Investigation of the Inhibitory Role of the Integrase Importin 7-Binding Domain on HIV-1 Replication

by

Meaghan Labine

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

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Abstract:

HIV integrase (IN) is a key viral protein involved in the early stage of viral replication, and requires the interaction of host factors to help carry out this process In particular, host karyopherin, importin 7, which facilitates nuclear import, has been shown to bind integrase and potentially assist with HIV-1 viral replication. Our lab showed the specific *in vivo* binding of integrase to host factor importin 7 (Imp7), and that transient knockdown of importin-7 in HIV-1 producing and target cells, resulted in a 3 fold inhibition of HIV infection. Therefore I hypothesize that Imp7 is a key host factor required for successful HIV-1 viral replication, and that by introducing the minimal IN Imp7 binding domain within the virus or cell prior to infection, that viral inhibition can be achieved, through competitive binding of both wild type and mutant forms of IN for binding with host Imp7.

Through the use of deletion and substitution mutational analysis we identified regions, ^{235}WKGPAKLLWKG and ^{262}RRKAK, within the IN C-terminal domain (CTD) as the sites which bind Imp7. To investigate the potential effect of the IN Imp7 binding domain on viral replication, we co-expressed the IN CTD with the wild type virus. Results revealed that transiently expressed IN CTD fused with T7 inhibited viral replication by roughly 50-80% , measured by luciferase and MAGI assays. More specifically, incorporation of the minimal Imp7 binding domain of IN into the viral particle prior to infection, impaired viral infectivity in C8166 cells by up to 18 fold in comparison with the control. The successful

completion of this project, will help lay the ground work for exploring the potential inhibitory properties of other HIV-1 viral proteins on HIV replication, and thus provide a basis for producing novel therapies to combat against HIV infection.

Acknowledgments:

I would like to extend my sincerest gratitude to my supervisor Dr. XiaoJian Yao, for his continual support and insight throughout my research, and for teaching me to the value of analytical thinking. In addition, without the continual guidance of my laboratory members, Zhujun Ao, Zaikun Xu, Guanyou Huang, Yuanyuan Gu and Lina Wang, my studies would not have been complete.

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Most importantly, without my family to support me throughout these past two years, I may have never completed my studies successfully, and for that I owe them everything.

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Abbreviations

AIDS	: Acquired immuno-deficiency syndrome
BAF	: Barrier to auto-integration factor
CA	: Capsid protein
CCD	: Catalytic core domain
cPPT	: Central polypurine tract
CypA	: Cyclophilin A
Env	: Envelope protein
FG	: Phenylalanine-glycine repeats
Gag	: Group specific antigen
H1	: Histone 1
HAART	: Highly Active Anti-Retroviral Therapy
HIV-1	: Human immunodeficiency virus
HMGI (Y)	: High mobility group type protein Y
IBB	: Importin beta binding domain
IN	: Integrase protein
IP	: Immunoprecipitation
Kap	: Karyopherin
LEDGF/p75	: Human lens epithelium-derived growth factor/transcription co-activatory p75
Luc	: Luciferase
MA	: Matrix protein
MAGI	: The multinuclear activation of galactosidase indicatory assay
NC	: Nucleocapsid protein
Nef	: Negative factor
NF-κβ	: Nuclear transcription factor κβ
NLSs	: Nuclear localization signals
NPC	: Nuclear pore complex
Nups	: Nucleoporins
PIC	: Preintegration complex
Pol	: Polymerase protein
PR	: Protease protein
RanGEF	: Ran guanine nucleotide exchange factor
RanGFP	: Ran guanine-phenylalanine protein
RRE	: Rev responsive element
RT	: Reverse transcriptase protein
RTCs	: Reverse transcription complex
SV40	: Simian virus 40
TSG101	: Human tumor susceptibility gene 101
Vif	: Viral infectivity factor
Vpr	: Viral protein R
Vpu	: Viral protein U
YFP	: Yellow – fluorescent protein

Chapter 1:

1.0 Introduction

The human immunodeficiency virus type 1 (HIV-1) is a retrovirus capable of infecting both dividing and non-dividing cells [1]. This unique characteristic distinguishes HIV-1 from other retroviruses, and it is one of the hallmarks of lentiviral infection [2]. HIV-1 replication is a complicated process, and requires the interaction of host factors with viral proteins to assist with the infectious cycle. This thesis will investigate and review the impact of host cellular factors on viral replication, and explore whether these interactions can be exploited to block viral replication. The primary focus will be on the interaction occurring between the viral protein integrase (IN), and host karyopherin importin 7 (Imp7). Previous research in the area has found this IN/Imp7 interaction to be important for viral replication, and therefore we seek to define the region of IN that binds Imp7, with the intent of utilizing the binding domain to study and inhibit viral replication.

The Imp7 fragment is of particular interest because it functions to assist with nuclear import of tagged compounds, from the cytoplasm to the nucleus. Passage of the viral cDNA from the cytoplasm to the nucleus through the intact nuclear pore complex, is essential for viral replication, and is carried out by the viral pre-integration complex (PIC) in an active and energy dependant manner [3]. The nuclear pore complex regulates passage into the nucleus, and therefore requires

interaction with specific cellular proteins such as Imp7 to facilitate entry of the PIC into the nucleus. The overall mechanism underlying nuclear import of the viral PIC is still not fully understood, as there are many viral and host factors that compose the complex, and assist with translocation [4, 5]. The structural arrangement of the PIC has not yet been identified, but the components of the complex include IN, Map17(MA), Vpr, reverse transcriptase (RT) , cDNA and host karyopherins such as importin α (Impα) , importin β (Impβ) and importin 7 (Imp7) [5]. Four of the PIC associated components IN, MA, Vpr and the cis-acting determinant cDNA Flap possess karyophillic properties, which allow them to facilitate nuclear import of the PIC by interacting with host karyopherins [6-8]. Karyophillic proteins mediate the nuclear import/export of a broad range of cellular proteins and RNA, in and out of the nucleus through the NPC [9].

Nuclear import can be mediated by several distinct nuclear import pathways, each utilizing different combinations of host karyopherins, thus allowing HIV-1 the ability to take advantage of the numerous import pathways available to ensure nuclear import [10]. The classical nuclear import pathway is mediated by the importin α/β heterodimer, which binds a cluster of basic amino acids or two basic clusters separated by 10-20 amino acids (bipartite nuclear localization signal [9-12] . Using this configuration, Impα functions as the adaptor molecule binding directly to the NLS, while Impβ interacts directly with the NPC [11, 13] . Impβ has been shown to interact with HIV-1 proteins Tat, Rev and HTLV, and transport cargo into the nucleus independently of Impα [14-19] . Imp7 and Imp8,

are two very similar nuclear import factors that share homology with Impβ, and function to mediate nuclear import of mammalian proteins [20].

Imp7 and Impβ also have the ability to form a heterodimer, and mediate non-classical nuclear import of such compounds as histone 1 into the nucleus [21]. As is evident, the host cell has numerous nuclear import pathways at its disposal, and therefore it is a matter of deciphering by process of elimination which pathway(s) the virus utilizes to carry out replication.

As mentioned previously, the preintegration complex (PIC) posses four main components harboring an NLS, which are IN, Vpr, the cDNA flap and MA. IN is 32 kDa, and is a key viral enzymatic protein required for replication, assisting with the different stages of early viral replication; reverse transcription [22, 23], nuclear import of the PIC and integration of the viral cDNA into the hosts chromosome [24, 25]. Mutational analysis of the structural conformation of IN, has identified three regions to the protein; N-terminal domain, catalytic core domain and the C-terminal domain, each possessing a characteristic function [26-28]. The C-terminal domain (CTD) of IN ranging from amino acids 212-288 , functions to bind non-specific DNA at amino acids L234 and R262 [29-32], and has been reported to participate in both nuclear localization and reverse transcription.

To elucidate further the role of IN during nuclear localization of the PIC, previous studies have explored the potential of IN to interact with host karyopherins. IN has been observed to associate with Impα *in vitro,* and to facilitate nuclear import through the Impα/β classical nuclear import pathway, but these results remain controversial [8, 33-35]. . Evidence has not yet shown whether IN *in vivo* binds Impα or Impβ directly, but there have been other host factors which have been rumored to act as adaptor proteins for these interactions To further define the mechanism of nuclear import, Fassati et al. wanted to identify which host karyopherins associate with the PIC [36]. It was concluded that the HIV-1 PIC utilizes the Imp7/β heterodimer complex to mediate nuclear import, and that Imp7 which binds IN is the major contributing factor [20, 36]. Zielske and Stevensen performed similar types of Imp7 siRNA knock-down experiments on HIV-1 and SIV-1 in macrophages, and their data did not reveal the functional importance of Imp7 on HIV-1 replication [37]. As a result, further investigation is required to define the role of Imp7 during viral replication.

In the present research project, I have focused on the interaction between IN and different cellular karyophilic proteins, and demonstrated that HIV-1 IN specifically interacts with Imp7, using a cell-based co-immunoprecipitation assay. I predict that by expressing the minimal Imp7 binding domain of IN simultaneously upon infection with wild type HIV-1 virus, that both the wild type and Imp7 binding fragment will compete for binding with essential replication

factors such as Imp7, and thereby the IN fragment will impair the proper function of the wild type IN and inhibit replication.

To determine the specificity of the IN/Imp7 interaction, binding assays were performed with other host cellular karyopherins and viral karyphillic proteins. Our results indicated that Impα and Imp8 did not bind to IN, while binding of Impα to the MA (Map17) viral protein, was observed. Prior to identifying the specific amino acid sequence of IN responsible for interacting with Imp7, we first defined which of the three IN domains was involved with Imp7 binding. Deletion of the various IN domains in comparison to wild type IN, highlighted that the IN/Imp7 interaction is localized to the C-terminal domain of IN. Mutational analysis of arginine/lysine rich domains defined two regions (^{235}WKGPAKLLWKG and ^{262}RRKAK) within the IN C-terminal domain, as the critical sites responsible for binding Imp7. After identifying the two Imp7 binding regions of the IN C-terminus, I then wanted to explored the potential HIV-1 inhibitory effect of the minimal Imp7 binding domain on wild type HIV-1 replication. To accomplish these sets of experiments, we used a two method approach of 1) expressing the IN CTD within the cell prior to infection and 2) expressing the IN CTD within the viral particle prior to infection. Prior to infection, the IN CTD was observed to impair viral replication by roughly 50-80%, and viral progeny derived from cells harboring the IN CTD fragment were impaired for infectivity by 75-80% as compared to the controls.

Expression of the IN CTD within the viral particle prior to infection also had a dramatic impact on viral replication, whereby we constructed IN CTD fragments possessing the minimal Imp7 binding domain. It was found that the IN CTD fragment R14-88 INc241-270 containing only the ^{262}RRKAK Imp7 binding domain, inhibited viral replication by roughly 2.5-18 fold. Whereas R14-88 INc228-270 possessing both ^{235}WKGPAKLLWKG and ^{262}RRKAK Imp7 binding domains exhibited the ability to restore viral replication to normal control levels. It is speculated replication inhibition is due to the competitive inhibition occurring between both the wild type and IN CTD fragments, for binding to essential replication factors such as Imp7. Due to the limited availability of the Imp7 substrate and the ease at which the smaller IN fragments can maneuver around the cell, it allows for substrate binding and inhibition of viral replication. Identification of a small HIV-1 inhibitory peptide has strong potential as potent anti-viral therapy, . whereby new drugs could be developed that mimic host cellular machinery that assists viral replication and block HIV-1 infection.

1.1 Study Objectives

The aim of this project, is to fully understand the relationship between integrase and importin 7, and define how this interaction can be exploited for therapeutic use. We predict that by constructing a peptide that mimics the Imp7 binding domain of IN, that it will disrupt through competitive inhibition, binding of Imp7 to wild type IN, and thereby block viral replication. The particular objectives of this research are listed below, and divide the project into its four main sections

1.) Define the integrase domain that binds host importin 7

2.) Define the specific amino acid regions of integrase responsible for binding importin 7

3.) Determining if the importin 7 binding domain of integrase inhibits HIV-1 viral replication

4.) Defining the minimal region of integrase that binds importin 7 and inhibits HIV-1 viral replication

1.2 Literature Review

Human immunodeficiency virus type 1 virus (HIV-1) is a retrovirus that causes acquired immune deficiency syndrome (AIDS), a disease in which the immune system fails to function, leading to infection and eventual death by opportunistic pathogens [26, 38-40]. HIV-1 was originally recognized in 1981, and to date 25 million people have died from AIDS, with currently over 40 million individuals infected worldwide. The virus primarily infects CD4+ T helper cells, but also macrophages and dendritic cells, leading to a gradual decline in cell mediated immunity [26, 41-44].

Currently, there is no vaccine or cure for HIV or AIDS, and the only known method of prevention is avoiding exposure. Antiviral therapies available consist of highly active antiretroviral therapy (HAART), and options include combinations of at least three drugs belonging to two or more classes of anti-retroviral agents such as; nucleoside analogue reverse transcriptase inhibitors, protease inhibitors or non-nucleoside reverse transcriptase inhibitors. HAART provides a means to stabilize the patient's symptom and suppress HIV-1 replication [45]. This therapy does not cure the patient nor alleviate the symptoms and must be taken life long, but due to high toxicity, metabolic disorders and emergence of drug resistant HIV strains, new, more patient friendly anti-viral therapies are required [46-49].

With increased understanding of the molecular mechanisms governing HIV-1 replication, and of the highly conserved viral-cellular protein interactions occurring, new therapies are being explored which are highly specific and potentially less toxic. Throughout the HIV-1 replication cycle, there are additional key proteins and processes which can be exploited and blocked to inhibit HIV-1 replication. Numerous groups have focused on IN inhibitors, prevention of translocation and definition of specific interactions occurring between viral and host proteins with the intent to block replication [23, 50, 51].

The following mini-review will provide an overview of the HIV-1 replication cycle, nuclear import within the host cell, structure and function of IN and other viral proteins and the structure and function of Imp7.

1.2.1 HIV-1 virion structure:

The mature HIV-1 virion particle is enveloped with a diameter of roughly 110nm, with gp41 protruding from and anchoring glycoprotein gp120 to the lipid envelope derived from the host membrane. Providing structural support to the envelope, is a thin layer of matrix protein lining the inner surface of the viral particle. Contained within the virion is the HIV-1 genome consisting of two identical 9 kb single stranded RNAs, surrounded by a conical capsid composed of roughly 2000 copies of viral protein, p24. To assist with early replication RT, IN, NC and various other accessory viral proteins, Vif, Vpr, p6 and Nef are present within the capsid (Fig. 1) [26, 52] .

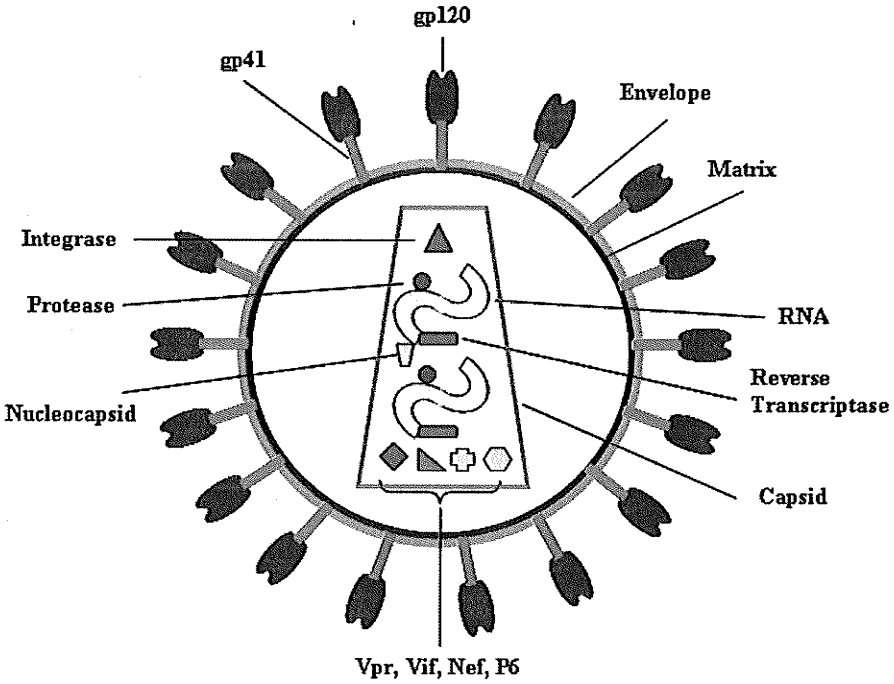

Fig 1. HIV-1 Virion Structure. A. Schematic diagram of the viral proteins and structural composition of HIV-1 mature virion. The HIV viral particle possess an outer envelope that is studded with gp120 and gp41 proteins which facilitate recognition and attachment to the host cell. The matrix protein is found just underneath the outer envelope, and housed within the entire structure is the conical core composed of capsid proteins that contains two copies of the positive single stranded RNA. In addition enzymatic proteins integrase, protease, reverse transcriptase are present to assist with early stage replication, and accessory proteins Vif, Vpr, Nef and P6 are present to enhance the efficiency of replication. The nucleocapsid is also present within the conical core and is a structural protein that assists with virion incorporation of viral RNA [26, 52, 240].

1.2.2 HIV-1 Genomic Organization:

The HIV-1 genome is a composition of nine structural and non-structural proteins encoded by the 9 kb RNA strand. The three structural genes gag, pol and env contain information required for synthesis of new virus particles. Gag encodes for $Pr55^{Gag}$, a single polyprotein, which is further cleaved by the viral protease into capsid (CA p24), matrix (MA p17), nucleocapsid (NC p7) and p6. During translation, Pr55 is myristoylated, allowing the protein to associate with all aspects of the host cell membrane. The membrane associated Pr55 polyprotein recruits two copies of cytoplasmic HIV-1 genomic RNA, along with other viral proteins to the cell membrane and stimulates virus budding. Following budding during viral maturation, virally encoded proteases cleave Pr55 into the other smaller proteins mentioned [26, 52, 53], as well two spacer proteins are also generated at the same time, P2 and P1.

The Pol gene encodes enzymatic proteins which are initially synthesized as part of a larger gag-pol polyprotein precursor $Pr160^{GagPol}$ [54]. During Pr55 translation, a frame shift event triggered by a cis-acting RNA motif, generates Pr160 which encodes for enzymatic proteins RT, IN and PR that are cleaved as well by viral protease [55] . The Env gene, which encodes for gp120 surface glycoprotein and gp41 transmembrane glycoprotien, is originally synthesized as polyprotein precursor gp160, which like gag and pol must undergo proteolytic processing by viral proteases within the cellular environment, to yield its protein derivatives. The Gag and Env proteins provide the structural framework for the virion,

composing the core of the particle and the outer member envelope. Essential enzymatic proteins are synthesized by the Pol gene, and are packaged within the viral particle, along with two strands of genomic RNA [56, 57]

1.2.3 HIV-1 Regulatory genes and their products:

HIV-1 also codes numerous regulatory genes aside from gag, pol and env, whose main function is to regulate transcription of the viral genome after insertion into the host chromosome. These regulatory genes include tat and rev (*Fig 2.*).

Tat is a small 30 kDa protein, and is encoded by separate exons located at the 3' end of the viral genome. The first exon which is 14 kDa ranging from 1-72 amino acids, proceeds the env gene, and contains the major functional domains which are capable of fully trans-activating transcription. The second exon is 2 kDa and encodes for an additional 14 amino acids, and is speculated to assist with tat binding to cell surfaces via integrin mediated cell adhesion. The overall function of this protein is to bind to the trans activating response RNA element (TAR) in the LTR promoter, and act as transcriptional regulator of viral gene expression. This protein also represses host cellular promotors to impair cellular processes, and allows for the production of full length HIV-1 transcripts [26, 52, 58]

Rev is another regulatory gene encoded by two separate exons which partly overlap with the tat exons. This is a 19 kDa protein that has a broad range of

functions, and facilitates export from the nucleus to the cytoplasm, splicing and stabilizing viral RNA transcripts. Rev directly binds to the RRE region located in the *env* coding domain, and prevents premature RNA splicing prior to nuclear export. Rev contains a strong leucine-rich nuclear export signal, which allows it to translocate between the nucleus and cytoplasm. This protein has also been reported to up-regulate the production of *gag* and *env* structural genes, and induce transition of viral replication from early to late phase [26, 52, 59]

1.2.4 HIV-1 accessory proteins:

HIV-1 accessory proteins Vpu, Vif, Vpr and Nef *in vitro* are not uniformly required for viral replication, and as such termed "accessory" proteins. During *in vivo* infection they act in varying degrees to increase the efficiency of viral replication and enhance disease progression [60-62] (*Fig 2.*).

Vpu is a 16 kDa oligomeric integral transmembrane protein, that associates with the host cellular and the endoplasmic reticulum (ER) membranes. Vpu has two primary functions 1) Promotes the degradation of CD4 allowing for the transport of gp120 and gp41 to the cell surface for incorporation into the budding viral membrane, from the ER. 2) Vpu enhances the release of viral particles, detaching them from the host cell surface [26, 52].

Vif is a basic 23 kDa protein that is spliced from a single mRNA transcript. This protein is packaged within the viral particle and as well localizes within the cell cytoplasm, and because its expression is dependant upon Rev, it is classified as a late gene product [59]. Vif has two primary functions 1) enhancing the infectivity of virus by 10 to 1000 fold and 2) promoting viral maturation. Borman et al. conducted a study in which they concluded that the effect of Vif deletion during viral replication was highly dependent on the cell type utilized, and potentially this was the result of specific host factors altering Vif function [63]. It is speculated that Vif may increase infectivity by interacting with specific anti-viral compounds such as APOBEC3G, and inducing degradation of the protein via a ubiquitination-dependent proteasomal pathway, thereby preventing inclusion of this anti-viral protein into the virion [26, 52, 64].

Vpr is a 14 kDa protein consisting of 96 amino acids. During cell culture infection, this protein is not required for viral replication, but is packaged into the virion through interaction with the p6 *gag* protein [65]. Vpr plays several major roles during the infectious cycle 1) Assisting with PIC nuclear import within non-dividing cells, by potentially interacting the classical Impα/β heterodimer pathway [66, 67]. 2) The presence of Vpr also induces cell cycle arrest in the G2 phase of the host cell cycle by activating the ATR cellular pathway [68]. 3) Modulates gene transcription through interaction with cellular factor p21(WAF1) [69], and induces apoptosis [69-71]. Mutations within the C-terminal domain of Vpr have also been associated with decreased virulence, as HIV-1 strains with mutant Vpr

have been found present in many long term non-progressors (LTNP), and may be the result of the proteins inability to induce cell cycle arrest, and prevent cellular division [68].

Nef, is a 27 kDa protein consisting of 206 amino acids that is contained in a single open reading frame overlapping the *env* gene [61], and is a membrane associated phosphoprotein. Myristoylation of Nef's N-terminus region facilitates membrane binding, and like that of Vpu, aids in the reduction of cellular CD4 and MHC-I by rerouting the molecules from the Golgi apparatus and cell surface to the lysosome for degradation [52]. Nef mutants lacking the gene experience decreased viral DNA synthesis [52], while in some situations Nef mutants with a 135 inner deletion induces enhanced virulence [72, 73]. The protein is present in low levels within the viral particle, and is thought to play a role in viral assembly, maturation or entry [52]. By binding to several Src-family proteins and regulating their tyrosine-kinase activities, Nef is also able to enhance viral infectivity, and prevent apoptosis by interacting with signal-regulating kinase (ASK1) protein [74].

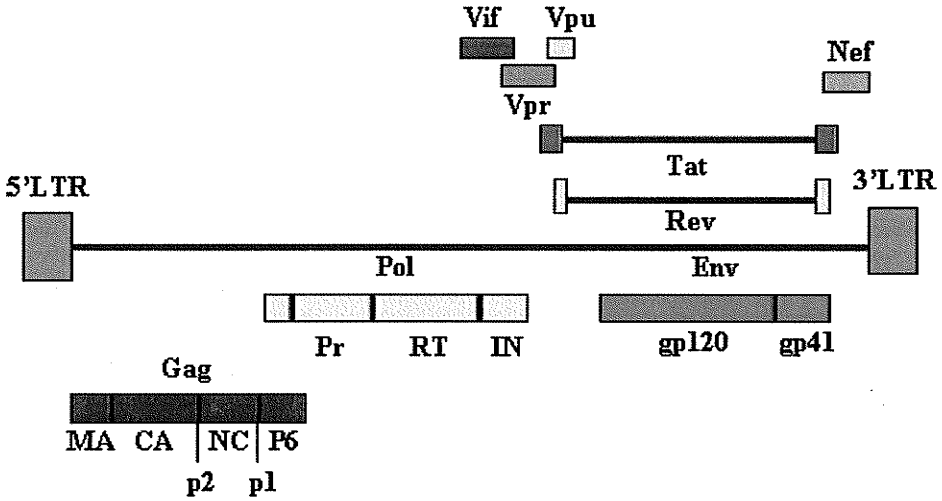

Fig 2. HIV-1 Genomic Organization: A. Schematic representation of the HIV-1 genome, structure and gene products. The HIV-1 genome consists of the Gag region which encodes structural proteins matrix (MA), capsid (CA), nucleocapsid (NC) and p6. Pol region encodes for enzymatic proteins protease (PR), reverse transcriptase (RT) and integrase (IN). The Env region encodes for envelope proteins gp120 and gp41. Spanning between between the Pol and Env regions is the area that encodes for the accessory and regulatory proteins. Tat and rev are regulatory proteins that assist with the process of transcription, while the accessory proteins Vif, Vpr, Vpu and Nef increase the efficiency of viral replication. The entire sequence is flanked by repeating LTR ends, which assist with integration of the viral cDNA into the host's chromosome [26, 29, 52].

1.2.5. Integrase structure and function:

HIV-1 integrase (IN) is a 32 kDa enzymatic protein, derived from the C-terminus of the Gag-Pol polyprotein by proteolytic cleavage (*Fig 2).* IN is organized as a tetrameric catalytic protein, which is involved throughout the early stages of viral replication to affect reverse transcription, nuclear import and integration [8, 27, 75, {Chiu, 2004 #8]. IN consists of three functionally and structurally distinct regions: N-terminal domain, spanning from residues 1-50 that possess a conserved HHCC zinc-finger binding site and is responsible for

stimulating multimerization of IN and contributing to IN's catalytic function. The catalytic core spanning from aa 51-212, is relatively unconserved and catalyzes the 3'OH end processing and DNA strand transfer of the viral cDNA, mediating integration. The C-terminus end of the protein runs from 212-288 aa of the protein, and is involved with non-specific DNA binding. Interestingly, although IN functions as a tetrameric complex throughout HIV-1 replication, the individual expression of each domain is able to form dimeric complexes consisting of two monomers [31, 56, 76] (*Fig 3.*).

The ability of IN to interact with cellular factors such as importin α, LEDGF/p75, EED, HSP60, Rad18 and HAT p300, has been associated with assisting various replication functions that the protein carries out [77-79]. Most importantly, the method by which host karyopherins facilitate nuclear import of the PIC remains unknown, and will be explored in a later section.

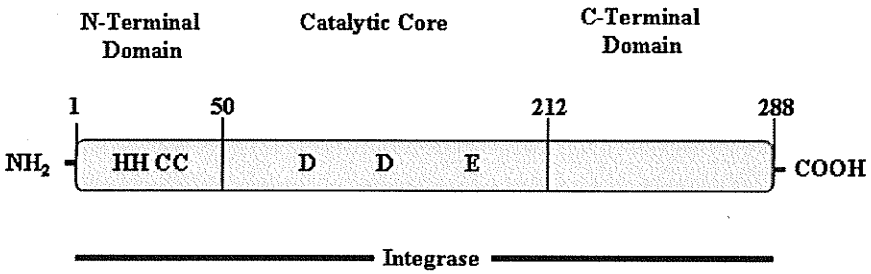

Fig 3. HIV-1 integrase domains: Schematic representation of the division between the three functional domains of IN. The N-terminal domain spans from 1-50 amino acids and is responsible for zinc binding. The catalytic core spanning from amino acids 51-212 catalyzes integration, in particular 3'OH end processing and DNA strand transfer. The C-terminal domain spanning between amino acids 212-288 is responsible for non-specific DNA binding and interaction with other host cellular factors [29]

1.2.6 Integrase C-terminal domain:

The IN CTD possesses five strands arranged in an anti-parallel orientation to form a β barrel which adopts an SH3-like fold, to facilitate IN-protein, IN-IN and IN-DNA interactions. The minimal solution structure of the CTD is composed of two monomers forming a dimmer, which is formed between two β sheets, and is orientated at a 90° angle to one another relative to their two fold axis and are bound to the core by helix α9. Dimerization of the CTD has also been shown to be essential for multimerization of the entire IN complex, and is essential for IN function [80, 81]. Mutational analysis of lysine residues within the CTD identified L241 and L242 as the key sites which mediate dimerization [29] (*Fig 4.*).

To accommodate for DNA binding, it is suspected that the cleft formed between the two CTD subunits is responsible for binding DNA, whereas another theory proposed that DNA binds along the outside surfaces. Amino acid residues arginine 262, lysine 264 and leucine 234 have been defined as the DNA binding regions, which are located along the outer regions and within the cleft respectively [27, 29, 31].

Within the CTD there are three highly conserved regions: Q, C and N. Each of which possess arginine and lysine rich domains, known to act as nuclear localization signals (NLSs), providing IN with karyophillic properties. It is within these domains that IN is suspected of interacting with host karyopherins, to

facilitate nuclear import of the PIC [8, 82]. In a recent report by Creseto et al they identified that host acetyltransferase factor, HAT p300 interacts with the CTD at specific lysines 264, 266 and 273. Acetylation of these residues was speculated to increase IN's affinity for DNA and promote DNA strand transfer [83]. In a more recent finding, acetylation of these residues was found to occur in a hierarchical manner, but it could not be confirmed that HAT p300 acetylation of the CTD played any significant role during IN catalyzed DNA strand transfer or integration in general [84].

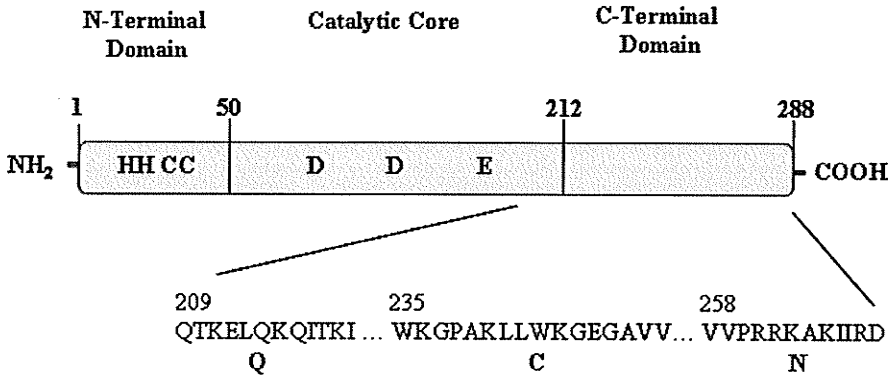

Fig 4. Conserved domains within the CTD of IN: The IN C-terminal domain is involved with non-specific DNA binding and contains numerous conserved domains. Region Q is a glutamine rich/basic region in all lentiviruses and spans from amino acids 209 – 220. Region C and N spanning from amino acids 235-250 and 258-270 respectively, are highly conserved throughout many strains of HIV-1, but not in all [29, 39].

1.2.7 Host cellular factors:

As was previously described, viral replication requires the assistance of numerous host replication factors and complexes in order to carry out successful replication. Described within this section are the main cellular components utilized by the virus to assist with nuclear import of the viral PIC. The nuclear pore complex (NPC) governs entry and exit of large complexes in and out of the nucleus, acting as the gate keeper of nuclear/cytoplasmic translocation. Passage through the NPC is provided to complexes harboring a nuclear localization signal (NLS), whereby that region is bound by host karyopherins such as Imp7, which function to mediate passage through the NPC. With regards to viral replication, the PIC containing the viral cDNA must be translocated from the cytoplasm to the nucleus through the NPC, in order to allow for viral cDNA integration into the hosts chromosome. The details surrounding the complete process of viral replication will be outlined later in the text.

1.2.8 Nuclear pore complexes:

To allow for communication between the cytoplasm and nucleus, cellular proteins and RNAs must be continually shuttled between the two compartments through nuclear pore complexes (NPCs). NPCs are the sole gateway for nucleocytoplasmic shuttling, these complexes provide a diffusion channel for small molecules and mediate active transport of larger proteins and RNAs. NPCs are large 44-60 MDa symmetric structures situated in the double lipid membrane

of the nuclear envelope, and are composed of roughly 30 nucleoporin proteins (Nups). Structurally, the NPC consists of a single flexible channel, that can expand between 10-25nm. The whole complex itself is composed of three separate structural elements: 1) Cytoplasmic fibers, 2) the central core and 3) the nuclear basket, which all function together to facilitate transport between the two lipid membranes [85]. Overall, NPCs have an 8- fold rotational symmetry perpendicular to the plane of the nuclear envelope [86, 87] (*Fig 5.*).

To bind directly to nuclear import compounds, Nups possess phenylalanine-glycine repeat units (FG), which forms a dynamic meshwork of filaments acting as a barrier at the cytoplasmic opening of the NPC. These compounds are situated symmetrically around the NPC, and constitute roughly half the mass of the NPC. When the karyopherin interacts with FG regions, the mesh reorganizes to allow passage through the NPC [88] (*Fig 5 and 6.*).

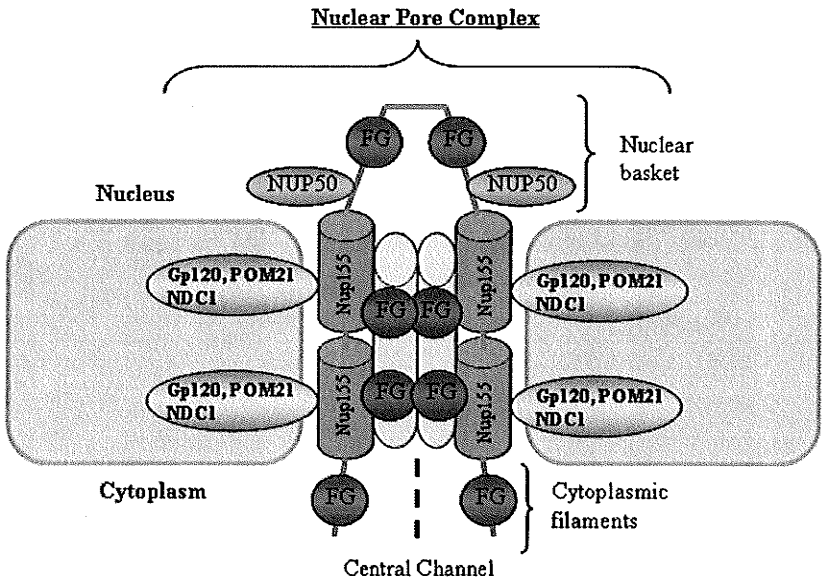

Fig 5. Organization of the nuclear pore complex: Schematic diagram of the mammalian nuclear pore complex (NPC). The NPC traverses the nuclear membrane and is composed varying nucleoporin proteins (Nups). The NPC functions to regulate passage in and out of the nucleus and requires interaction with specific host karyopherins to allow passage. The FG proteins interact with incoming cargo and realign to coordinate movement through the pore [86-89].

1.2.9 Nuclear localization signals:

Nuclear localization signals (NLSs) are specific sequences encoded within a protein that are recognized by protein import receptors, to shuttle the protein into the nucleus [90]. The NLS from Simian virus 40 large T-antigen (SV40), was the first NLS identified, and consists of basic amino acids (PKKKKRKV) [91, 92]. The classical NLS signal is well known for binding the importin α/β heterodimer complex and initiating nuclear import. The classical nuclear import pathway has a distinct protein interaction arrangement, as it requires an adaptor karyopherin such as importin α, which binds to the classical NLS on the cargo, followed by a transporter karyopherin such as importin β, which binds the adaptor, and interacts the ternary complex with the NPC to facilitate translocation into the nucleus [8, 12]. There are two main groups of classical NLSs, the monopartite signal which includes SV40 and the hydrophobic c-myc NLS (PAAKRVKLD). Bipartite NLSs consist of two stretches of basic amino acids as that seen in a monopartite signal, yet they are separated by a spacer region of roughly 10-20 amino acids [8, 18, 93-95].

Non-classical nuclear import pathways operate in a slightly different fashion from that of classical, whereby they express an amino acid NLS that binds directly and specifically with certain transporter proteins. Typically, karyopherins belonging to the importin β family act as the transporter protein, and currently 14 types have been discovered in yeast and over 20 in mammalian cells. In addition, Impβ is

able to engage other karyopherins to form heterodimeric complexes, such as the Impβ/7 heterodimer that imports histone H1 into the nucleus [21, 93].

1.2.10 Nuclear import into the nucleus:

There are two methods of translocation across the NPC, passive diffusion for compounds smaller than 40 kDa (9nm), and energy dependent active transport used for larger compounds up to 39nm in diameter. Active transport of larger molecules requires association with larger soluble import factors, including small GTPase Ran [9, 96]. NPCs mediate both nuclear import and export, and under certain circumstances *in vitro* transport is reversible [97, 98]. Karyopherins (Kap), are a group of soluble cellular transport proteins that work in association with the NPC, and function to shuttle cargo to and from the NPC for transport. These proteins recognize and bind specific sequence signals within a target protein, which are composed of arginine and/or lysine rich regions. There are two types of signals, a nuclear localization signal (NLS) which triggers nuclear import, and a nuclear export signal which stimulates removal or export of a compound out of the nucleus. Energy dependence and directionality of transport is dictated by the RanGTP gradient established across the cytoplasm and the nucleus. To date the only energetic requirement for transport is that linked with RanGTP hydrolysis [10, 16, 99] (*Fig 6.*).

During the initial stages of nuclear import, the karyopherin and NLS bearing protein are complexed together, and dock at the peripheral cytoplasmic region of the NPC, engaging the FG filaments. Upon reorganization of the FG regions via Kap interaction, the import complex passes through the NPC and into the nucleus. Liberation of the cargo from the Kap is catalyzed by RanGTP, which dissociates the two components [3]. During nuclear import RanGTP stimulates cargo release within the nucleus, while during nuclear export, RanGTP stabilizes the interaction between cargo and Kap, and migrates with the complex into the cytoplasm *(Fig 5.)*. To maintain the RanGDP/GTP gradient across the two compartments, RanGP1 a component of the RanGTPase system, mediates the RanGAP dependent disassembly of RanGTP to RanGDP once it enters the cytoplasm. Within the nucleus, RCC1 catalyzes the conversion of RanGDP to RanGTP in a series of reactions, whereby it is utilized for nuclear export [36, 87, 98, 99]. For retroviral replication, taking advantage of the host's nuclear import machinery is highly advantageous, as it increases the infection efficiency of the virus (*Fig 6.*).

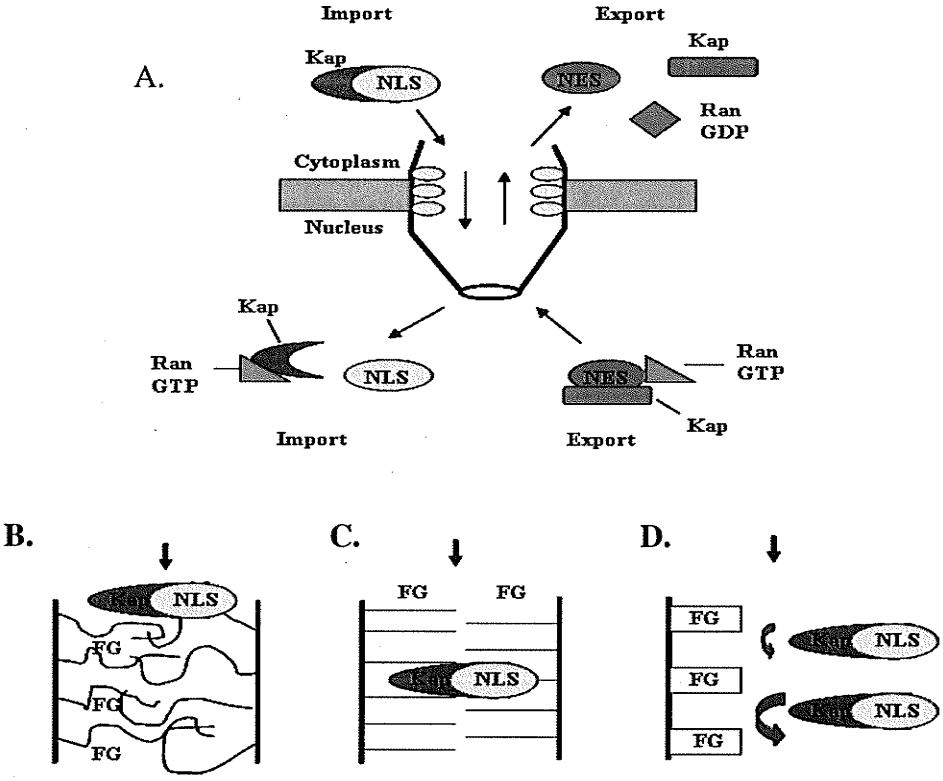

Fig 6. Nuclear import and export through the NPC and association with the FG filament region: A) Import of Kap and the NLS bearing cargo begins within cytoplasm, and is initiated once the import complex docks with the NPC. B-D) Kap interacts with the FG filaments, inducing a linear rearrangement to allow for passage, after passage through the NPC (A), RanGTP associates with the import complex and triggers release of the cargo into the nucleoplasm. A) The nuclear export complex interacts with RanGTP for stabilization, and upon export, RanGAP stimulates hydrolysis of RanGTP to form RanGDP, liberating the NES bearing cargo from the Kap [36, 97]. D) As the Kap and NLS complex move through the NPC, a gradient is established in which the complex is pulled through at a faster rate as it passes [10, 16, 99].

1.2.11 Host Karyopherins:

Within the host, cellular transport between the cytoplasm and the nucleus is an integral part of many processes such as, gene expression, cell-cycle progression and signal transduction. Nucleocytoplasmc shuttling is mediated by host karyopherins, which are a group of proteins that assist with nuclear import/export of NLS/NES bearing compounds, through the highly regulated NPC. They act as the key to nuclear translocation, and are essential for nucleocytoplasmic exchange and communication (*Fig 6.).*

Karyopherin α otherwise known as importin α (Impα), is a 60 kDa adaptor protein which binds the first identified classical NLS, such as that identified from SV40. The armadillo repeat region (ARM) of Impα binds the NLS of the cargo, while the Impβ binding domain (IBB) forms a heterodimer complex with Impβ, whereby they comprise the classical nuclear import pathway [9, 13, 95, 100, 101] (*Fig 7.*).

Karyopherin β otherwise known as importin β1 (Impβ1) is a 97 kDa protein, that is the first protein within a large super family of Impβ homologues. Impβ is able to function independently as an import factor binding to arginine rich NLSs, or in conjunction with other karyopherins such as Impα or Imp7 to form heterodimer import complexes [18]. Within the mammalian cell there are over 20 known Impβ family proteins, all of which are generally acidic, having in common an N-terminal Ran binding domain. These proteins function not only in nuclear import,

but assist with bidirectional translocation, nuclear export, assembly of the nuclear membrane and aiding maintenance of the cell cycle [93, 101-104].

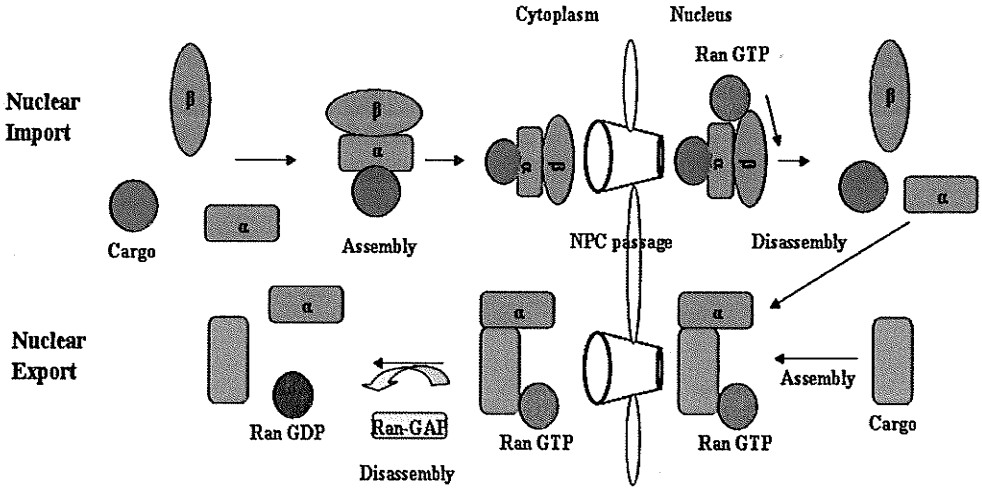

Fig 7. Importin α/β classical nuclear import pathway. Interaction of Impα with the cargo classical NLS, is facilitated by the cNLS binding pocket within Impα, and is composed of armadillo repeats (ARMs). The Impβ binding domain (IBB) within Impα interacts with Impβ to form the ternary nuclear import complex. The Impα/β heterodimer forms a complex with the cargo to facilitate classical nuclear import through the NPC. Impβ reorganizes the FG filament regions of the NPC, and mediates translocation through the pore. Once the complex has passed into the nucleus, Ran-GTP facilitates disassembly of the complex into its component parts. Impα and Ran-GTP are recycled and utilized during nuclear export of more another protein which harbors a nuclear export signal (NES). Impα, cargo and Ran-GTP form a complex that passes through the NPC, and upon entry into the cytoplasm Ran-GAP mediates disassembly and conversion of Ran-GTP into Ran-GDP. This process initiates disassembly of the nuclear export complex into its component parts [18, 93, 101-104].

1.2.12 Importin 7 function:

Importin 7 formerly known as Ran binding protein 7 (RanBP7), is a 110 kDa host karyopherin, that is part of the Impβ super family sharing a high degree of homology with Imp8. Imp7 is distantly related to Impβ, sharing only a similar N-terminal Ran binding domain [16, 103]. Like other karyopherins, Imp7 is affiliated with mediating nuclear import through the NPC, having the potential to function independently or in a heterodimer complex with Impβ1 [20, 37, 93]. There are numerous host proteins that Imp7 interacts with such as glucocoticoid receptor, ribosomal proteins and histone H1 to facilitate nuclear import. Interestingly, Imp7 has always been identified to function in concert with either the classical import pathway, or through formation of the Impβ/7 heterodimer complex [20, 21, 105].

With regards to HIV-1 viral replication, there is considerable controversy over the impact of Imp7 on nuclear import of the PIC. Fassati et al reported that Imp7 was the sole mediator of PIC nuclear import, while another more recent paper concluded that Imp7 was dispensable [36, 37]. There is the possibility that Imp7 mediated nuclear import is accomplished using the Impβ/7 heterodimer complex, as is the case with histone H1 [21]. Potentially, nuclear import of the PIC is a process mediated by numerous import pathways both classical and nonclassical, which coordinate in such a fashion as to increase the efficiency and success of this complicated mechanism.

1.2.13 HIV-1 Replication:

HIV-1 is a retrovirus that belongs to the genus lentiviridae. Replication of this virus is divided into early and late stages, and occurs through a series distinct events. Although there are many aspects of HIV-1 replication that are similar to other retroviruses, there are certain unique aspects that are distinct [106]. Like all viruses, HIV-1 requires interaction with host cellular machinery during both the early and late phases of replication, and throughout must down-regulate host defenses to ensure successful infection [107-109].

Early viral replication includes viral entry into the target cell, uncoating, reverse transcription of the viral RNA into cDNA, nuclear import of the PIC and integration of the cDNA into the host's chromosome. Late phase however includes all activities spanning from viral gene expression through to viral budding and particle maturation
[26, 106] (*Fig 8.*)..

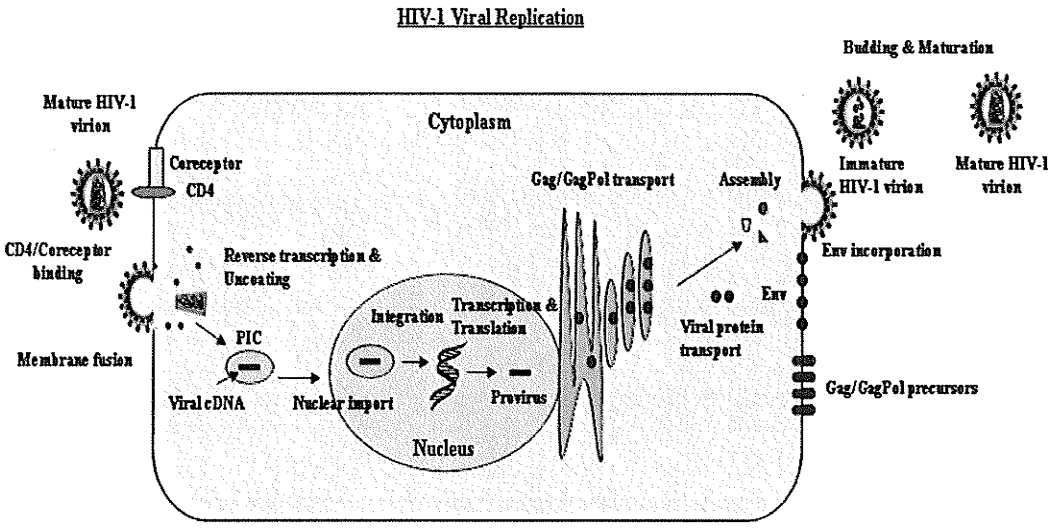

Fig 8. HIV-1 Replication: A. Diagram outing the major events during both early and late HIV-1 viral replication. The early stage of viral replication includes the processes of attachment, membrane fusion, reverse transcription, nuclear import and integration. The late stages of viral replication include transcription and translation of the viral genome, production and maturation late stage proteins, viral protein transport, assembly and encapsulation of viral proteins into the virion particle. After budding from the host cell, the virus is still immature, and must undergo further maturation to in order to produce a mature infectious viral particle [26, 106].

1.2.14 Viral Entry:

Entry into the host cell by the virus first requires receptor and co-receptor binding of gp120, leading to membrane fusion and penetration of the viral core into the host. Gp120 is highly specific for CD4 and binding to this protein induces conformational changes in gp120, which alters the specificity of the protein and increase it's affinity for co-receptors CCR5 and CXCR4 [43, 110]. Depending on the viral tropism determined by the V3 loop of gp120, this protein can bind either CCR5 or CXCR5 [111]. CCR5 is a β-chemokine receptor typically found on primary lymphocytes and macrophages, whereas it is not found on T cell lines [112-114] . CXCR4 is an α-chemokine receptor expressed on primary lymphocytes and T cell lines, but not on macrophages. Co-receptor binding allows HIV-1 to target and bind a broader host range of immune cells expressing variable receptors [115, 116] (*Fig 8.*).

Once gp120 interacts with CD4 and CXCR4/CCR5 receptors, the gp41 glycoprotein is activated to undergo an extended conformational change, in which the N-terminus of the protein is inserted into the host cell membrane [26, 106, 115, 117]. After insertion, a stable six-helix bundle is formed by the N and C helixes of gp41 tightly folding in, which positions the viral and host membranes in place to facilitate fusion and delivery of the HIV-1 core into the cytoplasm [106].

1.2.15 Viral uncoating:

Following fusion of the viral and host lipids membranes, the viral capsid enters the host cytoplasm. Uncoating is defined as the partial and progressive disassembly of the core to release the viral RNA genome and reverse transcription complexes (RTCs) into the cytoplasm [118]. This process of viral replication is very poorly understood. It is speculated that phosphorylation of both MA and CA proteins may influence uncoating, and the incorporation of theronine/serine kinases within the viral particle phosphorylates the MA and CA proteins [119]. The process of uncoating may be initiated by penetration itself, and assisted by other host and cellular factors not yet identified [26, 106, 118].

A recent study speculates that uncoating is not an immediate-early activity, and the process of reverse transcription occurs while inside an intact capsid shell on the way to the nucleus. They reported that the viral cDNA flap mediates uncoating once the PIC reaches the nucleus, and that in the absence of the cDNA flap, the linear viral DNA is trapped within the capsid [120] (*Fig 8.*).

1.2.16 Reverse transcription:

A hallmark of retrovirus infection, is the ability to convert their RNA genome into viral cDNA during the early stages of replication, using the process of reverse transcription [26, 121]. Reverse transcriptase (RT) is a 66 kDa enzymatic protein that consists of two subunits, p66 and p51, cleaved from the same region of the $Pr160^{GagPol}$ gene by viral protease [122]. Following uncoating,

viral RNA is reverse transcribed by virion-associated RT enzyme, to produce a linear full-length double stranded DNA molecule. Reverse transcription is carried out in a series of steps by various cis acting viral and host elements, to form the reverse transcription complex (RTC) [123, 124]. The precise composition of the RTC is still unknown, but it is the intermediate complex prior to forming the preintegration complex, and possess incompletely reverse transcribed viral RNA, NC and IN [26, 52, 106, 125] (*Fig 8 and 9.*).

To initiate reverse transcription, host tRNA must anneal to the 5' region of the viral RNA UTR, and function as a primer for the synthesis of the negative strand viral DNA [126]. In order for this to occur, Barraud et al postulated that methylated NC protein must associate with the viral particle, and chaperon the RNA rearrangement required for this event to occur [122]. The viral CA protein has been found to have a pronounced effect upon viral core maturation, and hence influences reverse transcription. The N-terminus region of the CA maintains the structure and functional activity of the protein, which when mutated, impaired the viruses ability to initiate reverse transcription [122, 124, 127]. Host protein cyclophilin A (CypA), is known to have the function of enhancing viral infectivity after initial post-entry, by counteracting the activity of host restriction factor Ref1. CypA interacts with HIV-1 CA protein to modulate the restriction enzyme's interaction potential, as to enhance viral infection [128, 129].

Several accessory viral proteins assist with reverse transcription, and potentially modulate the cellular environment. Vif is active during reverse transcription, and has the effect of degrading host cellular APOBEC3G, counteracting it's ability to impair reverse transcription [121] . It is not certain how Nef influences reverse transcription, but the viral protein is known to enhance viral DNA synthesis during either the uncoating process, fusion with the plasma membrane or reverse transcription itself [130] (*Fig 9.*).

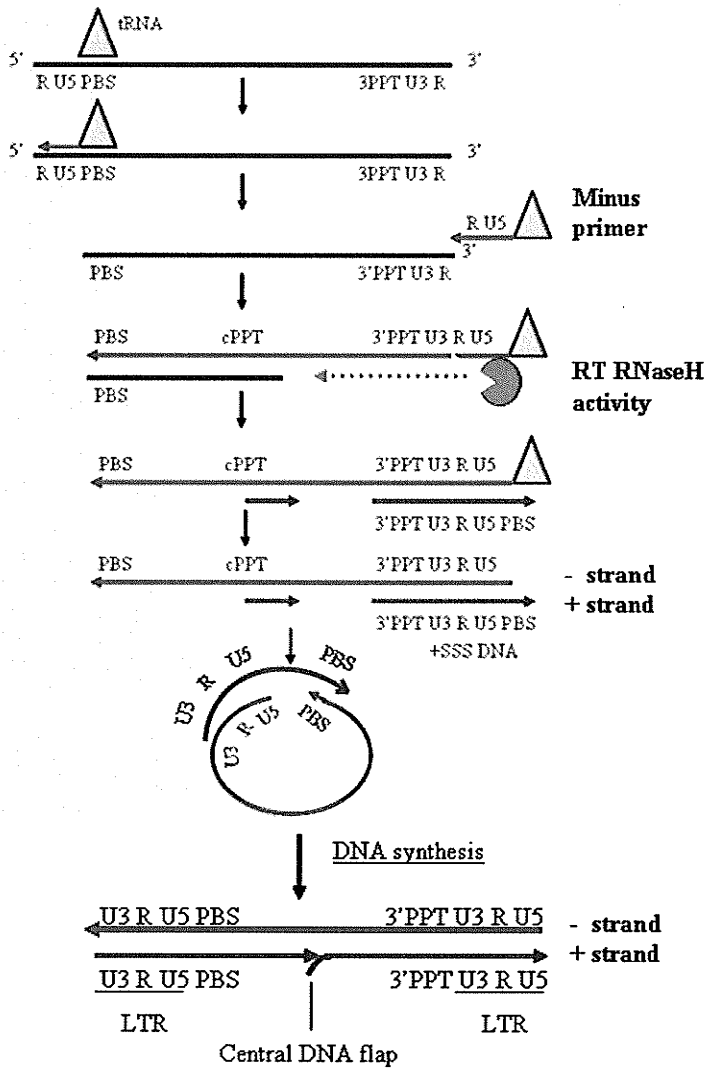

Fig 9. HIV-1 reverse transcription: Schematic diagram of reverse transcription, where host tRNA initiates the process by binding to the 5' end of the viral RNA molecule at the primer binding site (PBS). DNA synthesis begins from the 5' U5 region, generating a short DNA/RNA hybrid. Then the reverse transcriptase enzyme degrades the positive strand of RNA using its inherent RNaseH activity, to produce a single minus strand of primer DNA called the strong stop DNA. The minus primer DNA strand then jumps to the 3' end of the RNA binding to the homologous R region at each end, and is referred to as the first strand transfer. Full minus strand synthesis then occurs using the strong stop DNA as a primer for the process. Using RNA remaining from the minus-strand synthesis as primers, plus strand synthesis occurs, where priming begins from the polypurine tract (PPT) and from the central PPT (cPPT). The tRNA is then removed from the PBS by the RNaseH, allowing for plus strand synthesis along the minus strand [26, 52, 106].

1.2.17 Cytoplasmic shuttling:

Following reverse transcription, viral cDNA must reach its site of replication within the nucleus. Although the exact mechanism of cytoplasmic shuttling and nuclear import of the PIC are not fully understood, there is a fairly substantial body of evidence exploring the cooperation of host and viral factors to complete the task. Transport of the viral cDNA occurs using a preintegration (PIC) which contains RT, IN, MA, Vpr, viral cDNA, NC, CA and host proteins such as high mobility protein HMGI (Y), barrier-to-autointegration factor (BAF), lamina-associated polypeptide 2α (LAP2α) and human lens epithelium-derived growth factor/transcription coactivator p75 (LEDGF/p75) [131-134]. Miller et al. speculated that due to the disassociated nature of the CA and NC proteins from the PIC upon cofractionation, that the structural organization of the PIC consists of tightly condensed viral cDNA housed within a partially disassembled viral core, while the remaining viral proteins associate at the exposed opposing cDNA ends [26, 131]. Potentially, this organization of the PIC impacts the expression of various PIC associated viral proteins. CA has been reported to be highly expressed in the RTC immediately after early-post entry, but is barely detectable at later times within the PIC. Decreased CA expression allows for increased viral protein exposure and interaction with cellular import factors [35, 36, 127, 134].

Shuttling of the PIC within the cytoplasm to the nucleus, is thought to occur through the association of the PIC with dyanin and actin, which interaction with the hosts microtubule network. These findings suggest that cytoplasmic

trafficking of the PIC is a highly organized process, and is facilitated by host motor pathways which traverse the cytoplasmic environment [107, 135] (*Fig 10.*).

1.2.18 Nuclear import of the pre-integration complex:

A critical determinant during HIV-1 infection, is the virus's ability to translocate the viral cDNA associated with the PIC, from the cytoplasm to the nucleus through the nuclear pore complex (NPC) in non-dividing cells [3, 9, 26]. Although the exact mechanism underlying this interaction remains largely unknown, the individual components that govern this process have been identified and explored. It has been widely reported that the viral and host factors associated with the PIC possess karyophilic properties, which allow them to associate with nuclear import factors such as Impα, Impβ and Imp7, to undergo energy-dependant active import [8, 12, 36, 136]. As discussed previously, the PIC contains specific factors that possess nuclear localization signals such as MA, IN, Vpr and the central DNA flap, which coordinates nuclear import using classical and non-classical pathways [7, 77, 137, 138]. Having the ability to hijack host cellular factors and establish infection in both dividing and non-dividing cells, is a hallmark of HIV-1 replication. This characteristic is what distinguishes it from other oncoretroviruses, allowing it to establish infection within tissue macrophages, mucosal dendritic cells and quiescent T cells [135, 139, 140]. The defining factor behind this ability, lies in the fact that HIV-1 expresses viral proteins that possess karyophillic properties, allowing them to utilize the host's nuclear import machinery. Transport of the PIC through the

NPC is an energy dependent process, and requires the aid of host karyopherins and the RanGDP/RanGTP gradient [16, 96, 98]. The viral components of the PIC, includes RT, viral cDNA, Vpr, MA, CA and IN. Although the exact structural organization of the PIC had not been identified, there is much known about the individual components of the complex. IN, MA, Vpr and the cDNA flap are proposed to play a significant role in the nuclear import of the PIC, as they all possess karyophillic properties in the form of a functional NLS [7, 8, 33, 107, 136, 138]. It was also observed that Vpr is able to induce nuclear membrane herniations, by disrupting the nuclear lamina. Upon rupture of these herniations, both the nuclear and cytoplasmic contents were able to mix, thereby providing a potential bypass into the nucleus [8, 141]. So far many theories exist regarding the primary nuclear import pathway utilized by the PIC, but much controversy still remains (*Fig 10.*).

In a recent study exploring the impact of nucleoporin 98 on nuclear import of the PIC, it was found that RNA mediated gene silencing of the host nuclear pore protein, impaired nuclear import of viral cDNA in growth arrested cells [134, 142]. Similarily, LEDGF/p75 which has been linked with the PIC, is also suspected of enhancing nuclear import by improving the efficiency of IN nuclear targeting. LEDGF/p75 contains an NLS similar to that of SV40, and may contribute to the nuclear localization of the PIC in association with the other karyophilic viral proteins [134, 137, 143, 144].

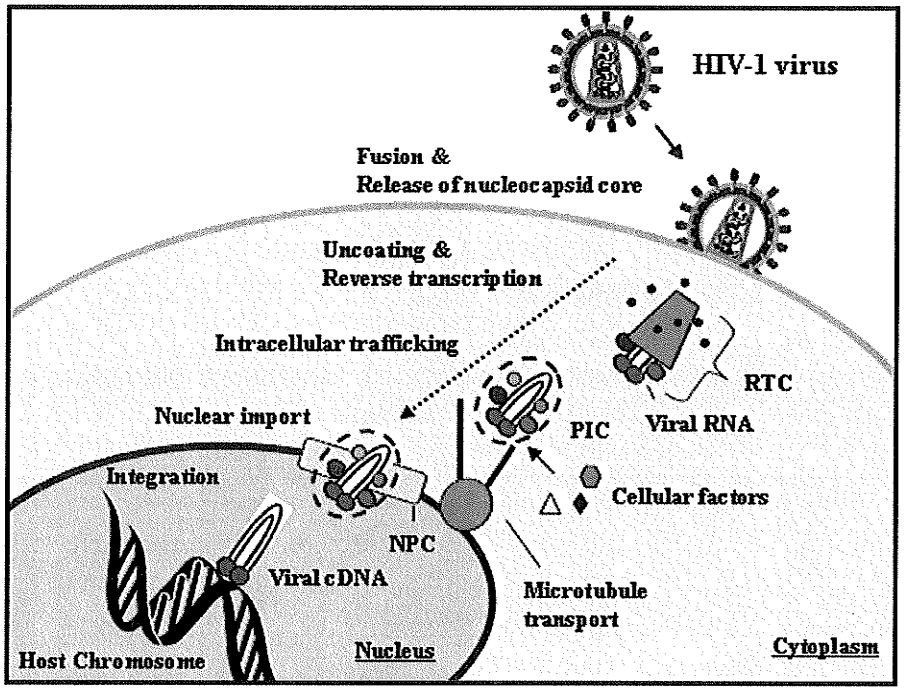

Fig 10. HIV-1 PIC nuclear import: Schematic representation of the process of nuclear transport of the PIC from the cytoplasm, through the NPC and into the nucleus. Prior to nuclear import, the viral PIC must first reach the NPC and is transported through the cytoplasm to the nucleus by interacting with microtubule filaments. Nuclear import of the PIC is accomplished through a coordinated effort between viral karyophillic compounds within the PIC (MA, IN, Vpr and cDNA Flap), and host karyopherins (Impα, Impβ and Imp7 [7, 8, 33, 134].

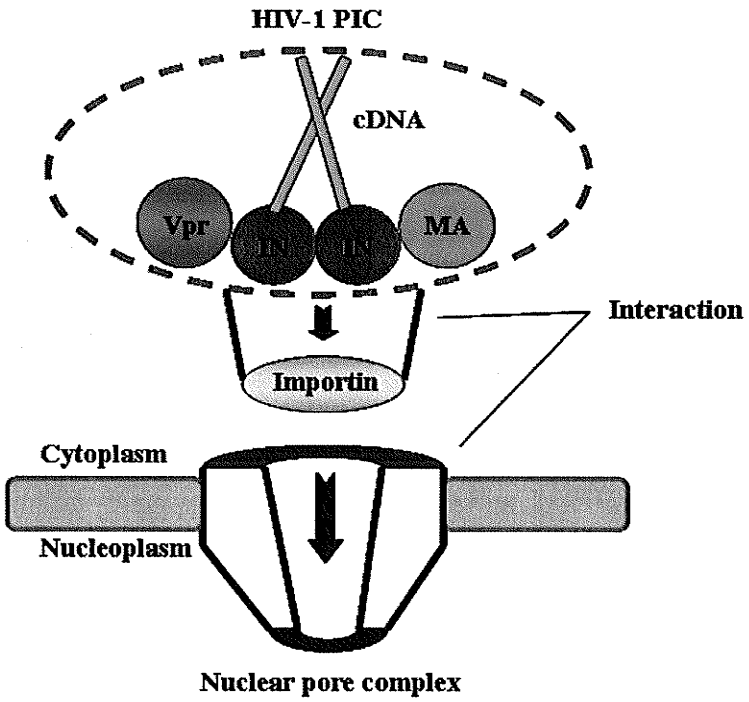

Fig 11. Organization of the PIC and NPC: Schematic representation of the composition of the PIC, possessing IN, Vpr, MA and viral cDNA, and its pathway through the nuclear pore complex, into the nucleus. Passage through the nuclear pore complex is achieved through association of the PIC with host importins [7, 8, 33, 134].

1.2.19 Viral factors involved with nuclear import:

As discussed previously there are several viral factors associated with the PIC (IN, MA, Vpr and viral cDNA Flap) that possess karyophillic properties, and mediate nuclear import of the PIC.

Integrase: Integrase is an essential enzymatic protein derived from the Pol gene, that is involved with reverse transcription, nuclear import and integration of early viral replication [27]. Investigations into the karyophillic properties of IN, identified that the protein *in vitro* is able to localize in the nucleus in absence of other HIV-1 viral proteins, and does not result from passive diffusion [77]. Studies investigating the impact of IN on nuclear import, observed that in the absence of both Vpr and MA NLSs, nuclear accumulation and viral replication was still successful with wild type IN, although to a lesser degree [145]. In attempts to identify the NLS within IN, and the possible karyopherins that the protein associates with, Galley et al discovered a bipartite NLS (^{186}KRK and ^{211}KELQKQITK), that when mutated induced a loss of IN nuclear import [145]. Another non-classical NLS was reportedly found in the catalytic core domain of IN [34], but in both reports, it could not be confirmed that either of the NLSs defined governed IN nuclear import, but that they were involved with reverse transcription and/or integration [146, 147].

Several studies have identified that IN binds to Impα, assisting with the overall nuclear import of the PIC, potentially through the classical importin α/β import

pathway [12]. As previously discussed, importin α is able to undergo nuclear import alone, or in association with importin β and utilize the classical nuclear import pathway [13]. There is still some discrepancy as to which IN NLS bearing region is solely responsible for binding importin α. Two independent studies have shown that amino acids 161-173 (IIGQVRDQAEHLK) and 186-188(KRK) possess NLS residues which bind Impα and mediate import [12, 77, 100]. Although it is was later found that nuclear import of IN was independent of Impα, Impβ1 and Impβ2 mediated pathways [77].

As discussed in a previous section, Imp7 mediated nuclear import of the PIC has been explored through various means, as it was identified that IN binds Imp7. In a pull-down study, IN was able to pull down Impα, Impβ, transportin and Imp7. The ability of IN to interact with these various karyopherins, draws to question whether IN interacts with them each separately or through heterodimer complexes, and if that is the case, which karyopherin interaction mediates nuclear import [15, 16, 20].

Recently, the IN domains responsible for binding LEDGF/p75 were identified. LEDGF/p75 is a transcriptional co-activator and part of the hepatoma derived growth factor family. As discussed previously, LEDGF/p75 plays a role in site directed cDNA integration chromosomal tethering and potentially impairing proteasomal degradation of IN [133, 143, 148, 149], and binds integrase within the catalytic core domain at a single amino acid Gln 168 [150]. Maertens et al

also identified an NLS domain within LEDGF/p75, and proposed that the cellular protein may contribute to the nuclear localization of the PIC [137]. But it was later revealed that LEDGF/p75 does not contribute to the nuclear localization of the PIC [133, 150, 151]. Overall, the nuclear import of the PIC as mediated by IN is still under exploration.

MA: was the first viral protein identified to posses an NLS at both its N-terminus and C-terminus domains, and was found to participate with the nuclear import of the PIC in non-dividing cells [136, 152, 153]. Mutation of the N-terminal domain NLS did not impair nuclear import of MA, but mutation of both the N and C-terminal NLSs resulted in a complete loss of PIC nuclear import. The C-terminus of MA regulates the binding to IN, and reportedly does not increase the karyophillic properties of the protein [152, 154]. Although MA is required by the virus for efficient nuclear import, it has been shown that the protein is not essential [33, 153]. The exact means by which MA contributes to the nuclear import of the PIC, is still unknown.

Vpr: Is a unique viral protein that has been shown to participate in the nuclear import of the PIC in non-dividing cells. Vpr is found present both in the cytoplasm and nucleus, and has the ability to enable virion incorporation of attached proteins via interaction with the p6 domain of the $Pr55^{Gag}$ polyprotein [65, 155]. Vpr has been found to also associate with Impα and in doing so increases the affinity of MA for Impα, thereby improving the overall karyophillic

nature of the PIC [8, 66, 67]. So far no conventional NLS has been identified within Vpr, but within both the N and C-terminal domains two independent signals have been elucidated [156, 157].

As discussed previously, Vpr has the ability to induce herniations within the nuclear membrane to induce membrane rupture, allowing mixing of the cytoplasmic and nuclear contents [141]. Potentially this mechanism may enable the PIC to translocate across the nuclear membrane without having to pass through the NPC.

The central DNA flap: This is a short triply stranded fragment of DNA which is transcribed by reverse transcriptase during the plus strand synthesis, forming the component through discontinuous synthesis [52, 158]. The flap is located within the center region of the DNA, and appears to have stabilizing and protective effects on viral DNA [158]. The cDNA provides a cis-acting structural determinant enhancing nuclear import of viral DNA [138, 159]. The effect of this cDNA component on the overall nuclear import of the PIC is still unknown.

1.2.20 Chromosomal tethering and integration:

Once the PIC has entered the nucleus, integration of the viral cDNA into the hosts chromosome is essential for replication [26]. Before integration can occur, the HIV-1 DNA must first be tethered to the hosts chromosomal DNA [24, 25]. Cellular factors such as LEDGF/p75, emerin, SWI/SNF, HMG 1 and BAF

within target cells mediate this process, and depletion of these components severely impairs integration and viral DNA association with the chromatin [25, 132, 160, 161]. Prior to integration, three forms of viral DNA can be found present within the nucleoplasm; linear DNA, 1-LTR or 2-LTR circles [52, 162-164] .

Integration catalyzed by IN is an essential step of viral replication, and once inserted, the viral cDNA is regarded as provirus and behaves like a cellular gene [27, 52]. Integration occurs in three steps; 3' end processing, DNA strand transfer and finally gap repair surrounding the inserted cDNA fragment. As with most intricate molecular processes of HIV-1 replication, there is still much research required to gain a better understanding of this action. Prior to integration of the cDNA into the host genome, the linear DNA strand must first undergo 3'-end processing whereby two amino acids are cleaved from both 3' DNA ends by IN, immediately behind a conserved CA motif [26]. This generates CA- 3'- hydroxy DNA ends that are the active intermediate of eventual DNA strand transfer [26, 27, 83, 164-166].

IN then catalyzes the staggered cleavage of cellular DNA, to generate a receptive DNA target for which to insert the viral strand. Strand transfer then results, where the CA-3' OH sticky ends of the viral DNA are annealed with the cleaved 5' ends of the host genome. Finally, gap repair is initiated whereby cellular enzymes repair the unfilled, 5' viral DNA ends . Studies exploring integration, discovered

that viral cDNA is preferentially inserted into transcriptionally active genes, particularly those that are active during HIV-1 infection [24, 25, 164, 167, 168].

Cellular factor LEDGF/p75 which interacts with the catalytic core of integrase, has been linked to tethering IN to cellular DNA near transcriptionally active genes, allowing for strand transfer to occur. Upon RNAi LEDGF/p75 interference within target cells, integration within virally infected cells was reduced by 74%, [148, 150]. In order to mediate concerted single strand transfer of the viral genome, LEDGF/p75 must remain at concentrations <1 compared to that of IN, to prevent disruption of IN-IN interactions [160]. LEDGF/p75 has proven to be a cellular protein that plays a dynamic role during HIV-1 infection, and potentially stimulates other steps during viral replication that have yet to be identified [143, 148, 168-170].

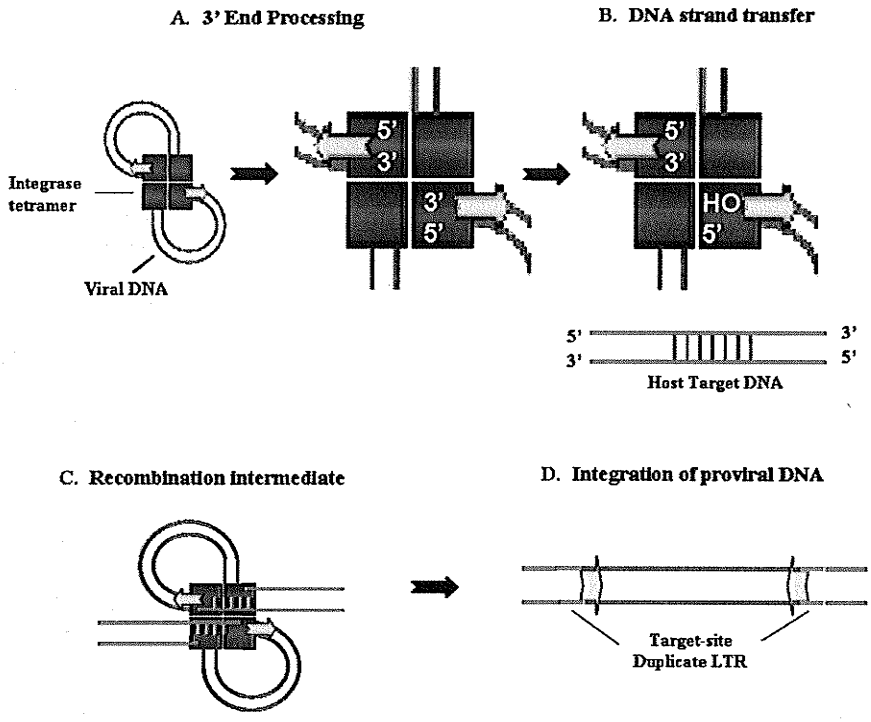

Fig 12. Integration of viral cDNA catalyzed by integrase: A) Integrase is catalyzing the hydrolytic 3' end processing of the viral cDNA to cleave off a conserved CA motif, and expose the 3' OH processed terminals. B) Once the correct position within the host's genome has been found, DNA strand transfer occurs whereby the 3' OH ends are used to cleave the target DNA in a staggered fashion, and annealing the 3' ends to the 5' phosphates of the host's genome. C) DNA strand transfer creates a recombination intermediate catalyzed by the integrase between the 3' and 5' viral ends. D) DNA gap repair fills in any missing nucleotides from the recombination event, to produce a fully integrated viral genome flanked by LTR repeats (5 bp repeats) [24, 25, 164].

1.2.21 Viral gene expression:

To complete the pathogenic life cycle of HIV-1, the integrated proviral genome must be transcribed for production of progeny virions. Ultimately, the viral genome serves as the template for synthesis of viral RNAs which are translated to yield the full viral genomic complement of structural, enzymatic and accessory proteins. Upon entry of the PIC and prior to integration, the viral genome exists in three forms within the nucleoplasm; linear DNA, 1-LTR or 2-LTR circles. The integrated DNA is homologous to the linear DNA, while the circularized forms are no longer able to integrate [107, 132, 162, 171]. Transcriptional regulation of the viral genome is directed by the 5' LTR promoter at the beginning of the proviral DNA, which harbors a number of *cis*-acting elements required for viral RNA production. Interaction of viral and host factors with viral *cis*-acting DNA elements within the LTR, is essential for proviral transcription [172]. Transcription of the HIV-1 provirus is divided into two stages 1) early tat-independent and 2) late tat-dependant [26, 52, 172-174].

Various host cellular factors such as nuclear phosphatase-1, AP-1, NF-AT, USF-1, Ets, IRF, Sp1 and most importantly NF-KappaB bind to the unique 3' LTR region and initiate transcription [172, 175-178]. During initial early tat-independent transcription, nuclear transcription factor κβ (NF-κβ), binds and activates the LTR to produce small incomplete RNA transcripts [179]. Because the HIV-1 promoter at the early stage is under strict control by local chromatin and the cellular transcription environment, RNA pol II functions efficiently and

cannot synthesize the full RNA strand . This process results in early Tat-independent transcription and the production of basal amounts of tat, nef and rev transcriptional activators, which function independently of the chromatin environment [26, 180]. Tat expression dramatically increases RNA synthesis by binding the TAR element at the 5' nascent end of the mRNA, and other cellular transcriptional elements to induce complete RNA transcription [26, 181, 182]. The ability of Tat to increase RNA synthesis lies in its ability to recruit the cellular complex positive-transcriptional elongation factor b (P-TEF-b) to the TAR element, and phosphorylate the RNA Pol II C-terminal domain, which stimulates transcription [26, 183, 184].Tat-dependent late transcription results in the production of numerous RNA transcripts that fall into three classes [26, 185, 186];

1) Fully spliced RNA: These are small 1.7- 2.0 kb fragments that have both HIV-1 introns removed, and have the potential to express Tat, Rev or Nef.

2) Partially spliced RNA: These are medium 5 kb fragments that retain one HIV-1 intron, and have the potential to express Env, Vif, Vpu, Vpr or the single intron form of Tat. Processing is achieved by utilizing the splice site nearest to the 5' end of the HIV RNA, in addition with splice sites found in the central domain of the virus. The partially-spliced env gene acts as the translational precursor protein gp160, which is further glycosylated within the endoplasmic reticulum

3) Unspliced RNA: This primary transcript consists of a 9 kb fragment that serves to generate the Gag and GagPol proteins, and be packed as the viral RNA genome into the progeny virions.

The majority of cellular mRNAs are spliced prior to nuclear export, although viral assembly requires unspliced and partially spliced mRNA to accumulate in the cytoplasm. To facilitate nuclear export of the unspliced and partially spliced mRNAs to the cytoplasm, rev binds the cis-acting rev responsive element (RRE) present within spliced and unspliced RNA located in the env gene [26, 59,188].

1.2.22 Viral Particle Assembly:

Viral assembly begins after the full complement of viral proteins has been synthesized and exported into the cytoplasm. The production of virion particles is achieved through a series of steps beginning with targeting viral proteins to a specific site in the lipid membrane, inclusion of the RNA genome, clustering of Gag-GagPol translated proteins and eventual release of the virus [189-192]. It is believed that viral assembly and release is a highly regulated process, and is dependant on both host and viral factors, most importantly Gag precursor polyprotein $Pr55^{Gag}$. $Pr55^{Gag}$ is a modular protein possessing six functional domains, Matrix (MA), capsid (CA), nucleocapsid (NC), p6 and two spacer regions p1 and p2. Throughout these functional domains there exist additional assembly domains of $Pr55^{Gag}$; membrane attachment (M) interaction (I) and late (L) domains . M is located in a myristylated region of the N-terminus glycine of

the MA domain, and was designed to target the Gag to the PM. The interaction region I is located within the C-terminus of CA, the N-terminus of NC and the p2 spacer, and is responsible for mediating Gag monomer interactions [193-195] (*Fig 8.).*

Multimerization of $Pr55^{Gag}$ is the driving force behind viral assembly, and is a multistep process initiated in the cytosol prior to membrane attachment, by the NC and CA domains of the protein. Host cellular factor Staufen1 (Stau1) mediates multimerization of Pr55Gag, by interacting the the NC domain of the protein, and it is further speculated that this host protein is involved with trafficking and packaging of viral RNA into the virion particles [190, 192, 196, 197].

Within minutes of Gag multimerization, the synthesized polyproteins target the lipid membrane to begin the process of assembly [192, 193]. $Pr55^{Gag}$ functions to target and bind determinants to the plasma membrane, promote Gag-Gag interactions, facilitate encapsidation of the full HIV-1 genome, interact with *Env* glycoproteins and mediate viral budding [189, 190, 198]. Plasma membrane targeting is accomplished via myristic acid modification of the MA Gag domain, which occupies 131 amino acids of the N-terminus of the $Pr55^{Gag}$ protein. To associate with the membrane, MA undergoes a conformational change to expose a positively charged basic acid moiety which allows it to bind to the negatively charged inner phosholipid membrane, thus stabilizing the Gag-PM interaction

[189, 191]. There is currently much debate over the pathway Gag utilizes to get to the plasma membrane (PM). It has recently been found that small amounts of Gag accumulates in intracellular compartments expressing late endosome (LE) or multivesicular body (MVB) markers [190]. It is suspected that the majority of synthesized Gag is localized to the PM to facilitate assembly, and that a small fraction is recycled back into internal LE/MVB compartments, potentially as a mechanism to stimulate more Gag PM accumulation. To denote the specific area within the PM that is targeted by Gag, Jolly et al proposed that localization is directed towards tetraspanin-enriched membrane domains (TEMs) within the PM, and that these regions stimulate viral assembly [189, 199].

Following translation directed by the Gag polyprotein, the Gag (Pr55), Gag-Pol (Pr160) proteins migrate to the PM and activate the assembly process. Within the PM Gag polyproteins accumulate at tetraspanin microdomains rich in CD9, CD63, CD81 and CD82, where components of the mammalian endosomal sorting complex, TSG101 and VPS28, also accumulate to assist with viral budding [198-200]. The Env polyprotein is transported through the endoplasmic reticulum and goli, where it is further processed and cleaved to form envelope glycoproteins gp41 and gp120. These glycoproteins are transported to the PM, where gp41 anchors gp120, and act as the site for viral budding [197, 201].

Encapsidation results when full-size genomic RNA consisting of two single strands of RNA is incorporated into the immature viral core, and is a key part of the assembly process [26, 202]. This process is mediated by the cis-acting

element found within the 5' leader sequence, known as E, between the primer binding site in the translation initiation site of the Gag- Pol- In gene, and by the p7 linker found with the NC portion of Gag. This packaging signal is important for ensuring RNA encapsidation specificity [26, 203-207].

Too complete the process of encapsidation, MA forms the inner shell of the particle, which is located just under the viral membrane, while CA forms the conical capsid to house the viral genomic RNA [119, 208]. Triggering capsid formation is the folding of the hairpin region within the immature Gag polyprotein, which stimulates proteolytic cleavage of the protein, activating subsequent activities [209]. Prior to completion of encapsulation, NC associates with viral RNA within the capsid [85]. To increase viral infectivity during early post-entry events, the Gag proline rich region within the N-terminus of the CA domain, recruits cellular cyclophilin A (CypA) into the viral particle during assembly [210, 211].

1.2.23 Viral Budding:

After viral assembly, the virion must separate from the host's lipid membrane to form individual viral particles. Budding of viral progeny is believed to occur at the PM of the majority of infected cell types, whereas in primary macrophages budding takes place from late endosomes which harbor endosomal/multivesicular body markers [212, 213]. More specifically budding is reported to occur at specialized sites within the PM called lipid rafts, which are

regions that are rich in cholesterol, glycolipids and sphingolipids [214]. Gag and Env polyproteins localize to these regions and associate with the membrane bound cholesterol via interactions with their acylated residues [215]. Depletion of membrane cholesterol and binding of Gag to non-lipid raft regions of the PM severely impairs viral particle production, and disruption of the PM causes Gag to be redirected to endosomal membranes and away from lipid rafts [26, 215, 216] (*Fig 8.*).

The L domain encoded by the PTAP sequence motif within the C-terminus of the p6 region of the Gag polyprotein, is the major viral mediator of budding, and catalyzes the pinching off of the viral particles from the host membrane. This factor coordinates with several host vacuolar-protein sorting complexes and the endosomal-sorting complex to facilitate viral release [57, 215]. The host tumor susceptibility gene (TSG101) has also been found to be critical in the release of viral particles from the plasma membrane, and to associate with the PTAP region within the Gag protein [217, 218]. The Gag protein also interacts with AP-2 and AP-3, components of the adaptor complexes, which control endocytic trafficking and potentially regulate the site of viral exit [26, 215, 219].

Another proposed method for viral budding includes the involvement of GTP binding proteins that interact with Gag, and their ability to associate with actin filaments to reorganize the actin cytoskeleton and allow for viral release [220].

The Vpu viral protein is also associated with stimulating viral release, in an Env and CD4+ independent manner. It was found that the protein does not localize in the same area as viral budding occurs, and is speculated to exert it's affect on viral release in an indirect manner. Studies found that although Vpu doesn't directly associate with Gag, it indirectly prevents the endosomal compartment accumulation of the protein, allowing Gag to localize in the PM instead [221]. The exact mechanism behind Vpu's action is still under debate, but it is thought that it acts as a multifunctional adaptor to alter adverse cellular actions [212, 221, 222].

1.2.24 Viral Maturation:

Viral maturation occurs shortly after the viral particles bud from the plasma membrane, and are released into the surrounding environment. This late step in viral replication includes viral protein cleavage to produce mature Gag and Pol proteins, stabilization of the viral RNA dimmer and condensation of the viral core [223]. The result, is an infectious particle that now contains mature viral proteins and an electron-dense conical core. Failure of viral maturation results in a loss in viral infectivity, altering the ability of the virus to adequately mediate fusion. Reports by Jiang et al. have linked the process of viral maturation with the activity of the gp41 cytoplasmic domain, whereby fusion to primary human CD4+ T cells is enhanced. Although it was observed that X4 tropic Env viruses were more dependent on viral maturation, than the X5 tropic Env viruses [224].

While in the viral particle, incorporated viral proteases cleave the Gag and GagPol polyproteins, to produce mature Gag and Pol proteins, and activate other sequential maturation events [225, 226]. However, PR cleaves each site with differing efficiency, and as a result, a stepwise cascade is formed of cleavage events which are termed primary, secondary and tertiary. Primary cleavage of the p2/NC region within the Gag polyprotein, mediates RNA dimer formation, while the liberated NC Gag region is essential for RNA incorporation within the core and stabilization of the dimer [227]. In particular, the NCp7 protein binds a specific 35bp RNA stem loop SL1, which is critical for RNA dimmer formation, it that it initiates refolding to produce a more stable linear particle [228].

Upon cleavage, multimerization occurs of viral Pol gene products; RT, IN and PR, to render the functional forms of the proteins. During this time reorganization of the viral core is initiated, where the CA spherical core is condensed to form the conical shell encasing NC and the RNA genome. HIV-1 also has a mechanism to down regulate proteolytic activity, and *in vitro* studies in bacteria identified the N-terminal domain Vif as possessing this function. Whereby *in trans* Vif interacts directly with PR, and induces a dose dependant inhibitory effect on the protein, inhibiting viral maturation [229-231].

Chapter 2:

2.0 Materials and Methods:

Outlined within this chapter are the various materials and methods utilized to carry out my research and conduct the experiments.

2.1 Construction of viral and cellular protein expressors:

The wild type full length HIV-1 IN cDNA was amplified using polymerase chain reaction (PCR), from the template HxBru strain which had an artificial start codon ATG inserted prior to the first amino acid of IN [32]. The amplification primers used were 5' - IN-HindIII-ATG (5'-CGCAAGCTTGGATAGATGTTTTTA GATGGAA-3') and 3' – IN- Asp718 (5'-CCATGTTGGTACCTCATCCTGCT-3'). To generate the IN-YFP fusion expressor, restriction enzymes HindIII and Asp718 were used to digest the PCR amplified products, which were then cloned in frame to the 5'end of the EYFP cDNAin a pEYFP-N1 vector (BD Biosciences clontech) [32].

SVCMV-YFP-IN fusion expressor constructs:

The SVCMV-YFP-IN fusion expressor was constructed from the amplified HxBru IN cDNA, that was amplified from the HIV-1 HxBru provirus. Polymerase chain reaction (PCR) was used to carry out the amplification process with 5' *Bgl*II (5'- GCCAGATCTTTCTTAGATGGAATAGATAAG-3') and 3' *Bam*HI primers (5' – CTAAACGGATCCATGTTCTAA-3'). Following the PCR fragment was digested with restriction enzymes *BglII* and *BamHI*. The amplified

fragment was then cloned in frame to the 3' end of EYFP cDNA in a pEYFP vector (BD Biosciences Clontech).

To generate the different SVCMV-YFP-IN deletion mutants, cDNA fragments encoding aa 1-212 and 1-240 of IN were produced using PCR with 5' *Bgl*II (5' – CAATTCCCGGGTTTGTATGTCTGTTTGC-3') and 3' *Xma*I IN212 (5'-CAATTCCCGGGTTTGTATGTCTGTTTGC-3) or 3' *XmaI* IN240 (5'-CCAGACCCGGGTTGCTGGTCCTTTCCA-3) respectively . This fragment was then inserted into the pEYFP-C1 vector at *Bgl*I and *Xma*I sites. To generate the IN50-288 cDNA, the primers used were IN50-HindIII-ATG-5' (GCGCAAGTTGGATAGATGCATGGACAAG-TAG-3) and 3'-IN-Asp718.

SVCMVin-IN-YFP substitution mutation constructs:

The substitution mutants INKK215,9AA-YFP, INKK240,4AA-YFP, INKK263,4AA-YFP, YFP-INKK249,50AA and YFP-INK258A, were generated using a two step PCR based method. Using a 5'-primer (5'-IN-HindIII-ATG) and 3'-primer (3'-IN-Asp718) and complementary primers containing the required mutations. The amplified IN cDNAs possessing the mutations were then cloned into the pEYFP-C1 or pEYFP-N1 vectors. By subcloning all the constructs into the SVCMV vector, the expression efficiency of each fusion protein was enhanced through the addition of the strong cytomegalovirus immediate early gene promoter.

SVCMVin-T7 INc206 construct:

The SVCMVin-T7 INc206 was constructed through PCR amplification of the of the IN CTD from the HIV-1 HxBru provirus DNA using 5' HindIII (5'- AGAATAGCGAAGCTTTAGCAACAGACAT- 3) and 3'-stop- BamHI primers (5'- CTAAACGGATCCATGTTCTAA- 3). Fragments were then digested with *HindIII* and *BamHI* restriction enzymes. The SVCMVin-T7 vector was generated by inserting a T7 promotor, T7 tag and multiple cloning sites derived from the pET21a *BglII-NotI* spanning region into the vector. The pET21a vector was digested with *BglII* and *NotI* restriction enzymes, and the fragment was inserted in frame into the 5' end *BglII* and *NotI* sites within the SVCMVin vector. Following that the SVCMVin-T7 vector was digested with HindIII and BamHI restriction enzymes, and the INc206 fragments were inserted in frame at the 5' HindIII site.

T7-Imp7 construct:

The T7-Imp7 fragment was constructed in a similar fashion to that previously for the T7-INc206 fragment. The Imp7 fragment was amplified by PCR using the pGEX-Imp7 Xenopus plasmids, which were generously provided by Dr. Yamamoto [20], using two-step PCR technique. The generated PCR products were then digested with BamHI and NotI and cloned into the 3' end of the T7-tag of the SVCMVin-T7 vector.

SVCMVinR14-88 fusion protein constructs:

The SVCMVinR14-88 Vpr-fusion protein constructs were generated by first amplifying the IN CTD fragments using PCR; R14-88 INc206-288 used 5'-*XbaI* (5'- ATAGCGGAATCTAGAGCAACAGACAT-3) and 3'-stop-*BglII* (5'- CTGTTCAGATCTCTAATCCTCATCCTG-3) primers, R14-88 INc228-270 used 5'-*XbaI* (5'- TTCGGGTTTCTAGAAGGGACAGCAG-3) and 3'-stop-*BamHI* primers and R14-88 INc241-270 used 5'-*XbaI* (5'-AAGGACCATCTAGACTCCTCTGGAA-3) primers. The exception was R14-88 INc206 240,4/263,4AA which was amplified using a two step PCR method utilizing the 5'-*XbaI and* 3'-stop-*BglII* primers already described, along with complementary primers containing the required mutations. To produce the SVCMVinR14-88 Vpr-fusion protein expressors, *XbaI* and *BamHI* or *BglII* restriction enzymes were used to digest the PCR products. The fragments were then cloned in frame to the *5' XbaI* and *BglII* sites within the SVCMVin-R14-88 vector *(Fig 13.)*. Fragments produced include R14-88 INc206, R14-88 INc206 240,4/263,4AA, R14-88 INc228-270 and R14-88 INc241-270.

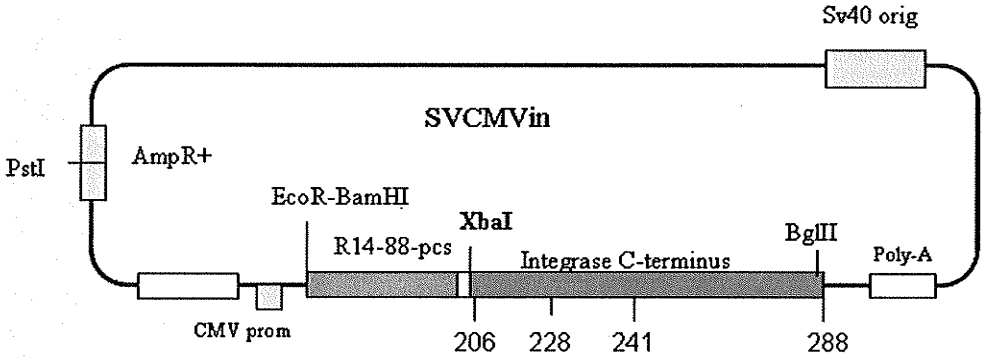

Fig 13. SVCMVinR14-88 Fusion protein expressor: Schematic representation of the construction and restriction sites used to generate the various SVCMVinR14-88 fusion protein expressor plasmids. SVCMVin was used as the vector backbone for the plasmid in order to insert both the Vpr 14-88 fragment as well as the IN CTD fragments. Vpr-fusion expressors include; R14-88, R14-88 INc206, R14-88 INc206 240,4/263,4AA, R14-88 INc228-270 and R14-88 INc241-270.

2.2 Virus production:

The 4.3-BruΔBgl/Luc (Luc+/env-) and 4.3- BruΔBgl/Luc/vpr- (Luc+/env-/vpr-) single cycle replication proviruses were constructed in a similar fashion, who's nef gene had been replaced by a firefly luciferase gene. To generate a proviral clones with genotypes 5' long terminal repeat *gag+ pol+ vif+ vpr+ tat + rev+ vpu+ env- nef-* and 5' long terminal repeat *gag+ pol+ vif+ vpr- tat + rev+ vpu+ env- nef-*, RT/IN/Env defective HIV-1 provirus was used as a backbone. From the backbone provirus the *ApaI* through to SalI (1556 to 5329, initiation of NL 4.3 transcription begins at +1 of 1556), were removed and replaced with a corresponding sequence from HIV-1 provirus HxBru. The 4.3 GFP+/nef- virus that was used was generously provided by Dr. Eric Cohen from the University of Montreal, and possess a GFP gene in place of nef. The NLlucΔBglD64E (4.3 D64E Luc+/env-) virus was generously provided by Dr. Irvin S.Y Chen.

Generation of SVCMVinR14-88 IN CTD expressor 4.3 Luc+/env-/R- virus:

Production of the 4.3 Luc+/env-/Vpr-/Vpr-fusion protein single cycle replication virus, resulted through co-transfecetion of 293T cells using the calcium phosphate precipitation method, with the VSV-G expressor, the SVCMVinR14-88 IN CTD (wt/mutant) expressors and 4.3- BruΔBgl/Luc/vpr- provirus. The following steps required for viral production are described below. The Vpr- provirus was used within this construct, to prevent the wild type Vpr from competing with the R14-88 fragment for incorporation into the viral particle. During viral assembly within the 293T cells the VSV-G, Vpr-fusion proteins and

proviral proteins were expressed, and packaged into the viral particle to create a VSV-G pseudotyped virus possess both wtIN and the IN CTD.

Viral expression:

General virus production resulted from the transfection of 293T cells with the various single cycle proviral vectors and the VSV-G expressor, using the calcium phosphate precipitation method. For the T7-INc206 and T7-INc206 240,4 luciferase and MAGI assay analyses, virus was generated through co-transfection of 293T cells with the IN CTD (wt/mutant) expressors and 4.3-BruΔBgl/Luc, 4.3-BruΔBgl/Luc D64E or 4.3 GFP+ 24hrs after transfection, the culture medium was changed from DMEM complete to RPMI complete medium to allow for improved viral production. 48, 72 and 96 hrs post-transfection, viral supernatants were collected and clarified using centrifugation at 3000rpm for 30min at 4°C . Then the viral supernatant was concentrated by ultracentrifugation for 2hrs at 35,000 rpm at 4°C. The viral pellets were re-suspended in 500ul of RPMI complete medium, and the amount quantified using the reverse transcription assay (*Fig 14.*).

2.3 Cell Lines and Transfection Method:

293 T human embryonic kidney cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (10% FCS) and 1% penicillin/streptomycin (1% P/S). HeLa-CCR5/CD4-β-Gal cells obtained from the AIDS Research and Reference Reagent Program, Division

of AIDS, NIAID, NIH, Bethesda, MD, USA, and also maintained in DMEM supplemented with 10% FCS and 1% P/S. The CD4+ C8166 and MT4 cells were maintained in RPMI-1640 medium supplemented with 10% FCS and 1% penicillin and streptomycin.

Transfection method:

DNA transfection into 293T cells was carried out using the standard calcium phosphate DNA precipitation method. After 48 hours of transfection, cells were harvested and lysed using either 0.5% RIPA lysis buffer for expression assays, or 0.5% CHAPS lysis buffer for binding assays between IN and Imp7.

2.4 Antibodies and Chemicals:

To carry out the immunoprecipitation and western blot analysis the following antibodies were used. The mouse anti-T7 antibody was obtained from Novagen Inc (Darmstsdt, Germany). The purified rabbit anti-GFP polyclonal, and mouse monoclonal anti-GFP antibodies were obtained from Molecular Probes Inc. The rabbit anti-Vpr antibody was generously provided by Dr. Eric Cohen. The ECL^{TM} HRP conjugated donkey anti-rabbit IgG and sheep anti-mouse IgG were purchased from Amersham Biosciences. CHAPS lysis reagent (3-((3-cholamidopropyl)- dimethylammonio) – 1- propane-sulfonate) was purchased from Sigma Chemical Co. RIPA lysis reagent.

2.5 Binding and Expression Assays Using Immunoprecipitation and Western Blot Analysis:

To explore the protein-protein interactions and protein expression in mammalian cells, 293T cells were transfected or co-transfected with specified expression plasmids. After 48hrs of transfection, cells were lysed with either 0.5% CHAPS lysis buffer (199 medium containing 0.5% CHAPS and a protease inhibitor cocktail (Roche)) or 0.5% RIPA lysis buffer (PBS containing 0.5% RIPA and a protease inhibitor cocktail (Roche)). Lysis was carried out on ice for 30min and centrifuged at 15, 000 rpm for 30min at 4° to clarify the samples. Immunoprecipitation (IP) was performed using the supernatant with rabbit anti-GFP, anti-Vpr or anti-HIV for 3hrs followed by Protein-A-sepharose pull down for 2hrs. For sequential -IP, cell lysates were treated consecutively with two different antibodies anti-GFP, and anti-T7 interspaced by Protein-A-sepharose pull down. 12.5% SDS-PAGE was used to resolve the immunoprecipitates, which was followed by western blot using rabbit anti-GFP, mouse anti-T7, rabbit anti-Vpr, rabbit anti-HIV and rabbit anti-p24 antibodies, depending on the protein type being selected for. Sequential immunoprecipitation was performed to measure the total amount of T7-tagged imp7 expression within the cell lysates, and was immunoprecipitated with mouse anti-T7 followed by western blot using the same antibody.

Trichloroacetic Acid protein precipitation method (TCA):

The TCA method is utilized to non-specifically precipitate protein out of cell lysate solution, without the use of IP. After the cells are lysed with 0.5% RIPA lysis buffer on ice for 30min, 100ul of the cell lysate is removed and used to carry out the TCA method. To the cell lysate, 100ul of cold 20% trichloroacetic acid is added, whereby the sample is incubated on ice for 30min. The sample is then centrifuged at 15 000 rpm for 15min at 4oc, and the supernatant removed. 300ul of cold acetone is added over the protein pellet, and again the sample is centrifuged at 15 000rpm for 5min at 4oC. The supernatant is then removed, and the pellet left to partially dry. The pellets are resuspended in SDS-Page loading buffer and boiled in water for 10mins. Samples are then ready to load in the 12.5% SDS-Page gel. The following western blot analysis is described below.

Western blot analysis:

Following protein resolution on an SDS-Page gel, the proteins are transferred overnight in a buffered transfer tank using 20% methanol transfer solution (20% methanol, tris and glycine), onto nitrocellulose membrane. The membrane is then blocked in a 5% milk bath solution for 1hr, and washed with PBS solution 2 to 3 times prior to putting it into the first antibody. The primary antibody is either anti-GFP, anti-T7, anti- HIV, anti-Vpr or anti-p24 which diluted in PBS according to concentration of each antibody, and is then incubated with the membrane on a rocker for 3hrs at room temperature. The membrane is then washed in PBS 5 times for 5min each, while being agitated on the rocker.

Following that, secondary anti-body which can be either anti-rabbit, anti-mouse or anti-human is again diluted with PBS depending on the antibody concentration, and incubated with the membrane for one hour. Finally the membrane is washed again in PBS 5 times for 5min each, and the membrane treated with Western Lighting Chemiluminescence Reagent Plus luminol reagent for 3min (Perkin Elmer™ Life Sciences). Developing film is then placed upon the membrane in darkness, and the final product is developed for visualization of the proteins.

2.6 Quantifying Viral Stocks:

Reverse transcriptase activity:

The reverse transcriptase (RT) activity test measures the amount of active reverse transcriptase activity present within a viral sample, and from that information allows for the quantification of how much virus is present within a given quantity. The process involves taking 50 ml of viral stock generated as previously described, and combining it with 50 ml of the RT reagent cocktail. The RT cocktail consists of 2.5ul of tris hydrochloride pH 7.9, 2.5ul of $MgCl^2$, 7.5ul of KCl, 2.5ul of EGTA, 2.5ul of Triton X-100, 2.5ul of ethylene glycol, 15ul of water, 2.5ul of dithiothreitol, 2.5 of reduced gluthatione, 4ul of poly-A-oligo and 6ul of 3H dTTP. The RT reagent cocktail and viral stock are incubated together overnight at 33oC. After incubating for 22hrs, the reaction is stopped with 1ml of cold 10% trichloroacetic acid (TCA) and precipitated on ice for 2hours. The solution is then collected on 2.4cm glass disks that are placed within the scintillator container. The glass filter disks are first soaked in 10% TCA and

then placed in the correct position. Then the reaction mixture is poured over the glass filter into the scintillator well, and the well rinsed with 5% cold TCA. 100% absolute ethanol is then used to rinse the wells again, after which the glass filters are allowed to be drained and partially dried. The glass filters are then removed and placed within glass scintillation vials and allowed to dry at 60°C for 30min. The vials are then filled with 5ml of scintillation fluid and the vials read using a B-counter to measure the radiation levels.

2.7 Viral Infection and Measuring Infectivity:

Luciferase Assay:

Infection of C8166 and 293T cells was achieved by introducing equal amounts of single cycle replicating (wt/mutant) viruses (4.3- BruΔBgl/Luc/vpr- or 4.3- BruΔBgl/Luc D64E) to the cell culture medium, and allowing the mixture to be agitated for 2hrs post-infection. After 24hrs post-infection the cells were washed 3 times, and the cell culture medium is changed with warmed RPMI complete. 48hrs post-infection, 1x106 cells are harvested from each separate sample and washed twice with PBS. Then 50ul of luciferase lysis buffer (Fisher Scientific Inc) is added to the cells, whereby 10ul of the cell lysate is removed to perform the luciferase assay. Luciferase activity is evaluated using the Top-Counter ® NXT™ Microplate Scintillation & Luminescence Counter (Packard, Meridian), whereby the activity is quantified as relative luciferase units (RLU). All samples were measured in duplicate, factoring in standard deviation into the calculations.

MAGI assay:

Viral infection of HeLa-CCR5/CD4-β-Gal cells is performed to measure viral infectivity using the MAGI assay. After the GFP+/nef- virus was generated through co-transfection with T7-INc206 or T7-INc206 240,4 expressors in 293T cells as previously described. Infectivity of the virus was then measured using HeLa-CCR5/CD4-β-Gal cells which were infected with equal amounts of generated virus (as determined by RT activity assay). HeLa-CCR5/CD4-β-Gal cells were plated $8x10^4$ in a 12 well plate, supplemented with DMEM complete medium. Then 48hrs post-infection, the number of infected cells were measured (β-Gal positive cells), using the MAGI assay as previously described [232].

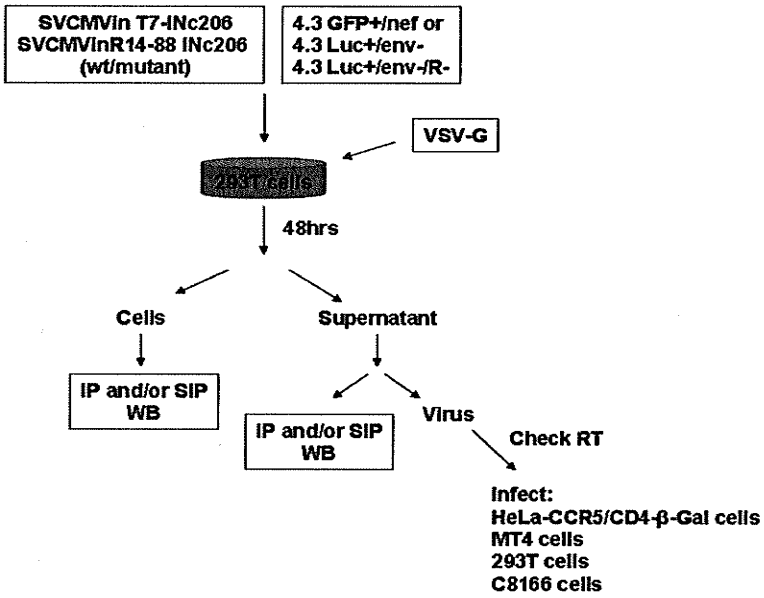

Fig 14. Overview of virus production: Schematic representation of the procedure involved with generating viral particles. 293T cells are co-transfected with the specified plasmids including VSV-G, HIV-1 provirus and an IN expressor plasmids. 48hrs post-transfection both the cells and supernatant are collected. The cells and virus are analyzed using IP and WB for the expression of the transfected IN plasmids, while the supernatant is checked for the presence of the IN proteins within the viral particle. The virus is collected and concentrated from the supernatant and quantified using the RT activity assay, and then used to infect the desired cell type.

Chapter 3:

3.0 Results:

The results outlined within this chapter include all the experiments performed within the context of this thesis, and highlight all the major findings throughout my research.

3.1 Binding of importin 7 to the C-terminal domain of integrase:

For this study, we wanted to delineate the specific domain(s) of integrase that bind importin 7. For our analysis in order to pull-down and detect our specific HIV-1 IN proteins, we constructed YFP tagged HIV-1 IN expressor plasmids within an SVCMV vector (CMV), that are able to cross react with anti-GFP antibody and allow for detection, Two types of YFP expressors were constructed at differing periods within our lab, and as a result either YFP-IN or IN-YFP plasmids are utilized throughout these experiments. Both YFP expressor types perform equally well, and the use of the differing constructs does not negatively impact my results. HIV-1 IN-YFP and YFP-IN (CMV-IN-YFP and CMV-YFP-IN) expressors were constructed bearing the required IN domain deletions. These constructs were generated by fusing full length HIV-1 IN cDNA (generated from the full-length HIV-1 HxBru clone) to the 5' or 3' end respectively of the YFP cDNA, as an SVCMVin-IN-YFP or SVCMVin-YFP-IN expressor as described in materials and methods. The molecular weights of the constructs are indicated within figure 15.

Truncated IN-YFP expressors were generated to delineate the IN domains involved with Imp7 binding. IN50-288-YFP, INY1-212-FP, IN1-240-YFP and YFP-IN206-288, were co-expressed with T7-Imp7 to determine binding specificity. Expression of these truncated IN-YFP expressors in 293T cells along with their molecular weights is presented in figure 15.

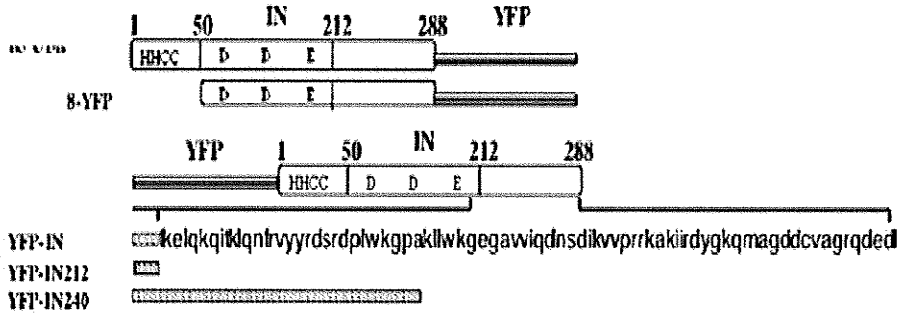

Fig. 15: Schematic representation of wild type of mutant truncations of IN-YFP: Schematic representation of the three domains of wild type IN, and the amino acid regions within the C-terminal domain where the YFP-IN1-212 and YFP1-240 truncations occur. The three domains of IN include the N-terminus spanning between amino acids 1-50, the catalytic core spanning between amino acids 51-212 and the C-terminal domain spanning between amino acids 213-288.

We next investigated which region of IN is responsible for binding Imp7 by using co-expression analysis. 293T cells were transfected with both the IN-YFP and Imp7 expressors in vivo , and after 48hrs the cells were lysed with 0.5% Chaps lysis buffer and subjected to co-immunoprecipitation as described in materials and methods. To rule out the possibility that the T7 or YFP tags attached to Imp7 and IN, were non-specifically inducing binding, T7 was co-expressed with IN-YFP and YFP was co-expressed with T7-Imp7 as controls. Western blot analysis using the corresponding antibodies to visualize the proteins, revealed that both wild type IN and IN50-288-YFP were able to bind T7- Imp7, while YFP, IN1-212-YFP and IN1-240-YFP that lacked the C-terminal domain of IN, were unable to interact with T7-Imp7 *((Fig 16.) upper panel, lanes 4 and 5)((Fig 17.) upper panel, lanes 4 and 5).* More specifically, IN-YFP lacking amino acids 240-288 exhibited the greatest T7-Imp7 binding inhibition, indicating that potentially the Imp7 binding domain of IN is located between amino acids 240 to 288. In addition, during the western blot analysis due to protein degradation and mild amounts of non-specific binding, additional bands can be identified within the diagram that do not impact the findings presented.

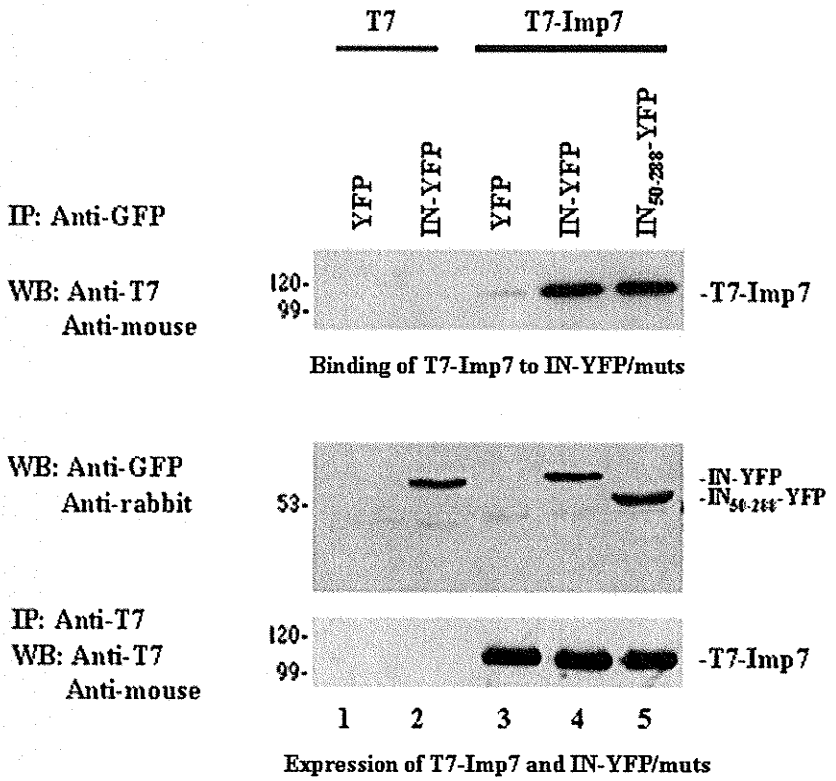

Fig. 16: Deletion mutation analysis of IN to determine the Imp7 binding domain; N-terminal domain binding: Western blot analysis of T7-Imp7 binding to YFP, IN-YFP and IN50-288-YFP, through co-expression within 293T cells. To further investigate the potential of non-specific binging between T7 and YFP and YFP-IN, T7 was co-expressed with both YFP and IN-YFP plasmids. Co-IP was used with anti-GFP which cross reacts with YFP to pull down any YFP tagged proteins, whereas anti-T7 pulls down T7 tagged proteins. The corresponding antibodies were used during the western blot analysis to visualize protein expression and binding. IN50-288-YFP lacking the N-terminus domain binds T7-Imp7 along with IN-YFP *(upper panel lanes 4 and 5)*, while a small amount of non-specific binding is observed between YFP and T7-Imp7 *(upper panel lane 3)*. No non-specific binding is observed between the T7 tag and YFP and IN-YFP (*lane 1 and 2*), but is not substantial enough to impact the results to induce excessive binding.

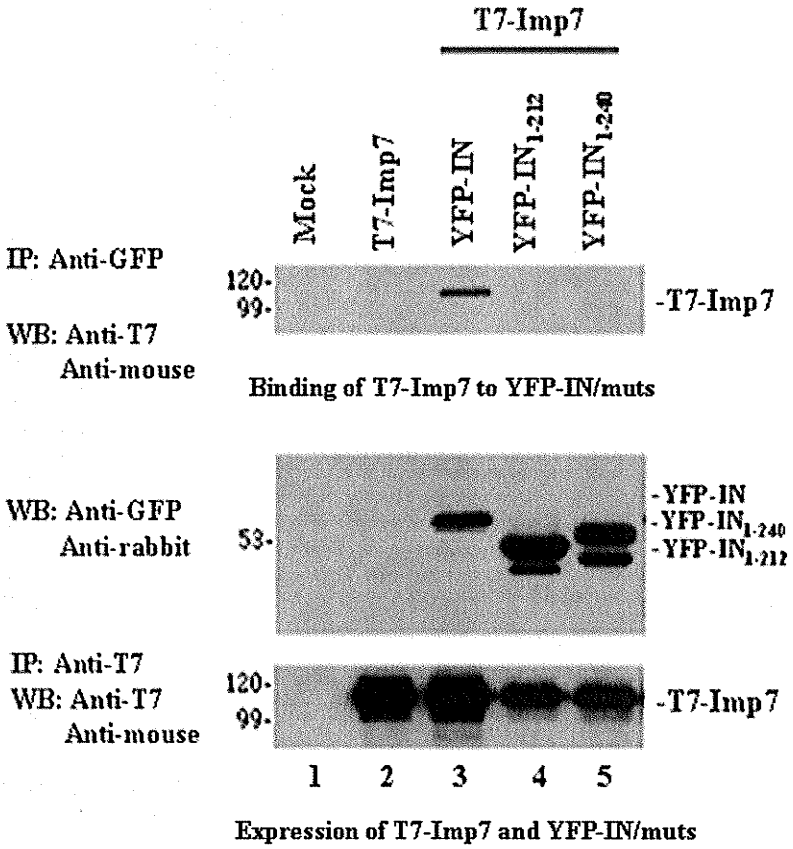

Fig 17. Deletion mutation analysis of IN to determine the Imp7 binding domain; C-terminal domain binding: Western blot analysis of T7-Imp7 interacting with YFP-IN, YFP-IN1-212 and YFP-IN1-240 was performed, by transfecting or co-transfecting 293T cells with either T7-Imp7 or T7-Imp7 and YFP-IN, YFP-IN1-212 or YFP-IN1-240. Binding was observed only for YFP-IN (*upper panel lane 3*), whereas constructs lacking the 240-288 CTD, YFP-IN1-212 and YFP-IN1-240, were unable to interact with T7-Imp7 (*upper panel lanes 4 and 5).*

To confirm that the IN C-terminus domain alone is able to specifically bind Imp7, co-transfection was performed within 293T cells with the Imp7 and YFP-IN206-288 expressors. After 48hrs transfection, cells were lysed with 0.5% Chaps lysis buffer and subjected to co-immunoprecipitation (co-IP) as described in materials methods. As mentioned previously, anti-GFP antibody was used to pull down the YFP tagged proteins through cross reactivity, and similarly anti-T7 antibody was used to pull down T7 tagged Imp7. Using western blot analysis as described in materials and methods with the corresponding antibodies, it was visualized that YFP-IN and YFP-IN206-288 associates specifically with T7-Imp7 *(Fig 18. lanes 2 and 3),* indicating that the IN CTD alone can bind Imp7. The YFP tag was expressed alone with T7-Imp7 to eliminate the possibility that binding is due to a non-specific interaction between YFP and T7-Imp7, but results revealed that no non-specific binding occurred, thus ruling out the possibility *(Fig 18. upper panel lane 1).* Within figure 18, expression levels of the various YFP expressors is not equal, and is the result differing expression efficiencies that differ due to plasmid construction and protein folding which influence protein stability.

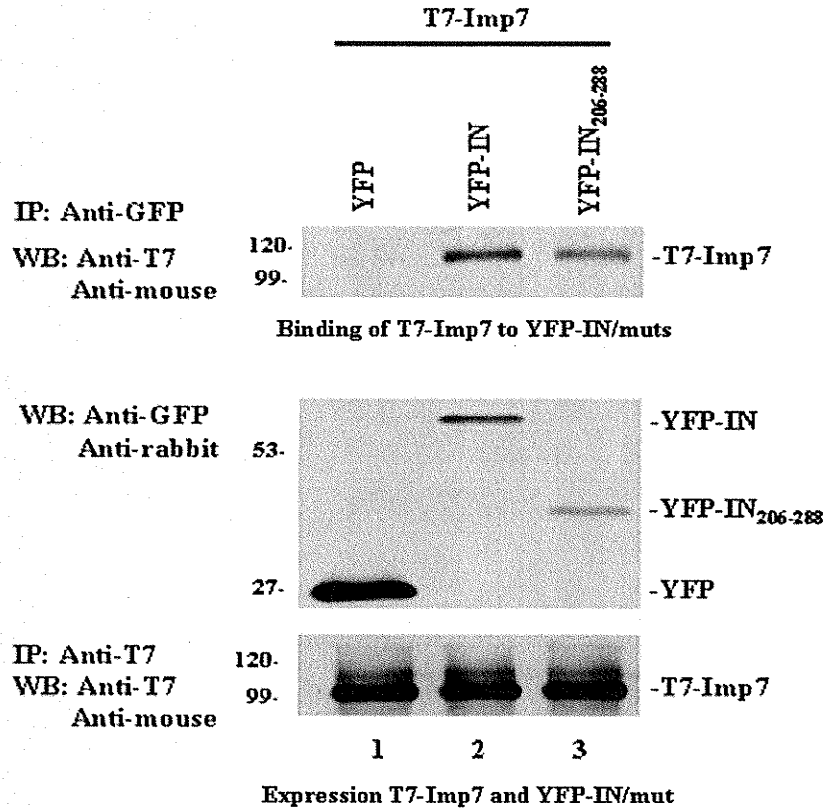

Fig 18: IN C-terminal Domain alone specifically interacts with Imp7:
To determine if the IN CTD can specifically interact with T7-Imp7, 293T cells were co-transfected with both T7-Imp7 and YFP-IN or YFP-IN206-288. Immunoprecipitation and western blot analysis using anti-GFP which cross reacts with YFP and anti-T7 antibodies, to specifically pull down and bind to YFP and T7 bound proteins, indicated that T7-Imp7 binds to YFP-IN and YFP-IN206-288 *(upper panel lanes 2 and 3)*, while no binding of T7-Imp7 to YFP was observed (*upper panel lane 1*). Expression of the YFP-IN and YFP-IN206-288 proteins was visualized using western blot analysis with Expression of T7-Imp7 was observed using anti-T7 antibody to pull down the T7 tagged protein and visual it using western blot analysis (*third panel lanes 1, 2 and 3*). Expression of YFP expressors (*middle panel lanes 1, 2 and 3*), shows uneven expression levels due to plasmid strength, while T7-Imp7 is expressed at equal levels (*lower panel lanes 1, 2 and 3*).

3.2 Defining the specific amino acids within the IN C-terminal domain that binds Imp7:

The IN C-terminal domain possess several highly conserved domains within the Q, C and N regions, that express NLS like characteristics, as they are rich in arginine and lysine. These regions are highly conserved amongst many different strains of HIV-1, and two of the domains, Q and C bare a high similarity with regard to their tri-lysine arrangement. Given their location to one another, ^{211}KELQKQITK and ^{236}KGPAKLLWK are named the proximal and distal tri-lysine regions respectively. Within the N domain there is a 3rd amino acid arginine and lysine rich sequence (^{262}RRKAK) that is also highly conserved, but is not similar to the Q and C domains with regards to number of lysines and spatial arrangement. To address whether the basic tri-lysine and basic amino acid regions function as either a mono or bipartite NLS, specific neutral alanine substitution mutations were introduced at two basic amino lysine and/or arginine residues within those domains (*Fig 19.*).

To investigate if Imp7 is binding to a single NLS within the IN C-terminal domain, two substitution mutations were introduced into each basic tri-lysine and basic amino acid sequences. IN215,9AA-YFP, IN240,4AA-YFP and IN263,4AA-YFP expressors were generated, by introducing a neutral alanine residue into either an arginine or lysine residue. IN-YFP mutant expressors were co-transfected with T7-Imp7 into 293T cells, and after 48hrs, cells were lysed with 0.5% CHAPS lysis buffer. Following, co-IP with anti-GFP and anti-T7

antibodies to pull down the desired products, samples were analyzed using SDS-page and western blot using the corresponding antibodies. Results revealed that both IN215,9AAYFP and IN240,4AA-YFP proteins bound to T7-Imp7 with equal affinity to that of IN-YFP, while T7-Imp7 binding to IN263,4AA-YFP was greatly impaired *(Fig 20. upper panel lanes 4,5 and 7).* Thus indicatig that region ^{262}RRKAK harboring the 263,4 amino acid mutations has the greatest impact on Imp7 binding to IN. In addition, within the western blot diagram there are traces of background lines which are the result of degradation and non-specific antibody binding.

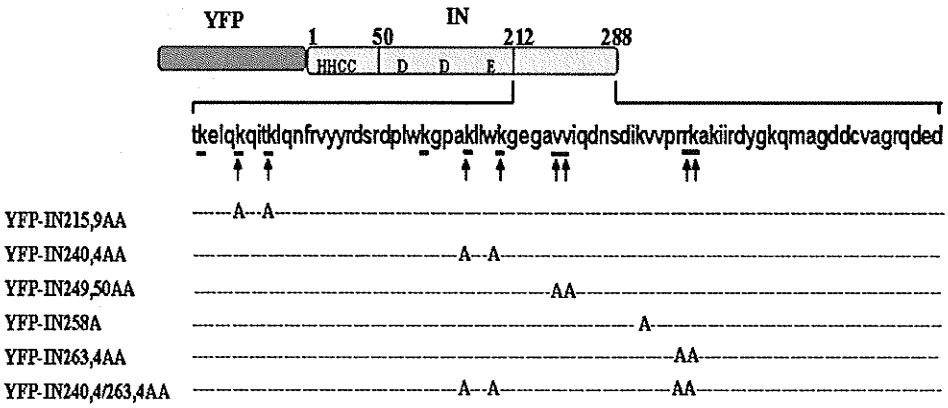

Fig 19. Substitution mutations within the IN CTD: Schematic diagram of the mutations induced within the IN CTD within highly conserved regions between amino acids 206-288, where the mutation sites are indicated at the bottom of the diagram.

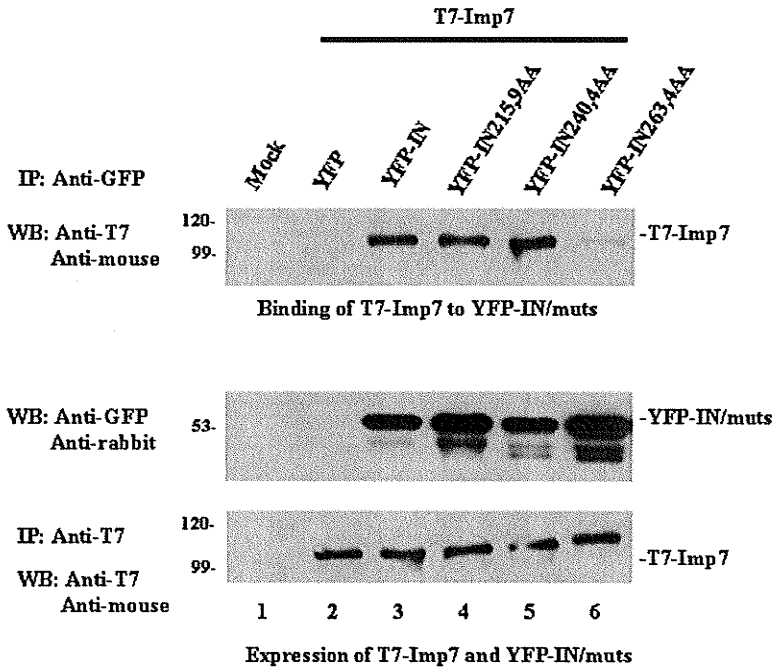

Fig 20. Defining the Imp7 binding regions within the IN CTD: To identify if T7-Imp7 interacts with a single NLS within the IN CTD, 293T cells were co-transfected with YFP-IN mutants harboring mutations within single NLS regions and T7-Imp7 plasmids. After 48hrs, the cells were lysed using 0.5% CHAPS lysis buffer followed by co-immunoprecipitation with anti-GFP and anti-T7 antibodies accordingly. The YFP-IN and T7-Imp7 interaction were analyzed using SDS-page and western blot analysis along with the corresponding antibodies, Binding of T7-Imp7 was observed between YFP- IN, YFP- IN215,9AA and YFP-IN240,4AA proteins, while the YFP-IN263,4AA protein showed a dramatic decrease in T7-Imp7 binding ability (*upper panel lanes 3,4,5 and 6*). Expression of YFP tagged proteins and T7-Imp7 was visualized using the above mentioned procedure (*middle and lower panels lanes 2, 3, 4, 5 and 6*).

Since it was identified previously that amino acids spanning between 240 and 288 of the IN CTD were responsible for binding Imp7, additional substitution mutations were focused within this region into highly conserved domains, to rule out the potential of additional binding locations. YFP-IN249,50AA and YFP-IN258A were constructed as previously described in materials and methods, were highly conserved valine and lysine residues at positions 249, 250 and 258 were substituted with a neutral alanine amino acid. Using co-IP and western blot analysis as previously described in materials and methods, binding of T7-Imp7 was explored with the above mentioned YFP-IN mutant expressors. Results indicated that the additional substitution mutations of the highly conserved valine and lysine residues of YFP-IN249,50AA and YFP-IN258A , did not impair the Imp7/IN interaction *(Fig 21. upper panel lanes 5, 6 and 11).*

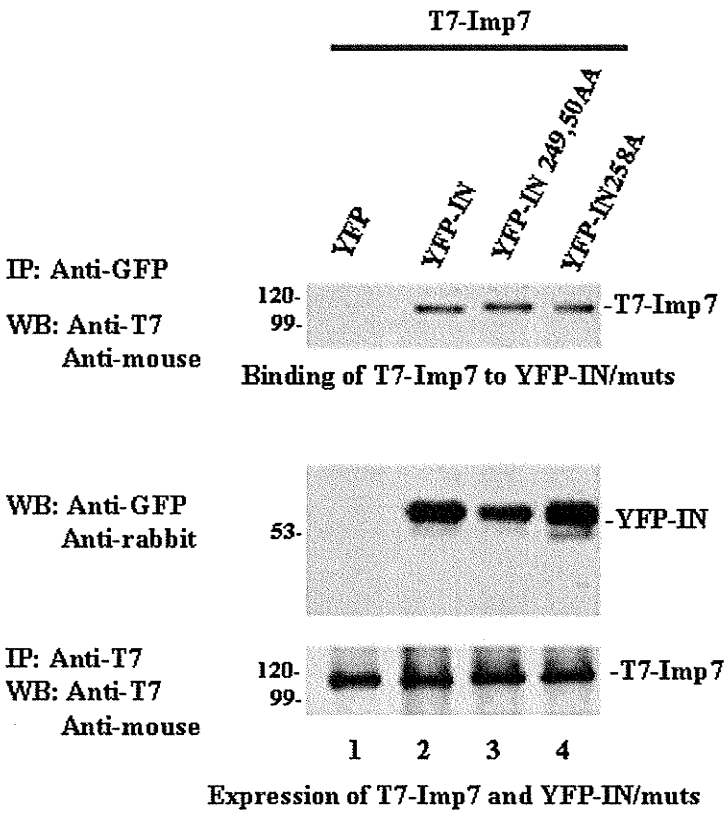

Fig 21. Substitution mutations of highly conserved amino acids within the IN CTD: To determine if additional conserved regions within the IN CTD aside from within the designated NLS regions assist with Imp7 binding to IN, additional substitution mutations were introduced into conserved amino acids 249, 250 and 258. To visualize T7-Imp7 binding to YFP-IN 249,50AA and YFP-IN258A,, 293T cells were co-transfected with the above mentioned plasmids. After 48hrs transfection, the cells were lysed using 0.5% Chaps lysis buffer and subjected to immunoprecipitation and western blot analysis as described in materials and methods. For the above mentioned procedures, anti-GFP and anti-T7 antibodies were used accordingly to visualize protein expression and binding. Results show that YFP-IN, YFP-IN 249,50AA and YFP-IN258A proteins all bind T7-Imp7 with roughly equal frequency (*upper panel lanes 2, 3 and 4*). Expression of the YFP and T7-Imp7 expressors is found in the middle and lower panels (lanes 1, 2 and 3), Within the middle and lower panels additional background bands can be detected which are due to non-specific antibody binding, and do not impact the results.

3.3 Impact of the IN C-terminal bipartite NLS mutation on Imp7 binding to IN:

As was previously observed, binding of Imp7 to IN occurs between regions 240-288, while mutation of amino acids 263 and 264 of the IN CTD significantly impairs Imp7 binding, but does not completely abolish it. With this understanding, it is presumed that Imp7 binds IN at two separate locations, one being at NLS ^{236}KGPAKLLWK, and the other which is defined by a stretch of basic arginine and lysine residues at region ^{262}RRKAK To render a NLS region non-functional, it is necessary to alter two arginine or lysine residues within that domain. During this assay substitution mutations were introduced simultaneously into two basic amino acid domains within the same mutant. YFP-IN215,9/240,4AA, YFP-IN240,4/263,4AA and YFP-IN215,9/263,4AA mutant expressors were generated, and co-transfected within 293T cells to explore their Imp7 binding potential. After 48hrs of transfection, cells were lysed using 0.5% Chaps lysis buffer and subjected to co-IP with anti-GFP and anti-T7 antibodies to pull down the corresponding proteins. Samples were then treated with laemli loading buffer and equal amounts of sample were loaded on an SDS-PAGE gel. Western blot analysis with the corresponding antibodies as described in materials and methods revealed that mutants harboring a double NLS mutation, impaired T7-Imp7 binding more efficiently that the single NLS mutants. It is observed that IN mutant YFP-IN240,4/263,4AA which when co-transfected with T7-Imp7 displayed strong binding inhibition in comparison to the wild type control (YFP-IN) (*Fig 22 ,upper panel lanes 8, 9 and 10*). Following the same method, double

mutant YFP-IN215,9/263,4AA moderately impaired T7-Imp7 binding as it possessed the 263,4 amino acid mutation, while YFP-IN215,9/240,4AA had very little to no effect on blocking T7-Imp7 binding to IN (*Fig 22. upper panel lanes 8, 9 and 10*). Within the diagram, a small amount of background bands can be identified, where non-specific binding is occurring, in particular between the YFP tag and T7-Imp7 (*Fig 22. upper panel lane 1*), but because all positive results are occurring at a binding intensity greater to that of the background binding, the non-specific bands do not affect the results. From these results it is evident that the ^{262}RRKAK binding domain is the primary site of Imp7 binding to IN, and region ^{236}KGPAKLLWK functions as the secondary binding domain to stabilize the primary interaction.

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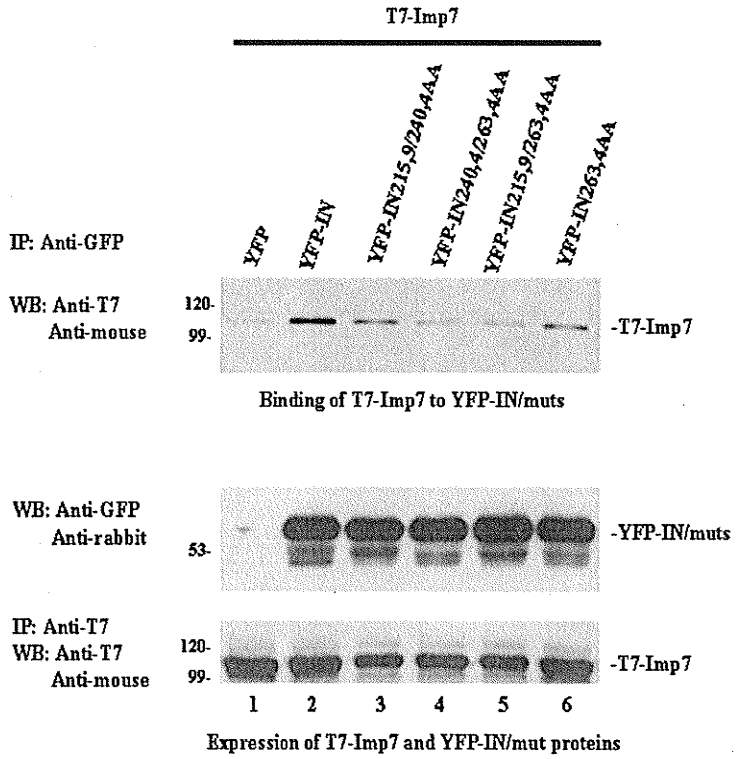


Fig 22. Defining the bipartite NLS within the IN C-terminal domain that binds Imp7: To determine which combination of NLS domains within the IN CTD are responsible for binding Imp7, mutants were constructed harboring double NLS mutated regions. 293T cells were co-transfected with both T7-Imp7 and YFP/mut expressors, and after 48hrs cells were lysed using 0.5% Chaps lysis buffer and subjected to co-IP. Samples were then treated with SDS-PAGE loading buffer, and equal amounts were loaded into the SDS-PAGE gel. Protein binding and expression was visualized using western blot analysis with the corresponding antibodies as described in materials and methods. Anti-GFP was utilized to cross react with and pull down YFP tagged proteins. Results show that YFP-IN215,9/240,4AA *(lane 3)* moderately impairs T7-Imp7 binding, YFP-IN215,9/263,4AA (lane 5) induces a greater amount of T7-Imp7 binding inhibition, whereas mutant YFP-IN240,4/263,4AA (*lane 4*) demonstrates the strongest T7-Imp7 binding inhibition, where only a small amount of interaction is occurring in comparison with control (upper *panel lanes 2, 3, 4 and 5*). Within the upper panel in lane 6, YFP-IN263,4AA single mutant is shown to express the same binding efficiency to T7-Imp7 as the YFP-IN215,9/240,4AA mutant. Expression of the YFP-IN/muts and T7-Imp7 expressors is demonstrated in the middle and lower panels (*lanes 1, 2, 3, 4, 5 and 6*). In addition a moderate amount of non-specific bands are observed within the middle and lower panels.

3.4 Effect of the IN CTD on HIV-1 replication within dividing 293T cells:

To assess more accurately the impact of the IN CTD on viral replication, it is necessary to use multiple assay types. Within this portion of the thesis I have evaluated two different methods for inhibiting HIV-1 viral replication. 1) Expression of T7-tagged IN CTD fragments within cells prior to infection with wild type HIV virus. 2) Expression of IN CTD fragments within the wild type viral particle prior to infection within cells. The luciferase assay is used to detect the early production of viral proteins and monitor viral replication, while the MT4 and MAGI assays monitor the later stages of viral replication by quantifying the virus's ability to infect neighboring cells. In addition, virus generated for use with the l

Luciferase assay:

The luciferase gene is derived from the firefly gene, and functions as a reporter gene to facilitate identification. The luciferase gene is put in place of the HIV-1 nef gene, which acts as a transcriptional regulator of viral genes. Expression of the luciferase gene indicates positive infectivity and replication of the virus. Virus used within the following experiments was generated by co-transfecting 293T cells with VSV-G and 4.3 Wt/Luc+/env- or Wt/Luc+/env-/D64E provirus plasmids and is described in materials and methods. The T7 tag was used within this assay, as it is small and would not interfere with protein folding and binding.

To address the question of whether the IN CTD is able to inhibit replication of 4.3 Wt/Luc+/VSV-G HIV-1 virus in trans, T7 and T7-INc206 expressors were transfected into 293T cells, and after 48hrs, cells were infected with VSV-G pseudotyped 4.3 Wt/Luc+/VSV-G HIV-1 single cycle replicating virus. Luciferase analysis, as described in materials and methods, indicated that the T7-INc206 protein was able to impair 4.3 Wt/Luc+/VSV-G viral replication by roughly 50-80%, in comparison to the T7 control (*Fig 23 and 24*). The results were generated from two separate experiments and cannot be compared statistically.

Another similar experiment was performed in parallel, using the VSV-G pseudotyped HIV-1 single replicating virus that was defective for cDNA integration, through inclusion of the IN class I mutant D64E (PNL 4.3 D64E/Luc+/env-/VSV-G). The 4.3 D64E/Luc+/VSV-G virus was produced as described previously. The intention of using an integration defective HIV-1 strain was to help identify which step during viral replication is affected by expression of the IN CTD. 293T cells were transfected with either T7-INc206 or T7 expressors, and after 48hrs they were infected with equal amounts of 4.3 Luc+/VSV-G pseudotyped D64E HIV-1 virus. Luciferase analysis, as described in materials and methods, indicated that the T7-INc206 protein was able to impair 4.3 D64E/Luc+/VSV-G viral replication by roughly 50% in comparison with the T7 control (*Fig 23 and 24.*). The results generated were derived from two separate experiments, and could not be compared statistically.

Inhibition of viral replication through the expression of the IN C-terminal domain within 293T cells prior to infection with both wild type and integration defective HIV-1 virus

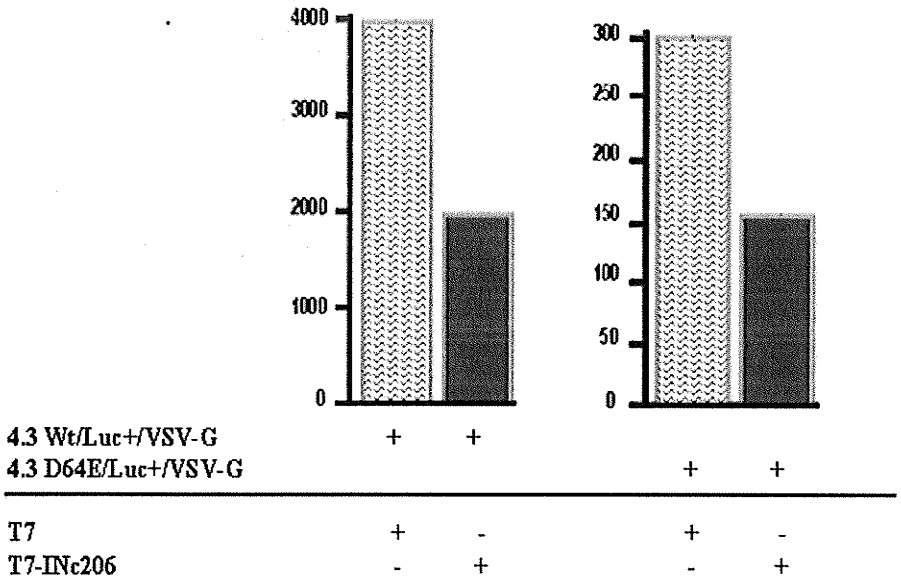


Fig 23. Impact of the IN CTD on HIV-1 viral replication measured by the luciferase activity assay: 293T cells were transfected with T7- INc206, and after 48hrs the cells were infected with equal amounts of either 4.3 Wt/Luc+/VSV-G or D64E/Luc+/VSV-G HIV-1 virus as measured by the RT activity assay. Results show that the T7-INc206 protein is able to impair viral replication of Wt/Luc+/VSV-G virus by 50%. While the T7-INc206 fragment impairs viral replication of the D64E/Luc+/VSV-G HIV-1 virus in comparison with the T7 control by roughly 50%. Figure 23 and 24 experiments were performed at different times, and therefore cannot be compared statistically, yet the results are similar and can be visually compared.

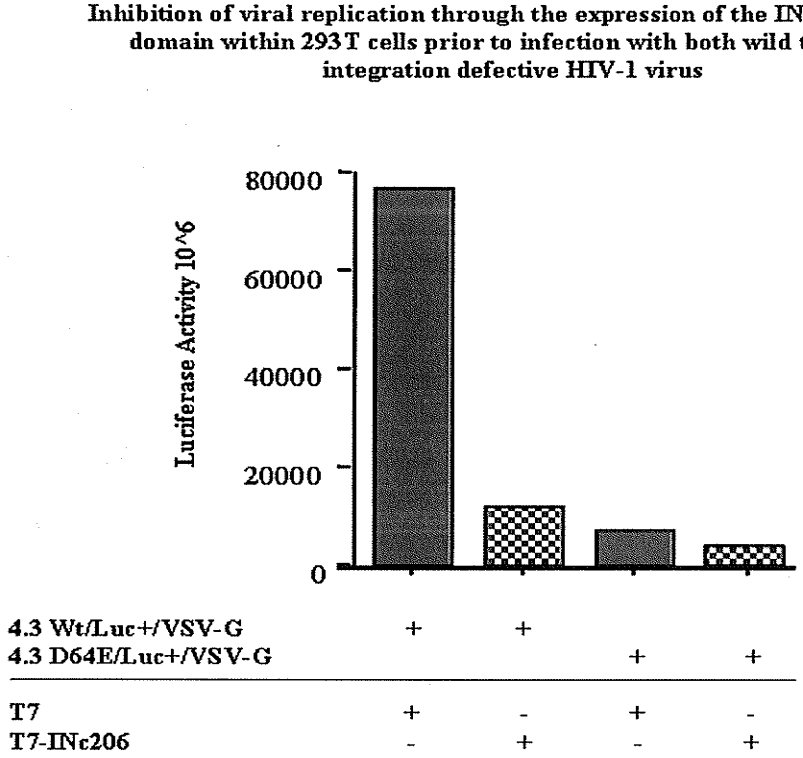


Fig 24. Impact of the IN CTD on HIV-1 viral replication measured by the luciferase activity assay (Second experiment): 293T cells were transfected with T7- INc206, and after 48hrs the cells were infected with equal amounts of either 4.3 Wt/Luc+/VSV-G or D64E/Luc+/VSV-G HIV-1 virus as measured by the RT activity assay. Results show that the T7-INc206 protein is able to impair viral replication of Wt/Luc+/VSV-G virus by 80%. While the T7-INc206 fragment impairs viral replication of the D64E/Luc+/VSV-G HIV-1 virus in comparison with the T7 control by roughly 50%. Figure 23 and 24 experiments were performed at different times, and therefore cannot be compared statistically, yet the results are similar and can be visually compared.

MAGI assay:

To further measure the impact of the T7-INc206 expressor on the early stages of viral replication, the amount of Tat regulated viral transcription was quantified using the MAGI and MT4 assays. Within the 4.3 GFP+/nef+ HIV-1 virus type, the GFP gene is placed prior to the Nef gene, which is under the transcriptional regulation of Tat. Tat expression induces transcriptional activation of both the GFP gene and other viral protein products, which can then be analyzed using the described methods. Briefly, the 4.3 GFP+/nef+ virus was generated through co-transfection of 293T cells with both the T7-INc206 and provirus expressors. After 48hrs the viral supernatant was harvested, clarified and concentrated as described in materials and methods, whereby the amount of virus was quantified using the RT assay. For the MAGI assay, an equal amount of HeLa-CCR5/CD4-β-Gal cells were infected for 2hrs with equal amounts of virus. After 2 to 3 days, the number of infected cells (β-galactosidase-positive cells) in each HeLa-CD4-β-Gal cell culture was determined as described in materials and methods. Results revealed that the transiently expressed T7-INc206 protein was able to impair viral infectivity by roughly 75%, as compared to the wild type virus (100%) *(Fig 25. A)*.

MT4 assay:

The MT4 assay (*Fig 25. B*) was carried out in much the same manner as the MAGI assay, were the 4.3 GFP+/nef + T7-INc206 virus that was generated as previously described, was used to infect equal amounts of MT4 cells. Early stage

viral replication was measured by quantifying the amount of GFP expression produced, under the transcriptional regulation of Tat. The amount of infected cells (GFP expressing cells) was measured using FACs analysis. Results revealed that the T7-INc206 expressor inhibited 4.3 GFP+/nef+ viral expression by roughly 85%, as compared with the T7 control (100%) (*Fig 25. B*).

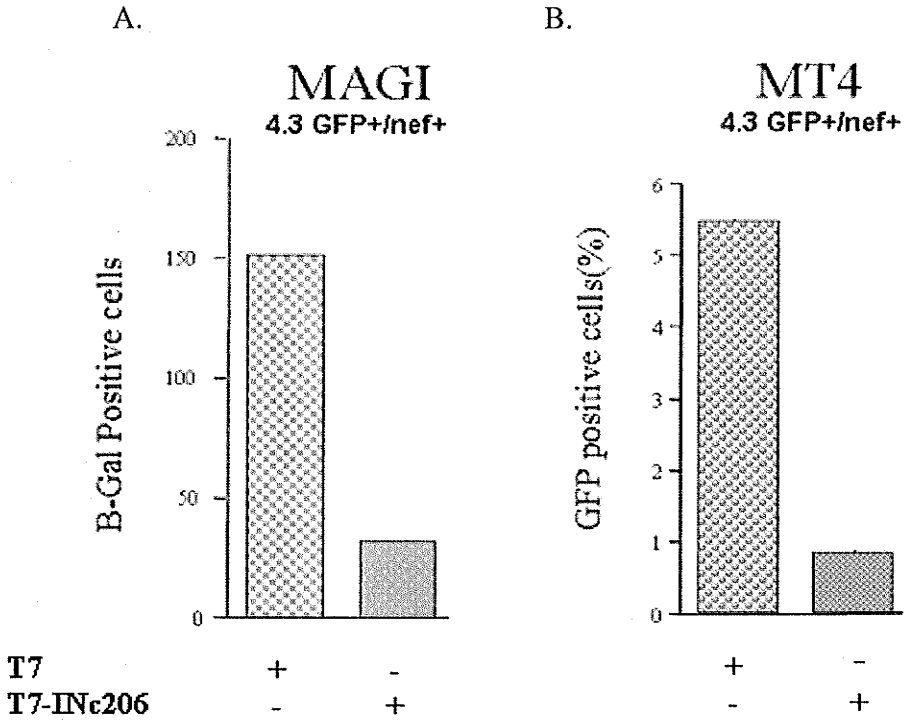


Fig 25. Impact of the IN CTD on HIV-1 viral infectivity measured by the MAGI assay: A) 293T cells were co-transfected with T7 or T7-INc206 and 4.3 Wt/GFP+/nef+ HIV-1 proviral plasmids, to generate virus which was then harvested and further clarified and concentrated as described in materials and methods. A) Hela-β-Gal cells were then infected for 2hrs with equal amounts of virus (determined by RT assay), and 2 and 3 days following, the % infectivity was calculated (β-Galactosidase positive cells). During the MAGI assay, the T7-INc206 impaired viral infectivity by 75% as compared to the T7 control (100%). B) The same virus that was generated from A), was used to infect MT4 cells. Infected cells were quantified using a FACs analysis machine, to measure the amount of GFP positive cells. As indicated, the T7-INc206 expressor inhibited viral infectivity by roughly 85%, as compared to the T7 control (100%). This experiment was only performed once and therefore no statistical relevance can be generated.

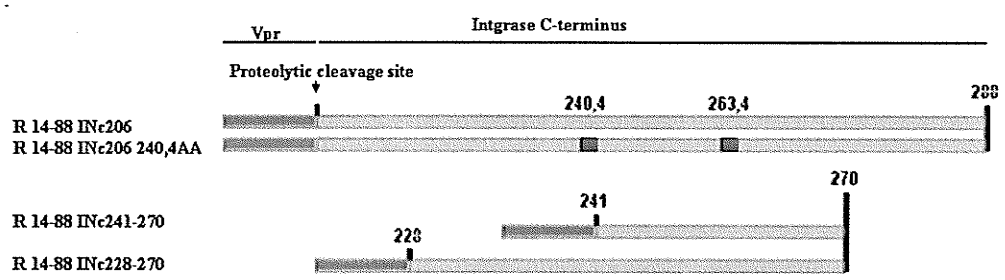
3.5 Expression of the Vpr-fusion protein within 293T cells:

To define the minimal region of the IN CTD required to inhibit viral replication within HIV-1 infected cells, we constructed a series Vpr-fusion proteins encompassing different stretches of the IN CTD. Vpr14-88 (R 14-88) which is able to facilitate virion incorporation of attached proteins, through interaction with the p6 domain of the $Pr55^{Gag}$ polyprotein, is fused to the N-terminus domain of the various IN CTD fragments which span between amino acids 206-288. The constructs were designed so as to possess different regions of the IN CTD that interact with Imp7, encompassing the minimal binding domains (*Fig 26. A*). The Vpr-fusion proteins are able to release their fusion partners into the virion particle in a native form, due to the inclusion of a protease amino acid cleavage sequence (SQNY/PIV), between the Vpr and attached IN CTD. This amino acid sequence corresponds to the viral protease processing site located between $p17^{Gag}$ and $p24^{Gag}$, which is cleaved *in vitro* by the HV-1 protease.

To verify that the Vpr-fusion proteins are being efficiently expressed within the target cells, we isolated the most important R14-88 IN CTD fusion proteins (R14-88, R14-88 INc206, R14-88c206 240,4/263,4AA, R14-88 INc228-270, R14-88 INc241-270), and subjected them to an expression assay, Briefly, 293T cells were transfected with the various R14-88 IN CTD fusion protein expressors. After 48hrs of transfection, cells were lysed using 0.5% RIPA and protein was eluted using the Trichloroacetic Acid method (TCA method). Results were visualized using SDS-Page and western blot analysis as described in materials and methods.

The desired proteins expressed within the expected ranges, although R14-88 INc206 and R14-88 INc206 240,4/263,4AA proteins which are supposed to express at similar molecular weights, do not match. This discrepancy is possibly due to altered folding induced by the substitution mutations, which could impact on the charge and molecular weight of the protein (*Fig 26. B) lanes 2 and 3).* In addition, R14-88 INc228-270 is expressing bands at various levels that are not expected. These bands are possibly due to altered folding as a result of the protein's mutated amino acid composition, which impacts on both multimerization of the protein and protein degradation yielding the differing bands *(Fig 26. B) lane 4).* Additional bands not described within the results are due to non-specific binding of the antibody, and do not impact on the results previously mentioned.

A.



B.

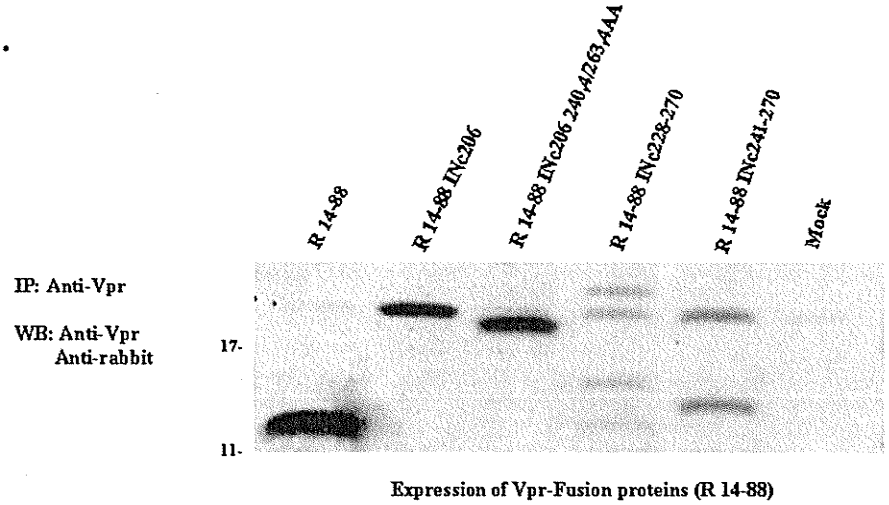


Fig 26. Expression of the SVCMVinR14-88 fusion proteins within 293T cells: A) Schematic representation of the Vpr-fusion protein constructs, possessing Vpr14-88 fused to the N-terminus domain of the IN CTD separated by a protease cleavage sequence. The Vpr-fusion constructs encompass the IN CTD, and include fragments R14-88 INc206 240,4/263,4AA which is unable to bind Imp7, R14-88 INc228-270 which contains both the primary and secondary Imp7 binding domains (^{236}KGPAKLLWK and ^{262}RRKAK) and R14-88 INc241-270 which possess only the primary binding domain (^{262}RRKAK) B) 293T cells were transfected with the various R14-88 IN CTD expressor plasmids, and after 48hrs transfection, samples were lysed using 0.5% RIPA lysis buffer. Protein was eluted using the tricholoric acid method (TCA method), and results visualized using SDS-Page and western blot analysis. Expression of the R 14-88 fusion proteins is as expected at the differing levels, although R 14-88 INc206 240,4/263,4AA should have a molecular weight that matches that of R 14-88 INc206. Due to the mutant's altered amino acid composition, the folding and molecular weight of the protein has also been changed. R 14-88 INc228-270 is expressing additional bands that are potentially the result of protein multimerization, and degradation due to the altered amino acide composition of the protein. Additional non-specific background bands can be identified within the diagram and are the result of non-specific antibody binding.

3.6 The effect of incorporated Vpr-fusion proteins on HIV-1 replication within CD4+ C8166 cells:

Delineation of the minimal domain of IN that binds Imp7, was conducted through construction of Vpr-fusion protein expressors. Constructs possessing Vpr segment 14-88, which facilitates virion incorporation, was fused to the N-terminal domain of the IN CTD fragments. Plasmids encoding R14-88 and R14-88 IN-Vpr fusion proteins, were co-transfected into 293T cells, along with PNL 4.3 Wt/Luc+/env-/Vpr- HIV-1 provirus and VSV-G plasmids to generate virus containing both the Wt and CTD forms of IN . After 48hrs of transfection, virus was collected at 48, 72 and 96hrs, clarified using ultracentrifugation, and quantified as previously described using the reverse transcriptase assay. To observe the impact of the incorporated IN CTD on viral replication, CD4+ C8166 cells were infected with equal amounts of the Vpr-fusion Wt HIV-1 virus over a 48hr period. After 48hrs, the C8166 cells were harvested, washed and lysed, and viral replication was analyzed using the luciferase assay as described in materials and methods. The results presented were generated from two different viral stocks, and therefore cannot be compared statistically, but never the less similar trends are observed and can be compared visually.

Results derived from the two separate experiments indicate that the R 14-88 INc206 Vpr-fusion protein impairs viral replication by roughly 1.6-1.7 fold (*Fig 27 and 28*). The R 14-88 INc206 240,4/263,4AA expressor partially restores viral infectivity in figure 26, but during the repeat experiment, infectivity was

completely restored with the mutation. The R14-88-INc228-270 Vpr fusion protein containing both the Imp7 binding sites impairs infectivity by 3.1 fold during the initial experiment *(Fig 27.)*, whereas in the second experiment no inhibitory effects were observed (*Fig 28.*). R14-88INc241-270 Vpr fusion protein displays the greatest inhibition of decreasing infectivity by 2.5-18, as observed within both assays fold (*Fig 27 and 28.*). These observations correspond with the previous findings that the ^{262}RRKAK Imp7 binding region is the major binding domain, and that small fragments containing this site provide the most pronounced inhibitory effect.

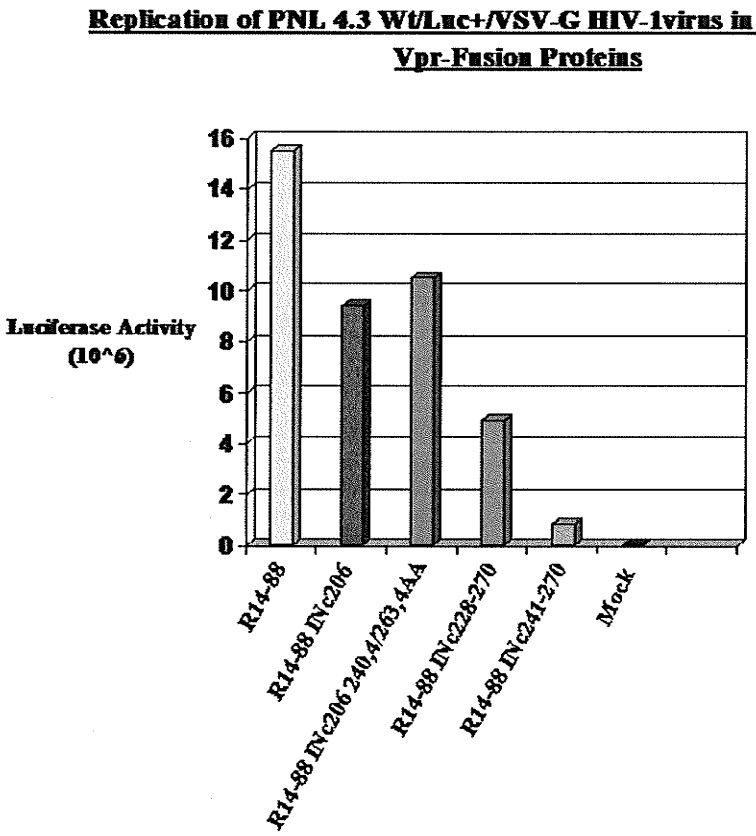


Fig 27. Effect of Vpr-fusion protein virion incorporation on HIV-1 replication: C8166 cells were infected with equal amounts of Vpr-fusion protein HIV-1 viruses (as determined by RT assay), derived from 293T cells transfected with Vpr fusion protein plasmids and PNL 4.3 Wt/Luc+/env-/Vpr- provirus. C8166 cells were infected for 48hrs, then harvested and lysed using the luciferase assay method as previously described. Results revealed that in comparison to the R 14-88 control, R 14-88INc206 impaired infectivity by 1.6 fold, R 14-88INc206 240,4/263,4AA impaired infectivity by 1.4 fold, R 14-88INc228-270 impaired infectivity by 3.1 fold and R 14-88INc241-270 impaired infectivity by 18 fold. This experiment was only performed once and cannot be compared statistically to figure 28.

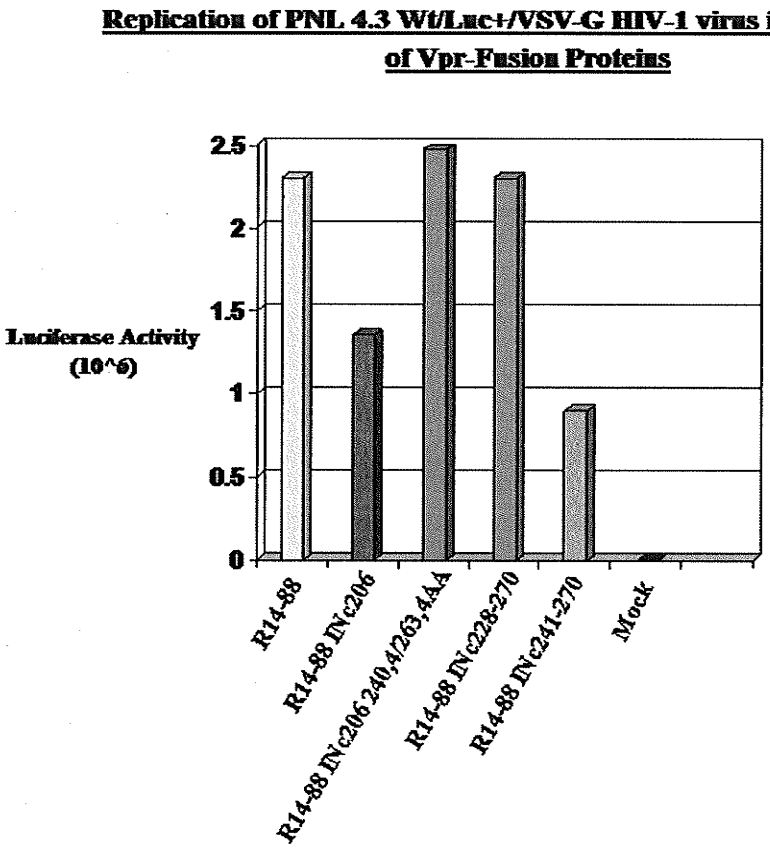


Fig 28. Effect of Vpr-fusion protein virion incorporation on HIV-1 replication (Second analysis): C8166 cells were infected with equal amounts of Vpr-fusion protein HIV-1 viruses (as determined by RT assay), derived from 293T cells transfected with Vpr fusion protein plasmids and PNL 4.3 Wt/Luc+/env-/Vpr- provirus. C8166 cells were infected for 48hrs, then harvested and lysed using the luciferase assay method as previously described. Results revealed that in comparison to the R14-88 control, R14-88INc206 impaired infectivity by 1.6 fold, R14-88INc206 240,4/263,4AA restored infectivity to above normal control levels by 0.7 fold. R14-88INc228-270 restored infectivity as well to the same level as the R14-88 control, while R14-88INc241-270 impaired infectivity by 2.5 fold. This experiment was performed once and cannot, be compared statistically to figure 27.

Chapter 4:

4.0 Discussion:

During the course HIV-1 viral replication, IN is expressed to act on the early stages of cellular infection where it is the key enzymatic protein involved in reverse transcription, nuclear import of viral cDNA and integration [26, 27]. It is clear that HIV-1 requires the interaction of host cellular factors to facilitate infection, but so far the mechanism underlying the coordination of viral and host proteins is unknown [77]. With regard to my work examined throughout this document, my intent was to explore the possible inhibitory effects of HIV-1 viral proteins on HIV replication. In particular, looking at viral protein integrase, and exploiting the Imp7 binding properties of the protein to inhibit viral replication. Various researchers have also sought to disrupt other important viral/cellular interactions to inhibit viral replication, such as the use of the truncated LEDGF/p75 integrase binding domain to competitively block binding of wild type LEDGF/p75 to integrase, which functions to assist with replication[233]. Other new therapies targeting viral/cellular interactions include; using mutant host cellular protein Tsg101 to inhibit Gag polyprotein transport into multivesicular bodies, using mutant INI1 cellular proteins to interact with the IN domain of the Gag-Pol polyprotein and prevent proper multimerization of Gag and Gag-Pol, and utilizing mutant cellular Sam68 nuclear export proteins to inhibit transactivation of RRE and Rev function [234, 235, 236]. Currently, there exist numerous methods to inhibit HIV-1 viral replication, yet still very few researchers are

investigating the inhibitory potential behind the host cellular interactions of integrase specifically, and its relation to host karyopherins.

Due to the compact nature of the HIV-1 virus, during viral replication it is necessary for HIV-1 to adapt itself the host's replication machinery in such a fashion, so as it is able to hijack cellular replication for its own purposes, while still maintaining cellular integrity, As previously mentioned, research has identified several host proteins which interact with IN to complement the viral protein's karyophillic and enzymatic functions, which function to assist the virus overcome various cellular regulatory components.

The nuclear pore complex (NPC), which acts as the gate keeper to the nucleus, regulates passage of compounds in and out of the nucleus. To facilitate active and energy dependent passage of the PIC through the NPC, karyophillic viral proteins must interact with host karyopherins, such as importin α (Impα) , importin β (Impβ) and importin 7 (Imp7) [20, 36, 93, 97, 99, 101, 237]. IN has been reported to interact with both Impα and Imp7, yet it is still unknown whether or not these karyopherins function in cooperation or independently to allow for NPC passage, or if they function to assist with another step of viral replication [12, 36, 77, 107]. Previous work already performed within our lab sought investigated the binding potential of both IN and Imp7 to other cellular and viral factors, and observed that the IN/Imp7 interaction is highly specific. There has been considerable controversy over the role Imp7 plays during viral replication, and as of yet it has still not been resolved [145, 154, 36, 37]. Within our lab we identified that Imp7

binding defective single-cycle replicating virus had impaired infectivity, and that both reverse transcription and nuclear import steps were affected. Assuming that Imp7 is essential for viral replication, the question arose how this interaction could be exploited to inhibit viral replication.

My research focused on isolating the particular domain of IN responsible for binding host Imp7, and then further defining the potential inhibitory effect of this region on viral infectivity. Binding analyses performed using IN deletion mutants co-expressed within 293T cells along with T7-Imp7, defined the IN CTD as the site responsible for interacting with Imp7. Substitution mutations induced within the highly conserved NLS regions within the IN CTD allowed us to pinpoint ^{235}WKGPAKLLWKG and ^{262}RRKAK , as the sites solely responsible for binding Imp7, where mutation of these regions (KK240,4AA/RK263,4AA) greatly impaired the IN/Imp7 interaction. Interestingly, it was observed that although ^{235}WKGPAKLLWKG and ^{262}RRKAK are both required for Imp7 binding, region ^{262}RRKAK is the critical site, and shows almost complete binding inhibition upon mutation, whereas mutation of ^{235}WKGPAKLLWKG only slightly impairs the Imp7 interaction. Therefore we concluded that Imp7, because it is required for viral replication, binds a bipartite NLS with ^{262}RRKAK being the primary site of interaction and ^{235}WKGPAKLLWKG being the secondary stabilizing site.

Experiments performed in our laboratory previously, sought to characterize the role of the nuclear localization signals within the IN C-terminal domain, those

being ^{211}KELQKITK, ^{235}WKGPAKLLWKG and ^{262}RRKAK. Findings indicated that aside from impacting the nuclear accumulation of viral cDNA, that activity of reverse transcription was also impaired. Mutation of the ^{235}WKGPAKLLWKG NLS had the most pronounced impact on reverse transcription, while regions ^{211}KELQKITK and ^{262}RRKAK decreased cDNA accumulation significantly. Fitting in with my analysis, it would appear that the Imp7 binding region within the IN C-terminal domain has the pleiotropic potential to negatively modulate viral replication at various time points during viral infection. This finding led us to question whether the IN Imp7 binding domain could inhibit viral replication, if expressed within cells and the viral particle prior to infection, and if this region conflicts with reverse transcription, nuclear import or both?

Two approaches were undertaken to answer this question, as both would differentially alter the impact of the IN CTD on viral replication. Firstly, I wanted to observe the impact of the IN CTD on viral replication when it was expressed within the cells prior to infection. From that point it allowed us to monitor the early stages of viral protein production using the luciferase assay, and simultaneously detect any later changes to viral infectivity with the use of the MT4 or MAGI assays. The second approach consists of introducing the IN CTD into HIV-1 virions prior to infection, and monitoring the resulting impact to viral replication upon initial contact.

With regards to the luciferase assay,T7 or T7-INc206 plasmids were both expressed within 293T target cells prior to infection with wild type virus. Results clearly show that the T7-INc206 protein is able to markedly impair viral infectivity by roughly 50-80%, compared to the T7 control, as visualized between the two separate experiments *(Fig 23 and 24)*. Similar results were also observed when T7-INc206 expressing 293T cells were challenged with the class I D64E mutant virus defective for integration, impairing infectivity by 50% throughout both experiments in figures 23 and 24. From these observations it is apparent that the IN CTD is affecting viral replication prior to the integration step, possibly during the step of nuclear import or DNA chromosomal tethering (*Fig 23 and 24*).

Comparing these results with that obtained from the MT4 and MAGI assays, it was observed that viral infectivity was greatly impaired within HeLa-CCR5/CD4-β-Gal cells, ranging between 75-80% between the two assays (*Fig 25.* A and B). From this information it is apparent that the IN CTD may be interfering with the early stages of viral replication in such a fashion, as to render progeny virions non-infectious.

The second approach addresses the possibility that IN maybe interacting with various unidentified viral or progeny incorporated cellular factors such as Imp7, prior to or upon infection. To expand on this concept, R14-88 IN CTD fusion expressors were constructed, in order to facilitate the incorporation of native IN CTD within wt HIV-1 virus particles, allowing us to monitor the inhibitory effects

induced immediately upon infection. As identified by Yao et al, Vpr is able to mediate viral incorporation of proteins within the viral particle through association with the p6 binding domain of the $Pr55^{Gag}$ polyprotein, and amino acids 14-88 is the minimal domain required for this activity [65, 155]. By including a viral protease cleavage site in between the Vpr and IN CTD fragments, these two regions are separated upon incorporation into the viral particle allowing for native IN CTD expression.

Results derived from two separate experiments performed at differing times revealed that the R14-88 INc206 fusion protein incorporated into the viral particle, was able to inhibit viral replication by roughly 1.6-1.7 fold in comparison with the R14-88 HIV-1 control. Surprisingly, between the two experiments (Fig 27 and 28), the R14-88 INc206 240,4/263,4AA mutant , had differing results whereby in figure 27, it had very little to no effect in restoring infectivity of the virus, whereas in figure 28, infectivity levels were restored to levels better than the control. Results derived from figure 28, match with the hypothesis that mutation of the Imp7 binding domain should restore infectivity, and impair the ability of the IN CTD to interact with host Imp7. Further investigation will have to be done to explore why this is occurring, as the two experiments were performed with different virus stocks

As previously discussed, ^{235}WKGPAKLLWKG and ^{262}RRKAK NLS regions each influence viral replication in a different fashion, and with regards to Imp7

binding, function as a unit to interact with the protein in a graded fashion. Throughout my research I identified that region ^{262}RRKAK functions as the dominant primary Imp7 binding domain, while ^{235}WKGPAKLLWKG acts as the secondary site to assist and potentially stabilize the interaction. Given the necessity of both domains for Imp7 binding and their differing functions throughout the replication cycle, it was important for me to further define the minimal Imp7 binding domain required to inhibit viral replication. Analysis revealed that the potent R14-88 IN CTD fragment was that spanning from amino acids 241-270 encompassing the ^{262}RRKAK region, which inhibited viral infectivity by 2.5-18 fold as measured between the two separate assays (*Fig 27 and 28.*). The IN CTD fragment spanning amino acids 228-270, possessing both ^{235}WKGPAKLLWKG and ^{262}RRKAK regions, only exhibited an inhibitory affect of 2.5 fold within figure 27, but displayed no inhibitory effect as compared with the control in figure 28. These observations lead us to consider several possibilities as to why this might occur. Upon deletion of both the N and C-terminal regions of the IN CTD, this alteration may have impacted both the folding and expression of the mutant, and slightly changed its functionality. Lutze et al identified regions L241 and L242 within the IN C-terminal, as the amino acids responsible for oligomerization of full length IN, while amino acids 262 and 234 were responsible for DNA binding [29]. Oligomerization and DNA tethering may impact the activity of the IN CTD, in that it still possess the ability to bind cellular Imp7, yet is impaired to carry out all other functions. Another possibility to account for the increased anti-viral activity of the R14-88 INc241-270

fragment, is that given its size, it has increased flexibility and ease of movement around the cellular environment in comparison with its larger R14-88 IN CTD counterparts.

Originally, the IN C-terminal domain was thought to participate solely in non-specific DNA binding, possibly contributing to chromosomal recognition and tethering of the viral cDNA during integration[26, 27, 52]. The role of the IN C-terminal domain during viral replication has since then been further examined, and numerous groups found it to be involved with various steps of viral replication including, as previously mentioned reverse transcription, nuclear import and post nuclear entry steps. In addition, Maertens et at explored the nuclear import potential of the IN CTD alone by fusing the fragment to the GFP, and found the protein to be exclusively localized within the nucleus [143]. Although as examined by Devroe et al, the nuclear accumulation ability of the IN CTD may be the result of it's DNA binding ability. Therefore the true role of the IN CTD domain during viral replication is still unknown. Given the pleiotropic nature of IN, and the fact that viral replication is a highly controlled and timed event, different regions of the IN CTD may be acting at specific times during infection. For example, ^{211}KELQKITK and ^{235}WKGPAKLLWKG may function immediately upon penetration to assist reverse transcription, while the ^{211}KELQKITK and ^{262}RRKAK regions only come into action once the PIC is assembled and on route to the nucleus, and requiring interaction with host karyopherins to assist with nuclear import. This possibility may alter the affect of

different anti-viral IN CTD peptides, as certain replication steps may be more restricted than others, and in the case of nuclear import, may require additional cellular factors to carry out the process. As investigated by Arhel et al, the uncoating process may not fully occur until the PIC has reached the NPC, therefore preventing interaction of the IN CTD with various host factors [120] such as Imp7, until the actual point of nuclear import when the PIC reaches the nucleus.

Another area to explore is the formation of the Imp7/Impβ heterodimer, which may contribute to the functionality of the R14-88 IN CTD fragment, whereby this two component system is facilitating nuclear import. As reported by Jakel et al, the Imp7/Impβ heterodimer mediates nuclear import of histone H1, and may act in a similar fashion to assist with nuclear import of the PIC by interacting with the IN CTD [15, 20]. The possibility also remains, that Imp7 does not bind directly to regions ^{235}WKGPAKLLWKG and ^{262}RRKAK, but rather requires Impβ to act as an adapter protein, as is the case of Impα in the classical import pathway[12]. Imp7 along with the Impα/β heterodimer, work in conjunction to mediate nuclear import of the glucocorticoid receptor [20], providing evidence the nuclear import may be due to the combined efforts off three or more host karyopherins. The overall mechanism employed by HIV-1 to execute nuclear import of the PIC is very complicated and involves the coordination of many host and viral factors, and as of yet, the exact mechanistic pathway responsible for nuclear accumulation of viral cDNA remains undefined. Overall we speculate, that the IN CTD

fragments are competing with wild type IN protein for binding with essential replication factors, and in the event that wt IN is unable to bind, replication is as a result attenuated. In addition, the size as well as folding potential of the IN CTD peptides also will impact their effectiveness, whereby the smaller peptides have the advantage of increased agility within the cell and viral particle to interact with both viral and cellular factors.

In the race to design more potent and specific anti-viral therapies, there are two major disadvantages working against the present therapies 1) Resistant mutations developing towards current anti-viral therapies within individuals undertaking HAART treatment. 2) HIV-1 viral reverse transcription is a highly error prone process, and as a result induces a high number of mutations during cDNA transcription [45, 49]. Consequently, new therapeutic approaches are continually required to maintain effective treatment. The IN CTD peptides that I am proposing, target domains within the IN C-terminal that are required to interact with host proteins to facilitate infection, and therefore must remain conserved. Proteins that mimic the binding domains of IN, lacking the functionality of full-length IN, will disrupt and compete with the functional protein-protein interactions required for replication. This research contributes to the overall body of knowledge by identifying a novel use for the IN CTD and its interaction with Imp7. So far no one else has sought to exploit the binding properties of host karyopherins for the purpose of designing new anti-virals. Considering host karyopherins act as key intermediates during viral replication, this approach will

provide a new means for identifying compounds that interact with highly conserved domains of viral proteins. By defining and understanding the effect of the IN/Imp7 binding domain on HIV-1 replication, we can design peptides to block this interaction, and further impair viral replication.

Chapter 5

5.0 Future Directions:

Immediately, the main areas that should be focused on in future work, are that which define the step(s) of viral replication affected by the Vpr-fusion protein within the viral particle. As was discussed previously, the IN CTD has the ability to influence various steps of viral replication such as reverse transcription, nuclear import and non-specific DNA binding. Given the complexity and efficiency of HIV-1 viral infection, it is likely that numerous replication activities are governed by the Imp7 binding domain, and that expression of the IN CTD may impact these steps at differing times during infection. It is also worth exploring the potential of other host karyopherins binding to the C-terminal domain such as Impβ, that possibly influence the binding of Imp7 to IN.

Once that has been accomplished, the inhibitory properties of the Vpr-fusion protein should be further examined to determine whether expression of the IN CTD is more potent when expressed within the cells, within the viral particle or both prior to infection. This will then provide insight into the best method of delivery, and possibly functionality of this protein. Addressing the question of peptide delivery is an interesting one, as it will allow for this research to be practically utilized and potentially explored from a clinical standpoint. By incorporating the INc241-270 fragment within a lentiviral vector, this peptide could be specifically delivered to infected cells, whereby the fragment would be incorporated into the host's genome and constitutively expressed, down regulating

viral infection. Hence once viral attenuation was established it would be important to understand the mechanisms underlying this action.

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