MODULATING THE EXPRESSION AND ACTIVITY OF THE NUCLEAR IMPORT PROTEIN, KARYOPHERIN β1, IN CANCER CELLS

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DECLARATION

I, Sarah Carden, hereby declare that the work on which this dissertation is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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CONTENTS

ABBREVIATIONS

~	Approximately
°C	Degrees Celsius
%	Percentage
APC	Adenomatous polyposis coli
APS	Ammonium Persulphate
BCA	Bicinchoninic acid
BRCA1	Breast Cancer 1
bp	Base pairs
BSA	Bovine Serum Albumin
CAS	Cellular Apoptosis Susceptibility Protein
Cdk	Cyclin-dependent kinase
cDNA	Complementary Deoxyribonucleic acid
CHX	Cycloheximide
cNLS	Classical Nuclear Localisation Signal
CRM1	Chromosome Region Maintenance 1
CSE1L	Chromosome Segregation 1 Like
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
DOX	Doxycycline

E.coli	Escherichia coli
EC_{50}	Half maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase
FACS	Fluorescent Activated Cell Sorting
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
FG	Phenylalanine-glycine
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GFP	Green Fluorescent Protein
H_2O	Water
HCl	Hydrogen chloride (hydrochloric acid)
HRP	Horse Radish Peroxidase
IκB	Inhibitor of Kappa B
IKK	IκB kinase
INI-43	Inhibitor of Nuclear Import-43
IRES	Internal Ribosome Entry Site
Kb	Kilobase
KCl	Potassium chloride
kDa	Kilodaltons
Kpnβ1	Karyopherin beta 1

Kpnα2	Karyopherin alpha 2
LA	Luria Agar
LB	Luria Broth
LMB	Leptomycin B
Log	Logarithm
Mcl-1	Myeloid cell leukemia 1
MCS	Multiple Cloning Site
MET	Mesencymal-epithelial transition
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
mRNA	Messenger Ribonucleic Acid
MTT	3'-(4',5'-Dimethylthiazol-2'-yl)-2',5'-diphenyltetrazolium bromide
Na_3VO_4	Sodium orthovanadate
NaCl	Sodium chloride
NES	Nuclear Export Signal
NFAT	Nuclear Factor of Activated T cells
NFκB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NLS	Nuclear Localisation Signal
NPC	Nuclear Pore Complex
NTF2	Nuclear Transport Factor 2
Nup	Nucleoporin
OD	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline

Ran	Ras-related nuclear protein
RanGAP1	RanGTP-activating protein 1
RanGDP	Ran-Guanosine Diphosphate
RanGEF	Ran-Guanosine Nucleotide Exchange Factor
RanGTP	Ran-Guanosine Triphosphate
RCC1	Regulator of Chromosome Condensation 1
RIPA	Radioimmunoprecipitation Assay
RNA	Ribonucleic Acid
RNAi	RNA interference
RNase	Ribonuclease
RPM	Revolutions per minute
rRNA	Ribosomal Ribonucleic Acid
SAF	Spindle Assembly Factor
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of the Mean
SINE	Selective Inhibitors of Nuclear Export
siRNA	Small interfering RNA
STAT	Signal Transducer and Activator of Transcription
TBE	Tris-Borate EDTA
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline-Tween-20
TE	Tris-EDTA
TEMED	Tetramethylethylenediamine
tRNA	Transfer Ribonucleic Acid

Units

M	molar
mM	milimolar
μM	micromolar
nM	nanomolar
g	gram
mg	miligram
μg	microgram
ng	nanogram
L	liter
ml	milileter
μl	microliter
cm	centimeter
mm	milimeter
μm	micrometer
nm	nanometer
sec	second
min	minute
hr	hour
V	Volts

ABSTRACT

Cancer is primarily a disease of disordered gene expression; the dysregulation of thousands of different genes has been associated with the progression of many types of cancer. Previous research from our laboratory aimed at identifying genes differentially expressed in cervical cancer compared to normal patient tissue, found Karyopherin β1 (Kpnβ1), the primary nuclear import protein, to be significantly overexpressed in cervical cancer tissue. Further studies showed that inhibition of Kpnβ1 expression by siRNA resulted in cancer cell death, while non-cancer cells were only minimally affected. These results suggest that Kpnβ1 has potential as an anti-cancer therapeutic target, thus warranting further research into the association between Kpnβ1 expression and cancer progression.

In this study, we investigated the biological effects associated with Kpnβ1 overexpression in order to further elucidate the relationship between Kpnβ1 and the cancer phenotype. Our data revealed that Kpnβ1 overexpression, above what was already detected in cancer cells, resulted in reduced proliferation and an associated delay in cell cycle progression. Additionally, overexpression of Kpnβ1 caused changes in the morphology and adhesion properties of cells. Co-expression of Ran, an important nuclear transport factor, binding partner and regulator of Kpnβ1, resulted in a further reduction in proliferation (greater than that of overexpression of either Kpnβ1 or Ran alone), suggesting that cells are particularly unable to handle an imbalance in the levels of Kpnβ1 and Ran.

Previous work from our laboratory using a newly identified small molecule, Inhibitor of Nuclear Import-43 (INI-43), showed that INI-43 significantly reduced the proliferation of cancer cells of different tissue origins and interfered with the nuclear import function of Kpnβ1. In order to investigate the specificity of INI-43 for Kpnβ1 in this study, we performed experiments to determine whether overexpression of Kpnβ1 could rescue cancer cells from the negative effects associated with INI-43 treatment. Results revealed that stable overexpression of Kpnβ1 was able to rescue cell viability, as well as the inhibitory effects that INI-43 had on the nuclear import of Kpnβ1 cargoes NFκB p65 and NFAT. Kpnβ1 overexpression was also able to rescue cells from an INI-43 induced G2/M cell cycle block. In addition, treatment of cells with INI-43 enhanced the degradation of Kpnβ1, indicating that INI-43 is likely acting by targeting Kpnβ1.

In order to determine the effects of Kpnβ1 dysregulation in HeLa cells, live cell time-lapse videomicroscopy was used to study cells as the progressed through the cell cycle. Inducible expression of Kpnβ1-EGFP, as well as the treatment of cells with the small molecule inhibitor INI-43, were used as mechanisms of dysregulation. Results revealed that modulation of Kpnβ1 by either overexpression or inhibition caused a disruption in mitotic progression, with the appearance of distinct mitotic abnormalities. The treatment of cells expressing Kpnβ1-EGFP with INI-43 resulted in a significant reduction in (or rescue of) the negative effects associated with either condition alone.

Taken together, this data suggests that a precise balance of Kpnβ1 expression is required for the correct functioning of cancer cells; when the balance is perturbed in either direction (i.e. with overexpression of Kpnβ1 or INI-43-mediated inhibition of Kpnβ1)

negative effects associated with a variety of biological processes are observed. In addition, results from rescue experiments conducted using Kpnβ1 overexpression in combination with INI-43 treatment suggest that that INI-43 is acting, at least in part, by targeting Kpnβ1.

CHAPTER 1

LITERATURE REVIEW

1.1 Cancer

Cancer is a leading cause of mortality worldwide. According to a report issued by the International Agency for Research on Cancer (IARC), in 2012 the global cancer burden was documented as 14.1 million new cases per year, with an expectation to rise to 22 million new cases within the next two decades. By the year 2032, cancer deaths are predicted to have risen from 8.2 million to 13 million deaths per annum, with a disproportionate 70% of these deaths occurring in less developed countries[1]. Increasing numbers in global cancer incidence and mortality stress the urgency and need for focussed research towards the identification of novel anti-cancer agents, improvement of diagnosis, and the implementation of effective prevention and treatment strategies.

1.2 Current anti-cancer treatment strategies

Over the past few decades, major advances in scientific research and medical practice have resulted in a dramatic improvement in the access, availability and effectiveness of anti-cancer treatment options. Conventional cancer treatment strategies such as surgery, radiation therapy and traditional chemotherapy are better understood and more widely used, while newer areas such as targeted therapy[2], immunotherapy[3] and gene therapy[4]

are emerging as promising alternatives in the field of precision medicine. Since reaching a peak in 1991, cancer death rates in the United States have declined by 22%, and an approximate 83% improvement in cancer survival rates can be largely attributed to new treatments[5,6]. Despite these figures, treatment options for a wide range of cancers are still limited and the need for novel anti-cancer therapeutics remains urgent.

Targeted therapies are currently the focus of many cancer research groups and pharmaceutical companies, whose aim is to identify novel anti-cancer therapeutics with higher efficacy and limited side effects. While traditional chemotherapy involves the use of chemical agents to target all rapidly dividing cells (normal and cancerous), targeted therapy refers to the use of drugs designed to interfere with specific molecular targets associated with cancer development and progression[2]. The intention is to limit the side effects commonly associated with conventional chemotherapy by specifically targeting and killing cancer cells, thereby limiting associated toxicities to non-cancer cells. Perhaps one of the most successful targeted therapies identified to date is the tyrosine kinase inhibitor imatinib mesylate (Gleevec), which targets and inhibits the kinase fusion protein Bcr-Abl in chronic myeloid leukaemia (CML). The constitutive activation of the Bcr-Abl fusion protein is both essential and sufficient for malignant transformation and CML, thus treatment with Gleevec allows for specific targeting of leukaemic cancer cells only[7,8].

Despite advances in cancer associated biomedical research, which have lead to the development of Gleevec and other successful therapeutics, the productivity of commercial drug research and development (R&D) pipelines is steadily declining, as a

large percentage of current research remains focussed on only a small subset of potential human target proteins, including the expansion and modification of previously validated targets such as the kinases[9,10]. Hence, there is a need for identification and validation of new, previously unexploited, anti-cancer targets.

1.3 Identification of novel anti-cancer therapeutic targets

The last decade has seen a pronounced increase in the use of high-throughput genomic and proteomic techniques to identify molecular targets, in an attempt to develop new and improved anti-cancer therapeutics[11]. Among many of the high-throughput methods used in cancer drug discovery efforts, transcriptional profiling remains an important tool in determining the genetic abnormalities and complexities associated with cancer. Genome-wide expression based microarrays, a powerful screening technique used to assess and compare the gene expression profiles of two different biological states (ie. cancer versus normal tissue), represent a technique commonly used in the identification of genes involved in cancer development and progression[12]. Genes that are differentially expressed in cancer compared to normal tissue present as potential targets for novel therapies. Over the past few years, there has been a rapid increase in the number of studies using DNA microarrays to identify critical genes involved in carcinogenesis, and many publications have revealed evidence for genes that play a role in cancer initiation, metastasis and survival. Furthermore, microarray technology has been useful in defining the gene expression changes associated with drug treatment and acquired drug-resistance in cancer cells, which has resulted in the discovery of novel molecular targets,

biomarkers of drug sensitivity, as well as factors that may aid in the prediction of treatment response/outcome[13].

In a study conducted by van der Watt *et al*. (2009), a cDNA microarray analysis profiling cancer versus normal cervical tissue from a South African cohort of patients was performed to identify genes that associate with the development of cervical cancer. Comparison of the expression profiles revealed a large number of genes showing differential expression, among them Karyopherin β1 (Kpnβ1), Karyopherin α2 (Kpnα2), and CRM1 – members of the Karyopherin superfamily of nuclear transport proteins – and Ran, were found to be significantly overexpressed in cervical cancer tissues. Subsequent research showed that inhibition of Kpnβ1 and CRM1 expression resulted in cancer cell death, revealing both proteins as potential anti-cancer therapeutic targets[14]. In recent years, targeting the nuclear transport machinery has received attention as a promising anti-cancer approach, as dysregulation of nuclear transport has been reported in a wide variety of cancers[15].

1.4 Nuclear transport

The nucleus in all eukaryotic cells is enclosed by the nuclear envelope, a double lipid bilayer membrane that divides the cells into two compartments, between which there is an interchange of proteins and RNAs into and out of the nucleus. This compartmentalisation of molecules is important, as it maintains cell homeostasis by allowing for spatial separation, and thus control, of important cellular processes; transcription factors and histones need to enter the nucleus in order to induce gene

expression and formation of nucleosome, respectively, while mRNAs, tRNAs and rRNAs that are transcribed in the nucleus need to be exported to the cytoplasm in order to function in translation[16].

Embedded in the nuclear membrane are the nuclear pore complexes (NPCs), which facilitate the bidirectional exchange of cargo proteins and RNAs between the nucleus and the cytoplasm. Large macromolecules move through these pores with the help of soluble transport factors known as the Karyopherin proteins, while smaller molecules (less than 40kDa in size) have the ability to passively diffuse through the NPC[16]. The NPC, which represents one of the largest protein structures in the eukaryotic cell, is composed of a set of approximately 30 different proteins called nucleoporins (Nups), which are constructed primarily of one or more of the following structural units: α-helical regions, β-propellar and α-solenoid folds, and tandem repeated phenylalanine-glycine (FG) motifs. These motifs (also called FxFG repeats) line the active transport channel of the NPC and serve as docking sites for transport receptors[17–19].

The NPC, in collaboration with the Ras-related nuclear protein (Ran) and Karyopherin nuclear transport proteins, collectively known as the nuclear transport machinery, are critically important for the shuttling of cargo molecules between the nucleus and the cytoplasm. Disruption of the nuclear transport process leads to inappropriate spatial arrangement of cargoes, which has been linked to various pathological conditions, including cancer[15,20].

1.5 The Karyopherin nuclear transport proteins

The Karyopherin protein superfamily consists of a large number of soluble transport factors that shuttle proteins and certain RNAs through the NPC. Studies have shown that in addition to their roles as nuclear transporters, the Karyopherins play vital roles in various mitotic processes, from spindle assembly and regulation to nuclear membrane and pore formation[21,22]. The Karyopherin superfamily can be divided into alpha (α) and beta (β) subfamilies. Members of the Karyopherin β (Kpnβ) subfamily function primarily as nuclear import proteins (importins) or nuclear export proteins (exportins), which are classified on the basis of the direction in which they carry their cargo. Members of the Karyopherin α (Kpnα) subfamily act as adaptor proteins, mediating recognition and binding of cargo molecules to certain Kpnβ proteins.

In the classical nuclear import pathway, recognition of a nuclear localisation signal (NLS), which most commonly consists of a number of clusters of basic amino acids and is termed the classical NLS (cNLS), by a Kpnα isoform is required for Kpnβ1-mediated nuclear import; Kpnβ1 is the main nuclear import protein in interphase cells [23,24]. After Kpnα binds to the NLS-containing cargo, forming a bridge between the cargo and Kpnβ1, Kpnβ1 interacts with FG-Nups on the NPC to allow for transfer of the cargo through the channel. Once in the nucleus, binding of RanGTP results in dissociation of the trimeric complex and release of the cargo[25]. Kpnβ1 and Kpnα are then recycled back to the cytoplasm for another round of nuclear import[24]. Alternatively to classical nuclear import, some cargoes are able to bind Kpnβ1 directly (independent of Kpnα), or utilise other members of the Kpnβ family for their nuclear import.

Nuclear export proteins, or exportins, bind to cargo molecules containing a nuclear export signal (NES). The classical nuclear export pathway involves the exportin CRM1 binding directly to NES-containing cargoes, which in the presence of RanGTP, exit the nucleus via the NPC. Hydrolysis of RanGTP to RanGDP promotes the dissociation of the CRM1 complex, and subsequent release of the cargo into the cytoplasm. CRM1 is then recycled back to the nucleus for another round of nuclear export[16]. For a simplified schematic showing the classical nuclear import and export processes, see Figure 1.1.

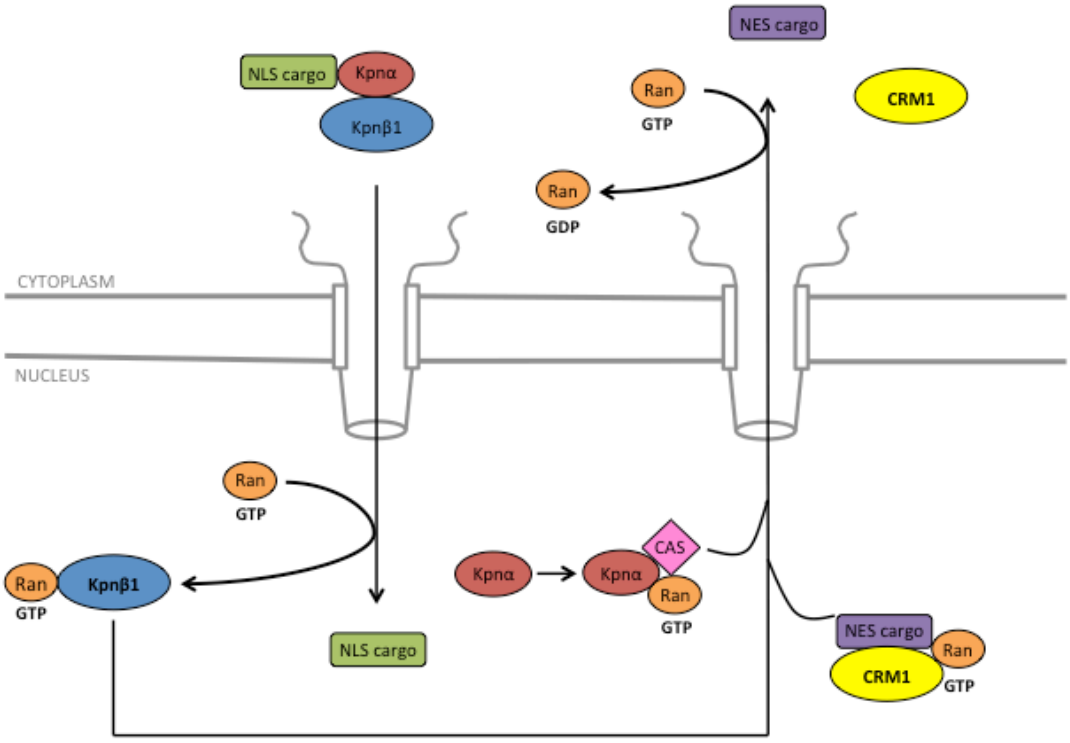

Figure 1.1. A simplified schematic showing the classical nuclear import and export pathways. NLS-containing cargo, Kpnα and Kpnβ1 form a trimeric complex and are transported through the NPC into the nucleus. Binding of RanGTP to Kpnβ1 results in dissociation of the complex and release of the cargo. Kpnβ1 and Kpnα exit the nucleus bound by RanGTP, and CAS and RanGTP, respectively. Nuclear export is mediated by CRM1, which recognises NES-containing cargo in the presence of RanGTP and shuttles it through the NPC. The hydrolysis of RanGTP to RanGDP in the cytoplasm results in dissociation of the complex and release of the cargo.

1.6 The RanGTP nuclear transport cycle

Ran, a small Ras-related nuclear guanosine triphosphatase (GTPase), is required for all forms of active nuclear import and export; it provides energy and determines directionality for the nucleo-cytoplasmic shuttling of cargoes based on whether it is bound to GTP or GDP[26]. The components of the RanGTPase system, including the RanGTP exchange factor RCC1 (Regulator of chromosome condensation 1), the GTPase activating protein RanGAP1, and the Ran binding protein RanBP1, are arranged in an asymmetrical manner to produce high levels of RanGTP in the nucleus, and high levels of RanGDP in the cytoplasm. This asymmetrical distribution of the different components is crucial for both NLS-cargo import and NES-cargo export[27–29].

In the classical nuclear import pathway, the cargo:Kpnα:Kpnβ1 trimeric complex dissociates in the nucleus when RanGTP binds to Kpnβ1 through its conserved Ran binding domain, thereby releasing the cargo and terminating nuclear import. Kpnβ1 and Kpnα are recycled to the cytoplasm; Kpnβ1 bound by RanGTP and Kpnα bound by its own nuclear exporter, the cellular apoptosis susceptibility protein, CAS (or CSE1L) and RanGTP[30]. Once in the cytoplasm, RanGTP is hydrolysed to RanGDP by RanGAP1 and RanBP1, resulting in release of both Kpnβ1 and Kpnα from their respective export partners. Kpnβ1 and Kpnα remain in the cytoplasm for another round of nuclear import, while CAS and RanGDP as well as the nuclear transport factor, NTF2, re-enter the nucleus so that RanGDP can be converted to RanGTP by RCC1[31].

For nuclear export to occur, RanGTP binds to exportin proteins such as CRM1 in the nucleus, which promotes assembly and docking of the export complex at the NPC. The hydrolysis of RanGTP to RanGDP in the cytoplasm, promoted by RanGAP1 and assisted by NTF2, results in the dissociation of the transport complex and release of the cargo, allowing for recycling of both factors for another round of function[31–33].

1.7 Karyopherin protein cargo molecules

While some proteins are able to enter or exit the nucleus without the help of the Karyopherin proteins, a considerably larger proportion of proteins rely on Karyopherin-mediated nuclear transport across the NPC. The Karyopherin proteins are responsible for the transport of multiple transcription factors, including NFκB[34], NFAT[35], c-Jun[36] and STAT[37]. These proteins are translated in the cytoplasm but are only active in the nucleus, where they can bind to the promoters of their target genes, resulting in activation or repression of gene expression.

NFκB, which is involved in a range of biological processes, relies primarily on nuclear import mediated by Kpnα2 in complex with Kpnβ1 to enter the cell nucleus to regulate expression of its target genes. Aberrant or constitutive NFκB expression leads to the uncontrolled expression of genes involved in the regulation of cellular processes such as proliferation, migration, apoptosis, and cell cycle control, which can ultimately lead to the development and progression of cancer[38–40]. In the classical NFκB pathway, the NLS is masked from recognition by the Kpnβ1:Kpnα2 complex due to binding of an inhibitor protein IκB, which holds it in an inactive state in the cytoplasm. Upon NFκB activation, via

processes such as stimulation by pro-inflammatory cytokines or viral infection, IκB is phosphorylated by the IKK complex, leading to its degradation, and thus allowing for the unmasking of the NFκB NLS and the resultant nuclear import of NFκB [34].

There are also many cell signalling networks that are dependent on the Karyopherins for the nuclear import of specific downstream proteins such as kinases, phosphatases and various transcription factors. For example, phosphorylated ERK, a component of the MAPK pathway, enters the nucleus via Kpnβ1-mediated import[41]. Many proteins involved in the cell cycle also rely on Karyopherin-mediated transport to fulfill their roles. For example, Cdk2/cyclin E and Cdc2/cyclin B1 are imported into the nucleus by Kpnβ1 to perform time-dependent phosphorylation of their substrates[42].

Various tumour suppressor proteins have also been shown to make use of the Karyopherin proteins for their nuclear import and export, including p53, BRCA1 and APC[43]. The tumour suppressor p53, which has multiple functions including the regulation of DNA replication, DNA repair and cell cycle checkpoint, exerts its tumour suppressor function by binding to specific promoter sequences in the nucleus[44]. p53 contains putative NLSs that interact with Kpnα2, allowing for its nuclear import in complex with Kpnβ1. Loss of p53 function in the nucleus due to dysregulated nuclear import has been shown to play a critical role in tumourigenesis[45].

It is evident that the Karyopherin proteins play a crucial role in the recognition and shuttling of a variety of cargo proteins across the NPC. Dysregulation of nuclear transport

has been reported in a broad spectrum of cancers, stressing the importance for tight regulation and control of Karyopherin protein expression and localisation.

1.8 Dysregulation of nuclear transport and cancer

Dysregulation of the nuclear transport process has been reported to be a characteristic of cancer cells. Transformed and cancer cells possess increased rates of nuclear transport due to elevated levels of the Karyopherin proteins, as well as Ran. Various studies have reported that members of the Karyopherin family, particularly Kpnβ1, Kpnα2, CRM1 and CAS, are overexpressed in cancer cells and tissue, and aid in the proliferation and survival of cancer cells[15]. Kuusisto *et al*. (2012) showed that accumulation of NLS-containing proteins is increased up to 7-fold in transformed cells, correlating with increased expression of the Karyopherin proteins[46]. It is likely that transformed and cancer cells have devised a mechanism to cope with their increased metabolic and proliferative demands by enhancing nuclear transport rates/efficiency.

Targeting of cargoes such as tumour suppressors, transcription factors and oncoproteins to their sites of action can be regulated at multiple points. Thus, any alterations or disruptions in the nuclear transport process may result in the uncontrolled cell growth that is associated with cancer. For example, the increased expression of nuclear import proteins likely allows for the increased nuclear entry of oncogenic or tumour-promoting proteins, such as ERK1/2 and c-Myc[41,47]. A diverse range of mechanisms exist to enable the regulation of nuclear transport, including the intermolecular and intramolecular masking of nuclear localisation and nuclear export signals (NLSs and NESs), modification

of the enzymes controlling the Ran gradient, modifications in the composition and architecture of the NPC, as well as changes in gene expression of members of the nuclear transport machinery, such as the nucleoporins and Karyopherins[48,49]. With particular reference to the Karyopherin proteins, altered expression and localisation are typically associated with the dysregulation of nuclear transport that is commonly observed in cancer cells[15]. For example, van der Watt *et al.* (2011) recently showed that overexpression of both Kpnβ1 and Kpnα2 in cervical cancer cells correlates with altered transcriptional regulation due to impaired E2F/Rb activity, which has been shown to be dysregulated in the vast majority of human cancers[50,51]. In terms of localisation, the ability of the Karyopherins to perform their function depends on their ability to interact appropriately with cargoes and other members of the transport machinery. Kim *et al.* (2000) described how a truncated from of Kpnα lacking part of its NLS-binding domain was unable to bind and transport its cargo (p53) into the nucleus in breast cancer cells. In fact, despite having an intact Kpnβ1-binding domain, the truncated form of Kpnα was predominantly located to the cytoplasm (as opposed to wild-type Kpnα being evenly distributed), suggesting that recognition and binding of cargo by Kpnα is necessary for Kpnβ1-mediated p53 nuclear entry. There are many other examples demonstrating how aberrant nuclear transport has been linked to cancer (for a detailed review see Stelma *et al.* (2016)[15]). Thus, not only is it evident that dysregulation of this process associates with cancer, but also that it presents as a promising target for the development of novel anti-cancer treatments.

1.9 Targeting Karyopherins for the development of anti-cancer therapeutics

The apparent association between dysregulation of Karyopherin expression/localisation and cancer suggests that the targeting or inhibition of this class of transporters may be an effective approach and addition to current anti-cancer therapeutic efforts. However, current drugs targeting these proteins are limited and further research needs to be conducted toward the development of improved Karyopherin inhibitors. There are a number of problems that arise when implicating drug design with targeting of the nuclear transport process, for example; normal and cancer cells share the same nuclear transport machinery, thus inhibition of certain components may have negative effects on normal cell proliferation. Research conducted in our laboratory, however, has shown that the inhibition of Kpnβ1 in cancer cells, but not normal cells, leads to cell death via apoptosis, suggesting that Kpnβ1 presents as a potential target for the development of novel anti-cancer therapeutics[14,52].

While evidence points to the potential of Kpnβ1 as an anti-cancer therapeutic target, CRM1 is currently the only Karyopherin protein that has been extensively investigated as a target for the development of novel anti-cancer therapies. Leptomycin B (LMB) was the first anti-CRM1 compound to display anti-cancer activity both *in vitro* and *in vivo*[53,54]. Despite promising initial findings however, LMB was eventually discontinued due to high toxicity in phase I clinical trials and subsequent analogues have shown very little clinical promise[55]. More recently, a number of compounds known collectively as selective inhibitors of nuclear export (SINE) have been developed and tested as more specific inhibitors of CRM1 that show fewer off-target effects. Their mechanism of action is

through irreversibly binding the cysteine 528 residue in the cargo-binding groove of CRM1, thereby preventing its nuclear export ability[56]. One particular compound, Selinexor (KPT-330), has been particularly successful in *in vitro* and *in vivo* studies for a wide variety of cancer types, and is now being evaluated in multiple clinical trials[57]. The success associated with the targeting of CRM1 using this group of compounds has highlighted the potential of targeting the Karyopherin proteins for the development of novel anti-cancer therapies.

Significantly less has been described with regard to the development of inhibitors of nuclear import; while a number of compounds have been reported on, none have been tested for their specificity and only a few have shown anti-cancer activity. More recent compounds that have been investigated for their ability to interfere with nuclear import are Ivermectin, Karyostatin 1A and Importazole. Ivermectin was first identified as a broad-spectrum anti-parasitic agent that only later was found to inhibit Kpnα/β-mediated nuclear import. No effect on other nuclear import pathways, such as those mediated by Kpnβ1 alone, has been found[58]. The first novel compounds found to interfere specifically with Kpnβ1-mediated nuclear import were the small molecule inhibitors Karyostatin 1A and Importazole, which have both been shown to disrupt the interaction between Kpnβ1 and Ran, thereby preventing cargo release in the nucleus. Both inhibitors, however, display suboptimal behaviour in cellular assays, and their off-target effects and potential anti-cancer activity remains to be investigated[59,60].

Overall, drugs targeting Karyopherin-mediated nuclear transport have shown potential for their use as anti-cancer therapeutics, but further research is required to aid in the development of human specific drugs that are safe and bioavailable.

1.10 Karyopherin β1: more than just a nuclear import protein

Karyopherin β1 (Kpnβ1; also known as Importin β or p97) is a member of the Karyopherin superfamily of nuclear transport proteins. It was first discovered by Adam and Adam (1994) as soluble factor required for NLS-mediated binding to the nuclear envelope, and Görlich *et al.* (1995) and Chi *et al.* (1995) later independently cloned it and characterised its mechanism of action using *in vitro* nuclear import assays[61–63]. Kpnβ1 is an 876 amino acid (or 97kDa) protein with a right-handed superhelical structure consisting of 19 HEAT repeat units. Each unit consists of an A and B helix that is connected by a short turn; in HEAT-8 this is replaced by an acidic loop that is critical for the regulation of Kpnβ1 substrate binding and release[64]. The N-terminal region of Kpnβ1 (comprising HEAT repeats 1-8) is crucial for binding to RanGTP, while the central and C-terminal regions spanning HEAT repeats 4-19 are important for binding of NPC/Nups and Kpnα/cargo, respectively (Figure 1.2)[65–67].

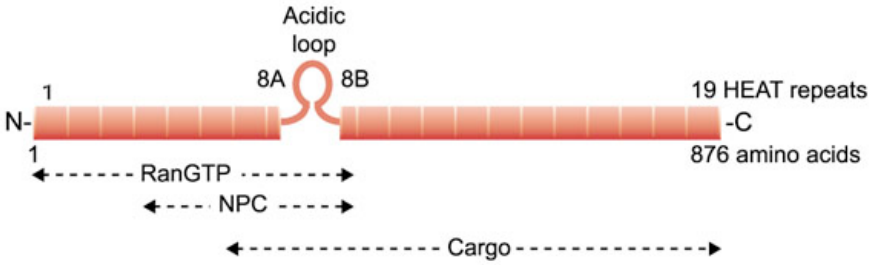

Figure 1.2. Structure of Kpnβ1. Figure from Ström and Weis (2001)[64]. Kpnβ1 is composed of 19 HEAT repeat units, each of which comprises an A and B helix separated by a short turn. In HEAT-8, the A and B helices are separated by an acidic loop that is critical for the regulation of substrate binding and release. The N-terminal region of Kpnβ1 is crucial for RanGTP binding, the central region for binding to the NPC, and the C-terminal region for Kpnα/cargo binding.

Kpnβ1 was initially identified and characterised as the major nuclear import protein responsible for transporting NLS-containing cargoes through the NPC into the cell nucleus, however, in more recent years a number of additional roles have emerged. During mitosis, Kpnβ1 adopts an entirely different (but equally vital) set of functions, by acting as a negative regulator for various mitotic processes from spindle assembly and regulation to nuclear membrane and nuclear pore reformation[22,68]. Moreover, Kpnβ1 has also been implicated in other cellular functions including actin cytoskeleton regulation[69], NPC permeability[70], and endoplasmic reticulum (ER)-associated degradation of misfolded proteins[71]. Due to the role that Kpnβ1 appears to play in multiple biological processes, it is not surprising that Kpnβ1 dysregulation has been linked to pathogenesis, including that of cancer.

1.11 Karyopherin β1: a potential cancer biomarker and therapeutic target

Targeting of the Karyopherin proteins for the development of novel anti-cancer therapeutics has received a significant amount of attention over the last decade, with a number of promising clinical candidates, such as the anti-CRM1 SINE compounds, emerging. This area of study is still however in its early stages; until quite recently the potential for Kpnβ1 as an anti-cancer target had been somewhat underexplored. Using a cervical cancer model, research from our laboratory was the first to show that Kpnβ1 is overexpressed in cervical cancer compared to normal cells and tissue, and that Kpnβ1 silencing by siRNA resulted in cancer cell death[14]. Subsequently, enhanced Kpnβ1 expression has been reported in a wide variety of human cancers, including ovarian cancer[72], breast carcinoma[73], myeloma[74], hepatocellular carcinoma[75], gastric cancer[76], head and neck squamous cell carcinoma and lung cancer[77], and a number of these studies have shown that inhibition of Kpnβ1 protein expression leads to cancer cell death. Moreover, van der Watt *et al.* (2009) and Kuusisto *et al.* (2015) both reported that siRNA-mediated inhibition of other members of the Karyopherin family, such as Kpnα1, Kpnα2, Kpnα3 and CAS, showed no anti-cancer activity or selectivity for malignant cells (compared to normal/non-transformed cells)[14,73]. These results provide evidence that Kpnβ1 plays a critical role in cancer cell biology, and suggest that targeting Kpnβ1 could have therapeutic potential for the treatment of cancer.

In addition to its potential as an anti-cancer target, there is evidence emerging that Kpnβ1 may be useful as a biomarker. A number of studies have revealed the possible prognostic use of Kpnβ1: Kuusisto *et al.* (2015) showed that the extent of Kpnβ1

overexpression correlates with diseased state in an MCF10 human breast tumour model; Zhu *et al.* (2016) and Yang *et al.* (2015) showed that Kpnβ1 expression levels positively correlate with tumour grade, infiltration and Ki-67 grade in gastric cancer, and histological grade, metastasis, vein invasion and tumour size in hepatocellular carcinoma, respectively[75,76]. No study to date has explored the potential of Kpnβ1 as a diagnostic biomarker. An ideal biomarker should be unique to cancer and easily obtained from patient samples such as saliva, blood or urine. A number of studies have revealed other members of the Karyopherin protein family, including Kpnα2[78,79] and CAS (or CSE1L)[80,81], as potential diagnostic biomarkers – both are found in serum or urine of patients with a variety of cancer types. Preliminary work from our laboratory has identified Kpnβ1 as one of several nuclear transport proteins that is secreted at higher levels in cancer versus normal cells (Wishart, MSc dissertation†). There is also evidence suggesting that it is present at high levels in exosomes (tumour cells have been shown to secrete excessive amounts of exosomes[82,83]), thus Kpnβ1 shows promise as a potential cancer biomarker.

1.12 Identification of a potential small molecule inhibitor of Karyopherin β1, INI-43

In order to identify small molecules with the potential to bind and inhibit Kpnβ1, a structure-based *in silico* screen was performed in collaboration with researchers at the Molecular Modeling Facility of the James Graham Brown Cancer Centre (University of Louisville, Kentucky, USA)[52]. Based on known crystal structures, a rational structure-based approach was used to identify compounds with the predicted ability to bind the

† A Wishart. "Investigating secreted biomarkers for cancer: the potential of the nuclear transport proteins." University of Cape Town, 2017

overlapping Ran and Kpnα2 binding region of Kpnβ1. This 33 amino acid region (spanning amino acids 331-363) has been reported to be essential for the nuclear import function of Kpnβ1, as Kpnβ1 requires both RanGTP and Kpnα2 binding for the nuclear import of classical NLS-containing cargoes[25]. Forty-seven of the top-scoring compounds were chosen according to their predicted Kpnβ1-binding affinity, and further tested for their ability to block nuclear import, as well as their effects on cancer versus normal cell viability. Of the forty-seven compounds that were tested, *3-(1H-benzimidazol-2-yl)-1-(3-dimethylaminopropyl)pyrrolo[5,4-b]quinoxalin-2-amine*), which was subsequently named Inhibitor of Nuclear Import-43 (INI-43), was identified as one of the best candidate inhibitors of nuclear import. This was due to the fact that, in addition to its potential to bind and inhibit Kpnβ1, INI-43 was found to be more effective at killing cancer and transformed cells than normal fibroblast cells, with an EC_{50} value (half maximal effective concentration) of approximately 10 μM, which is within the range of known chemotherapeutic agents such as cisplatin, when treating cancer cells in culture.

INI-43 is a quinoxaline derivative, a heterocyclic compound containing a benzene ring and a pyrazine ring, that has an additional benzimidazole and specific R-group attached (Figure 1.3). Interestingly, both benzimidazole and quinoxaline structures have been found to display anti-cancer activity[84,85]. Recent work conducted in our laboratory showed that INI-43 interfered with the nuclear localisation of Kpnβ1 and known Kpnβ1 cargoes including NFκB, NFAT, NFY and AP-1, and inhibited the proliferation of cancer cells of different tissue origins while only minimally affecting normal cells. In addition, intraperitoneal administration of INI-43 significantly inhibited tumour growth in cervical and oesophageal cancer xenograft models[52]. Thus, INI-43 shows promise a novel anti-

cancer therapeutic.

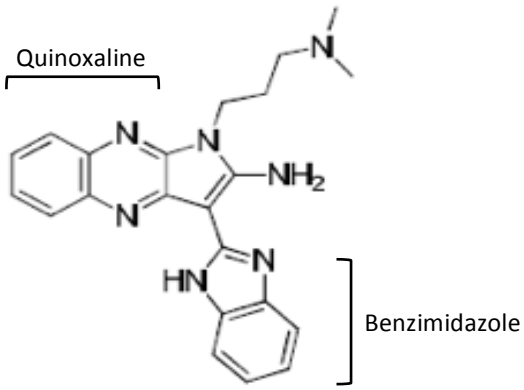

Figure 1.3. Structure of the small molecule Inhibitor of Nuclear Import-43 (INI-43). INI-43 is a 385.5 g/mole quinoxaline derivative with a benzimidazole and specific R-group attached.

1.13 Significance

Dysregulation of the nuclear transport process is directly associated with many human cancers, thus targeting members of the nuclear transport machinery, specifically the Karyopherin protein family, has shown potential for the development of novel anti-cancer therapeutics. While previous research has revealed that Kpnβ1 is overexpressed in various cancers, and is required for cancer cell proliferation, it remains unclear whether upregulation of Kpnβ1 induces any features of the transformed or cancer phenotype. This study further elucidates the relationship between Kpnβ1 and the cancer phenotype by investigating the biological effects associated with modulating Kpnβ1 expression using two approaches: overexpression and small molecule-mediated inhibition. The specificity of the small molecule inhibitor INI-43 for Kpnβ1 was also investigated.

1.14 Project aims

The aims of this study are:

I. To determine the biological implications of Kpnβ1 overexpression in cancer and normal cells.

II. To investigate the specificity of INI-43 for Kpnβ1 using expression of exogenous Kpnβ1 as a rescue mechanism.

III. To examine the effects of Kpnβ1 dysregulation on cell cycle progression using live cell time-lapse analysis.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Cell lines

The following cell lines were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA): human cervical carcinoma cell lines, HeLa and CaSki. FG0 normal skin fibroblasts were obtained from the Department of Medicine, UCT. The HeLa TET-ON Kpnβ1-GFP cell line was generated by Annalisa Verrico and Patrizia Lavia (Institute of Molecular Biology and Pathology, National Research Council of Italy, Rome, Italy). HeLa, CaSki and FG0 cells were maintained in complete medium: Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% Fetal Bovine Serum (FBS) (HyClone Laboratories, UT, USA). HeLa TET-ON Kpnβ1-GFP cells were maintained in DMEM supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% FBS (Tetracycline-free) (Takara Bio USA Inc, CA, USA). All cells were cultured at 37°C in a humidified chamber with 5% CO_2.

2.1.2 Plasmids

Kpnβ1 overexpressing HeLa and CaSki cells (pEFIRES-Kpnβ1-EGFP) and control cells (pEFIRES-EGFP) were established using the pEFIRES construct as previously described[52]. The pEFIRES plasmid was a kind gift from Yosef Shaul (Weizmann Institute of Science, Isreal)[86], and the Kpnβ1 sequence was released from the pEGFP-Kpnβ1 plasmid, which was kindly donated by Patrizia Lavia (Institute of Molecular Biology and Pathology, National Research Council of Italy, Rome, Italy)[87].

The Ran-EGFP plasmid for overexpression was created by Dr Pauline van der Watt, by amplifying the Ran coding sequence from CaSki cDNA using sequence specific primers (forward: 5' AGCTCGAGGATGGCTGCGCAGGGAGAG 3', reverse: 5' AGGGATCCTCACAGGT CATCATCCTCATCC 3'), and inserting it into the pEGFP-C2 vector (Clonetch, Takara Bio USA Inc, CA, USA).

2.1.3 Compounds

2.1.3.1 Inhibitor or Nuclear Import-43 (INI-43)

Inhibitor of Nuclear Import-43, INI-43 (*3-(1H-benzimidazol-2-yl)-1-(3-dimethylamino-propyl)pyrrolo[5,4-b]quinoxalin-2-amine*) was obtained in powder form from Chembridge (ZINC identification number 20547783, San Diego, CA, USA). The compound was

dissolved in DMSO to a stock concentration of 10 mM and stored at -20°C. Working solutions were kept at room temperature protected from light.

2.1.3.2 Importazole

The nuclear import inhibitor, Importazole, was purchased from Sigma (Sigma-Aldrich, St Louis, MO, USA) and dissolved in DMSO to a stock concentration of 25 mM and stored at 4°C protected from light.

2.1.3.3 Doxorubucin

The anthracycline antibiotic and anti-cancer drug, Doxorubicin, was purchased from Sigma (Sigma-Aldrich, St Louis, MO, USA) and dissolved in water to a stock concentration of 10 mM and stored at -20°C protected from light.

2.1.3.4 Puromycin

Puromycin was purchased from Calbiochem (Merck Millipore, Billerica, MA, USA), and dissolved in water to a concentration of 10 mg/ml. The solution was filter sterilised through a 0.22 µm filter and kept at -20°C protected from light.

2.1.3.5 Thymidine

Thymidine was obtained from Sigma (Sigma-Aldrich, St Louis, MO, USA), and dissolved in complete medium to a concentration of 100 mM. The solution was added to cells at a final concentration of 2 mM.

2.1.3.6 Doxycycline

Doxycycline was obtained from Sigma (Sigma-Aldrich, St Louis, MO, USA), and dissolved in water to a concentration of 10 mg/ml. The solution was filter sterilised through a 0.22 µm filter and kept at -20°C protected from light.

2.2 METHODS

2.2.1 Transformation and isolation of plasmid DNA

2.2.1.1 Transformation of competent cells with plasmid DNA

For transformation of competent cells with plasmid DNA, 20 ng of plasmid was added to 30 µl of *E.coli* JM109 competent cells (Promega, Madison, WI, USA), which were then incubated on ice for 10-30 minutes. The cells were heat shocked at 42°C for 2 minutes in order to aid plasmid uptake. 0.45 ml of Luria Broth (LB) medium was added to the heat-shocked cells, which were then incubated at 37°C for 1 hour, with shaking. 100 µl and 400 µl of the broth was then plated onto two separate 100 µg/ml ampicillin-containing LB agar plates, and incubated overnight at 37°C. The following day, a colony was picked from each plate and inoculated in 5 ml 100 µg/ml ampicillin-containing LB medium at 37°C for 6 hours. The starter culture was then inoculated in 100 ml LB medium containing 100 µg/ml ampicillin (Roche, Mannheim, Germany), and grown at 37°C for 12-16 hours with vigorous shaking.

2.2.1.2 Isolation of plasmid DNA

Plasmid extraction was performed according to the QIAGEN Plasmid Maxikit manufacturers instructions (Qiagen Inc, Valencia, CA, USA). Isolated plasmids were re-

suspended in TE buffer (pH 8.0), and quantified by use of a NanoDrop (NanoDrop 2000c, Thermo Fischer Scientific, MA, USA).

2.2.1.3 Restriction enzyme digest of plasmid DNA

To confirm amplification of the correct plasmid DNA, restriction enzyme digests were performed. The restriction enzymes *Sac*I and *Bam*HI (Fermentas, Thermo Fischer Scientific, Weltham, MA, USA) were used to linearise the plasmid, and in the case of Kpnβ1-EGFP, release the Kpnβ1-encoding fragment. For digestion, 1 µg plasmid DNA was digested by 1 µl *Sac*I and *Bam*HI, in a final volume of 20 µl dH_20 with 2 µl Fast Digest Green Buffer (Fermentas). The mix was incubated at 37°C for 2 hours, before running the digestion on a 1% agarose gel. The gel was run in TBE buffer at 100 V for 1 hour, and visualised by ethidium bromide staining. The size of the fragments were confirmed by running 10 µl KAPA Universal DNA ladder (Kapa Biosystems, MA, USA, Appendix II).

2.2.2 Generation of stable Kpnβ1 overexpressing cell lines

The plasmid used for overexpression of Kpnβ1 (pEFIRES-Kpnβ1-EGFP) was generated by inserting *Sac*I- and *Not*I-digested human Kpnβ1-EGFP, released from the plasmid pEGFP-Kpnβ1 (kind gift from Patrizia Lavia, Institute of Molecular Biology and Pathology, National Research Council of Italy, Rome, Italy), into the pEFIRES plasmid (kind gift from Yosef Shaul, Weizmann Institute of Science, Israel). pEFIRES-EGFP was used as a empty-vector control. For creation of stable cell lines, 500 ng plasmid DNA was transfected into

HeLa and CaSki cells using Genecellin Transfection Reagent (BioCellChallenge, Toulon, France), and thereafter positively transfected cells were selected using Puromycin (Calbiochem). Pools of stably transfected cells were maintained in 0.5 µg/ml Puromycin. Cloning of pEFIRES-Kpnβ1-EGFP and establishment of the HeLa pEFIRES-EGFP and pEFIRES-Kpnβ1-EGFP stable cell lines was achieved by Dr Kate Hadley and Dr Pauline van der Watt.

2.2.3 Western blot analysis

2.2.3.1 Antibodies

Antibodies and incubation conditions are shown in Table 2.1. Antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA), Bio-Rad (Hercules, CA, USA) and Pierce (Thermo Thermo Fischer Scientific, Weltham, MA, USA).

Table 2.1. Antibody concentrations and incubation conditions for western blotting

Primary antibody	Primary antibody conditions	Secondary antibody	Secondary antibody conditions	Substrate
Kpnβ1(H300) [sc-11367, Santa Cruz]	1:2000 in 5% milk	Goat anti-rabbit [Bio-Rad]	1:5000 in TBST	LumiGlo
GAPDH (0411) [sc-47725, Santa Cruz]	1:10 000 in TBST	Goat anti-mouse [Pierce]	1:5000 in 5% milk	LumiGlo
β-Tubulin (H-235) [sc-9104, Santa Cruz]	1:1000 in TBST	Goat anti-rabbit [Bio-Rad]	1:5000 in TBST	LumiGlo

Table 2.1. Antibody concentrations and incubation conditions for western blotting (continued)

Primary antibody	Primary antibody conditions	Secondary antibody	Secondary antibody conditions	Substrate
pHisH3 (Ser-10)-R [sc-8656, Santa Cruz]	1:1000 in TBST	Goat anti-rabbit [Bio-Rad]	1:5000 in 5% milk	LumiGlo
Mcl-1 (H-260) [sc-20679, Santa Cruz]	1:250 in TBST	Goat anti-rabbit [Bio-Rad]	1:5000 in 5% milk	LumiGlo
E-cadherin (H-108) [sc-7870, Santa Cruz]	1:500 in TBST	Goat anti-rabbit [Bio-Rad]	1:5000 in TBST	LumiGlo
Vimentin (V-9) [sc-6260, Santa Cruz]	1:2000 in TBST	Goat anti-mouse [Pierce]	1:5000 in TBST	LumiGlo
GFP (FL) [sc-8334, Santa Cruz]	1:500 in TBST	Goat anti-rabbit [Bio-Rad]	1:5000 in 5% milk	LumiGlo
Kpnα2 (C-20) [sc-6917, Santa Cruz]	1:1000 in 2.5% milk	Donkey anti-goat [Pierce]	1:5000 in 2.5% milk	LumiGlo
CRM1 (H-300) [sc-5595, Santa Cruz]	1:1000 in TBST	Goat anti-rabbit [Bio-Rad]	1:5000 in TBST	LumiGlo

2.2.3.2 Protein extraction

Cells in culture, grown to 80% confluency or subjected to the required treatment or transfection, were lysed in radioimmunoprecipitation assay (RIPA) buffer containing 1X protease inhibitor cocktail (Roche, Mannheim, Germany) and 0.1 M sodium orthovanadate (Na_3VO_4) to inhibit the action of phosphatases. For extraction of protein from adherent cells, the media was removed and cells were washed twice in cold, sterile

PBS, followed by the addition of RIPA buffer. Cells were harvested by scraping, and collected into sterile eppendorf tubes. Protein extracts were sonicated for 10 seconds to shear DNA, followed by centrifugation at 10000 x g at 4°C to pellet cellular debris. The supernatant containing the total cell protein was removed and placed into pre-chilled eppendorf tubes for storage at -80°C until ready for quantification and western blotting.

2.2.3.3 Protein quantification

Protein concentrations were quantified using a BCA protein assay kit (Pierce, Thermo Thermo Fischer Scientific, Weltham, MA, USA). Using a reagent containing bicinchoninic acid (BCA), this method allows for the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium (the biuret reaction). The colour change form green to purple that is associated with the reduction of Cu^{2+} to Cu^{1+} is read at 595nm, providing a quantitative method for determining total protein in a sample. A bovine serum albumin (BSA) standard curve with concentrations ranging from 25 µg/ml to 2000 µg/ml was constructed by adding 200 µl of the BCA working reagent to 25 µl of each concentration in a 96-well plate, followed by incubation at 37°C for 30 minutes, and absorbance values were read at 595nm. For each of the unknown protein samples, 5 µl of each protein extract was diluted into 20 µl dH_20 and the same procedure followed as for the standard curve to obtain OD_{595nm} readings for each sample. Using the BSA standard curve, the protein concentration for each sample was calculated.

2.2.3.4 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS polyacrylamide gels were prepared with 4% stacking gels set above 10% - 15% separating/resolving gels. Depending on the concentration of the sample, 10-30 µg of the extracted protein suspended in RIPA buffer, along with 1X Laemmli Loading Dye containing 10% β-mercaptoethanol, was denatured at 90°C for 5 minutes, before loading into the wells of the stacking gel. 5 µl of the Colour Prestained Protein Standard (New England Biolabs Inc, Ipswich, MA, USA; Appendix II) was loaded alongside the proteins as a size reference. The gel was run in 1X running buffer at 150 – 185 V for 60 - 90 minutes, or until the dye front had run off the bottom of the gel.

2.2.3.5 Immunoblotting

Proteins were transferred from the SDS polyacrylamide gel to a HyBond™-ECL™ nitrocellulose membrane (Amersham, Buckinghamshire, UK) in 1X transfer buffer at 100 V for 70 minutes. The membrane was then blocked in 5% non-fat dry milk (NFDM) in TBST, for 1 hour at room temperature with shaking, in order to prevent non-specific binding of the antibody to the membrane. Primary antibodies were added to the membrane as in Table 2.1, and incubated overnight at 4°C with shaking. Non-specific primary antibody was removed with three 10 minute washes in TBST, with shaking. Secondary antibody was added to the membrane as in Table 2.1, and incubated for 1 hour at room temperature with shaking, after which the membrane was washed a further three times with TBST before immunodetection.

2.2.3.6 Immunodetection

Protein bands were detected using LumiGlo chemiluminescent substrate (KPL Inc. Gaithersburg, MD, USA). The substrate was added to the nitrocellulose membrane, and converted by horseradish peroxidase (HRP), which is conjugated to the secondary antibody, to a luminescent product. Luminescent protein bands were detected by exposing X-ray film (AGFA CU-BP Medical X-ray film, Mostel, Belgium), for varying exposure times, to the membrane after addition of the substrate. The film was developed and fixed using AGFA developer (AGFA G128) and fixative (AGFA G333C).

2.2.3.7 Re-probing of western blots

For the re-probing of western blots, membranes were stripped using 1 M glycine (pH 2.5) for 6 minutes on each side of the membrane in order to remove bound antibodies, followed by neutralisation with 1 M Tris-Cl (pH 7.5). The membrane was then blocked in 5% NFDM in TBST for 30 minutes at room temperature with shaking in order to prevent non-specific binding of the antibody to the membrane. Primary and secondary antibodies were then added to the membrane as in Table 2.1.

2.2.4 Immunofluorescence

To analyse protein localisation using fluorescent microscopy, cells were grown to 60-70% confluency on glass coverslips and subjected to the appropriate treatments. Cells were then washed twice in ice-cold PBS, before fixation in 4% paraformaldehyde in PBS for 15 minutes at room temperature. Cells were then washed once with PBS, and permeabilised with 0.25% Triton-X-100 in PBS for 5 minutes at room temperature. After one wash in PBST, the cells were blocked using 1% BSA in PBST + 0.3 M glycine for 30 minutes at room temperature. Thereafter, cells were incubated with the primary antibody and secondary antibody diluted to appropriate concentrations in 1% BSA in PBST or 3% BSA in PBS (Table 2.2), each for 1 hour, with 3 x 5 minute washes in PBS in between and after the secondary antibody incubation. The cells were then incubated with DAPI (500 ng/ml in PBS) for 5 minutes to stain for nuclear DNA, before washing once with PBS. The coverslips were then mounted onto glass slides using Mowiol or Vectashield, and images were visualised using a Zeiss Inverted Fluorescence Microscope or a Nikon Eclipse 90i microscope, under 100 x oil immersion. Images were captured using the AxioVision 4.7 software (Zeiss, Oberkochen, Germany) or the NIS-Elements AR 3.22 software (Nikon, Minato, Tokyo, Japan).

Table 2.2 Antibody concentrations and incubation conditions for immunofluorescence

Primary antibody	Primary antibody conditions	Secondary antibody	Secondary antibody conditions	Mounting medium
NFκB/p65 x (H-286) [sc-7157, Santa Cruz]	1:200 in 1% BSA in PBST	Cy3 Goat anti-rabbit [Jackson Immunoresearch]	1:300 in 1% BSA in PBST	Mowiol [Calbiochem]
Kpnβ1 (H-300) [sc-11367, Santa Cruz]	1:100 in 1% BSA in PBST	Cy3 Goat anti-rabbit [Jackson Immunoresearch]	1:300 in 1% BSA in PBST	Mowiol [Calbiochem]
α-Tubulin (ab6161) [YOL1/34, Abcam]	1:3000 in 3% BSA in PBS	Texas Red Goat anti-rat [Vector Laboratories]	1:800 in 3% BSA in PBS	Vectashield [Vector Laboratories]

2.2.5 Phalloidin staining of F-actin

Cells were prepared similarly to that for immunofluorescence. After fixation, cells were washed twice in 0.04% PBST and blocked in 1% BSA for 30 minutes. Actin was labeled with 50 ng/ml Phalloidin-Tetramethylrodamine B isothiocyanate (Phalloidin) (Sigma-Aldrich, St Louis, MO, USA) in 1% BSA for 30 minutes at room temperature. After washing twice with PBS, cell nuclei were stained with 100 ng/ml DAPI in PBS for 10 minutes at room temperature. The stained cells were then washed twice in PBS and mounted on slides using Mowiol. Phalloidin images were viewed using the Zeiss Inverted Fluorescence Microscope under 100 x oil immersion and images captured using the AxioVision 4.7 software (Zeiss, Oberkochen, Germany).

2.2.6 Adhesion assays

To investigate cell adhesion, 20 000 cells were plated into each well of a 24-well plate (triplicate wells for 'unwashed' and 'washed') and allowed to adhere for 1 hour at 37°C. Thereafter, the medium was removed from all wells and 'washed' cells were rinsed twice with PBS before fixation of all cells in 0.5 ml fixation solution (acetic acid/methanol (1:7)) for 5 minutes followed by staining with 0.5% crystal violet solution for 2 hours at room temperature. Thereafter, crystal violet was removed and plates were rinsed in water and left to dry overnight. The number of cells over various fields of view were counted using a light microscope and normalised to the number of 'unwashed' cells, in order to control for total cells plated.

2.2.7 Proliferation assays

2.2.7.1 Anchorage-dependant MTT proliferation assays

Adherent growth as a result of Kpnβ1 and Ran overexpression was determined using the MTT proliferation assay, which analyses cell viability and proliferation by the addition of a yellow reagent, MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma-Aldrich, St Louis, MO, USA). The MTT reagent is reduced by metabolically active cells into purple formazan crystals, which can be solubilised by a solubilisation solution (10 % SLS in 0.01M HCl) and quantified by reading absorbance at OD_{595nm} using a BioTek microplate spectrophotometer (Winooski, VT, USA). A higher absorbance reading

correlates with a larger number of viable cells. Briefly, cells were plated in 96 well plates and allowed to proliferate for a period of 3 or 4 days, during which viable cells were measured by addition of the MTT reagent (Cell Proliferation Kit I, Roche, Mannheim, Germany) for 4 hours followed by solubilisation solution, at 37°C. The following day, absorbance readings were measured at OD_{595nm} and cell proliferation graphs were constructed using Microsoft Excel.

2.2.7.2 Anchorage-independent MTT proliferation assays

To allow for anchorage-independent growth, cells were plated onto Poly (2-hydroxyethyl methacrylate) (Poly-HEMA) (Sigma-Aldrich, St Louis, MO, USA) coated 96-well plates. Each well of the 96-well plate was coated with 30 µl of 12 mg/ml Poly-HEMA and left to dry overnight in the tissue culture hood under UV light. HeLa and CaSki cells (10 000 cells/well) were resuspended in 1% methyl cellulose-containing media and plated into the poly-HEMA coated wells. The increased density of the methyl cellulose-containing media ensures that individual cells don't adhere to each other, so that the ability of single cells to form a colony can be readily assayed. The number of colonies formed at various time points post plating were measured using the MTT reagent and solubilisation solution as described above, and absorbance readings were measured on a microplate spectrophotometer at OD_{595nm}.

2.2.8 Trypan Blue assays

Cell viability as a result of Kpnβ1 overexpression was assessed by means of a Trypan Blue assay. Cells were plated at 25 000 cells per well in a 24-well plate and allowed to adhere overnight. To assay for the number of viable cells, cells were trypsinised and incubated with 0.4% Trypan Blue (Merck Millipore, Billerica, MA, USA), which is taken up by non-viable/dead cells thereby staining them blue. Viable (white) and non-viable (blue) cells were counted using a haemocytometer, and the number of live cells at various time-points was recorded.

2.2.9 EC_{50} determination assays

For the determination of drug EC_{50} values, 5000 HeLa cells/well were plated into a 96-well plate and allowed to settle overnight. The following day, cells were treated with varying concentrations of the appropriate drug for a period of 48 hours, after which MTT and solubilisation solution were added as previously described. The following day, absorbance values were measured at OD_{595nm} and EC_{50} curves were generated using GraphPad Prism (GraphPad Software, Inc., CA, USA).

2.2.10 Cell cycle analysis

For analysis of the effects of Kpnβ1 dysregulation on the cell cycle, cells were plated in 60 mm dishes, synchronised/treated as required according to Table 2.3, and harvested at various time points. Cells were harvested using trypsin, after collecting floaters, and fixed using 8 ml ice-cold 100% ethanol. Cells in ethanol were stored at -20°C for up to two weeks. In preparation for FACS analysis, ethanol was removed from cells by centrifugation, and $0.5x10^6$ cells were transferred to a sterile eppendorf tube. RNA was removed by incubating cells with 50 μg/ml RNase in PBS for 30 minutes. 20 minutes before analysis the cellular DNA was stained using a stain solution containing propidium iodide (see Appendix I) and cell cycle profiles were analysed using the Accuri Flow Cytometer (Beckman Coulter, Fullerton, CA, USA). Quantification of the percentage of cells at different cell cycle stages was performed using the ModFit LT 3.3 software (Verity Software House, Topsham, ME, USA).

Table 2.3 Synchronisation and treatment conditions for cell cycle analysis

Synchronisation	Treatment conditions	Cell lines
G1/S phase	Thymidine (2 mM for 24 hours)	HeLa and CaSki pEFIRES EGFP and Kpnβ1-GFP
none	INI-43 (10 μM, 12 hours)	HeLa pEFIRES EGFP and Kpnβ1-GFP

2.2.11 Luciferase assays

To assay for NFAT luciferase activity, 25 000 cells were plated per well in 24-well plates and left to adhere overnight. The following day cells were transfected with 50 ng GFP-NFAT (Addgene plasmid #24219; gift of Jerry Crabtree[88]), 50 ng NFAT-luciferase (Addgene plasmid #10959; gift of Toren Finkel[89]), and 5ng pRL-TK (encoding *Renilla* luciferase; Promega, Madison, WI, USA), using 0.4 μl Genecellin Transfection Reagent (BioCellChallenge, Toulon, France). One day post transfection, cells were pretreated with 10 μM INI-43 for 2 hours and then stimulated with 0.5 μM PMA (Sigma-Aldrich, St-Loius, MO, USA) and 1.3 μM Ionomycin (Santa Cruz Biotechnology, Dallas, TX, USA) for 3 hours. Luciferase activity was assayed using the Dual-Luciferase Reporter assay system (Promega) according to the manufacturers instructions. Luciferase readings measured using the Veritas microplate luminometer (Promega) were normalised to *Renilla* luciferase readings.

2.2.12 Cycloheximide half-life experiments

For half-life determination experiments, cells were pretreated with 5 μM INI-43 for 3 hours before treatment with 40 μg/ml cycloheximide (CHX) (Sigma-Aldrich, St Louis, MO, USA). Protein was harvested at various time points after CHX treatment and quantitated using the BCA protein assay as previously described. 20 μg of each cell lysate was subjected to Western blot analysis and probed with anti-Kpnβ1, Kpnα2 and CRM1 antibodies. GAPDH was used a control for loading. Protein bands were subsequently quantitated by densitometric scanning using the ImageJ analysis software (NIH,

Bathesda, MD, USA), and normalised to GAPDH. In order to determine protein half-life, the band intensities were plotted in log scale relative to time, and a linear trendline drawn using GraphPad Prism (GraphPad Software, Inc., CA, USA). The half-life was equated to log2/slope.

2.2.13 Time-lapse videomicroscopy

Time-lapse videomicroscopy was used to analyse the cell cycle progression of HeLa cells with dysregulated Kpnβ1 levels (ie. Inducible Kpnβ1 overexpression and/or INI-43 mediated inhibition). HeLa TET ON Kpnβ1-GFP cells were seeded in 8-well micro slides (ibiTreat, code 80826, Ibidi, Klopferspitz, Martinsried, Germany), and subjected to various treatments with doxycycline (Sigma-Aldrich, St Louis, MO, USA) and/or INI-43. Just prior to recording, cell media was replaced with TET-free DMEM (no phenol red) (Thermo Fischer Scientific, MA, USA) supplemented with 0.5 μg/ml doxycycline to allow for the continuous stimulation of the cells. Cells were analysed for a period of 24 hours using an Eclipse Ti inverted microscope (Nikon, Minato, Tokyo, Japan) and a 60 x objective (Plan Apo, 1.4 N.A. Differential Interference Contrast (DIC), Nikon). During the time-course, cells were kept in a Basic WJ CO_2 microscope stage incubator (Okolab, Burlingame, CA, USA) at 37°C and 5% CO_2. DIC images were acquired every 15 minutes, and Fluorescein isothiocyanate (FITC) images every 60 minutes, over a period of 24 hours using a DS-Qi1Mc camera and the NIS-Elements AR 3.22 software (Nikon) for acquisition. Image and movie processing was performed using the NIS-Elements AR 4.2 software (Nikon).

2.2.14 Statistical analyses

Experiments were performed in triplicate or quadruplicate, and expressed as the mean ± standard error of the mean (SEM), unless otherwise stated. All experiments were performed at least two independent times. For data analysis, the Student's t-test was performed (using either Microsoft Excel or GraphPad Prism), and a p value of < 0.05 (indicated by * or #) was considered as statistically significant.

CHAPTER 3

INVESTIGATING THE BIOLOGICAL EFFECTS OF KPNβ1 OVEREXPRESSION

3.1 INTRODUCTION

The identification and further characterisation of Kpnβ1 as a potential anti-cancer therapeutic target is based on research from various studies showing that Kpnβ1 expression is elevated in a number of transformed and cancer cell lines, suggesting its association with the cancer phenotype[14,46,72]. Indeed, research by van der Watt *et al*. (2009) shows that inhibition of Kpnβ1 protein expression leads to cancer cell death via apoptosis[14], and Kuusisto and Jans (2015) recently showed using protein knockdown studies that faster growing tumour cells expressing high levels of Kpnβ1 are more dependent on, or "addicted to", Kpnβ1 than their non-transformed counterparts[73]. These results demonstrate that increased expression, and thus activity, of Kpnβ1 likely plays a role in cancer cell proliferation, making Kpnβ1 an attractive anti-cancer therapeutic target.

Regulation of protein levels (ie. by knockdown and/or overexpression) can be used to study protein function *in vitro*, by investigating the biological effects associated with altered expression. Previous studies in our laboratory revealed that siRNA-mediated knockdown of Kpnβ1 lead to a reduction in proliferation of cancer cells and

subsequent increase in apoptosis, while normal cells were relatively unaffected[14,90]. It is possible that cancer cells have devised a mechanism to cope with their increased metabolic and proliferative demands by enhancing expression of various proteins, among them Kpnβ1, potentially explaining why depletion of Kpnβ1 has such negative effects. In contrast, it appears as if normal cells are less reliant on Kpnβ1 for processes such as proliferation.

While many studies have revealed the importance of Kpnβ1 in cancer cell survival and proliferation, and have described its potential as an anti-cancer target, the precise role that Kpnβ1 plays in cellular transformation and cancer progression is largely unclear; the question remains as to whether Kpnβ1 in fact plays an oncogenic role and is able to induce features of the transformed or cancer phenotype? The aim of this chapter is therefore to further elucidate the relationship between Kpnβ1 and cancer progression by specifically studying the biological effects associated with Kpnβ1 overexpression. This study includes investigating the effects of stable expression of exogenous Kpnβ1 in cervical cancer cells (HeLa and CaSki), as well as the effects of transient overexpression of Kpnβ1 in non-cancer cells. In addition to previously published research verifying the importance of Kpnβ1 in a variety of biological processes, our data provides evidence that a tight regulation of Kpnβ1 is required for correct cell functioning.

3.2 RESULTS

3.2.1 Generating HeLa and CaSki cell lines stably expressing Kpnβ1-EGFP

HeLa and CaSki cell lines stably expressing Kpnβ1-EGFP were established to study the effects of Kpnβ1 overexpression on various biological phenotypes. The plasmid used for overexpression of Kpnβ1 (pEFIRES-Kpnβ1-EGFP; Figure 3.1 A) was generated by inserting *Sac*I- and *Not*I-digested human Kpnβ1-EGFP, released from the plasmid pEGFP-Kpnβ1 (kind gift from Patrizia Lavia, Institute of Molecular Biology and Pathology, National Research Council of Italy, Rome, Italy)[87], into the pEFIRES plasmid (kind gift from Yosef Shaul, Weizmann Institute of Science, Israel)[86]. The pEFIRES plasmid was used in order to allow for both the Kpnβ1-EGFP fusion gene and the puromycin resistance gene to be transcribed as a single mRNA transcript. The presence of an internal ribosome entry site (IRES) upstream of the puromycin resistance gene ensures that puromycin-resistant clones express high levels of the recombinant protein, Kpnβ1-EGFP. pEFIRES-EGFP was used as a control. Positive clones were identified by restriction digestion (Figure 3.1 B). For the establishment of cells stably expressing Kpnβ1-EGFP, HeLa and CaSki cells were transfected with the pEFIRES-EGFP and pEFIRES-Kpnβ1-EGFP constructs using the Genecellin Transfection Reagent (Celtic Molecular Diagnostics), and thereafter positively transfected cells were selected with Puromycin (Calbiochem, Merck). Pools of stably transfected cells were maintained in 0.5 μg/ml Puromycin.

To confirm the presence of EGFP and Kpnβ1-EGFP, cells were visualised using fluorescence microscopy. HeLa and CaSki cells expressing pEFIRES-EGFP (referred to as EGFP) showed nuclear and cytoplasmic fluorescence, due to the fact that EGFP is small (~32kDa) and can thus passively diffuse across the NPC. HeLa and CaSki cells expressing pEFIRES-Kpnβ1-EGFP (referred to as Kpnβ1-EGFP) show nuclear and cytoplasmic localisation of exogenous Kpnβ1, as well as a distinct perinuclear band (Figure 3.2 A). This makes sense, as the localisation of Kpnβ1 is related to its function; in interphase cells Kpnβ1 acts as a nuclear import protein and is thus present in the nucleus and cytoplasm, as well as around the nuclear envelope, due to its association with nuclear pores. Western blot analysis of protein lysates harvested from EGFP and Kpnβ1-EGFP cells revealed that Kpnβ1-EGFP was expressed at roughly equivalent levels to that of endogenous Kpnβ1 in both HeLa and CaSki cells (Figure 3.2 B).

Having established stable Kpnβ1-EFP expressing cell lines, these were next used in a variety of assays to investigate the biological significance of elevated Kpnβ1.

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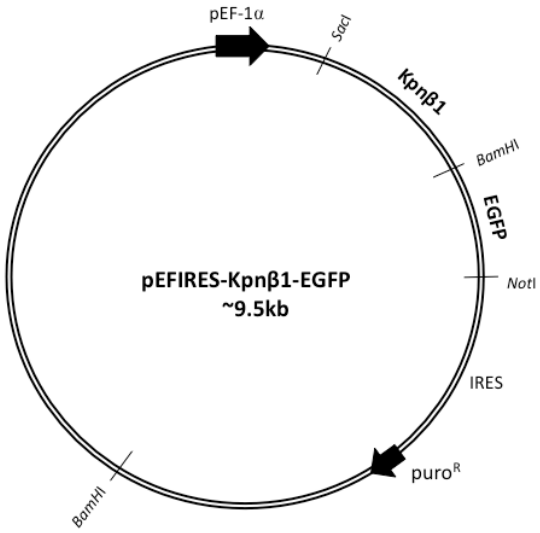

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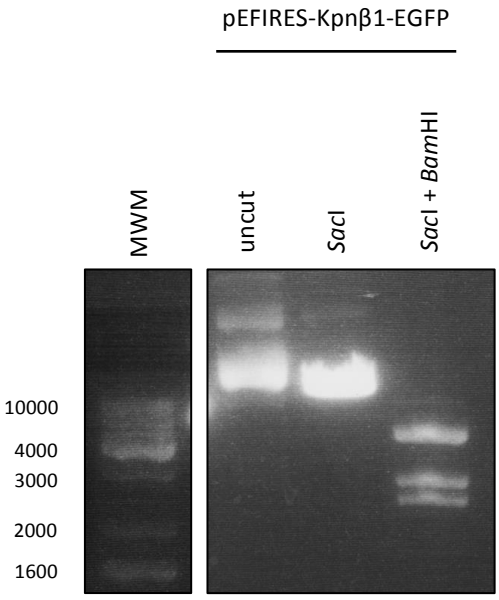

Figure 3.1. The pEFIRES-Kpnβ1-EGFP plasmid. **A:** Representation of the recombinant Kpnβ1-EGFP construct used for creation of HeLa and CaSki stable cell lines stably overexpressing Kpnβ1, into which the 2631 bp Kpnβ1-encoding fragment attached to a EGFP protein was cloned within the MCS of the pEFIRES vector. The Kpnβ1-EGFP fusion protein is under control of the human elongation factor 1α promoter (pEF-1α). **B**: Restriction enzyme digest of the pEFIRES-Kpnβ1-EGFP plasmid using the restriction enzymes *SacI* and *BamHI.* Digestion products were electrophoresed on a 1% agarose gel and the correct size products visualised for pEFIRES-Kpnβ1-EGFP (contains 2x *BamHI* sites)**.**

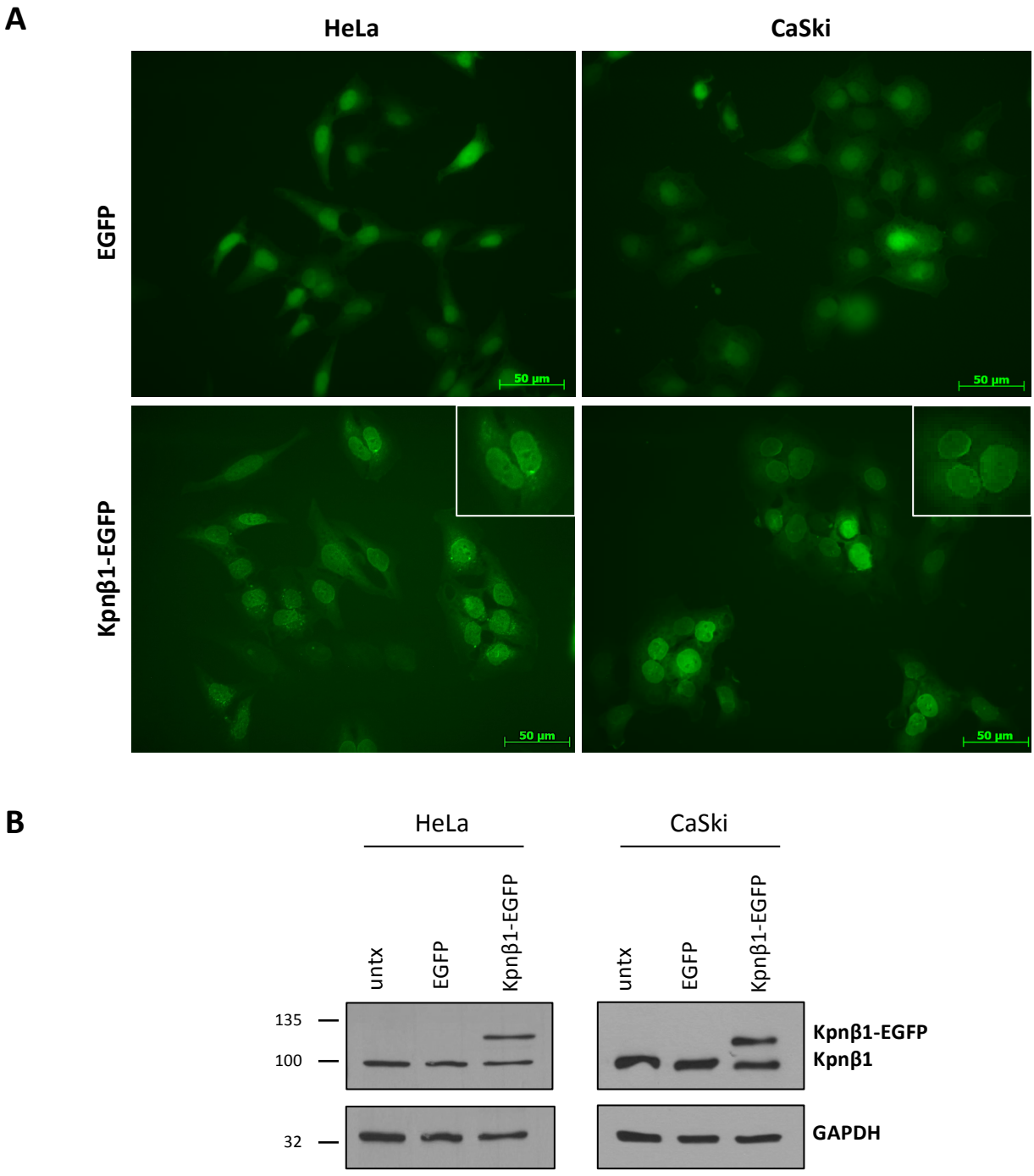

Figure 3.2. Stable expression of EGFP and Kpnβ1-EGFP in HeLa and CaSki cells. A: Immunofluorescence analysis was used to determine GFP expression across the HeLa and CaSki EGFP and Kpnβ1-EGFP stable cell lines. Representative GFP-Alexa 488 images are shown for each cell line. Enlarged images in the top right-hand corner show perinuclear localisation in representative HeLa and CaSki Kpnβ1-EGFP cells **B:** Western blot analysis was used to determine endogenous Kpnβ1 (97 kDa) and exogenous Kpnβ1-EGFP (129.7 kDa) expression levels across the HeLa and CaSki EGFP and Kpnβ1-EGFP cell lines. The first lane (untx) represents protein harvested from untransfected HeLa and CaSki cells. GAPDH was used as a control for loading.

3.2.2 Overexpression of Kpnβ1 results in a reduction in HeLa and CaSki cell proliferation

To determine the effect of Kpnβ1 overexpression on anchorage-dependent cell proliferation, cell viability assays were performed and live cells scored using Trypan Blue. Results revealed that overexpression of Kpnβ1 results in a significant reduction in the proliferation (or viability) of adherent HeLa and CaSki cells (Figure 3.3 A and B). When the effect of Kpnβ1 overexpression on cell proliferation was expanded to look at cells grown under anchorage-independent conditions using methyl cellulose, a similar result was observed; a significant reduction in proliferation of HeLa and CaSki cells stably expressing Kpnβ1-EGFP (Figure 3.4 A and B). This suggests that Kpnβ1 plays a role in cell proliferation, however expression of exogenous Kpnβ1 (over and above the levels already present in cancer cells) does not appear to provide a growth advantage in either adherent or non-adherent conditions. Rather, overexpression of Kpnβ1 results in a growth disadvantage, likely due to an imbalance in Kpnβ1-mediated processes such as nuclear import and mitotic progression. Interestingly, Kpnβ1 overexpression did not result in an increase in cell death, therefore the effects seen were due a reduction in rate of proliferation rather than an induction of cell death.

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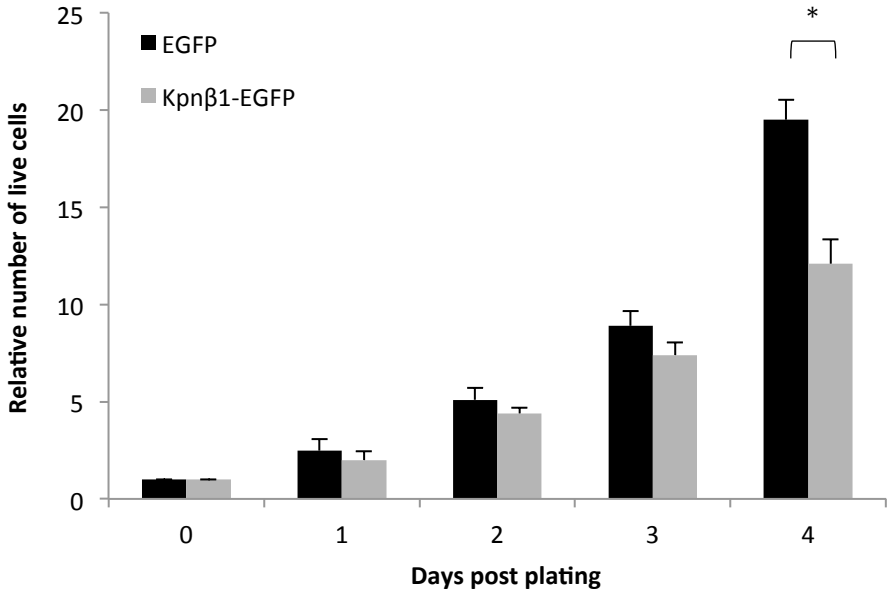

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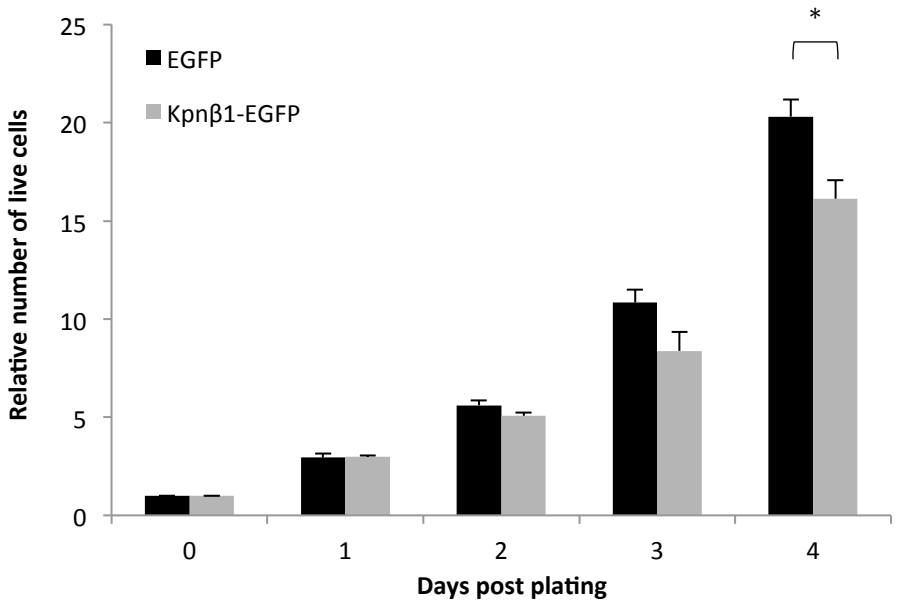

Figure 3.3. Stable overexpression of Kpnβ1 results in a reduction in anchorage-dependent proliferation of HeLa and CaSki cells. Trypan Blue assays revealed a significant reduction in the number of live HeLa (A) and CaSki (B) cells expressing Kpnβ1-EGFP compared to EGFP. Cells were counted for a period of up to 4 days post plating. Results shown represent the mean ± SEM for experiments performed in quadruplicate and repeated at least three times (*$p < 0.05$).

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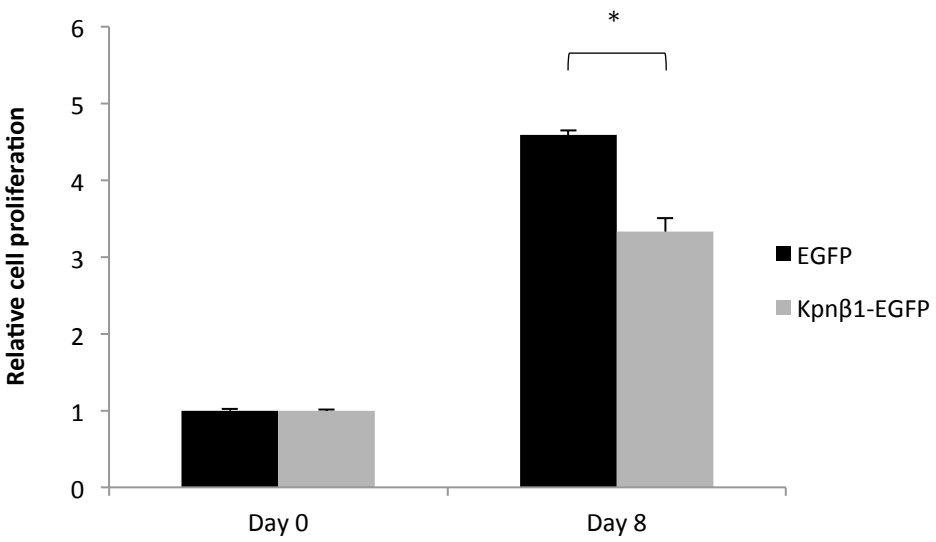

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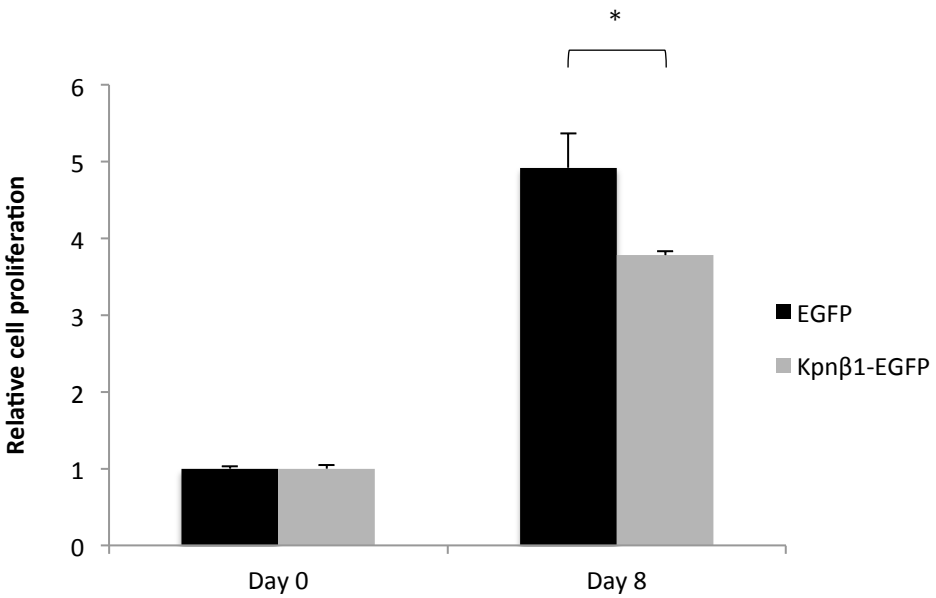

Figure 3.4. Stable overexpression of Kpnβ1 results in a reduction in anchorage-independent proliferation of HeLa and CaSki cells. MTT cell proliferation assays reveal a significant reduction in the anchorage-independent proliferation of HeLa (A) and CaSki (B) cells stably overexpressing Kpnβ1. Cells growing adherently were plated in 1% methyl cellulose containing media into 96-well plates coated in 12 mg/ml poly-HEMA. Cell proliferation was assayed by the addition of the MTT reagent for a period of up to 8 days. Results shown represent the mean ± SEM for experiments performed in quadruplicate and repeated at least two times (*$p < 0.05$).

3.2.3 Overexpression of Kpnβ1 causes a delay in cell cycle progression

To investigate the effects of Kpnβ1 overexpression on cell cycle progression, Fluorescence-activated cell sorting (FACS) analysis was performed to quantify percentage of cells in each phase of the cell cycle. Asynchronous cells revealed only minor changes when Kpnβ1 was overexpressed (data not shown), thus HeLa EGFP and Kpnβ1-EGFP cells were synchronised to late G1/early S phase using a thymidine block and harvested at 0, 2, 4, 6, 8, 10 and 24 hours post release into complete medium. FACS analysis of synchronised cells revealed that Kpnβ1 overexpression resulted in a prolonged delay or progression from the G1/S block, as Kpnβ1-EGFP expressing cells progressed through the cell cycle much slower than the control EGFP cells (Figure 3.5 A). Quantification of the percentage of HeLa EGFP and Kpnβ1-EGFP cells in each phase of the cell cycle showed that while 50% of control cells had exited G1 phase and entered S phase by approximately 3 hours, it took approximately 6 hours for the Kpnβ1-EGFP cells to reach the same stage (see arrows; Figure 3.5 B and C). These results suggest that Kpnβ1-EGFP expressing cells show delayed progression through G1/S and entry into G2/M phase of the cell cycle.

Western blot analysis of protein harvested from HeLa EGFP and Kpnβ1-EGFP cells at the same time points confirmed that Kpnβ1 overexpression resulted in a delay in cell cycle progression. After release from the thymidine block, pS10-Histone-H3 (pHisH3), a marker for cells in mitosis, peaked at 10 hours post release in EGFP cells, but remained low throughout the whole time-course in Kpnβ1-EGFP cells indicting that the cells did not reach M-phase within 10 hours post release (Figure 3.6 A). Mcl-1, an anti-apoptotic

protein that is regulated during the cell cycle, has been previously shown to increase during S and G2 phase and peak at mitosis[91], and this same trend was observed in EGFP cells. Kpnβ1-EGFP cells, however, showed a decrease in expression from the baseline levels observed in asynchronous cells, suggesting that Mcl-1 may be downregulated or degraded in response to Kpnβ1 overexpression, likely contributing to the observed delay in cell cycle progression. Interestingly, degradation of Mcl-1 has been shown to be induced under a number of cell stress conditions[91,92].

CaSki EGFP and Kpnβ1-EGFP cells harvested at various time-points post thymidine release were also analysed for changes in expression of cell cycle-associated markers. While not as pronounced as that observed in HeLa cells, CaSki cells overexpressing Kpnβ1 revealed a slight delay in progression through the cell cycle from the thymidine block. Expression of pHisH3 at 10 hours post release was reduced in Kpnβ1-EGFP cells compared to EGFP cells, indicating that fewer cells had reached mitosis by 10 hours (Figure 3.6 B). A decrease in expression of Mcl-1 was also observed in CaSki cells overexpressing Kpnβ1. These results suggest that CaSki cells overexpressing Kpnβ1, although showing a less marked effect revealed delayed entry into G2/M phase, similar to that observed in HeLa cells.

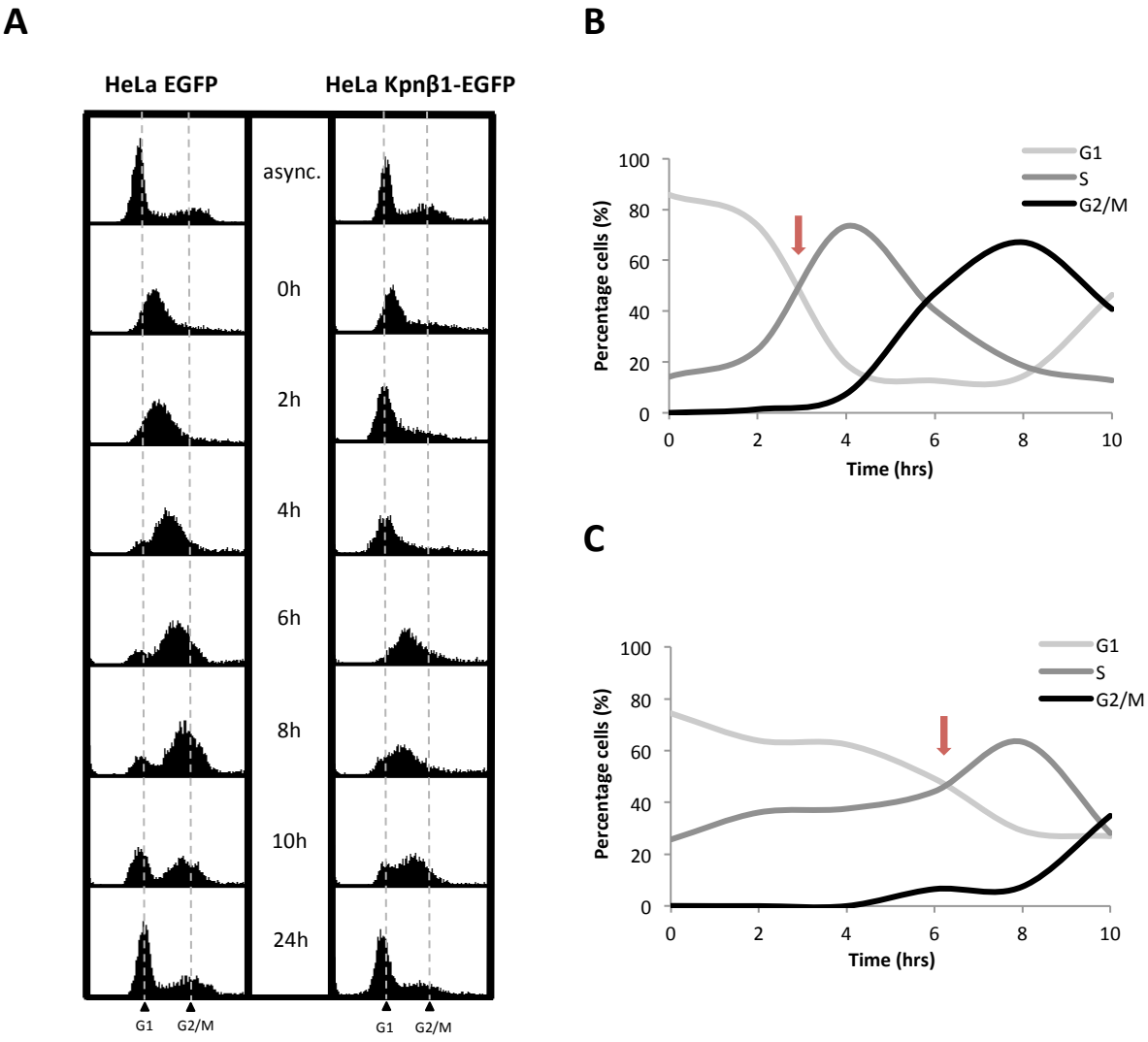

Figure 3.5. **Kpnβ1 overexpression results in a delay in cell cycle progression. A:** Flow cytometric analysis of HeLa EGFP and HeLa Kpnβ1-EGFP cells following synchronisation into late G1 and release into S phase. Cells were synchronised with 2 mM thymidine, released into complete medium, harvested at 0, 2, 4, 6, 8, 10 and 24 hours after release, and stained with propidium iodide (PI) to monitor their cell cycle distribution. An asynchronous culture of cells (async.) was used for comparison. Representative cell cycle profiles from data acquired using the BD Accuri Flow Cytometer from experiments performed in triplicate and repeated three times are shown. **B and C:** Line graphs represent quantification of the percentage of cells in each phase of the cell cycle for HeLa EGFP (B) and Kpnβ1-EGFP (C) cells. Data acquired by the BD Acurri Flow Cytometer was analysed using the ModFit software and graphs were constructed using Microsoft Excel. Arrows represent the approximate point where 50% cells had exited G1 phase.

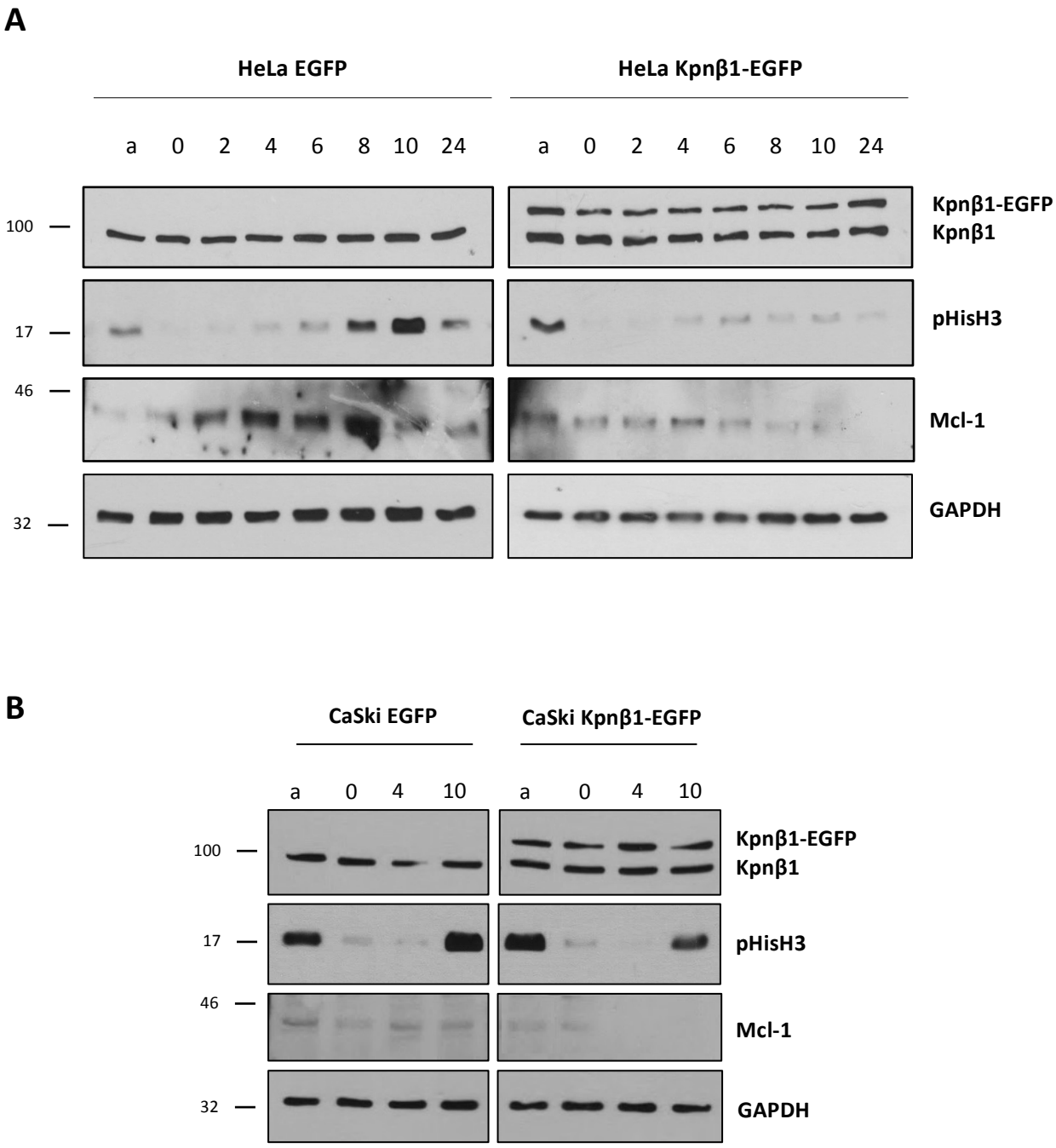

Figure 3.6. Kpnβ1 overexpression results in changes in expression of cell cycle associated proteins. A: Western blot analysis was used to determine the expression levels of various cell cycle associated proteins at 0, 2, 4, 6, 8, 10 and 24 hours post release following thymidine synchronisation, in HeLa EGFP and Kpnβ1-EGFP cells. Protein harvested from an asynchronous culture of cells (a) is shown for comparison. GAPDH was used as a control for loading. Representative data from experiments performed three independent times is shown. **B:** CaSki EGFP and Kpnβ1-EGFP cells were similarly harvested at 0, 4 and 10 hours after release following thymidine synchronisation, and compared to an asynchronous culture of cells (a). GAPDH was used as a control for loading.

3.2.4 Kpnβ1 plays a key role in cell morphology and actin reorganisation

The effects of Kpnβ1 overexpression on additional biological phenotypes, including cell morphology and actin organisation, were next analysed. Phase contrast microscopy of HeLa and CaSki cells stably overexpressing Kpnβ1 showed distinct changes in cell morphology, with cells becoming smaller and more tightly packed (Figure 3.7 A). Quantification of the changes in cell area showed that Kpnβ1 overexpression resulted in a significant decrease in cell area when compared to the area of control HeLa and CaSki cells (Figure 3.7 B and C). These results illustrate that Kpnβ1 plays a role in cell morphology; due to the vital role that it plays in multiple cellular processes, during both interphase and mitosis, it is likely that Kpnβ1 dysregulation has an impact on many cellular phenotypes or events.

Changes in cell morphology are often driven by rearrangement of the cytoskeleton; thus we next investigated whether the morphological changes associated with Kpnβ1 overexpression were linked to changes in actin redistribution. Immunofluorescence actin staining assays were performed using phalloidin, a high-affinity filamentous actin (F-actin) probe, to investigate actin reorganisation in HeLa and CaSki cells stably overexpressing Kpnβ1. Phalloidin staining revealed changes in cell shape, as well as a reduction in the number of actin-rich cytoplasmic extensions seen at the edges of the elongated, spindle-shaped control cells, more evident in HeLa cells (Figure 3.8 A). Quantification of these results showed that Kpnβ1 overexpression resulted in a significant decrease in cell area (Figure 3.8 B). This data confirms the changes in cell morphology and size seen using phase contrast microscopy, and indicates that Kpnβ1 plays a role in actin reorganisation.

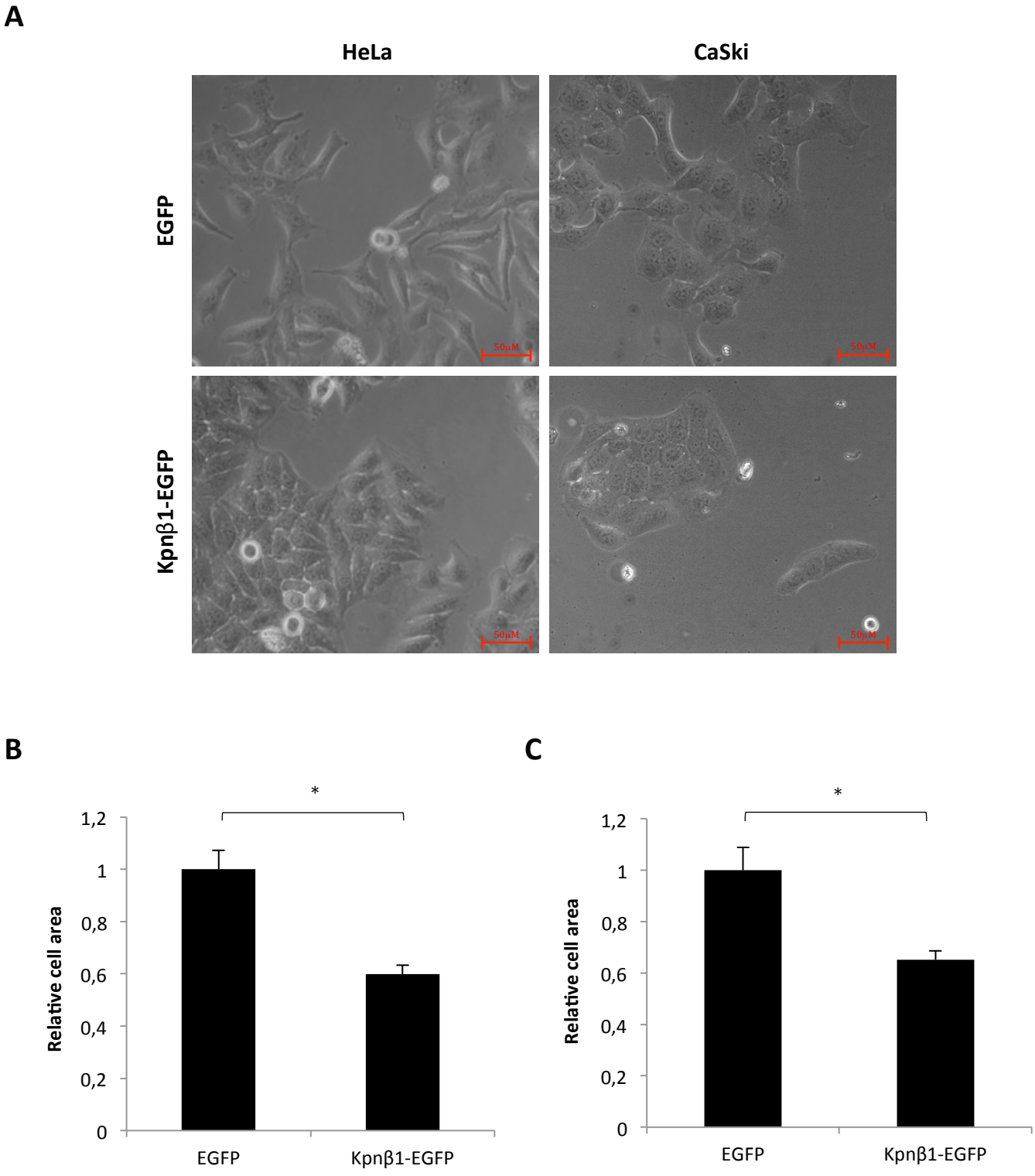

Figure 3.7. Overexpression of Kpnβ1 results in changes in the morphology and size of HeLa and CaSki cells. A: Phase contrast images showing control cells (EGFP) and cells overexpressing Kpnβ1 (Kpnβ1-EGFP), taken 48 hours post plating. Cells were viewed at 20 x magnification, using the Zeiss Primovert inverted phase microscope. **B and C**: Quantification of relative HeLa (B) and CaSki (C) cell area ± SEM of forty cells from each condition was performed using the AxioVision 4.7 software. Representative data from experiments repeated at least three independent times is shown (*$p < 0.05$).

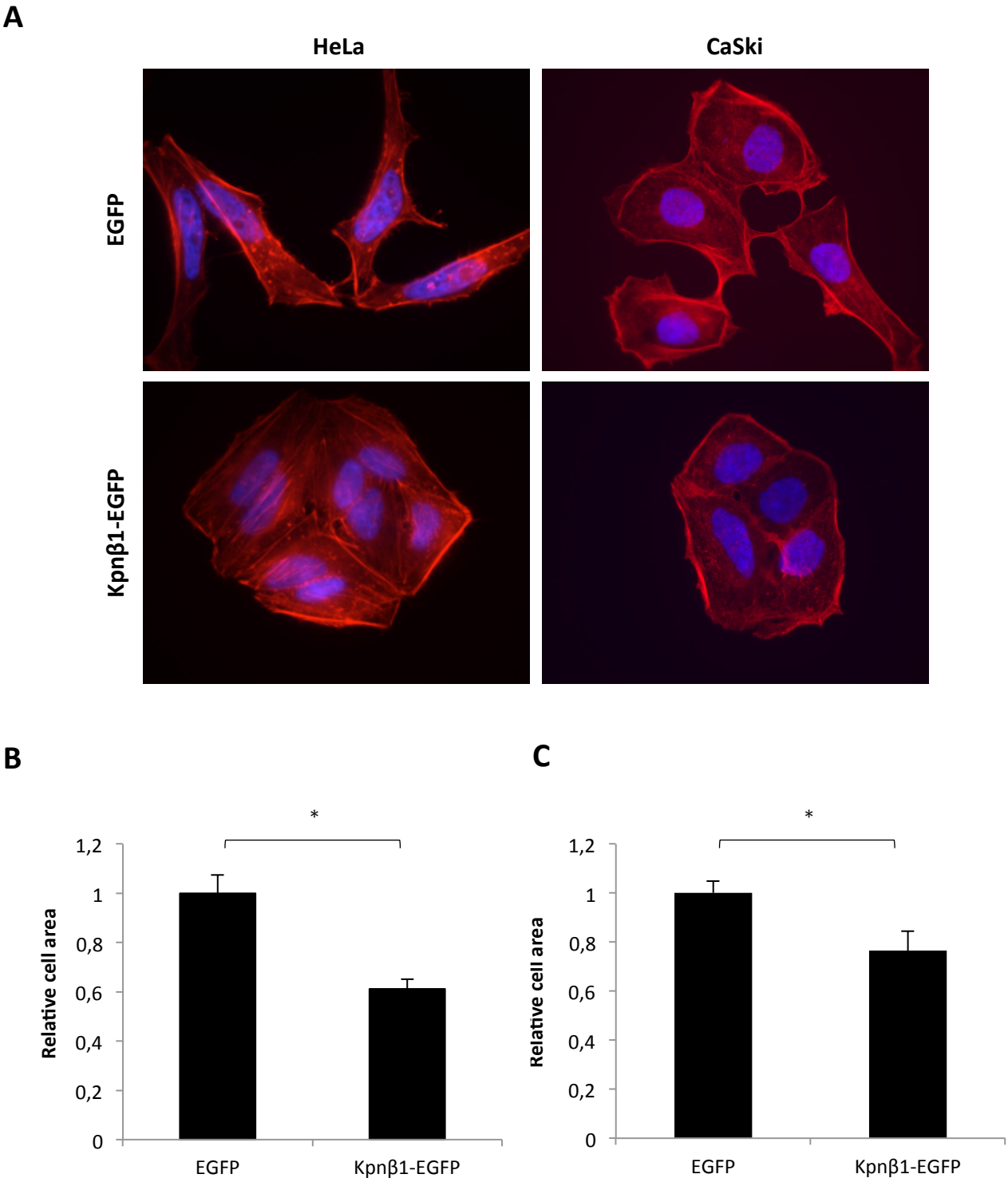

Figure 3.8. Overexpression of Kpnβ1 results in actin reorganisation in HeLa and CaSki cells. A: Fluorescent staining of polymeric F-actin using phalloidin (red) in EGFP and Kpnβ1-EGFP expressing cells. DAPI stain was used to visualise the cell nuclei (blue). **B and C:** Quantification of the changes in HeLa (B) and CaSki (C) cell area from the captured fluorescent images. Cell area was calculated using the AxioVision 4.7 Software. Results shown represent the mean ± SEM over twenty fields of view for experiments repeated at least two independent times (*$p < 0.05$).

3.2.5 Overexpression of Kpnβ1 results in changes in cell adhesion properties

The observed morphological changes between HeLa and CaSki EGFP and Kpnβ1-EGFP cells suggests that overexpression of Kpnβ1 causes cells to pack tighter and form clusters, which indicates the possibility of tight junction formation and increased cell adhesion. Thus, to investigate whether the observed morphological changes were associated with any changes in cell adhesion, adhesion assays were performed. Adhesion assays using uncoated cell culture dishes were used to determine overall changes in the adhesion properties of cells (particularly those involving cell-surface/cell-substrate interactions). Results show that HeLa and CaSki cells overexpressing Kpnβ1 were significantly more adherent compared to control cells (Figure 3.9 A and B). Furthermore, the expression levels of various proteins shown in the literature to be involved in adhesion, such as E-cadherin (plays an important role in promoting cell-cell adhesion[93]), and Vimentin (a marker of mesenchymally derived, non-adherent cells[94]), were also investigated. Western blot analysis revealed that E-cadherin levels were elevated in HeLa and CaSki cells overexpressing Kpnβ1, while Vimentin levels were decreased (Figure 3.9 C and D). These results suggest that dysregulation of Kpnβ1 expression plays a role in processes associated with cell-surface (ie. cell to extracellular matrix) and cell-cell adhesion.

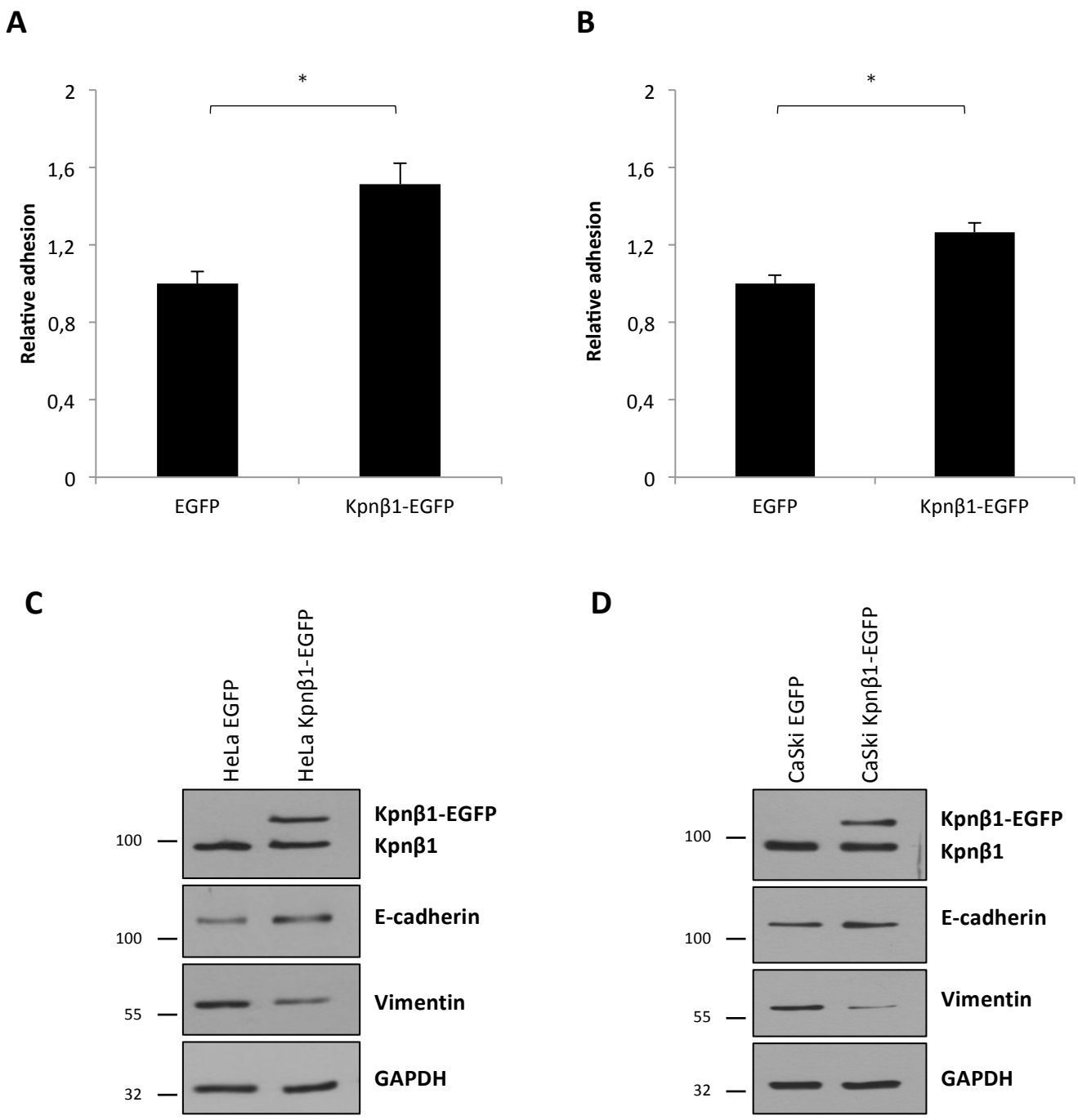

Figure 3.9. Overexpression of Kpnβ1 results in changes in the adhesion and expression of cell adhesion markers in HeLa and CaSki cells. A and B: Relative cell adhesion in control and Kpnβ1-EGFP HeLa (A) and CaSki (B) cells. Adherent cells were fixed (after removing non-adherent cells by washing) and stained with 0.5% crystal violet solution. Cells over ten fields of view were counted using ImageJ and normalised to unwashed cells. Results shown represent the mean ± SEM for experiments performed in triplicate and repeated at least two independent times (*$p < 0.05$). **C and D:** Western blot analysis was used to determine the expression levels of various proteins for HeLa (C) and CaSki (D) cells. GAPDH was used as a control for loading.

3.2.6 Overexpression of Ran results in a further reduction in proliferation of HeLa cells expressing Kpnβ1-EGFP

Various studies have revealed that a precise balance of Kpnβ1 to Ran is required for correct mitotic functioning, and have suggested that if one factor is overexpressed (ie. Kpnβ1), upregulation of the other (ie. Ran) should restore the balance and allow for rescue of the impaired functioning associated with overexpression of either factor alone[68,87,95]. We therefore hypothesised that the reduction in cancer cell proliferation associated with overexpression of Kpnβ1 could be rescued by the expression of exogenous Ran. Thus, in order to investigate the effects associated with overexpression of Ran, both in the absence and presence of stable Kpnβ1 overexpression, HeLa EGFP and Kpnβ1-EGFP cells were transfected with plasmid DNA containing Ran-EGFP and expression was confirmed by western blot analysis (Figure 3.10 A). Anchorage-dependent MTT proliferation assays revealed that similar to what was seen with Kpnβ1 overexpression, Ran overexpression resulted in a small but significant reduction in the proliferation of HeLa cells (Figure 3.10 B). Interestingly, when Ran-EGFP was expressed in cells already stably expressing Kpnβ1-EGFP there was a further reduction in proliferation, greater than that observed with overexpression of either Kpnβ1 or Ran alone. These results suggest that cells are particularly unable to cope with an imbalance in the levels of Kpnβ1 and Ran, factors that are important for multiple cellular processes, including nuclear transport and mitotic regulation.

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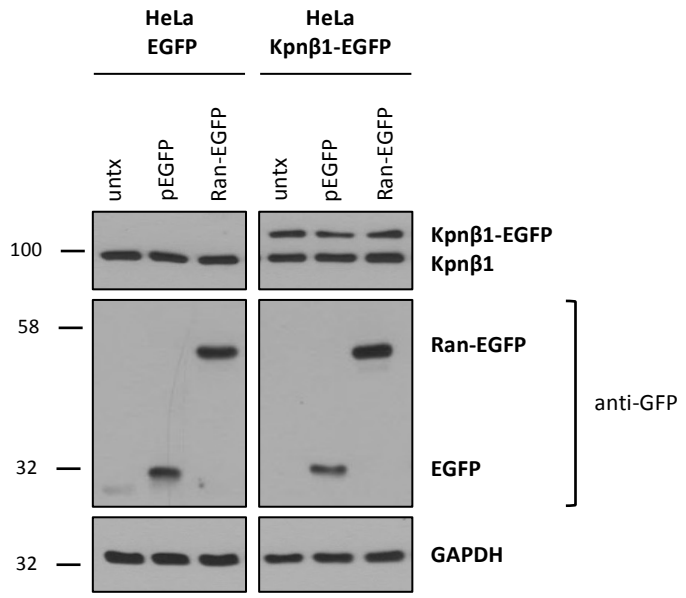

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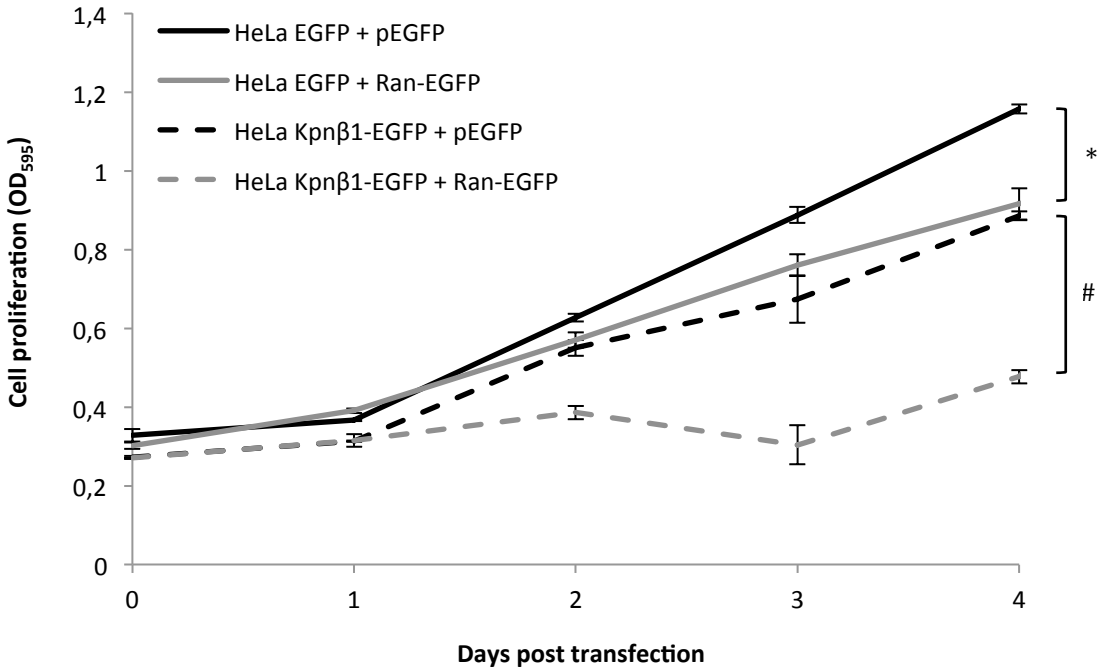


Figure 3.10. Expression of Ran-EGFP results in a further reduction in proliferation of HeLa cells overexpressing Kpnβ1. A: Western blot analysis was used to determine GFP expression levels at 24 hours post transfection of HeLa EGFP and Kpnβ1-EGFP cells with pEGFP-C2 and Ran-EGFP plasmids. First lane (untx) represents protein harvested from untransfected cells. GAPDH was used as a control for loading. **B:** MTT proliferation assays show a significant reduction in the proliferation of HeLa EGFP cells transiently expressing Ran-EGFP. Expression of Ran-EGFP in cells already stably expressing Kpnβ1-EGFP results in a further reduction in proliferation. Results shown represent the mean ± SEM for experiments performed in triplicate and repeated at least three times ($p < 0.05$; days 2,3 and 4 for HeLa EGFP cells (*) and HeLa Kpnβ1-EGFP cells (#) expressing pEGFP and Ran-EGFP).

3.2.7 Low expression of Kpnβ1-EGFP results in a minor reduction in the proliferation of non-cancer cells

In order to investigate the effects of Kpnβ1 overexpression on non-cancer cell proliferation, establishment of a cell line stably expressing Kpnβ1-EGFP was attempted. Despite optimisation of transfection conditions using various cell lines, transfection reagents, plasmid backbones, and amount of plasmid DNA transfected, generation of a non-cancer stable cell line was unsuccessful. As a result, FG0 normal skin fibroblasts (which were found to transfect most efficiently out of a number of non-cancer cell lines) were transiently transfected with the pEFIRES-EGFP and pEFIRES-Kpnβ1-EGFP constructs, and proliferation was assayed for a period of three days post transfection. Analysis of Kpnβ1 and GFP protein expression via western blot revealed that while FG0 cells transfected with pEFIRES-EGFP showed suitable EGFP expression (suggesting adequate transfection efficiency), levels of Kpnβ1-EGFP were very low in cells transfected with the equivalent amount of pEFIRES-Kpnβ1-EGFP (after much optimisation of transfection conditions, levels of Kpnβ1-EGFP could not be improved upon) (Figure 3.11 A).

MTT proliferation assays revealed that FG0 cells expressing Kpnβ1-EGFP showed a marginal but significant reduction in proliferation compared to cells expressing EGFP (Figure 3.11 B). It is possible that cells transfect less efficiently with pEFIRES-Kpnβ1-EGFP than pEFIRES-EGFP due to the plasmids large size (it contains the 2631bp Kpnβ1-encoding fragment), but that even low levels of Kpnβ1 overexpression are sufficient to result in a decrease in the proliferation of FG0 cells. However, given the poor

expression of Kpnβ1-EGFP in these cells it cannot be reasonably concluded that the minor effect observed was due to overexpression of Kpnβ1, and thus further optimisation to improve transfection efficiency is required.

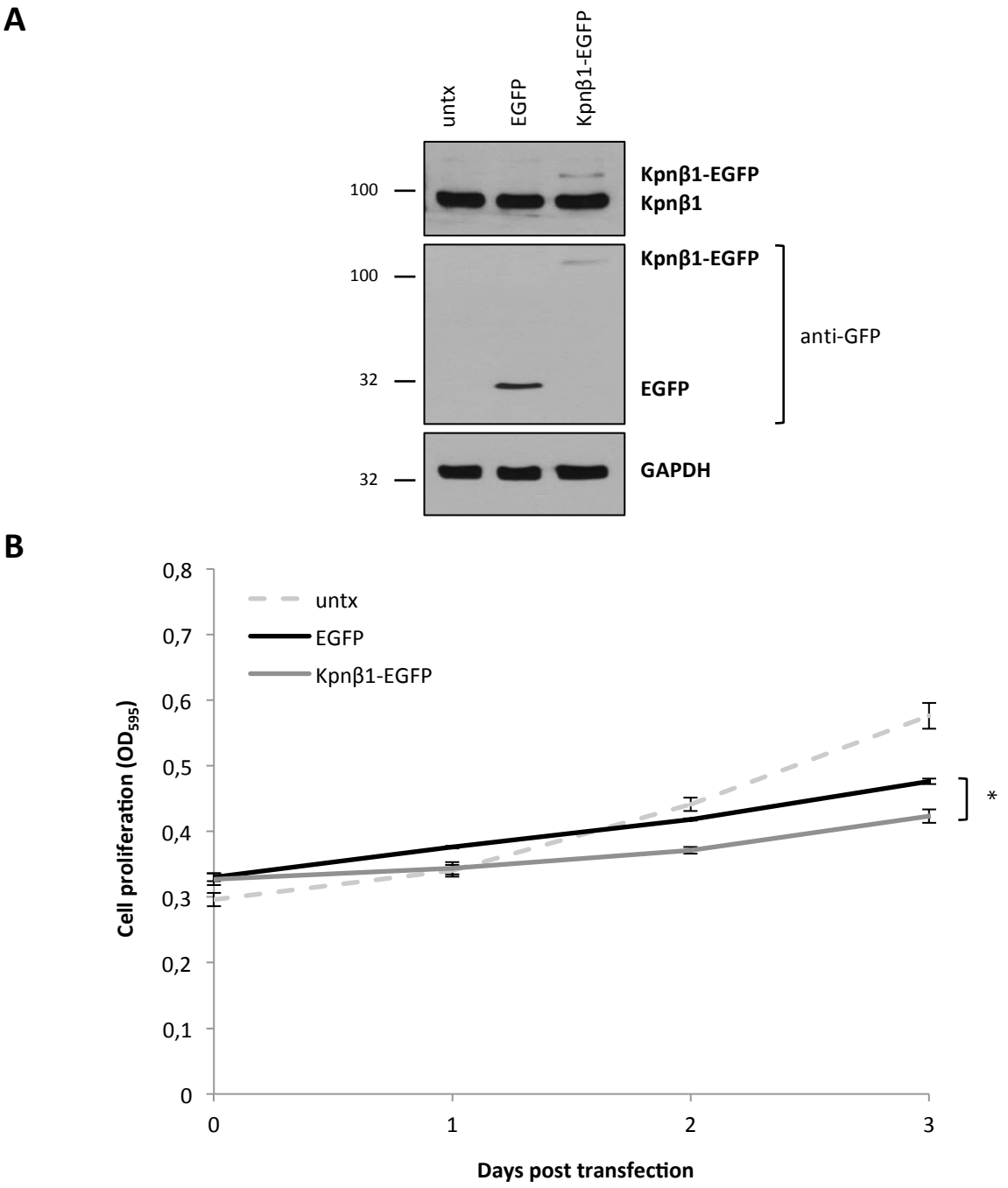


Figure 3.11. Low expression of Kpnβ1-EGFP results in a small but significant reduction in the proliferation of non-cancer cells. A: Western blot analysis of Kpnβ1 and GFP expression levels at 48 hours post transfection of FG0 normal skin fibroblasts with pEFIRES-EGFP and pEFIRES-Kpnβ1-EGFP plasmids. The first lane (untx) represents protein harvested from untransfected cells. GAPDH was used as a control for loading. **B:** MTT proliferation assays revealed a small but significant reduction in the proliferation of FG0 cells transiently transfected with Kpnβ1-EGFP. Representative data from experiments performed in triplicate and repeated two independent times is shown (* $p < 0.05$; days 1 - 3).

3.3 DISCUSSION

Changes in gene expression patterns are a significant driver of cancer progression. Thus, the identification of genes and pathways involved in and/or altered in this process will not only improve our understanding of cancer initiation and development, but will also provide evidence for the identification of novel anti-cancer drug targets. Previous studies have revealed Karyopherin β1 (Kpnβ1), the primary nuclear import protein, to be significantly overexpressed in a variety of cancer types including cervical[14], gastric[76], and breast cancers[73]. Furthermore, these studies reveal that inhibition of Kpnβ1 expression leads to cancer cell death, while van der Watt *et al*. (2009) showed that inhibition of Kpnβ1 protein expression in non-cancer cells has only a minor effect on cell viability[14]. These results suggest that Kpnβ1 has potential as an anti-cancer therapeutic target, thus warranting further research into the association between Kpnβ1 expression and cancer progression.

Of particular interest in this study is the role that Kpnβ1 plays in various processes associated with cellular transformation and cancer progression. When validating a protein as an anti-cancer target, identifying whether the protein is in fact a driver of cancer, or certain cancer phenotypes, is crucial (i.e. is it able to induce features of the transformed or cancer phenotype?). While the potential for Kpnβ1 as an anti-cancer target has been validated by protein knockdown studies in various normal, transformed and cancer cell lines, research into the role that Kpnβ1 plays in malignant transformation and tumour progression remains limited. The aim of this study was

therefore to investigate the effects of Kpnβ1 overexpression on various biological processes associated with cellular transformation.

In this study, the biological effects of Kpnβ1 overexpression in cancer and non-cancer cell lines were investigated. Through the use of a GFP-tagged expression vector system, stable Kpnβ1 overexpressing cell lines, HeLa and CaSki pEFIRES-Kpnβ1-EGFP were created, and the effects on various biological processes such as cell proliferation, cell cycle, cell morphology and adhesion were observed. In order to study the effects of Kpnβ1 overexpression in non-cancer cells, pEFIRES-Kpnβ1-EGFP was transiently transfected into normal human fibroblasts and the effects on cell proliferation assayed over a period of days.

Inhibition of Kpnβ1 protein expression through the use of RNA interference has been shown to result in a significant reduction in the rate of proliferation in cancer cells, but not normal cells, suggesting that increased levels of Kpnβ1 are likely essential to cancer cell biology[14]. Our findings described in this study show that stable overexpression of Kpnβ1 also resulted in a reduction in cervical cancer cell proliferation and viability. In addition to this, we showed that cell cycle progression as a result of Kpnβ1 overexpression was significantly delayed, as cells expressing Kpnβ1-EGFP revealed a prolonged progression through G1/S phase into G2 phase and mitosis. The reduction in proliferative rate and delay in cell cycle progression as a result of Kpnβ1 overexpression is likely due to the multi-role status that Kpnβ1 holds; in addition to its role as the primary nuclear import protein in interphase cells, Kpnβ1 has an entirely different set of functions in mitosis, acting as a regulator of various mitotic

processes from nuclear envelope breakdown and spindle assembly, to nuclear envelope and pore reassembly[22,68,95]. Previous studies by the Lavia group indicate that transient overexpression of Kpnβ1 results in the appearance of distinct mitotic defects, often accompanied by prolonged mitoses (mitotic delay) and/or arrest[87,96]. With such a significant set of roles in addition to its function as principle importer, it is easy to see why disruption of Kpnβ1 expression in cervical cancer cells (by inhibition or overexpression) has detrimental effects on cancer cell proliferation and cell cycle progression. In this study, we were unable to achieve successful synchronisation of cells to mitosis, but we hypothesise that Kpnβ1 overexpression would result in a significant delay in mitotic progression, accompanied by the appearance of mitotic abnormalities/defects. This remains to be further optimised and validated.

A wide range of cancer processes have been shown to rely on Kpnβ1-mediated nuclear import. For example, a recent review shows how the Karyopherin proteins are involved in controlling a large number of epithelial-mesenchymal transition (EMT) promoting pathways[97]. Kpnβ1 has been shown to be involved in the nuclear translocation of a number of EMT-promoting proteins including Snail[98], Smad[99], and Notch[100], all of which play a role in increasing cancer cell invasion and metastasis. In addition to this, Kpnβ1 has also been shown to play a role in actin cytoskeleton regulation, by regulating the interaction between an actin binding kinesin (NabKin) and F-actin, demonstrating that Kpnβ1 likely plays a role in influencing cytoskeletal elements and structure[68,69]. We showed that Kpnβ1 overexpression in HeLa and CaSki cells results in changes in cell morphology and actin reorganisation, with cells becoming smaller and more epithelial-like. In addition, Kpnβ1 overexpressing cells

showed enhanced adhesion, as well as an increase in E-cadherin and decrease in Vimentin protein expression. These results suggest that overexpression of Kpnβ1 does not enhance the EMT process, but rather results in a reversed EMT, or mesenchymal-epithelial transition (MET), where cells transition from motile, more spindle-shaped mesenchymal cells to adherent, more compact/columnar epithelial cells. There is much evidence linking the EMT process with tumour progression and malignant transformation, as transitioning to a more mesenchymal state promotes cell invasion and metastasis (or cancer cell dissemination)[101]. More recently, evidence has started to emerge supporting the concept that MET may be important for metastatic colonisation, but research remains limited. Thus, it appears as if overexpression of Kpnβ1 has negative consequences for the proliferation, progression and metastatic potential of cancer cells.

While Kpnβ1 overexpression alone appears to be insufficient to induce any of the biological phenotypes associated with cellular transformation, it is possible that overexpression of additional members of the nuclear transport machinery is required for cancer-associated changes. The GTPase Ran is one of the most important nuclear transport factors. Like Kpnβ1, it plays different roles during various phases of the cell cycle. In interphase cells, Ran is responsible for providing energy and determining directionality of transport and without it nucleo-cytoplasmic shuttling would be dysfunctional. In mitotic cells, Ran plays an important role in counter-regulating Kpnβ1 activity[22]. A number of studies have revealed that a precise balance of Kpnβ1 to Ran is required for the correct functioning of various mitotic processes, such as spindle formation[87] and nuclear envelope and pore assembly[68,95], suggesting that if one factor

is overexpressed (ie. Kpnβ1), upregulation of the other (ie. Ran) should restore the balance and allow for rescue of the impaired functioning associated with overexpression of either factor alone. We therefore hypothesised that the reduction in cancer cell proliferation associated with overexpression of Kpnβ1 could be rescued by the expression of exogenous Ran, however results revealed the opposite; proliferation was in fact further reduced when Kpnβ1 and Ran were co-expressed. It is possible that in terms of nuclear import, cancer cells have already reached their maximum capability and thus further enhancement of various factors such as Kpnβ1 and Ran, without strict control/regulation of all factors involved in nuclear transport (ie. a precise balance), results in further cellular dysfunction. It is also possible that wild type Ran, which can be bound by GTP or GDP depending on its cellular location[102], is insufficient to restore function and a higher ratio of RanGTP (RanGTP is required for the release of cargo molecules from Kpnβ1 in the nucleus, as well as counter-regulating the role that Kpnβ1 plays in various mitotic processes), would rescue the negative effects associated with upregulation of Kpnβ1 alone.

While various studies have provided evidence to suggest that enhanced Kpnβ1 expression is necessary for cancer cell survival and progression; van der Watt and colleagues (2011) showed that the Kpnβ1 promoter is more highly active in cancer and transformed cells compared to normal[50], and Kuusisto and Jans (2015) recently found that malignant cell types are more sensitive to Kpnβ1 protein knockdown than their non-transformed counterparts implying that increased levels (or activity) of Kpnβ1 likely plays a role in tumour progression[73], no research has yet emerged to show whether Kpnβ1 overexpression plays a role in the cellular transformation of non-

cancer cells. The question remains as to whether overexpression of Kpnβ1 alone is sufficient to induce/drive cellular transformation? A number of single genes that are able to induce cellular transformation due to mutation and/or increased expression (i.e. proto-oncogenes) have been identified to date, including p53[103], Ras[104], and Myc[105]. Thus, to answer this question, we performed experiments in which Kpnβ1-EGFP was transiently expressed in FG0 normal skin fibroblasts. Despite only achieving very low levels of overexpression in our experiments, our results indicate that Kpnβ1 overexpression resulted in a minor reduction in the proliferation of non-cancer cells, suggesting that Kpnβ1 alone is not able to drive cellular transformation. It is possible that if other members of the nuclear transport family such as Kpnα2, CRM1 and Ran (all of which have been shown to be overexpressed in a variety of cancers[15,106–108]) were also upregulated, enhanced proliferation and other effects associated with the transformed phenotype may be seen. Interestingly, Ly and colleagues (2010) demonstrated that an activated Ran mutant (that displays 5-fold greater levels of RanGTP to that of wild type Ran) was able to induce the cellular transformation of NIH-3T3 fibroblasts and that the injection of these cells into mice resulted in tumour formation. They also discovered that increasing levels of active Ran triggers the activation of a variety of signaling proteins including EGFR, Ras and ERK, and that active Ran-induced cellular transformation was dependent on activated EGFRs[109]. These lines of evidence suggest that as a consequence of Ran activation, loss of growth factor regulation gives rise to oncogenic transformation of normal cells. This leads to the possibility that Kpnβ1 overexpression alone is insufficient to induce the dysregulation of growth factor signaling that gives rise to cellular transformation and the development of cancer.

Taken together, these results suggest that overexpression of Kpnβ1 does not provide any growth advantage to normal or cancer cells, and is thus not sufficient to induce cellular transformation. In fact, it appears as if a precise balance of Kpnβ1 expression and activity is essential for the correct functioning of cancer cells, and that disruption of this balance in either direction has detrimental effects on many cellular processes. It is possible that while cancer cells may have reached the maximum of what they can handle in terms of Kpnβ1 expression levels, normal cells might require additional factors for oncogenic transformation. While it is known that Kpnβ1 expression is upregulated in transformed and cancer cells compared to normal, it still remains unclear as to at which point, and for all the reasons, along the transformation process Kpnβ1 becomes overexpressed.

CHAPTER 4

INVESTIGATING THE DRUG TARGET SPECIFICITY OF A SMALL MOLECULE INHIBITOR OF KPNβ1

4.1 INTRODUCTION

The discovery that Kpnβ1 is upregulated in cancer cells compared to normal, as well as the fact that it appears to be essential for cancer cell growth and survival (inhibition of Kpnβ1 protein expression in cancer cells leads to apoptosis[14]), suggested that it may be an effective target for the development of novel anti-cancer therapies. Thus, a structure-based *in silico* screen was performed to identify molecules with the potential to bind Kpnβ1, and the Inhibitor of Nuclear Import-43 (INI-43) was identified and further examined for its ability to block Kpnβ1-mediated nuclear import and decrease cancer cell viability. Recently published data from our laboratory reveals that INI-43 significantly reduced the proliferation of cancer cells and interfered with the nuclear localisation of Kpnβ1 and known Kpnβ1 cargo proteins, NFκB, NFAT, NFY and AP-1[52]. Only a minimal effect was observed when treating normal cells with the same concentration of INI-43 found to be cytotoxic to cancer cells, thus INI-43 shows promise as a novel targeted therapy for the treatment of cancer. Further investigation, however, is warranted to determine its specificity.

Validation of drug target specificity is an important step in drug discovery and development, however many challenges exist and countless drug candidates fail due to undesirable side effect profiles (partially due to off-target effects). For a protein to be a successful drug target it must be *druggable*, which essentially means that it should contain a binding site or region that favours interactions with small molecules[110,111]. Such a binding site should also be significantly different from regions on similar (closely related) proteins, so as to reduce the possibility of off-target binding. For the identification of compounds with the potential to inhibit Kpnβ1, a rational structure-based approach was used to identify small molecules that bind the overlapping binding region of Ran and Kpnα2 on Kpnβ1. This region, spanning amino acids 331-363 of the 876 amino acid structure of Kpnβ1, has been reported to be essential for Kpnβ1 functionality, as Kpnβ1 requires both RanGTP and Kpnα2 binding for the import of classical NLS-containing cargoes into the nucleus[28,112].

While the initial computational screen and subsequent research conducted in the laboratory suggest that INI-43 functions by targeting Kpnβ1 (INI-43 treatment interferes with the localisation of Kpnβ1 and its cargoes), little is known about the specificity of INI-43 for Kpnβ1. Biophysical assays and experiments to validate the region/site on Kpnβ1 where INI-43 binds are a priority for future research in the laboratory. The aim of this chapter is therefore to preliminarily assess the specificity of INI-43 for Kpnβ1 by performing a series of rescue experiments using exogenous Kpnβ1-EGFP. In addition, the effect of INI-43 on the stability of endogenous Kpnβ1 was investigated.

4.2 RESULTS

4.2.1 Cells overexpressing Kpnβ1 are more resistant to INI-43

Recently published data from the laboratory suggests that INI-43 may be acting by targeting Kpnβ1[52]. We therefore proposed that if INI-43 exerts its anti-cancer effects by targeting Kpnβ1, exogenous expression of Kpnβ1-EGFP should rescue the inhibitory effects induced upon INI-43 treatment. To examine this, the HeLa pEFIRES-EGFP and pEFIRES-Kpnβ1-EGFP cell lines described in Chapter 3 were used. Briefly, cells were treated with increasing concentrations of INI-43 and dose response curves were generated to calculate EC_{50} concentrations. Treatment of cells with Importazole, a recently identified small molecule inhibitor of Karyopherin α/β-mediated nuclear import[60], and Doxorubicin, a widely used anti-cancer chemotherapeutic[113], were used as positive and negative controls, respectively. Results revealed that HeLa pEFIRES-Kpnβ1-EGFP cells displayed an INI-43 EC_{50} value that was approximately 25% higher than that of control cells (10.4 μM compared to 8.3 μM) (Table 4.1). The positive control, Importazole showed similar results, with an additional ~30% of the small molecule inhibitor required to generate an EC_{50} in Kpnβ1-EGFP expressing cells (15 μM compared to 11.2 μM in control EGFP cells).

In addition, cell viability assays showed that cells expressing Kpnβ1-EGFP had significantly more viable cells after a 48-hour treatment with various concentrations of INI-43 (Figure 4.1 A). A significant rescue effect was also observed when Kpnβ1-EGFP expressing cells were treated with Importazole but not Doxorubicin (Figure 4.1 B and

C). These results suggest that HeLa cells overexpressing Kpnβ1 are more resistant to INI-43 treatment, implying that INI-43 is acting, at least in part, by targeting Kpnβ1.

While the rescue seen when Kpnβ1-EGFP expressing cells were treated with INI-43 was significant, it was not complete; there is a possibility that greater levels of overexpression (beyond the 1:1 ratio of endogenous to exogenous Kpnβ1 achieved in our stable cell line) are required to provide further resistance to INI-43.

Table 4.1. Representative EC_{50} values and 95% confidence intervals for INI-43, Importazole and Doxorubicin

	HeLa EGFP	***HeLa Kpnβ1-EGFP***
INI-43	8.3 μM (7.6 – 9.0)	10.4 μM (9.8 – 10.9)
Importazole	11.2 μM (8.0 – 13.0)	15 μM (13.4 – 16.8)
Doxorubicin	1.4 μM (0.7 – 2.1)	1.1 μM (0.6 – 1.2)

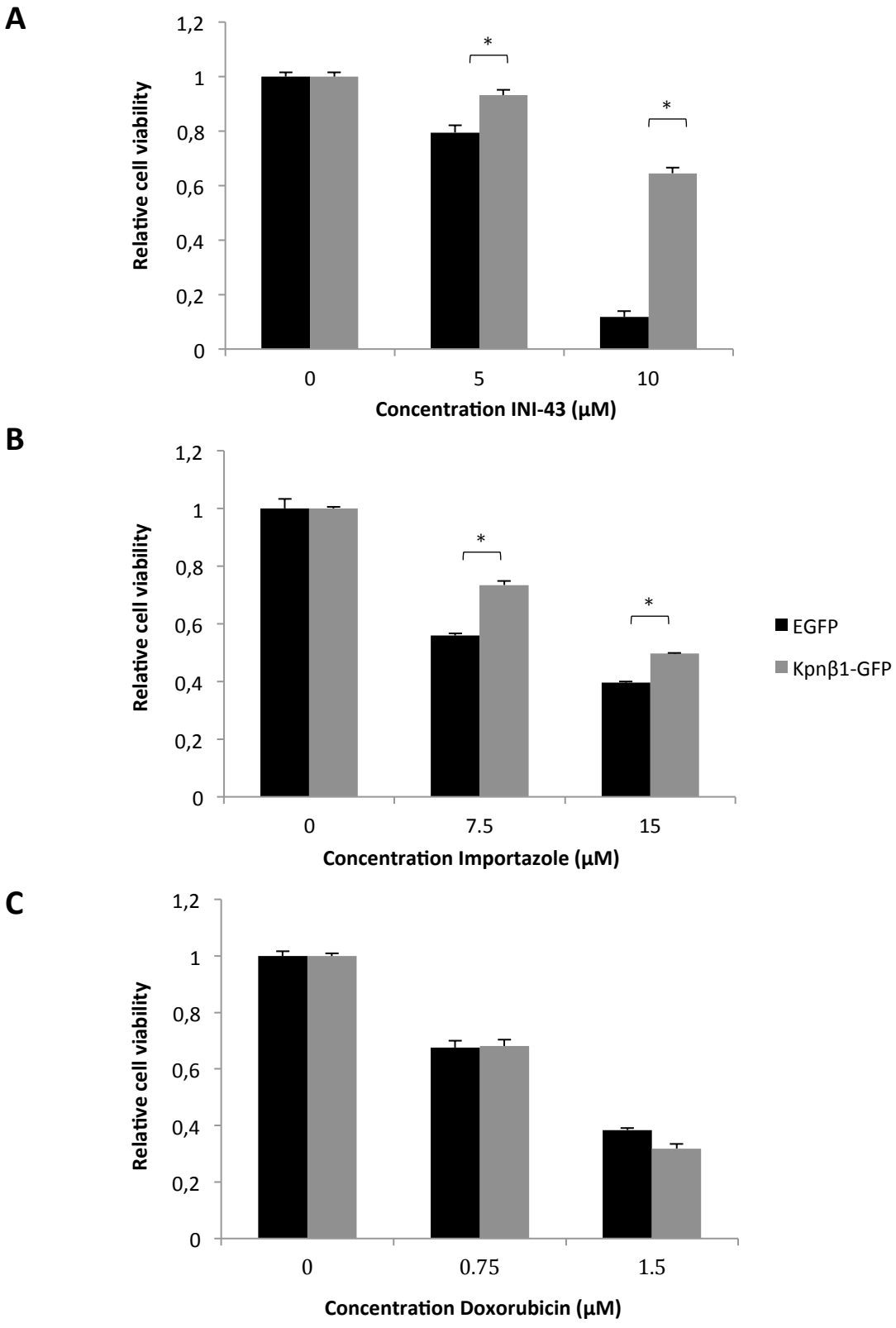


Figure 4.1. Kpnβ1 overexpression rescues HeLa cell viability in cells treated with the small molecule inhibitors, INI-43 and Importazole. MTT proliferation assays revealed reduced viability of EGFP expressing cells in response to 48-hour treatment with INI-43 (A) and Importazole (B), with significantly less cell death seen in Kpnβ1-EGFP overexpressing cells. Doxorubicin (C) was used as a negative control. Results shown represent the mean ± SEM of experiments performed in quadruplicate and repeated at least three times (*$p < 0.05$).

4.2.2 The inhibitory effect of INI-43 on nuclear import is associated with functional activity of Kpnβ1

4.2.2.1 Kpnβ1 overexpression rescues the inhibitory effects of INI-43 on the nuclear localisation of NFκB p65

To address the question of whether the inhibitory effects of INI-43 on nuclear import could be rescued by expression of exogenous Kpnβ1, the localisation of a known Kpnβ1 cargo protein, NFκB p65[34] was analysed by means of an immunofluorescence assay. Our results show that p65 is predominantly located in the cytoplasm of untreated EGFP and Kpnβ1-EGFP cells (Figure 4.2 A). In cells stimulated with the phorbol ester PMA, a nuclear translocation of p65 was seen. Pre-treatment of EGFP cells with 10 μM INI-43 prevented the nuclear translocation of p65. In the case of the Kpnβ1-EGFP cells, however, a nuclear localisation of p65 was observed despite pre-treatment with INI-43, suggesting that the inhibitory effects of INI-43 on the nuclear import of NFκB p65 can be partly rescued by Kpnβ1 overexpression. Quantification of the percentage of cells showing predominantly nuclear or cytoplasmic p65 localisation revealed that while only ~10% of EGFP cells showed nuclear localisation of p65 after treatment with INI-43, greater than 70% of Kpnβ1-EGFP cells showed nuclear p65 localisation after the same treatment (Figure 4.2 B and C).

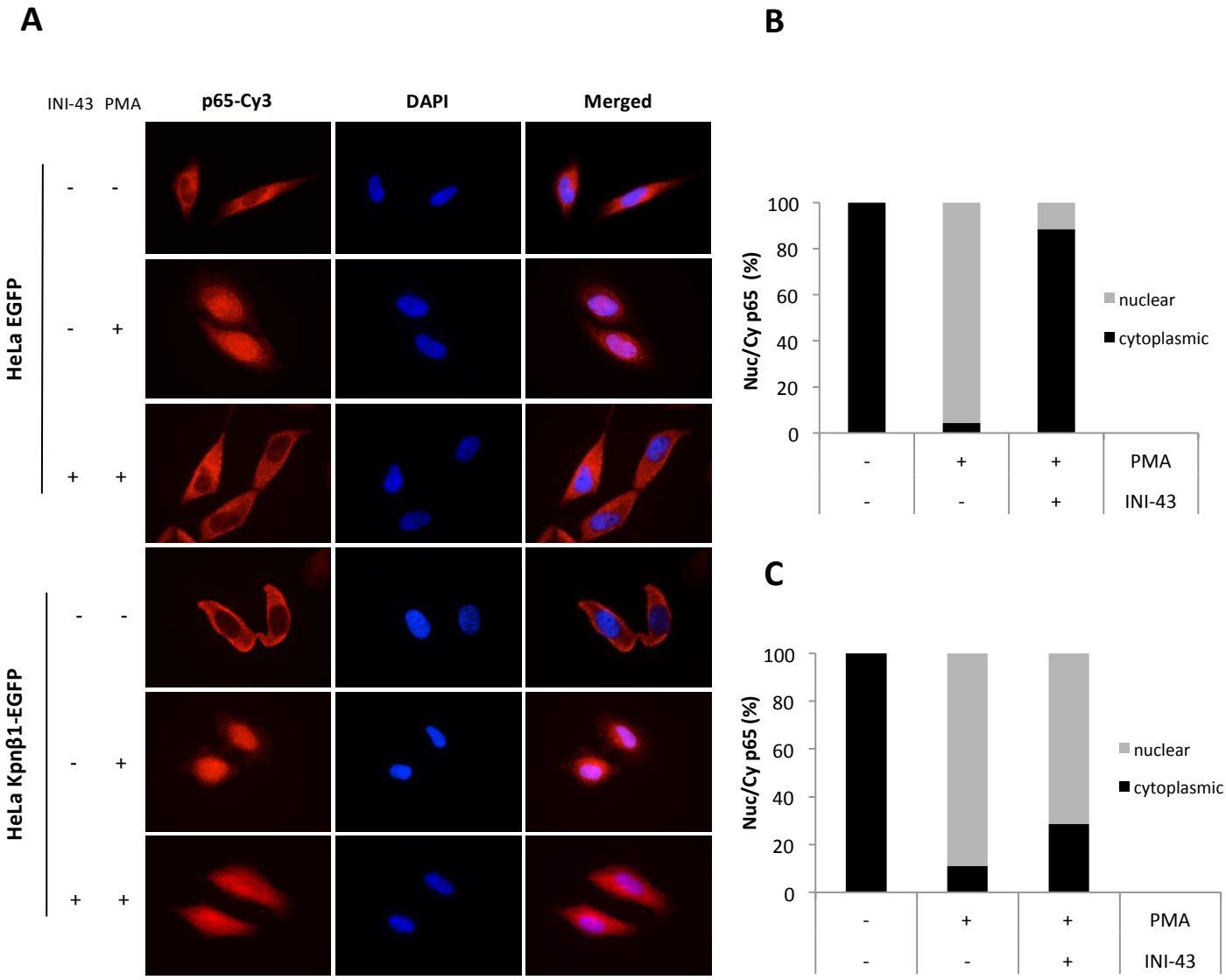


Figure 4.2. Kpnβ1 overexpression rescues the inhibitory effects of INI-43 on the nuclear import of NFκB p65. A: Immunofluorescence analysis of NFκB p65 localisation in control (EGFP) and Kpnβ1 overexpressing (Kpnβ1-EGFP) HeLa cells reveals predominantly cytoplasmic p65 localisation in untreated/unstimulated cells. Treatment of control and Kpnβ1-EGFP cells with 0.5 μM PMA results in a nuclear translocation of p65. Pre-treatment of control cells with 10μM INI-43 for 3 hours prevents p65 from entering the nucleus in response to PMA stimulation, whereas the nuclear accumulation of p65 is not prevented in Kpnβ1-EGFP cells ie. p65 is still able to enter the nucleus when Kpnβ1 is overexpressed. Representative images from experiments performed three independent times are shown. **B and C**: Quantification of nuclear and cytoplasmic p65 fluorescence in approximately 100 HeLa EGFP (B) and HeLa Kpnβ1-EGFP (C) cells. Cells were scored as having predominantly nuclear or predominantly cytoplasmic p65 fluorescence. Results shown represent the mean values of experiments performed three independent times.

4.2.2.2 Kpnβ1 overexpression rescues the inhibitory effects of INI-43 on NFAT activation

The ability of Kpnβ1 to rescue cells from the inhibitory effects of INI-43 was further investigated using a luminescence based screening assay in which a NFAT-luciferase reporter system was transfected into cells and used to measure nuclear NFAT activity. NFAT (Nuclear Factor of Activated T cells) is a transcription factor that shuttles between the nucleus and cytoplasm in a Kpnβ1-dependent manner[114]. NFAT is predominantly cytoplasmic, but can be stimulated by PMA and the calcium ionophore, Ionomycin, to be transported into the nucleus upon an increase in intracellular calcium levels. Nuclear NFAT activity can thus be used as a measure of functional Kpnβ1. To address the question of whether the inhibitory effects of INI-43 on NFAT activation[52] could be rescued by expression of Kpnβ1-EGFP, nuclear NFAT activity was assayed for following treatment with INI-43, and subsequent co-stimulation with PMA and Ionomycin. Results revealed that stimulation of NFAT transfected HeLa EGFP and Kpnβ1-EGFP cells with PMA and Ionomycin resulted in a significant increase in activation of the NFAT reporter, as a result of NFAT nuclear import (Figure 4.3). Pre-treatment with INI-43 significantly diminished NFAT activation in EGFP cells, but not in Kpnβ1-EGFP cells, suggesting that Kpnβ1 overexpression is partly able to protect cells from the inhibitory effects of INI-43 on Kpnβ1-mediated nuclear import of NFAT. Interestingly, basal NFAT activity was observed to be significantly higher in Kpnβ1-EGFP expressing cells, suggesting that overexpression of Kpnβ1 results in increased nuclear import of NFAT. Preliminary evidence suggests that cells expressing Kpnβ1-EGFP show increased nuclear import of additional cargoes, including AP-1; however further experimentation is required to confirm this.

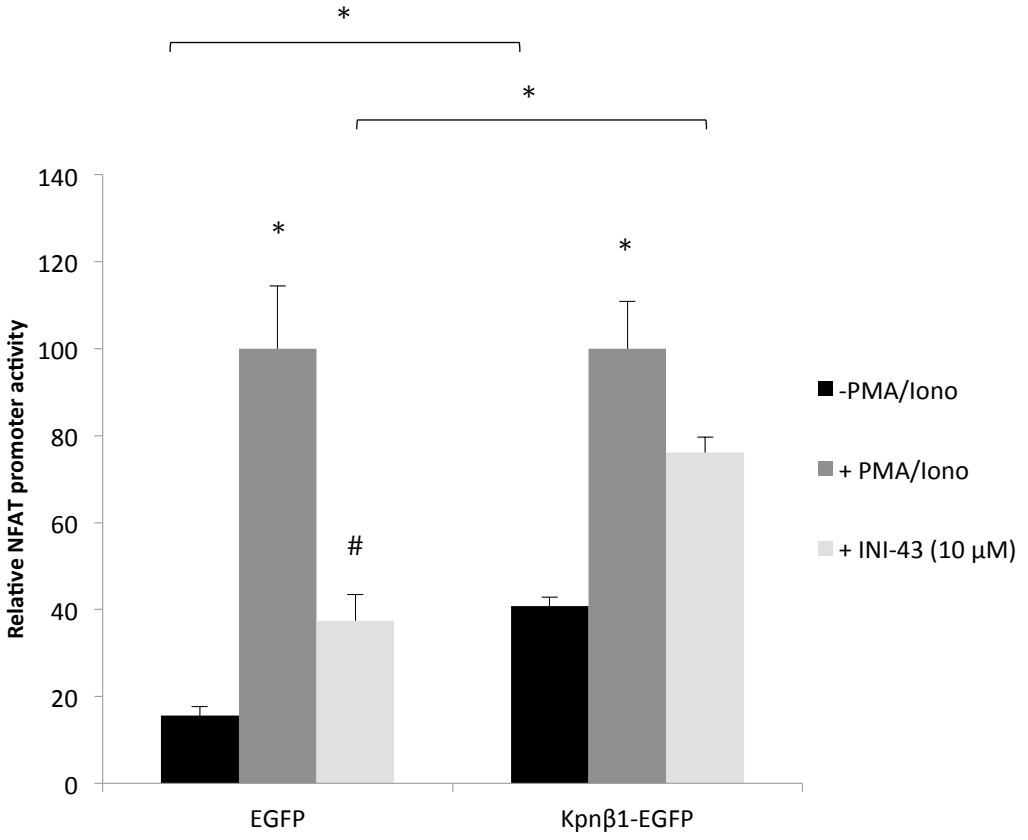


Figure 4.3. Kpnβ1 overexpression rescues the inhibitory effects of INI-43 on NFAT activation. Graph showing the effect of INI-43 on NFAT promoter activation in control (HeLa EGFP) and Kpnβ1 overexpressing (HeLa Kpnβ1-EGFP) cells. Cells were transfected with the NFAT-luciferase reporter and expression plasmids, pre-treated with 10 μM INI-43 for 2 hours and stimulated with PMA and Ionomycin for a further 3 hours. Results show a significant induction of NFAT promoter activity upon stimulation (*), followed by a significant reduction when EGFP cells (#), but not Kpnβ1-EGFP cells, were pre-treated with INI-43, indicating that there is still significant NFAT promoter activation in HeLa cells overexpressing Kpnβ1 despite the presence of INI-43. Relative NFAT promoter activity is represented as a percentage of PMA/Ionomycin stimulated cells (shown as 100%). Results shown represent the mean ± SEM of experiments performed in quadruplicate and repeated at least three times (*/ # $p < 0.05$).

4.2.3 Kpnβ1 overexpression rescues cells from an INI-43 induced cell cycle arrest

Previous research in our laboratory showed that INI-43 treatment at various time points induced a G2/M cell cycle arrest in HeLa cells[52]. In order to investigate whether Kpnβ1 overexpression rescued cells from an INI-43 induced cell cycle block in this study, FACS analysis was performed. Data reveals that the increase in G2/M observed in control cells after treatment with INI-43 (Table 4.2; Figure 4.4 A and B), was not observed in cells overexpressing Kpnβ1 (Table 4.2; Figure 4.4 C and D). These results suggest that overexpression of Kpnβ1 is able to rescue cells from the inhibitory effects that INI-43 has on cell cycle progression.

Table 4.2. Percentage of HeLa EGFP and Kpnβ1-EGFP cells in each phase of the cell cycle following INI-43 treatment (*p < 0.05)

Phase	***EGFP***		***Kpnβ1-EGFP***	
	0 µM	***10 µM***	***0 µM***	***10 µM***
% G1	**61.6**	**52.5** *	61.4	60.6
% S	17.2	16.4	18.0	16.5
% G2/M	**21.2**	**31.1** *	20.6	22.8

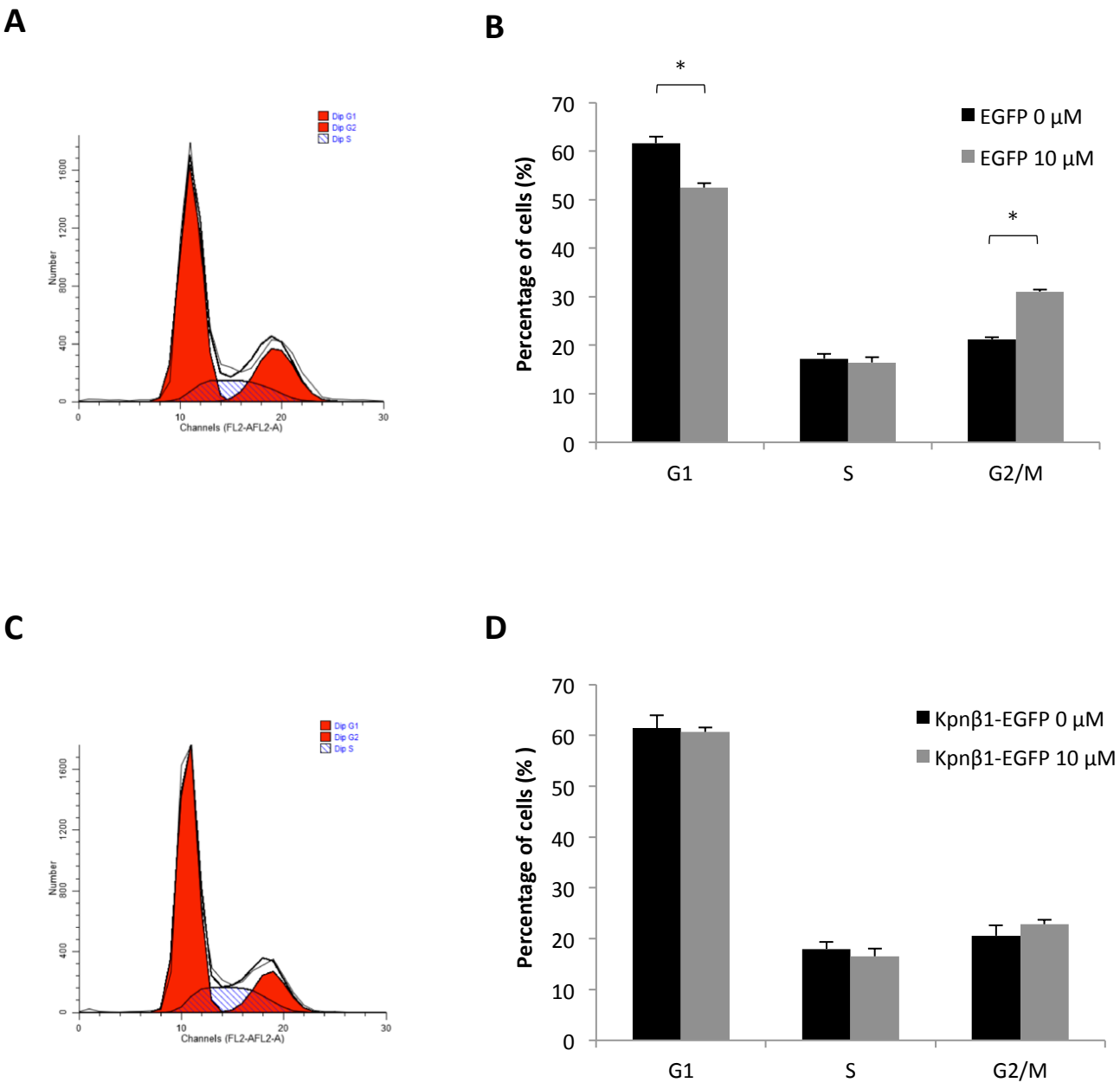


Figure 4.4. Overexpression of Kpnβ1 rescues cells from the INI-43 induced G2/M cell cycle arrest. A and C: Representative images showing the effect of INI-43 treatment on the cell cycle profiles of control EGFP (A) and Kpnβ1-EGFP (C) cells**. B:** Quantification of cell cycle data reveals a significant decrease in the percentage of control cells in the G1 phase of the cell cycle, accompanied by a significant increase in the percentage of control cells in the G2/M phase of the cell cycle 12 hours after treatment with 10 µM INI-43**. D:** Kpnβ1 overexpressing cells show no significant changes after a 12 hour treatment with INI-43. Results shown represent the mean ± SEM for experiments performed in triplicate and repeated at least two times (*$p < 0.05$).

4.2.4 INI-43 treatment induces degradation of Kpnβ1

Immunofluorescence analysis of HeLa cells showed that treatment with INI-43 resulted in a reduction in Kpnβ1 expression levels as well as a change in localisation of Kpnβ1, from predominantly nuclear in untreated cells, to cytoplasmic and nuclear in INI-43 treated cells (Figure 4.5 A). This result suggested that Kpnβ1 protein might be degraded in response to INI-43 treatment, which could be another indication that INI-43 is targeting Kpnβ1. Target protein degradation is emerging as a promising anti-cancer treatment strategy, and a number of potential drugs are in the early phases of development[115–117]. In order to investigate the possibility that INI-43 treatment results in Kpnβ1 protein degradation, cycloheximide half-life experiments were performed. HeLa cells were pre-treated with 5 μM INI-43 for three hours and then treated with 40 μg/ml cycloheximide (CHX), which inhibits *de novo* protein synthesis, therefore allowing for analysis of protein degradation over time. Protein lysates were harvested at various time points after CHX treatment and western blot analysis was performed to determine Kpnβ1 levels. In addition to analysing the stability of Kpnβ1, additional members of the Karyopherin protein family, CRM1 and Kpnα2, were also analysed. Western blot analysis revealed a reduction in Kpnβ1 protein stability after treatment with INI-43, indicated by a decrease in Kpnβ1 protein levels, whilst untreated cells exhibited a more stable Kpnβ1, which was not degraded during the time course (corresponding with the long Kpnβ1 half-life that has been reported in literature[118,119]) (Figure 4.5 B). CRM1 and Kpnα2, on the other hand, appeared to be largely unaffected by the treatment as untreated and INI-43 treated cells exhibited similar CRM1 and Kpnα2 degradation profiles. Densitometric scanning of Kpnβ1 levels relative to GAPDH

was performed, and band intensities were plotted in log scale relative to time. Protein half-life was equated to log2/slope. The half-life of Kpnβ1 in the absence of INI-43 treatment was approximately 33 hours, while in the presence of INI-43 treatment it was reduced approximately 4.7-fold to 7 hours (Figure 4.5 C). These results indicate that Kpnβ1 degradation is induced upon INI-43 treatment, suggesting that INI-43 is acting, at least in part, by targeting Kpnβ1.

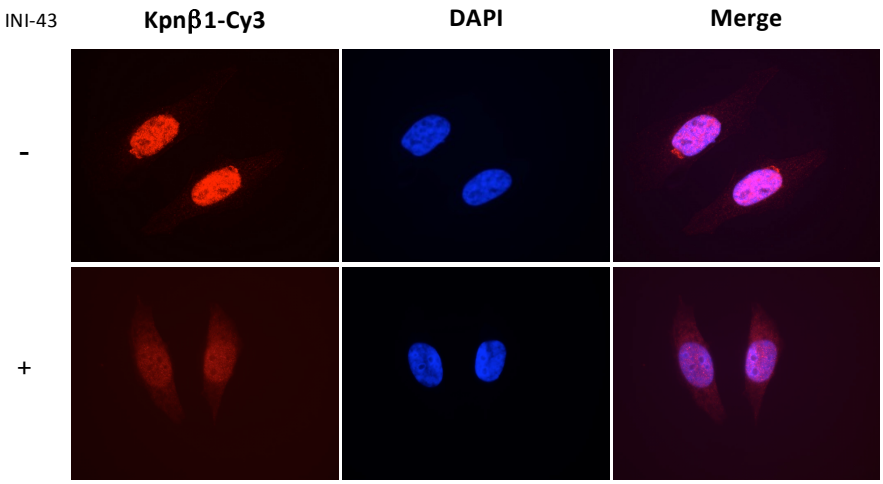


Figure 4.5 A. Treatment with INI-43 results in Kpnβ1 degradation. Immunofluorescence analysis showing Kpnβ1 protein expression in HeLa cells fixed 3 hours after treatment with 10 μM INI-43. Representative images from experiments performed three independent times are shown. Exposure time was kept constant for all images.

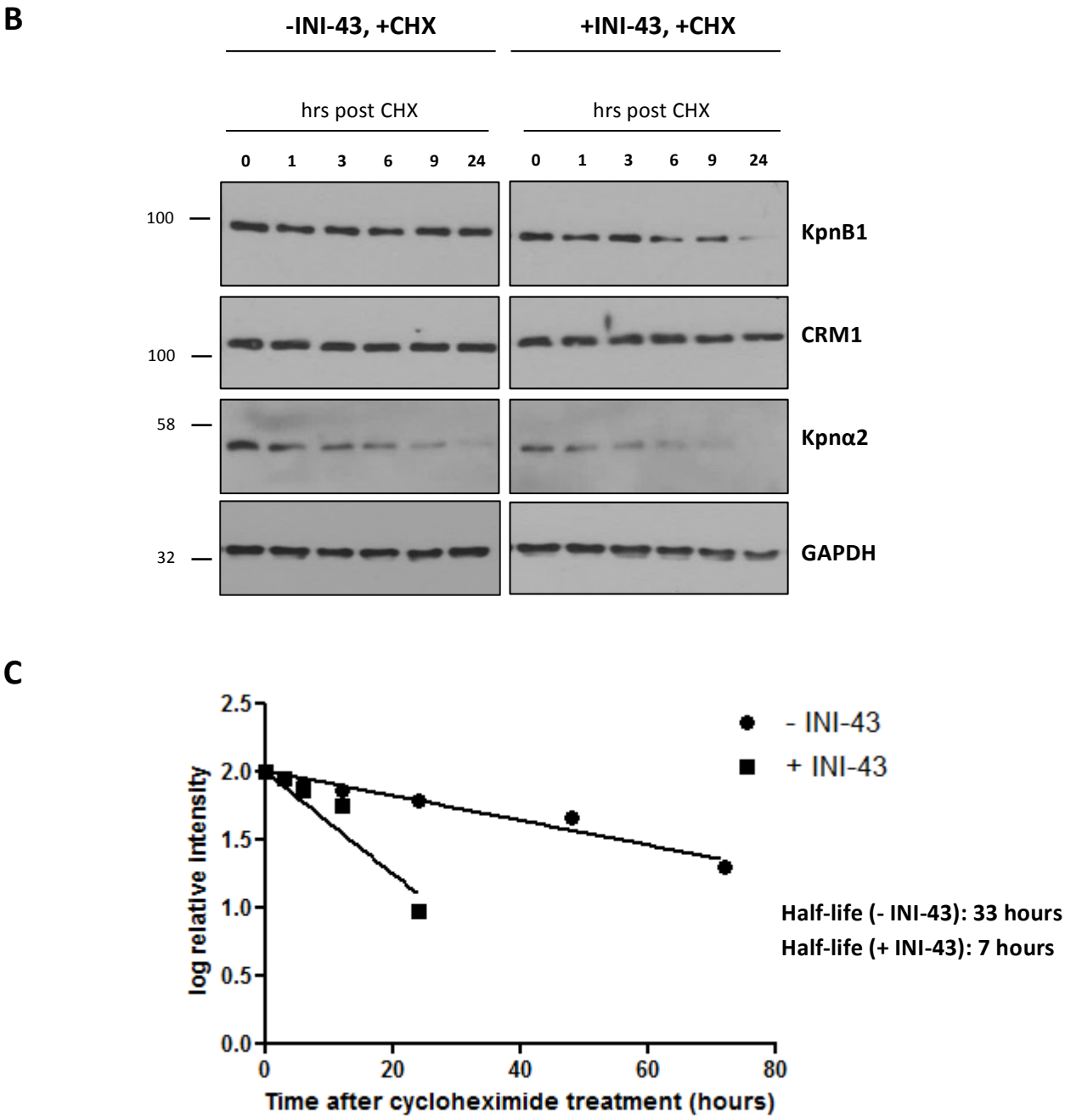


Figure 4.5 B and C. Treatment with INI-43 results in Kpnβ1 degradation. B: HeLa cells were pre-treated with 5 μM INI-43 for 3 hours and then treated with 40 μg/ml cycloheximide (CHX) and harvested for a period of up to 24 hours post CHX treatment. Western blot analysis shows a decrease in Kpnβ1 protein stability when cells are pre-treated with INI-43. Other members of the Karyopherin protein family, CRM1 and Kpnα2, were unaffected. GAPDH was used as a control for loading. **C:** Quantitative analysis of Kpnβ1 protein stability in the absence or presence of INI-43 treatment. Western blots were subjected to densitometric scanning and band intensities were plotted in log scale relative to Kpnβ1 levels at time zero. Kpnβ1 half-life was determined using log2/slope. Results shown are representative of experiments performed three independent times.

4.3 DISCUSSION

The past decade has seen a remarkable surge in the discovery and development of small molecule anti-cancer drugs. Conventional chemotherapy acts by targeting all rapidly dividing cells (including the skin, hair follicles and lining of the digestive system), thus patients experience severe and undesirable side effects. In recent years however, there has been a shift towards a more personalised approach when it comes to cancer treatment, with research focusing on the discovery and development of molecularly targeted therapeutics that act by exploiting the genetic abnormalities and dependencies of cancer cells. The aim for such treatments is to develop therapies that are more effective and have limited side effect profiles by increasing the specificity of these drugs to cancer cells.

The development of targeted anti-cancer therapeutics requires the identification of suitable target proteins, ideally ones that play a key role in cancer cell growth and survival. The main steps for successful discovery and development of novel anti-cancer drugs include: Identification and validation of a molecular target; high-throughput chemical screening, hit identification and lead compound generation; lead compound optimisation to develop a clinical drug candidate; and biomarker-driven clinical trials[120]. In recent years, targeting the nuclear transport machinery has received attention as a promising anti-cancer approach, with particular focus on the nuclear export protein CRM1 and the development of a class of novel Selective Inhibitors of Nuclear Export (SINE compounds)[121–125]. Our laboratory recently identified a novel inhibitor of nuclear import, INI-43, which was designed as a lead compound to bind

and inhibit Kpnβ1[52]. While recently published data supports the use of INI-43 as a cytotoxic agent that shows anti-cancer activity both *in vitro* and *in vivo*, limited research has been conducted toward the validation of INI-43 as a Kpnβ1 target.

Drug target validation is a crucial step along the drug discovery pipeline. Many biological tools exist to validate targets, including the use of RNAi, CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and gene overexpression, in combination with small molecule treatment. The idea behind these techniques is to elucidate whether perturbation of gene/protein function confers resistance to specific drugs. In this study, we used exogenously expressed Kpnβ1-EGFP to determine whether overexpression of Kpnβ1 confers resistance to INI-43. Results revealed that Kpnβ1 overexpression resulted in a significant increase in cell viability when cells were treated with varying doses of INI-43. In addition, the inhibitory effects of INI-43 on the nuclear import of Kpnβ1 cargo proteins NFκB p65 and NFAT, as well as the G2/M cell cycle arrest elicited by INI-43 treatment, were significantly reduced when Kpnβ1 was overexpressed. Despite the fact that a significant amount of rescue was seen in cells overexpressing Kpnβ1, a complete restoration of function was not observed. This was particularly apparent when comparing sensitivity to drug; control EGFP cells exhibited an INI-43 EC_{50} value of 8.3 μM, while Kpnβ1-EGFP expressing cells exhibited an EC_{50} value of 10.4 μM. While these values are significantly different, evident by their non-overlapping 95% confidence intervals, they both result in death of cancer cells within the micromolar range. It is therefore likely that while INI-43 is in part acting by targeting Kpnβ1, there may be uncharacterised off-target binding of the drug. Alternatively, the level of overexpression achieved using the HeLa pEFIRES-Kpnβ1-

EGFP cell line (approximately 2-fold) may be insufficient to completely rescue the effects of INI-43 on Kpnβ1.

While many targeted therapies have been successfully approved by the Food and Drug Administration (FDA) to treat different types of cancer, the clinical effectiveness of these targeted agents has been inconsistent (largely due to the development of drug resistant mutations in tumour cells resulting in disrupted drug:target binding), thus there is an urgent need to identify novel strategies to target drivers of oncogenesis. Recently, the selective targeting of oncogenic proteins for degradation has emerged as an attractive anti-cancer therapeutic strategy with the potential to overcome drug resistance[115,116]. While recent results from our laboratory suggest that treatment with INI-43 results in a change in Kpnβ1 subcellular localisation[52], we provide evidence in this chapter that treatment with INI-43 may be resulting in Kpnβ1 protein degradation as well. To explore this further, we performed cycloheximide half-life experiments and found that the stability of Kpnβ1 was reduced almost 5-fold in cells treated with INI-43. These results demonstrate that INI-43 appears to target Kpnβ1 protein for degradation, and highlights the need for further studies into elucidating the mechanism by which degradation occurs.

Taken together, these findings provide evidence that INI-43 targets Kpnβ1. However, off-target binding of the drug might be occurring as expression of exogenous Kpnβ1 results in just a 25% increase in INI-43 EC_{50} value, leading to only a partial rescue of cell viability. Alternatively, the partial rescue observed could be due to the extent of overexpression achieved (approximately 2-fold, or a 1:1 ratio of endogenous to

exogenous Kpnβ1, using the pEFIRES-Kpnβ1-EGFP cell line), and thus greater levels of overexpression might provide further resistance to INI-43. It is possible that stable overexpression of Kpnβ1 allows for an initial reduction in the toxicities and biological effects induced upon INI-43 treatment (at lower concentrations of INI-43 there is sufficient 'unbound' Kpnβ1 for cells to function normally), however at higher concentrations of drug (when all Kpnβ1 is bound by INI-43), a dysfunctional system emerges and cells die (Figure 4.6). This theory potentially explains why only a partial rescue in cell viability (and INI-43 EC_{50} value) is seen when HeLa pEFIRES-Kpnβ1-EGFP cells are treated with varying concentrations of drug - the stable expression of Kpnβ1-EGFP is quickly overwhelmed by an increasing amount of INI-43.

It will be important in the future to fully investigate and determine the exact mechanism of binding and action of INI-43 (including all off-target effects), so that lead compound optimisation and development into a clinical drug candidate can potentially occur.

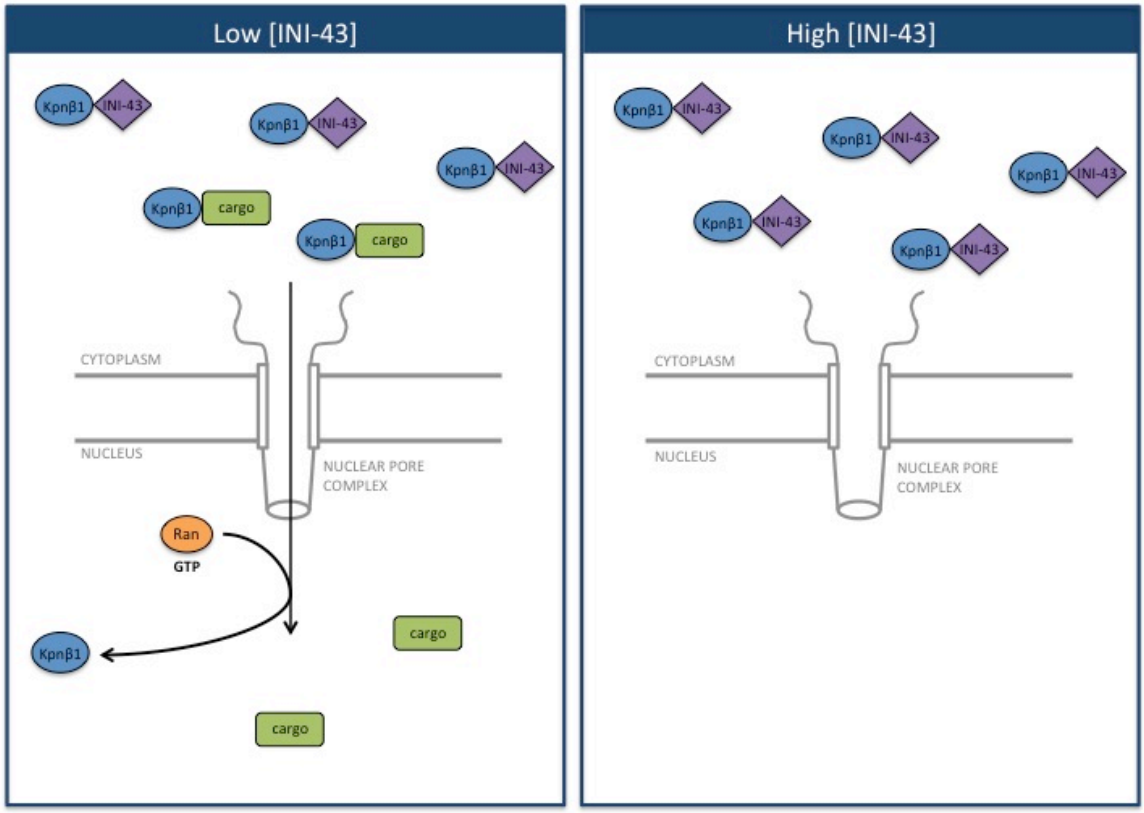


Figure 4.6. Proposed model for the partial rescue of Kpnβ1 function at low concentrations of INI-43. This figure depicts the role that Kpnβ1 plays as a nuclear import receptor in interphase cells. INI-43 binds Kpnβ1, thereby preventing the binding of Kpnα/cargo and subsequent nuclear import. The same theory holds true for the role of Kpnβ1 in mitosis: high concentrations of INI-43 hold Kpnβ1 inactive and unable to be released by Ran-GTP to perform various mitotic functions.

CHAPTER 5

USING LIVE CELL IMAGING TO STUDY THE EFFECTS OF KPNβ1 DYSREGULATION IN HELA CELLS

5.1 INTRODUCTION

In addition to its role as the main nuclear import protein in interphase cells, Kpnβ1 has been shown to play an important role in the regulation of various mitotic processes, such as mitotic spindle assembly and regulation, centrosome dynamics, nuclear membrane formation and nuclear pore assembly, where in most cases it is counter-regulated by the small regulatory GTPase, Ran[22].

Eukaryotic cells undergo division by cycling between interphase and mitosis. Kpnβ1 acts positively in interphase, as a nuclear import protein and potential motor adaptor for movement along the microtubules[22,68]. In contrast, Kpnβ1 plays a negative regulatory role in mitosis, preventing spatially inappropriate spindle formation, by binding and masking the activity of spindle assembly factors (SAFs) in cytoplasmic areas distant from the chromatin until high levels of RanGTP near the mitotic chromosomes result in release of the SAFs, thereby setting spindle assembly, and thus mitosis, in motion[68,126]. Kpnβ1 has also been shown to be involved in spatially controlling the major assembly events of late mitosis, such as nuclear membrane and nuclear pore assembly, with the Kpnβ1/RanGTP balance having been shown to be

crucial in the regulation of the vesicle-vesicle fusion reaction that is required for double nuclear membrane formation[95]. Excess Kpnβ1 in the system has an overwhelming effect on the amount of RanGTP present, resulting in the sequestration of SAFs (even those close to the mitotic chromosomes), thus preventing the formation of the mitotic spindle and inhibiting correct nuclear membrane and nuclear pore formation[22,95]. In contrast, excess RanGTP causes untimely release of Kpnβ1 which results in uncontrolled microtubule nucleation and formation of hyperabundant, invaginated nuclear membranes around the chromatin[68]. Thus, a precise balance of Kpnβ1/RanGTP activity is essential for correct mitotic functioning.

Previous studies have shown that disruption of Kpnβ1 expression (by loss of endogenous Kpnβ1 through siRNA-mediated knockdown, or transient overexpression of Kpnβ1 using plasmid-based transfection), resulted in the appearance of distinct mitotic defects, such as multipolar spindles, chromosome misalignment, and lagging or mis-segregation of chromosomes in anaphase/telophase[87,90,96]. In this study, we independently validated and expanded on this work by investigating whether dysregulation of Kpnβ1 (mediated by inducible expression of exogenous Kpnβ1-EGFP, or by drug-mediated inhibition of Kpnβ1 using the novel small molecule inhibitor, INI-43) resulted in the appearance of mitotic defects, by performing time-lapse analysis of HeLa TET-ON Kpnβ1-EGFP cells as they progress through the cell cycle.

This study was carried out as part of an international exchange in Dr Patrizia Lavia's laboratory at the Sapienza University of Rome. The work was completed over three months, between December 2015 and March 2016. Much of the work done, and

systems used (ie. the HeLa TET-ON Kpnβ1-EGFP cells), are part of a larger research effort in collaboration with Dr Lavia, most of which is still being investigated, and is thus largely unpublished at present.

5.2 RESULTS

5.2.1 Continuous induction of Kpnβ1 is toxic to HeLa cells

In Chapter 3, the effects associated with stable expression of Kpnβ1-EGFP were investigated. Kpnβ1-EGFP was overexpressed at an approximate 1:1 ratio, and this resulted in the various biological changes described. In this chapter, to investigate the effects of continuous induction of Kpnβ1 overexpression in HeLa cells, a stable HeLa cell line in which Kpnβ1-EGFP can be inducibly expressed was used, known as HeLa TET-ON Kpnβ1-EGFP (generated by Annalisa Verrico and Patrizia Lavia, Institute of Molecular Biology and Pathology, National Research Council of Italy, Rome, Italy). To confirm induction of Kpnβ1-EGFP, cells were incubated with 1 μg/ml doxycycline to stimulate the tetracycline responsive promoter, and live cell time-lapse fluorescence videomicroscopy was performed to analyse GFP intensity over time. Results revealed that in the presence of doxycycline, Kpnβ1-EGFP accumulated to approximately 1.5 times that of endogenous Kpnβ1 by 24 hours post induction (Figure 5.1). This was greater overexpression than that observed with stable expression of the pEFIRES plasmid, which expressed Kpnβ1-EGFP at levels roughly equivalent to endogenous Kpnβ1 (Chapter 3). Using time-lapse videomicroscopy to analyse cell death, our results showed that for the period up to and including 24 hours post induction (an approximate 1:1.5 ratio of endogenous to exogenous Kpnβ1 at 24 hours), there was a significant increase in recorded cell deaths compared to those recorded for the period up to and including 8 hours post induction (an approximate 1:1 ratio of endogenous to exogenous Kpnβ1 at 8 hours) (Figure 5.2). Cell deaths were recorded as either death

during interphase or first mitosis (Death in I or M), or death of daughter cells after first mitosis (Death after M). Cells overexpressing Kpnβ1 (up to 1.5 times that of endogenous Kpnβ1) underwent cell death during interphase or mitosis, as well as after first mitosis. These results suggest that HeLa cells cannot cope with expression of exogenous Kpnβ1 beyond a certain level (ie. once levels have exceeded a 1:1 ratio of endogenous to exogenous protein), and thus undergo cell death.

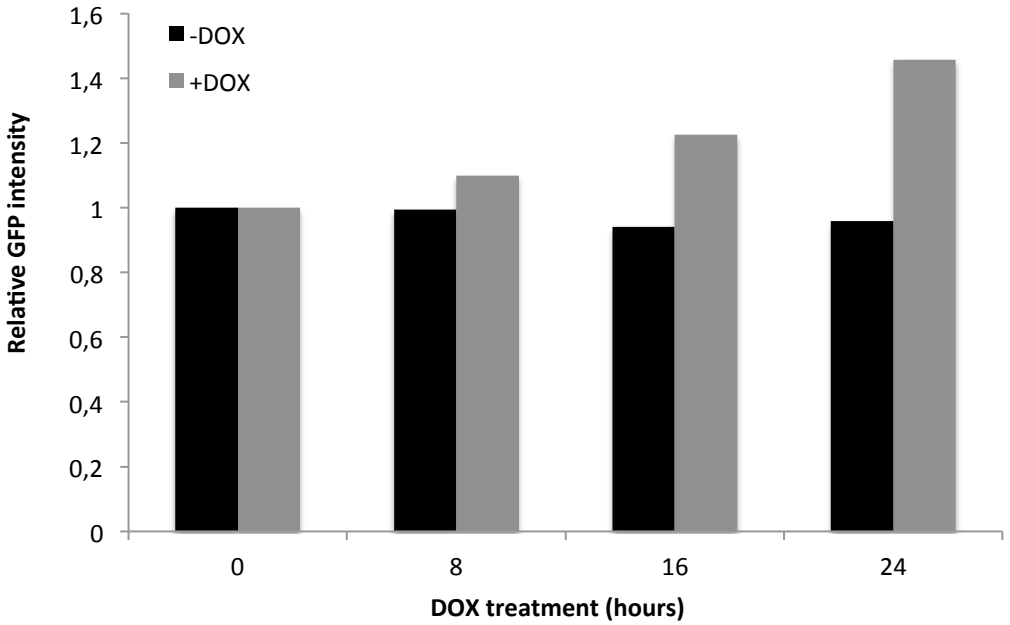


Figure 5.1. Kpnβ1-EGFP induction after doxycycline treatment is continous. Quantification of GFP intensity (measured in the FITC fluorescent channel) for up to 24 hours post induction of HeLa TET-ON Kpnβ1-EGFP cells with 1 μg/ml doxycycline (± DOX). Fluorescence intensity was recorded by analysing cells over a period of 24 hours using time-lapse videomicroscopy. FITC images were acquired every 60 minutes using a DS-Qi1Mc camera and the NIS-Elements AR 3.22 software for acquisition. Graphs were constructed by analysing data processed using the NIS-Elements AR 4.2 software.

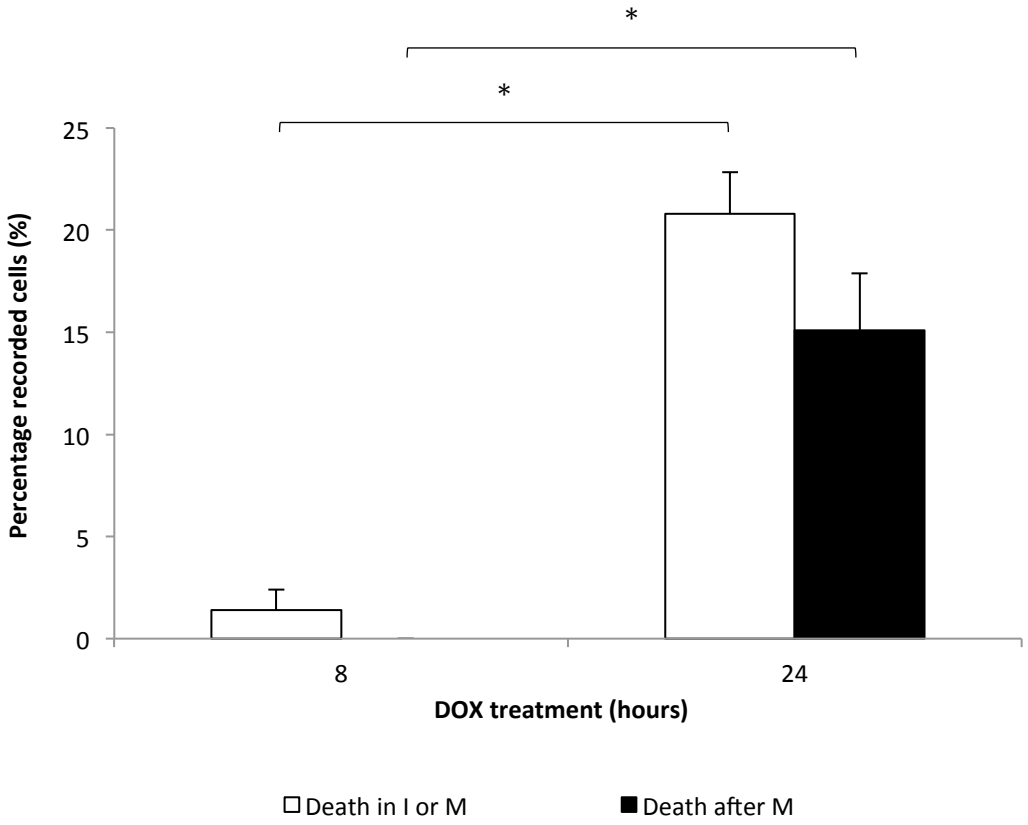


Figure 5.2. Continuous induction of Kpnβ1 results in significant cell death. Quantification of cell death, classified as either death in interphase or first mitosis (white bars), or death of daughter cells after first mitosis (black bars), and shown as a percentage of total recorded phenotypes observed up to and including 8 hours and 24 hours post induction with 1 μg/ml doxycycline. I - interphase, M - mitosis. Results shown represent the mean ± SEM for experiments repeated three independent times (*$p < 0.05$).

5.2.2 Dysregulation of Kpnβ1 results in the appearance of distinct mitotic abnormalities

Previous studies have shown that dysregulation of Kpnβ1 expression, by using transient overexpression of Kpnβ1-EGFP as well as siRNA-mediated knockdown of Kpnβ1, resulted in the appearance of distinct mitotic defects, suggesting the importance of maintaining a tight regulation of Kpnβ1 for correct mitotic functioning to occur[90,96]. To further our understanding of the links between Kpnβ1 dysregulation and genomic instability, we investigated the effects of inducible overexpression of Kpnβ1, as well as drug-mediated inhibition of Kpnβ1 (using the small molecule inhibitor, INI-43) in mitotic cells, by performing live cell time-lapse videomicroscopy as well as immunofluorescence analysis of fixed cells.

For the time-lapse analysis of HeLa TET-ON Kpnβ1-EGFP cells, 25 000 cells were seeded in 8-well micro slides and allowed to adhere overnight. The following day, cells were pre-treated with 1 μg/ml doxycycline for a period of 4 hours, in order to induce expression of Kpnβ1-EGFP. Following doxycycline treatment, cells were treated with INI-43 for a period of 3 hours (in the continued presence of doxycycline), as this had been previously established as being sufficient to cause inhibition of Kpnβ1 without resulting in significant cell death within the three hour period[52]. After treatment, cells were washed and media was replaced with TET-free DMEM (minus phenol red - to allow for better resolution imaging) supplemented with 0.5 μg/ml doxycycline to maintain stimulation. At this point cells were placed in a microscope stage incubator, which allowed for them to be maintained at 37°C and 5% CO_2 for the duration of the

time-lapse recording. Cells were recorded for a period of 24 hours; Differential Interference Contrast (DIC) images were acquired every 15 minutes, and FITC images (for analysis of GFP signal) every 60 minutes, using a DS-Qi1Mc camera and the NIS-Elements AR 3.22 software (see Figure 5.3 for the experimental workflow for time-lapse videomicroscopy).

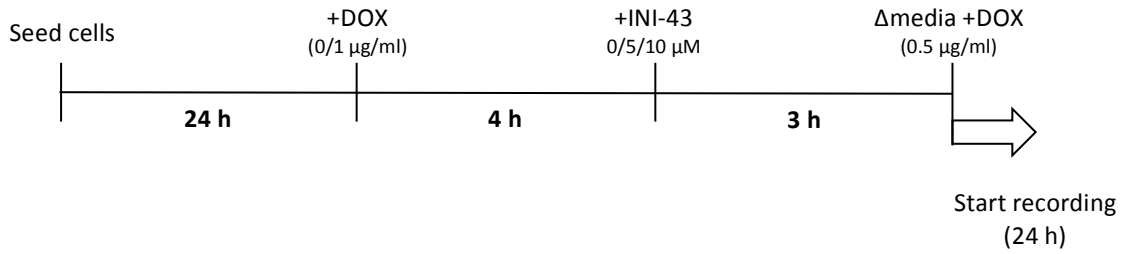


Figure 5.3. Experimental workflow for time-lapse vidoemicroscopy. Time-lapse analysis of HeLa TET-ON Kpnβ1-EGFP cells. 25 000 cells were seeded in 8-well Ibidi micro slides and allowed to adhere overnight. After 24 hours, cells were pre-treated with 1 μg/ml doxycycline (DOX) for a period of 4 hours, in order to induce expression of Kpnβ1-EGFP. Following doxycycline treatment, cells were treated with varying concentrations of INI-43 (0, 5 or 10 μM) for a period of 3 hours (in the continued presence of doxycycline), after which cells were washed and media was replaced with TET-free DMEM (no phenol red) supplemeted with 0.5 μg/ml doxycycline. Cells were then placed in a microscape stage incubator and maintained at 37°C and 5% CO_2 for the duration of the time-lapse recording (24 hours).

Results obtained by time-lapse analysis show that dysregulation of Kpnβ1 expression (by either doxycycline-induced expression of Kpnβ1-EGFP, or INI-43 mediated inhibition of Kpnβ1) resulted in the appearance of spindle and chromosome-associated mitotic abnormalities. Multipolar spindles, spindle axis rotation, and delay in prometaphase/metaphase progression were among the most common phenotypes observed in cells in which Kpnβ1 was dysregulated (Figure 5.4).

Inducible overexpression of Kpnβ1 resulted in a significant increase in the appearance of mitotic abnormalities, which were grouped into two broad categories: structural abnormalities (including multipolar division and spindle axis rotation) and abnormal mitotic progression and mitotic delay (Figure 5.5). Similarly to what was seen in cells induced to overexpress Kpnβ1, treatment of HeLa cells with 5 μM and 10 μM INI-43 resulted in a dose-dependent increase in the appearance of spindle and chromosome-associated mitotic abnormalities (Figure 5.6).

In addition to studying the negative effects of Kpnβ1 dysregulation on cell cycle progression using time-lapse videomicroscopy, cells fixed after induction of Kpnβ1-EGFP or treatment with 10 μM INI-43, were analysed using immunofluorescence (see Figure 5.7 A for experimental workflow). Various mitotic abnormalities were captured, including multipolar spindles in metaphase and anaphase/telophase, as well as some that were not identified by time-lapse analysis, such as chromosome misalignment and lagging chromosomes (Figure 5.7 B). These defects were quantified, and an approximate doubling in mitotic abnormalities was observed in cells in which Kpnβ1 was overexpressed (+ DOX) or inhibited (+ INI-43) (Figure 5.7 C).

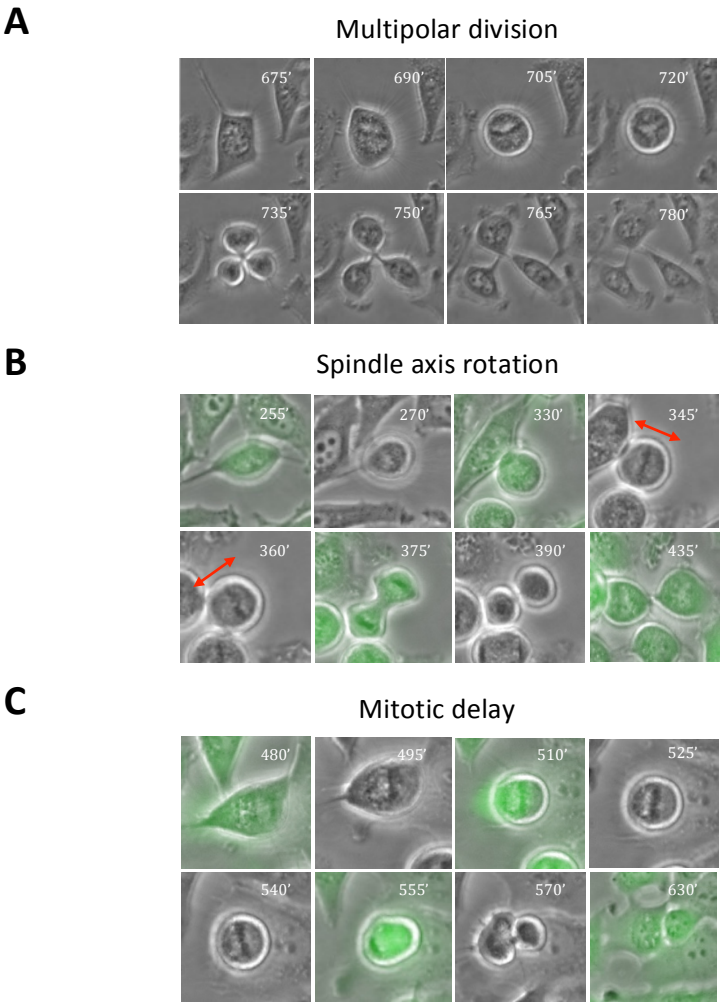


Figure 5.4. Kpnβ1 dysregulation results in the appearance of mitotic abnormalities. Examples of phenotypes recorded during time-lapse analysis of HeLa TET-ON Kpnβ1-EGFP cells induced to overexpress Kpnβ1 or treated with INI-43. Representative single frame images are shown; time from beginning of time-lapse is indicated in minutes. Various abnormal phenotypes were recorded and captured. The most common phenotypes are displayed, including: multipolar divison (A), spindle axis rotation (B) and mitotic delay (C).

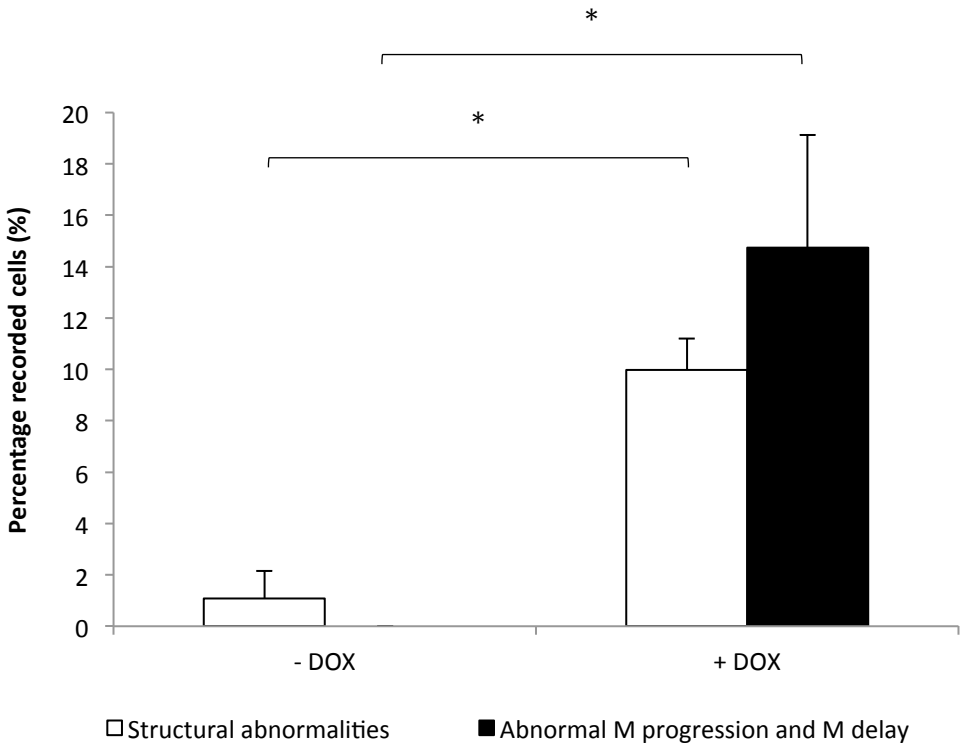


Figure 5.5. Kpnβ1 overexpression results in the appearance of mitotic abnormalities. Time-lapse videomicroscopy was performed using HeLa TET-ON Kpnβ1-EGFP induced to overexpress Kpnβ1. The frequency of mitotic abnormalities observed in each condition were counted, and recorded as a percentage of total recorded phenotypes over a period of 24 hours. Uninduced cells (- DOX) were compared to cells induced with 1 μg/ml doxycycline to express Kpnβ1-EGFP (+ DOX). M – mitotic. Results shown represent the mean ± SEM for experiments repeated three independent times (*$p < 0.05$)

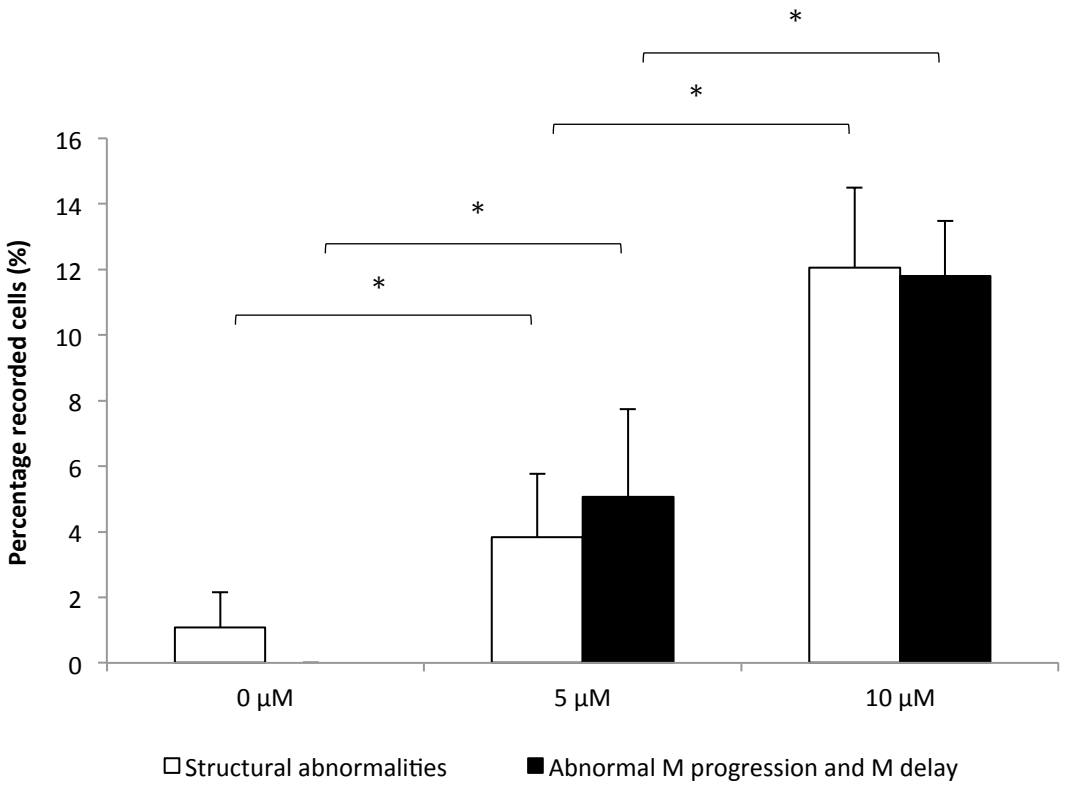


Figure 5.6. INI-43 treatment results in a dose-dependent increase in the appearance of mitotic abnormalities. Time-lapse videomicroscopy was performed using uninduced HeLa TET-ON Kpnβ1-EGFP treated with INI-43. The frequency of mitotic abnormalities was counted, and recorded as a percentage of total recorded phenotypes over a period of 24 hours. Untreated cells (0 μM) were compared to cells treated with various concentrations of INI-43 (5 μM and 10 μM). M – mitotic. Results shown represent the mean ± SEM for experiments repeated three independent times (* $p < 0.05$).

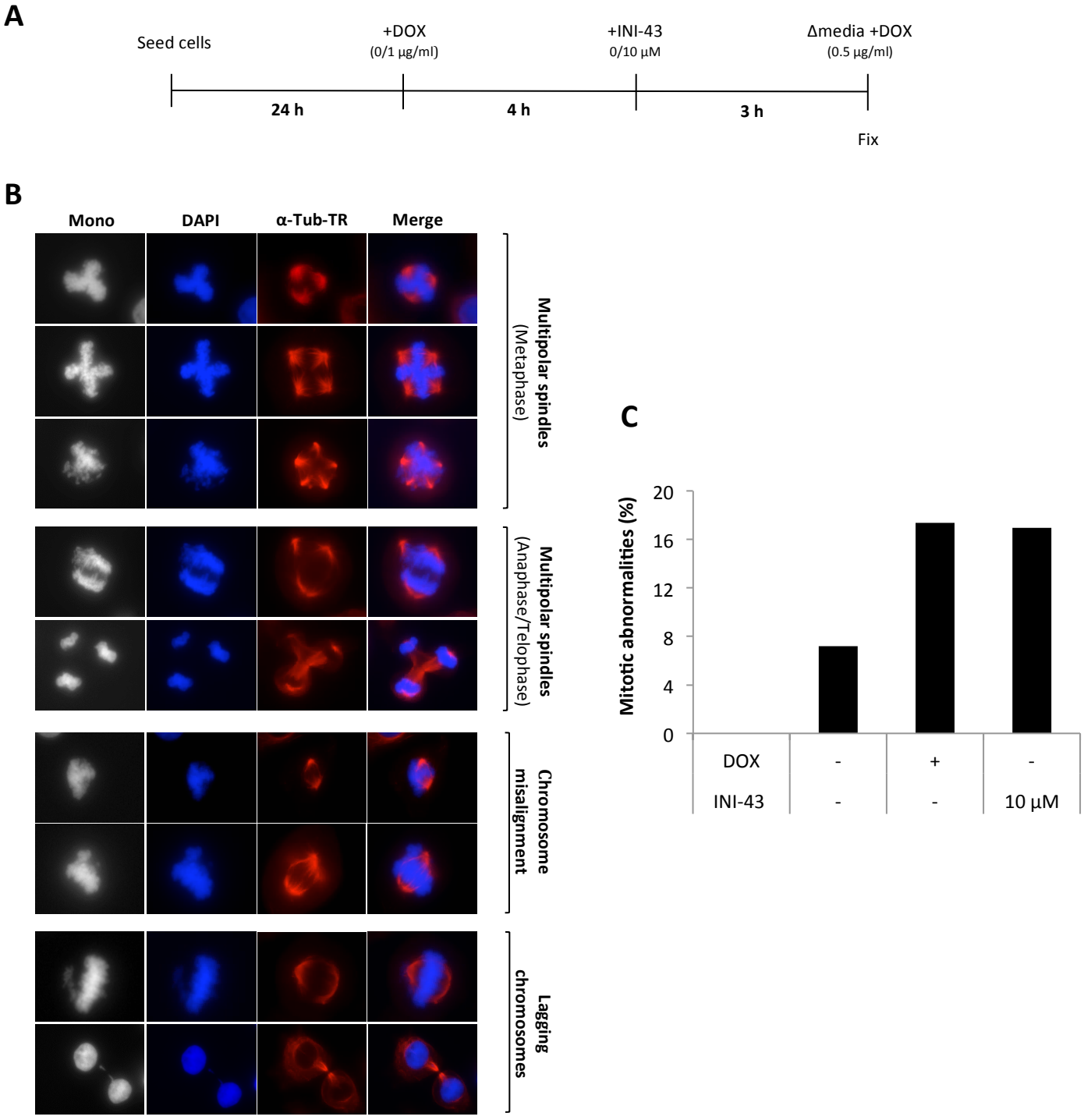
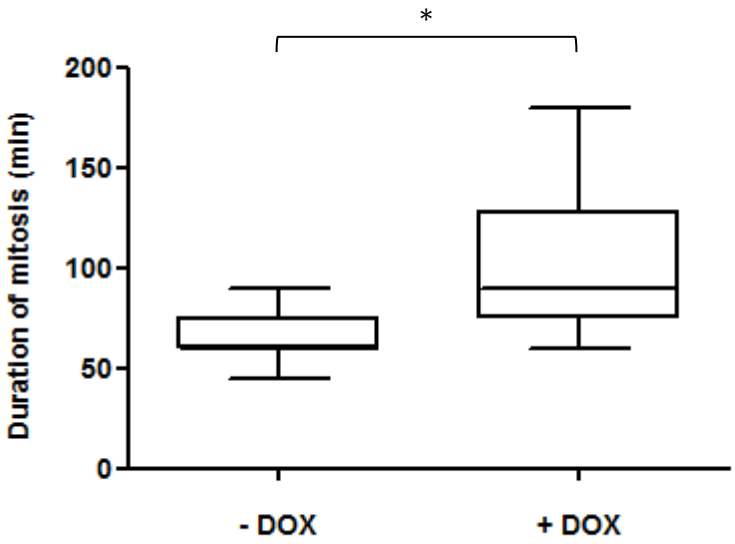


Figure 5.7. Dysregulation of Kpnβ1 results in the appearance of distinct mitotic abnormalities in fixed cells. A: Experimental workflow for immunofluorescence analysis of fixed cells. After induction with doxycycline or treatment with INI-43, cells were fixed using 4% paraformaldehyde. **B:** Representative images are shown for the mitotic abnormalities induced by dysregulation of Kpnβ1 (doxycycline-induced overexpression or INI-43 mediated inhibition) in HeLa TET-ON Kpnβ1-EGFP cells. Fixed cells were stained with DAPI (blue, panel 2) and α-Tubulin (red, panel 3). Merged images are shown in panel 4. **C:** Quantification of mitotic abnormalities recorded in fixed HeLa TET-ON Kpnβ1-EGFP cells. Mitotic abnormalities are displayed as a percentage of total number of mitotic cells (approximately 150 mitoses counted per condition).

Given the extent of mitotic disruption visualised in these cells, the duration of mitosis across the various treatment conditions was analysed. Using the data obtained by time-lapse analysis, the median time of cell division was calculated, and it was revealed that dysregulation of Kpnβ1 expression resulted in a significant increase in mitotic duration for both Kpnβ1 overexpressing and INI-43 treated cells (Figure 5.8 A and B).

Taken together, these results support data previously described in published literature, showing that the dysregulation of Kpnβ1 is associated with the disruption of mitotic events[90,96]. As the mitotic defects induced upon INI-43 treatment were similar to those observed upon overexpression of Kpnβ1 (as well as siRNA-mediated knockdown of Kpnβ1[90]), this provides further evidence as to the mode of INI-43-induced cell death; that is, via the targeting of Kpnβ1.

A



B

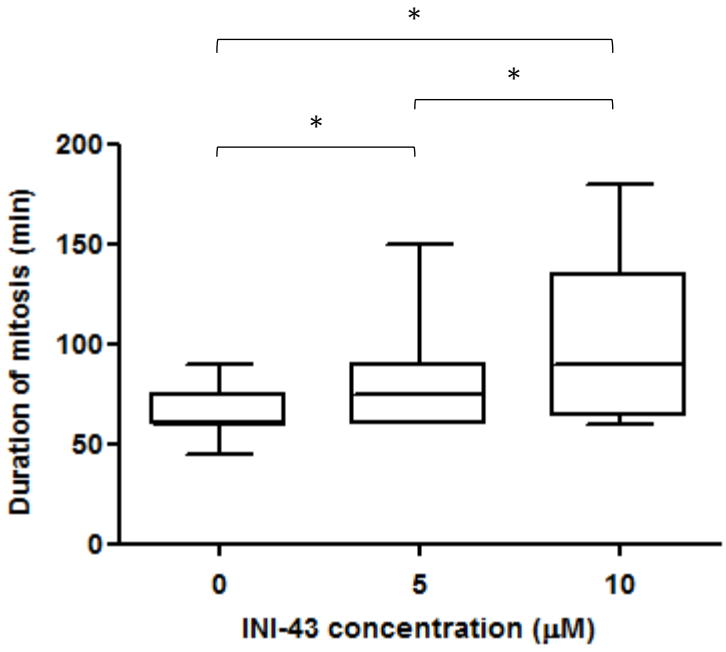


Figure 5.8. Kpnβ1 dysregulation leads to prolonged mitotic progression. Box and whisker plots display median duration of mitosis of HeLa TET-ON Kpnβ1-EGFP cells induced to overexpress Kpnβ1 using 1 μg/ml doxycycline (A) or treated with 5 μM and 10 μM INI-43 (B). At least fifty divisions from three independent experiments were scored for each condition (* $p < 0.05$).

5.2.3 Treatment of Kpnβ1 overexpressing cells with INI-43 results in a partial rescue of mitotic abnormalities

In order to investigate whether similar rescue effects to those seen in Chapter 4 (when HeLa cells stably overexpressing Kpnβ1 were treated with varying concentrations of INI-43) could be observed, HeLa TET-ON Kpnβ1-EGFP cells were induced with doxycycline for 4 hours, followed by treatment with INI-43 (in the continued presence of doxycycline) for a period of 3 hours. Analysis of time-lapse recordings revealed that induction of Kpnβ1-EGFP expression with doxycycline resulted in an increase in the appearance of mitotic abnormalities as previously described. Treatment of Kpnβ1-EGFP expressing cells with 5μM INI-43 resulted in a significant rescue in the percentage of mitotic abnormalities observed (Figure 5.9), thus a larger proportion of cells underwent normal division. Similarly, a significant reduction in mitotic duration was observed when cells overexpressing Kpnβ1 were treated with INI-43 (Figure 5.10). This data confirms that INI-43 is acting, at least in part, by targeting Kpnβ1 and suggests that a tight regulation of Kpnβ1 is required for normal functioning of cells.

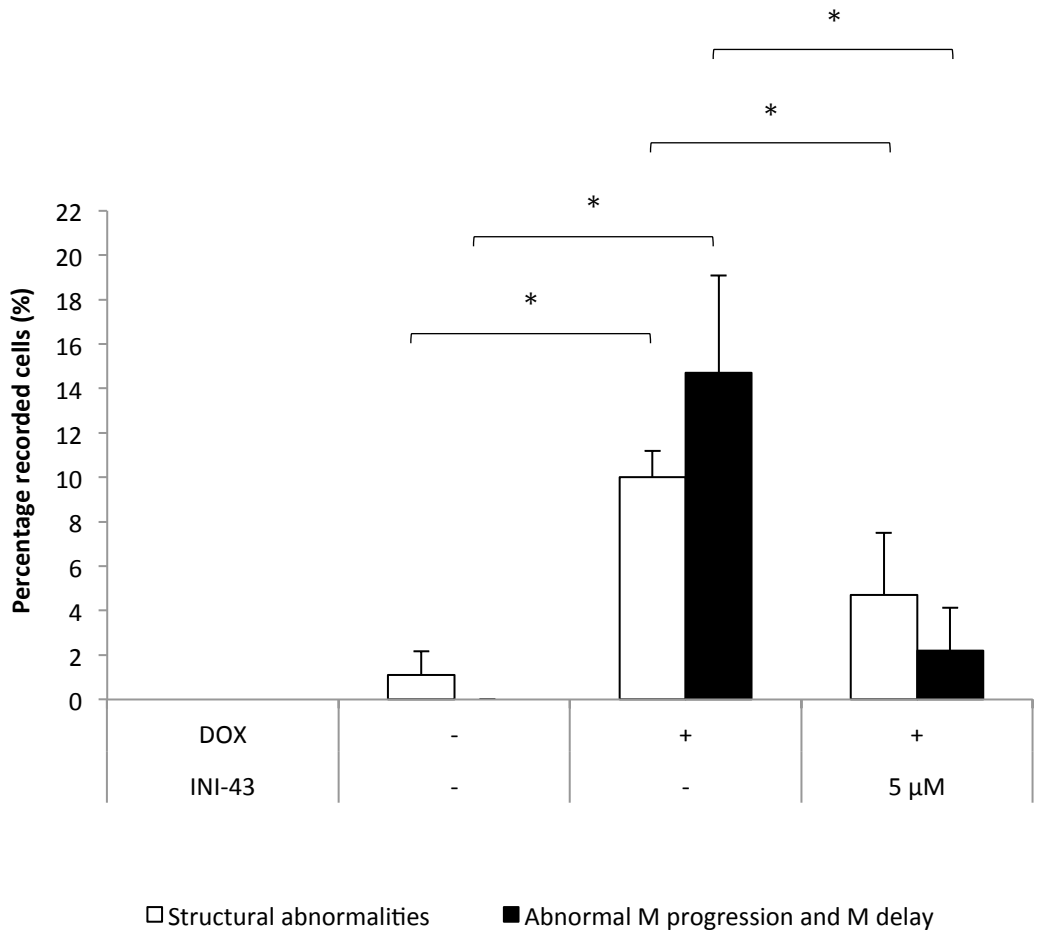


Figure 5.9. Treatment of Kpnβ1 overexpressing cells with INI-43 results in a reduction in the appearance of mitotic abnormalities. The frequency of mitotic abnormalities was counted, and recorded as a percentage of total recorded cells. Untreated HeLa TET-ON Kpnβ1-EGFP cells induced to overexpress Kpnβ1 (+ DOX) were compared to cells induced to overexpress Kpnβ1 and treated with 5 μM INI-43. Results shown represent the mean ± SEM for experiments repeated three independent times (* $p < 0.05$).

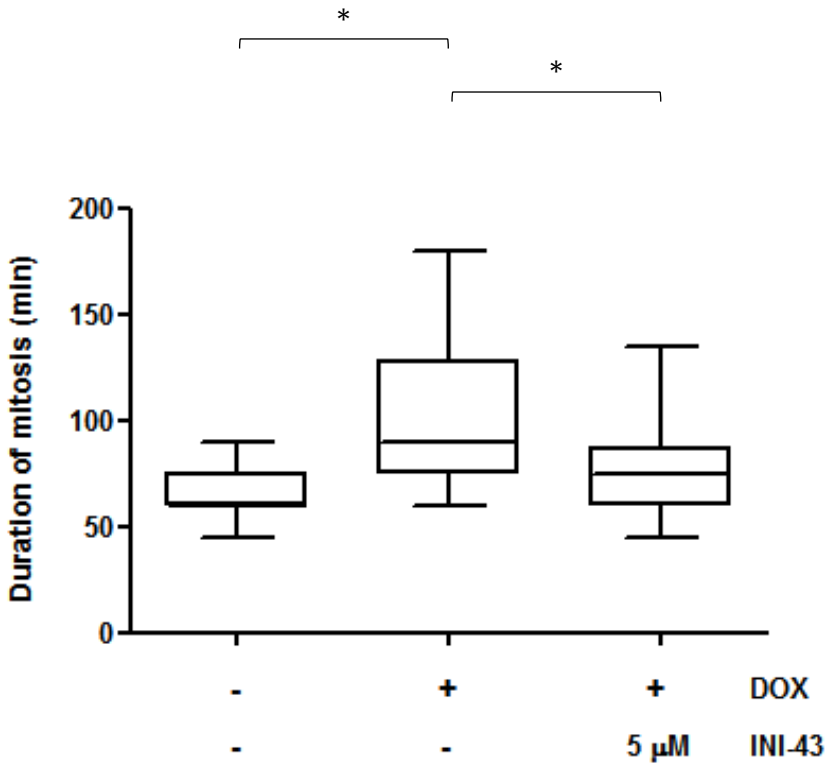


Figure 5.10. Treatment of Kpnβ1-overexpressing cells with INI-43 rescues cells from prolonged mitotic progression. The box and whisker plot displays median duration of mitosis of HeLa TET-ON Kpnβ1-EGFP cells induced to overexpress Kpnβ1 using 1 μg/ml doxycycline (+ DOX) and treated with 5 μM INI-43. At least fifty divisions from three independent experiments were scored for each condition (* $p < 0.05$).

5.3 DISCUSSION

For many years, Kpnβ1 was believed to act exclusively as the primary nuclear import protein. It was not until more recently that Kpnβ1 has emerged as playing roles in multiple other cellular processes. In addition to its function in nuclear transport, Kpnβ1 has a wide range of important roles in mitosis, spanning from the control of spindle assembly and centrosome dynamics, to the regulation of nuclear envelope and pore assembly. Research by various groups has revealed that Kpnβ1 dysregulation (by ectopic overexpression and RNAi-mediated protein knockdown) resulted in the appearance of distinct mitotic defects, suggesting that a tight balance of Kpnβ1 expression and activity is crucial for the correct functioning of cells[87,90,96].

Ciciarello *et al.* (2004) previously demonstrated that transient overexpression of Kpnβ1 resulted in defects in spindle pole organisation[87], and Roscioli *et al.* (2012) later showed that Kpnβ1 overexpression induced various mitotic abnormalities such as multipolar division, chromosome misalignment and significant mitotic delay in HeLa cells[96]. More recently in our laboratory, Angus *et al.* (2015) revealed that inhibition of Kpnβ1 protein expression by siRNA in cervical cancer cells resulted in a prolonged mitotic arrest, which was accompanied by the a similar set of mitotic defects to those observed with Kpnβ1 overexpression[90]. In this chapter, we set forth to independently validate these findings using two separate modes of Kpnβ1 dysregulation: inducible expression of exogenous Kpnβ1-EGFP, and INI-43 mediated inhibition of Kpnβ1. In addition, the specificity of INI-43 for Kpnβ1 was reassessed using the HeLa TET-ON Kpnβ1-EGFP cell line.

Previous studies indicate that depending on the levels of Kpnβ1 overexpression, various biological effects may be observed. For example, while high doses of exogenous Kpnβ1 in HeLa cells yielded a low mitotic index and arrest in G2/M phase of the cell cycle, moderate levels of exogenous Kpnβ1 (cells shown to express an approximate 1:1 ratio of exogenous to endogenous protein), despite resulting in no significant arrest, revealed distinct mitotic abnormalities[87,96]. The primary interest of these studies was in elucidating the role of Kpnβ1 in mitosis; hence the effects associated with varying exogenous Kpnβ1 dosage on other biological processes, such as cell proliferation, were not determined at the time. We showed in Chapter 3 that stably expressing Kpnβ1-EGFP at levels approximately equivalent to that of endogenous Kpnβ1 (ie. a 1:1 ratio) resulted in only a minor reduction in the proliferation of cancer cells, likely due to an observed delay in cell cycle progression. Here, we revealed that continuous upregulation of Kpnβ1 is toxic to HeLa cells, with significant cell death seen only when Kpnβ1 was overexpressed beyond levels achieved using the HeLa pEFIRES-Kpnβ1-EGFP cell line in Chapter 3. These results demonstrate that similarly to when Kpnβ1 was inhibited using RNAi[14], when Kpnβ1 expression was upregulated beyond a 1:1 ratio of endogenous to exogenous protein, significant cell death was observed. This leads to the conclusion that a tight regulation of Kpnβ1 expression is required, as cells appear to be unable to handle the negative effects associated with dysregulation of Kpnβ1 past a certain threshold.

While a few studies have shown evidence that dysregulation of Kpnβ1 leads to the accumulation of various mitotic defects, results are based only on transient overexpression or inhibition of Kpnβ1 protein using siRNA. In this chapter, we

independently verified the importance of Kpnβ1 in mitosis by studying the effects of inducible overexpression, as well as drug-mediated inhibition of Kpnβ1 in HeLa cells as they progress through the cell cycle in real time. Results revealed that inducible overexpression of Kpnβ1 resulted in the appearance of various mitotic abnormalities including multipolar spindles, spindle axis rotation, and prometaphase and metaphase delay, concurring with what has previously been described[87,96]. Similarly to what was seen in cells induced to overexpress Kpnβ1, treatment of HeLa cells with INI-43 resulted in a dose-dependent increase in the appearance of mitotic defects. Previous experiments in which Kpnβ1 was inhibited via RNAi showed similar results to that of INI-43 treated cells[90], suggesting that INI-43 is acting in a similar manner to Kpnβ1 protein knockdown, thus implying that it acts by targeting Kpnβ1.

In mitosis, Kpnβ1 acts as a negative regulator of various processes. For example, during spindle assembly it acts by sequestering SAFs at areas distant to mitotic chromosomes, thereby preventing untimely mitotic progression. RanGTP on the other hand acts as a positive regulator, counteracting the activity of Kpnβ1 by releasing SAFs and allowing spindle assembly to occur. Overexpression of Kpnβ1 results in sequestration of SAFs (even those close to the mitotic chromosomes) and thus prevents the formation of the mitotic spindle[22,95]. Inhibition of Kpnβ1 results in premature release of SAFs and activation of excessive mitotic spindle formation; overexpression of TPX2, a major SAF in mammalian cells, has been shown to result in significant mitotic spindle defects[87]. Therefore, the appearance of mitotic abnormalities as a result of Kpnβ1 dysregulation likely occurs due to a loss in the

regulatory function of Kpnβ1; SAFs are held inactive when Kpnβ1 is overexpressed or released inappropriately when Kpnβ1 is inhibited.

To expand on the results shown in Chapter 4, and to independently verify that INI-43 acts by targeting Kpnβ1 (at least in part), rescue experiments were performed. We found that the treatment of cells induced to express Kpnβ1-EGFP with INI-43 resulted in a significant reduction in the appearance of mitotic abnormalities seen with either condition alone. Research by the Lavia group suggests that Kpnβ1 overexpression leads to the sequestration of NLS-containing mitotic factors, which ultimately results in the appearance of certain structural (spindle organisation and chromosome segregation) abnormalities[87,96]. It appears as if these negative effects can to an extent be mitigated when cells are treated with INI-43, suggesting that INI-43 is targeting Kpnβ1 and thus allowing for restoration of activity of the cargoes.

Overall, our results suggest that when treating Kpnβ1 overexpressing cells with INI-43, a partial rescue effect/restoration of function is observed. Interestingly, treatment with higher concentrations of INI-43 did not have the same rescue effect, possibly due the fact that the system has become overwhelmed; this ties in with the proposed model for the partial rescue of Kpnβ1 function in Chapter 4 (Figure 4.7).

Ultimately, the results displayed in this chapter support previous data showing that dysregulation of Kpnβ1 is associated with the disruption of mitotic events, and suggest that a tight regulation of Kpnβ1 is essential for the correct functioning of cells. In addition, results from rescue experiments conducted using an inducible Kpnβ1

overexpression system, in combination with INI-43 treatment, independently validate that INI-43 is acting by targeting Kpnβ1.

CHAPTER 6

CONCLUSION

Dysregulation of the nuclear transport machinery has been directly linked to pathogenesis, including that of cancer. As a result, targeting this system for the development of novel anti-cancer therapeutics has received attention in recent years, with a particular focus on the targeting of the Karyopherin nuclear transport proteins. While previous research has revealed that the primary nuclear import protein, Kpnβ1, is required for cancer cell proliferation and shows potential as an anti-cancer target, it remains unclear as to whether Kpnβ1 induces any features of the transformed or cancer phenotype. In this study, we addressed the role that Kpnβ1 plays in various biological processes associated with cellular transformation and cancer progression, by studying the effects associated with dysregulation of Kpnβ1 (by exogenous overexpression and/or small molecule-mediated inhibition) in cancer cells. In addition, the specificity of the small molecule inhibitor, INI-43, for Kpnβ1 was investigated by using expression of exogenous Kpnβ1-EGFP as a rescue mechanism.

By investigating the effects of Kpnβ1 overexpression on various cancer cell phenotypes, we revealed that overexpression does not provide any growth advantage to cancer cells. In fact, stable expression of exogenous Kpnβ1-EGFP (at an approximate 1:1 ratio of endogenous to exogenous protein) resulted in a variety of negative effects, including a reduction in cell proliferation and delay in cell cycle progression after synchronisation. Previous research conducted using HeLa cells showed that depending

on the levels of Kpnβ1 overexpression, various effects (that increased in severity according to the level of overexpression) were observed[87,96]. In this study, stable expression of Kpnβ1-EGFP at levels higher than those achieved using the pEFIRES plasmid could not be attained due to high levels of cell death. It is likely that the effects seen in Chapter 3 are as a result of only moderate expression of Kpnβ1-EGFP in the pEFIRES stable cell lines (resulting in relatively mild phenotypic changes), and that if higher levels of overexpression were achieved, greater (or more severe) effects might be seen.

Kpnβ1 and Ran are known to counter-regulate each other during mitosis[22], and thus it is believed that a precise balance of their activity is essential for the correct functioning of various biological processes; indeed a number of studies have shown this[68,87,95]. We therefore hypothesised that the negative effects found to be associated with overexpression of Kpnβ1 might be rescued by the co-expression of exogenous Ran. This was not the case in our study however; we observed that overexpression of Ran in cells already overexpressing Kpnβ1 appeared to disturb the balance even further, resulting in a greater reduction in proliferation than what was seen with overexpression of either Kpnβ1 or Ran alone. Thus, correcting the balance appears to be more complex than just overexpressing both Kpnβ1 and Ran; it is unclear as to the extent at which Ran was upregulated in the Kpnβ1 overexpressing cells, and it is likely that additional factors (such as RCC1, which converts RanGDP to RanGTP[127] and NTF2, which transports Ran into the nucleus[31]) are required to maintain the precise balance required for correct cell functioning.

While previous research from our laboratory shows that Kpnβ1 is overexpressed in a variety of cancers and is essential for cancer cell proliferation[14], whether in fact Kpnβ1 upregulation is required for the cellular transformation of non-cancer cells is currently unknown. Preliminary results from this study suggest that overexpression of Kpnβ1 did not increase the proliferation rate of normal fibroblast cells, indicating that overexpression of Kpnβ1 alone is likely unable to drive cellular transformation. It is possible that Kpnβ1 upregulation is not a driver, but rather a product of cellular transformation and tumour progression; it is necessary but not sufficient for cancer development. A number of examples of genes that are required for cancer cell proliferation but are not sufficient to induce malignant transformation are present in the literature, including Cks1[128] and JNK[129]. There is a possibility that upregulation of the nuclear transport family as a whole might have greater ability to induce cellular transformation, as its appears as if the upregulation of Kpnβ1 in cancer cells does not occur in isolation, but rather alongside the upregulation of a myriad of other family members (unpublished observations).

Rescue experiments performed using exogenous Kpnβ1-EGFP revealed that overexpression of Kpnβ1 significantly rescued the negative effects observed when cells were treated with INI-43. Furthermore, overexpression of Kpnβ1 or treatment of cells with INI-43 resulted in a similar set of mitotic defects, that could be significantly rescued when cells expressing Kpnβ1-EGFP were treated with INI-43. These results imply that INI-43 exerts its effects, at least in part, by targeting Kpnβ1. Interestingly, when Kpnβ1 was overexpressed beyond levels achieved using the pEFIRES-Kpnβ1-EGFP stable cell line (by the use of a continuous Kpnβ1-EGFP expression system in

Chapter 5), a significant induction of cell death was observed. Inhibition of Kpnβ1 protein expression using siRNA[14], or treatment of cancer cells with INI-43[52], similarly resulted in increased cell death, suggesting that cancer cells cannot cope with dysregulation on Kpnβ1 expression beyond a certain threshold.

Taken together, this study provides evidence that a precise balance of Kpnβ1 expression (and activity) is crucial for cancer cell proliferation and survival. When the balance is perturbed in either direction (i.e. with overexpression of Kpnβ1 or inhibition of Kpnβ1 [that can be via siRNA-mediated knockdown[14] or small molecule-mediated inhibition, such as that with INI-43[52]], negative effects associated with various biological phenotypes are observed. These include: reduced proliferation (and induction of apoptosis when Kpnβ1 is dysregulated beyond certain levels), cell cycle delay or arrest, changes in the adhesion properties of cells, as well as the onset of mitotic instability (Figure 6.1). In addition, the negative effects observed as a result of Kpnβ1 dysregulation can be rescued upon the re-regulation of Kpnβ1 (using expression of exogenous Kpnβ1-EGFP or treatment with INI-43).

Ultimately, the results presented in this dissertation provide evidence that a tight regulation of Kpnβ1 is essential for the correct functioning of cancer cells, thus highlighting the potential for Kpnβ1 as a promising anti-cancer therapeutic target. Furthermore, rescue experiments confirm that the small molecule inhibitor, INI-43, is acting (at least in part) by targeting Kpnβ1. We propose that further modification and development of INI-43, and other potential small molecule inhibitors of Kpnβ1, may result in the emergence of novel anti-cancer therapies.

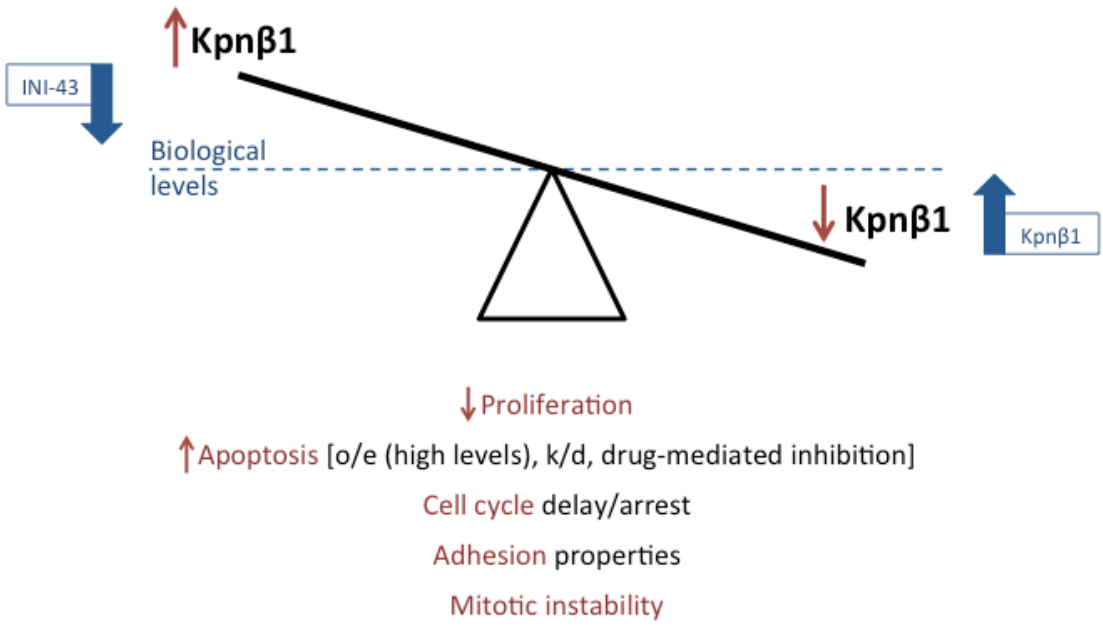


Figure 6.1. Cancer cells require a precise balance of Kpnβ1 expression and activity. Dysregulation of Kpnβ1 expression results in multiple disruptions to cellular processes, which can be rescued upon expression of exogenous Kpnβ1 or treatment with INI-43 (o/e – overexpression; k/d – knockdown).

6.1 Limitations and future perspectives

A major limitation of this study was the lack of data for the effects of Kpnβ1 overexpression in non-cancer cell lines. Despite optimisation of transfection conditions using various cell lines, transfection reagents, plasmid backbones, and amount of plasmid DNA transfected, generation of a non-cancer stable cell line was unsuccessful and the transient transfection achieved was poor, thus no solid conclusions could be drawn from this data. It would appear from these results that Kpnβ1 is not a driver of cellular transformation or the cancer phenotype (but is necessary for cancer cells). In the future we hope to be able to further investigate the role of Kpnβ1 in cellular transformation and tumourigenesis by studying the effects of Kpnβ1 dysregulation along various stages of the transformation process - i.e. through the use of a tumour progression model such as the MCF10 breast tumour progression series used by Kuusisto and Jans (2015)[73].

While rescue experiments using expression of exogenous Kpnβ1-EGFP suggest that INI-43 acts partly by targeting Kpnβ1, further investigation is required to determine binding and specificity. We propose performing molecular docking studies to identify potential binding site(s) for INI-43 on Kpnβ1, after which mutational studies and biophysical assays will be used to confirm binding by identifying potential resistance causing mutations. The mechanism of INI-43 mediated degradation of Kpnβ1 also needs to be further investigated. Ultimately, crystal structures of Kpnβ1 in complex with INI-43 will be necessary to fully elucidate the biochemical mechanism of action of INI-43.

There is sufficient evidence that targeting Kpnβ1 has therapeutic potential for the treatment of cancer, and that INI-43 may be useful as a lead compound for the future development of Kpnβ1 inhibitors that are clinically relevant[52]. We propose that modification of its chemical structure may result in compounds with improved pharmacokinetic profiles that can be taken further as potential clinical candidates.

APPENDIX I - SOLUTIONS

Cell culture solutions

Trypsin-EDTA

0.5g Trypsin

8g NaCl

1.45g $Na_2HPO_4.2H_2O$

0.2g KCl

0.2g KH_2PO_4

10mM EDTA, pH 8.0

Make up to 1L with PBS

Cell-freezing media

80% Complete medium

10% Fetal Bovine Serum

10% DMSO

MTT reagent (5mg/ml)

100mg MTT

20ml PBS

Vortex and incubate in 37°C waterbath for 15 minutes

Filter sterilise through 0.22µm filter

Store protected from light at 4°C for up to one month

Solubilisation Solution

25g SLS

Adjust volume to 250ml with dH_2O

Add 76.6µl concentrated HCl

1% methyl cellulose growth media

1g methyl cellulose

Autoclave in 100ml glass bottle

Add 100ml pre-warmed complete growth media

Vortex and shake at 4°C overnight

Poly-(HEMA) solution

2.4g poly(2-hydroxyethyl methacrylate)

200ml 96% ethanol

Dissolve with constant stirring for 5-6 hours in 65°C waterbath

Fixation Solution

12.5ml glacial acetic acid

87.5ml methanol

0.5% Crystal Violet Solution

500mg crystal violet

25ml methanol

75ml dH_2O

FACS Stain Solution

0.1% Triton X-100

2mM MgCl2

100mM NaCl

10mM PIPES, pH 6.8

10µg/ml Propidium Iodide

DNA solutions

TE Buffer

10mM Tris-Cl (pH 8.0)

1mM EDTA (pH 8.0)

TBE buffer (10X)

108g Tris

55g Boric Acid

7.4g EDTA

Make up to 1L with dH_20

Protein solutions

RIPA buffer

10mM Tris-Cl (pH 7.4)

150mM NaCl

1% Sodium deoxycholate

0.1% SDS

1% Triton-X100

Make up to 100ml with dH_20

PBS (10X)

40g NaCl

1g KCl

3.82g $Na_2HPO_4.2H_2O$

Make up to 500ml with dH_20

4% Stacking Gel

7.3ml dH_2O

1.25ml 1M Tris-Cl, pH 6.8

100µl 10 % SDS

1.3ml 30 % Acrylamide/bis-Acrylamide

120µl 10 % Ammonium Persulphate

12µl Temed

10% Separating Gel

2.85ml dH_2O

3.75ml 1M Tris-Cl, pH 8.8

50µl 10 % SDS

3.3ml 30 % Acrylamide/ bis-Acrylamide

175µl 10 % Ammonium Persulphate

17.5µl Temed

15% Separating Gel

1.15ml dH_2O

3.75ml 1M Tris-Cl, pH 8.8

50µl 10 % SDS

5ml 30 % Acrylamide/ bis-Acrylamide

175µl 10 % Ammonium Persulphate

17.5µl Temed

Laemmli Loading dye (4X)

2.5ml 1M Tris-Cl (pH 6.8)

6% SDS

0.005% Bromophenol blue

4ml Glycerol

Add 10% β-mercaptoethanol just before use

Running buffer (10X)

20g Glycine

31.6g Tris

5g SDS

Make up to 500ml with dH_20

Transfer buffer (10X)

72g Glycine

19g Tris

Make up to 500ml with dH_20

Transfer Buffer (1X)

100ml 10 X Transfer Buffer

200ml Methanol/Isopropanol

700ml dH_2O

TBST

50mM Tris-Cl (pH 7.5)

150mM NaCl

500µl Tween-20

Make up to 1L with dH_20

<u>Bacterial solutions</u>

Luria Broth (LB) medium

1g Tryptone

0.5g Yeast extract

1g NaCl

1mM NaOH

Make up to 100ml with dH_20

Luria Agar (LA)

Add 1.5g agar/100ml LB medium

Ampicillin (10 mg/ml)

1 mg Ampicillin

10ml dH_2O

Filter sterilise through 0.22µm filter

Store at -20°C

APPENDIX II – DNA AND PROTEIN MARKERS

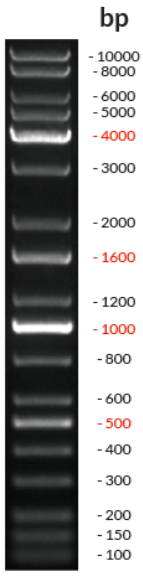


Figure A.1. KAPA Universal DNA ladder. Ladder used to determine the molecular weight of plasmid DNA after restriction enzyme digest.

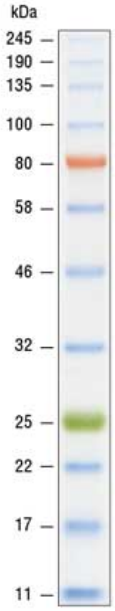


Figure A.2. Colour Prestained Protein Standard. Ladder used to determine the molecular weight of proteins electrophoresed on 10 -15% SDS-PAGE gels.

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