A. (2009) Increased telomere fragility and fusions resulting from TRF1 deficiency lead to degenerative pathologies and increased cancer in mice. *Genes Dev* **23**, 2060-2075
372. Kosugi, S., Hasebe, M., Entani, T., Takayama, S., Tomita, M., and Yanagawa, H. (2008) Design of peptide inhibitors for the importin alpha/beta nuclear import pathway by activity-based profiling. *Chem Biol* **15**, 940-949
373. Wagstaff, K. M., Sivakumaran, H., Heaton, S. M., Harrich, D., and Jans, D. A. (2012) Ivermectin is a specific inhibitor of importin alpha/beta-mediated nuclear import able to inhibit replication of HIV-1 and dengue virus. *Biochem J* **443**, 851-856
374. Peer, D., Karp, J. M., Hong, S., Farokhzad, O. C., Margalit, R., and Langer, R. (2007) Nanocarriers as an emerging platform for cancer therapy. *Nat Nano* **2**, 751-760
375. Glover, D. J., Ng, S. M., Mechler, A., Martin, L. L., and Jans, D. A. (2009) Multifunctional protein nanocarriers for targeted nuclear gene delivery in nondividing cells. *Faseb J* **23**, 2996-3006

376. Wang, T., Upponi, J. R., and Torchilin, V. P. (2012) Design of multifunctional non-viral gene vectors to overcome physiological barriers: dilemmas and strategies. *Int J Pharm* **427**, 3-20
377. Ma, Y., Nolte, R. J., and Cornelissen, J. J. (2012) Virus-based nanocarriers for drug delivery. *Adv Drug Deliv Rev* **64**, 811-825
378. Vacchelli, E., Eggermont, A., Sautès-Fridman, C., Galon, J., Zitvogel, L., Kroemer, G., and Galluzzi, L. (2013) Trial watch: Oncolytic viruses for cancer therapy. *OncoImmunology* **2**, e24612
379. Angus, L., van der Watt, P. J., and Leaner, V. D. (2014) Inhibition of the nuclear transporter, Kpnbeta1, results in prolonged mitotic arrest and activation of the intrinsic apoptotic pathway in cervical cancer cells. *Carcinogenesis*
380. Zhang, P., Garnett, J., Creighton, C. J., Al Sannaa, G. A., Igram, D. R., Lazar, A., Liu, X., Liu, C., and Pollock, R. E. (2014) EZH2-miR-30d-KPNB1 pathway regulates malignant peripheral nerve sheath tumour cell survival and tumourigenesis. *J Pathol* **232**, 308-318
381. Lu, Q., Lu, Z., Liu, Q., Guo, L., Ren, H., Fu, J., Jiang, Q., Clarke, P. R., and Zhang, C. (2012) Chromatin-bound NLS proteins recruit membrane vesicles and nucleoporins for nuclear envelope assembly via importin-alpha/beta. *Cell Res* **22**, 1562-1575
382. Schiffelers, R. M., Ansari, A., Xu, J., Zhou, Q., Tang, Q., Storm, G., Molema, G., Lu, P. Y., Scaria, P. V., and Woodle, M. C. (2004) Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle. *Nucleic Acids Res* **32**, e149
383. Song, E., Zhu, P., Lee, S. K., Chowdhury, D., Kussman, S., Dykxhoorn, D. M., Feng, Y., Palliser, D., Weiner, D. B., Shankar, P., Marasco, W. A., and Lieberman, J. (2005) Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors. *Nat Biotechnol* **23**, 709-717
384. McNamara, J. O., Andrechek, E. R., Wang, Y., Viles, K. D., Rempel, R. E., Gilboa, E., Sullenger, B. A., and Giangrande, P. H. (2006) Cell type-specific delivery of siRNAs with aptamer-siRNA chimeras. *Nat Biotech* **24**, 1005-1015
385. Hintersteiner, M., Ambrus, G., Bednenko, J., Schmied, M., Knox, A. J., Meisner, N. C., Gstach, H., Seifert, J. M., Singer, E. L., Gerace, L., and Auer, M. (2010) Identification of a small molecule inhibitor of importin beta mediated nuclear import by confocal on-bead screening of tagged one-bead one-compound libraries. *ACS Chem Biol* **5**, 967-979
386. Soderholm, J. F., Bird, S. L., Kalab, P., Sampathkumar, Y., Hasegawa, K., Uehara-Bingen, M., Weis, K., and Heald, R. (2011) Importazole, a small molecule inhibitor of the transport receptor importin-beta. *ACS Chem Biol* **6**, 700-708
387. Ambrus, G., Whitby, L. R., Singer, E. L., Trott, O., Choi, E., Olson, A. J., Boger, D. L., and Gerace, L. (2010) Small molecule peptidomimetic inhibitors of importin alpha/beta mediated nuclear transport. *Bioorg Med Chem* **18**, 7611-7620
388. Nebert, D. W. (2002) Transcription factors and cancer: an overview. *Toxicology* **181-182**, 131-141
389. Reddy, S. D. N., Gajula, R. P., Pakala, S. B., and Kumar, R. (2010) MicroRNAs and cancer therapy: the next wave or here to stay? *Cancer Biol Ther* **9**, 479-482
390. Li, K. K.-W., Yang, L., Pang, J. C.-S., Chan, A. K.-Y., Zhou, L., Mao, Y., Wang, Y., Lau, K.-M., Poon, W. S., Shi, Z., and Ng, H.-K. (2013) MIR-137 suppresses growth and invasion, is downregulated in oligodendroglial tumors and targets CSE1L. *Brain Pathol* **23**, 426-439
391. Tan, S., Ding, K., Li, R., Zhang, W., Li, G., Kong, X., Qian, P., Lobie, P., and Zhu, T. (2014) Identification of miR-26 as a key mediator of estrogen stimulated cell proliferation by targeting CHD1, GREB1 and KPNA2. *Breast Cancer Res* **16**, R40

392. Sun, X., Icli, B., Wara, A. K., Belkin, N., He, S., Kobzik, L., Hunninghake, G. M., Vera, M. P., Blackwell, T. S., Baron, R. M., and Feinberg, M. W. (2012) MicroRNA-181b regulates NF-kappaB-mediated vascular inflammation. *J Clin Invest* **122**, 1973-1990
393. Glinsky, G. V. (2008) An SNP-guided microRNA map of fifteen common human disorders identifies a consensus disease phenocode aiming at principal components of the nuclear import pathway. *Cell Cycle* **7**, 2570-2583

Appendices

Global enhancement of nuclear localization-dependent nuclear transport in transformed cells

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ABSTRACT **Fundamental to eukaryotic cell function, nucleocytoplasmic transport can be regulated at many levels, including through modulation of the importin/exportin (Imp/Exp) nuclear transport machinery itself. Although Imps/Exps are overexpressed in a number of transformed cell lines and patient tumor tissues, the efficiency of nucleocytoplasmic transport in transformed cell types compared with nontransformed cells has not been investigated. Here we use quantitative live cell imaging of 3 isogenic nontransformed/transformed cell pairs to show that nuclear accumulation of nuclear localization signal (NLS)-containing proteins, but not their NLS-mutated derivatives, is increased up to 7-fold in MCF10CA1h human epithelial breast carcinoma cells and in simian virus 40 (SV40)-transformed fibroblasts of human and monkey origin, compared with their nontransformed counterparts. The basis for this appears to be a significantly faster rate of nuclear import in transformed cell types, as revealed by analysis using fluorescence recovery after photobleaching for the human MCF10A/MCF10CA1h cell pair. Nuclear accumulation of NLS/nuclear export signal-containing (shuttling) proteins was also enhanced in transformed cell types, experiments using the nuclear export inhibitor leptomycin B demonstrating that efficient Exp-1-mediated nuclear export was not impaired in transformed compared with nontransformed cells. Enhanced nuclear import and export efficiencies were found to correlate with 2- to 4-fold higher expression of specific Imps/Exps in transformed cells, as indicated by quantitative Western blot analysis, with ectopic expression of Imps able to enhance NLS nuclear accumulation levels up to 5-fold in nontransformed MCF10A cells. The findings indicate that transformed cells possess altered nuclear transport properties, most likely due to the overexpression of Imps/Exps. The findings have important implications for the development of tumor-specific drug nanocarriers in anticancer therapy.—Kuusisto, H. V., Wagstaff, K. M., Alvisi, G., Roth, D. M., Jans, D. A. Global enhancement of nuclear localization-dependent nuclear transport in transformed cells. *FASEB J.* 26, 1181–1193 (2012). www.fasebj.org**

Key Words: importins · exportins · neoplastic transformation · isogenic · specific drug delivery

Tumor cells are characterized by a plethora of changes in signal transduction, which affect tumor cell metabolism, as well as proliferative potential and resistance to apoptosis. Ultimately, these cellular responses to extra- and intracellular signaling pathways converge at the level of the nucleus to effect phenotypic changes in transcription, chromatin structure, cell cycle control, DNA repair, *etc.* Protein transport into the nucleus through the nuclear envelope-embedded nuclear pore complexes (NPCs) is dependent on nuclear localization signals (NLSs), which are recognized by members of the importin (Imp) superfamily of cellular transport receptors, of which there are multiple α and β types (1, 2). A well-characterized example is Impβ1, which, either directly or through an Impα adapter, recognizes basic-type NLSs and mediates translocation of the Imp:cargo complex through the NPC into the nucleus, where the complex dissociates on binding to Impβ1 of the monomeric guanine nucleotide binding protein Ran in activated GTP-bound form (3). Nuclear protein export occurs analogously, whereby generally hydrophobic nuclear export signals (NESs) are recognized by exportins (Exps), homologs of Impβ1, in combination with RanGTP (3). The best-characterized Exps include Exp-1 (Crm-1), which recognizes leucine-rich NESs (4), and CAS (Cse1), which facilitates recycling of Impαs to the cytoplasm to enable subsequent rounds of nuclear import (5).

Nucleocytoplasmic transport can be regulated by a number of distinct mechanisms (see ref. 6), which are central to cellular processes such as differentiation and oncogenesis (7). One mechanism appears to be through alterations in the levels of expression of specific Imps. For example, the levels of different Impα

Abbreviations: CLSM, confocal laser scanning microscope; Exp, exportin; FRAP, fluorescence recovery after photobleaching; Imp, importin; LMB, leptomycin B; MRT, modular recombinant transporter; NES, nuclear export signal; NLS, nuclear localization signal; NPC, nuclear pore complex; ROI, region of interest; SV40, simian virus 40.

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isoforms alter during sperm development in the mouse testis (8, 9), embryonic stem cell (ESC) differentiation (10), and maintenance of pluripotency in ESCs (11) and in the central nervous system in the early postnatal and adult stages (12). Interestingly, recent reports indicate alterations in the expression levels of Imps/Exps in patient tumor tissue and in transformed cell lines, with elevation of Imp expression in breast (13–17), bladder (18, 19), esophageal (20, 21), cervical (22), hepatocellular (23), colon (24) and ovarian carcinomas (25), and also in simian virus 40 (SV40) or human papilloma virus-16-transformed cell lines (22), while Exps are also found overexpressed in carcinomas of the breast (26, 27), liver (28), and ovaries (29–31) and in numerous virally transformed and cancer cell lines (22, 26–28, 31). To date, however, no study has analyzed the effect of altered Imp/Exp expression on nuclear transport efficiency in transformed cells, with essentially nothing known regarding the comparative transport efficiency in tumor *vs.* normal cells.

Here we examine nuclear transport efficiency quantitatively in 3 isogenic pairs of transformed and nontransformed cells for the first time and relate this to levels of Imp/Exp expression. We show that Impα/β1- and Impβ1-dependent nuclear transport efficiencies and rates are increased in transformed cells compared with nontransformed counterparts, and that this correlates with increased levels of Impα1, Impβ1, and the Ran guanine nucleotide exchange factor RCC1 in transformed cells. Exp-1 levels were also elevated in the transformed cell types, which was associated with an increased responsiveness to the Exp-1-specific inhibitor leptomycin B (LMB) of NLS- and NES-containing cargo proteins, implying higher Exp-1 activity in transformed compared with nontransformed cells. The results overall have important relevance to the development of tumor-specific drug nanocarriers in anticancer therapy (32–34).

MATERIALS AND METHODS

Mammalian expression constructs

Plasmid pEGFP (Clontech, Palo Alto, CA, USA) encoding GFP alone was used as a control for transfection studies. The pEGFP-T-ag(114–135), pEGFP-T-ag(111–135), pEGFP-T-ag(111–135)NLSm (containing a $K^{128}T$ mutation), pEPI-UL54(1145–1161), pDEST53-UL54(1145–1161)NLSm (containing $R^{1157}A$ and $R^{1159}A$ mutations), pEPI-VP3 (74–121), pEPI-VP3(74–121)NLS1/2m (containing $KK^{86-87}NN$ and $RR^{117-118}NN$ mutations), pEGFP-PTHrP(1–141), and pEPI-Rev(2–116) expression constructs have all been described previously (33, 35–39). The TRF-1(337–440) fragment (40) was introduced into the Gateway system (Invitrogen, Carlsbad, CA, USA; ref. 35) by PCR using *att*B site-containing primers and subsequent BP and LR recombination reactions to generate plasmid pEPI-TRF-1(337–440). The bicistronic pIRES-mCherry expression plasmid was generated using a modified version of the pIRES-GFP plasmid (a kind gift from K. A. Steiner, Children's Medical Research Institute, Westmead, NSW, Australia), in which the GFP fragment was first removed by *Xba*I/*Sma*I digestion before ligation with PCR-amplified mCherry cDNA. pIRES-Imp-mCherry was generated by ligation of a PCR-amplified Impα1 or Impβ1 cDNA fragment using the *Not*I/*Bam*HI sites. The integrity of all plasmid constructs was verified by DNA sequencing.

Mammalian cell culture, transfection, and LMB treatment

The three isogenic nontransformed/transformed cell pairs used in this study are described in **Table 1**. In the case of the 1BR3/1BR3Neo (1BR3N) human skin fibroblast (41) and the CV-1/COS-7 African green monkey kidney fibroblast (42) cell pairs, the transformed derivative was generated by transfection with plasmids containing SV40 genomic sequences for

TABLE 1. *Isogenic cell pairs used in this study*

Cells	Transformed status	Origin	Derivation
1BR3	Nontransformed	Human skin	Fibroblast cells derived from a skin biopsy (41)
1BR3N	Transformed	1BR3 cell line	1BR3 cells stably transfected with plasmid pSV3neo that contains the early region of SV40 encoding for small, middle, and large tumor antigens (41)
CV-1	Nontransformed	Monkey kidney	Fibroblast cells derived from the African green monkey kidney (77)
COS-7	Transformed	CV-1 cell line	CV-1 cells stably transfected with an origin defective mutant of SV40 plasmid (−ori) that encodes for SV40 proteins, including the tumor antigens (42)
MCF10A	Nontransformed	Human breast	Spontaneously immortalized line from nonmalignant human breast epithelium (46)
MCF10CA1h	Transformed	MCF10A transfected with *Ha*-Ras	Derived from serial mouse xenograft implantations of MCF10A cells transfected with oncogenic *Ha*-Ras that progressed to a carcinoma; majority of MCF10CA1h cells form well-differentiated carcinomas with low metastatic potential in mice (43)

*T-ag, SV40 large T antigen. Numbers in parenthesis indicate references.

either the early region (1BR3N, precrisis stage) or a replication-defective (−ori) mutant (COS-7, postcrisis stage). In the case of the MCF10A/MCF10CA1h human breast epithelial cell pair, the nontransformed MCF10A cell line was isolated from a female patient with fibrocystic disease, whereas the tumorigenic MCF10CA1h line was generated from *Ha*-Ras transformed MCF10A cells, passaged serially *in vivo* in mice (43).

1BR3 and 1BR3N cells (both at passages 6–12) were cultured in DMEM supplemented with 15% FCS and 2 mM L-glutamine (all from Invitrogen). CV-1 and COS-7 cells were cultured in DMEM supplemented with 10% FCS and 2 mM L-glutamine (36, 44), while MCF10A (passage <72) and MCF10CA1h (passage <62) cells were cultured in DMEM/F12 medium supplemented with 5% horse serum (Invitrogen), 1 mM sodium pyruvate, 10 mM HEPES, 2 mM L-glutamine, 0.5 μg/ml hydrocortisone, 10 μg/ml insulin, 20 ng/ml human recombinant EGF, and 100 ng/ml cholera toxin (all from Sigma-Aldrich, St. Louis, MO, USA), as described previously (43). All of the cell line pairs were transfected using Lipofectamine 2000 transfection reagent (Invitrogen) as previously described (36). Where appropriate, transfected cells were incubated for 3–4 h in the presence of 2.8 μg/ml of the Exp-1-specific nuclear export inhibitor LMB before microscopic imaging.

Confocal laser scanning microscopy and image analysis

Cells were imaged live (100× oil-immersion objective, zoom at 1.0, 60-μm pinhole, 4 and 15% laser power for 488- and 568-nm lasers, respectively, heated stage) at 16–24 h post-transfection using a Nikon TSI 100 confocal laser scanning microscope (CLSM; Nikon, Tokyo, Japan) using a Kalman setting of 2. Image analysis was performed using the ImageJ 1.41 public domain software (U.S. National Institutes of Health, Bethesda, MD, USA) to determine the nuclear (F_n) cytoplasmic (F_c) and background (F_b) fluorescence. Briefly, a mean density measurement of pixel numbers was made on a nonsaturated region of interest (ROI) of equal size (ROI=30 arbitrary units) in the nuclear and cytoplasmic compartments, respectively, while Fb measurements were made by placing the ROI on a nontransfected, autofluorescent region near the cells outer perimeter. The ratio of nuclear to cytoplasmic fluorescence ($F_{n/c}$) was then determined according to the formula $F_{n/c} = (F_n - F_b)/(F_c - F_b)$ (44). Fold differences in nuclear accumulation between nontransformed and transformed cell lines were calculated according to the formula $[T_{Fn/c}]/[NT_{Fn/c}]$, where $T_{Fn/c}$ is transformed $F_{n/c}$, and $NT_{Fn/c}$ is nontransformed $F_{n/c}$.

Fluorescence recovery after photobleaching (FRAP)

FRAP experiments were performed using a modified method from Roth *et al.* (45). MCF10A/CA1h cells were transfected as above to express GFP-T-ag(114–135) and imaged live using an Olympus Fluoview 1000 CLSM (×100 oil-immersion objective, heated stage; Olympus, Tokyo, Japan) 16–24 h later. Before bleaching, 2 images using 3% laser power were taken at 8 μs/pixel and a zoom of 2-fold. By zooming 120-fold in the nuclear region and increasing the laser power to 100%, an area of ~5% of the nucleus was photobleached (1 scan, 100 μs/pixel, 2500 and 4000 ms for MCF10A and MCF10CA1h cells, respectively); the cells were then immediately scanned at 3% laser power (8 μs/pixel and zoom of 2-fold), and images were subsequently captured at 5-s intervals for up to 280 s to monitor the recovery of fluorescence in the nucleus and the decay of fluorescence in the cytoplasm. The level of specific nuclear (F_{n-b}) and cytoplasmic (F_{c-b}) fluorescence at each time interval was determined by image analysis as above, and values were expressed in terms of fractional recovery of fluorescence (values at each time interval divided by the maximal postbleach value), with the data for fractional recovery of specific nuclear fluorescence fitted exponentially according to the formula $y = (1 - a)e^{-bx}$ to determine the recovery half-times ($t_{1/2}$), as previously reported (45). The rate of exponential decay of cytoplasmic fluorescence was also determined for F_{c-b} values at 0–280 s postbleach using the equation $y = y_0 + ae^{-bx}$.

Preparation of cell extracts and Western blotting

Equal numbers of living nontransformed and transformed cells, as determined by hemocytometer/trypan blue staining, were harvested by trypsinization and centrifugation and washed once with PBS before freezing at −20°C for 30 min. Cell pellets were resuspended in ice-cold lysis buffer (150 mM NaCl; 50 mM Tris-HCl, pH 7.3; and 1% Triton X-100 with complete, EDTA-free protease inhibitor cocktail; Roche, Indianapolis, IN, USA), lyzed at 4°C for 30 min, and centrifuged at 14,200 rpm for 30 min at 4°C (Hereaus Instruments, South Plainfield, NJ, USA) to pellet insoluble material. The total protein concentration in each extract was estimated using the Bradford dye reagent (Bio-Rad, Hercules, CA, USA). Equal amounts (25 μg) of protein from each cell extract were then separated by SDS-PAGE (12% reducing gel), transferred onto nitrocellulose membrane, and probed with a predetermined (optimized) concentration of anti-Impα1 (1:2000 dilution), Impβ1 (1:500 dilution), Exp-1 (1:500 dilution), CAS (1:500 dilution), Ran (1:2000 dilution), RCC1 (1:250 dilution) monoclonal (all from BD Biosciences, San Jose, CA, USA), or anti-Impα3 (1:250 dilution) and Impα4 (1:500 dilution) polyclonal (Abcam, Cambridge, MA, USA) primary antibodies, followed by the appropriate host IgG-HRP secondary antibody (Chemicon, Temecula, CA, USA) according to the manufacturer's recommendations, and protein was visualized using the Chemiluminescence reagent (Perkin Elmer-Cetus, Wellesley, MA, USA or Millipore, Bedford, MA, USA). Where necessary, the membranes were then stripped of antibody using Western strip buffer (25 mM glycine and 1% SDS, pH 2) at 60°C, blocked, and reprobed with anti-α/β-tubulin (1:1000 dilution; Cell Signaling, Danvers, MA, USA) or anti-T-ag (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by the appropriate host-IgG-HRP secondary antibodies and visualized as previously. The intensity of the resulting bands for all proteins was estimated by densitometry using an Alpha Imager (Alpha Innotec, Santa Clara, CA, USA) for image capture and the 1D electrophoresis gel analysis module from ImageQuant TL software (Amersham Biosciences, Piscataway, NJ, USA) for analysis of the resulting images; results are given as the ratio of the signal for nuclear transport factor relative to that for α/β tubulin in transformed cells, relative to the respective value for the nontransformed cell line of the isogenic cell pair.

Statistical analysis

The significance of differences in results between transformed and nontransformed cells was determined using the Student's (or Welch corrected) *t* test for unpaired data (2-tailed *P* value) or Mann-Whitney test for nonparametric data, as appropriate.

RESULTS

To compare the efficiency of nuclear transport in transformed and nontransformed cells, we utilized 3

distinct nontransformed/transformed cell pairs, in which the transformed and nontransformed cell lines both share the same genetic (isogenic) background (see Table 1): the primary human skin fibroblast cell line 1BR3, and its SV40-transformed counterpart 1BR3N (41); the nontransformed monkey kidney fibroblast CV-1 cell line together with its SV40-transformed counterpart COS-7 (42); and the nontransformed, spontaneously immortalized MCF10A breast epithelial cell line (46) and its fully malignant counterpart, MCF10CA1h, generated by *in vivo* passaging through serial implantation of a *Ha*-Ras transfected MCF10A cell line in mice (43).

The cell lines were transfected to express either GFP alone or various NLS-containing GFP-fusion proteins (see **Table 2**), which are transported into the nucleus by either Impα/β1 (35, 47) or Impβ1 (40, 48), and subcellular localization was analyzed by quantitative live cell CLSM 16–24 h later. As expected, GFP localized uniformly throughout the nucleus and cytoplasm in all cell lines (**Fig. 1*A***) due to its lack of nuclear targeting signals and small size (27 kDa), which is well below the molecular mass cutoff for passive-type transport through the NPC (~50–70 kDa; ref. 49). In contrast, all of the Impα/β1- and Impβ1-recognized NLS-containing proteins localized more strongly in the nucleus of the transformed 1BR3N, COS-7, and MCF10CA1h cells, compared with their nontransformed counterparts (Fig. 1*A*). Image analysis was performed to determine at the single cell level the mean ratio of nuclear-to-cytoplasmic fluorescence $F_{n/c}$ (see Materials and Methods), statistical (*t* test) analysis confirming significantly ($P<0.0001$) higher levels of nuclear accumulation in the transformed cells compared with their nontransformed counterparts for all of the NLS-containing proteins (Fig. 1*B*), with the most pronounced differences in the levels of nuclear accumulation apparent for Impα/β1-recognized NLSs in 1BR3N (~7-fold) and MCF10CA1h (~6-fold) cells compared with nontransformed counterparts (Fig. 1*B*). The extent of nuclear accumulation was similarly assessed for GFP-fusion constructs in which the NLSs carry inactivating mutations (see Table 2); in all cases, the nuclear accumulation of the NLS-mutated fusion proteins, as well as that of GFP alone, was completely comparable between the transformed and nontransformed lines in each cell pair (Fig. 1*C*, MCF10A/CA1h cells not shown), indicating that the large-scale differences in nuclear accumulation efficiency were specific to NLS (and thus Imp)-dependent transport and not due to altered passive diffusion properties of the NPC in transformed cells.

Western blot analysis (not shown) indicated essentially identical levels of expression of the various GFP fusion proteins in the transformed and nontransformed lines, but to assess formally at the single cell level whether the results for increased nuclear accumulation in transformed compared with nontransformed cells may be attributable to increased expression levels of ectopically expressed proteins in the transformed compared with the nontransformed cells, we compared the $F_{n/c}$ values for several NLS-containing GFP fusion proteins in the MCF10A/CA1h cells, expressing the proteins at either high ($F_{n-b}>1500$ arbitrary fluorescence units), medium ($F_{n-b}=500–1500$) or low ($F_{n-b}<500$) levels (see Fig. 1*D* and Materials and Methods). Completely consistent with the whole population analysis (Fig. 1*B*), 3- to 6-fold higher levels of nuclear accumulation were observed in transformed cells regardless of the levels of expression, clearly indicating that the extent of nuclear accumulation is not related to the concentration of nuclear import cargo expressed.

To test whether the increased nuclear accumulation in transformed cells is associated with an increase in the rate of nuclear import, we performed FRAP experiments as previously (45) on the MCF10A/CA1h cell pair expressing GFP-T-ag(114–135), whereby the nucleus of a single cell was bleached, and the return of

TABLE 2. *Nuclear targeting sequences within the fusion proteins used in this study*

GFP-fusion protein	NLS	NES
Impα/β1-recognized		
T-ag(114–135)	P*KKKRK*V^{132} (47, 78, 79)	NA
T-ag(111–135)	As above	NA
T-ag(111–135) NLSm	P*KT KRK*V^{132} (36)	NA
UL54(1145–1161)	PA*KKR*A*R*^{1159} (35)	NA
UL54(1145–1161) NLSm	PA*KK*AAA^{1159} (35)	NA
Impβ1-recognized		
TRF-1(337–440)	*KKKK*ES*RR*^{356} (40)	NA
Impβ1- and Exp-1-recognized		
VP3(74–121)	*K*PPS*KKR*–12 aa spacer–*R*P*R*TA*KRR*I*K*L^{121} (33, 37)	VSKLKESLI^{105} (33, 44)
VP3(74–121) NLS1/2m	*K*PPSNN*R*–12 aa spacer–*R*P*R*TA*K*NNI*K*L^{121} (33)	As above
PTHrP(1–141)	RYLTQETNKVETYKEQPLKTPG*KKKKG*K*K*P^{94} (50, 51)	LSDTSTTSLEL^{136} (50, 51)
Rev(2–116)[a]	*R*QA*RR*N*RRRR*WR*E*R*Q*R*Q^{51} (53)	LPPLERLTL^{83} (52, 80)

For NLS, basic residues are in italics; for NES, hydrophobic residues are underscored. NA, not applicable; Exp, exportin; Imp, importin; NES, nuclear export signal; NLS, nuclear localization signal; NLSm, nuclear localization signal mutant; PTHrP, parathyroid hormone related protein; Rev, human immunodeficiency virus Rev protein; T-ag, SV40 large T antigen; TRF-1, telomeric repeat binding factor-1; UL54, human cytomegalovirus DNA polymerase catalytic subunit UL54; VP3, chicken anemia virus viral protein 3. [a]HIV-1 Rev NLS is also recognized by Imp7, Impβ3, Imp9 and Impβ2 import receptors (55, 56).

nonbleached fluorescence from the cytoplasm to the nucleus was monitored by CLSM for up to 280 s (**Fig. 2*A*, *B***; also see Materials and Methods). The fractional recovery for F_{n-b}, calculated from the initial (prebleach) fluorescence and maximal (postbleach) fluorescence recovery value, was then plotted for each time point to enable the $t_{1/2}$ value for 50% maximal recovery to be determined. While the mean maximal fractional recoveries of GFP-T-ag(114–135) did not differ significantly between MCF10A/CA1h cells (**Table 3**), the $t_{1/2}$ value was significantly ($P<0.05$) reduced by ~2-fold in transformed MCF10CA1h cells ($t_{1/2}=16.0\pm1.9$ s) compared with the nontransformed MCF10A cells ($t_{1/2}=27.4\pm4.9$ s; see Fig. 2*C* and Table 3). Consistent with this, the rate of loss of specific cytoplasmic fluorescence F_{c-b} was ~3-fold faster in the transformed ($t_{1/2}=28.2\pm3.7$ s) than in the nontransformed ($t_{1/2}=101\pm24.8$ s) cells, indicating significantly ($P<0.0154$) faster transport from the cytoplasm to the nucleus in transformed cells (Fig. 2*B*; Table 3). The results indicate that the enhancement of NLS-dependent nuclear import efficiency observed in transformed cells is due to a faster nuclear import rate.

We also tested the subcellular localization of a number of different NLS/NES-containing GFP-fusion proteins (see Table 2), able to shuttle between the nucleus and cytoplasm dependent on Impβ1 and Exp-1 (44, 48, 50–53) in the transformed/nontransformed cell pairs in the absence and presence of the Exp-1-specific inhibitor LMB (**Fig. 3*A***), where the extent of the increase in protein nuclear accumulation $F_{n/c}$ in the presence compared with absence of LMB was used as an indicator of the strength of nuclear export by Exp-1 in each cell line (see **Table 4**). We found that the NLS/NES-containing proteins generally showed significantly ($P\leq0.003$) higher levels of nuclear accumulation in the transformed cell lines compared with their nontransformed counterparts in both the absence and presence of LMB treatment (Fig. 3*B*), consistent with the idea that nuclear import efficiency is enhanced in transformed compared with nontransformed cells. GFP-Rev(2–116) was an exception, failing to show significantly enhanced nuclear accumulation in the presence of LMB in transformed COS-7 compared with nontransformed CV-1 cells, probably as a result of the fact that Rev nuclear import can be modulated by a number of different Imps, including Impβ1 (53, 54), Impβ7, Impβ3, Impβ9, and transportin (Impβ2) (55, 56). Most of the fusion proteins showed significantly ($P\leq0.03$) increased nuclear accumulation in the presence of LMB in both nontransformed and transformed cell lines, indicating robust nuclear export activity in its absence (Table 4). GFP-VP3(74–121) was the only exception to this, exhibiting nuclear accumulation that was not affected by LMB in any of the transformed cell lines, consistent with the fact that VP3-mediated nuclear export is inhibited in transformed cells as a result of tumor cell-specific phosphorylation near the VP3 NES (33, 44).

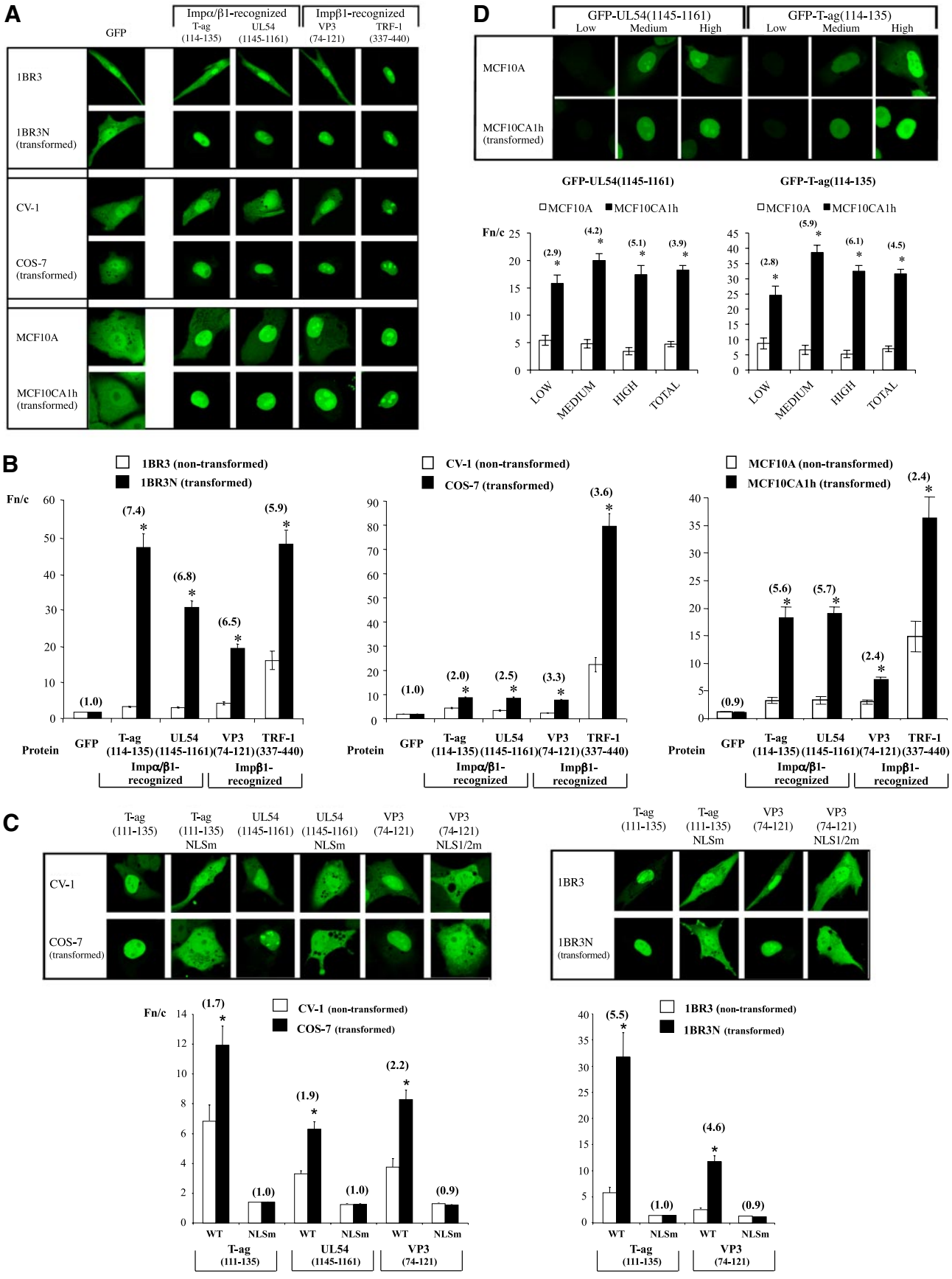
Notably, we found that the extent of the increase in nuclear accumulation of NLS/NES-containing proteins (apart from VP3) in the presence as compared with the absence of LMB $[(F_{n/c}+\text{LMB})/(F_{n/c}-\text{LMB})]$ was much greater in the transformed cell types compared with nontransformed cells (Table 4), implying enhanced Exp-1 activity in the transformed cell lines under normal conditions in the absence of LMB. Reduced nuclear export is clearly not the basis of higher nuclear accumulation of NLS-containing cargoes in the transformed compared with the nontransformed cell lines. Consistent with this, GFP-Rev(2–116), which preferentially localizes in the cell cytoplasm due to the presence of a dominant, Exp-1-recognized NES, showed significantly ($P<0.0001$) lower levels of nuclear accumulation in the absence of LMB treatment in transformed as compared with nontransformed cells, but either equivalent or significantly higher nuclear accumulation in its presence (Fig. 3*B*). The clear implication is that the various transformed cells show higher intrinsic levels of Exp-1-dependent export activity, in addition to the observed increased nuclear import efficiency (see above).

Since the globally enhanced nuclear transport efficiencies could relate to differences in the levels of components of the nuclear transport machinery, we assessed the levels of Imps, Exps, Ran, and Ran regulatory proteins in our nontransformed/transformed cell pairs by Western blot analysis/densitometry. After optimization, analysis was performed for a range of Imps (Impα1, Impα3, Impα4, and Impβ1), Exps (Exp-1 and CAS), Ran, and RCC1 in whole-cell extracts of 1BR3/1BR3N, CV-1/COS-7, and MCF10A/CA1h cells. We found that Impα1, Impβ1, Exp-1, CAS, and RCC1 proteins were all expressed at markedly higher levels in the transformed cells compared with their nontransformed counterparts (**Fig. 4*A***), with densitometric analysis (values expressed relative to the respective levels of the control protein α/β-tubulin) revealing ~2- to 4-fold higher levels of these components in transformed compared with nontransformed cells ($P<0.05$, $n\geq3$, for Impα1, Exp-1, and RCC1; see Fig. 4*B*).

To test whether enhanced Imp levels can enhance nuclear accumulation of proteins, we cotransfected nontransformed MCF10A cells to express a bicistronic pIRES-mCherry Impα1- or Impβ1-encoding plasmid and GFP-T-ag(114–135) and assessed nuclear accumulation in live cells at 40 h post-transfection (**Fig. 5*A***). Expression of Impα1 and Impβ1 significantly ($P<0.04$) increased GFP-NLS nuclear accumulation levels by ~5- and 2.5-fold, respectively, compared with the control plasmid in MCF10A cells (Fig. 5*B*), demonstrating formally that the overexpression of either Impα1 or Impβ1 can enhance nuclear import efficiency in nontransformed cells.

DISCUSSION

This study uses transformed/nontransformed cell pairs from various origins and high-resolution quantitative approaches to demonstrate for the first time that


A
GFP
Impα/β1-recognized
T-ag (114-135)
UL54 (1145-1161)
Impβ1-recognized
VP3 (74-121)
TRF-1 (337-440)
1BR3
1BR3N (transformed)
CV-1
COS-7 (transformed)
MCF10A
MCF10CA1h (transformed)
D
GFP-UL54(1145-1161)
GFP-T-ag(114-135)
Low
Medium
High
MCF10A
MCF10CA1h (transformed)
MCF10A MCF10CA1h
Fn/c
LOW
MEDIUM
HIGH
TOTAL
B
1BR3 (non-transformed)
1BR3N (transformed)
CV-1 (non-transformed)
COS-7 (transformed)
MCF10A (non-transformed)
MCF10CA1h (transformed)
Fn/c
Protein
GFP
T-ag (114-135)
UL54 (1145-1161)
VP3 (74-121)
TRF-1 (337-440)
Impα/β1-recognized
Impβ1-recognized
C
T-ag (111-135)
T-ag (111-135) NLSm
UL54 (1145-1161)
UL54 (1145-1161) NLSm
VP3 (74-121)
VP3 (74-121) NLS1/2m
CV-1
COS-7 (transformed)
1BR3
1BR3N (transformed)
Fn/c
CV-1 (non-transformed)
COS-7 (transformed)
1BR3 (non-transformed)
1BR3N (transformed)
WT
NLSm
T-ag (111-135)
UL54 (1145-1161)
VP3 (74-121)

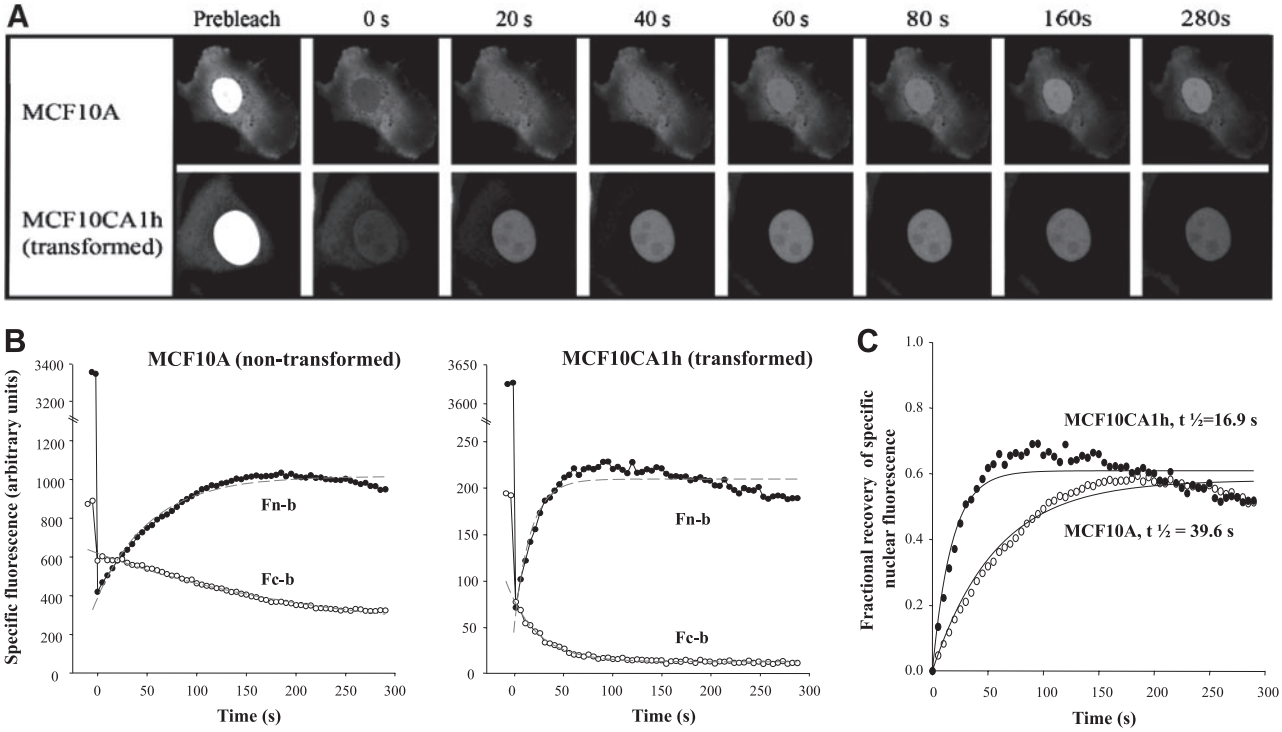


Figure 2. Rate of nuclear import is increased in transformed cells compared with nontransformed cells. *A*) Representative CLSM images (×100 oil-immersion objective) of photobleaching experiments in MCF10A/CA1h cells transfected to express GFP-T-ag(114–135). At 16–24 h post-transfection, a small (zoom 120-fold) area of the nucleus was photobleached (2500–4000 ms, 100% laser power) and monitored for the return of fluorescence by CLSM at 5-s intervals for a total of 280 s. *B*) Quantitative representation of the specific nuclear (F_{n-b}) and cytoplasmic (F_{c-b}) fluorescence values in prebleach (−10 to −5 s), bleach (0 s), and postbleach (5 to 280 s) images from *A* in single typical MCF10A and MCF10CA1h cells. Percentage loss of specific fluorescence in the nuclear and cytoplasmic compartments (F_{n-b} and F_{c-b} at 0 s time point, compared with initial F_{n-b} and F_{c-b} prebleach values) due to photobleaching was comparable between the 2 lines (data not shown). *C*) Quantitative representation of the fractional recovery of specific nuclear fluorescence (where values for F_{n-b} at respective time points were divided by the postbleach maximal value and normalized to the 0 s postbleach time point) at each time interval from digitized images such as those shown in *A*, as is shown for single typical cells from data sets obtained for MCF10A/CA1h cell pairs. Half-maximal time $t_{1/2}$ of recovery of specific nuclear fluorescence recovery is shown for MCF10A (r^2=0.84) and MCF10CA1h (r^2=0.97) cells.

conventional, NLS- and Imp-dependent nuclear protein import is globally enhanced in transformed cells compared with their nontransformed counterparts, correlating with elevated levels of specific components of the cellular nuclear transport machinery. Although elevated Imp and Exp levels have been previously reported in tumor and transformed cells and tissues (23, 31, 57, 58), this is the first study to document quantitative differences in nontransformed and transformed cell lines of isogenic origin and hence enables conclusions to be drawn with respect to the nuclear transport machinery and transformation status without the complications of confounding factors associated with the use of cells of different types, origins, and patients (59–61). Further, it is the first study to explore the kinetic parameters of nuclear transport in living transformed and nontransformed cells, establishing through fluorescence-based approaches and FRAP

Figure 1. Impα/β1- and Impβ1-dependent NLS-nuclear protein import is more efficient in transformed than in nontransformed cells. *A*) Typical CLSM images of 1BR3/1BR3N (top), CV-1/COS-7 (middle), and MCF10A/CA1h (bottom) isogenic cell line pairs (see Table 1), transfected to express GFP alone or the indicated NLS-containing GFP-fusion proteins (see Table 2) analyzed live at 16–24 h post-transfection (×100 oil-immersion objective). *B*) Quantitative analysis of the level of nuclear accumulation ($F_{n/c}$) of fusion proteins in digitized images such as those in *A* in 1BR3/1BR3N (left), CV-1/COS-7 (center) and MCF10A/CA1h (right) isogenic cell lines. Results represent means ± SE (n>33, from a series of ≥3 different experiments); fold change in $F_{n/c}$ ratio (indicated in parenthesis) represents the ratio of the $F_{n/c}$ values in transformed *vs.* nontransformed cells. *$P < 0.0001$ *vs.* nontransformed isogenic cells. *C*) Quantitative analysis of the relative $F_{n/c}$ of digitized images of CV-1/COS-7 (left) and 1BR3/1BR3N (right) cells transfected to express the indicated NLS-containing wild-type (WT) or NLS-mutated (NLSm) GFP-fusion proteins and analyzed live at 24 h posttransfection (×100 oil-immersion objective). Results represent means ± SE (n>22); fold change in $F_{n/c}$ ratio for each cell pair is indicated in parentheses. *$P < 0.0001$ *vs.* nontransformed isogenic cells. *D*) Quantitative analysis of the relative $F_{n/c}$ of digitized images of MCF10A/CA1h cells transfected to express the indicated NLS-containing GFP-fusion proteins in high-expressing (F_{n-b}>1500), medium-expressing (F_{n-b}=1500–500), and low-expressing (F_{n-b}<500) cell populations. Results represent means ± SE (n>14). *$P < 0.009$ *vs.* nontransformed cells.

TABLE 3. *Pooled data from a single representative fluorescence recovery after photobleaching experiment for GFP-T-ag(114–135) in populations of nontransformed MCF10A and transformed MCF10CA1h breast epithelial cells*

Parameter	MCF10A	MCF10CA1h	Normal/tumor
Half-time, $t_{1/2}$ (s)			
Nuclear recovery, F_{n-b}	27.4 ± 4.9 s	16.0 ± 1.9 s*	1.7
Cytoplasmic decay, F_{c-b}	101 ± 24.8 s	28.2 ± 3.7 s*	3.6
Maximal fractional recovery	0.82 ± 0.06	0.91 ± 0.05	0.9
Initial $F_{n/c}$	4.3 ± 0.60	16.7 ± 1.9*	NA
Initial fluorescence (arbitrary units)			
F_{n-b}	3218 ± 69	3466 ± 46*	0.93
F_{c-b}	964 ± 136	246 ± 31*	3.9

Results are presented as means ± SE (n=13). *$P < 0.05$; Student t test, unpaired data, 2-tailed.

studies that the efficiency and rate of nuclear import of various NLS-containing cargoes utilizing conventional, Imp-dependent pathways are enhanced in 3 different transformed cell lines compared with their nontransformed counterparts. The analysis of control molecules (GFP alone and NLS-mutated derivatives) established that the passive diffusion properties of the nuclear pore are not altered in the various lines and that the enhanced transport in the transformed lines is NLS dependent, with analysis of cell subpopulations with varying expression levels clearly indicating that differences between the transformed/nontransformed lines are not attributable to differing expression levels of the respective heterologous transport cargoes (62, 63). Rather, we propose that the basis of enhanced nuclear transport relates directly to increased levels of a subset of Imp/Exp molecules (Impα1, Impβ1, CAS, and Exp-1) and the Ran nucleotide exchange factor RCC1, wherein proof-of-principle experiments involving overexpression of specific Imps in nontransformed cells show increased nuclear transport efficiency. The clear implication is that increased nuclear transport efficiency may be a key feature of transformed cells.

Significantly increased nuclear import efficiencies and rates were observed for various different Impα/β1- and Impβ1-recognized NLS-containing proteins in 3 different transformed cell lines compared with their nontransformed counterparts; 2 of the lines were SV40 transformed (1BR3N and COS-7) and the other (MCF10CA1h) was representative of tumorigenic breast carcinoma cells. The observations with respect to the latter cell line represent the first report of enhanced nuclear import in nonvirally transformed tumor cell lines and tissues, but, consistent with our results here, enhanced nuclear import efficiency has previously been reported for SV40-transformed mouse fibroblasts (64, 65); enhanced nuclear import through conventional Impα/β1- and Impβ1-dependent path-

TABLE 4. *Quantitative analysis of nuclear accumulation of NLS/NES-containing GFP-fusion proteins in the absence as compared to the presence of LMB treatment in nontransformed and transformed cell lines; increased sensitivity to LMB treatment responsiveness in transformed cells*

	Nontransformed			Transformed			
GFP-fusion protein	−LMB	+LMB	+LMB/−LMB	−LMB	+LMB	+LMB/−LMB	$T_{[+LMB/-LMB]}$/ $NT_{[+LMB/-LMB]}$
	1BR3			1BR3N			
GFP alone	1.52 ± 0.04 (135)	1.57 ± 0.06 (58)	1.00	1.42 ± 0.02 (156)	1.70 ± 0.06 (41)	1.20	1.2
VP3(74–121)	2.89 ± 0.23 (114)	4.02 ± 0.54 (91)*	1.40	21.9 ± 1.94 (142)	24.8 ± 3.36 (110)	1.10	0.8
PTHrP(1–141)	2.85 ± 0.23 (112)	4.22 ± 0.58 (67)*	1.50	32.7 ± 5.73 (50)	91.6 ± 15.10 (21)*	2.80	1.9
Rev(2–116)	0.37 ± 0.04 (37)	18.2 ± 3.51 (50)*	50.00	0.16 ± 0.03 (67)	42.2 ± 5.00 (43)*	256.00	5.2
	CV-1			COS-7			
GFP alone	1.45 ± 0.062 (65)	1.38 ± 0.033 (51)	1.00	1.60 ± 0.05 (25)	1.68 ± 0.037 (41)	1.00	1.0
VP3(74–121)	2.34 ± 0.059 (205)	2.80 ± 0.123 (159)*	1.20	8.20 ± 0.40 (162)	7.52 ± 0.28 (80)	0.92	0.8
PTHrP(1–141)	4.78 ± 0.35 (44)	7.46 ± 1.22 (30)*	1.56	11.8 ± 0.60 (77)	21.9 ± 1.24 (77)*	1.85	1.2
Rev(2–116)	0.40 ± 0.013 (122)	26.8 ± 2.04 (102)*	67.30	0.28 ± 0.021 (75)	26.7 ± 2.23 (196)*	96.00	1.4
	MCF10A			MCF10CA1h			
GFP alone	1.24 ± 0.03 (41)	1.21 ± 0.02 (38)	1.00	1.14 ± 0.02 (61)	1.15 ± 0.02 (54)	1.00	1.0
VP3(74–121)	4.09 ± 0.43 (58)	3.73 ± 0.50 (63)	0.90	8.06 ± 0.61 (72)	9.71 ± 0.75 (85)	1.20	1.3
PTHrP(1–141)	3.67 ± 0.38 (75)	7.90 ± 0.56 (58)*	2.15	6.42 ± 0.47 (67)	20.14 ± 1.52 (60)*	3.14	1.5
Rev(2–116)	1.56 ± 1.79 (75)	9.17 ± 1.28 (59)*	5.90	0.50 ± 0.05 (69)	15.6 ± 1.37 (91)*	31.10	5.3

Values for +LMB and −LMB represent mean ± SE $F_{n/c}$; numbers in parentheses indicate n. $T_{[+LMB/-LMB]}$, transformed [+LMB/−LMB]; $NT_{[+LMB/-LMB]}$, nontransformed [+LMB/−LMB]. *$P < 0.0001$ *vs.* −LMB.

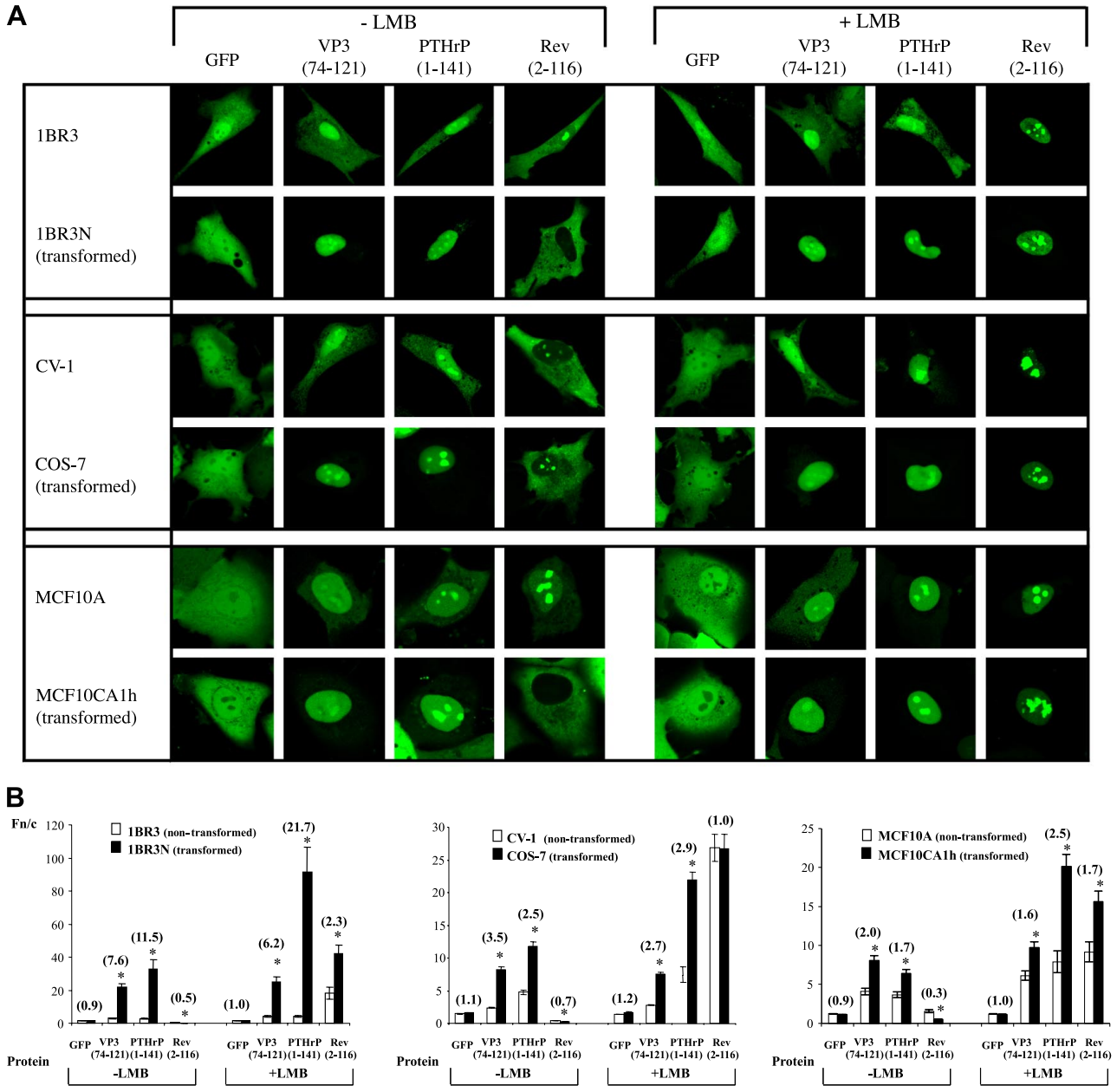


Figure 3. Reduced nuclear export is not the basis of enhanced nuclear accumulation of NLS/NES-containing proteins in transformed compared with nontransformed cells. *A*) Typical CLSM images of 1BR3/1BR3N (top), CV-1/COS-7 (middle), and MCF10A/CA1h (bottom) cells, transfected to express GFP alone or the indicated NLS/NES-containing GFP-fusion proteins (see Table 2), in the absence (−) or presence (+) of 2.8 μg/ml of the specific Exp-1 inhibitor LMB at 3–4 h before imaging and analyzed live at 16–24 h post-transfection (×100 oil-immersion objective). *B*) Quantitative analysis of the relative levels of nuclear accumulation $F_{n/c}$ between transformed *vs.* nontransformed cells from digitized images such as those shown in *A* in 1BR3/1BR3N (left), CV-1/COS-7 (center), and MCF10A/CA1h (right) isogenic cell lines. Results represent means ± SE (n>20, typical results from a series of ≥2 separate experiments); fold change in $F_{n/c}$ ratio (see Fig. 1*B* legend) for each cell pair is indicated in parentheses. *$P \leq 0.003$ *vs.* nontransformed cells.

ways may be a key feature of transformation by DNA tumor viruses such as SV40 to ensure the maximal nuclear concentration and activity of key host and viral gene products (see ref. 66). We also analyzed the efficiency of nuclear protein export for the first time in our 3 transformed/nontransformed cell pairs and found enhanced responsiveness to the Exp-1 inhibitor LMB in the transformed cells, suggesting higher intrinsic Exp-1 activity than in nontransformed cells. This was consistent with significantly increased Exp-1 protein levels in all of the transformed lines compared with their nontransformed counterparts.

Several studies in model cell types have shown that nuclear import efficiency can be influenced by Imp concentration (63, 67, 68). Here we observed significantly higher levels of both Impα1 and Impβ1 in transformed as opposed to nontransformed cells, in parallel with increased levels of the Ran guanine ex-

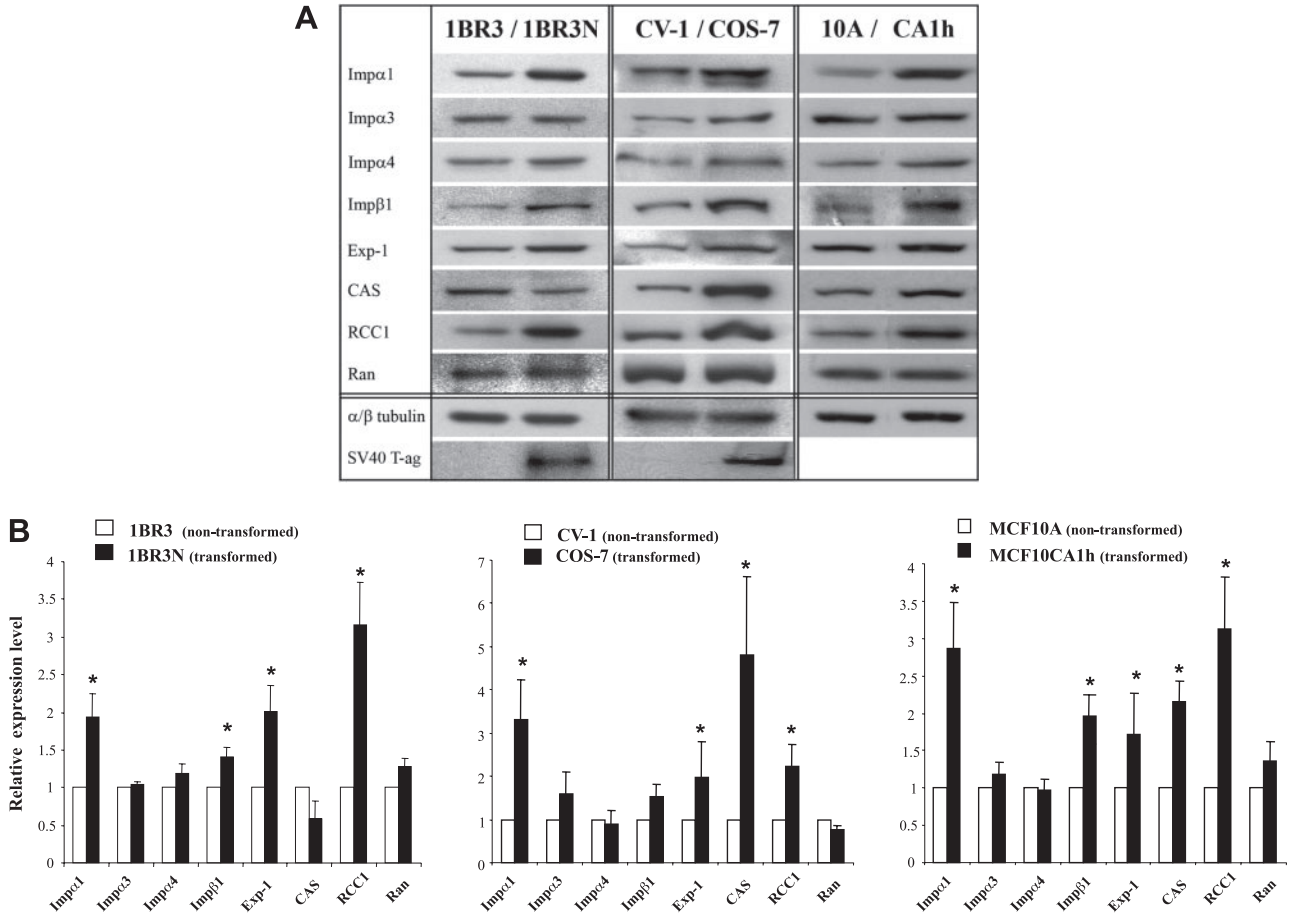


Figure 4. Impα1, Impβ1, Exp-1, CAS, and RCC1 are overexpressed in transformed compared with nontransformed cells. *A*) Endogenous nuclear transport factors in whole cell extracts of 1BR3/1BR3N (left panel), CV-1/COS-7 (middle panel) and MCF10A/CA1h (right panel) cell lines were detected by Western blot analysis using the indicated antibodies, with α/β-tubulin as a loading control, and SV40 T-ag control denoting transformed/nontransformed cell status as appropriate. Results are from a single typical experiment from a series of ≥3 separate experiments. *B*) Quantitative analysis of the relative expression levels of nuclear transport factors based on Western blot analysis as per *A* for 1BR3/1BR3N (left), CV-1/COS-7 (center), and MCF10A/CA1h (right) cell pairs, as determined by densitometry, is given as the ratio of the intensity of the signal obtained for the nuclear transport factor protein normalized to that for α/β tubulin in transformed cells, relative to nontransformed cells. Results represent means ± SE ($n \geq 3$). *$P < 0.05$ *vs.* nontransformed cells.

change factor, RCC1, which is important for unloading of import cargoes from Imps in the nucleus, as well as the exportin CAS, which is important for recycling Impα back to the cytoplasm after a round of nuclear import, and Exp-1. Since enhanced nuclear import efficiency in the transformed cell lines is dependent on a functional NLS, and ectopically expressed Imps clearly enhance nuclear transport efficiency in nontransformed MCF10A breast cells, it does not seem unreasonable to propose that the enhanced nuclear transport efficiency in the transformed cells observed here is a direct result of the increased levels of specific nuclear transport components, which are observed in many transformed cell lines and tumor samples (14, 19, 20, 22, 30, 31, 69, 70). Enhanced nuclear trafficking through higher levels of Imps and Exps could potentially affect the nuclear targeting of many growth- and proliferation-regulating signaling molecules and transcription factors, such as mitogen-activated protein kinase (58), or cytoplasmic localization of growth inhibitory factors such as $p27^{KIP}$ or $p21^{CIP}$, and thereby promote the transformed cell phenotype. The extent to which enhanced (amplified) signaling between the nucleus and cytoplasm may influence tumor biology and cancer pathology remains to be determined, but it is an intriguing possibility that enhanced nuclear transport may be a critical factor in tumor cell growth, proliferation, and progression, and hence a potential target for therapeutic intervention to treat cancer (*e.g.*, see refs. 71–73).

Notably, the results here raise the exciting possibility that the enhanced nuclear transport properties of transformed cells may be exploited to facilitate tumor cell-specific drug delivery. It is already known that the addition of NLSs to modular recombinant transporters (MRTs, comprised of assemblies of independently functioning protein domains capable of mediating cellular entry, endosomal escape, and efficient nuclear targeting of photosensitizing drugs; refs. 32, 34, 74) can improve cell killing >50-fold by inducing the localized

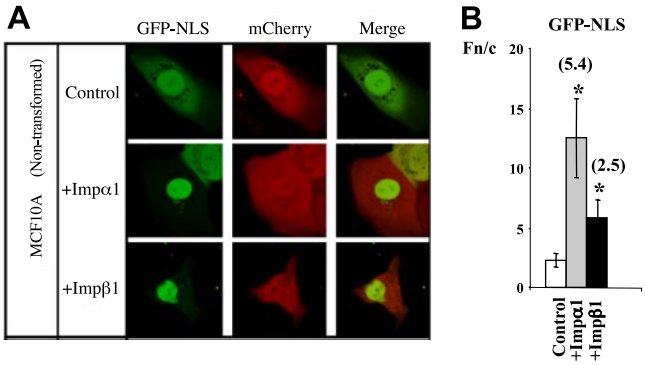


Figure 5. Overexpression of Impα1 or Impβ1 in nontransformed cells enhances protein nuclear accumulation. *A*) CLSM images of normal MCF10A cells cotransfected to express GFP-T-ag(114–135) and Impα1, Impβ1, or control (empty vector) imaged at 40 h post-transfection. mCherry expression from the pIRES-Imp-mCherry bicistronic plasmid was used to identify transfected cells. *B*) Quantitative analysis of the relative levels of nuclear accumulation $F_{n/c}$ of control (empty vector-transfected) or Imp-expressing MCF10A cells. Results represent means ± SE (n>41, results are from a single typical experiment in a series of 3 separate experiments). Fold changes in $F_{n/c}$ compared to MCF10A control are indicated in parenthesis. *$P < 0.05$ *vs.* MCF10A control.

generation of DNA-damaging reactive oxygen species (ROS) in the cell nucleus, the most sensitive site for ROS-induced damage (75, 76). Since the efficacy of cell killing through MRTs relies to a large extent on the efficiency of drug delivery to the nucleus, the fact that certain transformed cells possess intrinsically more efficient nuclear import implies that MRT killing action should be greater in transformed than nontransformed cells, supporting the idea of the utility of nuclear drug delivery in anticancer therapy.

Finally, the findings here have strong relevance to studies where SV40-induced transformation is used to increase cellular life span and growth potential to generate apparently "normal" model cell types, *i.e.*, from primary cells and mouse embryonic fibroblasts for a whole range of different applications (*e.g.*, studies of development). Based on the results here, it would seem judicious to be cautious in interpreting results using such cells, since SV40-transformed cells will almost certainly have significantly enhanced nuclear transport properties, which in turn may alter cell phenotype in various, yet-to-be-identified ways. In short, it is important to note that the use of SV40 in cell lines may well not be suitable for many studies for which they are currently used as accepted practice.

In summary, the results show that several transformed cell types from different origins possess enhanced Imp- and also Exp-dependent nuclear transport, correlated with increased levels of Imp and Exp proteins. The results are important for future anticancer therapies relying on nuclear drug delivery, as well as cell biological studies relying on transformation by SV40. Our future work will focus on how altered nucleocytoplasmic transport efficiency can be exploited optimally to achieve tumor-selective nuclear drug delivery using nonviral delivery approaches. FJ

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REFERENCES

1. Goldfarb, D. S., Corbett, A. H., Mason, D. A., Harreman, M. T., and Adam, S. A. (2004) Importin alpha: a multipurpose nuclear-transport receptor. *Trends Cell Biol.* **14,** 505–514
2. Harel, A., and Forbes, D. J. (2004) Importin beta: conducting a much larger cellular symphony. *Mol. Cell* **16,** 319–330
3. Pemberton, L. F., and Paschal, B. M. (2005) Mechanisms of receptor-mediated nuclear import and nuclear export. *Traffic* **6,** 187–198
4. Fukuda, M., Asano, S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M., and Nishida, E. (1997) CRM1 is responsible for intracellular transport mediated by the nuclear export signal. *Nature* **390,** 308–311
5. Kutay, U., Bischoff, F. R., Kostka, S., Kraft, R., and Gorlich, D. (1997) Export of importin alpha from the nucleus is mediated by a specific nuclear transport factor. [see comment] *Cell* **90,** 1061–1071
6. Jans, D. A., Xiao, C. Y., and Lam, M. H. (2000) Nuclear targeting signal recognition: a key control point in nuclear transport? *Bioessays* **22,** 532–544
7. Poon, I. K. H., and Jans, D. A. (2005) Regulation of nuclear transport: central role in development and transformation? *Traffic* **6,** 173–186
8. Hogarth, C. A., Calanni, S., Jans, D. A., and Loveland, K. L. (2006) Importin alpha mRNAs have distinct expression profiles during spermatogenesis. *Dev. Dyn.* **235,** 253–262
9. Loveland, K. L., Hogarth, C., Szczepny, A., Prabhu, S. M., and Jans, D. A. (2006) Expression of nuclear transport importins beta 1 and beta 3 is regulated during rodent spermatogenesis. *Biol. Reprod.* **74,** 67–74
10. Yasuhara, N., Shibazaki, N., Tanaka, S., Nagai, M., Kamikawa, Y., Oe, S., Asally, M., Kamachi, Y., Kondoh, H., and Yoneda, Y. (2007) Triggering neural differentiation of ES cells by subtype switching of importin-alpha. *Nat. Cell Biol.* **9,** 72–79; erratum, 479
11. Young, J. C., Major, A. T., Miyamoto, Y., Loveland, K. L., and Jans, D. A. (2011) Distinct effects of importin α2 and α4 on Oct3/4 localization and expression in mouse embryonic stem cells. *FASEB J.* **25,** 3958–3965
12. Hosokawa, K., Nishi, M., Sakamoto, H., Tanaka, Y., and Kawata, M. (2008) Regional distribution of importin subtype mRNA expression in the nervous system: Study of early postnatal and adult mouse. *Neuroscience* **157,** 864–877
13. Dahl, E., Kristiansen, G., Gottlob, K., Klaman, I., Ebner, E., Hinzmann, B., Hermann, K., Pilarsky, C., Dürst, M., Klinkhammer-Schalke, M., Blaszyk, H., Knuechel, R., Hartmann, A., Rosenthal, A., and Wild, P. J. (2006) Molecular profiling of laser-microdissected matched tumor and normal breast tissue identifies karyopherin alpha2 as a potential novel prognostic marker in breast cancer. *Clin. Cancer Res.* **12,** 3950–3960
14. Dankof, A., Fritzsche, F. R., Dahl, E., Pahl, S., Wild, P., Dietel, M., Hartmann, A., and Kristiansen, G. (2007) KPNA2 protein expression in invasive breast carcinoma and matched peritumoral ductal carcinoma in situ. *Virchows Arch.* **451,** 877–881
15. Gluz, O., Wild, P., Meiler, R., Diallo-Danebrock, R., Ting, E., Mohrmann, S., Schuett, G., Dahl, E., Fuchs, T., Herr, A., Gaumann, A., Frick, M., Poremba, C., Nitz, U. A., and Hart-

mann, A. (2008) Nuclear karyopherin alpha2 expression predicts poor survival in patients with advanced breast cancer irrespective of treatment intensity. *Int. J. Cancer* **123,** 1433–1438
16. Sotiriou, C., Wirapati, P., Loi, S., Harris, A., Fox, S., Smeds, J., Nordgren, H., Farmer, P., Praz, V., Haibe-Kains, B., Desmedt, C., Larsimont, D., Cardoso, F., Peterse, H., Nuyten, D., Buyse, M., Van de Vijver, M. J., Bergh, J., Piccart, M., and Delorenzi, M. (2006) Gene expression profiling in breast cancer: understanding the molecular basis of histologic grade to improve prognosis. *J. Natl. Cancer Inst.* **98,** 262–272
17. Wang, Y., Klijn, J. G. M., Zhang, Y., Sieuwerts, A. M., Look, M. P., Yang, F., Talantov, D., Timmermans, M., Meijer-van Gelder, M. E., Yu, J., Jatkoe, T., Berns, E. M. J. J., Atkins, D., and Foekens, J. A. (2005) Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. *Lancet* **365,** 671–679
18. Jensen, J. B., Munksgaard, P. P., Sorensen, C. M., Fristrup, N., Birkenkamp-Demtroder, K., Ulhoi, B. P., Jensen, K. M.-E., Orntoft, T. F., and Dyrskjot, L. High expression of karyopherin-alpha2 defines poor prognosis in non-muscle-invasive bladder cancer and in patients with invasive bladder cancer undergoing radical cystectomy. *Eur. Urol.* **59,** 841–848
19. Dyrskjot, L., Kruhoffer, M., Thykjaer, T., Marcussen, N., Jensen, J. L., Moller, K., and Orntoft, T. F. (2004) Gene expression in the urinary bladder: a common carcinoma in situ gene expression signature exists disregarding histopathological classification. *Cancer Res.* **64,** 4040–4048
20. Sakai, M., Sohda, M., Miyazaki, T., Suzuki, S., Sano, A., Tanaka, N., Inose, T., Nakajima, M., Kato, H., and Kuwano, H. Significance of karyopherin-α 2 (KPNA2) expression in esophageal squamous cell carcinoma. *Anticancer Res.* **30,** 851–856
21. Sato, F., Abraham, J. M., Yin, J., Kan, T., Ito, T., Mori, Y., Hamilton, J. P., Jin, Z., Cheng, Y., Paun, B., Berki, A. T., Wang, S., Shimada, Y., and Meltzer, S. J. (2006) Polo-like kinase and survivin are esophageal tumor-specific promoters. *Biochem. Biophys. Res. Commun.* **342,** 465–471
22. Van der Watt, P. J., Maske, C. P., Hendricks, D. T., Parker, M. I., Denny, L., Govender, D., Birrer, M. J., and Leaner, V. D. (2009) The Karyopherin proteins, Crm1 and Karyopherin beta1, are overexpressed in cervical cancer and are critical for cancer cell survival and proliferation. *Int. J. Cancer* **124,** 1829–1840
23. Yoshitake, K., Tanaka, S., Mogushi, K., Aihara, A., Murakata, A., Matsumura, S., Mitsunori, Y., Yasen, M., Ban, D., Noguchi, N., Irie, T., Kudo, A., Nakamura, N., Tanaka, H., and Arii, S. (2011) Importin-α1 as a novel prognostic target for hepatocellular carcinoma. *Ann. Surg. Oncol.* **7,** 2093–2103
24. Kirschbaum-Slager, N., Parmigiani, R. B., Camargo, A. A., de Souza, S. J., and x00E. (2005) Identification of human exons overexpressed in tumors through the use of genome and expressed sequence data. *Physiol. Genom.* **21,** 423–432
25. Zheng, M., Tang, L., Huang, L., Ding, H., Liao, W.-T., Zeng, M.-S., and Wang, H.-Y. Overexpression of karyopherin-2 in epithelial ovarian cancer and correlation with poor prognosis. *Obstet. Gynecol.* **116,** 884–891
26. Behrens, P., Brinkmann, U., Fogt, F., Wernert, N., and Wellmann, A. (2001) Implication of the proliferation and apoptosis associated CSE1L/CAS gene for breast cancer development. *Anticancer Res.* **21,** 2413–2417
27. Brinkmann, U., Gallo, M., Polymeropoulos, M. H., and Pastan, I. (1996) The human CAS (cellular apoptosis susceptibility) gene mapping on chromosome 20q13 is amplified in BT474 breast cancer cells and part of aberrant chromosomes in breast and colon cancer cell lines. *Genome Res.* **6,** 187–194
28. Wellmann, A., Flemming, P., Behrens, P., Wuppermann, K., Lang, H., Oldhafer, K., Pastan, I., and Brinkmann, U. (2001) High expression of the proliferation and apoptosis associated CSE1L/CAS gene in hepatitis and liver neoplasms: correlation with tumor progression. *Int. J. Mol. Med.* **7,** 489–494
29. Brustmann, H. (2004) Expression of cellular apoptosis susceptibility protein in serous ovarian carcinoma: a clinicopathologic and immunohistochemical study. *Gynecol. Oncol.* **92,** 268–276
30. Faggad, A., Darb-Esfahani, S., Wirtz, R., Sinn, B., Sehouli, J., x00F, nsgen, D., Lage, H., Weichert, W., Noske, A., Budczies, J., x00Fc, ller, B. M., Buckendahl, A.-C., x00F, ske, A., Eldin Elwali, N., Dietel, M., and Denkert, C. (2009) Topoisomerase IIalpha mRNA and protein expression in ovarian carcinoma: correlation with clinicopathological factors and prognosis. *Mod. Pathol.* **22,** 579–588
31. Noske, A., Weichert, W., Niesporek, S., x00F, ske, A., Buckendahl, A.-C., Koch, I., Sehouli, J., Dietel, M., and Denkert, C. (2008) Expression of the nuclear export protein chromosomal region maintenance/exportin 1/Xpo1 is a prognostic factor in human ovarian cancer. *Cancer* **112,** 1733–1743
32. Glover, D. J., Lipps, H. J., and Jans, D. A. (2005) Towards safe, non-viral therapeutic gene expression in humans. *Nat. Rev. Genet.* **6,** 299–310
33. Kuusisto, H. V., Wagstaff, K. M., Alvisi, G., and Jans, D. A. (2008) The C-terminus of apoptin represents a unique tumor cell-enhanced nuclear targeting module. *Int. J. Cancer* **123,** 2965–2969
34. Wagstaff, K. M., and Jans, D. A. (2009) Nuclear drug delivery to target tumour cells. *Eur. J. Pharmacol.* **625,** 174–180
35. Alvisi, G., Ripalti, A., Ngankeu, A., Giannandrea, M., Caraffi, S. G., Dias, M. M., and Jans, D. A. (2006) Human cytomegalovirus DNA polymerase catalytic subunit pUL54 possesses independently acting nuclear localization and ppUL44 binding motifs. *Traffic* **7,** 1322–1332
36. Moseley, G. W., Roth, D. M., DeJesus, M. A., Leyton, D. L., Filmer, R. P., Pouton, C. W., and Jans, D. A. (2007) Dynein light chain association sequences can facilitate nuclear protein import. *Mol. Biol. Cell* **18,** 3204–3213
37. Poon, I. K. H., Oro, C., Dias, M. M., Zhang, J. P., and Jans, D. A. (2005) A tumor cell-specific nuclear targeting signal within chicken anemia virus VP3/apoptin [comment]. *J. Virol.* **79,** 1339–1341
38. Roth, D. M., Moseley, G. W., Glover, D., Pouton, C. W., and Jans, D. A. (2007) A microtubule-facilitated nuclear import pathway for cancer regulatory proteins. *Traffic* **8,** 673–686
39. Ghildyal, R., Ho, A., Dias, M., Soegiyono, L., Bardin, P. G., Tran, K. C., Teng, M. N., and Jans, D. A. (2009) The respiratory syncytial virus matrix protein possesses a Crm1-mediated nuclear export mechanism. *J. Virol.* **83,** 5353–5362
40. Forwood, J. K., and Jans, D. A. (2002) Nuclear import pathway of the telomere elongation suppressor TRF1: inhibition by importin alpha. *Biochemistry* **41,** 9333–9340
41. Arlett, C. F., Green, M. H., Priestley, A., Harcourt, S. A., and Mayne, L. V. (1988) Comparative human cellular radiosensitivity: I. The effect of SV40 transformation and immortalisation on the gamma-irradiation survival of skin derived fibroblasts from normal individuals and from ataxia-telangiectasia patients and heterozygotes. *Int. J. Radiat. Biol.* **54,** 911–928
42. Gluzman, Y. (1981) SV40-transformed simian cells support the replication of early SV40 mutants. *Cell* **23,** 175–182
43. Santner, S. J., Dawson, P. J., Tait, L., Soule, H. D., Eliason, J., Mohamed, A. N., Wolman, S. R., Heppner, G. H., and Miller, F. R. (2001) Malignant MCF10CA1 cell lines derived from premalignant human breast epithelial MCF10AT cells. *Breast Cancer Res. Treat.* **65,** 101–110
44. Poon, I. K. H., Oro, C., Dias, M. M., Zhang, J., and Jans, D. A. (2005) Apoptin nuclear accumulation is modulated by a CRM1-recognized nuclear export signal that is active in normal but not in tumor cells. *Cancer Res.* **65,** 7059–7064
45. Roth, D. M., Moseley, G. W., Pouton, C. W., and Jans, D. A. Mechanism of microtubule-facilitated "fast track" nuclear import. *J. Biol. Chem.* **286,** 14335–14351
46. Soule, H. D., Maloney, T. M., Wolman, S. R., Peterson, W. D., Jr., Brenz, R., McGrath, C. M., Russo, J., Pauley, R. J., Jones, R. F., and Brooks, S. C. (1990) Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. *Cancer Res.* **50,** 6075–6086
47. Hubner, S., Xiao, C. Y., and Jans, D. A. (1997) The protein kinase CK2 site (Ser111/112) enhances recognition of the simian virus 40 large T-antigen nuclear localization sequence by importin. *J. Biol. Chem.* **272,** 17191–17195
48. Wagstaff, K. M., and Jans, D. A. (2006) Intramolecular masking of nuclear localization signals: analysis of importin binding using a novel AlphaScreen-based method. *Anal. Biochem.* **348,** 49–56
49. Cardarelli, F., Serresi, M., Bizzarri, R., Giacca, M., and Beltram, F. (2007) In vivo study of HIV-1 Tat arginine-rich motif unveils its transport properties. *Mol. Ther.* **15,** 1313–1322
50. Lam, M. H., Briggs, L. J., Hu, W., Martin, T. J., Gillespie, M. T., and Jans, D. A. (1999) Importin beta recognizes parathyroid

hormone-related protein with high affinity and mediates its nuclear import in the absence of importin alpha. *J. Biol. Chem.* **274,** 7391–7398
51. Pache, J. C., Burton, D. W., Deftos, L. J., and Hastings, R. H. (2006) A carboxyl leucine-rich region of parathyroid hormone-related protein is critical for nuclear export. *Endocrinology* **147,** 990–998
52. Askjaer, P., Jensen, T. H., Nilsson, J., Englmeier, L., and Kjems, J. (1998) The specificity of the CRM1-Rev nuclear export signal interaction is mediated by RanGTP. *J. Biol. Chem.* **273,** 33414–33422
53. Henderson, B. R., and Percipalle, P. (1997) Interactions between HIV Rev and nuclear import and export factors: the Rev nuclear localisation signal mediates specific binding to human importin-beta. *J. Mol. Biol.* **274,** 693–707
54. Truant, R., and Cullen, B. R. (1999) The arginine-rich domains present in human immunodeficiency virus type 1 Tat and Rev function as direct importin beta-dependent nuclear localization signals. *Mol. Cell. Biol.* **19,** 1210–1217
55. Arnold, M., Nath, A., Hauber, J., and Kehlenbach, R. H. (2006) Multiple importins function as nuclear transport receptors for the Rev protein of human immunodeficiency virus type 1. *J. Biol. Chem.* **281,** 20883–20890
56. Gu, L., Tsuji, T., Jarboui, M. A., Yeo, G. P., Sheehy, N., Hall, W. W., and Gautier, V. W. (2011) Intermolecular masking of the HIV-1 Rev NLS by the cellular protein HIC: novel insights into the regulation of Rev nuclear import. *Retrovirology* **8,** 17
57. Nakashima, T., Masuda, A., Sekiguchi, T., Nishimoto, T., and Uemura, T. (1994) Preliminary findings of chromosomal alterations and expression of cell cycle genes in head an neck tumors. *Eur. Arch. Otorhinolaryngol.* **251**(Suppl. 1), S87–S90
58. Smith, E. R., Cai, K. Q., Smedberg, J. L., Ribeiro, M. M., Rula, M. E., Slater, C., Godwin, A. K., and Xu, X.-X. Nuclear entry of activated MAPK is restricted in primary ovarian and mammary epithelial cells. *PLoS ONE* **5,** e9295
59. Nadler, S. G., Tritschler, D., Haffar, O. K., Blake, J., Bruce, A. G., and Cleaveland, J. S. (1997) Differential expression and sequence-specific interaction of karyopherin alpha with nuclear localization sequences. *J. Biol. Chem.* **272,** 4310–4315
60. Kohler, M., Ansieau, S., Prehn, S., Leutz, A., Haller, H., and Hartmann, E. (1997) Cloning of two novel human importin-alpha subunits and analysis of the expression pattern of the importin-alpha protein family. *FEBS Lett.* **417,** 104–108
61. Kohler, M., Fiebeler, A., Hartwig, M., Thiel, S., Prehn, S., Kettritz, R., Luft, F. C., and Hartmann, E. (2002) Differential expression of classical nuclear transport factors during cellular proliferation and differentiation. *Cell. Physiol. Biochem.* **12,** 335–344
62. Tachibana, T., Hieda, M., and Yoneda, Y. (1999) Up-regulation of nuclear protein import by nuclear localization signal sequences in living cells. *FEBS Lett.* **442,** 235–240
63. Timney, B. L., Tetenbaum-Novatt, J., Agate, D. S., Williams, R., Zhang, W., Chait, B. T., and Rout, M. P. (2006) Simple kinetic relationships and nonspecific competition govern nuclear import rates in vivo. *J. Cell Biol.* **175,** 579–593
64. Feldherr, C. M., Lanford, R. E., and Akin, D. (1992) Signal-mediated nuclear transport in simian virus 40-transformed cells is regulated by large tumor antigen. *Proc. Natl. Acad. Sci. U. S. A.* **89,** 11002–11005
65. Feldherr, C., and Akin, D. (1995) Stimulation of nuclear import by simian virus 40-transformed cell extracts is dependent on protein kinase activity. *Mol. Cell. Biol.* **15,** 7043–7049
66. Fulcher, A. J., and Jans, D. A. (2011) Regulation of nucleocytoplasmic trafficking of viral proteins: An integral role in pathogenesis? *Biochim. Biophys. Acta* **1813,** 2176–2190
67. Riddick, G., and Macara, I. G. (2005) A systems analysis of importin-α-β mediated nuclear protein import. *J. Cell Biol.* **168,** 1027–1038
68. Yang, W., and Musser, S. M. (2006) Nuclear import time and transport efficiency depend on importin beta concentration. *J. Cell Biol.* **174,** 951–961
69. Mortezavi, A., Hermanns, T., Seifert, H.-H., Baumgartner, M. K., Provenzano, M., Sulser, T., Burger, M., Montani, M., Ikenberg, K., Hofstadter, F., Hartmann, A., Jaggi, R., Moch, H., Kristiansen, G., and Wild, P. J. KPNA2 expression is an independent adverse predictor of biochemical recurrence after radical prostatectomy. *Clin. Cancer Res.* **17,** 1111–1121
70. Wu, M.-S., Lin, Y.-S., Chang, Y.-T., Shun, C.-T., Lin, M.-T., and Lin, J.-T. (2005) Gene expression profiling of gastric cancer by microarray combined with laser capture microdissection. *World J. Gastroenterol.* **11,** 7405–7412
71. Chahine, M. N., and Pierce, G. N. (2009) Therapeutic targeting of nuclear protein import in pathological cell conditions. *Pharmacol. Rev.* **61,** 358–372
72. Mutka, S. C., Yang, W. Q., Dong, S. D., Ward, S. L., Craig, D. A., Timmermans, P. B., and Murli, S. (2009) Identification of nuclear export inhibitors with potent anticancer activity in vivo. *Cancer Res.* **69,** 510–517
73. Lane, D. P., Cheok, C. F., and Lain, S. (2010) p53-based cancer therapy. *Cold Spring Harb. Perspect. Biol.* **2,** a001222
74. Sobolev, A. S. (2009) Novel modular transporters delivering anticancer drugs and foreign DNA to the nuclei of target cancer cells. *J. BUON* **14**(Suppl. 1), S33–S42
75. Akhlynina, T. V., Jans, D. A., Rosenkranz, A. A., Statsyuk, N. V., Balashova, I. Y., Toth, G., Pavo, I., Rubin, A. B., and Sobolev, A. S. (1997) Nuclear targeting of chlorin e6 enhances its photosensitizing activity. *J. Biol. Chem.* **272,** 20328–20331
76. Rosenkranz, A. A., Lunin, V. G., Gulak, P. V., Sergienko, O. V., Shumiantseva, M. A., Voronina, O. L., Gilyazova, D. G., John, A. P., Kofner, A. A., Mironov, A. F., Jans, D. A., and Sobolev, A. S. (2003) Recombinant modular transporters for cell-specific nuclear delivery of locally acting drugs enhance photosensitizer activity. *FASEB J.* **17,** 1121–1123
77. Jensen, F. C., Girardi, A. J., Gilden, R. V., and Koprowski, H. (1964) Infection of human and simian tissue cultures with rous sarcoma virus. *Proc. Natl. Acad. Sci. U. S. A.* **52,** 53–59
78. Kalderon, D., Richardson, W. D., Markham, A. F., and Smith, A. E. (1984) Sequence requirements for nuclear location of simian virus 40 large-T antigen. *Nature* **311,** 33–38
79. Kalderon, D., Roberts, B. L., Richardson, W. D., and Smith, A. E. (1984) A short amino acid sequence able to specify nuclear location. *Cell* **39,** 499–509
80. Hakata, Y., Yamada, M., Mabuchi, N., and Shida, H. (2002) The carboxy-terminal region of the human immunodeficiency virus type 1 protein Rev has multiple roles in mediating CRM1-related Rev functions. *J. Virol.* **76,** 8079–8089

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