Inhibitory Control of HIV-1 Infection by Cyclophilin A

By

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To Dad, Mom, Monet, Mommagene, and Grandmother, thank you for always believing in me, even and especially when I didn't believe in myself. To my husband, Avante thank you for every single minute you spent with me in lab, for picking me up when I stumbled, and for celebrating every victory, large and small.

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LIST OF ABBREVIATIONS

ADP	Adenosine Diphosphate
AIDS	Acquired immune deficiency syndrome
ATP	Adenosine Triphosphate
BBS	BES-buffered saline
BLAST	Basic Local Alignment Search Tool
BME	2-Mercaptoethanol
BSA	Bovine serum albumin
CA	Capsid protein, p24
CCR5	CC chemokine receptor 5
CD4+	Protein marker on surface of HIV-1 target cells
CDC	Centers for Disease Control
CPSF6	Cleavage Polyadenylation Specificity Factor 6
CsA	Cyclosporin A
CTD	Carboxy-terminal domain
CypA	Cyclophilin A
CXCR4	CX chemokine receptor 4
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
dNTP	Deoxynucleotide
DNA	Deoxyribonucleic acid

ELISA	Enzyme-linked immunosorbent assay EM
Env	Envelope glycoproteins
Gag	Group specific antigen
GFP	Green fluorescent protein
gp120	Envelope glycoprotein gp120;
HIV	Human immunodeficiency virus type 1
IN	Integrase
kb	Kilobases
LTR	Long terminal repeat
MA	Matrix protein, p17
MLV	Murine leukemia virus
MS	Mass Spectrometry
MuDPIT	Multidimensional Protein Identification Technology
MxB	Myxovirus resistance protein B
NC	Nucleocapsid protein, p7
Nef	Negative factor
Nup	Nucleoporin
nm	nanometer
ng	nanogram
NTD	Amino-terminal domain
OMK	Owl monkey kidney cells

PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PIC	Pre-integration complex
Pol	Polymerase gene
PR	Viral protease
Ref1	Restriction factor 1
REV	Regulator of virion protein expression
RNA	Ribonucleic acid
RNASeq	RNA Sequencing
RSV	Rous sarcoma virus
RT	Reverse transcriptase
RTC	Reverse transcription complex
qPCR	Quantitative PCR
SDS	Sodium dodecyl sulfate
shRNA	short hairpin RNA
Tat	HIV transactivator of transcription
TEM	Transmission electron microscopy TRIM Tripartite motif
TNPO3	Transportin 3
TRIM5α	Tripartite motif protein 5 isoform alpha
TRIMCyp	Tripartite motif fused with cyclophilin A
VCP	Valosin Containing Protein

VSV	Vesicular stomatitis virus
VSV-G	VSV glycoprotein

CHAPTER I

BACKGROUND AND RESEARCH OBJECTIVES

Viruses are obligate intracellular parasites whose existence depends on their ability to acquire molecular resources from the host. Some viruses obtain these resources through a commensal relationship with the host, but many viruses, especially those important in human disease, cause significant harm. In turn, hosts have evolved strategies to block viral replication leading to evolutionary arms races between viruses and hosts for consumption of cellular resources. I have investigated the ability of mammalian cells to inhibit HIV-1 infection by exploiting the interaction of the Human Immunodeficiency Virus type 1 (HIV-1) capsid protein with the host protein cyclophilin A.

HIV/AIDS

Human Immunodeficiency Virus (HIV) is the etiologic agent of Acquired Immune Deficiency Syndrome (AIDS)[1–4]. AIDS is characterized by progressive loss of immune function, susceptibility to opportunistic infections, and eventually death. More than 34 million people have succumbed to HIV/AIDS since the Centers for Disease Control first recognized and started tracking the disease in 1981[5]. Today, an estimated 37 million people are living with the HIV/AIDS globally, with roughly 2 million new cases per year. While there are effective antiviral treatments, and more recently prophylactic options, there is no cure for HIV/AIDS and the disease remains a serious worldwide healthcare burden [5].

HIV-1 is transmitted by sexual, perinatal, and percutaneous routes [6]. During sexual-mucosal transmission, the virus enters through small tears in the squamous epithelium where it encounters dendritic cells and is phagocytosed before migration to peripheral lymph nodes.[7]. In the lymph nodes, dendritic cells encounter and transmit HIV-1 to CD4+ T-cells, the primary target of infection. During the acute phase of infection, high levels of HIV genomic material and detectable levels anti-HIV-1 antibodies are present in the plasma, although no HIV-1 proteins can be detected. The infected individual may experience mild to severe flu-like symptoms including fever, sore throat and myalgia [7]. Infection then enters a period of clinical latency that can last from 1-20 years. During this time virus levels in the blood reach a “set-point” that is maintained throughout clinical latency. This set-point can vary between individuals and is linked to disease prognosis [8,9]. Throughout the chronic phase of infection, HIV-1 replicates in the CD4+ T-cell population, progressively killing cells by multiple mechanisms [10]. Ultimately, levels of CD4+ T-cells fall below 200 cells/ml in the plasma, marking the onset of AIDS and susceptibility to opportunistic infections and cancers (Figure 1-1). Without therapy, infected individuals will succumb to these diseases [7].

Anti-retroviral and Prophylactic Therapy

The first HIV/AIDS therapeutic strategies focused on treatment and prevention of opportunistic infections. In the early 1990s, the first therapies directly targeting HIV-1 were introduced [11]. These drugs target reverse transcription of the viral genome via chain-termination of nascent HIV-1 DNA strands. Although these

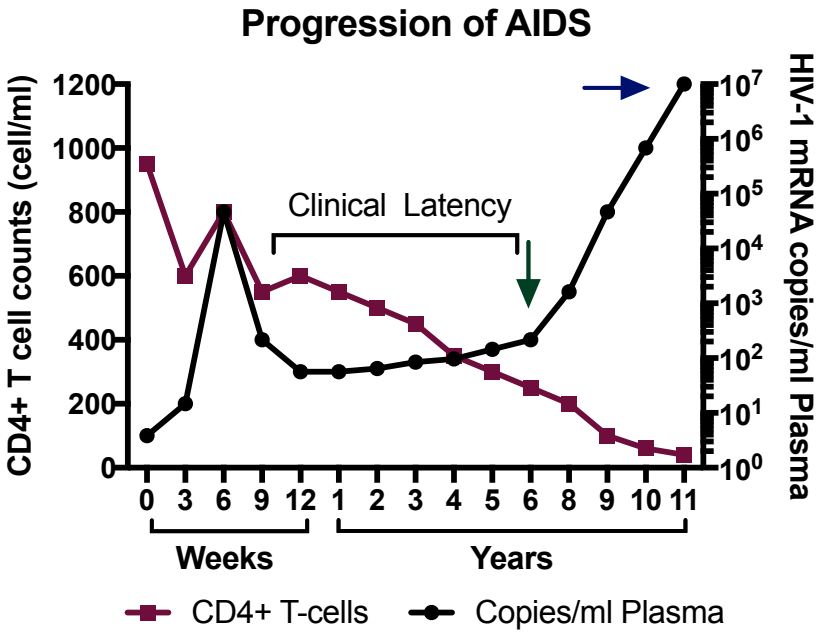

Figure 1-1: Clinical Progression of HIV-1/AIDS. In the acute phase of infection, shortly after exposure, levels of CD4+ T-cells are depleted while viral mRNA levels spike. As infection progresses to clinical latency, virus levels stabilize and CD4+ T-cells are progressively lost. Ultimately the levels of CD4+ T-cells fall below 200 cells/ml in the plasma (green arrow) increasing the likelihood of opportunistic infections and death (blue arrow). Adapted from Fauci *et al. Ann. Int. Med*. (1996) [12]

therapies provided some relief to patients, their effectiveness of was limited by high rates of drug resistance. A breakthrough in AIDS treatment came in the mid-1990s with the introduction of new classes of drugs targeting two key HIV-1 enzymes, reverse transcriptase and protease. These drugs were given in combination using a therapeutic protocol termed highly active antiretroviral therapy (HAART) [13–15]. This protocol combines three or more HIV/AIDS drugs, each inhibiting a unique viral target, dramatically reducing the emergence of drug resistant clones and viral load. Current treatment strategies have extended the combination protocol as prophylaxis for the groups most vulnerable to HIV/AIDS. This strategy, known as PreP or Pre-exposure prophylaxis, has been shown to reduce risk of infection up to 92% [16–18].

HIV-1 Structure and Replication

HIV-1 is a member of the Lentiviridae genus of the *Retroviridae* family of viruses[19]. Retroviruses are viruses that use reverse transcriptase enzyme to transcribe their single stranded positive sense genomic RNA into DNA before it is integrated into the genome of a host cell [19]. Lentiviruses are unique among retroviruses in the complexity of their genomes and in their ability to infect non-dividing cells. As with all retroviruses, the genome of HIV-1 contains canonical *gag*, *pol,* and *env* genes, but also encodes six additional genes *vif, vpr, vpu, tat, rev* and *nef* [19,20]. Transcription of the integrated HIV-1 DNA results in three classes of transcripts, which are singly or multiply spliced to produce the assortment of HIV-1 proteins. The first class, the unspliced full-length 9 kilobase (kb) transcript is used to produce the gag and gag-pol polyproteins [21]. The Gag

polyprotein is processed into structural proteins matrix (MA), capsid (CA), nucleocapsid (NC), and p6. The Gag-Pol polyprotein is processed into reverse transcriptase (RT), protease (PR) and integrase (IN) enzymes. The second class of transcript is singly spliced and results in the Vpr, Vif, Vpu, and envelope (Env) proteins [22,23]. Like *gag* and *gag-pol*, the Env protein is also transcribed as polyprotein, however it is processed by the host protein furin into gp41 and gp120. The third class of transcripts is doubly spliced and results in the regulatory proteins Tat, Rev, and Nef [21].

The HIV-1 particle is an enveloped particle containing a ribonucleoprotein complex housed within an icosahedral capsid [24]. The lipid bilayer is studded with trimers of the HIV-1 Env protein, a heterodimer of env proteins gp41 and gp120 [25,26]. Within the lipid bilayer, the matrix protein (MA) spherical scaffold. Within the MA scaffold, approximately 1500 molecules of capsid form a fullerene cone, enclosing two copies of the RNA genome coated with nucleocapsid protein. The enzymatic and accessory proteins, RT, IN, Vpr, Nef, Vif, and PR are also contained within the capsid along with several cellular proteins including cyclophilin A, tRNA, and APOBEC3G [27–30] (Figure 1-2). Together the capsid, genome, and the contained accessory proteins comprise a subviral complex known as the HIV-1 core [25].

HIV-1 Life Cycle

HIV-1 infection begins with the virus attaching to target cells via non-specific interactions of Env protein with heparan sulfate proteoglycans and integrins on the surface of the target cell[31]. Attachment brings the virus in close proximity with

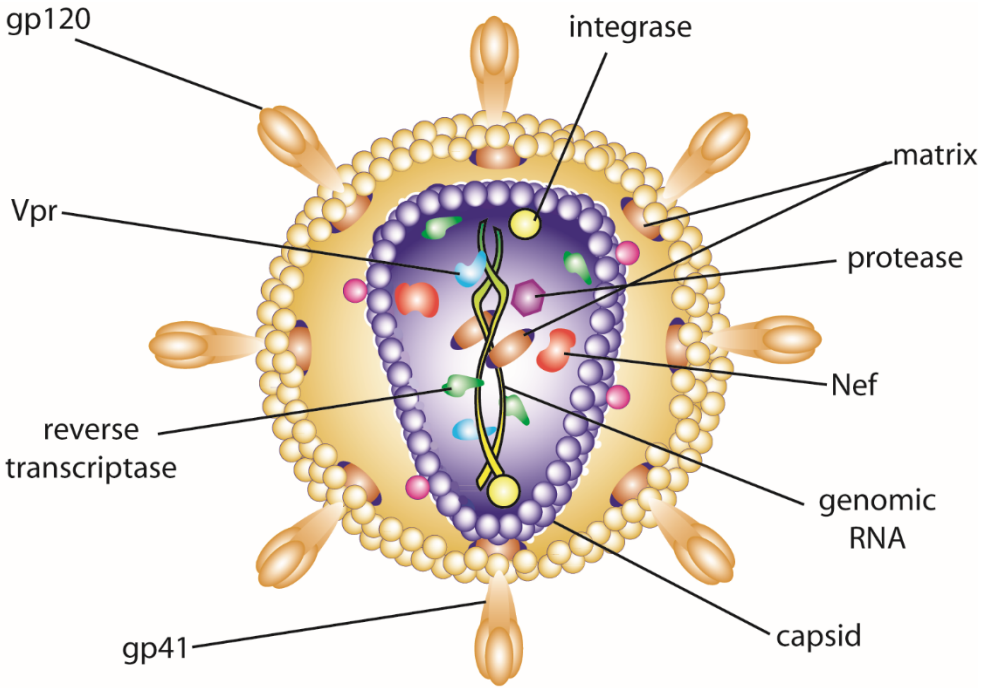

Figure 1-2: Structure of Mature HIV-1 Virion. The viral lipid membrane is anchored by the matrix protein and studded with g41/gp120 trimers. Enclosed within is the the mature HIV-1 conical core containing two copies of the RNA genome, reverse transcriptase, integrase, nef and cellular proteins.

the cellular receptors, allowing gp120 to bind the CD4 receptor and subsequently the CCR5 or CXCR4 co-receptors[32,33]. Co-receptor binding triggers exposure of the hydrophobic gp41 fusion peptide, which is inserted into the plasma membrane of the cell and triggers fusion of the viral membrane with the cellular membrane [31]. The viral core is then delivered into the cytoplasm of the cell where it interacts with the actin and microtubule networks for active transport to the nucleus [34–37]. As the core moves through the cytoplasm, the capsid undergoes a disassembly process known as uncoating, in which molecules of the CA protein are lost from the core [38–40]. Reverse transcriptase and nucelocapsid proteins direct synthesis of viral DNA from the RNA genome concurrently with uncoating. During this time the particle is known as a reverse transcription complex (RTC) [41]. Upon completion of reverse transcription and some degree of uncoating, the particle becomes known as the preintegration complex (PIC) (Fig 1-3). The PIC then enters the nucleus by interacting with components of the nuclear pore. Once inside the nucleus, HIV-1 integrase, in conjunction with cellular DNA repair proteins, direct integration of HIV-1 DNA into the host genome [42,43]. Tat and Rev are the first HIV-1 transcripts produced from the integrated DNA; they support transcription and nuclear export of HIV-1 transcripts, respectively [21]. During progeny virion assembly, the Env protein is glycosylated and proteolytically processed into gp41 and gp120 before transport to the plasma membrane where Gag directs clustering of Env within the plasma membrane. The Gag protein is targeted to specialized microdomains of the plasma membrane for assembly by myristolation of its MA subunit. The Gag-pol polyprotein is targeted the plasma membrane via CA-CA interactions between Gag and Gag-Pol. Interactions

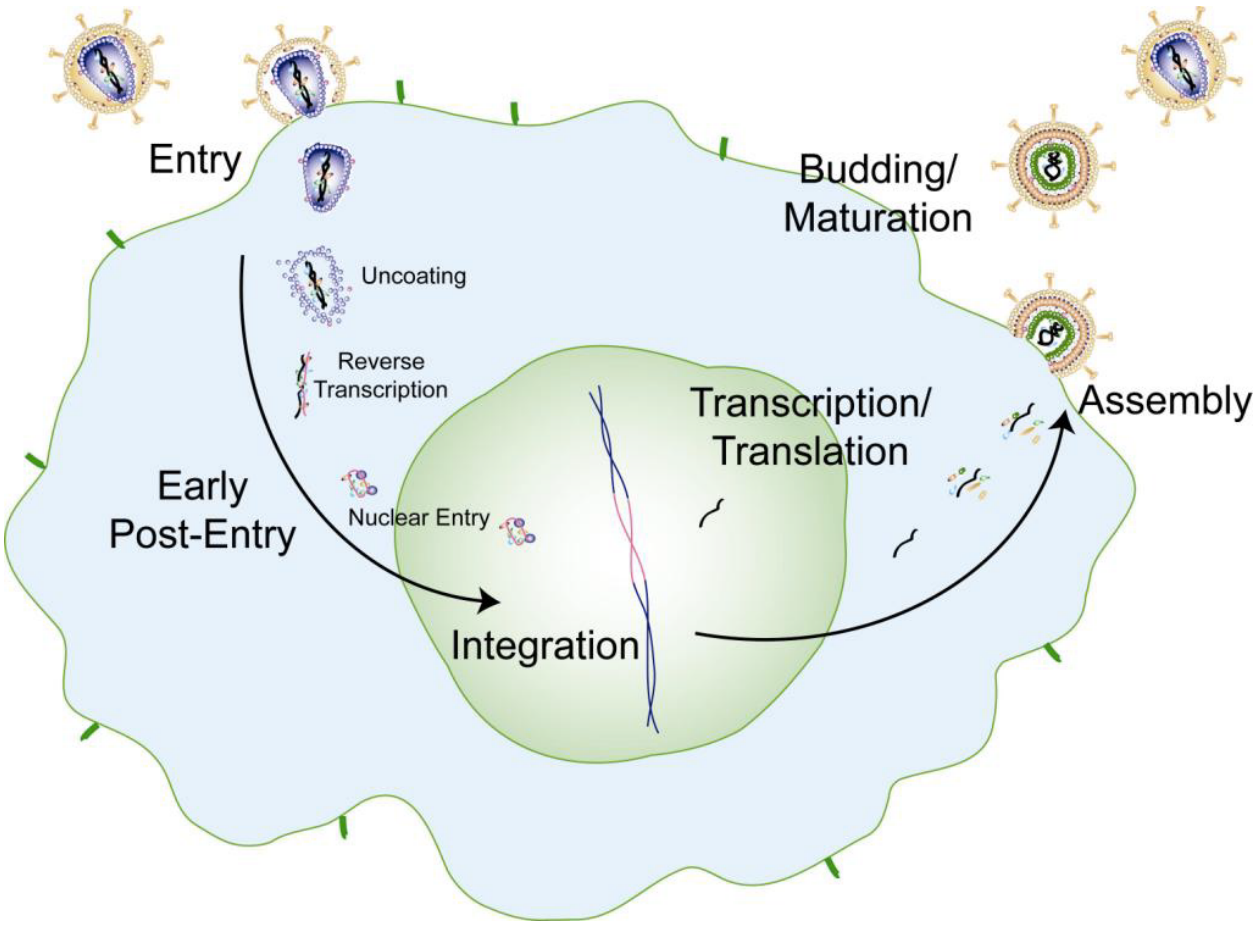

Figure 1-3: HIV-1 Life Cycle. The incoming particle attaches to and fuses with the plasma membrane of the target cell. The core enters cytoplasm and undergoes uncoating together with reverse transcription. The resulting preintegration complex is imported into the nucleus where HIV-1 integrase and cellular enzymes direct integration of HIV-1 DNA into the host chromosome. HIV-1 Tat and Rev are transcribed and translated first and promote transcription, synthesis, and export of HIV-1 mRNAs and genome from the nucleus. Newly translated viral proteins and polyproteins aggregate at assembly sites near at the plasma membrane and bud from the producer cell to generate immature progeny virions. The viral protease cleaves gag resulting in maturation of the HIV-1 core.

between the NC subunit of Gag and the full-length RNA transcript mediate incorporation of the genome into the nascent particle. The p6 region of Gag promotes incorporation of Vpr and Nef [25]. The incorporation of Nef promotes infectivity of HIV-1 by preventing incorporation of Serine Incorporator 5 (SERINC5) into the particle which can inhibit subsequent fusion of the particle [30,44]. Once a critical concentration of Gag protein has been reached at the assembly domain, the proteins rearrange to form a spherical structure and bud out from the cellular membrane [25]. PR is activated during budding and cleaves the Gag and Gag-pol polyproteins into their respective subunits in a process known as maturation, resulting in the conical capsid and encapsidation of the genome.

Structure of the HIV-1 Capsid

HIV-1 CA is made up of two domains, a 150 amino-acid-long amino terminal domain (NTD) and an 80 amino-acid-long carboxy-terminal domain (CTD) connected by a flexible linker[45–47]. Interactions between the NTD and CTD facilitate monomer assembly into hexamers and pentamers which serve as building blocks for the mature form of the capsid [26,47–49]. About half of HIV-1 CA's 231 amino acids lie within 11 structural helices and form the basis for the tertiary structure of CA. Considering HIV-1's low fidelity reverse transcriptase, these residues are remarkably intolerant of mutation. More than 70% of randomly introduced mutations result in non-viable particles; phenotypes which map to defects in particle assembly and maturation [50]. The early post-entry steps of infection are also highly dependent on the structural integrity of the capsid. Our lab has demonstrated that capsid stability, or the rate of uncoating, is dictated by the

sequence of CA. Mutations in CA, which make the virus hyperstable or unstable relative to the wildtype virus, often result in severe infectivity defects [51]. We and others have observed reduced efficiency of reverse transcription in cells infected with hyperstable or unstable capsid mutants [39,40,52]. Similarly, biochemical or genetic inhibition of reverse transcription, delays uncoating and increases capsid stability. Together these data highlight the importance of capsid structure to the early and late stages of infection.

HIV-1 Capsid as a Mediator of Virus-Host Interactions

In addition to its structural contribution, the HIV-1 capsid plays an increasingly appreciated role as a mediator of virus-host interactions. Study of these interactions has become an active area of research, both as a means of identifying novel therapeutic targets and to understand basic HIV-1 biology [53,54]. Nearly every step of early infection is determined in part by capsid-dependent host interactions. Uncoating, is determined in part by microtubule motor complexes dynein and kinesin-1. Dynein and kinesin-1 traffic cellular cargos throughout the cell using the microtubule network. Indirect interactions with capsid via adaptor proteins allow motor complexes to bind the viral capsid and promote disassembly by exerting opposing forces on the capsid [55–57]. Capsid-dependent interactions with dynein also support viral trafficking toward nucleus [35,58].

The later steps of early replication, nuclear import and integration, are also impacted by interactions between capsid-dependent host protein interactions [59]. In order to gain access to the host chromosome, HIV-1 enters the nucleus via the nuclear pore complex (NPC). The NPC is a large multiprotein channel comprised

of about 30 proteins that connect the cytoplasm to the nucleoplasm. Relatively small proteins passively diffuse through the NPC, however larger proteins or protein complexes like the HIV-1 PIC require active transport through the NPC [42]. Nucleoporins Nup153 and Nup358 support active translocation by binding to the capsid and promoting its entry into the nucleus [60,61]. Nup153 utilizes the common nucleoporin phenylalanine-glycine (FG) motif to specifically interact with HIV-1 CA, while Nup358 may rely on its cyclophilin A homology domain to bind to CA [60,62–64]. Following translocation of the HIV-1 particle into the nucleus, the HIV-1 genome is integrated into transcriptionally active regions of the host chromatin [65]. In addition to regulating nuclear trafficking, some nucleoporins regulate the structure of chromatin, including localizing transcriptionally active chromatin regions to the nuclear pore [66]. Accordingly, capsid-dependent interactions with Nup153 and Nup358 have also been shown to determine sites of viral integration [62].

Two additional proteins, cleavage and polyadenylation specificity factor 6 (CPSF6) and transportin 3 (TNPO3) bind to the capsid and promote infection during or after nuclear import. The the β-karyopherin TNPO3 has been implicated in a number of activities including accelerating uncoating *in vitro* and promoting nuclear import and integration [67–70]. However, the ability of TNPO3 to promote nuclear localization of CPSF6 appears to be most central to its effects on HIV-1 replication [70,71]. CPSF6, is a member of a complex of mRNA splicing factors that shuttles between the nucleus and the cytoplasm [72]. In the presence of TNPO3 is its primarily located in the nucleus. CPSF6 possesses multiple capacities to regulate HIV-1 infection depending on the target cell type and strain

of HIV-1. For lab adapted wildtype strains of HIV-1, CPSF6 promotes integration site selection by an unknown mechanism [73]. Depletion studies have revealed that CPSF6 is essentially dispensable for infectivity of HIV-1 in culture, yet primary isolates and lab adapted strains of the virus retain CPSF6 binding under selective pressure in culture and in primary T-cells suggesting that CPSF6 performs an important function for the virus [74,75].

In an interesting twist, HIV-1 has also evolved a capsid-dependent stealth mechanism to escape innate immune recognition in macrophages[76]. Wild type HIV-1 replicates normally in macrophages despite its generation of immunogenic double-stranded DNA. HIV-1 achieves this escape through a capsid-dependent interaction with CPSF6. Mutant capsids which lack the ability to bind to CPSF6, trip cellular DNA sensors and trigger the innate immune response, preventing replication in macrophages. This activity has only been observed in macrophages, but may explain why the virus chooses to maintain interaction with CPSF6 under conditions that might typically inhibit its replication [76].

In contrast, a small but growing number of cellular proteins have been demonstrated to bind to the capsid and inhibit HIV-1 infection. Tripartite motif protein five alpha (TRIM5α) was one of the first capsid-binding restriction factors discovered to block infection [77]. TRIM5α binds to the capsid lattice via its SPRY domain and inhibits reverse transcription and nuclear import of HIV-1 [78,79]. TRIM5α can also synthesize ubiquitin chains upon recognition of CA, activating NF-κB genes and inducing an antiviral state within T-cells to indirectly control infection [80,81]. TRIMCyp, another capsid-binding restriction factor uses a similar

mechanism to restrict HIV-1 although it relies on a CypA domain for capsid binding [82,83]. Most recently, the interferon stimulated gene, myxovirus resistance protein B (MxB) was shown to bind to capsid and inhibit HIV-1 infection at steps during and after nuclear import [84–88]. Humans encode two myxovirus resistance genes MxA and MxB, while MxA has been show to inhibit infection of a number of viruses including influenza A, MxB selectively inhibits HIV-1 replication [89].

Cyclophilin A and HIV-1

Despite their disparate functions, the capsid-binding proteins described above share one unifying feature; their ability to regulate infection of HIV-1 is determined in part by the host protein cyclophilin A (CypA) [62,67,74,76,90,91]. CypA was one of the first host proteins discovered to interact with HIV-1 CA and to regulate its infection [28,92,93]. Cyclophilins are a ubiquitous, highly-conserved family of proteins present in both prokaryotic and eukaryotic cells. All cyclophilins have a conserved cyclophilin-like domain (CLD) comprised of 109 amino acids with peptidyl-prolyl isomerase activity (PPiase) [94]. PPIases, catalyze *cis* to *trans* isomerization of proline bonds [95]. There are 16 structurally distinct human cyclophilins, which are expressed in a variety of cellular compartments and tissues. CypA is the most abundant cyclophilin, and one of the most abundant proteins in the cell representing 0.1-0.6% of the total cytosolic protein [96]. CypA depletion is well tolerated by many cell types, limiting our ability to assign its primary biological function, and suggesting functional redundancy [97]. In yeast cells, CypA is enriched in the nucleus and helps regulate meiosis [98]. In humans, misregulation of CypA promotes pathology in variety of inflammatory diseases including cardiovascular disease, diabetes, artherosclerosis, Alzeheimer's disease,

and rheumatoid arthritis[99]. At the cellular level, human CypA has been implicated in protein folding and trafficking, and in intracellular and extracellular signaling [97]. In addition to HIV-1, CypA promotes the activity of a number of viruses, including Hepatitis C Virus, Hepatitis B Virus, and Vesicular Stomatitis Virus (VSV). In contrast, CypA inhibits the replication of Influenza A and Rotavirus [100,101].

CypA binds the amino-terminal domain of HIV-1 CA, via an unstructured loop between helices 4 and 5 known as the CypA-binding loop. The glycine at position 89 and the proline at position 90 are required for CypA binding [102]. CypA binding can be inhibited by mutating these residues or by treatment with a number of biochemical inhibitors, most notably, cyclosporine A (CsA) [103,104]. A second site for CypA binding within HIV-1 CA was recently discovered. This site spans two molecules of CA, bridging two CA hexamers, and potentially stabilizing the hexamers [105]. HIV-1 CA binds to the active site of CypA, and is subject to *cis-trans* isomerization of the Pro90 bond [106]. The relevance of isomerization to HIV-1 replication remains unknown due to the lack of enzymatically dead CypA mutants which retain the ability to bind HIV-1 CA.

In addition to isomerizing the Pro90 bond, binding of CypA also alters the dynamics of the capsid. Both the assembled capsid, and the CypA binding loop are highly dynamic structures. CypA binding limits the movement of both the CypA binding loop and distal residues, resulting in an overall reduction in the movement of the assembled capsid [107]. These changes in dynamics are phenocopied by capsid mutants whose replication is highly sensitive to the presence of CypA, suggesting that changes in structure underlie the ability of CypA to regulate infection.

Binding of CypA to HIV-1 CA typically leads to higher levels of infection. This effect has been attributed to increased efficiency of reverse transcription, and modification of uncoating [67,103,108,109]. Interestingly, CypA is not required for replication by all HIV-1 strains. Main group viruses (M-group, comprising the vast majority of circulating strains), require CypA for full infectivity, while outlier group (O-group) isolates tend to be independent of CypA for replication [110]. The requirement for CypA among M-group viruses varies according to cell type. Some cells such as Jurkat and 293T cells impose a strong requirement for CypA, while others, such as HeLa cells do not [111]. These data suggested that CypA may also affect extrinsic properties of the virus, such as virus-host interactions, in addition to intrinsic properties like capsid stability and efficiency of reverse transcription. Consistent with this hypothesis, several studies have demonstrated that CypA promotes the dependence on NPC associated proteins that support nuclear steps of infection [62,63,67,74].

Somewhat paradoxically, CypA can also inhibit HIV-1 infection depending on the cell type and strain of virus. In primate cells, binding of CypA promotes restriction of wildtype HIV-1 by TRIM5α, such that blocking CypA binding partially rescues HIV-1 replication in these cells [91,112,113]. Several reports have confirmed this observation, and further shown that CypA acts early to enhance TRIM5α restriction. In human cells, CypA inhibits some O-group viruses, and a specific class of HIV-1 capsid mutants, known as cyclosporine resistant/dependent mutants (CsA R/D) [109,114]. These mutants are resistant to cyclosporine (CsA) treatment in cells in which CsA inhibits the wildtype virus, but they also require for cyclosporine for full infectivity.

Research Objectives

While extensive studies have characterized the interaction of CypA with HIV-1 CA, and revealed multiple mechanisms by which CypA promotes infectivity of HIV-1 little is known about the mechanisms underlying the ability CypA to inhibit HIV-1. At the start of this work, two examples of CypA-dependent inhibition had been reported, restriction of HIV-1 by TRIM5α, and the inhibition of CsA R/D mutants. In this work, I sought to identify novel and shared mechanisms of CypA-dependent inhibition of HIV-1 by achieving two aims; 1) identifying the mechanism by which CypA promotes TRIM5 restriction of HIV-1 and 2) identifying putative capsid-binding restriction factor responsible for restriction of CsA R/D mutants. In this dissertation, I describe a method for capturing and identifying CA binding proteins, and demonstrate in collaboration with the Yamashita Lab at Aaron Diamond AIDS center, that CPSF6 is the host protein responsible for CypA-dependent restriction of CsA R/D mutants. I also provide evidence that CypA potentiates TRIM5 restriction of HIV-1 namely by inhibiting nuclear import of HIV-1, suggesting that a shared mechanism underlies the ability of CypA to promote infection in some cells and inhibt infection in others. Unexpectedly, I find that although CypA has the ability to alter the structure of capsid, it does not promote binding of CPSF6 or TRIM5α to the capsid. I propose that the ability of CypA to promote engagement of specific nuclear import proteins promotes sensitivity to capsid-binding restriction factors. These results, are the first descriptions of the mechanisms of CypA-dependent restriction of HIV-1.

CHAPTER II

CPSF6 IS REQUIRED FOR CYPA-DEPENDENT RESTRICTION OF CYCLOSPORINE RESISTANT/DEPENDENT MUTANTS

Introduction

The discovery of cyclosporine resistant/dependent mutants arose from the desire to use cyclosporine (CsA) to treat HIV-1 infection[114]. It was widely known that the immunosuppressive fungal metabolite CsA blocked replication of HIV-1, but the mechanism of action was unclear. Early studies linked antiviral activity to the ability of CsA to prevent activation of T-cells, a necessary step in HIV-1 infection. By forming a complex with CypA, CsA sequesters the serine/threonine phosphatase calcineurin in the cytoplasm, preventing cytokine transcription and cellular activation. The discovery of non-immunosuppressive analogs of cyclosporine that retain the ability to block infection later discredited this hypothesis. Instead, the anti-HIV-1 activity of CsA and its analogs was found to correlate with their ability to bind CypA. In an independent line of work, the interaction between HIV-1 CA and CypA was discovered and characterized. Together these observations led to a model in which CsA inhibits HIV-1 by blocking its interaction with CypA.

To explore the potential of CsA and its analogues as antiviral therapy for HIV/AIDS, studies were completed to examine ability of HIV-1 to escape CsA treatment [114]. From these studies, a novel class of CA mutants were discovered which were not only resistant to CsA, but also dependent on the presence of the drug for high levels of infection (known as CsA resistant and dependent, CsA R/D) [114]. Many of these

mutations are located in or near the CypA-binding loop, including A92E, G94D, and T54A. Ablation of the CypA-CA interaction by mutating the viral capsid, or by RNAi-mediated depletion of CypA also permitted normal replication of CsA R/D mutants indicating that CypA inhibits infection of these mutants. Subsequent studies revealed that CypA-dependent inhibition is cell type specific, occurring in HeLa and CEM cells, but not 293T or Jurkat cells[109,114–116]. Our lab demonstrated that the cell-type inhibition is dominant, such that heterokaryons of restrictive cells, which demonstrate the CsA R/D phenotype and permissive cells which do not retain a restrictive phenotype suggesting that a dominantly acting factor prevents infection of CsA R/D mutants. We also observed inhibition of the CsA R/D mutants in non-dividing cells, where wildtype HIV-1 replicates normally. Lastly, our lab identified a suppressor mutation for the CsA R/D phenotype, a substitution of threonine for the alanine at position 105 in HIV-1 CA (A105T) restores the ability of CsA R/D mutants to replicate in restrictive cells. As outlined in table 1 (and reviewed in [117]), this set of observations correlates closely with the features of known HIV-1 restriction factors. The CsA R/D phenotype is evaded by the wildtype virus, dominantly acting, cell-type specific, and counteracted by specific mutations in the capsid[118]. Therefore, I hypothesized that a cellular restriction factor was responsible for inhibition of the CsA R/D mutants. Specifically, I hypothesized that the presence of CsA R/D mutations together with CypA, promote binding of a restriction factor that blocks infection by CsA R/D mutants. To identify this putative restriction factor, we performed a mass spectrometry screen using recombinant HIV-1 CA tubes as bait.

Table 2-1. Shared Features of HIV-1 Restriction Factors

Known Restriction Factors	CsA R/D Phenotype
Wildtype virus evades restriction	Yes
Dominantly acting	Yes
Germline encoded	??
Counteracted by a viral protein	??/ Specific CA escape mutants
Species specific control of infection	Cell-type specific
Hallmarks of positive selection	??

*Adapted from Malim and Bienasz, *Cold Spring Harb Perspect Med*. 2012 May;2(5) [119]

Results

CsA R/D Phenotype is Not Saturable

Due to the finite quantities of protein in cells, inhibition of HIV-1 by host restriction factors can typically be saturated by high viral inoculum. To assess the saturability of the CsA R/D phenotype, I assayed the ability of a panel sensitive and resistant viruses to abrogate restriction of the CsA R/D mutant, A92E. To first confirm the CsA R/D phenotype, I inoculated HeLa cells with WT, A92E, A92E/P90A and A92E/A105T HIV-1 GFP reporter viruses. A92E/P90A and A92E/A105T viruses were included as negative controls, as both secondary mutations provide escape from the CsA R/D phenotype. As previously reported, infectivity of A92E was significantly enhanced by treatment with CsA or by the presence of the non-CypA binding mutant, P90A. In contrast, infection of WT, A92E-P90A or A92E-A105T viruses was increased by less than 2-fold by CsA treatment (Fig 2-1). Inclusion of the A105T mutation increased infection of A92E to levels comparable with WT HIV-1. Together these observations confirm the CsA R/D dependent phenotype of A92E in HeLa cells. Having confirmed the CsA R/D phenotype of A92E, I next assessed the saturability of CsA R/D inhibition. HeLa cells were inoculated with a fixed, sub-saturating dose of A92E GFP reporter virus (A92E-GFP), and increasing quantities of unlabeled A92E, A92E/P90A, or A92E/A105T decoy particles (Figure 2-2A). As a negative control, an identical assay was completed using wildtype GFP reporter virus (WT-GFP) (Figure 2-2A). Wildtype HIV-1 is not subject to the CsA R/D phenotype and therefore should not be enhanced by the addition of decoy particles. To confirm the infectivity of decoy particles, the luciferase reporter cell-line, TZM-bl was infected with the increasing quantities of decoy particles (Figure 2-2B). This

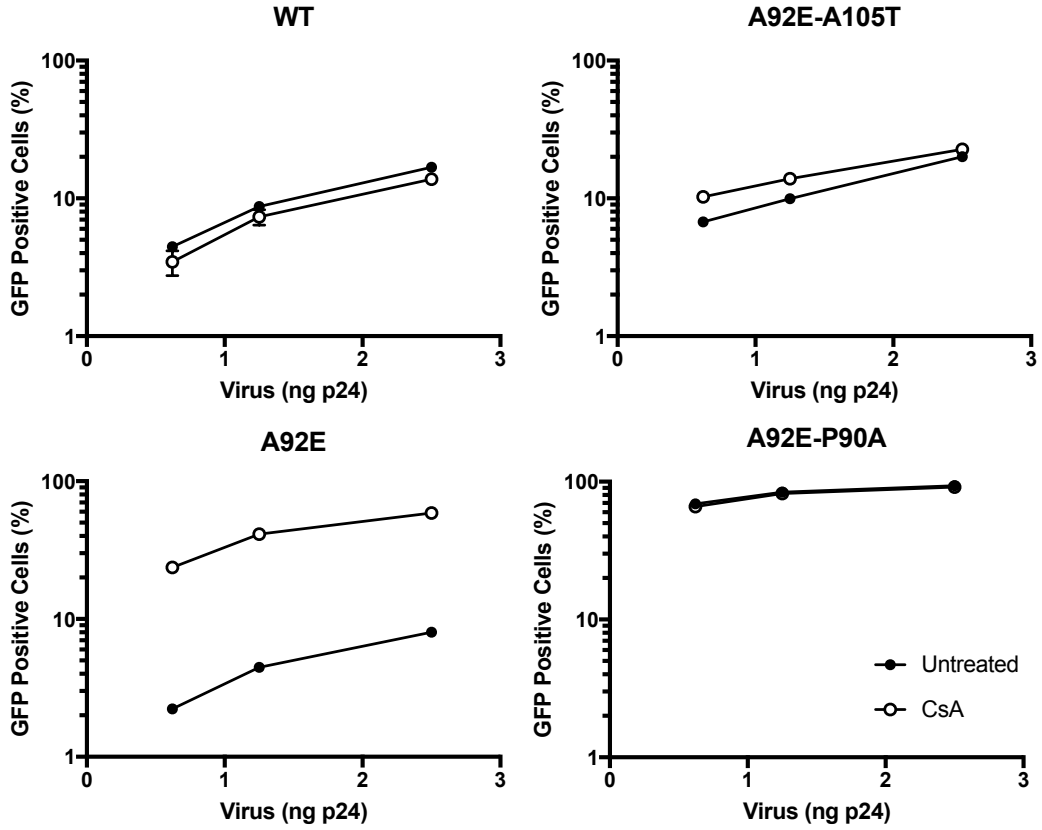

Figure 2-1: CsA R/D mutant A92E is enhanced by inhibiting CypA-CA interactions. HeLa cells were inoculated with indicated quantities of wildtype or mutant viruses in the presence (open circles) or absence (closed circles) of CsA, extent of infection was determined by flow cytometry 48hrs after infection. Results shown are the mean values from 2 independent experiments; error bars reflect the standard error of the mean.

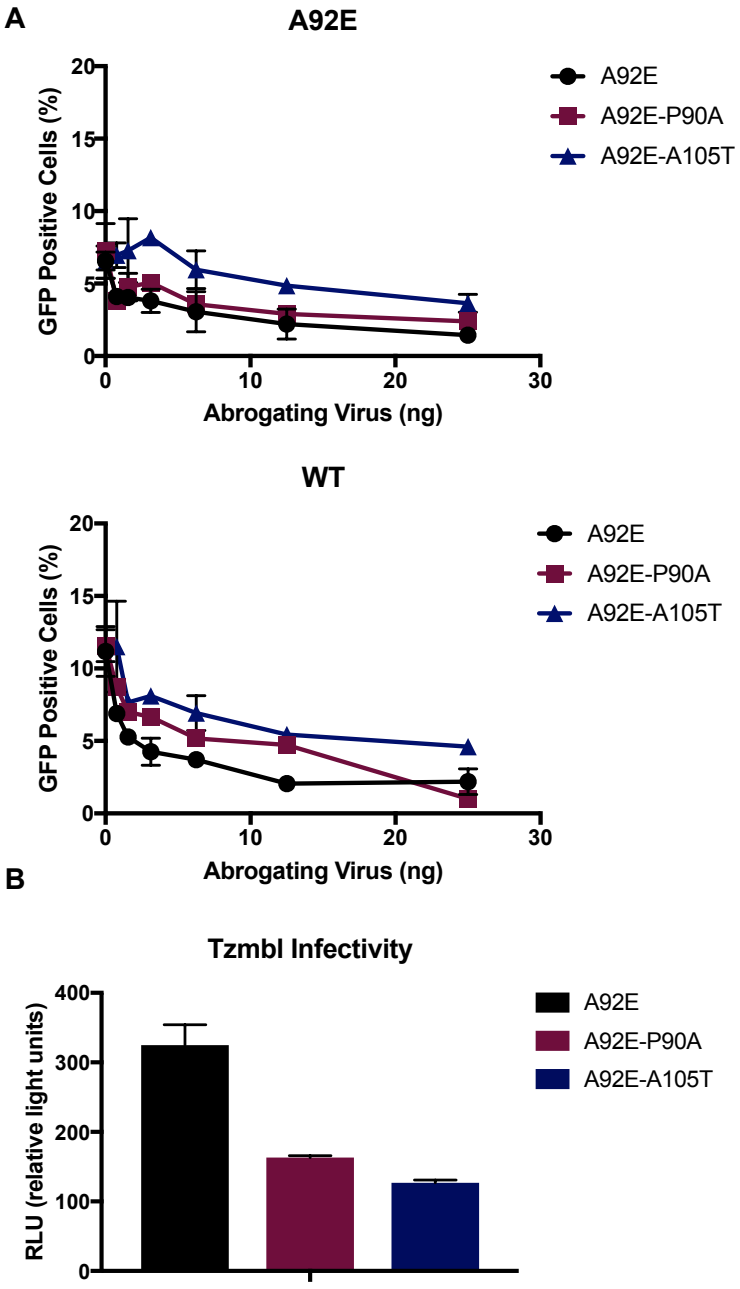

Figure 2-2: CsA R/D Phenotype is not saturable in HeLa cells. **(A)** HeLa cells were inoculated with a fixed dose of A92E-GFP or wildtype-GFP reporter virus and titrating quantities of A92E, A92E-P90A, or A92E-A105T decoy particles. Extent of infection was quantified by flow cytometry 48hrs after infection. **(B)** TZM-bl cells were infected with A92E, A92E-P90A, or A92E-A105T decoy particles, extent of infection was quantified luminometer detection of luciferase activity. Results shown are the mean values from 2 independent experiments; error bars reflect the standard error of the mean.

cell line encodes a luciferase reporter gene that is activated by the expression of the HIV-1 protein Tat upon viral integration and thus can score infectivity of HIV-1 viruses which do not carry a reporter signal (Fig 2-2B). I found that infection of A92E was not increased by the addition of restriction-sensitive A92E decoy particles or by the addition of resistant A92E-P90A, A92E-A105T particles. Rather, I observed a marked decrease in replication in the presence of high concentrations of decoy particles, suggesting competition between the A92E-GFP and the decoy particles for cellular resources rather than saturation of a restriction factor. Similarly, the addition of decoy particles to cells infected with WT-GFP decreased infection in a dose-dependent manner. These data indicate that the CsA R/D phenotype is not saturable, and suggest a more complex mechanism of CsA R/D inhibition, one that may not solely rely on binding to capsid to for inhibition.

Development and Validation of MuDPIT Screen for HIV-1 CA Binding Proteins

To identify the putative factor host factor responsible for the CsA R/D phenotype, I developed an assay to capture and identify proteins specifically bound to restriction sensitive HIV-1 capsids. The choice of bait in this screen was critical, as binding to capsid is not only influenced by the sequence of the HIV-1 CA protein, but also by the higher order structure of the capsid. A previous mass spectrometry screen designed to identify novel HIV-1 binding proteins utilized monomeric CA as bait and did not report any CA-binding proteins [120]. Thus, to increase the likelihood of identifying relevant capsid-binding proteins, I utilized recombinant HIV-1 CA tubes as bait. These tubes are disulfide-stabilized arrays of recombinant capsid containing cysteine substitutions at

codons 14 and 45 formed by dialysis in a high salt buffer. The tubes retain the sequence specific binding observed with monomeric CA, and bind proteins that require an intact lattice like TRIM5α (Figure 3-4).

Due to their large size, assembled HIV-1 tubes can be pelleted by low-speed centrifugation. Therefore, to isolate CA-binding proteins from cellular extracts, CA tubes were incubated with extracts from HeLa cells and pelleted by low-speed centrifugation. Pellets were then rinsed with buffer and analyzed by non-reducing SDS-PAGE to resolve bound proteins from the large quantity of cross-linked CA present in the sample. I hypothesized that the putative restriction factor would specifically bind to CsA R/D sensitive mutants. Accordingly, I used CA tubes bearing the sensitive A92E mutation as the primary bait for the putative factor and included WT and A92E/A105T reasoning that the putative factor would not bind these tubes as they are not subject to CsA R/D restriction. I also hypothesized that CypA would regulate the binding of the putative factor and therefore included A92E/P90A tubes and CsA treatment as negative controls for CypA binding. To validate the assay, I assessed two variables 1) tube assembly 2) specific capture of cellular proteins. To analyze assembly, a 5μM solution of each CA mutant was prepared by diluting assembled capsid in binding buffer and pelleted by low-speed centrifugation. I quantified the amount of CA in the pellet and the supernatant by enzyme linked immunosorbent assay (ELISA). I found that more than 99% of CA was present in pellets for both wildtype and mutant tubes, indicating the assembly was nearly complete for each mutant (Fig 2-3). Next, I assayed the specificity of protein capture. I wanted to confirm that proteins could be detected in pellets due to specific interactions with CA and not simply due to co-pelleting within aggregates of tubes. I

assayed binding of CypA to CA tubes as a measure of specific protein capture. As shown in Figure 2-3, CypA is captured by each bait except the non-CypA binding A92E-P90A tubes, or when tubes were treated with CsA. Importantly, CypA did not pellet in the absence of CA tubes indicating that proteins can be specifically captured from cellular extracts.

Functional Analysis of HIV-1 CA Proteome

I utilized the workflow outlined in Figure 2-3 to generate samples for mass spectrometry protein identification. The samples were submitted to the Vanderbilt Mass Spectrometry Research Center for multidimensional protein identification (MuDPIT). The abundance of each protein was reported using a relative unit known as spectral counts. In light of the limited knowledge of capsid-binding proteome, I first wanted to analyze the full complement of proteins captured by our screen. In total 447 proteins were identified across all samples. However, many of these proteins were detected in only one sample and with spectral counts as low as 1. To focus on the proteins most likely to genuinely bind to the HIV-1 capsid, I curated the list by discarding hits detected with spectral abundances less than 5 that were detected in fewer than 2 samples. The resulting list of 141 proteins (Table 2-1) was then subjected to functional annotation using Protein ANalysis THrough Evolutionary Relationships Classification tool (PANTHER http://pantherdb.org). This analysis groups genes according to

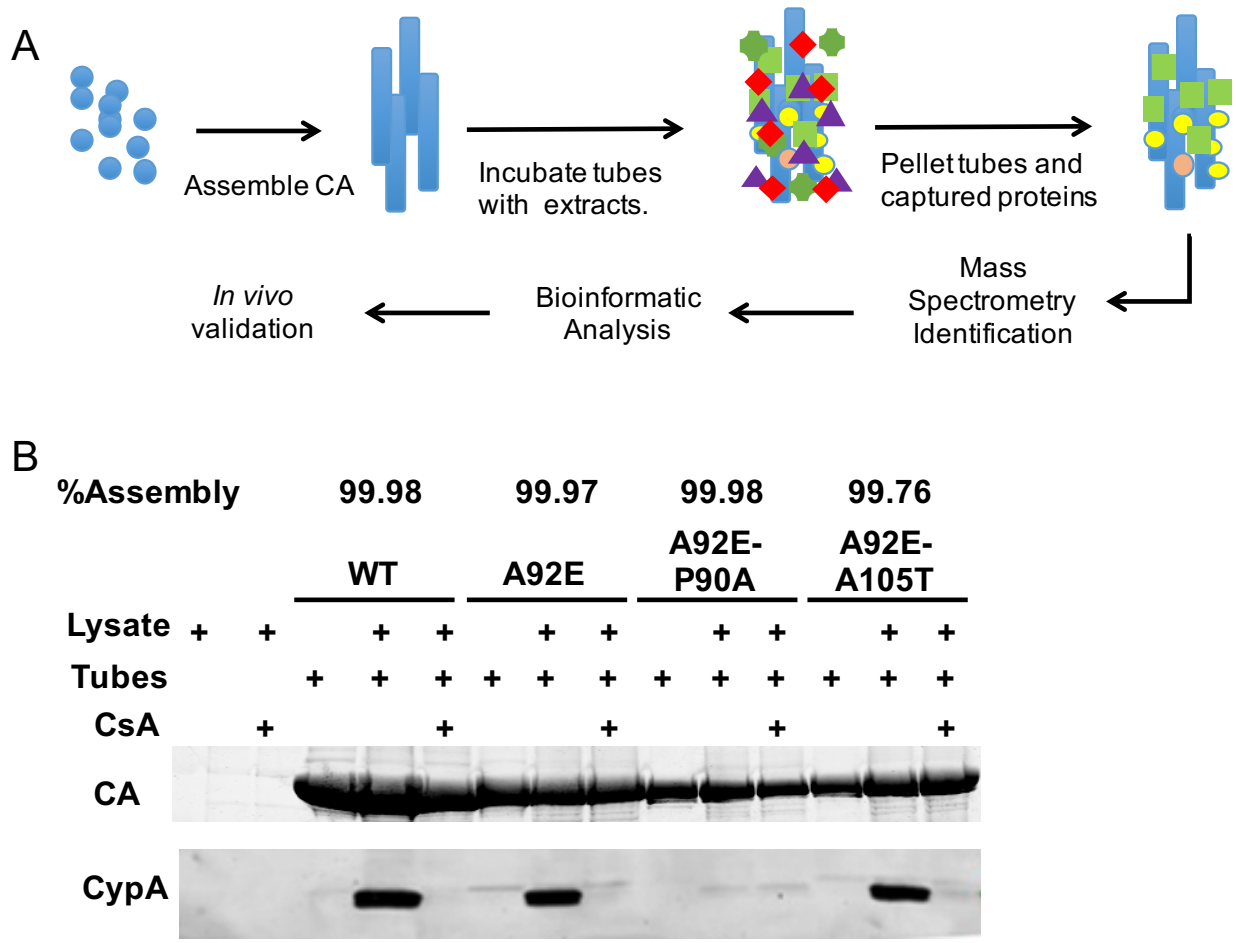

Figure 2-3: *In Vitro* Capture Assay. (A) Experimental scheme. Recombinant CA tubes are assembled into tubes by dialysis into non-reducing buffer containing 1M NaCl. Tubes are then incubated with extracts from HeLa cells. Tubes and associated proteins are pelleted by centrifugation, and resolved by SDS-PAGE before MuDPIT to identify captured proteins. **(B)** Indicated CA tubes were incubated with HeLa extracts as in in the presence and absence of CsA, pellets were analyzed by SDS-PAGE and immunoblotting for CypA; % assembly indicates the percent of CA present in pellets compared to total CA as determined by ELISA.

ontological function (i.e. biological process, cellular component, or protein class), thereby simplifying assessment of large groups of genes [121,122]. The most abundant protein class by far were nucleic acid binding proteins (Fig 2-4A). Perhaps unsurprisingly, many of these proteins were ribosome associated proteins however, mRNA processing proteins also represented a significant portion of these proteins including several members of the Cleavage Factor I (CFIm) complex, CSPF5, CPSF6 and CPSF7. RNA Helicases also represented a portion of the nucleic acid proteins including a number of DEAD-box helicases. DEAD-box helicase 17 (DDX17) has been shown to increase extracellular virus production [123]. Cytoskeletal proteins (both tubulin and actin components), hydrolases, transferases and enzyme modulator proteins were the next most abundant protein classes by function. To assess enrichment, or overrepresentation of functional groups, I employed the open-source gene ontology tool, Enrichr. This tool completes ontological grouping as above, then compares the number of genes present in each group to an expected value based on proportional representation within the human genome. Using a modified version of Fisher's exact test, Enrichr (http://amp.pharm.mssm.edu/Enrichr/) then determines the statistical significance of the overlap between the two groups, providing a statistical measure of the relevance of detecting a given gene ontology term. In contrast to PANTHER, Enrichr uses a full library of GO terms, while PANTHER uses an abbreviated library (termed GO-Slim) which is useful for binning a large group genes into high-level groups and acocunts for the slight differences in naming. The top 10 most enriched ontology groups are depicted in Fig 2-5B, the

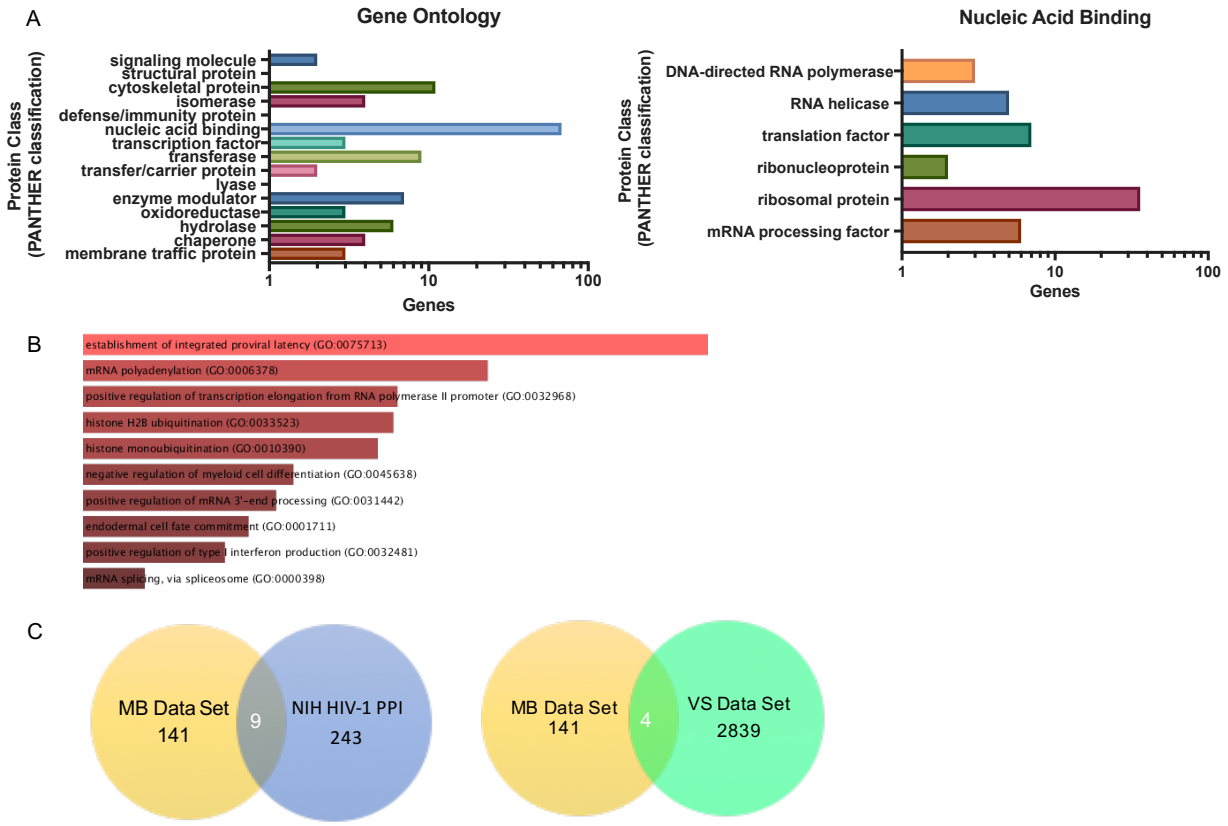

Figure 2-4: Qualitative Analysis of Captured Proteins (A) Functional annotation of curated prey proteins. After removing known contaminants and low abundance hits, prey proteins were subjected to gene ontology analysis using the PANTHER GOSlim Protein Class assignment tool. Bar graphs indicate 'Protein Class' GO term and the number of genes in each class. **(B)** Enrichment analysis of GO terms, the set of curated proteins were subject to enrichment analysis using Enrichr. The 10 most highly enriched GO terms according "Biological Function" are indicated in bar graph. The composite score of statistical enrichment is indicated by the length of the bar. **(C)** Overlaps between MB data set and the NIH HIV-1 CA-host interactions database, and VS microarray data set.

most enriched functional group were genes associated with the establishment of viral latency. In agreement with the PANTHER analysis, a number of mRNA associated functional groups were also statistically enriched in our set. Two functional groups associated with innate immunity were also enriched in our set: positive regulators of type I interferon production, and negative regulation of myeloid cell differentiation.

I next compared the proteins identified in our screen to two existing databases; genes in the NIH HIV-1 Interactions database (NIH HIV-1 PPI), and genes upregulated in cells exhibiting the restrictive CsA R/D phenotype (VS data set) (Fig 2-4C). Of the selected 141 proteins, 9 proteins were previously identified in the NIH HIV-1 PPI database as proteins that interact with capsid or regulate infection in a manner that affects capsid. In previous attempt to identify this CsA R/D putative factor, our lab conducted comparative gene expression analysis in permissive and non-permissive cells using a microarray (VS data set)[124]. I compared the genes detected in my screen to the 2839 genes upregulated by at least two-fold in the VS data set, and found 4 proteins shared between the data sets. A list of the overlapping proteins from the NIH HIV-1 PPI database and the genes upregulated in non-permissive cells are indicated in Table 2-1.

Characterization of interaction of VCP with HIV-1 Capsid

The most abundant protein isolated in our screen was the ATP-driven chaperone valosin-containing protein/p97 (VCP). VCP is a highly abundant AAA ATPase expressed in the majority of eukaryotic cells. Within mammals, VCP is primarily cytoplasmic and in keeping with its sheer abundance has been implicated in a number

of cellular functions including ubiquitin-dependent protein quality control and intracellular signaling. Of particular interest to our purposes, VCP also functions within the innate immune system. Together with the innate immune sensor TRIM-21 and virus-specific antibodies, VCP blocks replication of adenovirus in a process known as antibody dependent intracellular neutralization (ADIN). Therefore, I considered the possibility that VCP could regulate infection of HIV-1 by binding to capsid. To test this hypothesis, I first confirmed the interaction of VCP with CA tubes. I completed the *in vitro* capture assay as previously described with the panel of CA baits in the presence and absence of CsA, and probed for VCP in pellets (Fig 2-5). VCP bound to each of the baits comparably, but was not detected in the absence of CA tubes. These data confirm that VCP co-pellets with tubes, but suggests that it does not selectively bind to CsA R/D mutants. CsA treatment did not affect co-pelleting of VCP suggesting that CypA does not affect VCP binding to capsid (Fig 2-5A). To determine whether VCP binds directly to CA tubes, tubes were incubated with recombinant VCP as previously described. At the highest concentrations, a large quantity of VCP pelleted in the absence of CA. However, at lower concentrations VCP pelleted selectively in the presence of CA suggesting that VCP directly binds to CA (Fig2-5B). Next, I measured the ability of a biochemical inhibitor of VCP, N2, N4-dibenzylquinazoline-2,4-diamine (DBeQ) to prevent binding of VCP to CA, by incubating extracts and recombinant VCP with CA tubes in the presence and absence of DBeQ. Both endogenous and recombinant VCP pelleted in the presence of the inhibitor suggesting that DBeQ does not block VCP binding to CA tubes.

In an effort to assess the specificity of VCP binding, I assessed the role of ATP hydrolysis in VCP binding to CA tubes. The conformation of VCP changes dramatically in response to ATP binding and hydrolysis. I hypothesized that this conformational change would alter VCP binding to capsid. To test this hypothesis, tubes were incubated with VCP as above in the presence and absence of ATP. As a negative control for ATP hydrolysis, identical reactions were prepared with the non-hydrolyzable ATP-γS and with ADP (Fig 2-6). The presence of ATP prevented co-pelleting of endogenous VCP with CA tubes. In contrast, the presence of ADP or ATP-γS did not inhibit co-pelleting of VCP with CA tubes indicating that an interaction with ATP limits VCP co-pelleting with CA tubes. The effects of ATP on recombinant VCP co-pelleting were less pronounced, although the presence of ATP did reduce binding of recombinant VCP to CA tubes, ADP also slightly reduced the co-pelleting of recombinant VCP (Fig 2-6).

Disassembly of the incoming capsid is a necessary feature of HIV-1 replication. I hypothesized that VCP could promote uncoating of HIV-1. To test this hypothesis, Vaibhav Shah, a post-doctoral fellow in our lab completed studies to assess the role of VCP in HIV-1 uncoating, using an *in vitro* uncoating assay. In this assay, purified HIV-1 cores, intact capsids containing accessory proteins and the viral genome, are incubated for 1hr at 37C, prompting spontaneous disassembly or uncoating of the cores. The extent of disassembly is measured by pelleting the reactions and quantifying the CA present in pellet and supernatant fractions. Purified cores were incubated in the presence and absence of recombinant VCP for 45 min at room temperature. Identical reactions were completed in the presence of ATP, in order to

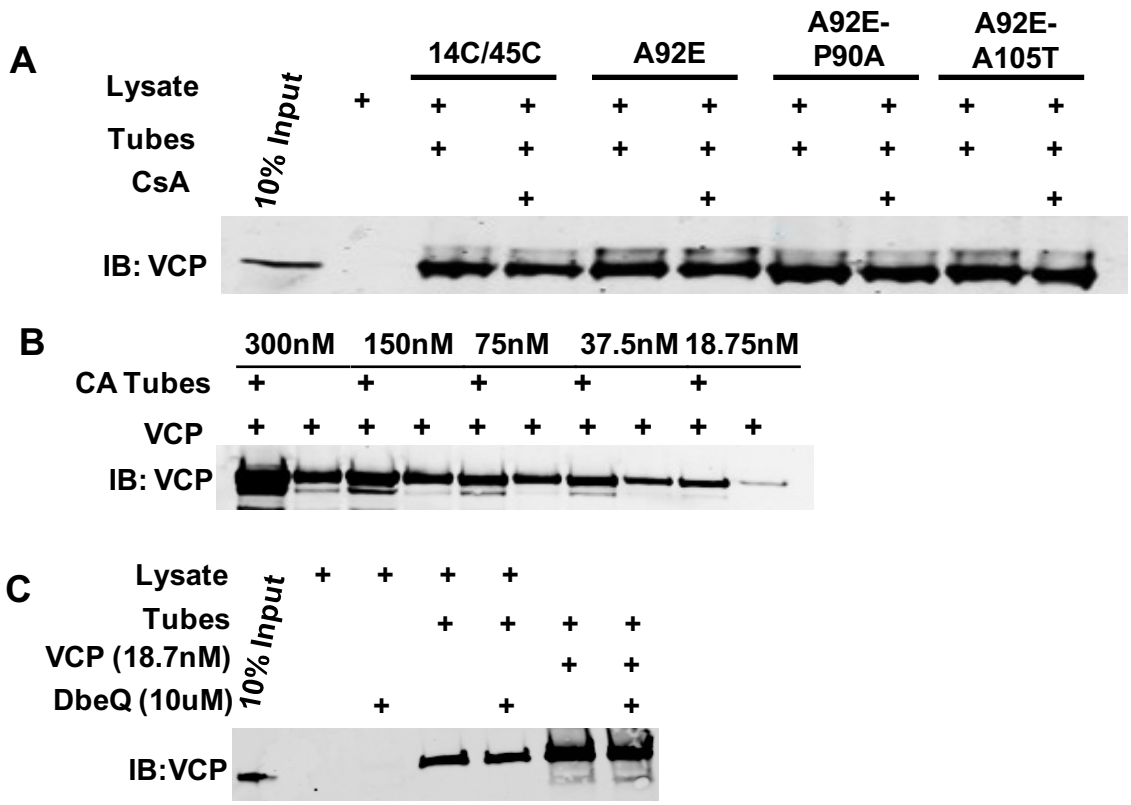

Figure 2-5: VCP binds directly to HIV-1 CA Tubes. Indicated CA tubes were incubated with **(A)** extracts from HeLa cells in the presence and absence of CsA **(B)** Indicated concentrations of recombinant VCP or **(C)** Recombinant and endogenous VCP in the presence and absence of VCP inhibitor DBeQ and pelleted by centrifugation and analyzed by SDS-PAGE and immunoblotting for VCP.

assay the contribution of ATP to uncoating. As indicated in Fig 2-6B, the addition of VCP significantly increased the quantity of free CA from 22% of the total CA in the control sample to 42% of the total CA in the presence of VCP. Notably, the addition of ATP prevented the VCP-dependent increase in uncoating, while the addition of ATP alone did not affect uncoating of the cores. Together these results indicate that VCP binds to cores, and promotes uncoating of the capsid in an ATP-dependent manner.

To directly assess the ability of VCP to regulate infection of HIV-1 we infected HeLa cells depleted of VCP by RNAi (VCP) and control cells shRNA (NS) with wildtype HIV-1 GFP reporter virus. Depletion of VCP was confirmed by immunoblotting and RT-qPCR of VCP transcripts. Depletion of VCP slightly increased replication of HIV-1 when cells were infected 48 or 72 hours after transduction, suggesting that VCP imposes a small barrier to replication of HIV-1 in HeLa cells. Together, these results confirm that VCP is specifically isolated from cellular extracts through an ATP dependent interaction with capsid. Moreover, these results support the utility of the *in vitro* capture assay for identifying *bona fide* capsid-binding proteins. However, these results do not indicate a significant role for VCP in infectivity of HIV-1, and highlight the necessity for functional corroboration of any candidate protein identified using this method.

N74D Permits Escape from CsA R/D Phenotype

Prior to this work, a truncated version of CPSF6 ($CPSF6_{358}$) was identified from a cDNA screen for novel host proteins that block HIV-1 infection. $CPSF6_{358}$ was shown to inhibit nuclear import of HIV-1, and to more potently restrict HIV-1 when when the cell-cycle was arrested, mirroring a key feature of the CsA R/D phenotype. Since I had

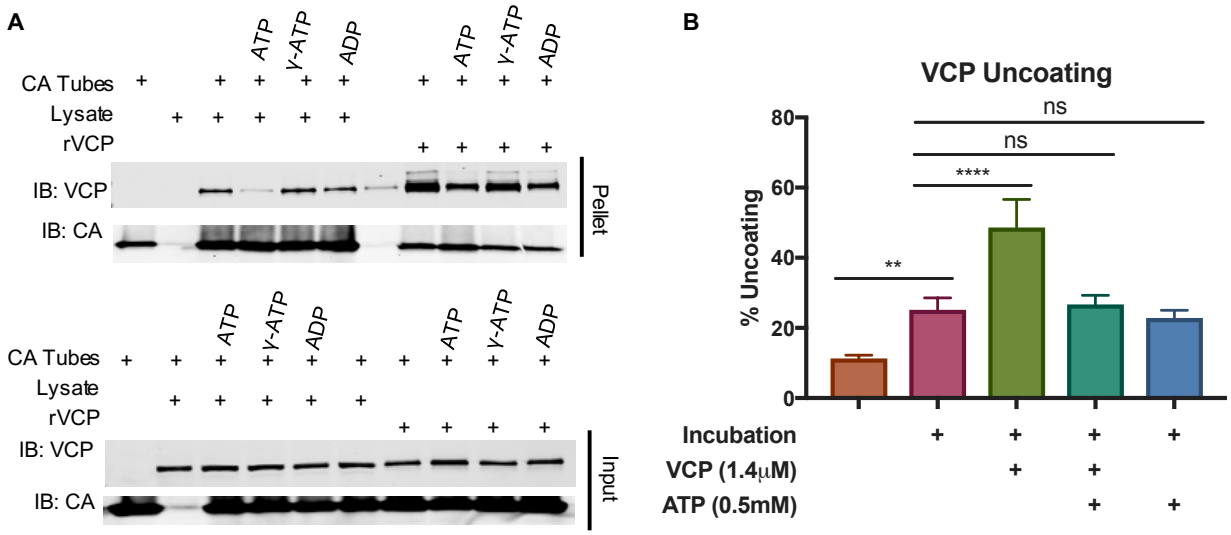

Figure 2-6: VCP binds to and promotes uncoating of HIV-1 capsid in an ATP dependent manner. (A) CA tubes were incubated with endogenous and recombinant forms of ATP in the presence and absence of ATP, ADP, or gamma-ATP, 10% of reactions were analyzed separately by SDS-PAGE as loading controls. **(B)** In vitro uncoating assay, purified HIV-1 cores were incubated with recombinant VCP, ATP, or both, % Uncoating refers to the quantity of pelletable CA present following incubation (Vaibhav Shah).

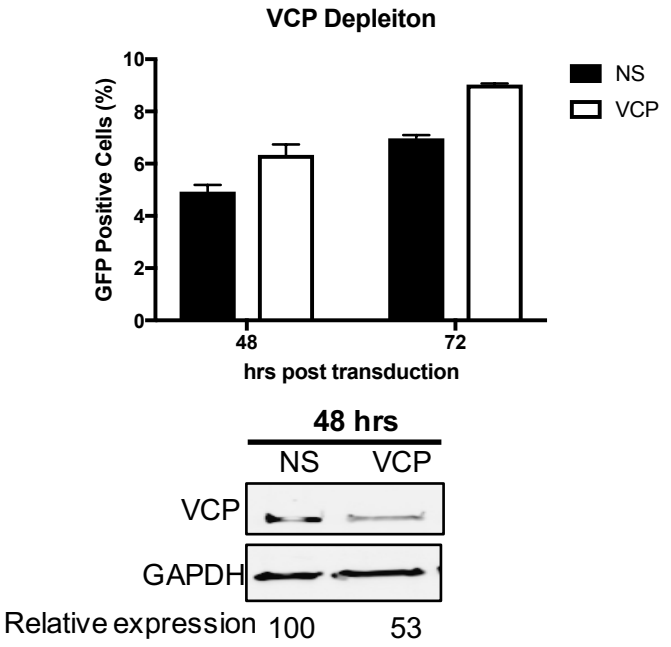

Figure 2-7: VCP does not alter replication of HIV-1. HeLa cells transduced with control shRNA or shRNA directed against VCP were inoculated with WT HIV-1 48 or 72 hrs after transduction. Extent of infection was measured 48hrs ater infection by flow cytometry. Extent of depletion was measured by RT-qPCR of mRNA (relative expression) or by immunoblotting. (Vaibhav Shah).

observed reproducible capture of CPSF6 from cellular extracts using HIV-1 CA tubes, we hypothesized that CPSF6 might play a role in the CsA R/D phenotype. A substitution of aspartic acid for asparagine at position 74 (N74D), allows escape from restriction by $CPSF6_{358}$ by preventing its binding to capsid [60,125]. To assess the ability of the non-CPSF6 binding mutation to permit escape from CsA R/D phenotype I generated double mutant viruses bearing containing both the A92E and N74D mutations and inoculated non-permissive HeLa cells with WT HIV-1, A92E or A92E/N74D GFP-reporter viruses in the presence and absence of CsA (Fig 2-8). As previously described, infection of wildtype HIV-1 was minimally enhanced by CsA treatment, while A92E infection was increased nearly 5-fold. The introduction of N74D increased the infectivity of A92E and relieved sensitivity to CsA. Together, these data indicate that N74D suppresses the CsA R/D phenotype and suggest that binding of CPSF6 contributes to the CsA R/D phenotype.

CPSF6 is necessary for Inhibition of CsA R/D mutants

To directly determine the requirement for CPSF6 in the CsA R/D phenotype we collaborated with the Yamashita Lab at the Aaron Diamond AIDS Research Center. Control HeLa cells, HeLa cells transfected with a non-targeting siRNA (nt-siRNA), and HeLa cells transfected with an siRNA directed against CPSF6 (CPSF6 k/d) were infected with a panel of CsA R/D mutants or wild type HIV-1 (Fig 2-9A). Depletion of CPSF6 increased the infectivity of CsA R/D mutants, while having no effect on replication of the wildtype virus. To determine whether the CsA R/D suppressor mutations permitted escape from CPSF6 dependent restriction, control and CPSF6-depleted HeLa cells were infected with the CsA R/D mutant T54A and the double

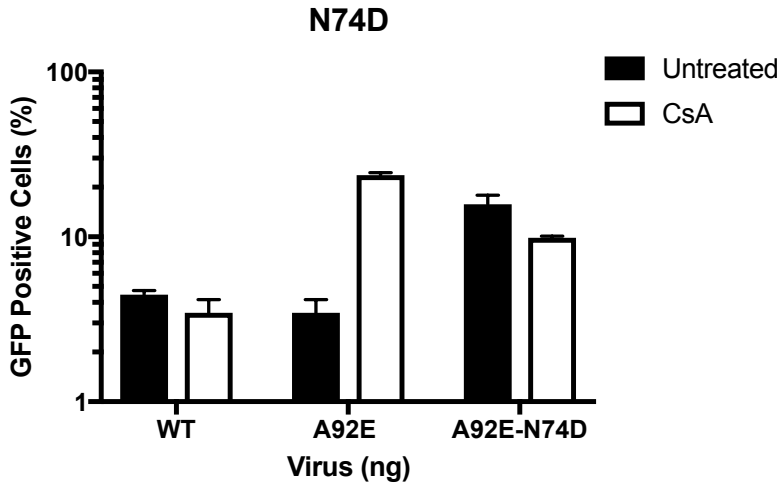

Figure 2-8: The N74D substitution rescues infectivity of A92E HIV-1**.** HeLa cells were inoculated with indicated viruses in the presence and absence of CsA. Extent of infection was determined by flow cytometry 48 hours after infection.

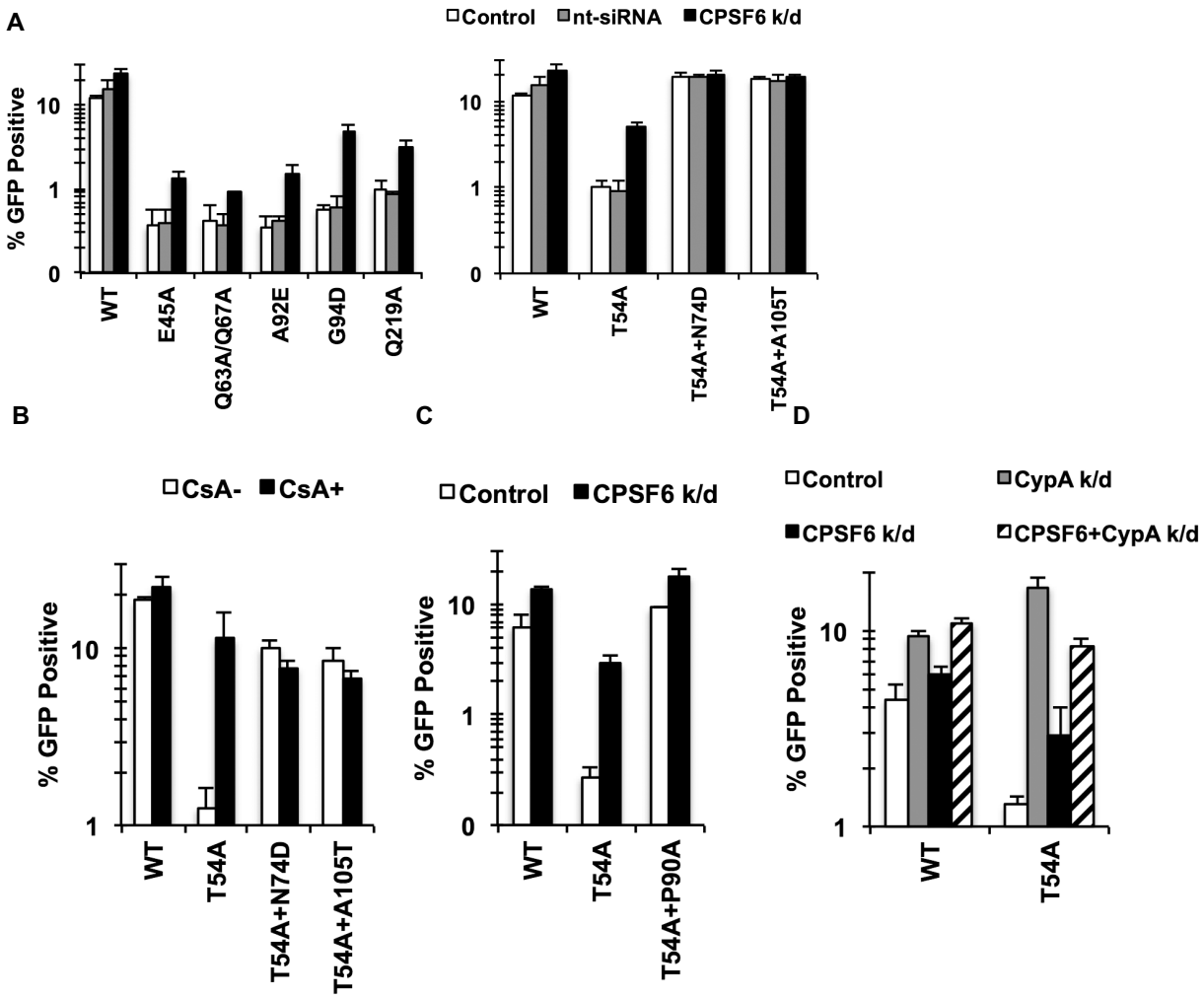

Figure 2-9: CPSF6 is required for CsA R/D restriction of HIV-1. (A) Control and HeLa cells depleted of CPSF6 were inoculated with indicated viruses, extent of infection was quantified by flow cytometry. **(B)** HeLa cells were inoculated with WT T54A, T54A/A105T or T54A/N74D viruses in the presence and absence of CsA. **(C)** Control and HeLa cells depleted of CPSF6 were inoculated with WT, T54A, OR T54A/P90A HIV-1. **(D)** Control, HeLa cells depleted of CypA, CPSF6 or both were inoculated with WT and T54A reporter viruses. Extent of infection was measured by flow cytometry. *Henning et al* [74]

mutants unaffected by CPSF6 depletion. Together these observations, indicate that endogenous CPSF6 inhibits infection by CsA R/D mutants.

To confirm that CypA collaborates with CPSF6 to inhibit the mutants, HeLa cells were inoculated with wildtype, T54A, T54A/A105T, and T54A/N74D in the presence and absence of CsA (Fig 2-9C). Similar to my results the addition of CsA specifically rescued replication of T54A while infection of wildtype or the double mutants was unaffected by CsA treatment. Infection of T54A and T54A/P90A was also compared in HeLa cells depleted of CPSF6 (Fig 2-9C). While CPSF6 depletion rescued replication of T54A, neither WT HIV-1 nor the double mutant were enhanced in CPSF6 depleted cells. Finally, our collaborators examined infection cells depleted of both CPSF6 and CypA (Fig 2-9D). Depletion of CypA potently rescued replication of T54A and moderately increased infection of wildtype HIV-1. Depletion of CPSF6 also rescued infection of T54A but to a lesser extent than depletion of CypA, while depletion of both proteins did not enhance infection beyond that observed with CypA alone. Together these results indicate that CypA and CPSF6 collaborate to inhibit infection of T54A.

Since we had observed CPSF6-dependent inhibition of CsA R/D mutants, I wanted determine if the A105T mutation reduced CPSF6 binding as a mechanism for suppression of the phenotype. I generated CA tubes bearing the T54A, T54A/A105T mutations and incubated these tubes with extracts from HeLa cells as previously described. I found that introduction of A105T reduced co-pelleting CSPF6 with T54A tubes, indicating that A105T is a determinant for CPSF6 binding to capsid. I next wanted to address the hypothesis that CypA promotes binding of CPSF6 to CsA R/D sensitive capsids. I assessed co-pelleting of CPSF6 with WT, T54A,

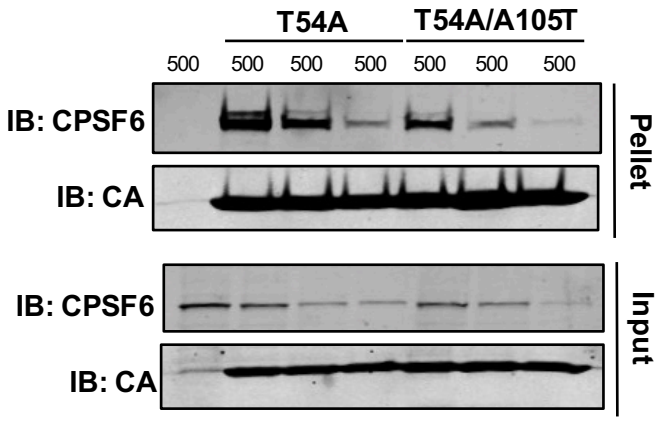

Figure 2-10: The A105T substitution reduces CPSF6 binding to HIV-1 CA tubes. T54A and T54A-A105T CA tubes were incubated with indicated quantities of HeLa cell extracts for 1hr with gentle mixing followed by centrifugation. Pellets were analyzed by SDS-PAGE and immunoblotting for CPSF6 and CA. Input represents 10% of initial reaction.

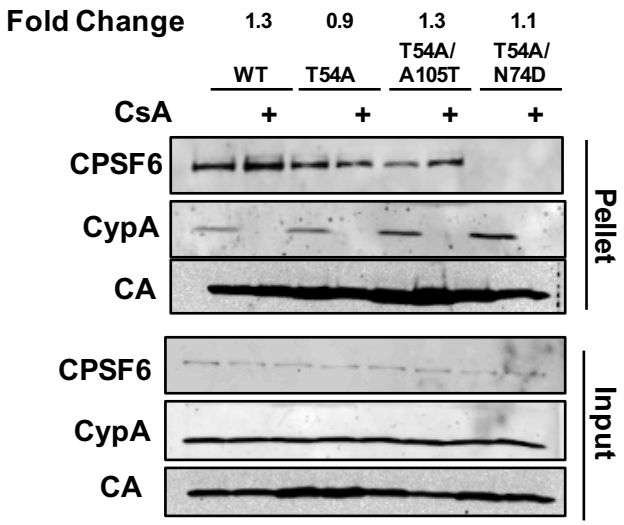

Figure 2-11: CsA treatment does not block CSPF6 binding to tubes. WT, T54A, T54A-A105T, and T54A-N74D tubes were incubated with HeLa cell extracts in the presence and absence of CsA, pelleted and analyzed by SDS-PAGE and immunoblotting for CSPF6, CA, and CypA. Input represents 10% of initial reaction.

T54A/A105T, and T54A/N74D tubes in the presence and absence of CsA. We observed equivalent binding of CPSF6 to WT, T54A, T54A/A105T tubes in the presence and absence of CsA treatment. No CPSF6 was detected in T54A/N74D pellets. CsA treatment blocked CypA binding to all tubes. This observation suggests that binding of CypA to CA tubes does not influence the extent of CPSF6 binding to tubes. In summary these data indicate that CPSF6 is necessary for restriction of CsA R/D dependent mutants, and that suppressor mutations A105T and N74D prevent restriction by reducing CPSF6 binding tubes. These data also indicate that while CypA is necessary for restriction of CsA R/D mutants, it does not act by promoting binding of CPSF6 to the capsid.

Discussion

The aim of this study was to identify the putative restriction factor implicated in the CsA resistant/dependent phenotype (CsA R/D) of the HIV-1 CA mutant A92E. Prior reports from our lab and others demonstrated a dominant, cell-type specific restriction that is evaded by the wildtype virus and enhanced in non-dividing cells. This phenotype is mediated by CypA binding to capsid, as evidenced by the loss of restriction when the CypA-CA interaction is biochemically or genetically inhibited. To identify the putative factor responsible for this phenotype, I developed an *in vitro* capture assay using assembled recombinant CA tubes as bait to capture and identify proteins specifically bound to restriction sensitive CA. As “proof-of-concept” I demonstrated genetically and biochemically selective capture of CypA with CA tubes. Using the validated *in vitro* capture assay, proteins captured by the restriction sensitive A92E tubes and the

negative control tubes WT, A92E/P90A and A92E/A105T were identified by mass spectrometry. Inspection of the top 15 most abundant proteins captured in the screen revealed that CPSF6 reproducibly co-pelleted with CA tubes. Although, CPSF6 was not selectively captured by A92E tubes, the inhibitory phenotype of its truncated form, $CPSF6_{358}$, prompted us to investigate the possibility that the full-length protein inhibits infection of CsA R/D mutants. We generated A92E GFP reporter viruses bearing a mutation known to prevent sensitivity to $CPSF6_{358}$ binding and restriction, N74D, and found that inclusion of N74D rescued the infectivity of A92E, and prevented sensitivity to CsA treatment. Unfortunately, our own attempts to directly examine the role of CPSF6 in restriction using RNAi were unsuccessful. However, in collaboration with the Yamashita lab we were able to confirm that depletion of CPSF6 by RNAi specifically increased infection of A92E and other CsA R/D mutants. They also confirmed that CypA and CPSF6 collaborate to block infection. Using the *in vitro* capture assay I was able to demonstrate that A105T, the suppressor mutation previously identified in our lab, rescued replication by inhibiting interactions with CPSF6. Together these results establish CPSF6 as the host protein responsible for the CsA R/D phenotype.

I was initially puzzled by the observation that the CsA R/D phenotype is not saturable. The discovery that CPSF6 is underlies the phenotype helps to clarify this result. Typically, HIV-1 restriction factors are saturated by high doses of virus, allowing for rescue of infection *in trans*. However, in the absence restriction, the addition of viral particles only serves as competition for a finite quantity of cellular resources. At the time this work was completed little was known about the contribution of CPSF6 to HIV-1 replication, and even now many details remain unclear. In culture, CPSF6 is

dispensable for HIV-1 replication, however *in vivo* the virus maintains interaction with CPSF6 even under negative selective pressure. These data suggest that CPSF6 plays an important role in viral infection, and therefore does not function as a typical restriction factor. Further explanation for the lack of saturability may also lie in the specific mechanism of CPSF6-dependent inhibition. Depletion of the karyopherin TNPO3, inhibits HIV-1 infection by mislocalizing CPSF6 to the cytoplasm of target cells, leading to inhibition of infection[70,71]. Thus nuclear CPSF6 is not inhibitory to infection of wildtype HIV-1, however when localized to the cytoplasm CSPF6 blocks infection. This suggests a kinetic component for CPSF6 inhibition such that engagement of CPSF6 at the nuclear pore or after translocation is beneficial while earlier engagement is detrimental. I propose that improper engagement of CPSF6 by CsA R/D mutants blocks interaction with nuclear pore proteins that support nuclear import and integration of HIV-1. Accordingly, attempts to rescue restriction in *trans* are unsuccessful because the addition of decoy particles simply compounds the problem of accessing to nuclear pore proteins.

Despite the fact that we were able to successfully identify the host protein involved in CsA R/D restriction, my initial approach of utilizing the mass spectrometry screen to achieve that goal proved to be ineffective. Although many of the features associated with CsA R/D restriction mirror those of canonical HIV-1 restrictions, we made two assumptions in the design our study which precluded our ability to identify CPSF6 using this method 1) we assumed that the protein would solely function as a restriction factor and therefore selectively bind restriction sensitive capsids and 2) that CypA would play a major role in the interaction of the putative factor with the capsid. Upon identification

of the factor, both of these assumptions proved to be incorrect. However, this assay does represent a useful method for identifying *bona fide* capsid-binding proteins and for measuring capsid binding *in vitro*. In addition to recovering previously reported capsid-binding proteins like cyclophilin A, and cyclophilin B, Nup153, and CPSF6 we also identified a number of proteins reported by the NIH HIV-1 protein-protein Interactions Database to regulate infection of HIV-1 in a manner that depends on or affects the capsid protein (Fig 2-4). As further validation of the method, we analyzed the interaction of the most abundant protein detected in our screen, VCP with the capsid. We found that VCP directly binds to CA tubes in an ATP dependent manner. When VCP was assayed for its ability to stimulate uncoating we found that VCP significantly increased the extent of uncoating of HIV-1 cores in a manner that is sensitive to the presence of ATP. These results indicate that VCP functionally interacts with the capsid. However, our findings *in vitro* did not corroborate with the relevance of this interaction in cells. When cells depleted of VCP were infected with wildtype HIV-1 no significant change in infection was observed suggesting that VCP does not play a major role in HIV-1 infection. It should be noted that we only achieved a marginal reduction in VCP expression as indicated by protein expression and quantification of mRNA transcripts, therefore we cannot rule out the possibility that significant depletion of VCP would demonstrate an effect on infection. Future studies should consider a gene knockout approach to achieve complete depletion of the protein, although its high levels and ubiquitous functions in the cell may preclude this approach.

Due to the early identification of CPSF6, analysis of the results of the screen were limited to primarily qualitative methods. Future screens employing this method

would be improved by several alterations in order to permit meaningful quantitative analysis of resulting gene lists.

1. **Reagent Selection and Design**: In order to focus on the most likely candidate proteins, I relied on reproducible binding to across samples, however future studies should include 'extract-only' samples to provide a background for detection of contaminant or non-specifically pelleted proteins. Biological replicates will allow for analysis of statistically significant changes spectral abundance even when spectral counts are low. For studies designed to address specific binding hypotheses, as described here, this step is critical, as uniquely binding proteins or proteins with low abundances can be confirmed by replicate and statistical analysis.
2. **Stable Isotope labelling Methods**: The label-free quantitation method used here is sufficient for detecting changes in protein abundances, and when used in combination with sufficient biological replicates provides statistical confidence in differences in relative protein abundance. However, alternative methods such as stable isotope labelling offer improved protein identification and quantitation. Future studies employing this method should consider the multiplex iTRAQ labelling system which allows high throughput identification of up to 8 separate samples. This method reduces biases in detection due to fragmentation and allows more thorough identification of proteins present in a sample
3. **Quantitative Bioinformatic Workflow:** The qualitative analyses described here are useful for initial examination, future studies using this method should

be committed to a discrete bioinformatic workflow. A candidate protein list should first be compared to background or bait-free samples for removal of non-specifically pelleted proteins, the open source Contaminant Repository for Affinity Purification (CRAPome http://www.crapome.org/?q=about) provides bioinformatic tools to compare candidate gene lists to user provided background lists and for comparison with lists submitted by other users utilizing identical cell lines or methods of purification. Next, low abundance (as determined by a Z-score based on the needs of the study) should be removed. Then samples can be compared for quantitative differences in spectral abundance in addition to the qualitative analyses described here.

Table 2-2: Spectral Counts and Previous Detection of Most Abundant Captured Proteins

	Spectral Counts				Cross Detection	
Accession Number	**WT**	**A92E**	**A92E/P90A**	**A92E/A105T**	**NIH HIV-1 CA PPI**	**VS Data Set**
sp|P55072	57	52	39	52		
sp|P07437|	35	36	62	58		
sp|P68371|	34	34	59	56		
sp|Q13885|	30	32	51	50		
sp|P68363|	24	19	46	36		
sp|Q13509|	21	20	35	32		
sp|P62937	20	20	5	6	yes	
sp|P68366	18	16	41	30		yes
sp|Q16630	18	14	8	14	yes	
sp|P08670	17	18	20	23		
sp|P23284	15	10	4	2	yes	
sp|Q9BUF5	13	12	27	28		
sp|P11142|	12	10	8	18		
sp|Q12768|	12	11	11	14		
sp|P36578	12	15	11	8		
sp|Q99613|	12	7	7	7		
sp|P63261|	11	13	20	20		
sp|P05388|	11	11	11	8		
sp|Q8N684|	11	4	5	8		
sp|P08238|	10	9	12	18	yes	yes
sp|P68032|	10	10	15	13		
sp|Q8N7H5|	10	7	8	6		
sp|Q01813|	9	2	25	7		
sp|P63244|	9	7	6	7		
sp|P53999|	9	10	8	6		
sp|O43719|	9	4	7	6		
sp|Q15029|	8	11	16	10		
sp|P08195|	8	5	6	3		
sp|P62263|	8	8	7	8		
sp|P11940|	8	3	4	3		
sp|Q2M389|	8	3	6	5		
sp|P53621|	8	2	0	0		
sp|P68104|	7	3	14	13		
sp|P07900|	7	6	11	13		
sp|P19338|	7	11	16	7		

sp|O43809|	7	11	10	13	
sp|P62424|	7	10	11	7	
sp|P46777|	7	16	12	10	
sp|P08107|	7	5	12	12	
gi|136429	7	9	8	5	
sp|Q02878|	7	14	9	6	
tr|Q5JR95|	7	7	7	5	
sp|P18124|	7	5	7	4	
sp|P62244|	7	4	2	5	
sp|P30876|	7	5	4	3	
sp|P62906|	6	8	8	6	
sp|P49959|	6	6	3	6	
sp|P39023|	6	17	9	8	
sp|P49902|	6	9	5	3	
sp|Q01105|	6	7	11	6	
sp|P61247|	6	11	11	7	
sp|C4AMC7|	6	5	4	5	
sp|Q9H0A0|	6	9	4	3	
sp|P23396|	5	5	9	11	
sp|P30050|	5	8	10	10	
sp|P22626|	5	6	9	9	
sp|P62701|	5	12	10	9	
tr|B4DY08|	5	6	7	4	
sp|P26373|	5	8	8	5	
sp|Q9Y265|	5	4	1	3	
sp|Q9UKD2|	5	6	10	5	
sp|P61353|	5	6	8	2	
sp|P07195|	5	6	3	7	yes
sp|P62280|	5	1	3	4	
sp|Q9Y3U8	5	4	4	2	
sp|Q9NR50|	5	7	6	6	
sp|O75533|	5	4	1	3	
sp|O43172|	5	1	1	0	
sp|Q16531|	4	8	4	10	
sp|O00267|	4	6	6	4	
sp|P62805|	4	7	6	10	
sp|P14618|	4	5	7	10	
sp|P46781|	4	6	7	4	
sp|P62888|	4	4	4	3	
sp|P78406|	4	2	2	7	

sp|P62829|	4	5	4	8		
sp|O60664|	4	4	4	3		
sp|Q9BUQ8|	4	4	5	3		
sp|P08865|	4	5	4	3		
sp|P17858|	4	0	7	7		
sp|Q99536|	4	2	1	2		yes
sp|Q8WVC0|	4	4	3	4		
sp|P62910|	4	4	8	1		
sp|P49770|	4	6	4	4		
sp|Q8TDN6|	4	5	8	2		
sp|P61254|	4	3	6	3		
sp|P62750|	4	3	2	1		
sp|Q9UI10|	4	9	6	4		
sp|Q99848|	4	7	6	3		
sp|P11021|	4	2	2	6		
sp|P42766|	4	3	5	4		
sp|Q15393|	3	4	3	3		
sp|Q9Y230|	3	7	8	14		
sp|P15880|	3	8	5	6		
sp|P35606|	3	4	6	10		
sp|P67809|	3	6	5	5		
sp|Q00839|	3	3	3	0		
sp|Q92841|	3	3	6	3	yes	
sp|P52272|	3	2	5	9		
sp|P46778|	3	3	5	4		
tr|F8VV04|	3	5	1	3		
sp|P84098|	3	5	4	2		
sp|Q6P1J9|	3	3	5	3		
sp|P50914|	3	5	3	1		
sp|P14625|	3	3	5	5		
sp|P29401|	3	5	6	6		
sp|Q07020|	3	4	4	5		
sp|P04406|	3	4	5	7		
sp|P61313|	3	1	3	7		
sp|P62249|	2	2	5	5		
sp|P31943|	2	2	4	6		
sp|P18621|	2	8	9	3		
sp|P17844|	2	6	6	4	yes	
sp|P31689|	2	1	4	8	yes	
sp|P62277|	2	1	2	5		

sp|P62269|	2	6	1	4		
sp|O60814|	2	3	7	3		
sp|P62913	2	3	5	3		
sp|Q9UQ80|	2	8	10	3		
sp|Q08211|	2	2	7	6		
sp|P00558|	1	5	11	15		
sp|Q9H6W3|	1	5	7	8		
sp|P52597|	1	0	2	3		
sp|P01891|	1	7	2	7		
sp|P46776|	1	4	7	3		
sp|P09429|	1	5	4	2		
sp|P09651|	1	3	5	3		
sp|O75396|	1	2	3	2		
sp|P62487|	1	4	12	7		
sp|P13639|	1	2	6	4		
sp|P08237|	1	0	8	3		yes
sp|Q9BQE3	0	0	35	29		
sp|P12956	0	9	8	10		
sp|Q2TAY7	0	0	1	8		
sp|P13010	0	2	8	8		
sp|Q8WVV9	0	4	3	6		
sp|P06748	0	5	9	5		
sp|P60842	0	1	4	6		
sp|Q92688	0	2	2	6		
sp|Q9Y2X7	0	1	0	6		
sp|O60506	0	5	8	4	yes	
Total Shared Genes					9, p = 0.315	4, p = 0.002

p value indicates statistical significance of detecting the indicated number of shared genes between data sets.

CHAPTER III

CYCLOPHILIN A POTENTIATES TRIM5α RESTRICTION OF HIV-1 WITHOUT PROMOTING TRIM5α BINDING TO HIV-1

Introduction

Tripartite motif protein 5 isoform alpha (TRIM5α) is a species-specific retroviral restriction factor that blocks infection by binding to the capsid [77,126–129]. TRIM5α is comprised of 4 parts: the 3 domains of the canonical TRIM RBCC motif, the RING domain, one or more B-box domains, and a coiled-coil domain, and the SPRY (B30.2) domain. The SPRY domain confers species specificity to TRIM5α restriction [77,126,130–132]. TRIM5α binds to retroviral capsids via an array of low affinity binding sites on the surface of the viral capsid, necessitating an intact lattice for retroviral recognition. This binding pattern has to date precluded the identification of point mutations within HIV-1 capsid which block TRIM5α binding.

TRIM5α inhibits retroviral infection by primarily by accelerating uncoating and disrupting reverse transcription [77,78]. As the retroviral capsid enters the cytoplasm of a target cell, it is bound by TRIM5α. TRIM5α is degraded in response to binding restriction sensitive capsid which is believed to accelerate capsid disassembly and block reverse transcription[133]. Biochemical inhibition of the proteasome, or specific mutations within the RING domain uncouple the block to reverse transcription from inhibition of infection, and reveal the ability of TRIM5α to inhibit nuclear import of HIV-1 [134,135]. In addition to restricting infection, TRIM proteins have also been implicated in innate immune signaling, indicating an broader role for these proteins in host defense

[136,137].

CypA enhances the ability of TRIM5α to block HIV-1 infection [91,112,138] although the mechanism is poorly understood. Initial reports demonstrated that depletion of CypA, or treatment of cells with CsA, promotes HIV-1 infection of cells expressing old world monkey orthologues of TRIM5α. Infection of such cells by HIV-1 CA mutants that do not bind CypA, such as G89V and P90A, is not enhanced by CsA, indicating that CypA mediates its effect through its binding to the HIV-1 capsid [91,113]. Multiple orthologues of TRIM5α including $TRIM5\alpha_{agm}$ and TRIM5α from rhesus macaques ($TRIM5\alpha_{rh}$), exhibit stronger restriction of HIV-1 in the presence of CypA. However, only HIV-1 is subject to CypA-dependent restriction, despite the ability of other retroviruses such as SIV from tantalus monkeys (SIV_{agmtan}) to bind CypA [113,139]. Here I have examined the mechanism of CypA-dependent TRIM5α restriction.

Results

Cyclophilin A interaction with the HIV-1 capsid potentiates TRIM5α restriction

To confirm the previously reported ability of CypA to potentiate TRIM5α restriction in old world monkey cells, I examined the effect of inhibiting CypA-CA interactions in Vero cells [91,112,113,140]. Vero cells were stably depleted of TRIM5α (shT5α) or CypA (shCypA) using shRNAs directed against the corresponding mRNAs. As a control, cells were transduced with the corresponding vector lacking an shRNA (shEmpty) (Fig 3-1). Depletion of the mRNAs was confirmed by quantitative RT-PCR. To assess functional depletion of proteins, we used N-tropic (N-MLV) and B-tropic MLV

(B-MLV). N-MLV is potently restricted by TRIM5α while B-MLV is not, and neither virus binds CypA [110,141]. As expected, depletion of TRIM5α markedly enhanced N-MLV infection, while B-MLV infectivity was unaffected in both cell lines (Fig 3-1A). Depletion of CypA moderately increased N-MLV infection (Fig 3-1A). I then inoculated the Vero cell lines with a GFP-encoding HIV-1 reporter virus pseudotyped with VSV-G in the presence and absence of CsA. The non-CypA binding mutant P90A HIV-GFP was included as a negative control. As previously reported, CsA treatment or CypA depletion markedly increased wildtype but not P90A HIV-1 infection (Fig 3-1B and D). Depletion of TRIM5α also increased infection by wild type HIV-1, and markedly reduced the enhancement of infection by CsA (Fig 3-1C). P90A infectivity was enhanced less than 2-fold by CsA treatment in control and TRIM5α-depleted cells (Fig 3-1B and C). CypA depletion also increased infectivity of P90A HIV-1 although to a much less extent than wild type HIV-1 (Fig 3-1D). These results indicate that binding of CypA to the incoming viral capsid inhibits HIV-1 infection of Vero cells by a mechanism that depends on expression of TRIM5α.

Transcriptome Mining for TRIMCyp in Vero cells

TRIMCyp is a closely related family member of TRIM5α that arose as the result of LINE-1 catalyzed retrotransposition of CypA into the *TRIM5* gene locus in owl monkeys (39). TRIMCyp consists of the RBCC domains of TRIM5α with CypA replacing the SPRY domain, was first identified in an owl monkey kidney cell line (OMK). Since the first reporting, 4 separate TRIMCyp genes have been reported, all arising from similar retrotranspositions into TRIM5 genes in macaque species, suggesting that

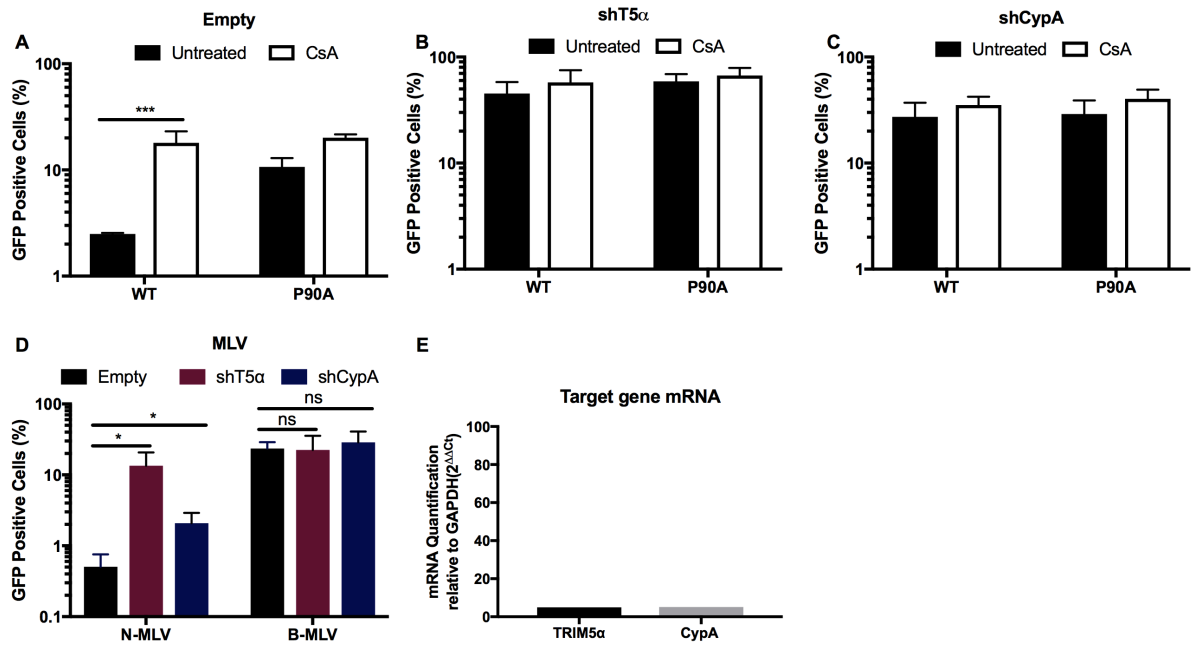

Fig 3-1: CypA potentiates TRIM5α restriction through its interaction with the HIV-1 capsid. Vero cells depleted of TRIM5α (shT5α) or CypA (shCypA), and control (Empty) were assayed for infection by the indicated HIV-1 **(A-C)** or MLV **(D)** reporter viruses in the presence and absence of CsA. Results shown are the mean values from 3 independent experiments, error bars reflect the standard error of the mean. **(E)** Quantification of target gene mRNA to confirm protein depletion. Statistically significant differences as determined by the two-way ANOVA test are indicated as follows ns = not significant, *= $p<0.05$, **= $p<0.01$, ****= $p<0.001$, ****= $p<0.0001$

TRIMCyp genes could exist in a variety of old world monkey cell lines [82,83,143]. Restriction by TRIMCyp occurs via binding of the CypA domain of the protein to the viral capsid, and is prevented by CsA treatment. Therefore, we considered the possibility that the apparent ability of CypA to promote restriction of HIV-1 in Vero cells might be a consequence expression of a previously unreported TRIMCyp protein. To determine whether a TRIMCyp mRNA is present in Vero cells, we analyzed the transcriptome of Vero cells by RNAseq. OMK cells were included as a positive control for the presence and recovery of TRIMCyp transcripts. Total RNA was isolated from Vero and OMK cells in triplicate and subject to Illumina HiSeq sequencing, which yielded an average of 68.3 million reads for OMK samples and 73.6 million reads for Vero samples (Table 3-1). These reads were subject to *de novo* assembly using CLC Genomics Workbench 10. The contigs, continuous sequences resulting from overlapping reads, generated from assembly of the triplicate Vero and OMK samples were pooled to create BLAST databases for each species. These databases were queried for contigs that aligned to *Aotus trivirgartus* TRIMCyp mRNA (OMK TRIMCyp) (Fig. 3-2A, and summarized in Tables 3-1, 3-2, and 3-3). Alignment of the top 4 BLAST hits from Vero and OMK contig databases are shown in Figures 3-2B and 3-2C respectively. Two OMK contigs, OMK_contig_150 and OMK_contig_276 respectively, aligned to TRIMCyp regions that span the junction between TRIM5α and CypA homology regions, indicating that TRIMCyp transcripts could be detected and assembled using the CLC Genomics *de novo* assembler algorithm. However, none of the Vero contigs aligned with the junction between TRIM5α and CypA homology regions, suggesting that a fusion transcript is not present in the transcriptome of Vero cells. I also generated two BLAST libraries for

Figure 3-2: TRIMCyp transcripts are not detected in Vero cells by RNAseq. (A) RNASeq workflow. Total RNA was isolated from triplicate samples of Vero and OMK cells and subject to Illumina HiSeq PE75 sequencing. Sequencing reads were assembled using the CLC Genomics Workbench 10 *de novo* assembler. The resulting contigs were used to create BLAST databases to assess the presence of TRIMCyp transcripts. Alignment of top BLAST hits for OMK **(B)** or Vero **(C)** to TRIMCyp sequence.

each Vero and OMK that contained contigs which aligned to either CypA or TRIM5α. The TRIM5α libraries were then queried for CypA alignment, and the CypA libraries were queried for TRIM5α alignment. As outlined in Table 3-1, only two sequences scored as hits in these searches. The previously identified OMK_contig_150 and OMK_contig_276 aligned with both TRIM5 αnd CypA queries, however no sequences with alignment to both TRIM5 and CypA were identified from the Vero BLAST libraries. Finally, I assessed read coverage within the TRIM-Cyp junction by mapping the read libraries directly to OMK TRIMCyp. Although the read coverage in the junction region was similar between Vero and OMK samples, there were qualitative differences (Fig. 3-3), such that the Vero reads primarily aligned to either side of the TRIM-CypA junction but did not span it, while the OMK reads mapped across the junction. Together, these results indicate that Vero cells do not express a TRIMCyp protein and that the inhibition of HIV-1 infection by CypA does not result from TRIMCyp restriction.

CypA-CA interactions do not promote TRIM5α binding to the capsid

TRIM5α must bind to the incoming viral capsid to inhibit infection. Previous studies demonstrated that CypA acts shortly after virus fusion to promote HIV-1 restriction by TRIM5α [113]. Together with the well-known ability of CypA to isomerize the Gly89-Pro90 bond, and to alter the dynamics of HIV-1 I hypothesized that CypA enhances TRIM5α binding to the capsid in order to potentiate restriction. To test this hypothesis, I assayed TRIM5α binding to recombinant tubular assemblies of CA that mimic the hexagonal lattice structure of the capsid. Extracts were generated from 293T cells ectopically expressing hemagluttinin-tagged African green monkey TRIM5α

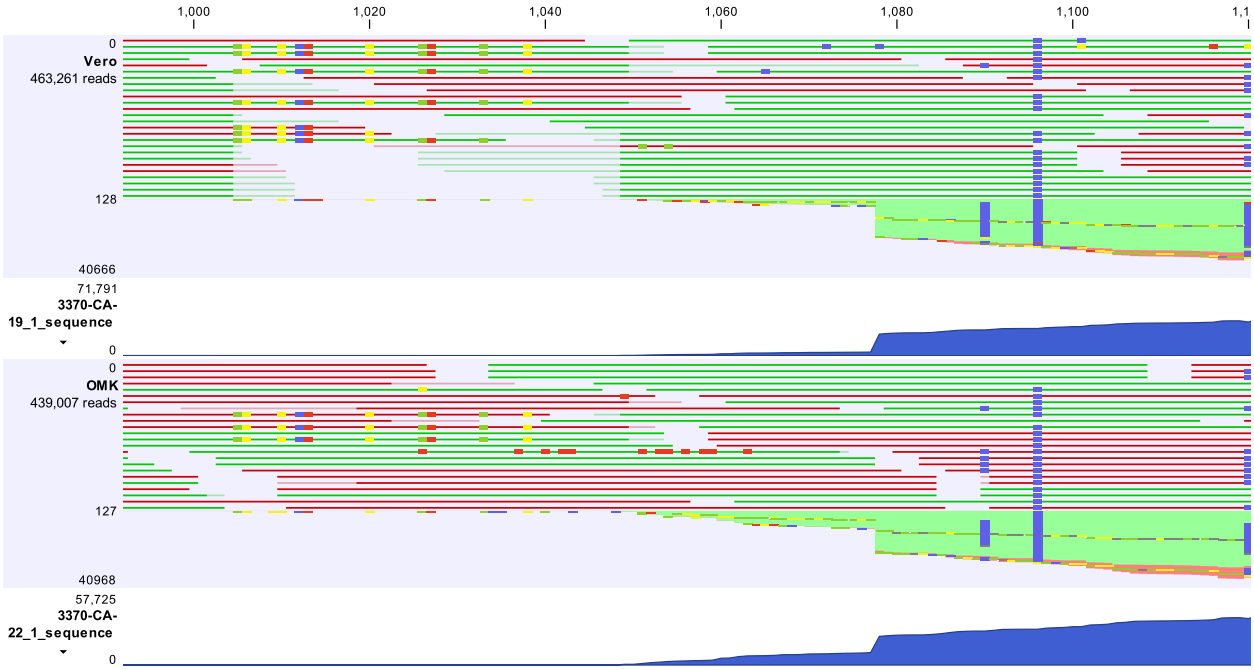

Figure 3-3: Track view read mapping for each Vero and OMK samples to OMK TRIMCyp. (A) and **(C)** Alignment of reads across a 400bp section of OMK TRIMCyp containing the TRIM and CypA homology junction. Green and red lines indicate forward and reverse reads. Multicolored blocks indicate mismatches with the main sequence. Faded red or faded green portions indicate regions of the read which do not align to reference sequence. Arrows denote the junction site and the beginning of the CypA homology region. **(B)** and **(D)** Graphical representation of read coverage across the junction region

(TRIM5α-HA). CA tubes were incubated with cell extracts, and the complexes were pelleted by centrifugation. The pelleted complexes were subjected to SDS-PAGE and analyzed by immunoblotting for the HA-tagged TRIM5α protein. First, I compared the association of TRIM5α with WT CA tubes and with mutant P90A tubes, which are impaired for binding to CypA. I observed similar levels of TRIM5α-HA co-pelleting with WT and P90A tubes, however, with an intermediate quantity of cell extract, P90A bound slightly more TRIM5α-HA (Fig. 3-3A). Next, I asked whether biochemical inhibition of CypA binding would alter the quantity of TRIM5α-HA bound to the tubes. Using the intermediate quantity of cell extract (250 μg), I performed binding assays in the presence and absence of CsA. CsA did not affect the level of TRIM5α-HA associated with the wild type or P90A tubes (Fig. 3-4B). Probing the blot with a CypA-specific antibody confirmed that the CypA protein in the cell extract was bound to the wild type but not the P90A CA tubes, and that CsA prevented CypA association with the former. Thus, neither genetic nor biochemical inhibition of CypA binding to CA decreased TRIM5α-HA binding to CA tubes *in vitro*. The affinity of CypA for CA is approximately 16 μM [144]. Because the concentration of CypA in the cell extracts was necessarily lower than that in intact cells, I considered the possibility that the concentration of CypA present in the binding reactions is insufficient to promote the binding of TRIM5α to the capsid. To test this, I added recombinant CypA at concentrations of 8, 16, or 32 μM to the reactions and determined the effects on TRIM5α binding (Fig. 3-4C). The addition of recombinant CypA did not increase the binding of TRIM5α at any CypA concentration tested. These *in vitro* data suggested that CypA does not enhance TRIM5α binding to the capsid.

To probe the effects of CypA on TRIM5α-capsid binding in cells, I exploited the ability of HIV-1 particles to overcome TRIM5α restriction *in trans* by saturation. The sequence and stability of the capsid dictate the ability of a virus to saturate TRIM5α restriction, making some viruses more efficient at saturation than others [145,146]. By comparing the ability of different viruses saturate restriction *in trans*, one can infer viruses, I employed N-MLV and B-MLV retroviruses in order to dissociate the effect of CypA on the abrogating virus from an effect on the reporter virus. B-MLV, which is not restricted by TRIM5α, was included as a negative control for loss of restriction at higher doses of abrogating virus. A fixed, sub-saturating dose of these viruses was inoculated together with increasing doses of wild type or P90A HIV-1 decoy particles. I also tested the ability of CsA to enhance type infection. Addition of wild type HIV-1 differences in TRIM5α binding. I measured the infectivity of a fixed dose of GFP-encoding reporter virus when co-infected with increasing quantities of “decoy” particles. As reporter wild increased the infection by N-MLV from 0.5% to 15% infection, reflecting an ability to saturate restriction (Fig. 3-5A); however the addition of CsA did not reduce the ability of HIV-1 to saturate restriction (Fig. 3-5B). Similarly, P90A HIV-1 decoy particles abrogated restriction of N-MLV as effectively as wild type HIV-1 (Fig. 3-5B). Infection by B-MLV particles was not enhanced by HIV-1 particles at any dose tested, consistent with the known selectivity of TRIM5α for restriction of N-MLV (Fig. 3-5A and B, right-hand panels). Together with the *in vitro* binding results, these data indicate that CypA does not promote the binding of TRIM5α to the HIV-1 capsid.

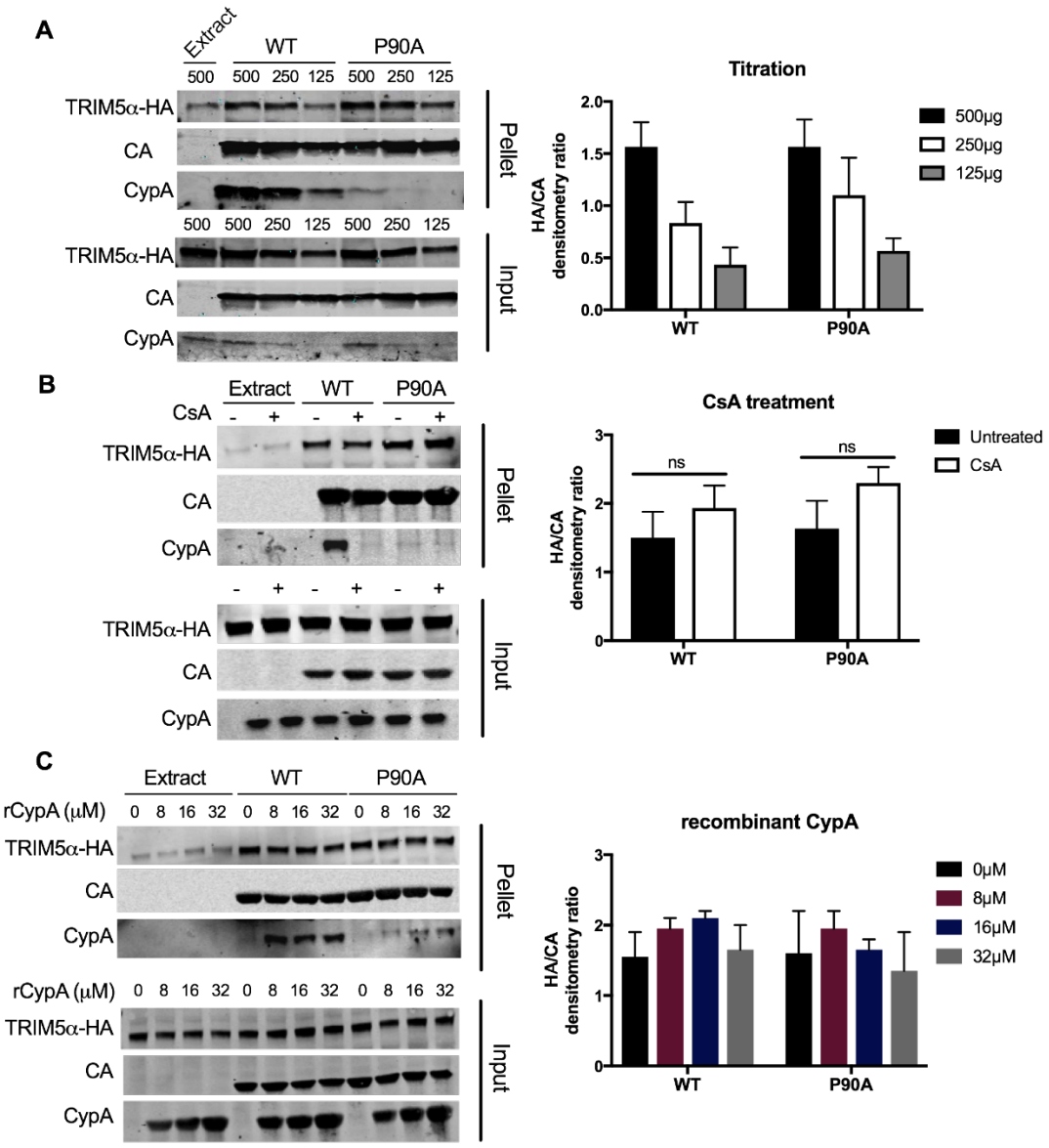

Fig 3-4: CypA does not enhance TRIM5α association with recombinant CA tubes. Recombinant CA tubular assemblies were incubated with extracts from 293T cells expressing TRIM5α-HA. CA tubes and associated proteins were pelleted and analyzed by SDS-PAGE and immunoblotting for HA, CA and CypA. Graphs shown to the right of each blot represent the quantification of blots. Data shown are the mean values of three independent determinations; error bars represent the standard error of the mean. **(A)** WT or P90A CA tubes were incubated with the indicated quantities of TRIM5α-HA cell extracts. **(B)** WT or P90A tubes were incubated with TRIM5α-HA cell extract (250 µg protein) in the presence or absence of CsA. **(C)** WT or P90A tubes were incubated with 250 µg TRIM5α-HA extracts and the indicated added concentrations of recombinant CypA in the presence or absence of CsA. Results shown are the mean values from 3 independent experiments, error bars reflect the standard error of the mean.

Capsid stability does not influence CypA-dependent TRIM5α restriction

CypA has been shown to stabilize capsids in cells and *in vitro*. A recent report demonstrating a non-canonical second binding site for CypA within the capsid which stabilizes capsid hexamers provides a potential mechanism for this activity. Our lab had previously reported that capsid stability alters viral sensitivity to TRIM5α. Since I had not observed differences in TRIM5α binding in the presence and absence of CypA, I wanted to determine whether CypA enhanced TRIM5α restriction by promoting capsid stability. To test this hypothesis, I measured the ability of CsA rescue infection of hyperstable and unstable capsid mutants. I hypothesized that hyperstable mutants would not be enhanced by CsA treatment due to limited increases in stability afforded by CypA binding**.** In contrast, unstable mutants would retain sensitivity to CsA treatment. I inoculated Vero cells with two unstable capsid mutants P38A and Q63/67A and two hyperstable mutants E128/R132A and E45A in the presence and absence of CsA (Fig 3-6). I also included R143A, a CA mutant with capsid stability similar to wildtype HIV-1 (Fig 3-6). WT and P90A were included as positive and negative controls respectively. In contrast to my hypothesis, I observed enhancement of both unstable and hyperstable mutants. WT, R143A, P38A, and E45A each enhanced more than 5-fold by CsA treatment. Interestingly, both hyperstable (E128/R132T) and unstable (Q63/67A) capsids escaped CsA-dependent enhancement of infection and were enhanced less than 2-fold by CsA treatment. These data indicate that intrinsic capsid stability does not correlate with sensitivity to CypA-dependent restriction.

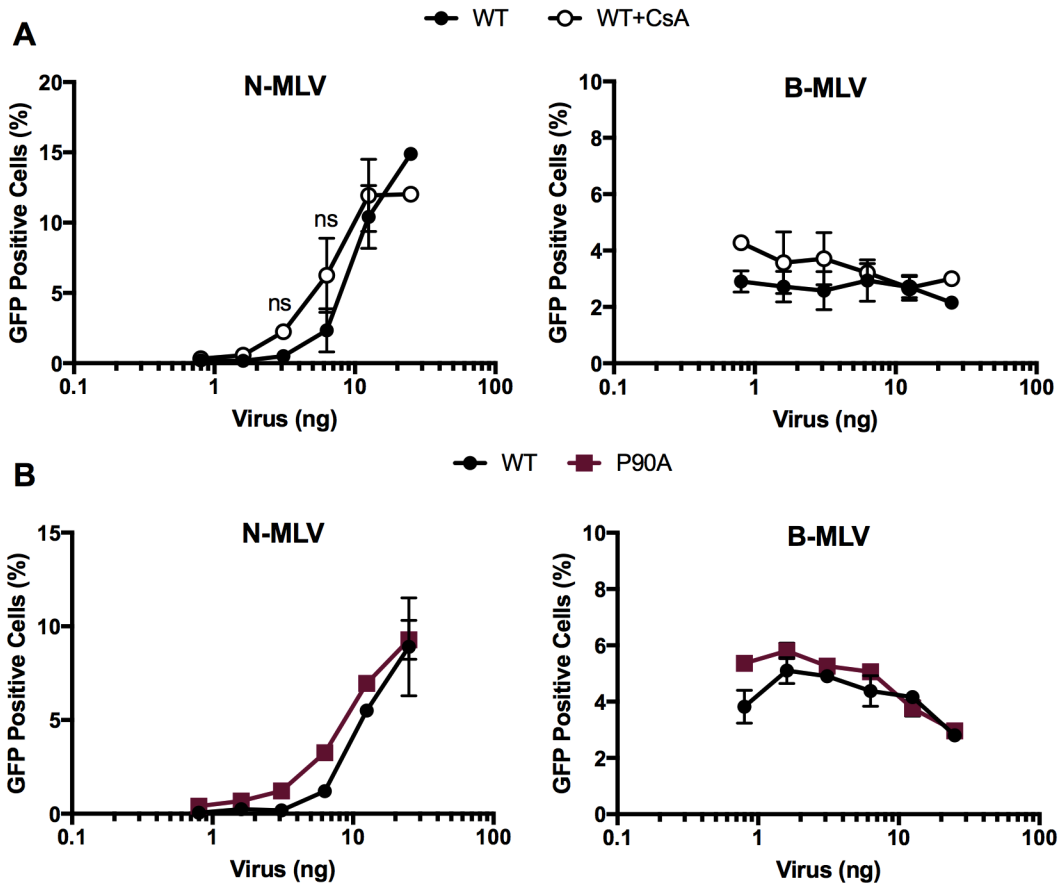

Fig 3-5: CypA-CA interactions do not enhance saturation of TRIM5α *in trans*. Vero cells were inoculated with a fixed dose of N-MLV or B-MLV GFP reporter viruses and titrating quantities of wild type HIV-1 in the presence and absence of CsA **(A**), or with the P90A mutant virus **(B).** Extent of infection was quantified by flow cytometry. Results shown are the mean values from 3 independent experiments; error bars reflect the standard error of the mean. Statistically significant differences as determined by the two-way ANOVA test are indicated as follows ns = not significant, *= $p<0.05$, **= $p<0.01$, ****= $p<0.001$, ****= $p<0.0001$

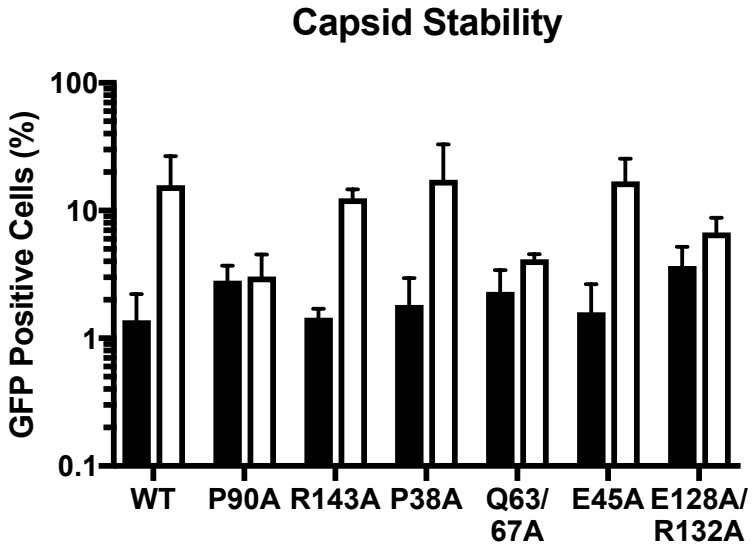

Figure 3-6. CsA enhances infectivity of HIV-1 viruses with altered capsid stability. Vero cells were inoculated with indicated WT or mutant viruses in the presence and absence of CsA. Extent of infection was quantified by flow cytometry. Values shown are mean values from 2 independent experiments; error bars represent the standard error of the mean.

Proteasome activity is dispensable for CypA inhibition of HIV-1 infection

A hallmark of TRIM5α restriction of retroviruses is the inhibition of reverse transcription. Previous studies demonstrated that proteasome activity is necessary for TRIM5α to inhibit reverse transcription. To ask whether proteasome activity is required for CypA-dependent inhibition of infection, I assayed HIV-1 infection in Vero cells treated with the proteasome inhibitor MG132, with CsA, and with both compounds. As expected, MG132 treatment alone only moderately enhanced infection of both wild type HIV-1 and the P90A mutant (Fig. 3-7A). MG132 did not affect the extent to which CsA treatment stimulated infection of wild type HIV-1 in Vero cells (Fig. 3-7A), indicating that proteasome activity is dispensable for HIV-1 inhibition by CypA. To confirm functional inhibition of the proteasome by MG132, I measured the accumulation of late reverse transcription products in these cells (Fig. 3-7B). As a control for contamination of the virus with plasmid DNA, I included a parallel infection of Vero cells treated with Efavirenz, an inhibitor of HIV-1 reverse transcriptase. As expected, MG132 increased the levels of both WT and P90A reverse transcription products, indicating that proteasome activity was inhibited [135]. CsA treatment increased the accumulation levels of both WT or P90A reverse transcripts by less than 2-fold in MG132-treated and untreated cultures (Fig. 3-7B). These data indicate that proteasome activity is dispensable for CypA-dependent inhibition of infection, and are in agreement with a previous study reporting that CypA has a limited effect on TRIM5α inhibition of reverse transcription [113].

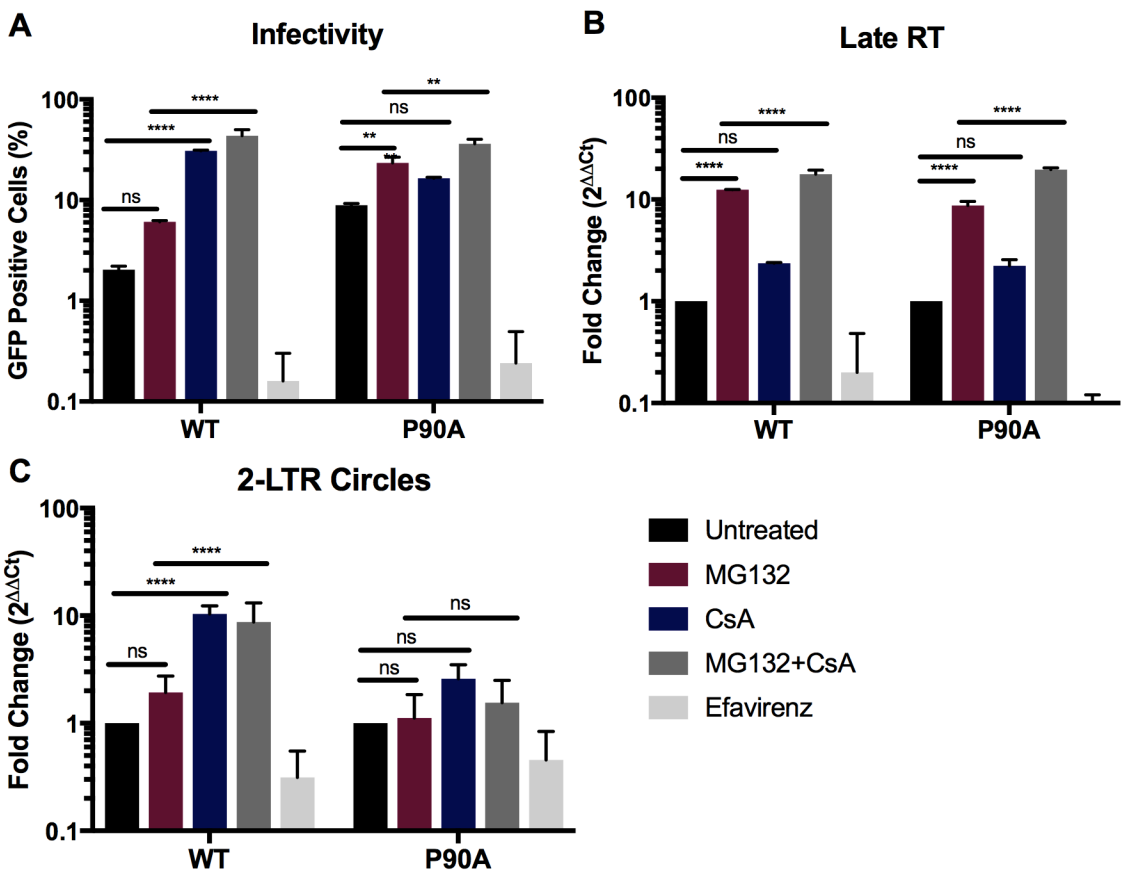

Fig 3-7. Proteasome activity is dispensable for CsA sensitive replication of HIV-1 in Vero cells. Vero cells were infected with wild type or P90A HIV-1 reporter viruses and assayed for **(A)** infection, **(B)** late RT DNA synthesis or **(C)** 2-LTR circle formation. Infection was carried out in the presence of CsA, MG132 or both as indicated. Results shown are the mean values from 3 independent experiments; error bars reflect the standard error of the mean. Statistically significant differences as determined by the two-way ANOVA test are indicated as follows ns = not significant, *= $p<0.05$, **= $p<0.01$, ****= $p<0.001$, ****= $p<0.0001$

To test the hypothesis that CypA affects nuclear import in Vero cells, I monitored 2-LTR circle formation, both when reverse transcription was rescued by proteasome inhibition, and in the absence of the proteasome inhibitor. 2-LTR circles are a product of host DNA repair mechanisms, and are commonly used as markers for HIV-1 nuclear import as they are selectively formed in the nucleus. I observed a 10.3-fold increase in accumulation of 2-LTR circles in cells infected with wildtype HIV-1 in the presence of CsA. By contrast, CsA enhanced the accumulation of 2-LTR circles formed by the P90A mutant by approximately 2-fold (Fig. 3-7). When proteasome activity was blocked by MG132 treatment, 2-LTR circle formation increased moderately for the wild type and P90A virus (2.9 fold). The addition of MG132 and CsA together enhanced wild type 2-LTR circle formation by 8.6 fold, indicating that CypA inhibits 2-LTR circle formation in the both the presence and absence of proteasome activity. Together, these observations suggest that CypA and TRIM5α cooperate to inhibit HIV-1 nuclear import and that this effect does not require proteasome activity.

Residues within the CPSF6-binding domain determine sensitivity to CypA-dependent TRIM5α restriction.

Since I had observed a CypA-dependent decrease in nuclear import, I next considered how CypA might promote TRIM5 inhibition of nuclear import. CypA is known to alter the dependence of HIV-1 infection on several nuclear pore complex (NPC) proteins, including Nup153, Nup358, CPSF6, and the transportin TNPO3. Several HIV-1 CA

substitutions, including N74D, A105T, and T107N, reduce the dependence of HIV-1 on one or more of these proteins while maintaining normal levels of infectivity [60,62,70,73,147]. I hypothesized that these host proteins were necessary for CypA-dependent restriction by TRIM5α. To test this hypothesis, I determined the ability of CsA to promote infection by the N74D, A105T, and T107N mutant viruses in Vero cells. I observed that CsA enhanced infection by these mutants 2-fold or less, similar to that of P90A HIV-1 (Fig. 3-8A). To confirm that these mutants are restricted by TRIM5α, I repeated the infection in the presence and absence of CsA in Vero cells depleted of TRIM5α or CypA. I observed 5-fold or greater increase in infection by the mutants in cells depleted of TRIM5α, indicating that the mutants are inhibited by TRIM5α despite the apparent resistance to the CypA-dependent component of restriction (Fig. 3-8B). As previously observed with P90A HIV-1 and N-MLV, CypA depletion also moderately increased infection by the other CA mutants. (Fig. 3-8C). To confirm that these mutants are able to bind CypA, I assessed incorporation of CypA into mutant HIV-1 particles by SDS-PAGE. CypA was detected in wild type and mutant virions, with the exception. of the P90A mutant (Fig 3-8D). These results indicate that CypA binding is necessary but not sufficient to confer sensitivity to CypA-dependent restriction, and suggest that these residues specifically contribute to restriction by TRIM5α

To determine the requirement for NPC proteins in CypA-dependent restriction, I depleted Vero cells of the four NPC associated proteins which are dependent on CypA, Nup153, Nup358, CPSF6 and TNPO3 using RNAi. Extent of depletion was confirmed by RT-qPCR of mRNA transcripts isolated from shRNA-treated Vero cells (Fig 3-9). In order to determine any phenotypes resulting from protein depletion that are independent

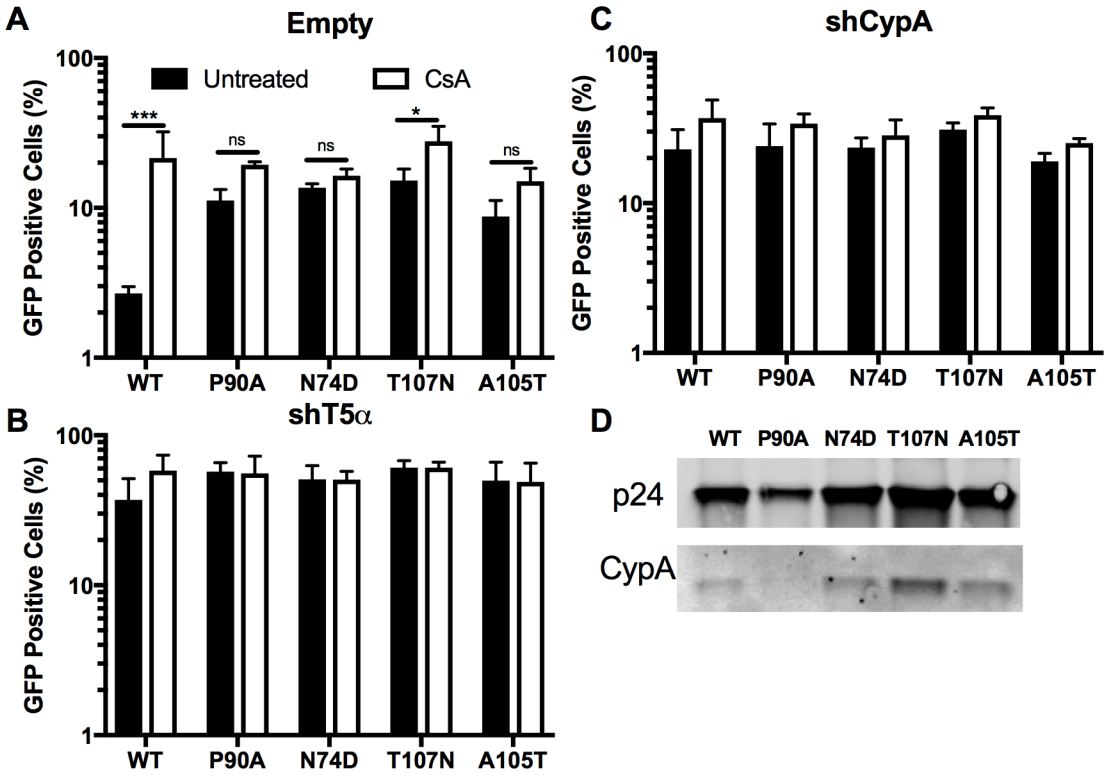

Fig 3-8 Selected CA mutants escape CsA-dependent TRIM5α restriction. Vero cell lines were assayed for infection by wild type HIV-1 and indicated CA mutants in the presence or absence of CsA. **(A)** Vero-shEmpty, **(B)** Vero-shT5α, or **(C)** Vero-shCypA were inoculated with WT or mutant HIV-GFP viruses. **(D)** Incorporation of CypA into HIV-1 virions. Virions were pelleted and analyzed by immunoblotting for CA protein and CypA. Values shown are mean values from 3 independent experiments; error bars represent the standard error of the mean. Statistically significant differences as determined by the two-way ANOVA test are indicated as follows ns = not significant, *= $p<0.05$, **= $p<0.01$, ****= $p<0.001$, ****= $p<0.0001$

of TRIM5α restriction, I first compared the infectivity of the TRIM5α sensitive N-MLV and infectivity of N-MLV was reduced by more than 10-fold compared to B-MLV regardless of which protein was depleted, suggesting that TRIM5α restriction in general is not affected by depletion of CPSF6, TNPO3, Nup153 or Nup358 (3-9A). To address the contribution of these NPC proteins to CypA-dependent TRIM5α restriction, I inoculated control and knockdown cells with wildtype HIV-1 in the presence and absence of CsA. Depletion of Nup358 resulted in the greatest reduction in HIV-1 infection, reducing infection from 2% to 0.5%. Depletion of CPSF6, TNPO3, or Nup153 did not significantly alter the replication of HIV-1. However, depletion of CPSF6 did prevent enhancement by CsA suggesting that CPSF6 is important for CypA-dependent TRIM5α restriction (Fig 3-9B). In contrast, CsA treatment enhanced wildtype HIV-1 infection to the same extent in cells depleted of TNPO3 or Nup153 as in control cells. Depletion of Nup358 actually increased the enhancement of CsA in a manner proportional to the decrease in infectivity of untreated cells. In contrast to my hypothesis, these results suggest that neither CPSF6, TNPO3, Nup153 nor Nup358 promote restriction by TRIM5α. However, CSPF6 appears to contribute to the sensitivity to CsA treatment. Together these data indicate that specific residues within the resistant B-MLV GFP reporter viruses in transduced cells. As previously observed, CPSF6 binding interface determine sensitivity to CsA, however the contribution of proteins known to bind to this region do not appear to be necessary for restriction.

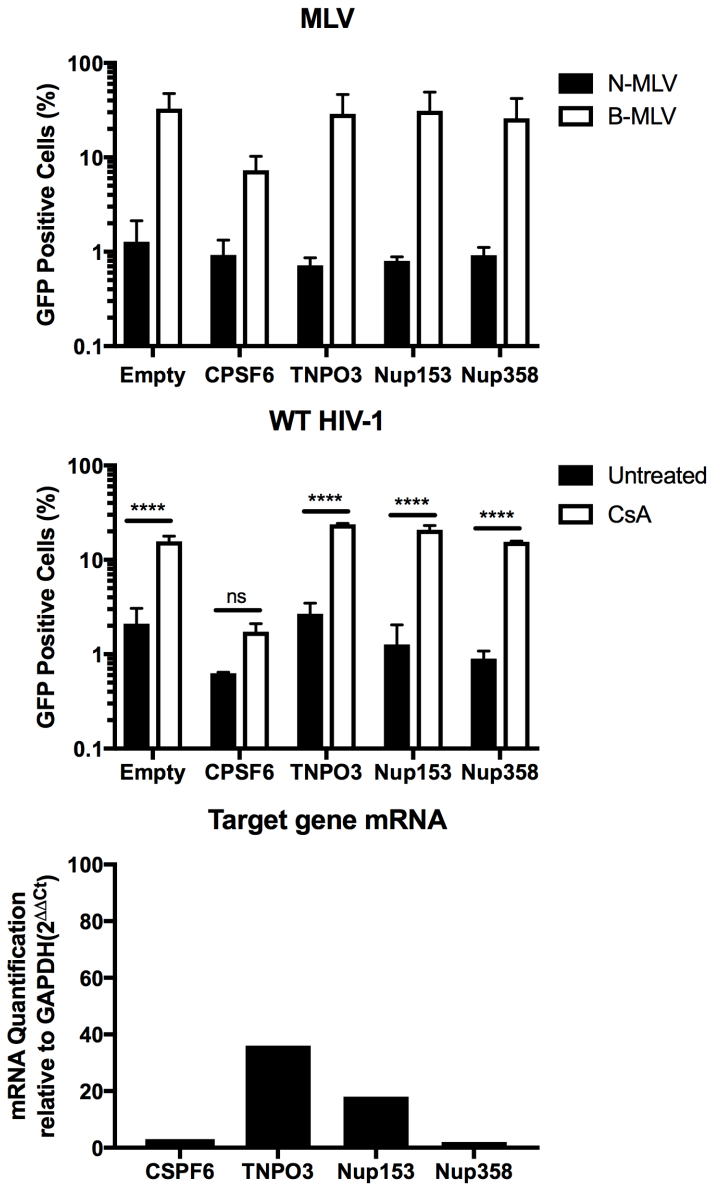

Fig 3-9. Depletion of CSPF6 prevents enhancement of HIV-1 infectivity by CsA. Control or Vero cells depleted of CPSF6, TNPO3, Nup153 or Nu358 were inoculated with **(A)** N or B-MLV or **(B)** WT HIV-1 in the presence and absence of CsA. Values shown are mean values from 2 independent experiments; error bars represent the standard error of the mean. **(C)** Quantification of target gene mRNA to confirm protein depletion. Statistically significant differences as determined by the two-way ANOVA test are indicated as follows ns = not significant, *= $p<0.05$, **= $p<0.01$, ****= $p<0.001$, ****= $p<0.0001$

Discussion

The aim of this study was to identify the mechanism by which CypA potentiates TRIM5α restriction of HIV-1. Initial observations of CypA-dependent TRIM5α restriction showed that depletion of CypA, or treatment with CsA, promotes HIV-1 infection of cells expressing old world monkey alleles of TRIM5α. These studies also showed that CypA can act as early as 30 minutes after HIV-1 entry to enhance restriction (11, 35). To achieve this goal, I measured the contribution of CypA to each step of TRIM5α restriction. First, I confirmed that CypA promotes TRIM5α restriction of HIV-1 in cells depleted of TRIM5α or CypA by RNAi. Next, I assessed the possibility that a novel TRIMCyp protein was responsible for the CypA-dependent phenotype observed in Vero cells. Analysis of the Vero transcriptome did not reveal any sequences with homology to both TRIM5α and CypA. While these results do not strictly rule out the possibility that a TRIMCyp protein exists in Vero cells, together with the observation that depletion of TRIM5α is sufficient to rescue replication of HIV-1 and prevent enhancement by CsA treatment, we conclude that TRIMCyp protein is not responsible for the CypA-dependent phenotype observed in these cells.

TRIM5α binding to capsid is a necessary first step in inhibition of HIV-1. I hypothesized that CypA promoted binding of TRIM5 to capsid in order to potentiate restriction. Neither genetic nor biochemical inhibition of CypA binding reduced TRIM5 binding to capsid. The addition of recombinant CypA also did not affect co-pelleting of TRIM5α. When TRIM5α binding in cells was measured using the abrogation of restriction assay, no significant differences in the ability of HIV-1 to abrogate restriction

of N-MLV were observed, indicating that CypA does not promote binding of TRIM5α to the capsid. Since capsid stability is also known to affect the ability of TRIM5α to bind capsid, we also measured the sensitivity of panel of CA mutants to CsA treatment, however I found that both hyperstable and unstable capsids were stimulated by CsA treatment. Together these observations indicate that CypA promotes TRIM5α restriction at a step after binding of TRIM5α. My observation that inhibition of CypA increased the levels of 2-LTR circles, but not total reverse transcripts confirmed this hypothesis (Figs 3-6B and C, [113]). Inhibition of cellular proteasome activity did not alter the ability of CypA to potentiate TRIM5α restriction, consistent with an effect mediated after reverse transcription (Fig 3-6A). Previous reports have suggested that the ability of TRIM5α to block nuclear import of HIV-1 is revealed only upon the inhibition of proteasome activity [79,135,148]. As CsA treatment rescues nuclear import in the presence and absence of proteasome activity, my results suggest that inhibition of nuclear import is a distinct step in TRIM5α restriction of HIV-1, and depends on CypA.

How might CypA promote TRIM5α inhibition of nuclear import? In permissive human cells CypA promotes nuclear entry of HIV-1 [62,149]. Depletion of CypA, or mutation of the CA binding site, reduces HIV-1 dependence on specific components of the nuclear pore complex (NPC) [60,62,63,69,70]. We observed that CsA did not stimulate infection of Vero cells by CA mutants exhibiting reduced dependence on canonical HIV-1 NPC components, yet the mutants were sensitive to TRIM5α restriction and retained the ability to bind CypA (Fig 6). These results suggest engagement of the nuclear pore determines sensitivity to TRIM5α. However, when Nup153, Nup358, or TPNO3 were depleted from host cells, CsA still stimulated infection of wildtype HIV-1

suggesting that interaction with these proteins is dispensable for CypA-dependent restriction. Intriguingly, depletion of CPSF6 did prevent enhancement of infection by CsA suggesting that CPSF6 is necessary for CypA sensitivity but not for restriction by TRIM5α. At first glance this result is unexpected. If CPSF6 is necessary for restriction then preventing its interaction with capsid should rescue infection in addition to preventing enhancement by CsA. However, this conclusion is based on the assumption that the mechansim of escape by provided by N74D, T107N, and A105T is solely inhibition of CPSF6 binding. In addition to preventing the interaction with CPSF6 and relieving the dependence on canonical Nup proteins, N74D also gains the ability to utilize non-canonical nucleoporins Nup85 and Nup155. If inhibition of specific NPC components is necessary for CypA-dependent TRIM5α restriction, then introduction of the N74D mutation provides an alternative route of entry into the nucleus in addition to blocking the interaction with CPSF6. I propose a model in which CypA directs HIV-1 to engage specific nucleoporins, namely Nup153 and Nup358. In the presence of TRIM5α those interactions are sterically blocked, leading to a decrease in nuclear import and infection. Providing an alternate pattern of nucleoporin engagement or preventing the interaction with CypA allows escape from this restriction.

Table 3.1 *De Novo* Assembly Statistics

		Total number of reads	% Matched Reads	Total number of contigs	Longest contig length (bp)
OMK	**1**	78,022,554	64.060	14,030	14,986
	2	70,520,226	62.992	12,638	13,931
	3	56,613,962	60.466	11,126	13,692
Vero	**1**	77,766,242	78.951	10,473	19,578
	2	73,627,466	75.059	11,408	17,631
	3	69,573,622	73.425	12,430	19,555

Table 3.2 OMK TRIMCyp BLAST Summary Statistics

	Aligned to OMK TRIMCyp	Aligned to Junction	E Value	% Query Overlap	% Identity
OMK_Contig_150	Yes	Yes	0	94.4	98.6
OMK_Contig_276	Yes	Yes	0	79	99.6
Vero_Contig_236	Yes	No	0	40.7	94.3
Vero_Contig_364	Yes	No	0	40.8	94.3

Table 3.3 Mapping Statistics for OMK TRIMCyp

	Total mapped reads	% Total Reads	Reads in Pairs	Broken Paired Reads
Vero	264,747	0.12%	120,348	144,399
OMK	292,061	0.14%	175,720	116,341

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

The host protein CypA can both stimulate and inhibit HIV-1 infection. In cells in which CypA promotes infection, it has been shown to promote to viral uncoating, reverse transcription, nuclear import, and integration site-selection [62,67,108,150]. The mechanisms by which CypA inhibits HIV-1 are less clear. At the time that I began this work there were two well-established examples of CypA-dependent inhibition of HIV-1. I investigated these phenotypes with the goal of identifying novel and shared features of CypA-dependent inhibition of HIV-1. My first aim was to identify the host protein responsible for the CsA R/D phenotype observed in human cells [114,151,116]. My second aim was to identify the mechanism of CypA-dependent TRIM5α restriction [91,91,140]. My work has accomplished both of these goals by identifying CPSF6 as the host protein responsible for CsA R/D phenotype, and demonstrating that CypA specifically contributes to TRIM5α inhibition of nuclear import [74]. The observations presented here suggest two conserved features of CypA-dependent inhibition. First, that a conserved interface within HIV-1 CA determines sensitivity to CypA-dependent inhibition, and second that restriction factor binding is necessary but not sufficient to inhibit HIV-1. I hypothesize that CypA inhibits HIV-1 infection using an unusual mechanism that does not rely on binding of the restriction restriction factor, but on indirect interference perhaps by preventing access to nuclear pore co-factors.

In addition to serving as the delivery vehicle for the genomic payload, the HIV-1 capsid mediates important virus-host interactions. The growing list of capsid-dependent

proteins, including Nup153, Nup358, CPSF6, TRIM5α, MxB, and KIF5B, can promote or inhibit the infectivity of HIV-1 at a variety of steps during early infection. Intriguingly, CypA regulates the dependence of HIV-1 on a number of these proteins including those known to inhibit infection, CPSF6, TRIM5α and MxB. One explanation for the ability of CypA to regulate the activity of this diverse group of molecules is that CypA controls host protein binding to capsid. CypA significantly alters the structure of HIV-1 CA, locally by catalyzing *cis-trans* isomerization of the proline rich CypA-binding loop of HIV-1 CA, and en masse by stabilizing CA hexamers [95,105,107,152]. Consequently, I hypothesized that CypA promotes binding of inhibitory capsid-binding molecules in order to block HIV-1 infection. The work presented here argues against that hypothesis. I measured binding of CPSF6 and TRIM5α to CA tubes in the presence and absence of CypA using the *in vitro* capture assay and found no ability of CypA to promote host protein binding to tubes. With respect to TRIM5α, I also assessed the ability of CypA to enhance HIV-1 saturation of restriction *in trans* and found that CypA also did not affect TRIM5α binding in cells. These observations indicate that CypA does not affect binding of CypA-dependent host proteins, CPSF6 and TRIM5α. This conclusion is corroborated by reports regarding the recently identified, myxovirus resistance protein B (MxB). MxB is an interferon-stimulated protein, that binds to the capsid and inhibits infection in a CypA-dependent manner [84,85,90]. Similar to my results regarding CPSF6 and TRIM5α, CypA did not affect the ability of MxB to bind to capsid [153]. These results suggest that CypA does not promote binding of inhibitory capsid-binding proteins to the capsid in order to inhibit HIV-1 infection. One caveat to this conclusion is the amount of CypA present in *in vitro* binding reactions conducted here and elsewhere. Generation of

cellular extracts necessarily lowers the concentration of CypA, and even when added exogenously, as described in Fig 3-4, concentrations of CypA may still be insufficient to regulate factor binding to tubes. With respect to TRIM5α, I confirmed that binding to capsid was also unaffected in cells, however for CPSF6 and MxB, CypA-dependent binding in cells has not been ruled out. The utilization of recombinant forms of CA rather than native HIV-1 cores is also limitation for measuring and interpreting changes in capsid binding. If CypA is inducing structural changes in CA in order to regulate binding of host proteins, the use of disulfide stabilized forms of capsid may limit the ability to observe changes in CA structure.

If CypA does not promote binding of restriction factors to the HIV-1 capsid, how does it potentiate their activity? The answer may lie in the mechanisms underlying the ability of CypA to promote infectivity in permissive cells. My studies indicate that CypA primarily contributes to TRIM5α-dependent inhibition of nuclear import. When cytoplasmic and nuclear forms of HIV-1 DNA were quantified by qPCR, CsA treatment specifically rescued accumulation of 2-LTR circles in Vero cells, suggesting that CypA-dependent inhibition primarily occurs at nuclear import. Similarly, previous studies from our lab and others demonstrate that inhibition of CsA R/D mutants also occurs at nuclear import [154,155]. Parallel observations have been made regarding MxB, which accumulates near nuclear pores and blocks nuclear import of HIV-1 [86]. These data indicate that CypA-dependent inhibition relies in part on inhibiting viral entry into the nucleus. In permissive cells, CypA is known to enhance nuclear import of HIV-1 by promoting the dependence on two nucleoporins, Nup153 and Nup358. Several reports have demonstrated that the requirement for Nup153 or Nup358 is dictated by CypA.

Together, these data suggest that CypA can both promote and inhibit nuclear import, and suggested to me that CypA could be utilizing a common mechanism to achieve both effects.

Inhibition of wildtype HIV-1 by MxB or the CsA R/D mutants by CPSF6 can be relieved by the introduction of one of three mutations, N74D, T107N, or A105T. These mutations block CPSF6 binding to capsid, and reduce viral dependence on nucleoporins Nup153 and Nup358. Surprisingly, I found that these same mutations partially relieved CypA-dependent TRIM5α restriction. These observations suggest that engagement of specific host factors is important for enhancement of infection in permissive cells and for inhibition of infection in restrictive cells. I tested this hypothesis directly in the context of CypA-dependent TRIM5α inhibition of HIV-1 by depleting Vero cells of Nup153, Nup358, CPSF6 and TNPO3. I predicted that inhibiting expression of these proteins would relieve sensitivity to CypA-dependent TRIM5α restriction. In contrast, I still observed potent enhancement of infection by CsA treatment in cells depleted of Nup153 or TNPO3 (Fig 3-9). While in cells depleted of Nup358, infection appeared to become slightly more sensitive to CsA treatment. Interestingly, depletion of CPSF6 also did not rescue infection, but did relieve sensitivity to CsA treatment. Initially, I found these results puzzling, because I predicted that the mechanism of escape provided by the N74D, A105T, and T107N substitutions was solely related to reducing the dependence on Nup153, Nup358, CPSF6 or TNPO3. Accordingly, I expected depletion of these proteins to phenocopy the introduction of the rescue mutations. However, the N74D substitution has also been reported to confer the ability to utilize a unique set of nucleoporin proteins. While WT HIV-1 infection is inhibited by depletion of

Nup153 and Nup358, N74D infection is inhibited by depletion of Nup85 and Nup155[60]. This observation suggests that the mechanism of escape from CypA-dependent TRIM5α restriction could be related to the ability to utilize a separate pathway for entry into the nucleus. Thus, N74D could provide escape from inhibition by reducing dependence on restriction sensitive nuclear proteins yet maintain an efficient route into the nucleus. Alternate patterns of NPC engagement of have not been demonstrated for A105T or T107N, but it is plausible that these mutants share this capacity since they also demonstrate a reduce affinity for CPSF6.

Based on these observations, I propose a new model for CypA-dependent inhibition, in which CypA coordinates with inhibitory capsid-binding proteins in order to prevent access to specific nuclear co-factors. In this model, the incoming capsid is bound by CypA shortly after fusion. When the preintegration complex (PIC) reaches the nuclear pore, CypA directs the virus to engage Nup153 and Nup358. This activity is positive in permissive cells, increasing the efficiency of nuclear import. In the presence of an inhibitory capsid-binding protein (i.e. TRIM5α, MxB, or CPSF6) the PIC is unable to interact with Nup153 or Nup358, inhibiting nuclear import due to steric hindrance (Fig 4-1). When CypA binding is inhibited, or if the N74D, T107N or A105T CA substitutions are present in the capsid the virus is no longer obligated to utilize Nup153/Nup358 for import and gains access to the nucleus using non-canonical nucleoporins. Thus, CypA binding promotes infectivity in permissive cells by enhancing nuclear import, but inhibits infection in restrictive cells by forcing the virus to engage the NPC in a manner that is sensitive to the presence of inhibitory capsid-binding proteins.

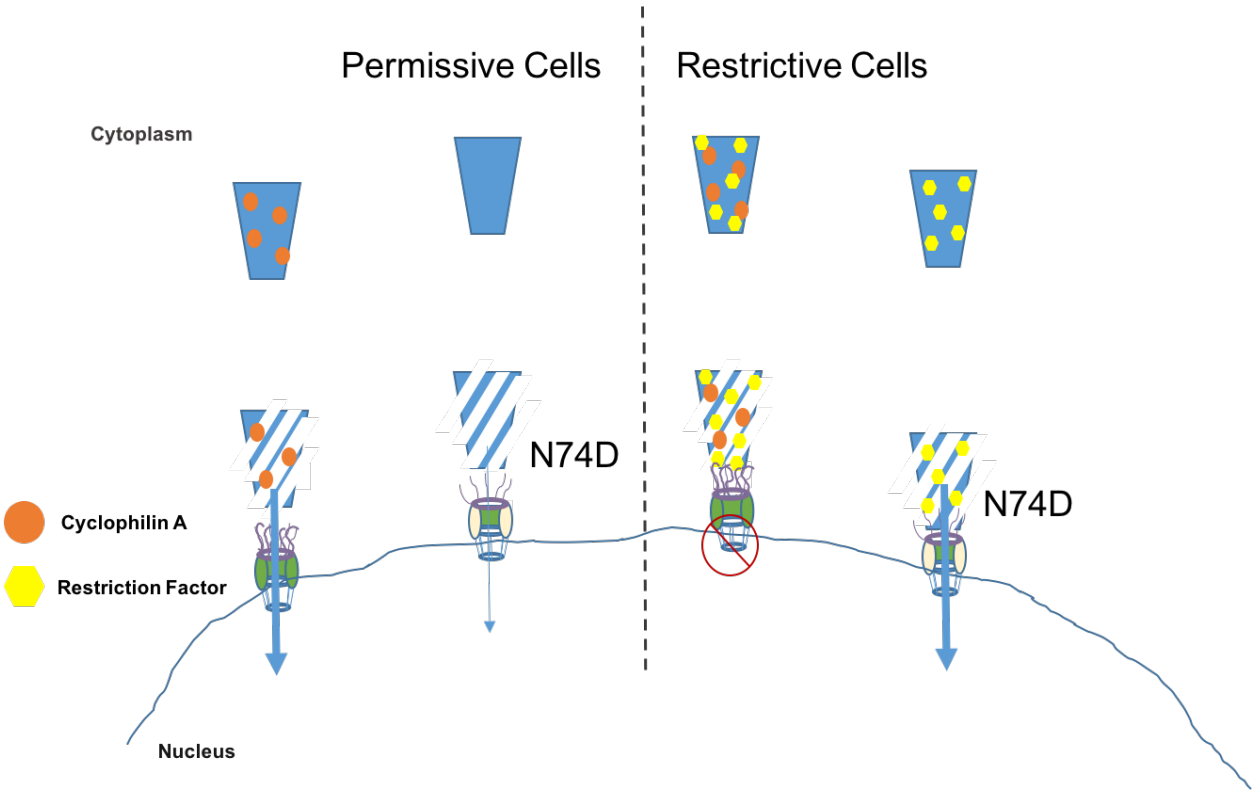

Figure 4-1: Model for CypA-dependent inhibition of HIV-1 by inhibiting nuclear import. The incoming viral capsid is bound by CypA (orange circles). The viral core undergoes uncoating and the viral PIC engages the nuclear pore. In the presence of CypA, Nup358 and Nup153 more efficiently bind the capsid and promote nuclear import. In its absence the HIV-1 PIC less efficiently engages the capsid resulting in reduced nuclear import. In restrictive cells CypA directs the virus to interact with Nup153 and Nup358, however these interactions are blocked by the presence of the restriction factor. Inhibiting CypA allows interaction with alternative nuclear pore components (indicated in yellow) and escape from restriction.

This model relies on the premise that inhibiting interactions with specific NPCs inhibits HIV-1 infection in a CypA-dependent manner. At least two reports have already demonstrated that HIV-1 sensitivity to depletion of Nup153 or Nup358 is relieved in the absence of CypA, suggesting that CypA directs the virus to engage these proteins. The next step would be to determine if TRIM5α and MxB compete with components of the NPC for binding to the capsid. This could be completed *in vitro* by measuring the ability of HIV-1 CA tubes pre-incubated with recombinant forms of TRIM5α or MxB to bind Nup153 and Nup358. In cells, this is a much more complicated task becuase components of the NPC cannot easily be added *in trans* in order to rescue nuclear import. However, an approach using “mixed core” viruses one might be able demonstrate a dose-dependent relationship between the amount of restriction factor present on the capsid and inhibition of nuclear import. In this assay, plasmids expressing sensitive and resistant CA proteins are co-transfected to generate viruses with capsids containing both wildtype and mutant CA. Our lab previously utilized this approach to demonstrate that particles containing as little 25% of restriction sensitive CA are subject to TRIM restriction [146]. Therefore it may be possible to find a ratio in which the virus is inhibited for infection, but not subject to CypA-dependent inhibition of nuclear import.

I hypothesize that steric inhibition of NPC binding also determines CPSF6-dependent restriction of CsA R/D mutants. In this context, CPSF6 serves as the host protein which blocks access to the NPC, however it is not clear why wildtype HIV-1 escapes inhibition. One hypothesis is that CsA R/D mutants have increased affinity for CPSF6. An alternative explanation for this phenotype is that CsA R/D mutations perturb

normal viral uncoating. There are two models of uncoating, one which supposes continuous disassembly en route to the nucleus, and another which suggests that uncoating is triggered at the nuclear pore. The CsA R/D mutations may alter capsid stability in a manner that inhibits uncoating. In the same way the presence of TRIM5α or MxB may also interfere with uncoating by stabilizing the capsid, leading to aberrant uncoating. In this model of CypA-dependent inhibition, preventing CypA binding to capsid or the presence of the suppressor mutations serves to destabilize the capsid. With the recent discovery of second binding site within the HIV-1 capsid for CypA binding which stabilizes hexamers *in vitro,* and reports demonstrating that the N74D and A92E substitutions can alter uncoating in cells, any future studies should address these hypotheses [105,157]. However, based on the observation that CypA-dependence is cell-type specific, I doubt that an intrinsic viral property such as capsid stability mediates CypA-dependent inhibition.

What cell specific features could mediate the cell-type specificity of this phenotype? Differences in protein localization or expression, or the presence of genetic isoforms of CypA or CPSF6 with different activities could explain the cell-type specificity of these phenotypes. Several reports demonstrating heterogeneous function and composition of nuclear pore complexes also hint at an interesting explanation. Nuclear pore complexes are often thought of as being homogenously composed, however new data suggests that NPCs can be composed of different nucleoporins and function in a tissue specific manner or under different conditions within one cell. [reviewed in 148]. NPC composition and or function could vary significantly between permissive and restrictive cells, leading to different rates of nuclear import and or availability of NPC

proteins for capsid-interactions. Future studies of this phenotype should include an analysis of the potential differences in NPC function or composition in permissive and restrictive cells.

In hindsight, the description of this set of observations as a “restriction” may be a misnomer. In general, HIV-1 restriction factors are defined as proteins whose primary function is to block viral replication. These activities can be direct such as the action of TRIM5α and tetherin to inhibit reverse transcription and budding respectively, or indirect such as inhibition by APOBEC3G and SAMHD1 by sequestering and degrading host nucleic acids. In contrast, the mechanisms investigated in my work, feature the human proteins CypA and CPSF6 which promote infection in some contexts and are inhibitory in others. CPSF6 is especially puzzling because of the ostensibly positive role it plays during infection *in vivo*. Although dispensable in culture, in primary T-cells CPSF6 plays an important role in replication of HIV-1 as evidenced by maintenance CPSF6 binding during replication assays. In macrophages, HIV-1 binding to CPSF6 is absolutely required for infection in order to evade detection by innate immune surveillance. I hypothesize that the inhibitory actions of CypA are a consequence of a typically positive action to direct viral engagement with specific host proteins. Thus enhanced inhibition of HIV-1 by TRIM5α or CPSF6 in the presence of CypA is a consequence of this activity rather than an evolutionary effort by the cell to control infection. In exchange for fitness advantages conferred *in vivo,* the virus tolerates a level of vulnerability to inhibition of nuclear import. However, even if the “restriction” does not represent a biologically relevant mechanism of inhibition, the vulnerabilities exposed by study of this phenotype do represent a therapeutic opportunity. The region of the capsid that regulates

interaction with the NPC proteins and CPSF6 described here has already been pharmaceutically targeted with a compound known as PF-3450074 (PF74) [125,159,160]. PF74 was discovered and characterized in a small-molecule screen for HIV-1 inhibitors. Initial studies focused on its ability to accelerate uncoating by destabilizing the capsid. At low concentrations, PF74 mirrors the CypA-dependent restrictions described here [159]. The activity of PF74 is potentiated by the presence of CypA while specific capsid mutants in the CPSF6 binding interface, including N74D and A105T display partial resistance to drug treatment. In culture, sensitivity to these drugs is easily overcome by evolution of mutations within the CPSF6 binding interface, however *in vivo* inhibition may not be easily overcome in light of extensive selective pressure to maintain interaction with CPSF6 [76]. Screens to identify potent and selective inhibitors that improve upon the vulnerability targeted by PF74 could lead to novel inhibitors of HIV-1.

In summary, my work has identified CPSF6 as the host protein responsible for inhibition of the CsA R/D mutants and has revealed that CypA potentiates TRIM5α restriction of HIV-1 by inhibiting nuclear import. Together these findings suggest novel mechanisms for inhibition of HIV-1 that rely on inhibiting engagement of the nuclear pore complex and the engagement of CypA.

CHAPTER V

MATERIALS AND METHODS

Cells and Viruses

HeLa, CRFK, Vero and Owl Monkey Kidney (OMK) cell lines were purchased from the American Type Culture Collection (ATCC cat. nos. CCL-2, CCL-94, CCL-81 and CRL-1556, respectively). The 293T-pLPCX-TRIM5α_{agm} cell line was previously described [133]. The Tzm-bl cell line was obtained from the NIH AIDS Reagent program. All cells were cultured at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (10%), penicillin (100 U/ml), and streptomycin (100 µg/ml) (D10). Virus stocks were generated by calcium chloride transfection of 293T cells for infectivity assays, or with TransIT-293 Transfection Reagent (Mirus) for use in experiments involving real-time PCR [161]. HIV-1 reporter virus was produced from HIV-GFP, an Env-defective HIV-1 proviral construct encoding green fluorescent protein (GFP) in place of Nef [162], or from the *corresponding* mutants encoding a substitution for proline at position 90 (P90A), alanine at position 92 (A92E), for asparagine at position 74 (N74D), for alanine at position 105 (A105T), and for threonine at position 107 (T107N). Double mutants included both A92E and N74D, A92E and A105T, A92E and P90A. Particles were pseudotyped by vesicular stomatitis virus glycoprotein (VSV-G) via cotransfection of the proviral constructs (20 µg) with pHCMV-G (5 µg). VSV-G-pseudotyped HIV-1 particles used in abrogation-of-restriction assays were produced from Env-defective R9 (R9ΔE) proviral construct and the corresponding P90A, A92E, A92E/P90A, A92E/A105T mutant proviral

constructs. HIV-1 stocks were quantified using a p24 enzyme-linked immunosorbent assay (ELISA) or titrated on CRFK cells to normalize infectious dose, aliquoted and stored at -80°C prior to use. N-tropic murine leukemia virus (N-MLV) and B-tropic MLV vector particles were produced by cotransfection of pVPack-GP-N (Agilent Technologies) or pVPack-GP-B (encoding N-tropic or B-tropic MLV Gag-Pol respectively) (20 µg) with pBABE-EGFP (15 µg) and pHCMV-G (5 µg).

Infectivity Assays

Vero, HeLa, or CRFK cells (15,000 per well) were seeded in a 96-well plate. The next day, cells were inoculated with GFP-encoding HIV-1 for 16 h in the presence of polybrene (8 µg/ml) and cyclosporine A (CsA, Calbiochem, 5 µM), or MG132 (Sigma, 5 µM) where indicated. 16 h after virus inoculation, virus and drugs were removed and replaced with fresh media. 48 h after infection, cells were detached with trypsin-EDTA and fixed with an equal volume of phosphate-buffered saline (PBS) containing 4% paraformaldehyde. The extent of infection was assayed by flow cytometry for GFP expression using an Accuri C6 flow cytometer. A minimum of 5,000 cells was analyzed for each sample.

Abrogation-of-Restriction Assay

VSV-G pseudotyped N-MLV-GFP and B-MLV GFP reporter particles were titrated onto Vero cell monolayers to determine the dose corresponding to approximately 1% infection, Or A92E-GFP and WT-GFP reporter particles were titrated on HeLa

cells to determine a sub-saturating dose. Vero or HeLa cells (15,000 cells per well in 96-well plates) were inoculated with a fixed quantity of reporter virus together with titrating concentrations of VSV-G-pseudotyped HIV-1 particles in the presence of polybrene (8 μg/ml). 16 h after inoculation, virus and media were removed and replaced with media. 48 h after inoculation, cells were detached with trypsin-EDTA and fixed with an equal volume of PBS containing 4% paraformaldehyde. Extent of infection was quantified by flow cytometry with an Accuri C6 flow cytometer. At least 5,000 cells were analyzed for each sample.

Engineering, Purification, and Assembly of recombinant HIV-1 CA

Mutations were introduced into pET21a(+) vectors encoding cysteine-mutated HIV-1 CA at positions 14 and 45, using the QuikChange Multi Site-Directed mutagenesis kit (Agilent) according to manufacturer's direction. The original 14C/45C HIV-1 CA plasmids were a kind gift from Jinwoo Ahn. Recombinant CA was prepared from *E.coli* as follows. Isopropyl β-D-1-thiogalactopyranoside (IPTG 1mM, GoldBio) was added to cultures of *E. coli* expressing wildtype or mutant CA proteins entering the exponential growth phase, OD_{600}= 0.6. Cultures were then shifted to 25°C and grown for 12hrs before pelleting at 5.5krpm for 5 min. Cells were lysed by sonication, using the Sonic Dismembrator Ultrasonic Processor with the following settings: Amplitude: 20, Process Time: 2:30 m, Pulse-ON time: 30 s, Pulse-OFF time: 30 s. Sonicated lysates were centrifuged for 1hr at 15,000 krpm at 4°C in 60ml tubes. Pellets were discarded and supernatants were subject to 25% ammonium sulfate precipitation for 45 min at 4°C with stirring. Precipitates were collected by centrifugation for 20min at 8krpm at 4°C, and then resuspended

in buffer containing 50ml Tris pH8 and 200mM BME for 15minutes with nutation. Protein solutions were then dialyzed overnight in S-column buffer using 10,000 MWCO dialysis tubing. The next day solutions were cleared by centrifugation for 20min at 8krpm at 4C, before cation exchange chromatography using Bio-Scale UNOsphere S Cartridges and BioRad Bio-logic DuoFlow FPLC. Fractions containing CA as determined by gel electrophoresis were combined and dialyzed in Q-column buffer (20mM Tris pH 8, 60mM NaCl, 60mM BME) using 10000 MWCO dialysis tubing. Dialyzed protiens were cleared by centrifugation for 20min at 8 krpm at 4C before anion exchange chromatography using Bio-Scale UNOsphere Q Cartridges. Fractions containing CA as determined by gel electrophoresis were combined and concentrated using the Amicon Ultra-15 Centrifugal Filter Units for 30 min at 3500rpm according to manufacturer directions. Once concentrations reached at least 10mg/ml proteins were aliquoted and flash frozen using liquid nitrogen and stored at -80C until further use. Purified proteins were thawed on ice then assembled by dialysis into Buffer 1 (1M NaCl, 50mM Tris pH 8, 20 mM 2-mercaptoethanol) for three hours, followed by dialysis into Buffer 2 (1 M NaCl, 50 mM Tris pH 8) overnight. Dialysis was completed using Slide-A-Lyzer MINI Dialysis Cups, 10000 MWCO.

In Vitro Capture Assay

Control HeLa cells or 293T cells stably expressing African green monkey TRIM5α tagged with hemagglutinin epitope extracts were prepared by seeding 2 million cells in each of (20) 100 cm dishes. After culturing for 2 days, cells were detached with trypsin. Cells were pelleted, washed with 5 volumes of cold PBS, and

resuspended in 5 volumes of lysis buffer (10 mM Tris-HCl, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM EDTA [pH 8], 1:100 mammalian protease inhibitor [Sigma]). Lysates were sonicated using a Sonic Dismembrator Ultrasonic Processor with the following settings: Amplitude: 7, Process Time: 10 s, Pulse-ON time: 5 s, Pulse-OFF time: 5 s. Sonicated lysates were centrifuged for 30 min at 14,000× g at 4°C to pellet cellular debris. The remaining supernatant was assayed by BCA to determine concentration and aliquots were flash frozen in liquid nitrogen and stored at −80°C. Each aliquot was thawed once prior to use in binding assays. CA assemblies (5 μM) were then incubated with extracts for one hour with gentle mixing in 100 μl reactions in binding buffer (50 mM Tris pH 8, 150 mM NaCl, 5 mM MgCl2, and 0.5 mM EDTA). When indicated, cyclosporine A (Calbiochem) was included at 5 μM concentration. Reactions were centrifuged for five minutes at 5,000× *g* and the pellets washed with 100 μl of binding buffer before pelleting again. The pellets were analyzed by non-reducing SDS-PAGE. Electrophoresis gels were stained with colloidal coomassie (Invitrogen, cat. No. LC6025), and sample lanes were cut just below the high molecular weight CA band before submission to the Vanderbilt MSRC. For binding reactions membranes were probed with antibodies against CA (183-H12-5C), cyclophilin A (Millipore), and CPSF6 (Novus Biologicals). For reactions completed for TRIM5α binding, membranes were immunoblotted for CA, cyclophilin A, or HA epitope (Roche, 100 ng/ml). Band density was quantified using Licor Image Studio with the top and bottom background subtraction. TRIM5α-HA binding was determined by calculating the ratio of the TRIM5α-HA band intensity to that of CA.

Uncoating Assay

Samples of purified wild-type HIV-1 cores (50 µl) were diluted in 1x STE buffer (pH 7.4) containing bovine serum albumin (10 µg/ml) without or with recombinant proteins. The cores were then incubated at 37°C for 45min, followed by ultracentrifugation at 125,000xg using the Beckman TLA-55 rotor at 45,000 rpm for 20 min at 4°C. Supernatants were removed and set aside for analysis. Pellets were dissolved in sample diluent (10% donor calf serum and 0.5% Triton X-100 in phosphate-buffered saline [PBS]). The CA content of supernatants and pellets were determined by p24 ELISA. The extent of uncoating was determined as the fraction of the total CA present in the supernatant.

shRNA-mediated depletion

Lentiviral vectors expressing Mission short hairpin RNA against each TRIM5α, CypA, CPSF6, TNPO3, Nup153, or Nup358 or Mission pLKO.1-puro empty (sequences below) were prepared by calcium chloride transfection of 293T cells. pLKO.1 (20ug) was cotransfected with psPAXII (15 µg) and pHCMV-G (5 µg) to produce VSV-G pseudotyped particles [163]. Subconfluent cultures of Vero cells were then inoculated with each of these vectors; 48 h later, the transduced cells were selected in puromycin (Vero, 10 µg/ml) for an additional 48 h. Depletion was confirmed by harvesting mRNA from puromycin-resistant cell populations using an RNeasy RNA Isolation kit (Qiagen). Isolated RNA was subject to cDNA synthesis using iScript cDNA synthesis kit (Life Technologies). cDNA transcripts were

quantified by real-time PCR using SYBR green for detection with a Stratagene MX3000p instrument. Primers used to detect depleted transcripts are indicated in table below. GAPDH mRNA was quantified to normalize quantification of target mRNAs. Target gene mean threshold cycle (Ct) values were normalized to the Ct value for GAPDH of each sample using the ΔΔCt method.

RNA sequencing and transcriptome analysis

Total RNA was isolated in triplicate from 60% confluent, 6 cm dishes of Vero or OMK cells using the RNeasy RNA Isolation kit (Qiagen) according to manufacturer's instructions. The quality of RNA was assessed by capillary electrophoresis using the Bioanalyzer 2100 (Agilent Genomics). Samples that received a RNA integrity (RIN) score lower than 8 were discarded. Total RNA libraries were prepared for each sample using the TruSeq Stranded Total RNA kit (Illumina) and subject to Illumina HiSeq PE75 sequencing. The resulting sequencing reads were imported into CLC Genomics Workbench 10 (**https://www.qiagenbioinformatics.com/)** as paired-end reads and analyzed by two workflows 1) de novo assembly and BLAST with the following parameters automatic word size: 45, bubble size: 98, minimum contig length: 1000 and read mapping back to contigs or 2) read mapping to TRIMCyp sequences. Assembled contig sequences resulting from de novo assembly (workflow 1) were pooled to create BLAST databases for Vero and OMK samples using CLC Genomics 'Create BLAST database' feature. BLAST databases were queried for contigs aligning to OMK TRIMCyp, TRIM5, or CypA. BLAST Parameters were as follows, Match cost 1, Mismatch cost 1, Gap Existence 2, Gap Extension 2, Expectation

value = 10.0, Word size = 11, Mask lower case = No, Filter low complexity = Yes, Maximum number of hits = 250, Number of threads = 4. To assess read alignment in the TRIM and CypA junction region of TRIMCyp we mapped each read library to OMK TRIMCyp using CLC Genomics WorkBench 'Map Reads to Reference' Tool. Coverage analysis of junction regions were assessed using the 'Coverage Analysis' Tool.

The complete high throughput sequencing dataset has been submitted to the Sequence Read Archive (SRA) (http://www.ncbi.nlm.nih.gov/sra) under accession number.

Real Time PCR of HIV-1 Transcripts

Vero cell cultures were inoculated with DNase I-treated stocks of Env-defective HIV-1 particles pseudotyped with VSV-G in the presence or absence of CsA (5 μM), MG132 (5μM), both, or Efavirenz (1 μM) as indicated. 12h post infection, cells were detached with trypsin-EDTA, pelleted, and washed once with 1X PBS. The cells were then treated with lysis buffer (50mM KCl, 1.5mM $MgCl_2$, 1.5mM Tris-HCl pH 8.0, 0.45% NP40 and 0.45% Tween-20) containing proteinase K (1 mg/ml) for 1 hr at 57°C. To inactivate proteinase K, samples were incubated at 95°C for 15 minutes then stored at -80°C until further use. HIV-1 DNA in the samples was quantified by real-time PCR using primers specific for second strand transfer products LateRT-FWD 5′-AGCAGCTGCTTTTTGCCTGTACT-3′ and LateRT-Rev 5′-CCTGCGTCGAGAGATCTCCTCTGG-3′ and 2-LTR circle products were detected with J2 5′–C AGTGTGGAAAATCTCTAGCAGTAC–3′ and JRev 5′–GCCGTGCGCGCTTCAGCAAGC–3′using SYBR green detection in a Stratagene

MX3000p instrument. GAPDH was also quantified in each sample to normalize HIV-1 cycle threshold (C(t)) values by GAPDH C(t) values to generate delta delta Ct (ΔΔCt) values.

Table 5-1 shRNA and Primer Sequences Used in this Study

Target Gene	shRNA sequence	qPCR Primers
GAPDH	CCGGGTGGATATTGTTGCCATCAATCT-CGAGATTGATGGCAACAATATCCACTTTTT	**FWD:**ATGACATCAAGAAGGTGGTG **REV:** CATACCAGGAAATGAGCTTG
VCP	CCGGATATAGAGAGTAGGTTGA-TTTCTCGAGAAATCAACCTACTCTCTATATTTTTTTG	**FWD:** GGTCATTTGCTGGCTTTGTG **REV:**TCGTAGTCTATTTGAATCTTCCAGG
TRIM5	CCGGGCACTGTCTCATTCTT-CAATACTCGAGTATTGAAGAATGAGACAGTGCTTTTT	**FWD:** CTGGAGATGCTGAGGCAGAAGC **REV:** GTCCAGGATGTCTCTCAGTTGC
CypA	CCGGCATCAAACCATTCCTTCTGTACT-CGAGTACAGAAGGAATGGTTTGATGTTTTTG	**FWD:** CCAGGGTTTATGTGTCAGGG **REV:**CCATCCAACCACTCAGTCTTG
CPSF6	CCGGGGTGATTATGGGAGTGCTATTCTCGAG-AATAGCACTCCCATAATCACCTTTTTG	**FWD:** CCCCTGGAAGAGAAATGGATAC **REV:**CAGGAACTGTTTATTGCATGGAG
TNPO3	CCGGCCTGGTGAACTTCTTTCTAAACTCGA-GTTTAGAAAGAAGTTCACCAGGTTTTTG	**FWD:** CCCACAGTGTTCTTAGATCGC **REV:** AAGGTACAGGAAGCAGGAATG
Nup153	CCGGGCCCTTACATTGACAGTGGT-TCTCGAGAACCACTGTCAATGTAAGGGCTTTTTG	**FWD:** TCTCCATCACCCATCAATTCG **REV:** CATCTGCCTCAGTAGACTTTACG
Nup358	CCGGGCTTGTCAGAATCCAGGTAAACTC GAGTTTACCTGGATTCTGACAAGCTTTTTG	**FWD:** GGATTTGGAAGTAGCACAGGG **REV:**CTCTTTTGCTTTGTTCTCAGGC

Statistical analysis

Analysis was performed using GraphPad Prism Software (GraphPad, La Jolla, CA, USA). Comparisons were analyzed with the two-way Analysis of Variance (ANOVA) test unless otherwise indicated. All tests were considered statistically significant at $p<0.05$.

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