

The Role of Karyopherin β 1 in the Nuclear Import of HIV-1 Proteins

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Abbreviations

AIDS	Acquired Immunodeficiency Syndrome
Amp	Ampicillin
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
bp	Base pairs
°C	Celsius
CD4	Cluster of Differentiation 4
cDNA	Complementary DNA
DAPI	4'6-diamidino-2-phenylindole·2HCl
dH ₂ O	Distilled water
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide Triphosphate
<i>E. Coli</i>	<i>Escherichia coli</i>
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
GFP	Green fluorescent protein
G418	Geneticin
HIV	Human Immunodeficiency Virus
Hr	Hour
IN	Integrase

IC ₅₀	50% inhibitory concentration
kD	kilo Daltons
Kpnβ1	Karyopherin β1
Kpnα	Karyopherin α
LB	Luria broth
M	Molar
mg	milligrams
ml	millilitre
mRNA	Messenger ribonucleic acid
NES	Nuclear exit signal
NLS	Nuclear localisation signal
NPC	Nuclear pore complex
Nm	nanometre
P	probability value
PARP	Poly (ADP-ribose) Polymerase
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PIC	Pre-integration Complex
RNA	Ribonucleic acid
RPM	Revolutions per minute
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS- polyacrylamide gel electrophoresis
SD	Standard deviation
siRNA	Small interfering RNA
Tat	Transactivator of Transcription

TBE	Tris-borate- EDTA
TBP	TATA-binding Protein
TBST	Tris-buffered saline with Tween20
TFIID	Transcription Factor II D
UCT	University of Cape Town
UV	Ultraviolet
μ	micro
V	Volts
vDNA	Viral DNA
Vpr	Viral Protein R
X-gal	5-bromo-4-chloro-3-indolyl- -galactopyranoside

Abstract

The HIV-1 retrovirus has the ability to infect both dividing and non-dividing cells of its human host. In order to gain access into the nucleus, the viral nucleoprotein pre-integration complex (PIC) requires carrier molecules for its transport through the nuclear pore. Following integration of the viral DNA into the host genome, regulatory proteins such as HIV Tat also require import into the nucleus to perform their functions. It has been suggested that members of the Karyopherin superfamily of nuclear transporters may aid in the transport of both PIC and regulatory viral proteins. The exact viral determinants that direct nuclear import, as well as which Importins are involved in their import remains very controversial in HIV research to date.

In this study, the role of the Importin, Karyopherin $\beta 1$ (Kpn $\beta 1$), in the nuclear import of the HIV-1 proteins Tat, Viral Protein R and Integrase was investigated. To determine its importance in the nuclear import of HIV proteins, Kpn $\beta 1$ was inhibited using siRNA and a small molecule inhibitor, c43. Tat transactivation as an indicator of Tat nuclear localisation was then assayed using the HIV LTR promoter, containing a Tat responsive element, fused to the luciferase reporter gene. In addition, the localisation of the recombinant HIV-1 proteins after Kpn $\beta 1$ knockdown was also determined via fluorescence microscopy and Western blot analysis of nuclear and cytoplasmic protein fractions. Our data show that Tat transactivation capability was significantly reduced after inhibiting Kpn $\beta 1$ using siRNA. The same result was found when Kpn $\beta 1$ was inhibited with a previously undescribed inhibitor under investigation in our laboratory, c43. Fluorescent microscopy analysis of GFP-Tat

showed marginal cytoplasmic accumulation following Kpn β 1 knockdown in HeLa cells. Analysis of the PIC proteins GFP-Vpr and GFP-IN showed no effect on GFP-Vpr localisation following Kpn β 1 inhibition, while a significant decrease in the ratio of nuclear/cytoplasmic fluorescent intensity for GFP-IN was observed. To confirm the change in GFP-IN localisation, we performed Western blot analysis on nuclear and cytoplasmic protein fractions. We saw a significant decrease in the nuclear GFP-IN protein levels. Together, our results show that while HIV Tat transactivation is decreased when Kpn β 1 is inhibited in TZM-bl cells, its subcellular localisation is partially affected in HeLa cells. We observed no effect on GFP-Vpr localisation, while IN localisation was significantly decreased in the nucleus via fluorescent microscopy and Western blot analysis, following Kpn β 1 knockdown. These preliminary findings suggest Kpn β 1 is involved in the nuclear import of the HIV proteins Tat and Integrase. The literature describes various other nuclear transporters shown to import these proteins, suggesting the virus has evolved in flexibility and redundancy in the nuclear import pathways of its proteins.

CHAPTER ONE:

INTRODUCTION

1.1 The Human Immunodeficiency Virus (HIV)

1.1.1 Epidemiology of HIV

HIV was first isolated in 1984 and determined to be the causative agent of Acquired Immunodeficiency Syndrome (AIDS). AIDS is a disease characterised by a compromised immune system that if attacked by an opportunistic infection, can lead to death (1, 2).

According to the World Health Organisation, 34 million people were living with HIV in 2011, with 2.5 million new infections and 1.7 million deaths that year alone (3). HIV is therefore a global health threat, with the highest percentage of infected people living in Africa, particularly sub-Saharan Africa (Fig 1.1).

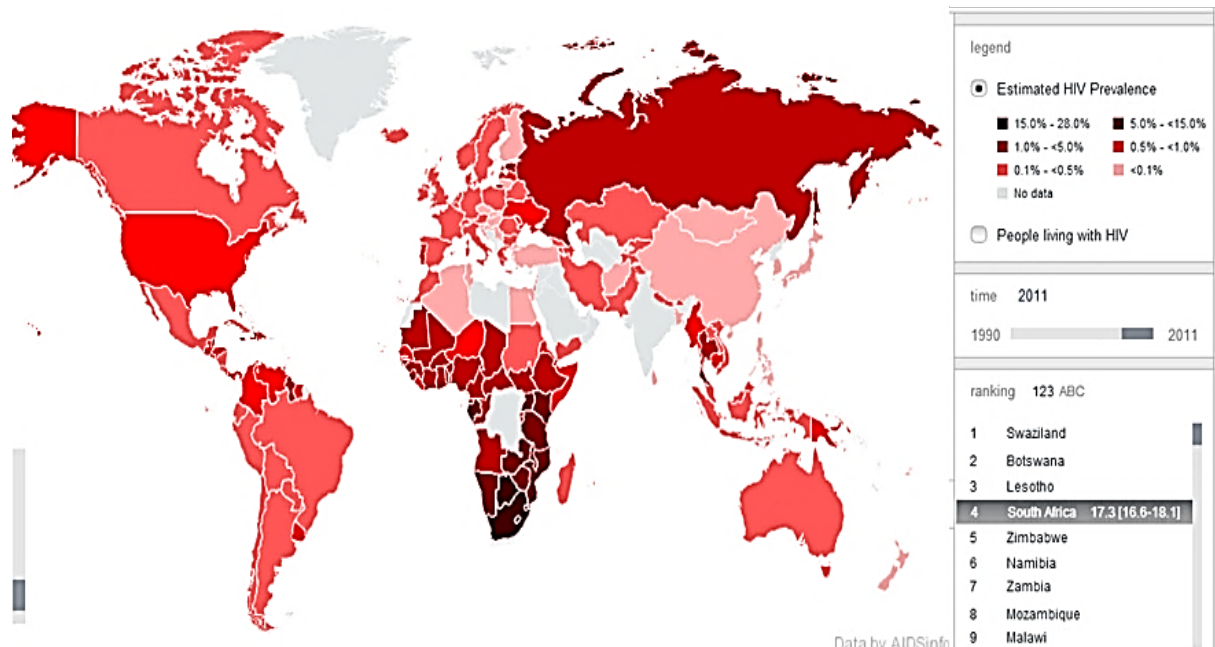


Figure 1.1. Global HIV Prevalence for 2012. HIV is most prevalent in Sub-Saharan Africa and is therefore a major health concern for South Africa. Taken from (4).

In 2011, 5.6 million people living in South Africa were infected with HIV, accounting for 16% of the global disease burden. Unprotected sex with multiple partners has been described as the highest risk factor in this region (3). With the introduction of Highly Active Antiretrovirals (HAART), a combination of 3-5 drugs that target various stages of the HIV life cycle, and prevention strategies, South Africa has reduced its new infections by 41% from 2001 to 2011(4).

Although progress is being made with the introduction of HAART, there is currently no widely available cure for AIDS. The current medications prevent the virus from replicating and therefore allow for an extended patient lifespan. As can be seen from figure 1.2, life expectancy has increased drastically since the introduction of highly active antiretroviral therapy (HAART) (Figure 1.2) (5).

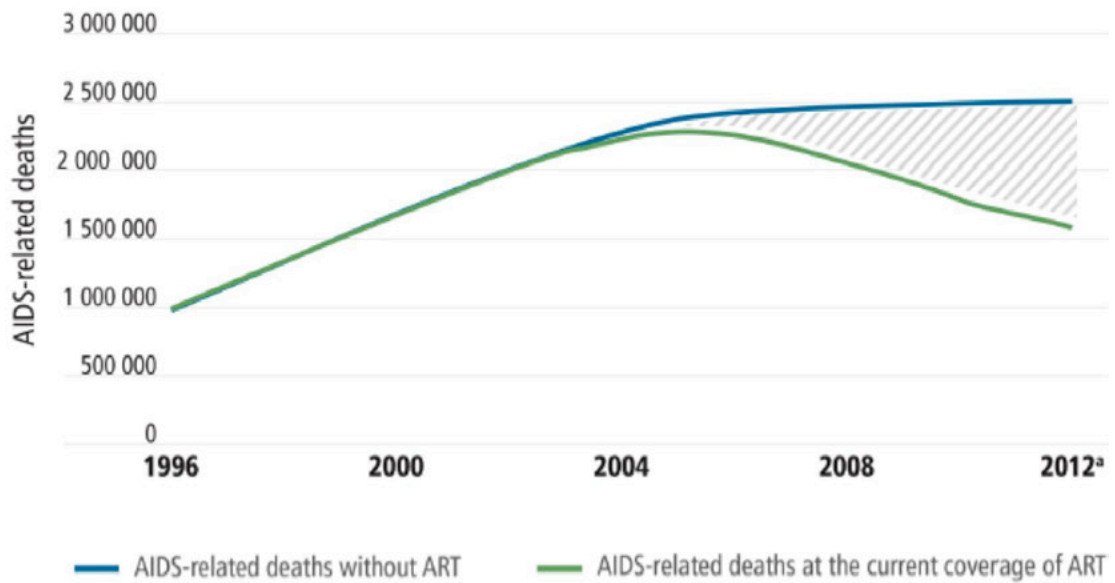


Figure 1.2. The annual number of people dying from AIDS-related causes globally compared to a scenario of no antiretroviral therapy. The graph spans 1996-2012. Taken from (162).

HAART targets many stages of the viral life cycle to effectively reduce the circulating viral load to undetectable levels. This combination therapy has reduced HIV infection from a deadly disease into a manageable, chronic condition (6). However, the virus persists in the body due to its ability to hide in viral reservoirs such as resting CD4⁺ T cells, leading to viral rebound following interruption of therapy (7, 8).

Traditionally, HIV drugs have been targeted at viral proteins. The most common being Reverse Transcriptase inhibitors (zidovudine, didanosine, zalcitabine, stavudine, lamivudine, abacavir, emtricitabine and tenofovir, nevirapine, delavirdine, efavirenz and Etravirine), Protease inhibitors (saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, lopinavir, atazanavir, fosamprenavir, tipranavir and darunavir)

and recently the Integrase inhibitor (raltegravir) (9). However there are high mutation rates associated with viral proteins due to the lack of a proofreading function of the HIV-1 Reverse Transcriptase enzyme (10, 11). This has led to host factors are now becoming increasingly more viable candidates. Host proteins represent a much wider pool of possible targets, whereas the viral genome only encodes 15 proteins (12, 13).

A prime example of a host protein being an excellent target is the CCR5 co-receptor. In order for HIV to enter target cells, it must bind either CCR5 or CXCR4 co-receptors. In February 2007, a patient received complete stem cell transplantation with cells that contained a 32 base pair deletion in both alleles of the CCR5 gene, termed CCR5 Δ 32/ Δ 32. These cells possess non-functional CCR5 receptors that resulted in viral replication ceasing despite discontinuation of antiretroviral therapy (14). No evidence of HIV replication has been observed in the patient in up to 5 years of follow up, in the absence of combination antiretroviral therapy (15). Although promising, the cost of the procedure results in it not being a viable 'cure' for the millions of poverty stricken patients.

The best hope of eradicating the virus permanently is a prophylactic vaccine. However, the variability in the viral epitopes has resulted in difficulty with vaccine development. The HIV virus is highly diverse and dynamic, mainly due to the high mutation rates (16).

There are two main types of HIV worldwide, HIV type 1(HIV-1) and HIV type 2 (HIV-2) that were transferred from Simian Immunodeficiency Viruses (SIV) hosted

To elicit broadly neutralising antibodies that can detect all these diverse phenotypes from a single vaccine will be a great challenge for HIV researchers. The answer will help not only to eradicate HIV but also other viruses that evolve at a similarly rapid rate.

1.1.2 The HIV-1 Viral Genome and Life Cycle

HIV belongs to the lentivirus subfamily of the retroviridae family of viruses, characterised by their RNA genomes and reverse transcription capability. These viruses differ from the oncovirus subfamily by possessing the ability to infect non-dividing cells such as resting T cells and macrophages (18, 19). These non-dividing cells then act as viral reservoirs, evading the immune system and leading to recurrence of infection if HAART is discontinued. HIV is described as a ‘budding virus’ whose progeny are encapsulated by the host cell membrane and literally ‘bud off’ to infect susceptible cells. The viral life cycle consists of a series of steps that lead to integration into the host genome, enabling another round of replication and infection of fresh cells. The genome of HIV-1 encodes only 15 proteins, forcing the virus to exploit many host proteins for successful replication (20). The various stages of the life cycle will be discussed, with reference to each viral protein’s role.

The HIV-1 genome consists of the reading frames gag, pol and env (Figure 1.4). These are responsible for producing the viral core, Reverse Transcriptase, Integrase and Protease enzymes and the surface Envelope, respectively. The virus also encodes the regulatory genes tat and rev and the auxiliary genes nef, vif, vpr, vpx and vpu(21).

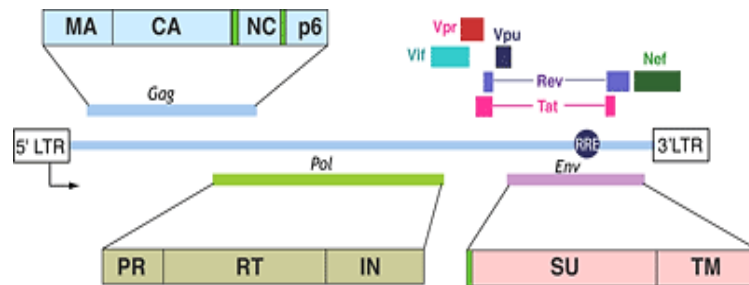


Figure 1.4: Schematic outlining the organisation of the HIV-1 genome. The genome is made of three large open-reading frames that express structural (Gag), enzymatic (Pol) and envelope (Env) proteins, surrounded by terminal repeated sequences LTR (Long Terminal Repeat). The genome also encodes regulatory (Tat and Rev) and auxiliary (Nef, Vif, Vpr et Vpu) proteins. Taken from http://www-ibmc.u-strasbg.fr/arm/Marquet/them2_marq_en.html.

The HIV-1 virus particle consists of an outer lipid membrane containing membrane glycoproteins and an inner cone-shaped core containing the viral RNA genome as well as proteins necessary for the journey to the cell nucleus (22, 23) (Figure 1.5A).

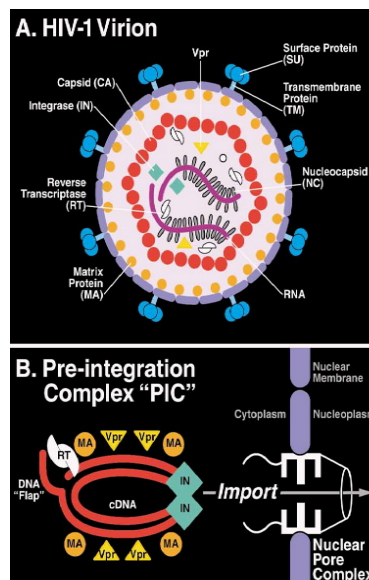


Figure 1.5: Diagram showing (A) The structure and organisation of the HIV-1 virion, (B) The contents of the preintegration complex (PIC) and its requirement to be imported through the NPC. Taken from (21)

Successful infection requires overcoming various obstacles in order to integrate the viral genome into the host chromosome. There are various known steps that lead to productive viral infection and each HIV protein is responsible for different steps in the life cycle (Figure 1.6).

Firstly, the virus must enter the cell, crossing the cell membrane. It achieves this through a series of steps that ultimately lead to fusion of the viral and cellular membranes and the emptying of the viral contents into the cell. The target cells of HIV contain a cell surface receptor, named cluster of differentiation 4 (CD4), previously termed T4, that was discovered in 1986 to be present on the surface of cells of the immune system and the brain (24). This group also showed that after T4 receptor: HIV gp120 (encoded by the env gene) interaction, the virus enters the cell via receptor mediated endocytosis. However, only later did it become clear that additional binding to a chemokine co-receptor, either CCR5 or CXCR4 depending on the strain and cell type, was necessary for cellular entry. Also, binding of CD4 to gp120 induces binding to CCR5 via a conformational change in the protein (25).

After endocytosis into the cell, the virus sheds the capsid layer, releasing the viral genome, along with other proteins required for the journey into the nucleus. It has long been thought that this uncoating process occurs directly after entry into the cell, but recent evidence suggests it occurs closer to the nuclear membrane and that Capsid (CA) plays a pivotal role in various subsequent steps (reviewed in 25).

After shedding, the viral RNA is reverse transcribed by the Reverse Transcriptase enzyme into double stranded cDNA that forms a high molecular weight nucleoprotein

complex with other viral proteins present in the core. This complex is then targeted to the nucleus and has been named the pre-integration complex (PIC) (27) (figure 1.5B).

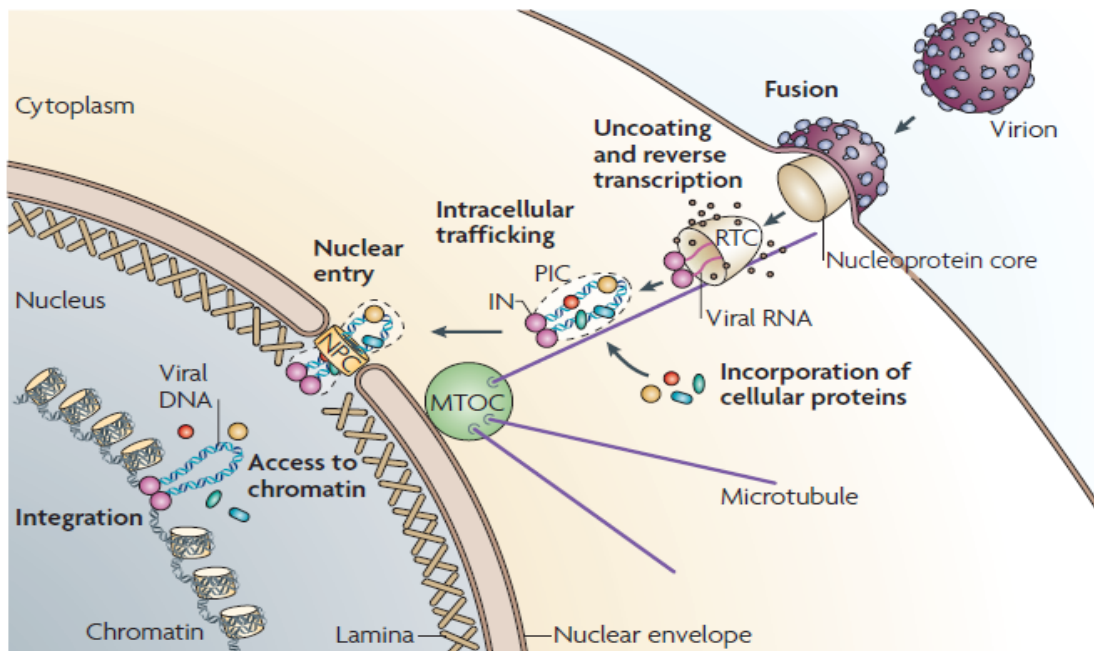


Figure 1.6: Diagram depicting transport of the HIV virion components from the extracellular space to the nucleus, through the nuclear pore complex(NPC) Taken from (154).

Tracking of virus particles in live cells with the green fluorescent protein (GFP) tag has shown the PIC to make use of the microtubule network to migrate towards the nuclear membrane (Figure 1.6) (28). The HIV-1 Capsid(CA) protein is the viral determinant that interacts with the microtubule network to transport the PIC towards the nucleus (29). Within 4-6 hours of infection, the viral DNA is imported into the nucleus through active transport mechanisms (30, 31). These import mechanisms form the context of the focus of this dissertation. There is contention within the literature regarding the exact PIC proteins necessary for mediating import through the nuclear pore complexes as well as which host proteins are involved.

The nuclear membrane forms a physical barrier between the contents of the nucleus and the cytoplasm. In order for the viral PIC to traverse this barrier, it needs to interact with the nuclear pore complexes (NPCs) that stud the nuclear membrane.

A NPC is a 125 MDa complex composed of multiple proteins called nucleoporins that form a channel that allows for the selective passage of certain molecules across the nuclear membrane (32). The NPC allows ions and small proteins (<40kDa) to travel through this channel, while larger proteins require active, receptor mediated transport. The direction of cargo transport through the NPC is governed by a conserved signal in the cargo. A nuclear localisation signal (NLS) directs import into the nucleus, while a nuclear export signal (NES) directs export out of the nucleus. The PIC is estimated at roughly 56nm in diameter and so requires active, facilitated transport through the pore complexes (33). The nuclear import of HIV-1 complexes will be discussed in the next section but, briefly, the main PIC proteins identified as potential mediators of nuclear import are Integrase, Viral Protein R and Matrix Protein. The accessory proteins Tat and Rev also require import after the initial integration step and once they have been translated in the cytoplasm.

Once inside the nucleus, the double stranded HIV genome is targeted to be integrated into the host genome, and serve as the template for another round of viral replication. The sites within the human genome for integration to occur have been correlated with regions of active gene transcription, especially genes activated by HIV infection. It has also been revealed after global analysis that certain regions are also favoured for integration, with a 2.4Kb region containing 1% of integration sites (34). The preference for integration within active genes is not surprising, considering that the chromatin in these regions is less tightly packed, allowing for easier accessibility (35).

Integration is mediated by a large (~160S) nucleoprotein complex, with the main viral protein that catalyses this process being Integrase (27). As its name suggests, this protein is involved in integrating the viral DNA into the host genome to allow for productive viral replication (36, 37). Integrase is a 32 kDa protein encoded by the pol gene. In the first step of integration, nucleotides are removed from the 3' end of the viral DNA in a 3' end processing event. The DNA is then cleaved at the CA sequence of the 3' end, resulting in a free OH that is to be bound to the chromosomal DNA. This processed viral DNA then attacks a phosphodiester bond present in the target DNA. The 3' end processing and the strand transfer reactions are both mediated by Integrase. Cellular repair and ligation enzymes then carry out the remaining steps to fully integrate the foreign DNA (38). Once this process has been completed, HIV-1 transcription from the long terminal repeat (LTR) promoter can commence and new virions can be assembled and released from the infected cell.

The transcription from the LTR promoter is greatly enhanced by the HIV Tat protein, which is a potent trans-activator and once in the nucleus it binds an RNA element termed the transactivation responsive element (TAR element) and enhances the initiation and elongation steps of HIV-1 transcription (39–41). Tat not only acts as a transcription factor, but also causes induction of apoptosis in CD4⁺ T cells, thereby aiding in the depletion of CD4⁺ cells seen in HIV positive patients (42, 43).

The newly transcribed, unspliced RNA is transported out of the nucleus by the HIV Rev protein (44). Speculation exists as to whether RNA export is the only function of the Rev protein, as viral infectivity can be stimulated >1000 fold by its presence, an effect possibly not only due to nuclear export (45). However, mRNA transport appears to be the main function of the Rev protein. When bound to its mRNA, Rev utilises the nuclear exporter protein, Chromosome region maintenance 1 (CRM1), to shuttle to the cytoplasm with its cargo (46)(47). Here the viral mRNA is translated by the host translation machinery to produce viral proteins to be packaged into new virus particles.

The late stages of the HIV-1 life cycle include intracellular transport, assembly, maturation and budding. The HIV Gag polyprotein is crucial to these late stage events and is capable of singlehandedly assembling non-infectious virus like particles (48). The assembly of new virions occurs at the plasma membrane at specific membrane microdomains such as lipid rafts and tetraspanin-enriched microdomains (TEMs)(49). These are cholesterol enriched regions of the plasma membrane known to be entry and exit sites for pathogens (50). The Gag protein consists of four domains; the Matrix protein (MA), capsid (CA), nucleocapsid (NC) and p6. The MA protein is known to target assembly to the plasma membrane as well as being implicated in nuclear import of the PIC. Myristoylation of Matrix's N terminal glycine appears to be the key event targeting Gag to the plasma membrane. The p6 protein is responsible for viral budding as the viral lipid membrane separates from the host cell membrane. Once assembly and budding have been completed, the newly formed virus particles are free to infect neighbouring susceptible targets. The auxiliary proteins Nef, Vif,

Vpr, Vpx and Vpu aid in increasing overall infectivity and viral replication through a variety of functions throughout the life cycle (51).

All the steps of the HIV-1 life cycle have been extensively studied and drug targets identified at most of the stages. Less well understood stages at this point are the nuclear import of the viral PIC, as well as the nuclear import of the regulatory proteins.

1.1.3 HIV-1 Nuclear Import

HIV-1 consists of only 9.2 kb of unspliced genome, which encodes 15 proteins in total. This limited size has led the virus to evolve mechanisms of exploiting host factors and pathways in order to achieve successful replication. For example, the HIV LTR promoter makes use of common host transcription factors for optimal transcription of its inserted genome. These transcription factors include NF- κ B, Sp1, AP-1 and many more (52). In fact, an interaction between host factors and HIV-1 are widespread and the 'HIV-1-Human Protein Interaction Database' has been constructed from the literature (53, 54).

During the early preintegration stage of infection, the PIC requires nuclear import across the nuclear envelope to complete integration and subsequent replication within the nucleus. In the following postintegration stage, regulatory proteins such as Tat and Rev require nuclear import to perform their functions in the nucleus. The virus makes use of multiple host proteins during these steps.

The main viral candidates identified in the literature as being involved in the nuclear import of the PIC are the HIV proteins Matrix Antigen (MA), Integrase (IN) and Viral Protein R (Vpr), as well as the central ‘DNA flap’/ central polypurine tract (cPPT) (21) (Figure 1.7). Their precise roles and the mechanisms in this process are, however, controversial and contradictory.

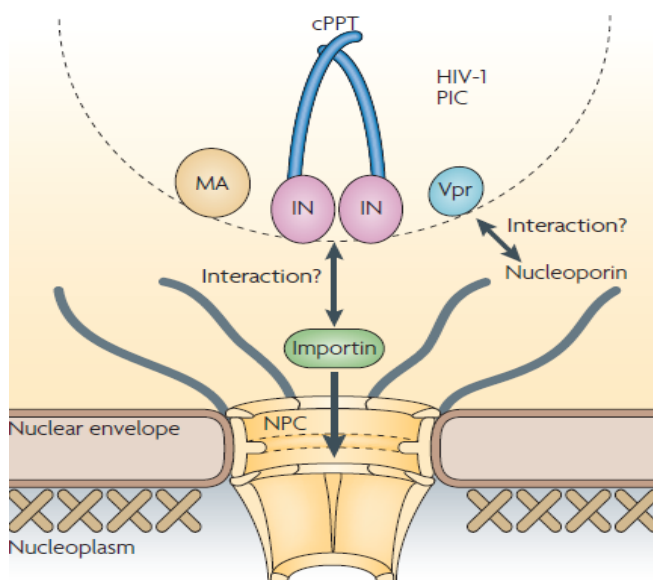


Figure 1.7. A schematic of the NPC and the proposed import of the HIV-1 PIC containing the proteins Matrix, Integrase and Vpr. These three viral proteins have been identified in the literature as possible mediators of nuclear import but much controversy still exists. Adapted from (154).

All facilitated transport into the nucleus is regulated by the macromolecular nuclear pore complexes, as well as transporter proteins. In order to be recognised for import by a transporter, a nuclear localisation signal (NLS) is required to be present within the cargo. This NLS interacts with a transporter protein that then navigates interactions through the lumen of the nuclear pore complex to successfully ferry the

cargo across the nuclear membrane. Nucleoporins are by no means passive bystanders in the nuclear import process. They actively interact with both the cargo and nuclear transporters to support nuclear import, through docking and undocking reactions (55). Recent research revealed that selective depletion of nucleoporins 153 and 98 from human Jurkat lymphocytes resulted in a marked decrease in HIV-1 infection. Nup 153 and 98 were also shown to bind the HIV-1 core, further implicating them as important in PIC import and possibly even integration (56, 57). Karyopherins α and β have been seen to bind FXFG repeat regions within the nucleoporins *in vitro* and *in vivo*. The NPC is the stationary phase in this process and facilitates docking and release reactions along the lumen of the nuclear pore (reviewed in (58)). Nuclear import can be competitive, with members of the same family competing for binding to nucleoporins as well as cargo (59).

Functional NLSs have been discovered in the HIV proteins Matrix (MA) and Integrase (IN) that could facilitate passage of the PIC through the nuclear pore complex (60). The Vpr protein, however, does not seem to contain an NLS but has been shown to be important for PIC nuclear transport as seen when a mutant virus lacking Vpr failed to enter the nucleus *in vitro* (61). The presence of a putative NLS, however, is not sufficient to confer nuclear import. For instance, Hearps et al (2008) showed that MA fails to enter the nucleus and they propose its “NLS” serves other purposes such as forming higher order structures and DNA binding (62). Additionally, MA may be dispensable for nuclear import, as seen by the fact that efficient viral replication can occur in the complete absence of the Matrix protein (63).

Once the PIC has entered the nucleus and transcription is underway, accessory proteins such as Tat and Rev also require entry and exit from the nucleus. Both proteins contain arginine-rich sequences that function as NLS's (64). Rev has shown to utilise different nuclear import pathways in different cellular environments (65), while Tat has also been described as able to diffuse passively through the NPC due to its small size (14kDa) (66). Apart from these proteins, a 'DNA flap' created during reverse transcription has also been implicated as a cis-determinant of HIV nuclear import (67). This project will focus on the nuclear import pathways employed by the PIC proteins IN and Vpr, as well as the regulatory protein, Tat.

HIV nuclear import poses an attractive novel therapeutic target. In order for the virus to integrate and replicate, various key proteins must be actively imported into the nucleus. However, the host proteins that perform this function are intricately intertwined in a web of host processes that if tampered with could lead to toxicity.

1.2 The Nuclear Transporter Karyopherin β 1 (Kpn β 1)

Multiple soluble nuclear transport receptor proteins exist in the cell that shuttle cargo to and from the nucleus. The main family of transporters is the Karyopherin superfamily of proteins (59). The first discovered family member that has been extensively studied is Karyopherin β 1 (Kpn β 1), also known as Importin β , p97 and yeast Kap95 (69). This particular Importin works either in concert with an adapter protein, Karyopherin α or alone to import cargo bearing a basic nuclear localisation signal (NLS) into the nucleus, via the nuclear pores (70).

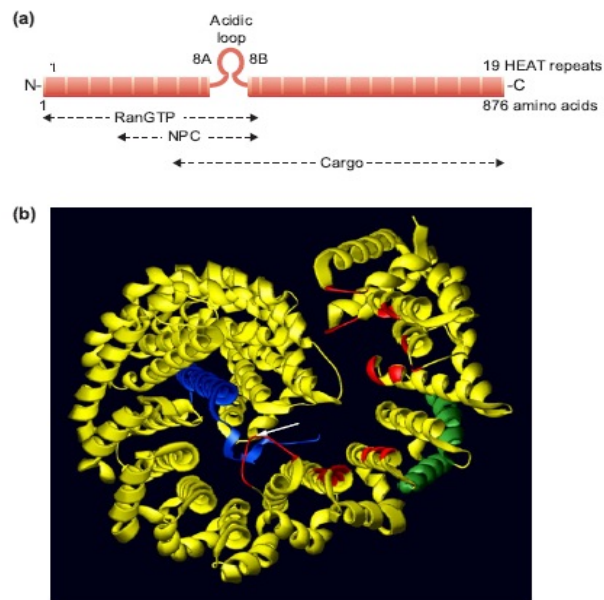


Figure 1.8. (a) Schematic of 19 HEAT repeats of Kpnβ1 showing binding sites for RanGTP, the NPC as well as cargo. (b) Structure of Kpnβ1 (yellow) bound to the IBB domain of Kpnα (blue). Derived from (163).

It was first discovered in 1994 that a cytosolic protein was responsible for selective protein import into the nucleus (71). A year later researchers identified that the process of transporting NLS containing proteins into the nucleus involved association and dissociation reactions driven by a RanGTP gradient (72). To allow directionality of transport, there exists a concentration gradient of the small GTPase, Ran. The binding site on Kpnβ1 for Ran overlaps the binding site for its cargo, thereby releasing the cargo upon Ran binding (Figure 1.8a and Figure 1.9). Ran is present at a high concentration in the nucleus and this allows for cargo release upon entry into the nucleus.

Kpnβ1 is a 97kDa protein consisting of 876 amino acids that form 19 helical repeat motifs (HEAT repeats) of ~40 residues in length (Figure 1.8a). These HEAT repeats consist of right handed superhelical structures that twist and contract upon cargo or subunit binding (73). Kpnβ1 contains 23 well distributed cysteine residues (68). The

protein is able to bind RanGTP at residues 1-364 (HEAT repeats 1-8) while residues 331-876 (HEAT repeats 7-19) are responsible for Karyopherin α binding (69).

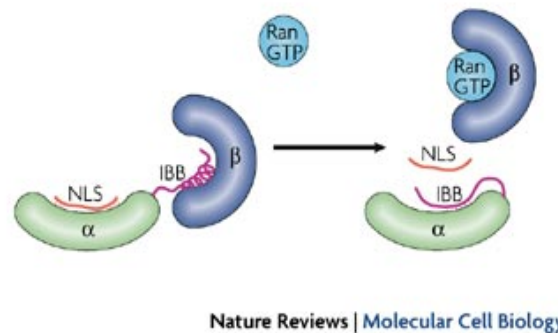


Figure 1.9. Cartoon showing the dissociation of Kpn α and Kpn β , as well as the cargo NLS, upon RanGTP binding in the nucleus. Picture adapted from (164).

Kpn β 1, together with Kpn α bound to the cargo, then facilitates transport through the nuclear pore complexes into the nucleus (Figure 1.9) (74). Kpn β 1 also contains binding sites for the NPCs that allow for sequential binding reactions to facilitate translocation through the nuclear pores (69). Zinc ions are essential for the nuclear envelope binding activity of Kpn β 1 (p97) (68).

The binding site present on Karyopherin α for β is termed the Importin β -binding (IBB) domain and the crystal structure of the two proteins intimately bound has been solved (73). Thus, once the trimeric cargo: Kpn α : Kpn β 1 complex enters the nucleus, the high concentration of RanGTP causes the cargo to dissociate from Kpn α as RanGTP binds Kpn β 1 at the position of the IBB domain (72, 75). Kpn β 1 and Kpn α are exported from the nucleus, to await another round of import.

There is therefore a constant cycling of the transporter proteins and a release of their cargo in the nucleus. Of interest, overexpression in yeast of fragments containing the binding regions of Karyopherin α or β resulted in sequestration of the Importin proteins to these fragments, as well as inhibition of cellular growth, highlighting the importance of this process to cellular function (76).

In order for Kpn β 1 to recognise its cargo proteins, they must contain a short sequence of highly basic amino acids termed a nuclear localisation signal (NLS) (77). The first NLS to be characterised was the lysine rich 'classical' NLS, PKKKRKV (78). This canonical NLS is similar to that of the SV40 Large T antigen and is recognised by the 60kDa protein Karyopherin α (Kpn α), which then binds to Kpn β 1 (74, 78–80). Kpn β 1 brings the complex to the nuclear envelope and interactions with the nucleoporins of the NPC to allow for nuclear import of the heterotrimer (71,76). This 'classical' pathway is the best characterised nucleocytoplasmic transport pathway to date. Distinct NLS sequences have since been identified, such as the bipartite NLS discovered in nucleoplasmin that also requires the Kpn α -Kpn β heterodimer, as well as other adapters being utilised by Karyopherin β . The bipartite sequence consists of two clusters of basic amino acids, separated by a linker region (82).

Recently, it has come to light that Kpn β 1 performs many other functions in the cell apart from nuclear transport (83). These various roles are prevalent in the cell cycle, where it appears to be a negative regulator of nuclear envelope and spindle assembly (84). After nuclear envelope breakdown, Kpn β 1 is transported to the spindle poles. A disturbance in the balance of the expression of Kpn β 1 affects cellular division and pole assembly(85). The organisation of the microtubules into spindles is controlled by

special proteins termed spindle assembly factors (SAFs). Examples of these are TPX2, NuMA, and the kinesin XCTK2. Kpn β 1 and Kpn α inhibit spindle formation by sequestering these SAFs. The small GTPase, Ran, acts in an antagonistic manner by displacing SAFs from Kpn α or β , thereby inducing spindle assembly (83, 86). Kpn β 1 has also been seen to negatively regulate nuclear membrane assembly by inhibiting NPC assembly as well as membrane fusion, also in concert with Ran (87). Additionally, nuclear pore proteins do not efficiently localise to the membrane in Kpn β 1 mutants, further emphasising its crucial function in correct NPC assembly (88).

1.3 Karyopherin β 1 and HIV nuclear import

Karyopherins have been shown to exhibit flexibility in their import pathways and targets, depending on the cellular context (89, 90). The HIV-1 virion may therefore utilise a variety of Karyopherins to achieve integration. Many different host factors have been suggested to import elements of the PIC. Importin 7 was seen to be involved in the active nuclear import of purified Reverse Transcription complexes (RTCs), as the PIC was previously named, in primary macrophages (91). Pull down studies revealed that Importin 7 binds directly to the C terminus of HIV Integrase, suggesting a mechanism for Importin 7's interaction with the PIC (92).

The cellular protein Lens-epithelium derived growth factor (LEDGF) has been implicated in facilitating nuclear import of the PIC by interacting with Integrase (93, 94). LEDGF/p75 binds directly to Integrase and aids in its nuclear import, as well as

integration activity by enhancing the DNA tethering of the enzyme (93, 94). One group showed that IN formed complexes with TNPO3/Importin 3 as well as Importin α in cultured cells transfected with Integrase and disruption of either of these complexes was seen to abrogate nuclear import *in vitro* (95).

Previously, Karyopherin β 1, together with Karyopherin α 2, was proven to bind and import a Capsid protein from HPV 11, suggesting it is a pathway commonly hijacked by invading viruses (96).

There is conflicting evidence in the literature regarding whether Kpn β 1 imports the karyophilic PIC proteins Matrix, Integrase and Vpr, as well as regulatory proteins such as Tat and Rev. In this study we focused on the regulatory HIV protein Tat and the PIC proteins Integrase and Vpr to determine whether Karyopherin β 1 mediates their nuclear import.

1.3.1 Tat Nuclear Import

Investigation into interactions of the HIV-1 Tat protein with the Karyopherin family of transporters has yielded varying results in previous studies. In particular, Tat has been shown to contain a non-classical NLS that conferred nuclear accumulation in the absence of an intact nuclear envelope, suggesting that this NLS targets binding to nuclear components *in vitro* (97). The same study found that in the absence of ATP, this nuclear accumulation was abrogated. This suggested that Tat was utilising an as yet discovered method to enter the nucleus. In contrast, Truant et al. (1999)

demonstrated that the arginine rich NLS in the Tat protein failed to interact with the conventional Karyopherin α adapter, but instead bound directly to Karyopherin β 1 and that this interaction was sufficient to direct nuclear import (64). This is not surprising as it has since been shown that arginine rich NLSs interact directly with Kpn β 1(70). Interestingly, this same NLS region has been shown to confer the RNA binding properties to the protein necessary for its transactivation of the HIV LTR promoter (98). Truant and colleagues also investigated other β -like transporters, such as Transportin, and found that they were not involved in Tat nuclear import (64). Other studies have also shown that Tat is able to interact with Kpn α and Kpn β , albeit in the absence of other competitors (100). The small size of the Tat protein also suggests that it may passively diffuse through the nuclear pore complex. This is indeed the case as seen by *in vivo* analyses of GFP tagged Tat peptide. FRAP analysis of nuclear/cytoplasmic exchange in energy depleted cells showed that no active transport mechanism contributed to the diffusion profile, which corresponded to benchmark passive diffusion proteins. These results prove that Tat nuclear accumulation is an energy independent process (66). However, GST-pull down studies performed in 2013 showed that Tat interacts directly with Importin 13. Residues 49R50K51K were identified as essential for import by this factor (100).

It is possible that Tat does all of the above depending on the cellular context. Redundant pathways most likely exist to ensure replication and as a result of years of evolutionary battles between virus and host.

1.3.2 Integrase Nuclear Import

Similarly, there is much controversy regarding the requirement of the Karyopherin proteins for the nuclear import of HIV-1 Integrase protein. The Integrase NLS has been suggested to be located at residues 161-173 due to nuclear import being abrogated by the presence of a synthetic peptide of this region. This region was sufficient to cause nuclear import of attached bovine serum albumin (BSA). This sequence alone, as well as full length IN interacted with Kp α and this interaction could be out competed by the IN NLS as well as the SV40 NLS (101). While Integrase seems to contain nuclear localisation signals, its interaction with the Karyopherins is still under debate.

HIV-1 Integrase is transported actively into the cell nucleus via a saturable mechanism, suggesting a cellular factor is involved. The first studied viral determinant of nuclear import was the Matrix protein. However, focus also shifted onto the Integrase protein when it was discovered that there is a direct interaction between tyrosine phosphorylated MA and the central domain of IN which results in MA incorporation into virions (102). However, it was observed that the karyophilic properties of IN alone are not responsible for nuclear targeting of the PIC (103). Mutagenic studies also point to the C-terminal tri-Lysine region of Integrase being integral in nuclear targeting of viral DNA, in addition to reverse transcription and integration (104).

1.3.3 Viral Protein R (Vpr) Nuclear Import

There is also conflicting evidence for the role of active nuclear import in Vpr nuclear transport. An early study showed that Vpr contains an as yet characterised NLS that does not interact with Karyopherin alpha and suggested it utilises a distinct nuclear import pathway (105). A contradictory study argued that Vpr does indeed bind to Karyopherin α as well as being competed from this binding by the molecular chaperone, heat shock protein 70 (Hsp70). The binding of either Vpr or Hsp70 to a novel binding site present on Kpn α resulted in nuclear import of the HIV-1 PIC. This binding also enhanced interaction between Kpn α and MA, a crucial requirement for import according to these authors (106). Although the PIC proteins appear to be individually karyophilic in nature, one cannot underestimate the power of their interactions with one another to ensure import of the PIC. While useful information can be obtained from studying the individual proteins, one must always bear in mind the true *in vivo* situation.

1.4 Project Aim

Much controversy surrounds the precise proteins and mechanisms involved in the entry of the HIV-1 viral proteins into the nucleus of target cells. In this study, the aim was to investigate if the nuclear transporter protein Karyopherin β 1 is involved in the nucleocytoplasmic translocation of key HIV-1 proteins.

1.5 Project Objectives

- (1) To investigate the transactivation capability of Tat after Kpn β 1 inhibition using siRNA and a small molecule inhibitor.

- (2) To determine if targeted inhibition of Karyopherin β 1 affects nuclear import of the HIV-1 proteins Tat, Viral protein R and Integrase.

CHAPTER TWO:

MATERIALS AND METHODS

2.1 Cell Culture

2.1.1 Cell lines

In order to study the transactivation capability of the HIV Tat protein, TZM-bl (JC53-bl) cells, a HeLa derived cell line stably expressing a firefly luciferase (*Photinus pyralis*) construct under the control of the HIV LTR promoter, were cultured (107). This cell line was kindly donated by Professor Carolyn Williamson, Medical Virology, UCT. HeLa cells are a human cervical cancer cell line and were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA).

2.1.2 Tissue cell culture methods

TZM-bl and HeLa cell lines were maintained at 60-80% confluency in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, Scotland) supplemented with 10% (v/v) fetal calf serum (FCS) (Highveld Biologicals, Modderfontein, Johannesburg, RSA), penicillin (100 units/ml) and streptomycin (P/S) (100 µg/ml).

Cells were incubated at 37°C in a 5% CO₂ incubator and were split using a 0.05% Trypsin-EDTA solution, which was then neutralised with DMEM. The cells were then pelleted by centrifugation and resuspended in 8ml fresh DMEM. 1ml of this solution was then added to 10ml fresh DMEM and plated in a 100mm dish. The remaining solution was re-centrifuged and suspended in 2ml cell freezing media (Appendix B). 1ml aliquots were transferred to cryovials for freezing. Cells were stored at -80°C for two weeks prior to long term storage in liquid nitrogen. This slow freezing process was to prevent ice crystal formation.

2.2. Short interfering RNA (siRNA)

The siRNA used to silence expression of Kpn β 1 was Karyopherin β 1 siRNA (h) (sc-35736, Santa Cruz Biotechnology, Santa Cruz, CA, USA). This siRNA consists of pools of three to five target-specific 19-25 nucleotide siRNAs designed to knockdown gene expression. As a control, non-silencing siRNA, MISSION® siRNA universal negative control (sic001, Sigma-Aldrich Inc., St Louis, USA) not complementary to any known mRNA sequence was used. siRNAs were received as lyophilised powders that were resuspended in RNAase-free water to yield working stocks of 10 μ M.

2.3. Drugs and Inhibitors

The small molecule inhibitor used to block Kpn β 1 function was compound 43(c43). c43 was the result of a functional screen for small molecules that could bind Kpn β 1 performed by our laboratory and was purchased from Molport (Riga, Latvia).

The phorbol ester, 12-o-tetradecanoylphorbol-13-acetate (TPA), was purchased from Sigma (Sigma-Aldrich Inc.). Ionomycin was obtained from Santa Cruz (sc-3592).

2.4 Transformation and Isolation of plasmid DNA

2.4.1 HIV expression vectors

As a means of studying the HIV-1 proteins Tat, Integrase, Vpr and Matrix, their respective expression vectors were acquired according to Table 2.1.

Table 2.1. Description and sources of HIV-1 expression vectors.

HIV protein	Expression vector	Source	Catalogue #	Tag	Selection Marker
Tat	pSV2tat72	NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: pSV2tat72 from Dr. Alan Frankel(108).	294	-	Ampicillin
GFP-Tat	pEGFP-Tat	Constructed in this study.	-	GFP	Kanamycin
GFP-Integrase	pEGFP-IN	Prof Zeger Debyser, Division of Molecular Medicine Katolieke Universiteit, Belgium (109).	-	GFP	Kanamycin
GFP-Vpr	pEGFP-Vpr	NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: pEGFP-Vpr from Dr. Warner C. Greene(110).	11386	GFP	Kanamycin

2.4.2 Transformation

Competent JM109 bacterial cells (Promega, Madison, WI, USA) were used to amplify plasmid DNA. 10-50 ng plasmid DNA was added to 30 µl JM109 cells and incubated on ice for 10-30 minutes. Thereafter the cells were heat shocked at 42°C to encourage DNA uptake. Cells were allowed to recover and growth was encouraged by the addition of 450 µl LB media and incubation at 37°C for 1 hour with shaking.

Thereafter, cells were ready to be spread onto agar plates containing either 30 µg/ml Kanamycin or 100 µg/ml Ampicillin, depending on the selection marker present in the plasmid. Only those cells that selectively took up the plasmid will survive in the antibiotic, yielding positive colonies. Plates were incubated at 37°C overnight and the following day individual colonies were picked and grown up in a starter culture of 5ml LB containing either 60 µg/ml Ampicillin or 30 µg/ml Kanamycin for 6-8 hours. Thereafter the starter cultures were transferred into 100ml LB containing either 100 µg/ml Ampicillin or 60 µg/ml Kanamycin and allowed to grow to late log phase overnight at 37°C with shaking.

2.4.3 Isolation of Plasmid DNA

In order to isolate and purify plasmid DNA from transformed JM109 cells, plasmid preparations were performed. For small scale purification for screening purposes, minipreps using the PureYield™ Plasmid Miniprep system (Promega) were performed. For medium to large scale purification for subsequent transfections, midi and maxi-preps were carried out. For midipreps, the PureYield™ Plasmid Midiprep System (Promega) was used, while for the maxipreps, the Qiagen Plasmid Maxi Kit was used. DNA was purified as per the manufacturer's instructions.

2.4.4 Plasmid sequence confirmation

To confirm isolation of the correct plasmids, restriction digests were performed to check if the correct insert was released. Digests were performed using conditions

optimal for each restriction enzyme and double digests carried out in compatible buffers only.

2.4.5 Agarose gel electrophoresis

Digests were run on 1% (w/v) agarose gels to confirm the correct sizes. The 1% agarose was melted in 1XTBE buffer for two minutes in a microwave oven. Once cooled, a final concentration of 0.5µg/ml ethidium bromide was added to the gel to visualise the DNA bands under ultraviolet (UV) light. The gel was then allowed 20 minutes to set before samples were loaded. The gel was then run at 100V for ~1 hour or until desired bands were separated adequately.

2.4.6 Glycerol Stocks

Transformed JM109 cells were stored long term in glycerol at -80°C. A suspension of 50% cells, 50% glycerol was made that once frozen could be used again by simply scraping off a portion of the glycerol/cell mixture and inoculating into LB media again.

2.5 Molecular Cloning of Tat cDNA from pSV2-tat72 into pEGFP-C2

To allow for visualisation of HIV-1 Tat, it was necessary to construct a GFP-tagged recombinant plasmid. Tat cDNA was thus cloned from its original pSV2-tat72 vector into the pEGFP-C2 vector to generate a GFP-Tat construct that would allow tracking of GFP-Tat localisation via fluorescence microscopy.

2.5.1 Tat Primer Design

To amplify Tat cDNA, primers were designed according to the sequence for the Tat gene provided with the pSV2-tat72 vector map. To allow for in frame ligation into pEGFP-C2, restriction sites (KpnI and XhoI) corresponding to sites present in the multiple cloning site (MCS) of pEGFP-C2 were incorporated into the primers. These sites were chosen as they were not present in the pGEM-T Easy plasmid used for sub cloning and sequencing. The sites were also not present within the Tat cDNA sequence. The primers were designed with the aid of the Primer Blast program on the NCBI website (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>).

The resulting primer sequences, with restriction sites KpnI and XhoI underlined were:

F: 5' ATC TCG AGA ATG GAA CCG GTC GAC CCG 3' and

R: 5' CGG GTA CCT CAC TGT TTA GAC AGA GAA ACC TG 3'

2.5.2 Polymerase Chain Reaction (PCR)

PCR was performed using the above primers to amplify the tat gene from 50ng of template pSV2-tat72 plasmid DNA. The reaction consisted of 1X PCR buffer (1.5mM MgCl₂), 20pmol of each primer, 0.2mM dNTP's and 1 Unit of high fidelity Pfu DNA polymerase (Thermo Scientific). The following conditions were then applied for the PCR reaction: 95°C for 5 mins, 35 cycles of 95°C for 30 seconds, 50°C for 30 seconds and 72°C for 30 seconds and finally an extension step at 72°C for 5 minutes. The resulting PCR product was then subjected to electrophoresis on a 1% agarose gel. The size of the bands was determined using the O'Gene Ruler DNA Ladder Mix Marker (Fermentas Inc., Waltham, MA, USA).

2.5.3 Purification of DNA from agarose gel

DNA bands were visualised under low UV light (230-50Hz, Ultratec Ltd) in a Uvitec Light box and excised from the agarose gel using a sterile blade. The DNA band was purified using the Wizard® SV gel and PCR Clean-up Kit (Promega) according to the manufacturer's protocol.

2.5.4 A-tailing Reaction

The gel purified PCR product was then subjected to an A-tailing reaction in order to ligate into the T-tailed region of pGEM-T-Easy (Promega). A low fidelity polymerase, Taq Polymerase, was thus employed. The reactions was performed using

6µl of the purified PCR product and 5 Units of Taq Polymerase (Stratagene, USA) in a reaction containing 1 X Taq buffer, 2.5mM MgCl₂ and 0.2mM dATP. This reaction was incubated for 30 minutes at 70°C in a PCR machine and the resulting product directly ligated into the pGEM-T-Easy cloning vector (Promega). For this ligation, 3µl of PCR product, 50ng of pGEM-T-Easy, 1 X Rapid Ligation Buffer, and 3 Units T4 DNA Ligase (Promega) were incubated at 4°C overnight.

The next day 5µl of this ligation mix was transformed into 30µl competent *E.Coli* JM109 cells. The cells were first left on ice for 30 minutes and thereafter heat shocked at 42°C for 2 minutes. Subsequently 0.45ml Luria broth was added and the cells incubated for 37°C. After this incubation the cells were spread onto agar plates containing 100µg/ml Ampicillin, 100µl of IPTG and 20µl of X-gal. Successful cloning results in the disruption of the lacZ gene, resulting in white colonies as the X-gal cannot be cleaved in the absence of β-galactosidase, which usually results in a blue product. Thus, white colonies were picked and grown up overnight in 5ml of LB media containing 100µg/ml Ampicillin. Glycerol stocks were stored the following day before proceeding with isolation of the plasmid DNA via minipreps, as described above.

2.5.5 Plasmid mapping and sequence confirmation

In order to verify the correct plasmid had been purified, restriction enzyme digestions were performed. Enzymes present in the PCR primers that would release the insert were used. KpnI and BamHI, both FastDigest Enzymes (Fermentas), were added to

1µg of each miniprep clone as well as 1X FastDigest Green Buffer (Fermentas) and allowed to react for 1 hour. The digests were then loaded directly onto a 1% agarose gel and electrophoresed for ~1hr. All clones were seen to release the 240bp Tat insert from the pGEM-T-Easy vector.

One correct glycerol stock was then grown up and large-scale plasmid preparations performed using the Qiagen Maxi Kit as per the manufacturer's instructions. Thereafter, sequencing was performed to validate that the insert was indeed the tat gene. The sequencing reaction involved 500ng template DNA (pGEM-T-Easy with insert), 2µl Ready Reaction Premix, 2µl 5X sequencing buffer BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) sequencing buffer, 6.4pmol either T7 or SP6 Primers and dH₂O up to 20µl. The PCR reaction for each sample was then performed as follows: 96°C for 1 minute followed by 25 cycles of 96°C for 10 seconds, 45°C for 5 seconds, and 60°C for 4 minutes, after which the PCR products were sent to the UCT Human Genetics Sequencing Unit.

2.5.6 Ligating the Tat insert into pEGFP-C2

After sequencing confirmed the correct insert was sub cloned into pGEM-T-Easy, Tat cDNA was cloned into the final plasmid. Firstly the insert was released from pGEM-T-Easy by digestion with the Fermentas FastDigest restriction enzymes KpnI and XhoI. The final pEGFP-C2 vector was linearised with the same restriction enzymes. The digests were then run on a crystal violet stained gel to prevent Ultraviolet (UV) damage to the sticky ends. The gel and 1X TAE buffer contained crystal violet dye at

a concentration of 10µg/ml and the DNA was then visualised using a light box in the absence of UV. The released Tat insert and the linearised pEGFP-C2 plasmid were both gel purified using the Wizard® SV Gel and PCR clean-up System (Promega) as per the manufacturer's instructions.

The DNA was then quantitated on a NanoDrop 2000 machine and the ligation performed according to the following equation,

$$\frac{ng\ vector}{kb\ insert} \times kb\ insert \times molar\ ratio \frac{insert}{vector} = ng\ insert$$

The ng of vector used is normally 100ng and the molar ratio of insert to vector is usually within the range of 1:1 to 1:10. After many attempts at optimising this ratio, it was found that a ratio of 60:1 worked, despite this being outside the usual range found in the literature. The vector and insert DNA were combined at 100ng vector and 250ng insert DNA. Thereafter 1X Ligase buffer, 1µl 50% PEG and 5 Units T4 DNA Ligase, and dH₂O up to 10µl were added to the DNA and the reaction was incubated at room temperature overnight. The following day, the entire ligation mix was transformed into 40µl JM109 *E.coli* cells as described previously, and plated onto agar plates containing 30µg/ml Kanamycin. Ten colonies were picked and grown up in 5ml LB containing 30µg/ml Kanamycin and minipreps performed. Once a correct clone was identified by restriction enzyme digestion to release the insert, this clone was amplified and purified using the Qiagen MaxiKit.

2.6 Transfection

2.6.1 Transient Transfection of siRNA into cells

TZM-bl and HeLa cells were plated at approximately 40-60% confluency (100-120 000 cells/ 35mm dish). Once the cells had adhered overnight, short-interfering RNA (siRNA) was transfected using Transfectin (Bio-rad) Transfection Reagent. For a 35mm dish, media was aspirated off and replaced with 1ml of fresh complete DMEM. For transient transfection, 0.625 μ l Transfectin was added to 50 μ l serum and penicillin/streptomycin free DMEM and allowed to incubate for 5 minutes. Thereafter, 2 μ l of 10 μ M siRNA (Kpn β 1 or non-silencing control) was added to the mixture and the reaction incubated for 15 minutes at room temperature to allow complex formation. The transfection mix was then added to the cells in a drop-wise manner, resulting in a final concentration of 20nM siRNA. Transfection mixes were left on the cells overnight.

2.6.2 Transient Transfection of HIV-1 expression plasmids

TZM-bl and HeLa cells were plated at 60-80% confluency the day before transfection with plasmid DNA. The HIV-1 expression vectors pSV2-Tat72, GFP-IN, GFP-Vpr, GFP-Gag and pEGFP-Tat were then transfected into the cells according to the conditions listed in Table 2. Transfection mixes were left on the cells overnight and the media was changed the following day.

Table 2.2. Table of transfection conditions used for HIV expression plasmids.

HIV-1 Expression Vector	Transfection Reagent	µg plasmid DNA/35mm
pSV2-Tat72 (Luciferase assays)	Transfectin™ Lipid Reagent (Bio-Rad)	0.2
pEGFP-Tat (Fluorescent microscopy)	Genecellin Transfection Reagent (BioCellChallenge, Toulon, France)	2.0
GFP-IN	Genecellin Transfection Reagent (BioCellChallenge, Toulon, France)	2.0
GFP-Vpr	Genecellin Transfection Reagent (BioCellChallenge, Toulon, France)	2.0

2.7 Luciferase Assays

2.7.1 NFAT assays

To confirm the ability of the Karyopherin β 1 small molecule inhibitor c43 to block nuclear import, NFAT luciferase assays were performed. Nuclear factor of activated T cells (NFAT) is a transcription factor that translocates into the nucleus in the presence of intracellular calcium(111). Nuclear translocation of NFAT occurs by recognition of its NLS by the Karyopherin α/β pathway (112–114). The phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) and the calcium salt Ionomycin work synergistically to release intracellular calcium stores and activate NFAT nuclear import. The addition of a drug that blocks nuclear import would see a decrease in NFAT import and a resulting decrease in luciferase expression from its target gene.

To perform NFAT assays, 25 000 HeLa cells per well were plated in a 24 well plate. The following day, 50ng GFP-NFAT (AddGene, Cambridge, MA, USA), 50ng NFAT-Luciferase (AddGene) and 5ng pRLTK-Renilla (*Renilla reniformis*) (Promega) were transfected per well using the GeneCellin™ (BioCellChallenge) Transfection Reagent. For this transfection, 50µl of DMEM media, 50ng GFP-NFAT, 50ng NFAT-Luciferase and 5ng pTK-Renilla and 0.4µl GeneCellin™ (BioCellChallenge) Transfection Reagent were added together in a 1.5ml microcentrifuge tube and incubated for 15 minutes. Thereafter the mixture was added drop wise to the cells.

The following day the Karyopherin β1 small molecule inhibitor c43 was added to the cells at its IC₅₀ concentration, as well as 15µM. Cells were incubated in the presence of c43 overnight. The following day, TPA and Ionomycin were added to the cells at a concentration of 100nM TPA and 1.3uM Ionomycin per well, in the continued presence of c43. After 5 hours, luciferase and Renilla luminescence were measured using a Glomax Luminometer (Promega).

2.7.2 Tat Transactivation in TZM-bl cells

In order to test Tat transactivation capability, luciferase luminescence was measured from TZM-bl cell lysates after transient transfection of pSV2-tat72 following siRNA transfection. TZM-bl cells containing the HIV LTR promoter attached to the luciferase reporter gene were plated in a 24 well dish at 40 000 cells/well. Renilla- ptk was used as a transfection control at 5ng per well. Cells were washed twice with 1 X

PBS before addition of 1X Passive Lysis Buffer (Promega) for 15 minutes on a shaker. Thereafter luminescence was read on a Glomax Luminometer (Promega) using the Dual-Luciferase® Reporter Assay System (Promega). According to this protocol, 40µl cell lysate was combined with 40 µl of the luciferase substrate, Luciferase Assay Reagent II (LAR II), and subsequent firefly luminescence read. In order to control for variations in plating and transfection, Renilla was read from the same wells by addition of its substrate, Stop & Glow® (Promega), which effectively quenched the luciferase reading and read the luminescence produced by Renilla. Values for each treatment were taken in quadruplicate.

2.8 Immunocytochemistry and Fluorescent Microscopy

In order to track the subcellular localisation of GFP-tagged Tat, IN and Vpr, fluorescent microscopy experiments were performed. Cells were plated at 200 000 cells per 35mm well on coverslips in a 6 well dish. The following day the relative plasmids were transfected in using conditions specific to each plasmid. The next day cells were treated with siRNA against Kpnβ1. Cells were allowed to incubate overnight to ensure knockdown and media was refreshed the next day. To allow visualisation of Kpnβ1 in order to confirm knockdown as well as study co-localisation of HIV-1 proteins and Kpnβ1, the protein was stained with the cy3 secondary antibody. It could then be detected in the red channel on the confocal microscope.

Briefly, cells were washed twice with 1XPBS and subsequently fixed in 4% paraformaldehyde for 20 minutes at room temperature. Following this incubation the

cells were washed three times with 1ml of 1XPBS. After the washing steps, the cells were permeabilised with 0.5% Triton X-100 for 5 minutes. Thereafter, the cells were washed with 1X PBS then quenched with 50mM NH₄Cl for 5 minutes. The cells were then blocked in 0.2% gelatin in PBS for 30 minutes. Following this blocking step, 100µl of the primary antibody (rabbit anti-Kpnβ1) diluted in blocking solution (1:200) was added to each coverslip and covered with a square of parafilm to prevent evaporation. The cells were incubated for 45 minutes in primary antibody before being washed a further three times with 1X PBS for 5 minutes each. The secondary antibody, Cy3-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch, USA), was then added in a similar manner diluted in blocking solution (1:300) and incubated for 45 minutes at room temperature. After washing once with 1XPBS, the cells were stained with the DNA binding dye, DAPI (100ng/ml in PBS) for 5 minutes. After a final wash with 1XPBS, the coverslips were rinsed in dH₂O and mounted in 15µl Mowiol (Calbiochem, MerckMillipore, USA). The mounted coverslips were allowed to dry for a minimum of 5 hours at room temperature before viewing under the microscope. Slides were examined using the X100 objective under oil immersion. Images were captured using an LSM 510 AxioObserver (Zeiss, Oberkochen Germany) and Zen 2009 software (Zeiss). The Excitation wavelength was 405nm for DAPI, 488nm for GFP and 570nm for Cy3. In order to compare control and Kpnβ1 siRNA treated cells, GFP fluorescent intensity in individual cells was quantitated and the nuclear/cytoplasmic fluorescent intensity analysed.

2.9 Western Blot analysis

2.9.1 Antibodies

All primary antibodies and incubation conditions used are shown in Table 2.3.

Antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA),

The NIH AIDS Research and Reference Reagent Programme or Bio-Rad.

Table 2.3. Primary and Secondary Antibody Conditions. (Kpn β 1; Karyopherin β 1, GFP; Green fluorescent protein, TBP; Tata-binding protein, IN; Integrase, milk; Elite fat free milk powder, NIH; NIH AIDS Research and Reference Reagent Program).

Primary Antibody	Primary Antibody conditions	Secondary Antibody	Secondary Antibody conditions	Substrate
Kpn β 1(H300) [sc-11367, Santa Cruz Biotechnology]	1:1000 in TBST	Goat anti-rabbit [Bio-rad]	1: 5000 in TBST	LumiGlo [®] (Kirkegaard & Perry Laboratories, Inc.(KPL), Gaithersburg, MD, USA)
GFP(FL) [sc-8334, Santa Cruz Biotechnology]	1:1000 in TBST	Goat anti-rabbit [Bio-rad]	1:5000 in 5% milk	Supersuper (Pierce, Thermo Scientific, Rockford, IL, USA)
TFIID(TBP) [sc-204, Santa Cruz Biotechnology]	1:500 in TBST	Goat anti-rabbit [Bio-rad]	1 in 5000 in TBST	LumiGlo [®] (KPL)
β -Tubulin(H-235) [sc-9104, Santa Cruz Biotechnology]	1:1000 in TBST	Goat anti-rabbit [Bio-rad]	1 in 5000 in TBST	SuperSignal West Pico Chemiluminescent Substrate (Pierce)

2.9.2 Protein Extraction

Protein was extracted from cells using radioimmunoprecipitation assay buffer (RIPA) containing a 1X complete protease inhibitor cocktail (Roche, Mannheim, Germany) and 0.1M sodium orthovanadate phosphatase inhibitor. The cells are thus lysed while preventing degradation from endogenous proteolytic and phospholytic enzymes. Cell lysates were sonicated for approximately 10 seconds and then centrifuged for 10 minutes at 10 000 rpm at 4°C to remove cell debris.

2.9.3 Protein quantification

Protein samples were quantitated using the Thermo Scientific™ Pierce™ BCA Protein Assay which uses bicinchoninic acid (BCA). This assay is based on the principle that proteins will reduce Cu^{2+} to Cu^{1+} in an alkaline medium and the reaction product is purple in colour and can be read at a wavelength of 595nm. This purple colour is formed by the chelation of two molecules of BCA with one Cu^{1+} ion and its intensity is directly proportional to the amount of protein present. The absorbance is read at 595nm on a Bio-Tek EL800 (Bio-Tek Instruments, Winooski, Vermont, Canada) reader using Gen5 software (Bio-Tek). A bovine serum albumin (BSA) dilution series is read in parallel to produce a standard curve of known concentrations plotted against absorbance values; from this the protein concentration of unknown samples can be determined.

2.9.4 Sodium Dodecyl Sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl-sulphate (SDS) polyacrylamide gels were set to a thickness of 1.5mm. Protein samples (20 or 30µg) were electrophoresed initially on a 4% stacking gel and then separated on either a 10% or 15% resolving gel. The Spectra™ Multicolor Broad Range Protein Ladder (Thermo Scientific) was loaded to determine the sizes of the separated proteins. The gels were run at ~150V for 1 hour in 1X running buffer (recipe in Appendix B).

2.9.5 Immunoblotting

Proteins were then transferred horizontally onto a Hybond™-ECL™ nitrocellulose membrane (Amersham Life Sciences, Amersham, UK) on ice at 100V for 70 mins in 1X Transfer buffer (recipe in appendix). The membrane was then blocked in 5% fat free milk in TBST for 1 hour at room temperature on a shaker at 70rpm. This blocking process was to prevent non-specific binding of the antibody to the membrane. After blocking, the antibody was added as in table 2.3 and the membrane left overnight on a shaker at 4°C.

The next day the membrane was washed three times for 10 minutes each with TBST. Secondary HRP antibody was added as in Table 2.3 for 1 hour at room temperature with gentle shaking. Thereafter the membrane was washed an additional three times for 10 minutes each. The blot was then either detected or stored at 4°C in TBST for no longer than two days.

2.9.6 Immunodetection

For detection of specific protein bands, LumiGLO Chemiluminescent Reagent (KPL) or SuperSignal West Dura/ west pico (Pierce) were applied to the membrane according to the manufacturer's instructions. These reagents contain the substrate, luminol to 3-aminophthalate, that the horse radish peroxidase enzyme oxidises to produce a light emitting product. This light causes the silver halide crystals in the x-ray film to change metallic black in colour, indicating where the protein is located as well as the amount.

2.9.7 Densitometric quantification of Western Blot bands

In order to compare protein levels, quantification of protein bands was performed by densitometry using Image J software (NIH, USA). Protein expression levels were normalised to β -tubulin.

2.10 Fractionation of Nuclear and Cytoplasmic compartments

As a means of studying subcellular localisation of HIV-1 proteins via Western blot analysis, nuclear and cytoplasmic fractions were separated. Cells were plated in 10cm dishes and protein was harvested by trypsinisation. Cells were then lysed and cellular compartments separated using the Thermo Scientific™ NE-PER™ Nuclear and Cytoplasmic Extraction Reagents kit (Pierce). Nuclear and cytoplasmic protein samples were then stored at -80°C until subjected to Western blot analysis to monitor HIV protein localisation following Kpnβ1 inhibition.

2.11 Statistical Analysis

All statistical analyses were performed using GraphPad Prism® Software. All figures were also generated using this programme. A p value of <0.05 was considered statistically significant. The ANOVA statistical tests with the Tukey post-test were used to determine significance between multiple samples. The Student's t test was used to determine significance between two paired samples.

CHAPTER THREE:

THE EFFECT OF KPN β 1 INHIBITION ON HIV-1 TAT TRANSACTIVATION AND LOCALISATION

3.1 Introduction

The HIV Trans-activator of transcription (Tat) is an essential protein to HIV replication and is active in the cell nucleus. Tat functions as a unique transcriptional activator by binding a cis acting RNA enhancer element, the transactivating-responsive region (TAR), present at the 5' end of all viral transcripts (nt +1 to +59) (41, 115). The TAR binding domain within Tat corresponds to its Arginine-rich presumed NLS. Arginine-rich sequences are found in many RNA binding proteins and are thought to aid in RNA hairpin recognition (98, 116). Tat also enhances elongation of HIV transcripts by interacting with positive transcription elongation factor b (p-TEFb), which is known to release the paused RNA polymerase machinery, allowing for the elongation of the already initiated mRNA chain. It does this by phosphorylating the C-terminus of RNA polymerase II (98, 117, 118).

HIV infection is characterised by depletion of CD4⁺ target cells, leading to immune deficiency. Although not fully understood, one such mechanism proposed as the leading cause of this depletion is apoptosis induced by the regulatory HIV protein, Tat (42, 43). These are not the only functions of the Tat protein, however. It appears to be a pluripotent protein with as yet undiscovered effects on host cells.

The aim of this chapter was to investigate whether Karyopherin β 1 mediates the translocation of the Tat protein from the cytoplasm to the nucleus, where it performs its trans-activating function. Inhibition of Tat nuclear import would result in a dramatic decrease in HIV replication rate and this pathway therefore represents a novel therapeutic target. Elucidating the mechanisms of this pathway would also add to our current understanding of HIV biology.

In order to test whether Karyopherin β 1 (Kpn β 1) is important for Tat nuclear import, we employed a functional approach to determine whether inhibition of Kpn β 1 affects Tat transcriptional activation activity. For Tat to exert its transactivation effects, it has to be present in the cell nucleus and a block to its nuclear import pathway would therefore cause a decrease in HIV-1 LTR promoter activity (119). To assess Tat transactivation capability, we employed TZM-bl cells that have been stably transfected with the HIV LTR promoter attached to the firefly luciferase reporter gene (Figure 3.1).

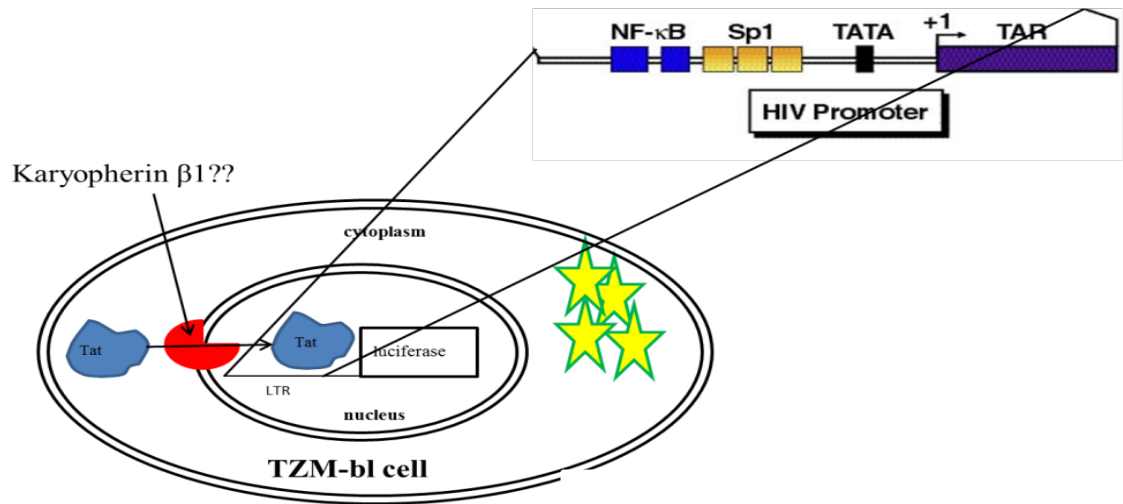


Figure 3.1. Diagram depicting TzM-bl cells used for Tat transactivation luciferase assays. TzM-bl cells are HeLa cells stably transfected with the HIV LTR promoter attached to the luciferase reporter gene. They also stably express the cell surface receptors CD4 and CCR5 and therefore more closely mimic HIV target cells.

3.2 Results

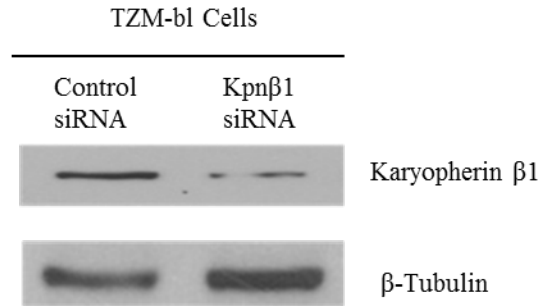
3.2.1 Tat transactivation capability after inhibition of Karyopherin β 1 with siRNA

We hypothesised that if Kpn β 1 is necessary for Tat nuclear import, it is expected that Tat transactivation capability will be reduced when Kpn β 1 is inhibited. To determine the requirement of Kpn β 1 for Tat transactivation, we silenced expression of Kpn β 1 using siRNA technology complementary to the Karyopherin β 1 mRNA sequence. Western blot analysis was performed to confirm effective knockdown of Kpn β 1 protein levels in Kpn β 1siRNA transfected TZM-bl cells. Control siRNA (Sigma, sc001) composed of a scrambled non-specific sequence was used as a control for transfection effects. Our results show that Kpn β 1 protein expression was effectively inhibited with the siRNA (Figure 3.2A).

To address whether Kpn β 1 was necessary for the transactivating activity of HIV-1 Tat, a luciferase reporter assay was performed to measure the HIV-1 LTR promoter's activity in TZM-bl cells. Cells were transfected with siRNA followed by transfection with the HIV-1 Tat expression vector (pSV2-Tat72). Luciferase activity was monitored 48 hours post Tat transfection and the HIV-1 LTR luciferase activity corrected for normalised using pTK-Renilla as an internal control.

The results of this experiment showed that the addition of recombinant Tat protein resulted in a substantial and significant increase in LTR promoter activation (Figure 3.2B). This increase was significantly abrogated by inhibition of Kpn β 1 using siRNA for 48 hours (Figure 3.2B). Addition of exogenous Tat resulted in a more than 5000 fold induction in LTR promoter activity, highlighting the potency of Tat as an HIV-1 transcriptional activator. Control experiments without the addition of Tat accounted for basal activity of the HIV-LTR promoter in TZM-bl cells. It also accounted for those host transcription factors such as NF κ B and Sp1 that activate the LTR promoter and are also imported by Kpn β 1. A slight reduction in basal promoter activity in the absence of Tat was therefore expected after Kpn β 1 siRNA. In the presence of Tat, Kpn β 1 siRNA significantly inhibited activation of the HIV LTR promoter. These results suggest that Kpn β 1 is necessary for Tat transactivation of the HIV LTR promoter.

(A)



(B)

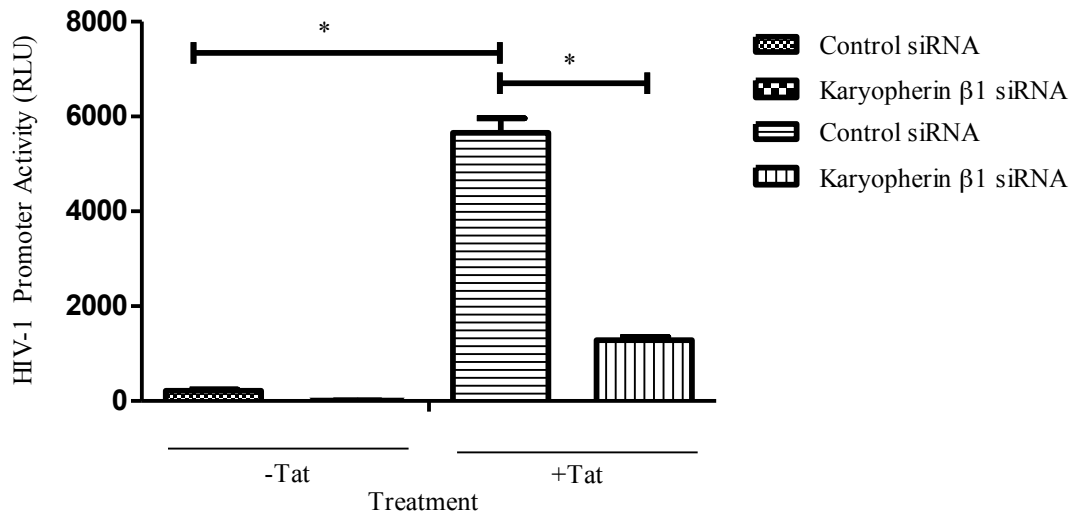


Figure 3.2. Effect of Karyopherin β1 inhibition using siRNA on Tat activity (A) Western blot analysis confirming knockdown of Kpnβ1 protein levels after transfection with Kpnβ1 siRNA for 48 hours in TzM-bl cells. **(B)** Relative luciferase activity as a measure of the HIV LTR promoter activity after inhibition of Kpnβ1 with siRNA. Addition of Tat significantly enhances promoter activity, while Kpnβ1 siRNA treatment significantly inhibits this effect. Results shown are the mean ± SEM of experiments performed in quadruplicate and repeated at least two independent times. *p<0.05. RLU: Relative Luciferase Units.

Interestingly, we noted that Kpn β 1 siRNA treatment resulted in a tenfold increase in activity of the transfection control, Renilla luciferase (Figure 3.3A, Experiment 1), suggesting an increase in transfection efficiency upon Kpn β 1 knockdown. We hypothesised that this could be due to the inhibition of Kpn β 1 resulting in aberrant nuclear membrane reassembly as Karyopherin β 1 is integral in nuclear membrane assembly processes (83, 88). This would allow easier entry of cDNA into the nucleus, resulting in increased transfection efficiency.

Under these assumptions, we speculated that transfecting with Kpn β 1 siRNA first may result in inflated HIV Tat transfection efficiency and in this way impact the interpretations of our results. We therefore repeated the experiment and all subsequent experiments transfecting the Tat plasmid at least 24 hours before the siRNA to ensure the Tat construct and pTK-Renilla would be transfected evenly throughout. When the experiment was performed in this order, there was no longer a significant increase in Renilla luciferase after Kpn β 1 siRNA transfection (Figure 3.3A, experiment 2). When the experiment was performed in this manner, a significant reduction in Tat transactivation capability upon Kpn β 1 knockdown was still observed (Figure 3.3B). These results suggested that Kpn β 1 mediates the nuclear transport of HIV Tat.

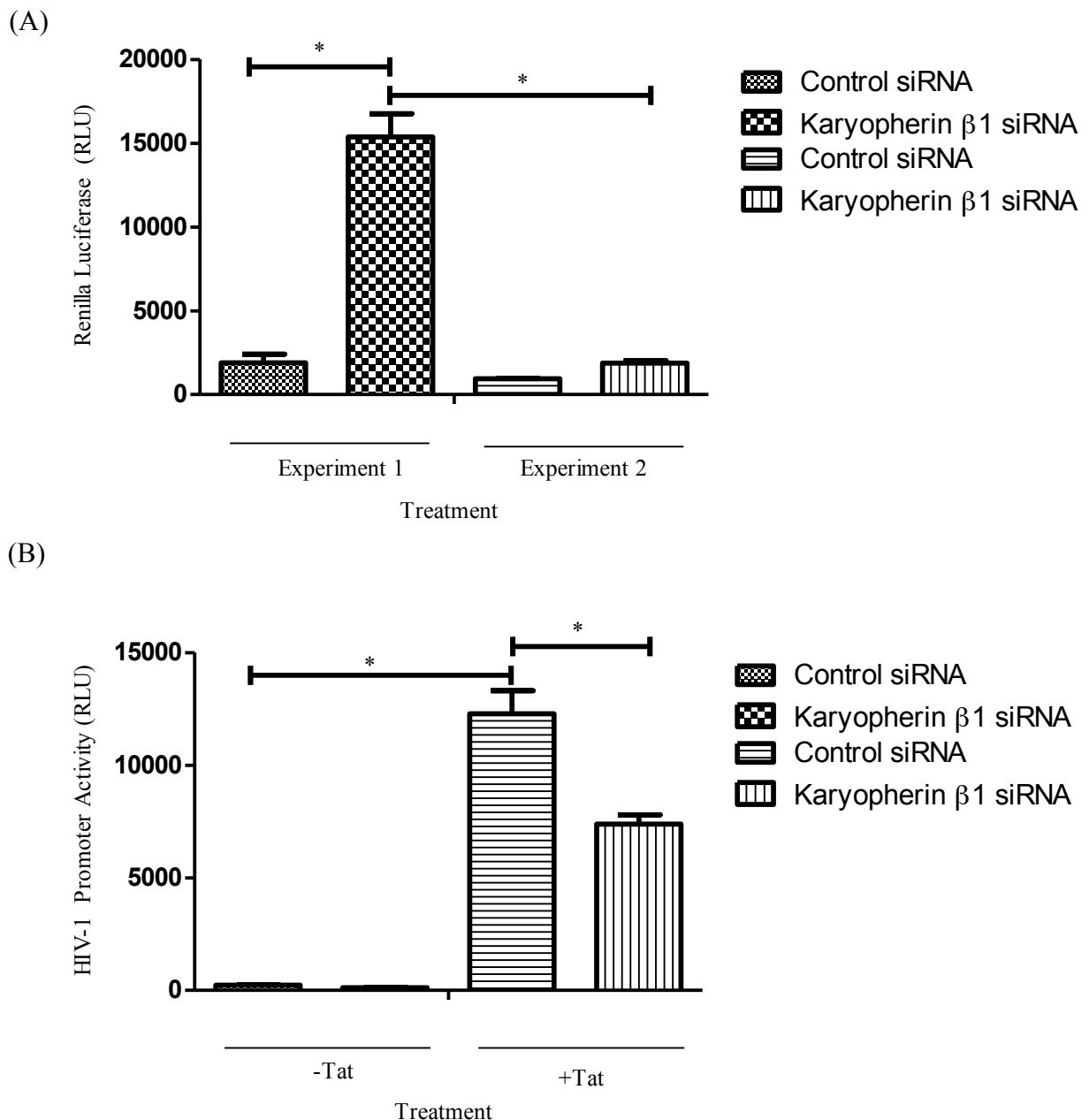


Figure 3.3 (A) Graph showing Renilla luciferase values from promoter assays performed in two different ways. Experiment 1: siRNA transfected before Tat and Renilla plasmids resulted in an increase in transfection and resulting expression of these plasmids as see by the significant increase in Renilla following Kpn β 1 siRNA transfection. Experiment 2: When siRNA was transfected after Tat and Renilla, this increase was no longer significant. All subsequent experiments were performed in the order of experiment 2. (B) Luciferase assay performed by transfecting Tat plasmid DNA before siRNA. A significant decrease in Tat transactivation capability after Kpn β 1 inhibition is still observed. Results shown are the mean \pm SEM of experiments performed in quadruplicate and repeated at least two independent times, * p <0.05. RLU: Relative Luciferase Units.

3.2.2 Tat transactivation capability after inhibition of Karyopherin β 1 with the small molecule inhibitor, c43

Our laboratory has an interest in identifying small molecule inhibitors of Kpn β 1. Research in our group identified a small molecule, named compound 43 (c43), with potential to inhibit nuclear import. The c43 compound was identified using a computational screening approach undertaken in our laboratory that aimed to identify compounds that would bind the 331-364 amino acid residue region within Kpn β 1 that binds RanGTP, Kpn α 2, as well as cargo (Figure 3.4). Compound 43 has been studied extensively in our laboratory as an anti-cancer drug that blocks nuclear import and the functions of Kpn β 1.

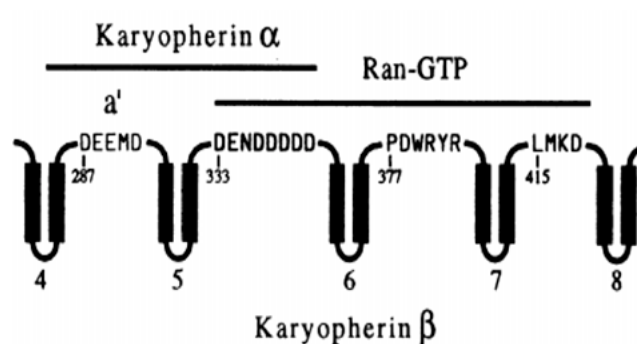


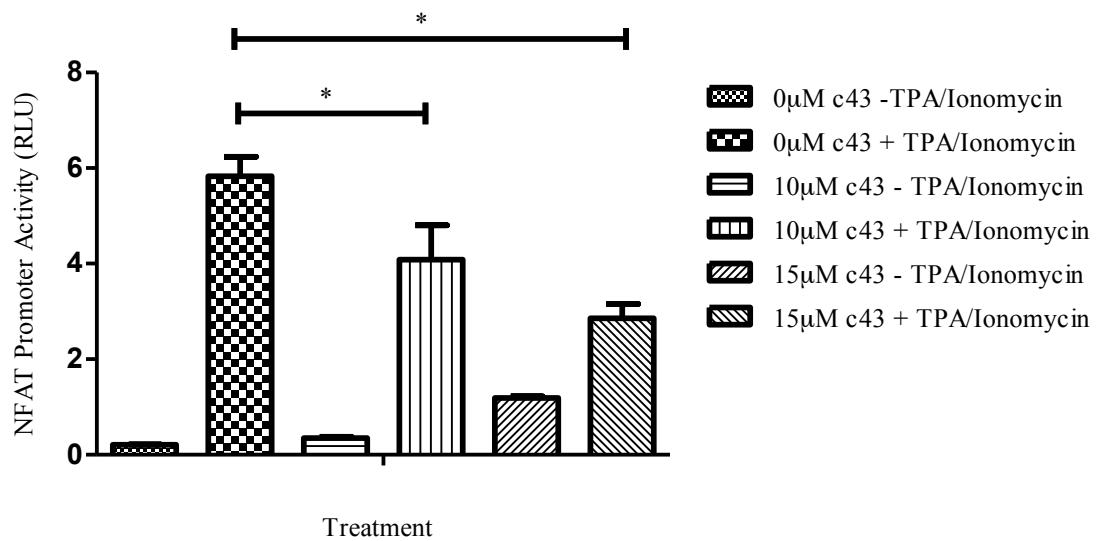
Figure 3.4. Schematic depiction of the overlapping binding sites on Karyopherin β 1 for Karyopherin α and Ran-GTP. Compound c43 binds in the region of residues 331-364. Adapted from (75).

This small molecule inhibitor of Kpn β 1 was thus used to further evaluate the requirement of the Kpn β 1 mediated nuclear import system in Tat transactivation. To confirm that c43 was functional at blocking nuclear import via Kpn β 1, the nuclear translocation of NFAT was analysed. NFAT is a transcription factor that is imported into the nucleus via Kpn β 1 in response to intracellular calcium release (112). A small

molecule inhibitor of Kpn β 1 would therefore interfere with NFAT nuclear translocation and activation of an NFAT-responsive promoter construct. To stimulate NFAT translocation to the nucleus, TPA and Ionomycin were used to release intracellular calcium and the effect of Kpn β 1 inhibition using c43 monitored by measuring NFAT promoter activity. In this manner, c43 was shown to significantly block NFAT transcriptional activity in a dose dependent manner (Figure 3.5A). This result served as validation that c43 inhibits Kpn β 1 mediated nuclear transport.

To investigate the effect of c43 on Tat transactivation capability, TZM-bl cells transfected with a Tat construct were treated with 10 μ M (the IC₅₀ of c43) and 15 μ M of c43 and the relative luciferase activity analysed as a measure of HIV LTR promoter activity. Our results showed that c43 caused a significant decrease in Tat transactivation function at both 10 μ M and 15 μ M concentrations (Figure 3.5B). These results further support the role of Kpn β 1 in Tat nuclear import.

(A)



(B)

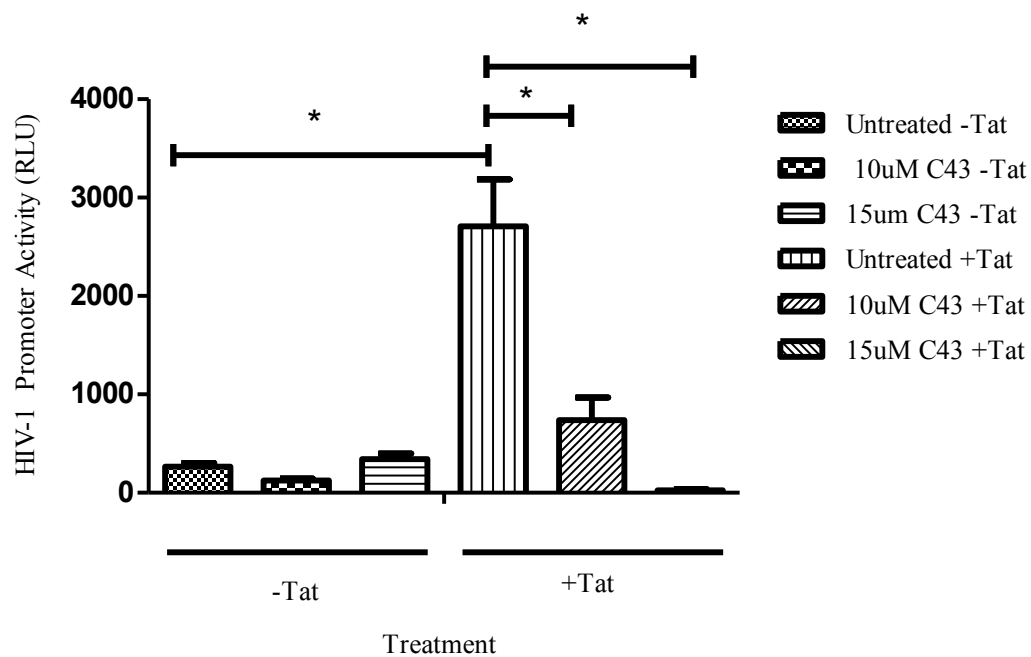


Figure 3.5 (A) NFAT assays showing inhibition of NFAT nuclear translocation after treatment with the Kpnβ1 inhibitor c43 at 10µM and 15µM. (B) Promoter assay showing a decrease in Tat transactivation capability after inhibition of Kpnβ1 with c43 treatment in TZM-bl cells. Results shown are the mean ± SEM of experiments performed in quadruplicate and repeated three independent times.*p <0.05, *** p < 0.01. RLU: Relative Luciferase Units.

3.2.3 Cloning of HIV-1 Tat into the expression vector pEGFP-C2

Having shown that inhibition of Kpn β 1 reduces Tat transactivating activity, we next wanted to further assess whether Kpn β 1 mediates Tat nuclear import using a complementary technique, like fluorescence microscopy. In order to visualise Tat, a GFP-Tat fusion protein was required. To insert the Tat gene into the GFP expression vector pEGFP-C2, the cDNA from the pSV2tat72 plasmid was first PCR amplified. As the restriction sites present in the final vector and the insert were not compatible, a PCR approach was selected that would allow addition of restriction sites into the primers that would then be compatible with the final vector.

Firstly, primers were designed that flanked the start and stop codons of the Tat gene as well as restriction sites being introduced that corresponded to the MCS of the pEGFP-C2 plasmid and allowed for in frame insertion of the insert into the final vector. The restriction sites chosen were KpnI and XhoI as these created sticky ends and were not present within the coding region of either the Tat gene or the final vector. A PCR product of approximately 240 bp was retrieved from a 1% agarose gel electrophoresis of the PCR reaction (Figure 3.6A, Lane 2). This product was then excised from the gel and purified using the Wizard® SV gel and PCR Clean-up Kit (Promega).

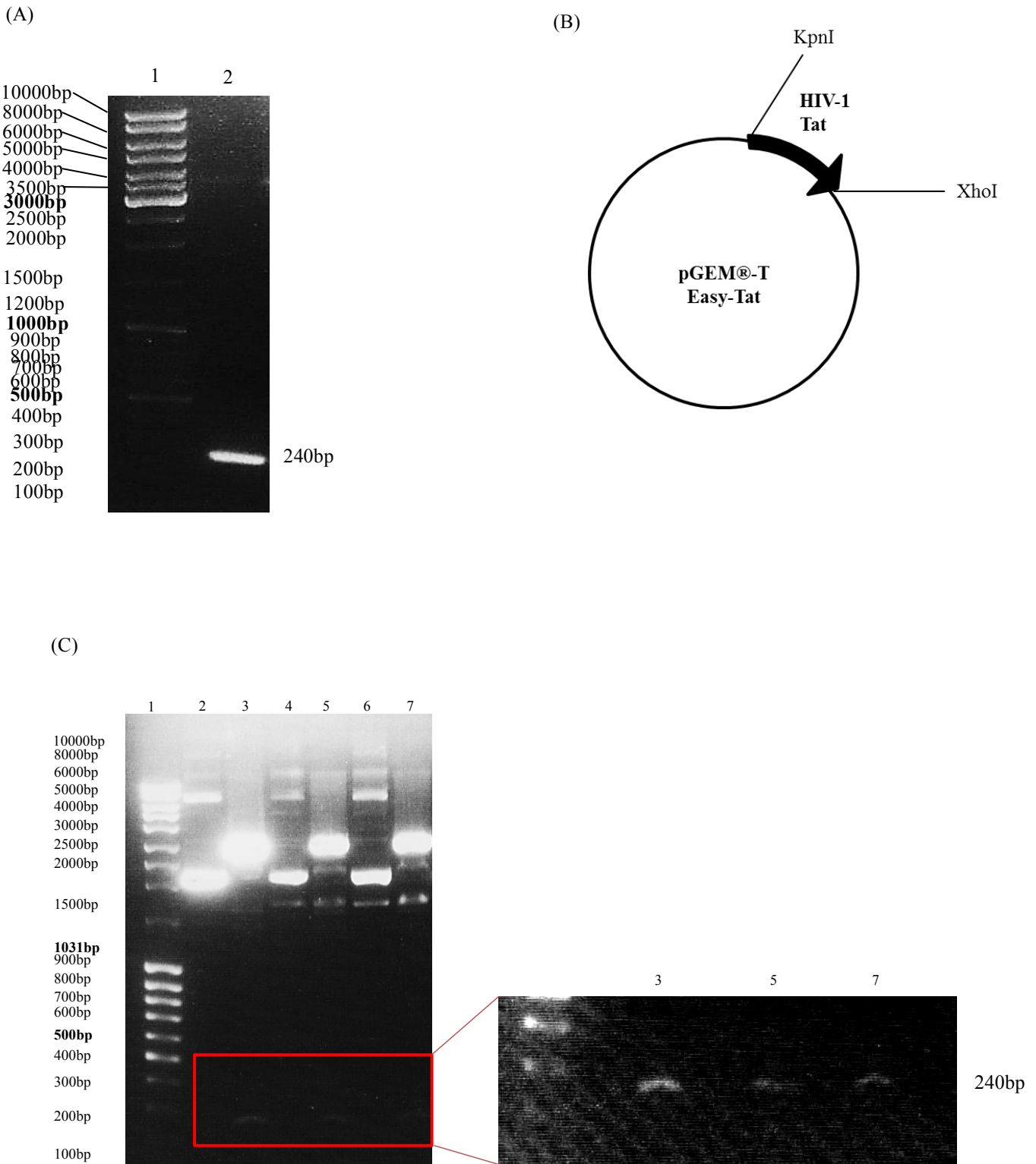
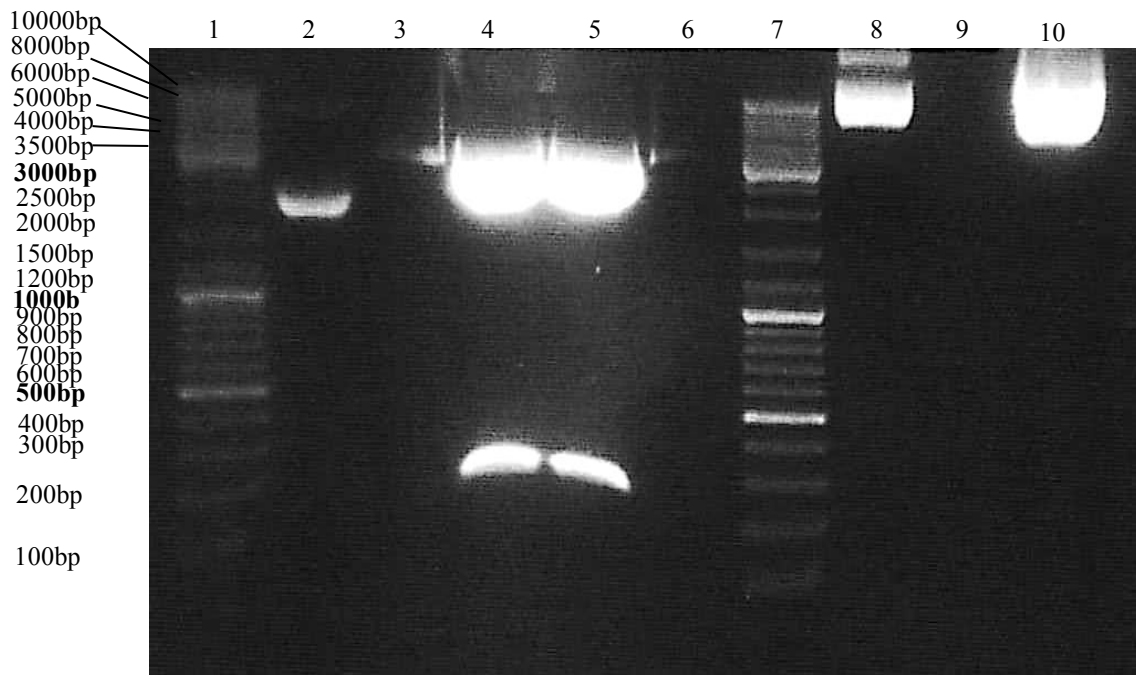


Figure 3.6 (A) 1% agarose gel showing the PCR product obtained from PCR amplification of the HIV-1 Tat gene from the pSV2-tat72 plasmid using primers flanking the gene and containing restriction sites for further cloning into the final pEGFP-C2 vector. Lane 1: O'Gene Ruler DNA ladder mix (Fermentas) Lanes 2: PCR amplified Tat cDNA product (240bp) (B) Schematic depiction of pGEM-T Easy containing the Tat insert cDNA. (C) 1% agarose gel showing digests to screen for the Tat insert. Lane 1: Mass Ruler DNA ladder mix(Fermentas), Lanes 2, 4 and 6: 1µg undigested pGEM-T Easy-Tat. Lanes 3, 5 and 7: 1µg PGEM-T Easy-Tat digested with KpnI and XhoI overnight to release the Tat insert cDNA.

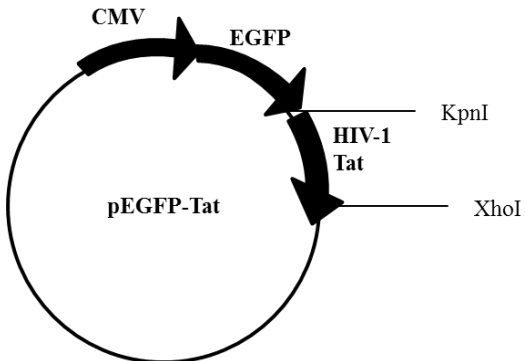
Following purification and quantification, the PCR product was subjected to an A-tailing reaction that would allow ligation into the pGEM-T Easy shuttle vector (Figure 3.6B). This then allowed for selection of colonies that contained the insert via blue/white screening, as well as sequencing to confirm the insert was correct. Clones were also screened by restriction digestion with KpnI and XhoI to release the insert (Figure 3.6C). A correct pGEM-T Easy-Tat clone was grown up for large-scale plasmid DNA preparation using the Qiagen Maxi Plasmid Extraction Protocol. The purified plasmid DNA was then sequenced to confirm the correct insert had been ligated and that no errors were introduced in the PCR reaction.

Plasmid DNA from the sequenced clone was then digested with KpnI and XhoI enzymes to release the Tat cDNA for subsequent cloning into the pEGFP-C2 expression vector (Figure 3.7A, Lanes 4 and 5). The pEGFP-C2 final vector was similarly digested to allow linearisation (Figure 3.7A, Lane 10) and ligation of compatible sticky ends of the Tat insert into the MCS of the final vector. These digests were then run on a 1% agarose gel stained with crystal violet dye to prevent degradation of sticky ends by ultraviolet light exposure. Figure 3.7A is representative of the digestion as we were unable to capture the crystal violet gel optimally for visualisation. The appropriate bands were then excised from the gel and purified and the insert ligated into the KpnI/XhoI cut MCS of the pEGFP-C2 vector (Figure 3.7B).

(A)



(B)



(C)

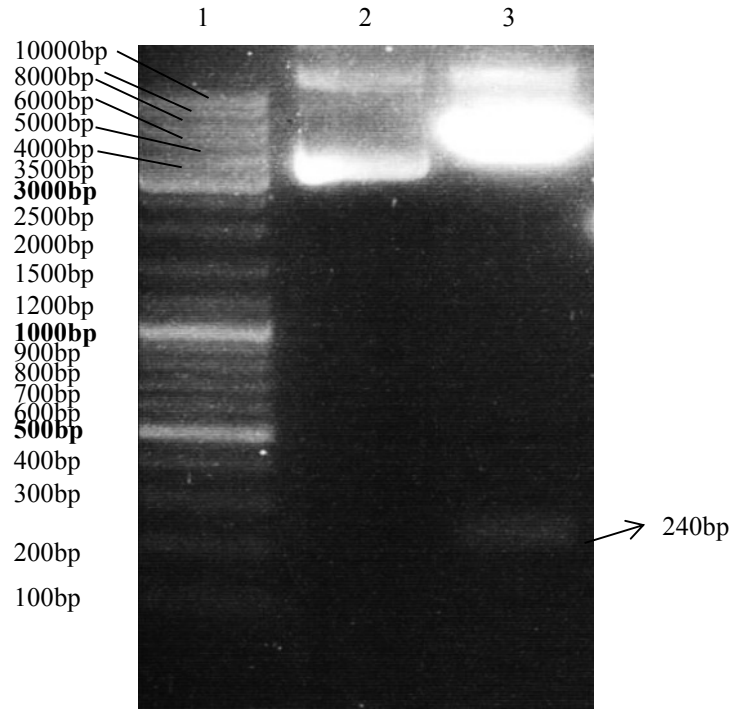


Figure 3.7 (A) 1% agarose gel containing digestions of pGEM-T Easy-Tat and pEGFP-C2 with KpnI and XhoI. Lane 1: O'Gene Ruler DNA Ladder Mix (Fermentas), Lane 2: Undigested pGEM-T Easy-Tat, Lane 3: Empty, Lanes 4-5: pGEM-T Easy-Tat digested with KpnI and XhoI to release the 240bp Tat cDNA insert. Lane 6: Empty, Lane 7: O'Gene Ruler DNA Ladder Mix (Fermentas). Lane 8: Undigested pEGFP-C2. Lane 9: Empty, Lane 10: pEGFP-C2 digested with KpnI and XhoI. (B) Schematic depiction of the recombinant pEGFP-C2-Tat plasmid, pEGFP-Tat. The Tat insert was inserted into the MCS of pEGFP-C2 by restriction digestion with the enzymes KpnI and XhoI. The GFP-Tat fusion protein is under the control of the CMV promoter. (C) 1% agarose gel electrophoresis picture of pEGFP-Tat digested with KpnI and XhoI restriction enzymes to release the Tat insert. Lane 1: O'Gene Ruler DNA ladder mix (Fermentas). Lane 2: Undigested pEGFP-Tat (4940bp). Lane 3: pEGFP-Tat digested with KpnI and XhoI, releasing the 240bp insert.

The ligation mix was then transformed into *E.Coli* JM109 cells and plated onto agar plates containing the pEGFP-C2 selection antibiotic, Kanamycin. The resulting colonies were subsequently screened by digestion with KpnI and XhoI to release the 240 bp Tat insert (Figure 3.7C, Lane 3). The recombinant pEGFP-Tat plasmid would therefore express the HIV-1 Tat protein fused at the N-terminus to the fluorescent protein GFP under the control of the CMV promoter and allows for monitoring of Tat by confocal microscopy.

3.2.4 Analysis of the subcellular localisation of GFP-Tat after Kpn β 1 inhibition in HeLa cells.

The cloned pEGFP-Tat recombinant plasmid was transfected into HeLa cells to monitor subcellular localisation of GFP-Tat. HeLa cells were chosen as they are known for their high transfection efficiency and they are the parent cell line to TZM-bl cells. They also exhibit an elongated morphology that makes them superior for studying cytoplasmic localisation over TZM-bl cells that are much smaller and more rounded in morphology.

pEGFP-Tat was transfected into HeLa cells before treatment with either control or Kpn β 1 siRNA. The small molecule, c43, could not be used in this instance as it is a fluorescent compound making fluorescent data analysis in the presence of c43 difficult to evaluate.

Immunocytochemical staining for Kpn β 1 expression was done using a cy3-conjugated secondary antibody. Our results show that the Importin is concentrated on the nuclear membrane (Figure 3.8, red channel). This is in line with its function in importing cargo through the nuclear pores. Knockdown was observed upon Kpn β 1 siRNA treatment as shown by a decrease in intensity of the cy3 fluorophore (Figure 3.8, red channel). This knockdown was quantitated for at least 20 individual cells that were also successfully transfected with the GFP-Tat plasmid, using Image J software.

Fluorescent images were captured forty eight hours after siRNA transfection, to ensure adequate Kpn β 1 knockdown (the Kpn β 1 half-life is approximately 24 hours). Variation in intensity of fluorescence from cell to cell was observed due to the nature of transient transfections. To account for this, the ratio of nuclear/cytoplasmic fluorescent intensity was determined for individual cells using the Zen Software (Zeiss) and the mean nuclear/cytoplasmic ratio for GFP-Vpr and GFP-IN determined for twenty cells in each experimental condition. These values were then compared between control and Kpn β 1 siRNA treated groups. To visualise the nuclear localisation in each cell the nucleic acid stain, DAPI, was used. In order to study the localisation of the GFP-tagged HIV proteins in relation to the nucleus, the GFP and DAPI signals were merged to determine the extent of the GFP nuclear localisation.

It was observed that in control siRNA transfected cells, the recombinant GFP-tagged Tat protein localised predominantly in the nucleus. Interestingly the protein concentrated in the nucleoli of the cell nucleus (Figure 3.8, green channel). This is not a surprising observation due to Tat being an RNA-binding protein (98). Inhibition of Kpn β 1 resulted in a marginal increase in GFP-Tat cytoplasmic localisation (Figure

3.8, green channel). Quantification of Kpn β 1 fluorescence in control and Kpn β 1 siRNA transfected cells showed a significant decrease in its expression upon Kpn β 1 knockdown (Figure 3.9A). Quantification of GFP-Tat fluorescence and analysis of the ratio of nuclear/cytoplasmic GFP-Tat fluorescence revealed a decrease in Tat nuclear/cytoplasmic fluorescence when Kpn β 1 was inhibited, suggesting that it favours localisation to the cytoplasm upon Kpn β 1 knockdown. This decrease, however, was not significant (Figure 3.9B).

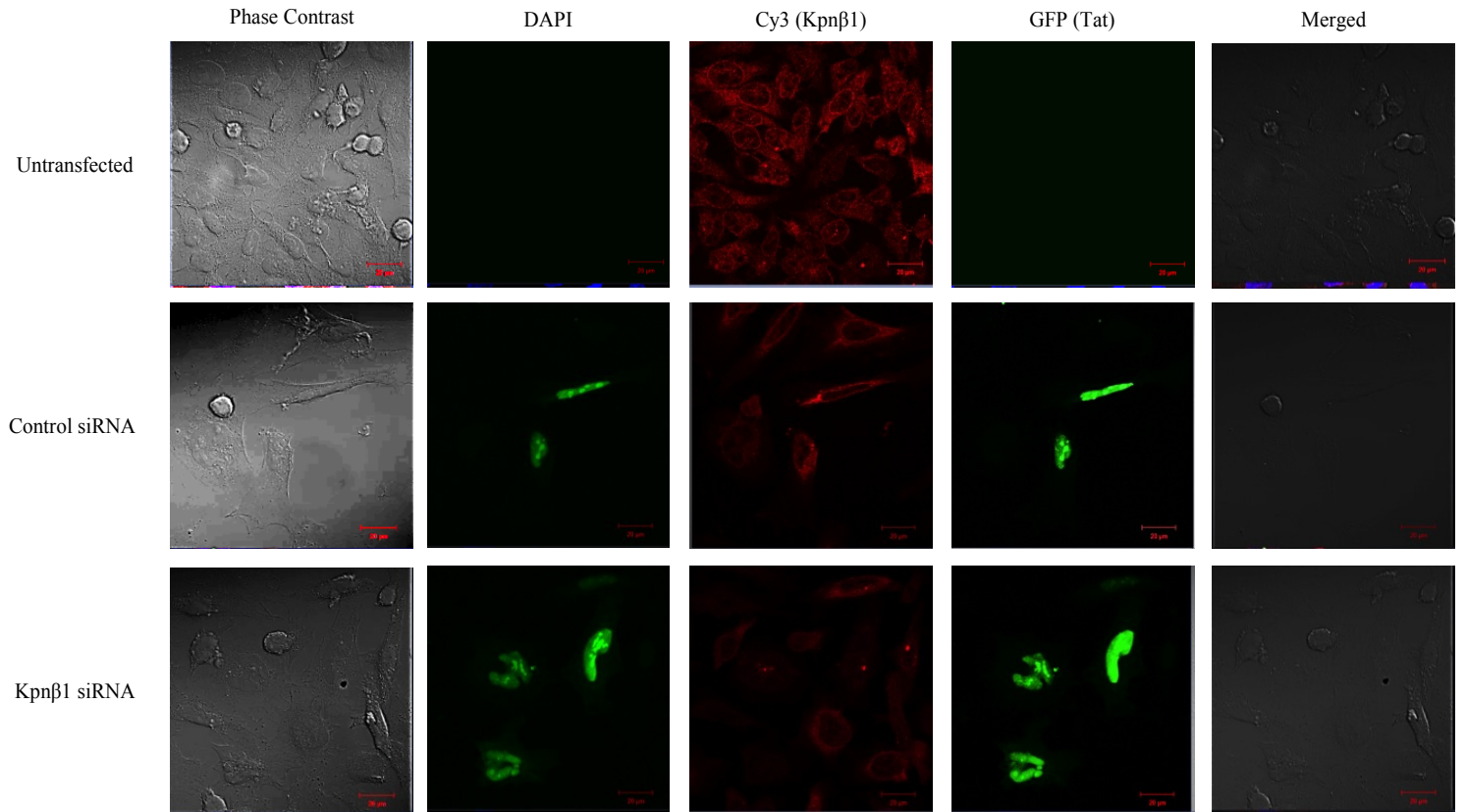
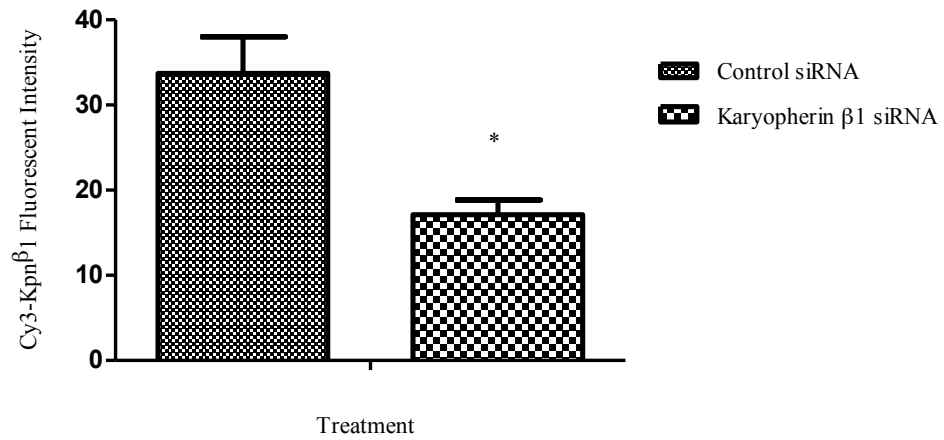


Figure 3.8. Confocal Microscopy images of HeLa cells transfected with the pEGFP-Tat plasmid and subsequently treated with either control or Karyopherin β 1 siRNA. Cells were viewed 48 hours after siRNA treatment at 100X magnification under oil. Images were taken using a Zeiss LSM 510 AxioObserver confocal microscope. DAPI: denotes the nucleus; Cy3: Kpn β 1; GFP: GFP-Vpr.

(A)



(B)

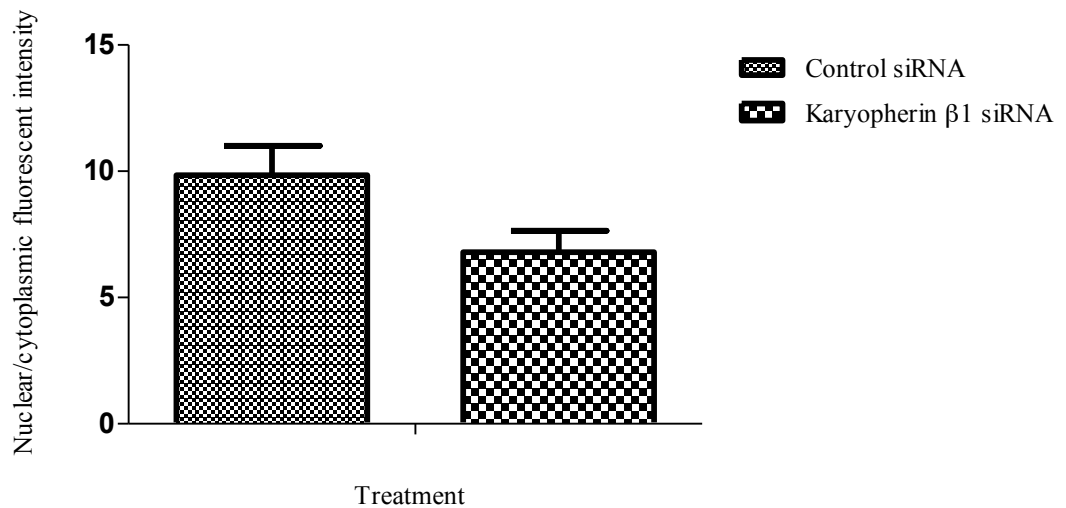


Figure 3.9. (A) Confirmation of Karyopherin β 1 knockdown via siRNA technology. Quantitation of Cy3 fluorescent intensity was performed using Zen Software (Zeiss) for control and Kpn β 1 siRNA treated cells. (n=20) (B) Quantification of the ratio of nuclear/cytoplasmic intensity for control and Kpn β 1 knockdown cells (n=20). Results shown are the mean \pm SEM (n=20 cells) of experiments performed at least two independent times. *p<0.05.

3.3 Discussion

The work presented in this study was aimed at determining whether Karyopherin $\beta 1$ mediates the nuclear import of the HIV-1 Tat protein. The small size of Tat (14kDa) suggests that passive diffusion through the nuclear pore complexes could account for its nuclear import. Indeed, recent studies in HeLa cells showed that Tat can enter the nucleus via passive diffusion (120). Tat, however, bears an arginine rich nuclear localisation signal (NLS) that directs the import of heterologous proteins when fused to the NLS (98). This NLS has also been shown to bind Kpn $\beta 1$ directly in a single study (64), as well as perform the TAR RNA binding function during transactivation of the HIV LTR promoter (98). Recently, evidence for the nucleocytoplasmic shuttling of Tat by Importin 13 has also been shown via co-localisation and pull down assays (100). Due to the controversy surrounding nuclear import of the HIV proteins, additional studies need to be undertaken to conclusively elucidate the import mechanisms of Tat. A growing body of evidence is suggesting that HIV utilises more than one pathway to enter the cell nucleus, implying redundant pathways may have evolved to allow for nuclear entry in a more controlled and timely manner (90, 121).

This study found that when the nuclear transporter, Karyopherin $\beta 1$, was inhibited using siRNA technology, as well as a small molecule inhibitor, c43, Tat nuclear function was significantly abrogated. Our findings also showed a trend towards a decrease in the ratio of nuclear/cytoplasmic fluorescent intensity of GFP-Tat, following Kpn $\beta 1$ inhibition in HeLa cells.

In analysing Tat transactivation using luciferase assays, we found that Kpn β 1 inhibition appeared to affect (significantly increase) the subsequent transfection efficiency of the cells. The fact that the transfection efficiency of Renilla luciferase as well as Tat was affected by Kpn β 1 siRNA could be due to the role of Karyopherin β 1 in nuclear membrane assembly, as well as the cell cycle. It has been shown that mitosis is intricately linked to efficiency of cationic lipid-mediated transfection of DNA, as when the nuclear membrane is broken down, DNA can more readily enter the nucleus (122). The role of Kpn β 1 in the cell cycle is well documented. Work in our laboratory showed that inhibition of Kpn β 1 expression results in a prolonged mitotic arrest (123). It is thus plausible that Kpn β 1 inhibition causes cells to arrest in mitosis, allowing for increased entry of transfected DNA. Due to this effect we performed all subsequent experiments transfecting the Tat plasmid DNA before Kpn β 1 siRNA.

To validate the ability of the c43 molecule to inhibit nuclear import via Kpn β 1, we utilised an NFAT assay. NFAT is a transcription factor that is imported by Kpn β 1 and the inhibition of its transcriptional activity was an indicator that its nuclear import was being inhibited. However, it is important to note that this same effect would be seen if c43 was a general inhibitor of transcription. To account for this, Renilla luciferase was used as an internal control during subsequent luciferase assays. The transcription of Renilla is controlled by the thymidine kinase minimal promoter and therefore if c43 was a general inhibitor of transcription, it would have caused a decrease in Renilla luciferase levels. We found this not to be the case and could safely conclude that c43 selectively blocked nuclear import and not transcription. Although a further control that could have been included would have been a luciferase reporter construct

not requiring nuclear import of a factor/s for its activation. Inhibition of Kpn β 1 with c43 would then not have affected transcription of this promoter and proved that this is indeed a Kpn β 1 specific effect.

Previous studies of GFP-tagged Tat showed that the GFP tag had no effect on the protein's nuclear localisation or function as a transactivator of transcription (124). Our results showed that Tat localised to the nuclei, specifically the nucleoli of HeLa cells. The nucleolar localisation of Tat is probably due to the RNA binding capability of this protein, which also happens to be the Arginine-rich NLS region of the protein (98). Our observation of the nuclear and nucleolar localisation of Tat is in line with that observed in other studies (120, 124, 125).

To account for basal promoter activity due to host transcription factors that activate the HIV LTR promoter such as Sp1 and NF κ B, we performed our promoter assays in the absence of Tat as well. It was seen that the decrease in promoter activity after Kpn β 1 knockdown in the absence of Tat was marginal in comparison to the decrease in the presence of Tat. This slight decrease is probably due to the fact that these host transcription factors are also imported by Kpn β 1 and the decrease in promoter activity could also in part be due to a decrease in their nuclear import. We could therefore account the significant decrease in the LTR promoter activity following Kpn β 1 inhibition in the presence of Tat, to a decrease in Tat transactivation function and not host factors alone.

Taken together, these promoter results show that Kpn β 1 is necessary for optimal Tat nuclear function and could possibly be involved in Tat's nuclear import in TZM-bl

cells. Tat function, however, was not completely abolished following Kpn β 1 siRNA. These results suggest that while Kpn β 1 may be one pathway the Tat protein employs to enter the nucleus, it is likely not the only one. An alternative explanation is that knockdown of Kpn β 1 protein expression using siRNA is not always absolute and residual Kpn β 1 protein is still expressed and could continue to import Tat into the nucleus to perform its functions.

Similarly, we observed a trend towards a change in GFP-Tat subcellular localisation following Kpn β 1 siRNA knockdown in HeLa cells. The fact that this was not significant could also be due to the unsilenced Kpn β 1 proteins continuing to import GFP-Tat into the nucleus. Alternatively, Tat could not rely as heavily on Kpn β 1 in HeLa cells than in TZM-bl cells. TZM-bl cells are HeLa cells that have been stably transfected with key HIV specific proteins, as well as the LTR promoter attached to a luciferase construct. Although they should thus behave similarly, it is noted that these stable transfections led to a significant change in the morphology of these cells, suggesting further reaching biochemical changes as well that could account for this discrepancy. The fact that these results show a trend towards decreased nuclear import of Tat in HeLa cells following Kpn β 1 siRNA suggests that Kpn β 1 may be one of many importers in this cell line able to import HIV Tat into the nucleus.

Our results support current literature reporting that Tat is imported by Karyopherin β 1. A key study showed that Tat binds directly to Kpn β 1, without the need for the Kpn α adapter molecule (64). Our findings do not, however, distinguish between direct binding to Kpn β 1 or binding via the adapter protein, Karyopherin α (Kpn α). Further

investigation would be needed, possibly inhibiting Kpn α selectively, to draw conclusions regarding which sub-pathway the Tat protein utilises.

These results are in contrast to a study suggesting Tat nuclear import is ATP-dependent but independent of the Karyopherin pathway (97). However, this study utilised HTC rat hepatoma cells and analysed a Tat-NLS- β -galactosidase fusion protein. It could be that Tat uses a range of import pathways depending on the cell type and context and this could explain why our results are in contrast to this finding. A recent study also showed that Tat can bind Kpn α /Kpn β in the absence of competitors, whereas this property is silenced in the cellular environment (126). Unless these specific competitors are less active in the TZM-bl cell line we used, our findings are in contradiction to this hypothesis.

In conclusion, we present evidence that Tat transactivation function is significantly decreased when Kpn β 1 is knocked down. This could be due to a decrease in the nuclear import of Tat. There is controversy in the literature regarding the exact mechanisms involved in Tat import and this could be due to many redundant pathways operating simultaneously, as well as variations depending on the cell type and context. The nuclear import of HIV Tat is therefore a complex and diverse process and thoughts on designing a therapy to block this step should possibly target a common entity such as the nucleoporins or the common NLS domain.

CHAPTER FOUR:

**THE EFFECT OF KPN β 1 INHIBITION ON
LOCALISATION OF HIV-1 PIC PROTEINS
INTEGRASE AND VIRAL PROTEIN R**

4.1 Introduction

In order for productive viral infection to occur, the HIV Pre-integration complex (PIC), composed of double stranded viral DNA and proteins, requires entry into the host cell nucleus. Too large for passive diffusion, PICs must utilise the cellular nuclear transport machinery, as well as the Nucleoporins (NUPs), to cross the nuclear membrane via the nuclear pore complexes (NPC) (127). The details of this process are, however, still unclear. The main viral proteins identified as possibly importing the HIV PIC into the nucleus are Viral Protein R, Integrase and Matrix (21). However, evidence in the literature is contradictory on this matter, with studies both supporting and refuting the role of each of these components in the nuclear translocation of the PIC (128, 129). There is also disagreement on whether the nuclear transporter, Karyopherin β 1, is involved in the import of the PIC via these proteins (21, 60, 61, 95, 130–133).

We aimed to investigate this process in our laboratory and focused on the PIC proteins, Viral Protein R (Vpr) and Integrase (IN) and their individual nuclear transport when Karyopherin $\beta 1$ is inhibited. Although the Matrix protein has also been proposed to be involved in importing the PIC, it requires the viral Protease (PR) enzyme for its post-translational processing. HIV-1 Protease cleaves the Gag polyprotein precursor (Pr55^{Gag}) into the mature p17 Matrix (MA), p24 Capsid (CA), p7 Nucleocapsid (NC) and p6 proteins (48, 134, 135). These constructs were not available to us hence Matrix nuclear transport was not included in this investigation.

To determine the role of Karyopherin $\beta 1$ in Vpr and IN nuclear import, we investigated the subcellular localisation of the GFP-tagged proteins of interest following Kpn $\beta 1$ siRNA mediated knockdown. If Kpn $\beta 1$ is necessary for their nuclear import, we anticipated a decrease in the nuclear localisation of these proteins, following Kpn $\beta 1$ inhibition.

4.2 Results

4.2.1 Optimisation of transfection conditions for pEGFP-Vpr and pEGFP-IN

GFP expression plasmids containing the HIV-1 Vpr and IN cDNA were obtained from the NIH Aids Reagent Programme and Dr Zeger Debyser (University of Leuven, Belgium), respectively.

To study the localisation of GFP-Vpr and GFP-IN, the plasmids were transfected into HeLa cells and GFP fluorescence examined by fluorescence microscopy. Initially, the GFP signal was poor for both plasmids, suggesting non-optimal transfection conditions. This was unlike the GFP-Tat plasmid we used which did not require optimisation. Transfection conditions were thus optimised using varying cell lines, transfection reagents, amount of plasmid DNA transfected, as well as the confluency of the cells upon transfection. Transient transfections were therefore performed with either pEGFP-Vpr or pEGFP-IN plasmids at 0.5 μ g, 1 μ g and 2 μ g concentrations using the Genecellin Transfection Reagent (BioCellChallenge) (Figure 4.1 and 4.2). 2 μ g plasmid DNA was determined to yield optimal transfection of both plasmids and was used in subsequent experiments. The localisation of GFP-Vpr fluorescence was found to be cytoplasmic with some nuclear fluorescence (Figure 4.1), while GFP-Integrase fluorescence localised predominantly to the cell nuclei (Figure 4.2). This greatly improved the transfection efficiency, although we only ever observed approximately 30% GFP positive cells.

To improve the percentage of GFP positive cells, we attempted to construct a stable cell line stably expressing the GFP-Integrase fusion protein. Selection for cells containing the plasmid was performed with G418 (Geneticin) and cell sorting via FACS analysis sorted GFP positive cells (Addendum III, page 100). Unfortunately very few GFP fluorescent cells were obtained, despite the cells being grown for weeks in G418 antibiotic. It was suspected that the cells had retained the antibiotic resistance gene, whilst removing the unnecessary GFP-IN cDNA.

We therefore performed all subsequent experiments using the optimised conditions for transient plasmid transfections. We found the experimental conditions that worked best for this study involved the use of Genecellin as a transfection reagent, 2 μ g of plasmid DNA and 20 μ M control and Kpn β 1 siRNA.

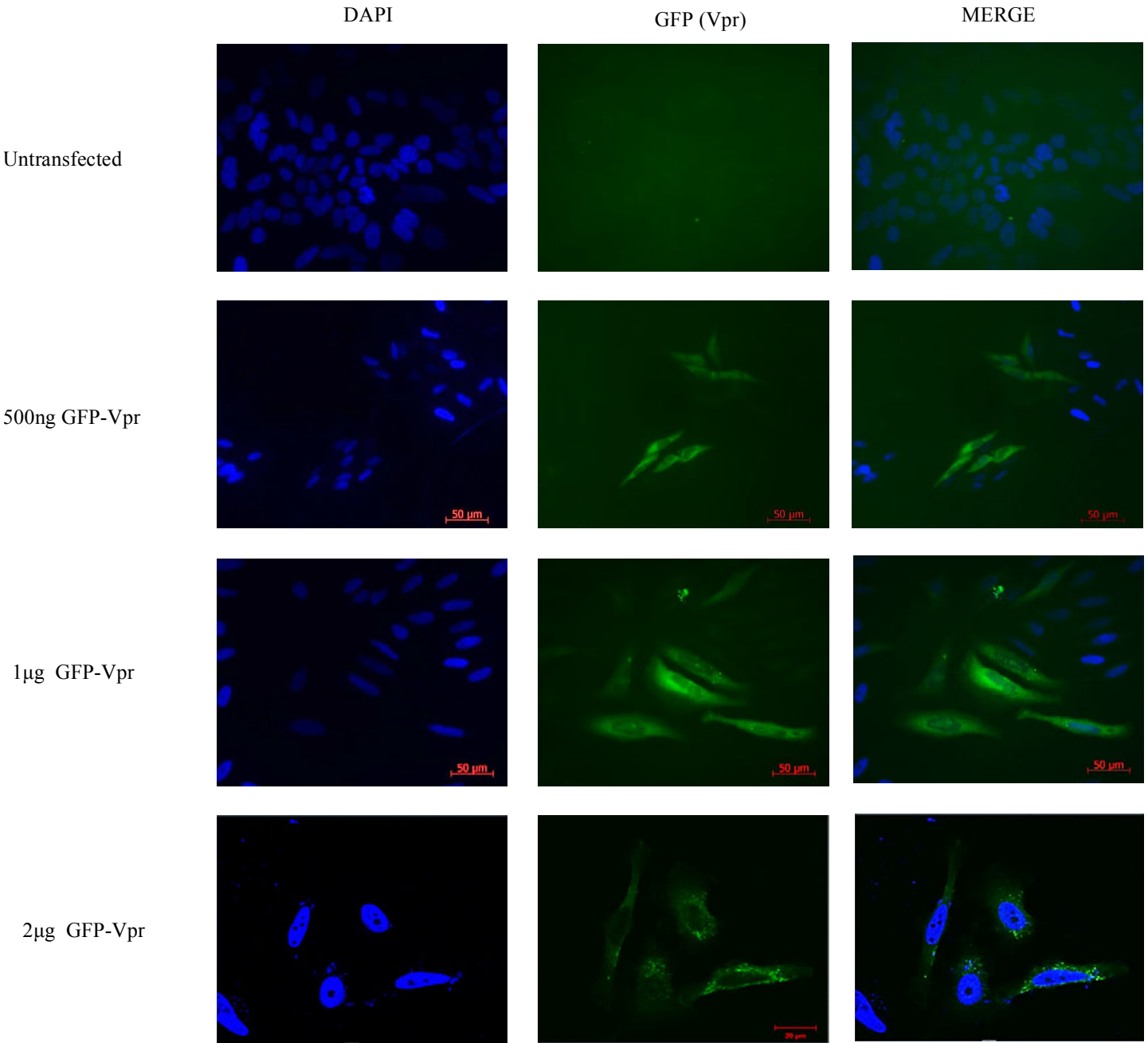


Figure 4.1. Optimisation of transfection conditions for HIV protein expression plasmid pEGFP-Vpr transfected into HeLa cells and visualised via fluorescence microscopy. A titration was performed using 0.5, 1 and 2 µg plasmid DNA transfected into 35mm plates containing 120 000 cells using the transfection reagent, Genecellin (BioCellChallenge). 2µg was determined to yield the best transfection. GFP-Vpr localised predominantly to the cytoplasm. DAPI: nuclear stain, GFP: GFP-Vpr.

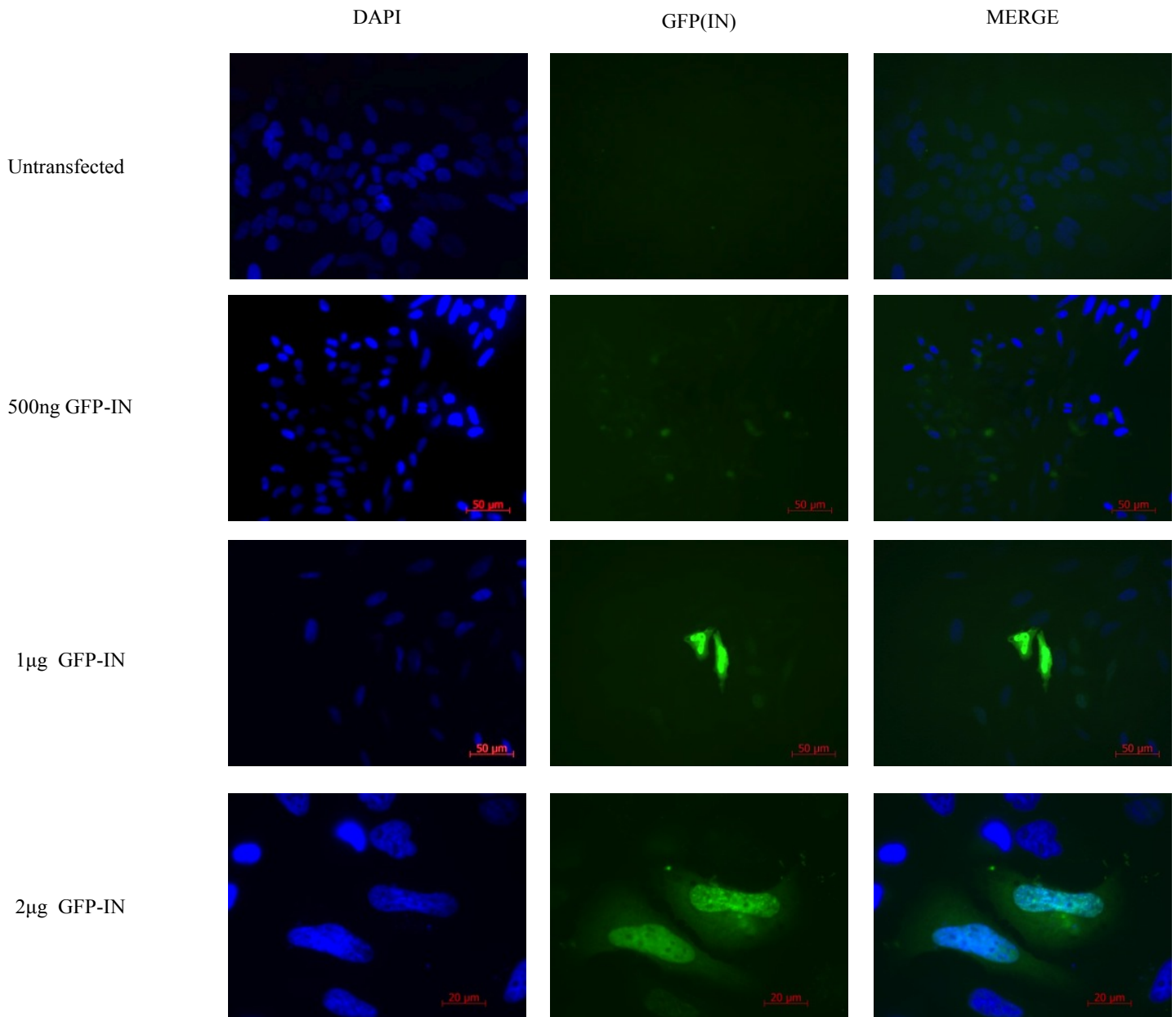


Figure 4.2. Optimisation of transfection conditions for HIV protein expression plasmid pEGFP-IN transfected into HeLa cells and visualised via fluorescence microscopy. A titration of 0.5µg, 1µg and 2µg of plasmid DNA/35mm plate was transfected into 120 000 HeLa cells using the transfection reagent Genecellin (BioCellChallenge). 2µg was determined to yield the best transfection. Top two panels were taken at 40X magnification, bottom panel was taken at 100X magnification. GFP-IN localised predominantly to the nuclei. DAPI: nuclear stain; GFP: GFP-IN.

To determine the effect of Kpn β 1 inhibition on Vpr and Integrase subcellular localisation, confocal microscopy was performed following Kpn β 1 inhibition with siRNA. The Kpn β 1 inhibitor, c43, could not be used as it fluoresces in the green channel due to its quinoxaline side chain and would therefore interfere with the signal.

Plasmids were transfected into HeLa cells using the optimised transfection conditions, followed by transfection of control or Kpn β 1 siRNA the following day. We noted that after transfection the number of cells present was significantly lower in the transfected dishes than in the untransfected dishes. However, sufficient transfected cells were obtained such that we could analyse the results. As can be observed in Figure 4.3, the number of cells present after transfection is significantly lower than the untransfected images, with the cells appearing more flattened and elongated. It was clear that many of the cells were affected by the transfection conditions.

4.2.2 Subcellular localisation of GFP-Vpr following Kpn β 1 inhibition

The GFP-Vpr protein localised diffusely between the cytoplasmic and nuclear compartments in control siRNA transfected HeLa cells (Figure 4.3, control siRNA green channel). Although GFP-Vpr appears predominantly cytoplasmic upon visualisation, quantification of the mean fluorescent intensity of the whole cell based on its phase contrast outline revealed that the mean intensity was roughly equal for the nuclear and cytoplasmic compartments.

To visualise Kpn β 1, immunocytochemistry staining was performed using a Cy3-conjugated secondary antibody and the intensity levels evaluated between control and knockdown cells (Figure 4.3, red channel). As in chapter 3, we observed Kpn β 1 localisation concentrated to the nuclear membrane. A decrease in fluorescent intensity in the Red Cy3 channel was observed in the Kpn β 1 siRNA-transfected cells, confirming Kpn β 1 knockdown. This decrease in intensity was quantified using the Zen Software (Zeiss) (Figure 4.4A).

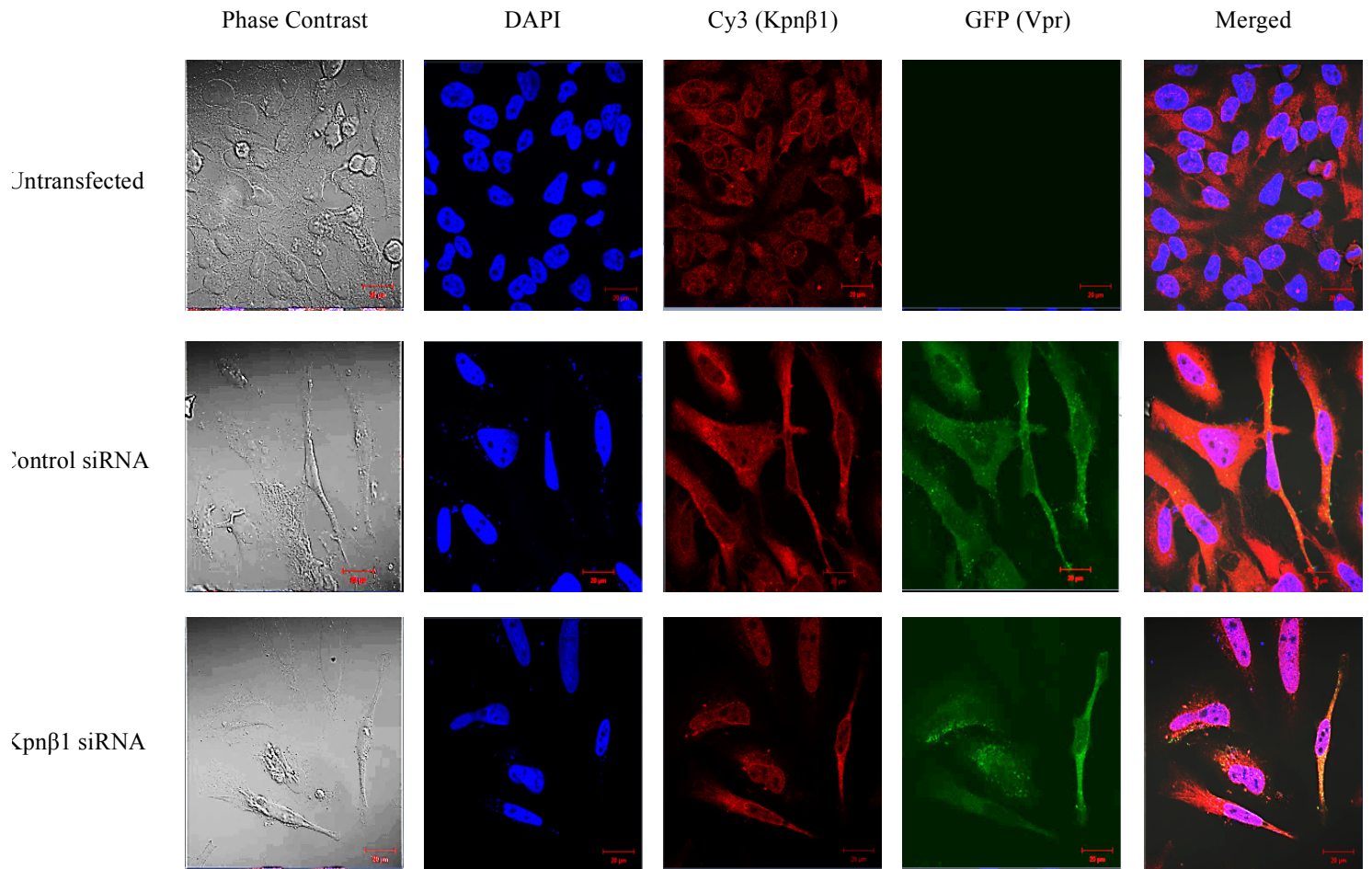
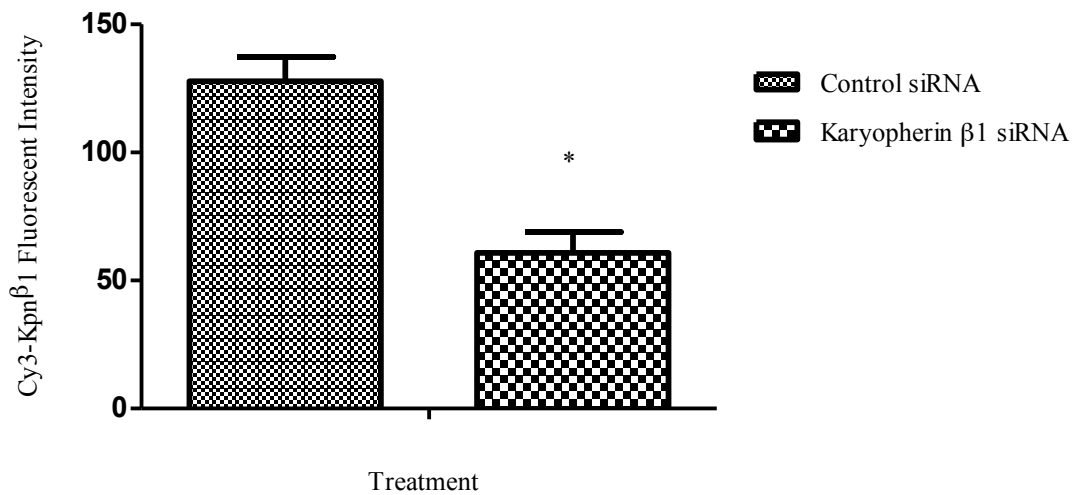


Figure 4.3. Confocal microscopy images of HeLa cells transfected with GFP-Vpr plasmid and either untreated (top panel), control siRNA treated (middle panel) or Kpnβ1 siRNA treated (bottom panel). Images were taken on a Zeiss LSM 510 AxioObserver confocal microscope at 100X magnification under oil, 48 hours following siRNA transfection. GFP-Vpr was seen to localise diffusely between the cytoplasm and nucleus. DAPI: denotes the nucleus; Cy3: Kpnβ1; GFP: GFP-Vpr.

Quantification of the ratio of nuclear/cytoplasmic GFP-Vpr fluorescent intensity showed that Kpn β 1 knockdown had no effect on GFP-Vpr localisation (Figure 4.4B). While our results showed no change in GFP-Vpr localisation, we had concerns regarding the pEGFP-Vpr plasmid construct as the subcellular localisation we observed is in contradiction to the literature. Several studies have shown that Vpr localises mainly to the nuclei and nuclear envelope of transfected and infected cells (136–139).

(A)



(B)

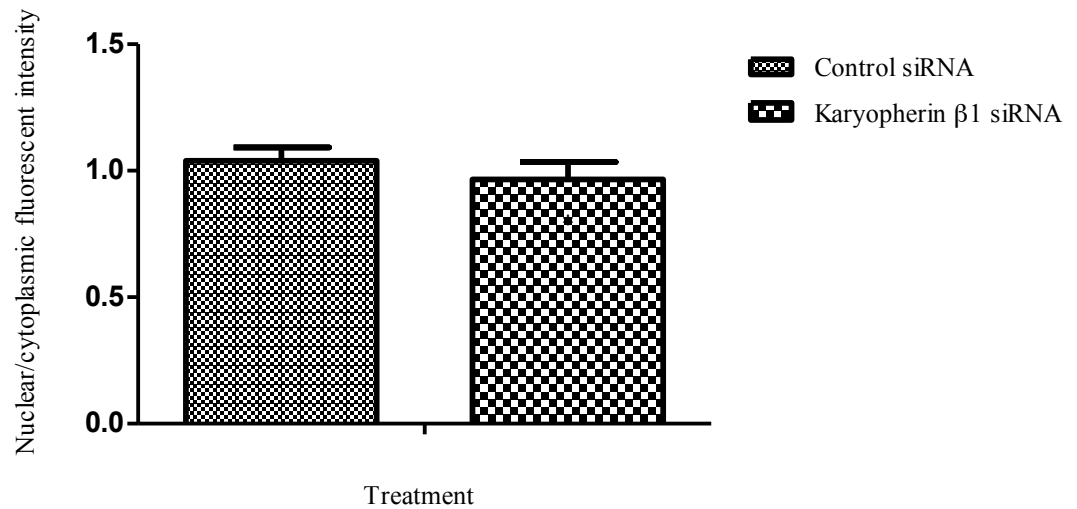


Figure 4.4. (A) Confirmation of Kpnβ1 siRNA knockdown. Cy3 fluorescent intensity was quantitated using Zen Software (Zeiss) for control and Kpnβ1 siRNA treated cells. (B) Quantitation of nuclear/cytoplasmic fluorescent intensity in cells transfected with GFP-Vpr (as shown in Figure 4.2) using Zen Software (Zeiss). N=20. No change was observed after Kpnβ1 siRNA treatment. Results show are the mean \pm SEM (n=20 cells) for experiments performed at least two independent times. *p<0.05.

4.2.3 Subcellular localisation of GFP- Integrase following Kpn β 1 inhibition

As was observed in previous experiments, the number of cells after transfection with GFP-IN plasmid and siRNA was significantly lower than the untreated images (Figure 4.5, phase contrast and DAPI channels).

In control siRNA transfected cells, GFP-Integrase (GFP-IN) was seen to localise predominantly to the nuclei of cells, with some cytoplasmic localisation observed. Of particular interest, the pattern of GFP-IN localisation appeared punctate, with small spheres of GFP-Integrase aggregating within the cells (Figure 4.5, green channel).

Kpn β 1 siRNA knockdown was confirmed by quantitation of the Cy3 fluorescent intensity. A comparison between control siRNA and Kpn β 1 siRNA treated cells showed a significant reduction in Kpn β 1 fluorescent signal (Figure 4.6A). Upon Kpn β 1 siRNA knockdown, we observed an increase in cytoplasmic accumulation of GFP-IN but not complete exclusion from the nucleus (Figure 4.5). When the nuclear/cytoplasmic intensity ratios were quantitated and analysed, a significant decrease in the nuclear/cytoplasmic fluorescent intensity ratio in the Kpn β 1 siRNA treated cells was observed (Figure 4.6B). This suggested that Kpn β 1 is required, in part, for the nuclear import of HIV-1 Integrase.

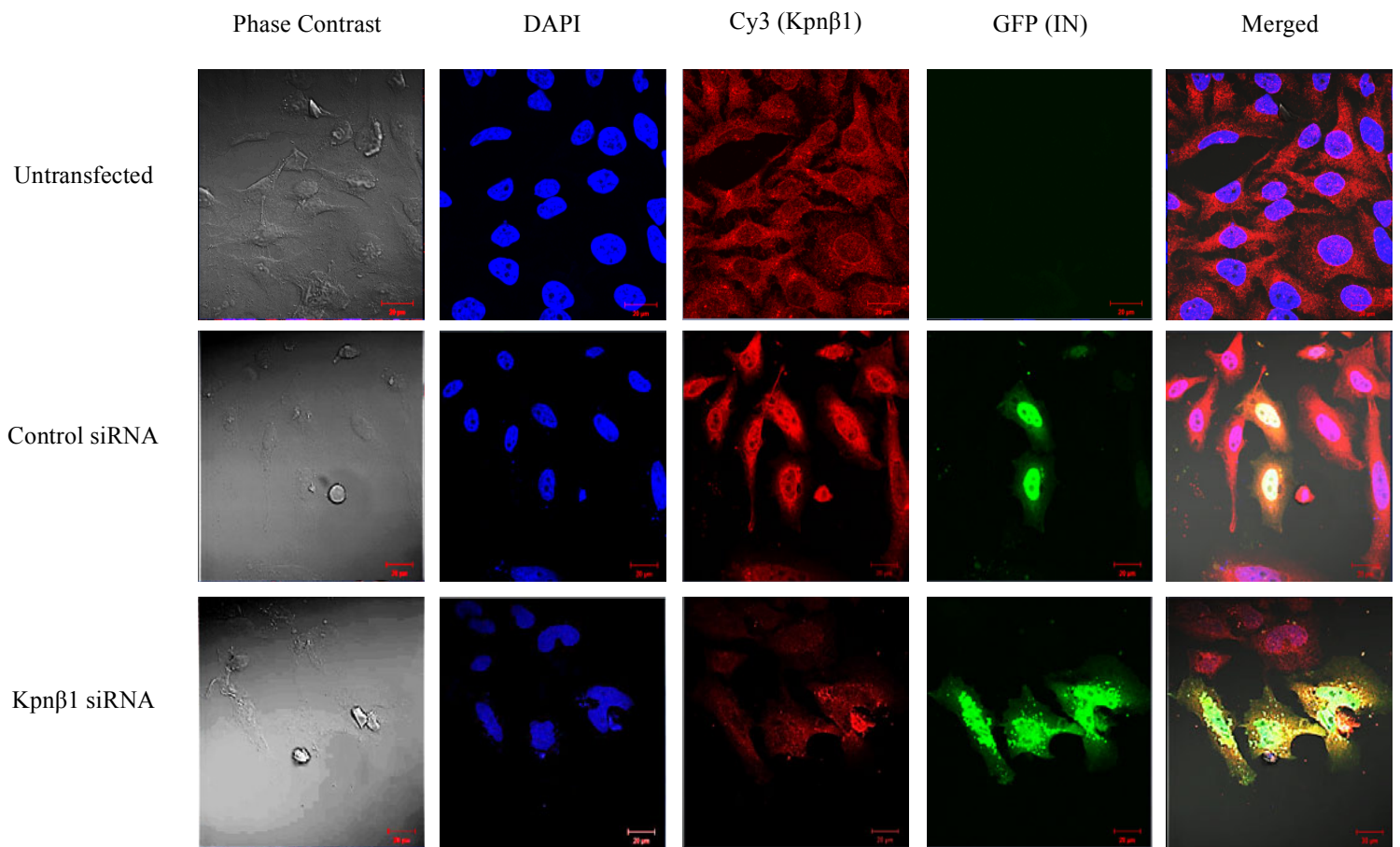
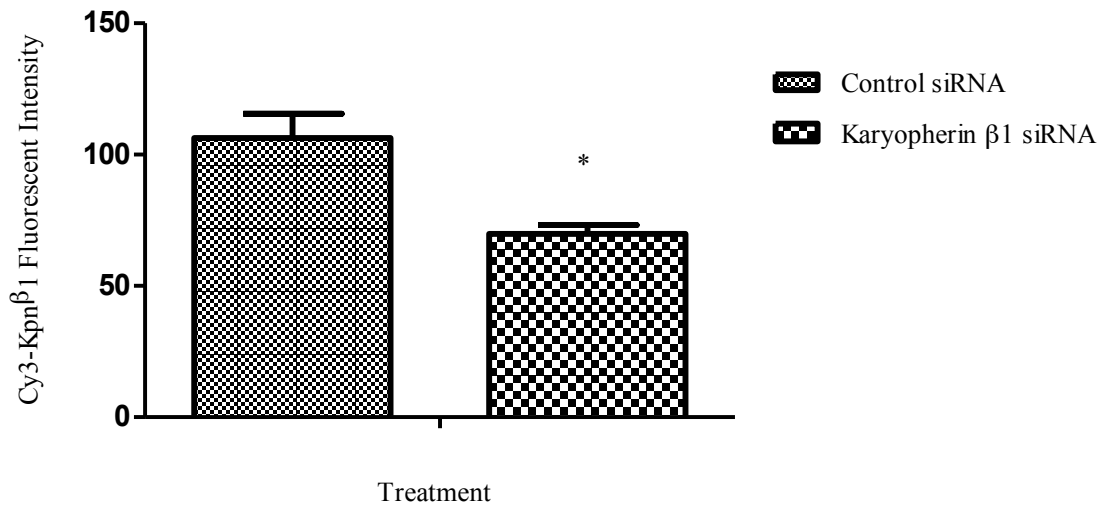


Figure 4.5. Confocal microscopy images of GFP-Integrase transfected into HeLa cells. Top panel: Untreated, middle panel :Control siRNA treated, bottom panel: Kpn β 1 siRNA treated.). Images were taken on a Zeiss LSM 510 AxioObserver confocal microscope at 100X under oil, 48 hours following siRNA transfection. Experiments were performed at least two independent times. DAPI: denotes the nucleus; Cy3: Kpn β 1; GFP: GFP-IN.

(A)



(B)

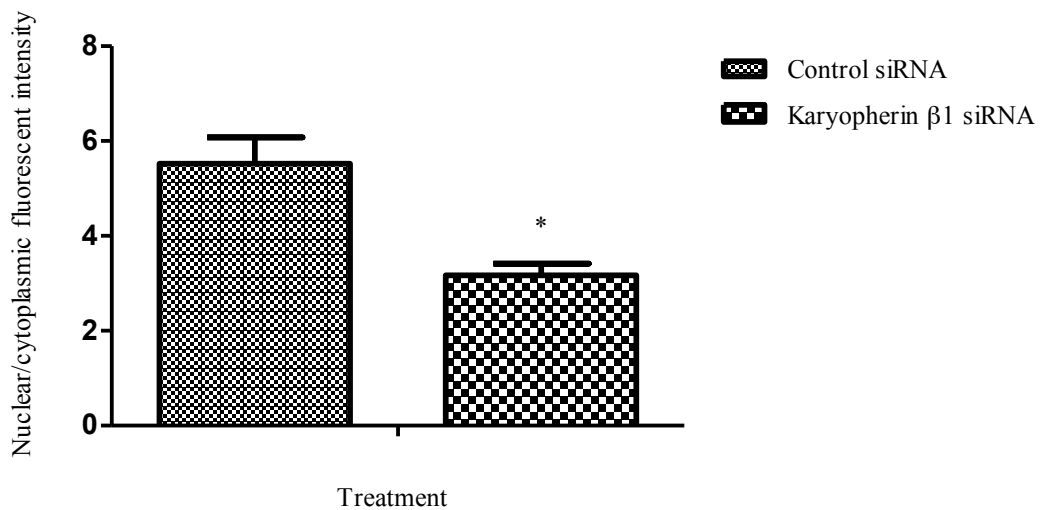
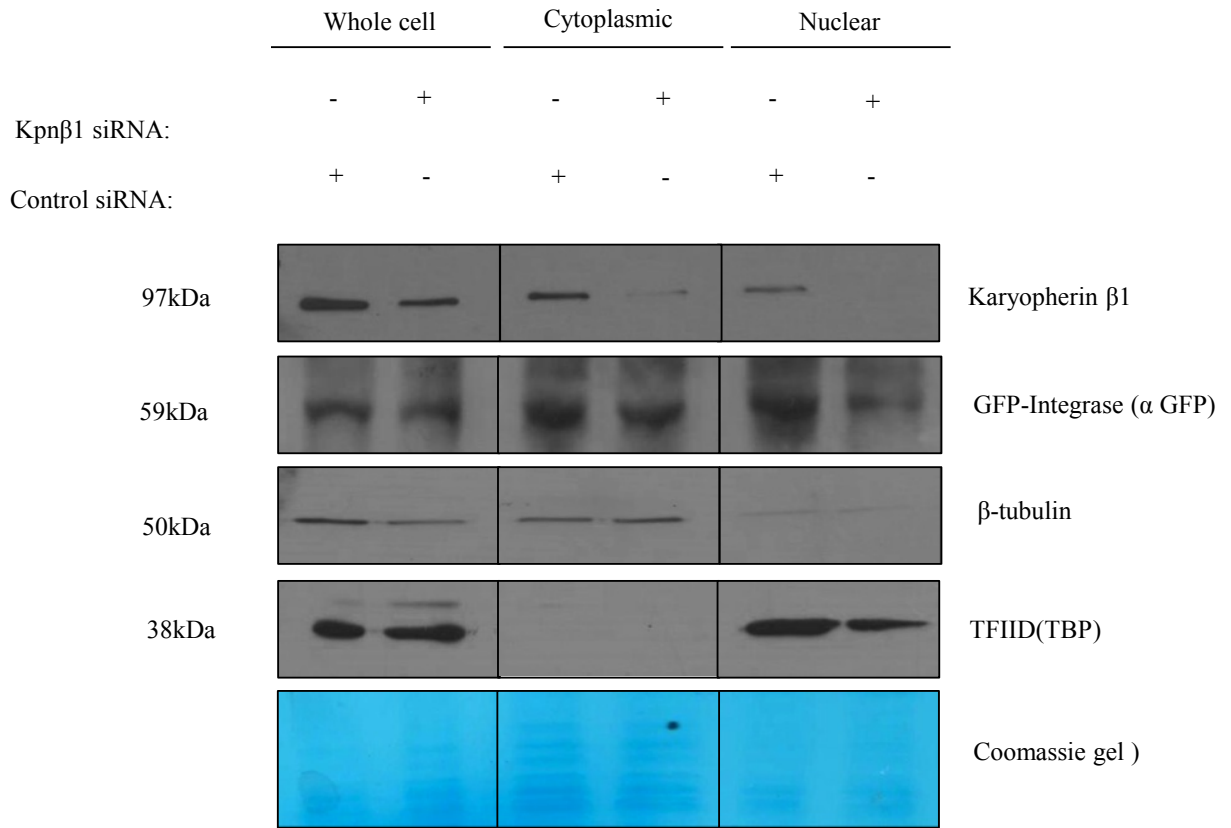


Figure 4.6. (A) Confirmation of Kpnβ1 siRNA knockdown. Cy3 fluorescent intensity was quantitated using Zen Software (Zeiss) for control and Kpnβ1 siRNA treated cells. (B) Quantitation of the ratio of nuclear/ cytoplasmic mean fluorescent intensity in cells transfected with GFP-IN. Kpnβ1 siRNA caused a significant decrease in nuclear/cytoplasmic ratio of fluorescent intensity of GFP-IN. Results show are the mean \pm SEM (n=20 cells) for experiments performed at least two independent times. *p<0.05.

The significant change observed by confocal microscopy in GFP-IN localisation upon Kpn β 1 knockdown, was confirmed using an independent experiment. Western blot analysis, using protein extracts after nuclear and cytoplasmic separation, was therefore performed to validate the microscopy result. HeLa cells were transfected with the pEGFP-IN plasmid, followed by transfection with control or Kpn β 1 siRNA for 48 hours. Nuclear and cytoplasmic protein fractions were then isolated from the cells and subjected to Western blot analysis. A ‘whole cell’ protein sample was also harvested from a dish treated in parallel. Kpn β 1 knockdown was confirmed by Western blot analysis using the Kpn β 1 antibody (Figure 4.7A, top panel). HIV-1 Integrase was detected using a GFP specific antibody as we were unable to detect IN using the IN antibody obtained from the NIH repository (Figure 4.7A, second panel from top). Although GFP-IN localised predominantly in the nucleus of transfected cells in our fluorescent studies, it also localised to the cytoplasm. Thus we observed GFP-IN in the nuclear and cytoplasmic protein fractions, albeit slightly more in the nuclear fraction (Figure 4.7A).

Analysis of GFP-IN in the nuclear protein compartment revealed a decrease in the nuclear localisation after Kpn β 1 inhibition compared to control siRNA transfected cells. Interestingly, the nuclear loading control, TBP, also decreased (Figure 4.7A). This decrease in TBP following Kpn β 1 knockdown has been seen previously in our lab and appears not to be due to uneven loading and could be due to a decrease in TBP nuclear import. TBP has been shown to be imported into the nucleus by the Karyopherins, Kap114p (mammalian Importin 9), Kap123p (Karyopherin β 2) and Kap121p (Kpn β 3) (140).

(A)



(B)

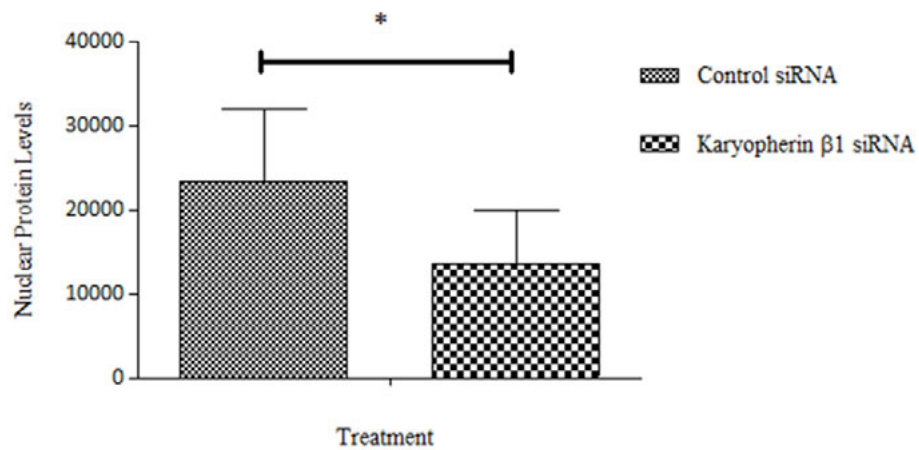


Figure 4.7. (A) Representative Western blot analysis of whole cell, cytoplasmic and nuclear protein fractions. Karyopherin β1 protein knockdown was seen in all cellular compartments. GFP-Integrase decreases in the nuclear fraction after Kpnβ1 inhibition with siRNA. Tata-binding protein (TBP) also decreases. Coomassie staining of the gel shows even loading. (B) Quantitation of the GFP-IN bands in the nuclear protein fractions from four independent Western blots shows a significant reduction in the nuclear GFP-IN protein levels of Kpnβ1siRNA treated cells compared to control siRNA treated cells. Results show are the mean ± SEM for experiments performed four independent times. * $p < 0.05$.

It has been seen to utilise alternative pathways when one of these is absent and could quite possibly also utilise Kpn β 1, although this has not been shown. The other known nuclear loading controls are also imported by Kpn β 1, namely Histones H1 and H3 (141, 142) and PCNA (143). To ensure that the results obtained were not due to loading differences, a coomassie stain of the gel was included to show similar protein levels across the lanes.

We did not observe an increase in the cytoplasmic GFP-IN following Kpn β 1 knockdown possible due to the fractionation method excluding the nuclear membrane or the accumulated GFP-IN could be targeted for degradation. B-tubulin was used as the whole cell and cytoplasmic loading control (Figure 4.7A).

Quantification of the nuclear GFP-IN levels was performed on four separate experiments and compared between the control and Kpn β 1 siRNA treated lanes. Levels could not be normalised to the loading control, TBP and so loading was seen to be roughly even between control and Kpn β 1 via coomassie staining. The power of this result is derived from the decrease in nuclear GFP-IN following Kpn β 1 knockdown being observed four independent times. This decrease was found to be statistically significant (Figure 4.7B).

These results show via two independent experiments that the nuclear localisation of GFP-Integrase decreases following knockdown of Kpn β 1. Taken together, these results suggest that Integrase is imported, in part, by Kpn β 1.

4.3 Discussion

The results presented in this chapter were aimed at elucidating whether the nuclear transporter protein, Kpn β 1, mediates the nuclear import of the PIC proteins Viral Protein R (Vpr) and Integrase (IN). The precise mechanisms of Vpr and Integrase nuclear transport are yet to be determined as there is much controversy surrounding this topic in the literature.

To determine whether Kpn β 1 mediates the import of Vpr or IN, we performed knockdown studies, inhibiting Kpn β 1 using siRNA technology. HeLa cells were transfected with the HIV expression vectors pEGFP-Vpr and pEGFP-IN and the localisation of GFP-Vpr and GFP-IN observed, respectively. We then studied viral protein localisation following inhibition of Kpn β 1 with siRNA via confocal microscopy. Kpn β 1 knockdown was also confirmed by quantitation of the Cy3 fluorescent intensity between the control and Kpn β 1 siRNA treated cells. We also performed Western blot analysis on nuclear and cytoplasmic protein fractions following Kpn β 1 siRNA transfection to analyse GFP-IN subcellular localisation.

GFP-Vpr was seen to localise diffusely throughout the cell with a slight cytoplasmic accumulation. This is in contrast to the literature where most studies observe HIV-1 Vpr to localise to the cell nuclei as well as the perinuclear region (144). Interestingly, our observation is more in accordance with the Simian Immunodeficiency Virus (SIV) Vpr which has been seen to localise uniformly between the cytoplasm and the nucleus of transfected cells (30). Although the C-terminus of Vpr contains several arginine residues that resemble a basic NLS (146), this sequence has not been found

to function as an NLS; instead it seems that the nuclear import function of Vpr is determined by the N-terminal region that is required for protein–protein interactions (147, 148). The N terminus of Vpr has been recognised as being crucial to the nuclear targeting and import abilities of this protein (145). As our construct contained GFP fused to the N terminus of Vpr, this could have possibly reduced the nuclear targeting functions of the protein. This contradiction with the literature was also initially thought to be due to our use of HeLa cells to perform our transient transfections. However, previous work using a GFP-tagged Vpr construct transiently transfected into HeLa cells saw GFP-Vpr localising to the nucleus of transfected cells (30). It is also possible that the integrity of the plasmid has been compromised, leading to an alteration in subcellular localisation that is more similar to that of GFP itself, known to be diffusely localised throughout the cell. We did have trouble with transfection efficiency with this construct and it is also possible that the poor transfection led to overexposure of background fluorescence not entirely due to GFP-Vpr.

In this study we found that inhibition of Kpn β 1 had no effect on the nuclear localisation of GFP tagged Vpr, however this conclusion requires further validation, possibly with an alternative construct whose fusion protein's localisation matches other reports.

Vpr has been suggested to utilise the classical Kpn α / β pathway as it associated with Kpn α in a solution binding assay (61). Indeed, Vpr has consistently been shown to interact with Kpn α (106, 132, 149). Alternatively, Vpr contains novel NLSs and is able to utilise two import pathways distinct from the classical Kpn α mediated

pathway (150). The precise mechanisms of Vpr nuclear import via the host machinery are therefore still being investigated.

The role of Vpr in directing the nuclear import of the PIC is still uncertain. However, multiple reports attest to its vital role in this process (61). Some studies have, however, shown that Vpr is not needed for nuclear import of the PIC in HeLa cells specifically (30). This could be the case in this study as we performed our experiments in HeLa cells and found Vpr not to concentrate to the nuclei or be imported via Kpn β 1. It has also been suggested that Vpr acts together with Matrix to determine import of the PIC. It was observed that when either Vpr or Matrix were independently mutated, viral infectivity was not affected. When both were mutated, nuclear localisation of viral nucleic acids was greatly attenuated, suggesting a redundancy in PIC nucleophilic determinants (149).

The GFP tagged Integrase protein localised predominantly to the nucleus in HeLa cells transiently transfected with the pEGFP-IN plasmid. This localisation is in accordance with previous studies supporting its karyophilic nature (30, 94, 109). We also noted aggregates of GFP fluorescence that made the signal appear punctate. This could possibly be due to Integrase spontaneously forming multimers as it does during the Integration reaction. Indeed, Integrase has been shown to form stable tetramers in human cells to accomplish integration of vDNA into the host chromosome (151, 152)

While Integrase seems to contain nuclear localisation signals (153), they were proven not to be transferable and it is speculated that its karyophilic properties stem from its interaction with cellular factors, such as LEDGF/p75 (154). The nature of Integrase's

NLSs is also under debate. It has been proposed to contain a non-canonical NLS that is not recognised by the classical import pathway (153), as well as an NLS that interacts directly with Kpn α (60, 155).

In this study we observed GFP-IN localisation to be significantly affected by knockdown of Kpn β 1 using siRNA technology. The ratio of GFP-IN nuclear/cytoplasmic fluorescent intensity was calculated for individual cells due to the differences in fluorescent intensity between cells. This ratio significantly decreased following Kpn β 1 knockdown, suggesting a role for Kpn β 1 in Integrase nuclear import. Possible reasons for the fact that GFP-IN was not entirely excluded from the nucleus could be that the inhibition of Kpn β 1 was not always 100% and the residual protein left is still available to import GFP-IN into the nucleus. Alternatively, some cells may have taken up the plasmid DNA but not the siRNA.

The result for GFP-IN was further validated by Western Blot analysis. A significant reduction in nuclear protein levels of GFP-IN was observed following knockdown of Kpn β 1. These findings support studies showing that GFP-IN is imported by Kpn β 1 (60), while contrasting evidence that shows that IN nuclear localisation does not involve the Karyopherin superfamily (156). Depienne et al (2001) observed IN to interact directly with Importin α and that import of the PIC can be blocked by an NLS-IN peptide (155). However, Bukrinsky et al (1992) suggest that HIV Integrase is not essential to the process of PIC nuclear import (31). The controversy surround this issue is possibly due to the flexible use of multiple alternative pathways that exist for nuclear import of PIC proteins. Integrase has also been shown to be imported by Transportin 3, Importin 7 and LEDGF/P75 (94, 95, 157).

In conclusion, this study suggests that Vpr does not require Kpn β 1 for its nuclear import in HeLa cells. Through two independent methods we show that Integrase localisation is affected by an inhibition of the nuclear transporter, Kpn β 1. This suggests Kpn β 1 is involved in the import of recombinant Integrase protein. We are cognisant that this may not be the case *in vivo* in HIV target cells and that the effects of the PIC entity as a whole may alter this process.

These results further suggest that the nuclear import of HIV proteins is a complex and multifaceted process that requires future investigation and novel techniques of study (90). It also appears that, like many viral proteins, Vpr and IN utilise multiple pathways to ensure import of the PIC.

CHAPTER FIVE:

CONCLUSIONS AND FUTURE PERSPECTIVES

5.1 Conclusions

Nuclear import is a crucial step in the viral life cycle. Unique but redundant NLS signals reside within the viral PIC proteins Vpr, MA and IN that could potentially direct nuclear import through the nuclear pores. To date, understanding of this process has been impeded by a failure to establish links between viral and host proteins involved in this process. This study aimed to determine whether Karyopherin $\beta 1$ is involved in the nuclear import of the HIV regulatory protein Tat and the pre-integration complex proteins Viral Protein R and Integrase.

Initially, HIV was thought to require nuclear membrane dissolution in order to enter the host cell nucleus, much like the other oncogenic retroviridae family members. Since the discovery that HIV can infect non-dividing cells, the active nuclear import of the viral components through an intact nuclear membrane has been a subject of intense research. The karyophilic nature of these proteins has allowed researchers to exploit their nuclear targeting properties. For example, HIV Tat has become a widely used molecular carrier, allowing nuclear targeting of a variety of cargoes (158, 159).

Despite this intense research, an agreement on a precise model of PIC, as well as HIV regulatory protein, nuclear import remains elusive. This may be due, in part, to the redundant nature of this process. It appears that HIV uses the multiple nuclear import pathways present in the host cell in a flexible manner (89, 90).

The flexible nature of this import process is observed in the multitude of transporter proteins able to bind and import HIV proteins. The HIV Tat protein has been shown to bind Karyopherins α/β (64, 99), Importin 13 (100), as well as enter the cell via passive diffusion (99, 120). Studies suggest Integrase nuclear import is mediated by numerous host proteins, including Kpn α/β (131) (60, 101), Importin 7(92), LEDGF/p75 (94) and Transportin 3 (95). Similarly, Vpr has been suggested to enter the nucleus via Kpn α/β (61, 132), as well as direct binding to the nucleoporins (150). Further redundancy exists in that the karyophilic determinants of PIC nuclear import all function to ensure PIC so that if one fails to direct nuclear import, the others will compensate. For example, mutation of either Matrix or Vpr is inconsequential to nuclear import, as replication is still consistent with wild-type levels. When both were mutated, however, viral replication was severely attenuated (149). This redundancy and flexibility allow the virus to replicate in a range of cell contexts.

Our findings suggest that Kpn β 1 is necessary for Tat transactivation capability and possibly its import. The importance of Kpn β 1 for HIV LTR activation is a novel finding. However, its inhibition did not result in complete abrogation of promoter activation via Tat. This suggests that Tat is still able to enter the nucleus via other pathways. Alternatively, this could be due to siRNA knockdown of protein expression rarely being 100%. Low levels of Kpn β 1 not silenced by the siRNA could still have

been present that could continue to import Tat into the nucleus to activate the LTR promoter. Similarly, although inhibition of Kpn β 1 resulted in a significant decrease in the nuclear/cytoplasmic ratio of GFP-IN, this protein was not excluded from the nucleus altogether. Again this suggests that IN was still able to enter the nucleus by other means or that the residual Kpn β 1 continued to import these proteins, albeit at a reduced efficiency.

A prophylactic vaccine against HIV has proven problematic due to the high mutation rate of the virus. This has led to a high rate of emergence of strains resistant to current drugs. There is therefore a need for novel targets and therapies until a cure can be obtained. Nuclear import represents an attractive novel target (160). However, due to the importance of the nuclear import pathways to cell functioning, toxicity could be a concern when inhibiting these host pathways. Agents should possibly be designed to target the interface between viral proteins and host Importins.

In summary, our findings support other studies showing Kpn β 1 to be necessary for the nuclear import of key HIV viral proteins, specifically Tat and Integrase.

5.2 Future Perspectives

Future work stemming from this study includes studying the interactions between Kpn β 1 and the HIV proteins Tat and Integrase to determine the mode of binding and whether the classical Kpn α /Kpn β pathway is utilised for their import or if they bind directly to Kpn β 1. Pseudovirus studies using GFP tagged virions could also answer the question of whether Kpn β 1 inhibition affects replication rates of the virus. To further determine Kpn β 1's role in overall viral infectivity, overexpression of Kpn β 1 and the effects of this on HIV protein nuclear import, as well as viral replication rate could be studied. These experiments could determine whether targeting a host nuclear importin such as Kpn β 1 could have a negative impact on HIV replication. Inhibiting the host nuclear import machinery could, however, have an adverse effect on the normal functioning of host cells. An ideal target would involve selecting one that has limited effects on normal host cells. Experiments in our laboratory investigating Kpn β 1 as an anticancer target have shown that selective inhibition of this protein via siRNA and a targeted drug approach is toxic to cancer cells but to a significantly lesser extent to normal cells (161), Van der Watt, unpublished), suggesting that Kpn β 1 could be such a target.

Targeting host nuclear import pathways necessary for the HIV life cycle in combination with current HAART therapies could result in even better management strategies.

Appendix I: Solutions

Bacterial Solutions

Luria Broth Medium (1L)

10 g Tryptone
5 g Yeast Extract
1mM NaOH
Up to 1l with dH₂O
Autoclave

Luria Broth Agar (1L)

10 g Tryptone
5 g Yeast Extract
1mM NaOH
15g Agar
Up to 1l with dH₂O
Autoclave
Pour into bacterial plates

IPTG (0.1M)

0.072 g IPTG
3ml dH₂O
Filter sterilised (0.2µM filters)
Store at 4°C

X-Gal (50mg/ml)

0.05g X-gal
1ml DMSO
Store at -20°C in the dark

Ampicillin (10mg/ml)

1 mg Ampicillin
10ml dH₂O
Filter-sterilise
Store at -20°C

Kanamycin (50mg/ml)

50mg Kanamycin
1ml dH₂O
Vortex
Store at -20°C

DNA Solutions

TE buffer

10mM Tris-Cl, pH 8.0
1mM EDTA, pH 8.0

10X TBE

108g Tris
55g Boric Acid
7.4g EDTA
Up to 1l with dH₂O

1 % agarose gel

1g Agarose
100ml 1 X TBE
Microwave until melted and clear
Allow to cool for 10 minutes
5µl Ethidium Bromide
Pour into gel cast

Tissue Culture Solutions

Cell freezing media (50ml)

5ml FCS
40ml DMEM complete
5ml DMSO

10X PBS

40g NaCl
1g KCl
5.75g Na₂HPO₄ · 7H₂O
1g KH₂PO₄
Up to 500ml with dH₂O

Protein Harvesting Solutions

RIPA Buffer

150mM Sodium Chloride
1% Triton X-100
1% Sodium Deoxycholate
0.1% SDS
10mM Tris-Cl, pH 7.4

Harvesting RIPA buffer

1mM Na₂VO₃
1 X Protease Inhibitor Coctail (PI) (Roche)

Western Blot Solutions

4% Stacking Gel

7.3ml dH₂O
1.25ml 1M Tris-Cl pH 6.8
100µl 10% SDS
1.3ml 30% Acrylamide
120 µl 10% Ammonium Persulphate
12µl Temed

10% Separating Gel

5.5ml dH₂O
7.5ml 1M Tris-Cl, pH 8.8
200µl 10% SDS
6.7ml 30% Acrylamide
400µl 10% APS
40µl Temed

1M Tris pH 6.8

60.5g Tris
300ml dH₂O
pH with concentrated HCl to pH 6.8
Up to 500ml with dH₂O

1M Tris pH 8.8

60.5 g Tris
300ml dH₂O
pH with concentrated HCl to pH 8.8
Up to 500ml with dH₂O

4 X Loading Dye

250 mM Tris-Cl, pH 6.8
6% SDS
0.005% Bromophenol Blue
40% Glycerol
10% β-mercaptoethanol

10X Running Buffer

20g Glycine
31.6g Tris
5ml 10% SDS
Up to 500ml with dH₂O

10X Transfer Buffer

72g Glycine
19g Tris
Up to 500ml with dH₂O

1X Transfer Buffer

100ml 10X Transfer Buffer
200ml Isopropanol/Methanol
700ml dH₂O

Coomassie Staining Solution

0.024% Coomassie Brilliant Blue
90% dH₂O
10% Acetic Acid

Destain Solution

10% Acetic Acid in dH₂O

1 X TBST

30ml 5M NaCl
50ml 1M Tris, pH 7.5
500µl Tween-20
920 ml dH₂O

1 M Glycine, pH 2.5

37.54 g Glycine
500ml dH₂O
pH with concentrated HCl to pH 2.5

Immunofluorescence Solutions

50mM NH₄Cl

0.267g NH₄Cl
100ml 1XPBS
Autoclave

0.5% Triton X-100

500µl Triton X-100
100ml 1 X PBS

0.2% Gelatin

0.04g Gelatin
20ml 1 X PBS

Appendix II: Protein and DNA markers

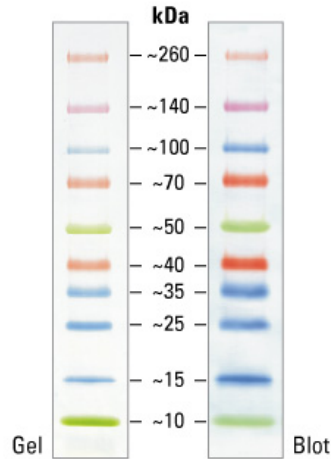


Figure IIA. SDS-PAGE band profile of the Thermo Scientific Spectra Multicolour Broad Range Protein Ladder. Images are from a 4-20% Tris-glycine gel (SDS-PAGE) and subsequent transfer to membrane. This ladder was used to determine the molecular weight of proteins separated by an SDS-PAGE gel for Western blot analysis.

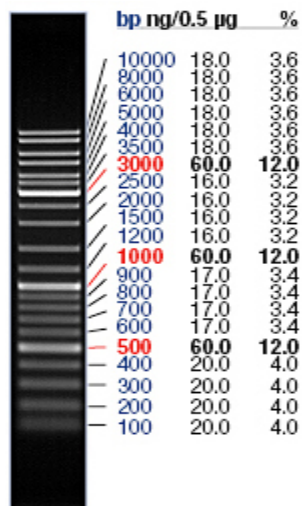


Figure IIB: DNA Ladder (O'Generuler DNA ladder mix, ready to use (Fermentas)). This ladder was used in molecular cloning to determine sizes of plasmids after manipulation.

Appendix III: FACS Cell Sorting

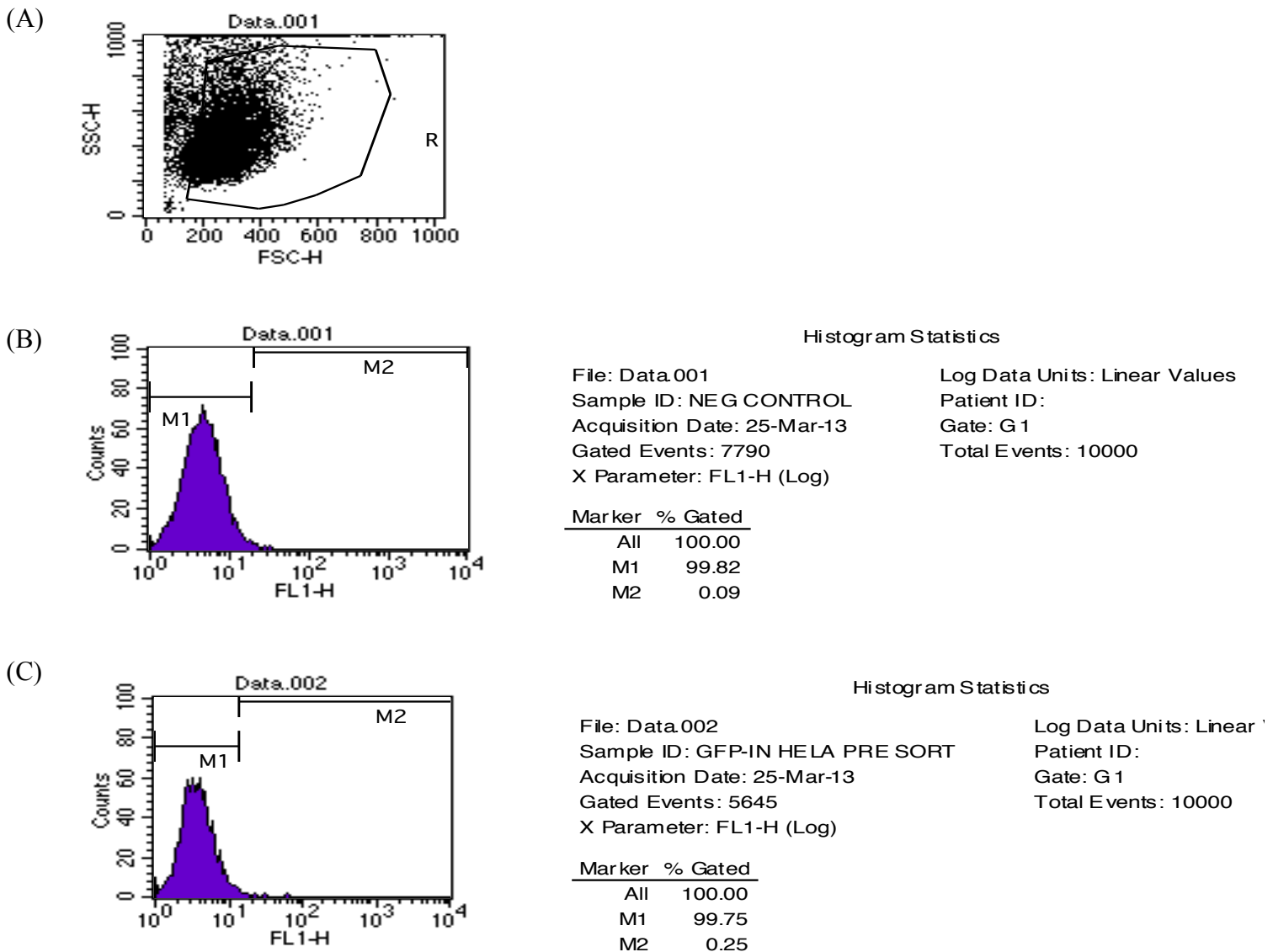


Figure III: Data from FACS cell sorting of GFP-IN transfected HeLa cells. (A) Scatter plot showing gating of cell parameters. (B) Negative control histogram of GFP negative HeLa cells. (C) GFP-IN transfected HeLa cells pre sorting. M1 represents the GFP negative population and M2 represents the GFP positive cell population. We were unable to produce a stable cell line as too few GFP positive cells could be obtained from cell sorting.

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