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Investigating the Roles of NEDD4.2s and Nef in the Release and Replication of HIV-1: A Dissertation

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INVESTIGATING THE ROLES OF NEDD4.2s AND NEF IN THE RELEASE AND
REPLICATION OF HIV-1

A Dissertation Presented

By

Eric Richard Weiss

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

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INVESTIGATING THE ROLES OF NEDD4.2s AND NEF IN THE RELEASE AND
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Eric Richard Weiss

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Dedication

To my wife, Alexandra, without whose constant love and support this would not have been possible.

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ABSTRACT

Replication of HIV-1 requires the assembly and release of mature and infectious viral particles. In order to accomplish this goal, HIV-1 has evolved multiple methods to interact with the host cell. HIV-1 recruits the host cell ESCRT machinery to facilitate the release of nascent viral particles from the host cell membrane. Recruitment of these cellular factors is dependent on the presence of short motifs in Gag referred to as Late-domains. Deletion or mutation of these domains results in substantial decrease in the release of infectious virions. However, previously published work has indicated that over-expression of the E3 ubiquitin ligase, NEDD4.2s is able to robustly rescue release of otherwise budding-defective HIV-1 particles. This rescue is specific to the NEDD4.2s isoform as related E3 ubiquitin ligases display no ability to rescue particle release. In addition, rescue of particle release is dependent on the presence of the partial C2 domain and a catalytically active HECT domain of NEDD4.2s. Here I provide evidence supporting the hypothesis that a partial C2 domain of NEDD4.2s constitutes a Gag interacting module capable of targeting the HECT domains of other E3 ubiquitin ligases to HIV-1 Gag. Also, by generating chimeras between HECT domains shown to form poly-ubiquitin chains linked through either K48 or K63 of ubiquitin, I demonstrate that the ability of NEDD4.2s to catalyze the formation of K63-polyubiquitin chains is required for its stimulation of HIV-1 L-domain mutant particle release. In addition, I present findings from on-going research into the role of the HIV-1 accessory protein Nef

during viral replication using the culture T-cell line, MOLT3. My current findings indicate that downregulation of CD4 from the host cell membrane does not solely account for the dramatic dependence of HIV-1 replication on Nef expression in this system. In addition, I present evidence indicating that Nef proteins from diverse HIV-1 Groups and strains are capable of enhancing HIV-1 replication in this system. Analysis of a range of mutations in Nef known to impact interaction with cellular proteins suggest that the observed replication enhancement requires Nef targeting to the host cell membrane and may also require the ability to interact with select Src-kinases. Lastly, we find that the ability of Nef to enhance replication in this system is separate from any increase in viral particle infectivity, in agreement with current literature.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
AIP1	Actin Interacting Protein 1
AIP4	Atrophin-1 interacting protein 4
ALIX	ALG Interaction Protein X
ALV	Avian Leukosis Virus
AP-1	Activator Protein 1
APC	Antigen Presenting Cells
APOBEC3G	Apolipoprotein B mRNA Editing Enzyme Catalytic Polypeptide-like 3G
ARRDC	Arrestin domain-containing
ART	Arrestin-related Trafficking
ATP	Adenosine-5'-triphosphate
CA	Capsid
CCR5	C-C Chemokine receptor type 5
CDK9	Cyclin-dependent Kinase 9
cDNA	Complimentary DNA
CEP55	Centrosomal protein of 55 kDA
cET	Cryoelectron Tomography
CHMP	Charged Multivesicular Body Protein
CIN85	Cbl-interaction protein of 85 kDA
CMS/CD2AP	CD2 associated protein
CRM1	Chromosome Region Maintenance 1
CTD	C-terminal Domain
CTL	Cytotoxic T-lymphocyte
CXCR4	C-X-C Chemokine receptor type 4
CYCT1	Cyclin T-1
CypA	Cyclophilin A
DNA	Deoxyribonucleic Acid
DRM	Detergent Resistant Membranes
E6-AP	Human Papillomavirus E6-associated protein
EIAV	Equine Infectious Anemia Virus
ELISA	Enzyme-linked immunosorbant assay
ENV	Envelope
ESCRT	Endosomal Sorting Complex required for Transport
FACS	Fluorescence Associated Cell Sorting
GAG	Group-specific antigen
GFP	Green Fluorescent Protein
Glyco-Gag	Glycosylated Gag
GM1	Monosialotetrahexosylganglioside

gp41	Glycoprotein of 41 kDA
gp120	Glycoprotein of 120 kDA
GTP	Guanosine-5'-triphosphate
HA	Human Influenza Hemagglutinin
HAART	Highly Active Anti-retroviral Therapy
HBR	Highly Basic Region
HECT	Homologous to E6-AP Carboxyl Terminal
HECW	HECT, C2 and WW domain-containing protein
HERC	HECT domain and RCC1-like domain-containing protein
HIV-1	Human Immunodeficiency Virus type 1
HLA	Human Leukocyte Antigen
HRS	Hepatocyte growth factor-regulated tyrosine kinase substrate
hTE	Human Thioesterase
HTLV-1	Human T-cell Leukemia Virus type 1
HUWE	HECT, UBA and WWE domain-containing
IN	Integrase
ISG15	Interferon-induced 15 kDA protein
ITCH	E3 ubiquitin ligase Itchy
K48	Lysine 48
K63	Lysine 63
KIF4	Kinesin Family member 4
Ld	Liquid disordered
Lo	Liquid ordered
LTR	Long terminal Repeat
MA	Matrix
M β CD	Methyl-Beta-cyclodextran
MHC-I	Major Histocompatibility Complex class 1
MLV	Murine Leukemia Virus
MoMLV	Moloney Murine Leukemia Virus
mRNA	Messenger RNA
MSCV	Murine Stem Cell Virus vector
MVB	Multivesicular Body
NC	Nucleocapsid
NFAT	Nuclear Factor of Activated T-cells
NF- κ B	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NEDD4	Neuronal Expressed Developmental Downregulated ubiquitin ligase
NEF	Negative Effector
NTD	N-terminal Domain
PAK2	p21 associated kinase 2
PBMC	Primary Blood Mononuclear Cells
PFV	Prototypic Foamy Virus
PI [4,5] P2	phosphatidylinositol 4,5 bis-phosphate

PIC	Pre-Integration Complex
PM	Plasma Membrane
PR	HIV-1 protease
PRD	Proline Rich Domain
P-TEFb	Positive Transcription Elongation Factor B
rER	Rough Endoplasmic Reticulum
REV	Regulator of Virion Expression
RFP	Red Fluorescent Protein
RING	Really Interesting New Gene
RLD	Recognizer of Chromatin Condensation Like Domain
RNA	Ribonucleic Acid
RNA POLII	RNA polymerase II
RNP	Ribonucleoprotein
RRE	Rev-Response Element
RSP5	Reverses SPT-phenotype protein 5
RSV	Rous Sarcoma Virus
RT	Reverse Transcriptase
RTC	Reverse Transcription Complex
SFK	src family kinase
SIV	Simian Immunodeficiency Virus
SMURF	SMAD ubiquitination regulatory factor
SP1	Spacer peptide 1
SP2	Spacer peptide 2
TAR	Trans-activation Response Region
TAT	Trans-activator of Transcription
TCR	T-cell Receptor
TIRF	Total Internal Reflection Fluorescence microscopy
TNF α	Tumor necrosis factor alpha
tRNA	Transfer RNA
TSG101	Tumor Susceptibility gene 101
Ub-Gag	Ubiquitin- conjugated Gag
UEV	Ubiquitin E2-Variant
UTR	Untranslated Region
VIF	Viral Infectivity Factor
VLP	Virus-like particles
VPR	Viral Protein R
VPS	Vacuolar Protein Sorting-associated protein
VPS4	Vacuolar Protein Sorting-associated protein 4
VPU	Viral Protein U
VS	Virological synapse
VSV-G	Vesicular Stomatosis Virus protein G
VTA1	Vps20-associated homolog
WWP1	WW domain-containing protein 1
Y2H	Yeast Two-Hybrid

CHAPTER I

Rationale and Introduction

Rationale and Objectives of Thesis Research

Human Immunodeficiency Virus type I (HIV-1), the etiological agent of AIDS, is responsible for approximately 34 million current infections worldwide, and in 2010 contributed to the deaths of 1.8 million infected individuals (2011 UNAIDS report). While this figure is staggering, the number of AIDS related deaths has fallen in recent years due to improvements in antiretroviral therapies as well as increased dissemination of treatments. However, this information is tempered by the fact that more than 2.7 million new infections were reported for the same year (2011 UNAIDS report). This statistic, as well as the proven ability of HIV-1 to circumvent multiple retroviral treatments, highlights the continued need for scientific research in this field. Without pharmaceutical intervention to limit virus production, individuals infected with HIV-1 often develop Acquired Immune Deficiency Syndrome (AIDS) (205). This disease is characterized by a decrease in CD4+ T-cells over the course of infection due to various cytopathic mechanisms, reducing the ability of the immune system to recognize and contain other pathogens, often resulting in opportunistic infection and death (205). CD4+ T-cells are one of the earliest populations of cell affected, as they are the cells initially infected upon exposure to the virus, either through mucosal transfer or direct introduction of the virus into the blood stream through transfusion.

Depletion of CD4+ T-cells is the result of directed cell killing by cytotoxic T-lymphocytes, increased levels of apoptosis in HIV-1 infected cells, and defective regeneration of new T-cells (205). Additional cell types such as dendritic cells and macrophages are also targeted by HIV-1 due to their expression of CD4 and the viral co-receptor, the chemokine receptor CCR5 (205). During the course of infection, mutations in the viral envelope alter the cell preference from CCR5-expressing cells to CD4+ cells that express CXCR4, an indicator of T-cell activation, further depleting the CD4+ T-cell population (57). Ultimately, this attack on the CD4+ cells leaves the infected individual susceptible to opportunistic infections otherwise contained by a healthy immune system (205).

Symptomatically, the initial exposure to HIV-1 results in normal flu-like symptoms as the immune system fights to keep the virus infection in check (205). During this time the first noticeable T-cell decrease is observed as infected cells are cleared by the immune system, or die due to virus-induced cytopathic effects (205). Cells infected during this time may also return to a resting state as the viral genome enters a state of latency, where the viral genome has integrated into the host cell genome, but no active viral replication is detectable (274). These latently infected cells, containing an integrated but epigenetically silenced viral genome, can serve as reservoirs for the virus, capable of replicating and releasing virus upon appropriate cell stimulation (142, 274). Currently, no therapeutic intervention is available to clear these reservoirs.

Current therapies are directed against the activity of the HIV-1 enzymes - reverse transcriptase, integrase and protease- each of which are necessary for HIV-1 replication (11). However, due to the high mutation rate of the HIV-1 genome, the virus has shown a remarkable ability to circumvent directed therapies (11). Current treatment protocols call for a combination of drugs that target one or more of these enzymes simultaneously, but the regimen is difficult to maintain and can adversely affect patients, leading to discontinuation of treatment and return of high viral titers (11). Thus, continued investigation into the replication cycle of HIV-1 may provide new targets for antiviral agents.

While the devastation caused by the HIV-1 epidemic is clear, it must be remembered that the negative effects HIV-1 has on an infected individual are not the goal of the virus per se. Although HIV-1 has debilitating effects on the host in terms of immune suppression, the virus evolved these mechanisms for one purpose, to enable it to replicate and infect new hosts/cells. In truth, the virus exists solely to copy and transfer its genome to a new host; any other deleterious effects in the cell or host are corollary to this end.

In order to transfer its genome to another host, the virus must package it into a delivery system capable of protecting the genome and targeting it to another host. HIV-1 has developed multiple strategies to not only create this delivery system, the virus particle, but to optimize the assembly, release and infectivity of the particle to maximal efficiency. In doing so, HIV-1 has evolved to

utilize host cell machinery not just to transcribe and translate the viral genome, but also to shuttle viral proteins to assembly sites, create virus particles of the correct stoichiometry for release, and enhance the ability of these particles to be taken up by the next host (80, 283).

In this thesis I address several mechanisms by which HIV-1 interacts with the host cell to generate maximally infectious particles. In the first chapter, I introduce general strategies that HIV-1 uses during the course of the viral replication cycle from initiation of transcription, trafficking of viral components and assembly of viral particles, to release and particle maturation. In the second chapter, I discuss the role that the host cell Endosomal Sorting Complex Required for Transport (ESCRT) machinery plays in the release of viral particles from the host cell, with particular emphasis on my research investigating the mechanism of Nedd4 E3 ubiquitin ligases in rescuing the release of otherwise defective viruses. Previously publications reported that over-expression of the NEDD4.2s E3 ubiquitin ligase demonstrated a specific ability to rescue the release of Late-domain deficient HIV-1 particles (56, 292). The focus of my research was directed at investigating the role of NEDD4.2s in this release, o

I then address on-going research into the role that the HIV-1 protein Nef plays in enhancing the infectivity and replication of HIV-1 in a specific cultured cell line. Experiments performed in the Gottlinger lab previously demonstrated that HIV-1 replication in the MOLT3 cell line, a T-cell line derived from a leukemia

patient, is highly dependent on the expression of the lentiviral Nef proteins, in stark contrast to HIV-1 replication in other cultured T-cell lines as well as primary blood mononuclear cells (PBMCs). The focus of on-going research is directed at determining the role that Nef plays in the replication of HIV-1 in this system, with the intent to identify a restriction factor present in these cells that maybe functionally counteracted by Nef. Lastly I discuss how the activities of Nef and ESCRT recruitment by HIV-1 may perform similar function in the production of infectious viral particles.

INTRODUCTION

Hiv-1 Virus and Replication Cycle

Replication and transmission of the HIV-1 genome requires timely and specific interactions with proteins and other components of the host cell. In this chapter, I discuss several interactions between HIV-1 proteins and host cell factors that occur during the course of the HIV-1 replication cycle. The HIV-1 replication cycle can be divided into two distinct stages often referred to as the early and late events, which together compose the complete cycle. The early events can be considered as those interactions between the components of the infecting virus particle and the host cell, and originate with the recognition of host cell CD4 by the virion, and end with integration of the viral genome into the genome of the host cell. Those activities considered to be part of the late events of the replication cycle include interactions between viral proteins and the host cell required for the production of the mature viral particle. These activities include, transcription of the viral genome, translation of viral proteins, processing of viral RNA, trafficking of viral particle components and genomes to the plasma membrane, assembly and release of the viral particle, and finally, maturation of the particle into an infectious virion.

A brief summary of the viral genome and proteins is necessary to provide an introduction into the relevant molecules discussed in more detail in the

following sections reviewing the early and late stages of replication. However, as the early events can be considered as interactions that occur between the virion and the host cell, an introduction into the morphology and components of the mature and infectious virus is also appropriate.

Introduction to HIV-1: Viral Genome and Structure

Mature HIV-1 virions average 145 nm in diameter and are characterized by multiple electron dense layers when visualized by electron microscopy (schematic Fig. 1.1) (33). An electron dense ring of viral envelope supported by viral matrix protein (MA) can be seen, from which extend 8-10 trimers of the viral Env protein composed of non-covalently interacting gp120 and gp41 proteins (326). The interior of the virus is dominated by another electron dense cone structure composed primarily of capsid (CA) proteins. Biochemical studies have shown that this cone structure, referred to as the viral core, contains molecules of the viral proteins Vpr (33), Nef (151), and the HIV-1 integrase and reverse transcriptase enzymes, which are required by the virus for initiation of the next round of infection (33). In addition, condensed within the viral core are two copies of the HIV-1 genome bound to the viral nucleocapsid protein (194).

The HIV-1 genome consists of a single-strand positive sense RNA molecule of approximately 9kb, with two molecules being packaged into each virus (194). This genome contains coding information for only 15 genes with

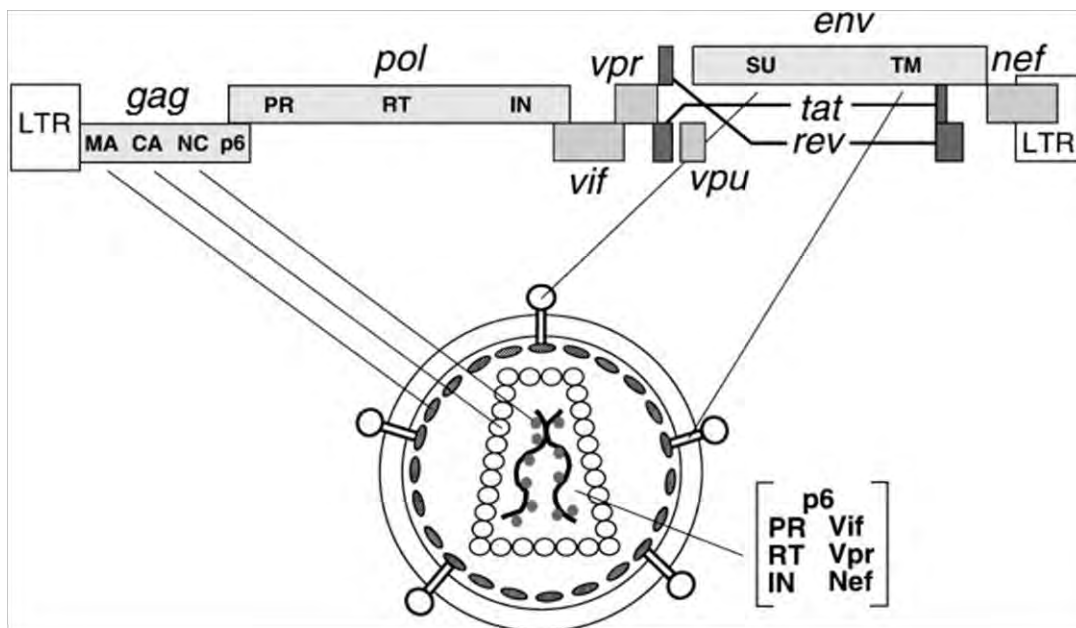


Figure 1.1 Schematic Representation of the HIV-1 genome and virus particle. Reproduced with permission from Frankel and Young, 1998.

several arising from multiply spliced transcripts (Fig. 1.1, (82)). Each gene plays an important role during the course of the infection, and a partial summary of those roles is enumerated below.

Introduction to HIV-1:Viral Proteins

Gag polyprotein: The Gag polyprotein contains the structural proteins required to form a new virus particle. Expression of Gag alone is sufficient to

produce non-infectious virus-like particles (VLP), indicating that all of the information required for virus formation is contained within the sequences of this protein (95). The components of Gag (matrix-MA, capsid-CA, nucleocapsid-NC, p6 as well two spacer peptides SP1 and SP2 the flank the NC protein) are responsible for interaction with the host cell membrane, formation of higher order Gag multimers through self interaction, recruitment and packaging of the viral genome and recruitment of cellular factors involved in particle release (41, 67, 68). These components are cleaved by the HIV-1 protease to generate the subunit proteins that form the mature viral particle (283).

Gag-Pol polyprotein: The Gag-Pol polyprotein arises from a -1 shift in the reading frame of Gag that occurs approximately once out of every 15 -20 translation events (125). Gag-Pol contains all of the enzymes required by the virus to reverse transcribe the viral RNA genome into DNA (Reverse Transcriptase-RT), and integrate the viral cDNA into the host cell genome (Integrase-IN). Also contained in this coding sequence is gene encoding the viral protease (PR), which is required to cleave the viral Gag structural proteins during particle maturation. These enzymes, RT, IN and PR, are the primary targets for most current antiviral therapies (11).

Envelope glycoprotein: HIV-1 envelope is responsible for initiating the next round of virus infection through recognition of CD4 and the chemokine receptors CCR5 and CXCR4. Also transcribed as a polyprotein, the HIV-1

envelope glycoprotein (Env-gp160) is cleaved by cellular furin-like proteases during trafficking through the secretory system resulting in a membrane anchoring subunit, transmembrane –TM, referred to as gp41, as well as a surface exposed glycoprotein, SU-gp120 (48). In addition, the HIV-1 envelope protein has also been shown to play a role in downregulating CD4 from the host cell surface, thus playing a role in preventing superinfection of the host cell and potentially enhancing particle infectivity (19, 312).

Tat: Transcription of the HIV-1 genome by cellular RNA polymerase II is one of the first rate-limiting steps to infection. Basal levels of transcription from the promoters contained within the 5' UTR of the integrated HIV-1 genome are inefficient due to cellular regulation of RNA Pol II (254). However, Tat, one of the first viral gene products produced and absolutely required for replication, helps the virus overcome this limitation, by recruiting cellular transcription factors CycT1 and CDK9 to increase the processivity of the polymerase (254). Tat interacts with a three dimensional motif found within the mRNA of the stalled transcript and helps to increase the processivity of Pol II, resulting in an increase of full length viral RNA (142).

Rev: Similarly to Tat, the HIV-1 Rev protein is also essential for replication. Full length viral RNA serves not only as a template for production of viral proteins, but is also required to be the genome of the progeny virions. The Rev protein encoded by HIV-1 recognizes and binds to full-length RNA through a

region of the viral genome termed the Rev Response Element (RRE) and directs it, and other RRE containing viral mRNA out of the nucleus (26). Levels of this protein play a role in determining the translation of other retroviral proteins that rely on interaction with Rev for nuclear export (142).

Accessory proteins (Vif, Vpr, and Vpu): In addition to the structural and regulatory proteins listed above, HIV-1 encodes a series of other proteins which have been termed accessory, because they are not needed to productively infect most cultured cells. However, in the natural targets of HIV-1, these proteins perform several necessary functions that assist in productive replication. For instance, the Vif accessory protein protects the viral genome from cell directed mutation by counteracting a specific innate immune mechanism, the cytidine deaminase APOBEC3G which packages into viral particles and stimulates hypermutation of the package viral genome (103, 149). The Vpr protein assists in targeting the viral genome to the nucleus of non-dividing cells following infection, and a role for G2 arrest has been proposed (103). The protein Vpu disrupts multiple interactions between viral and cell host proteins that would otherwise impact particle infectivity and release, by down regulation cell surface tetherin and CD4 (103, 149).

Nef: An additional accessory protein, transcribed early in HIV-1 infection, is Nef, which performs a myriad of tasks throughout the course of replication. A partial list of functions attributed to this protein include: 1) down-regulating of cell

surface molecules such as CD4 and MHC-I (80, 161); 2) lowering the threshold of activation of infected CD4+ T-cells (294); and 3) increasing the infectivity of viral particles (201). This protein will be discussed in greater detail in Chapter III of this thesis.

HIV-1 Replication Cycle

To appreciate the complexities of the interactions the HIV-1 makes with the host cell, it is necessary to provide background regarding the viral replication cycle. The viral replication cycle can be divided into two stages: the first stage encompasses the period from interaction of the virus with the host cell and culminates with the insertion of the viral genome into that of the host cell; the second stage is initiated by the transcription of the integrated viral genome ultimately resulting in the formation and release of the next round of virus. These two stages are also referred to as the early and late events of HIV-1 replication.

Viral infection-Early Events

The early events of HIV-1 infection consist of interactions between the host cell and the invading HIV-1 virion (Fig. 1.2). Infection of the target cell by HIV-1 can occur via two separate mechanisms: either virus-cell transmission or cell-cell transmission (50). Infection of target cells from free-floating virus is

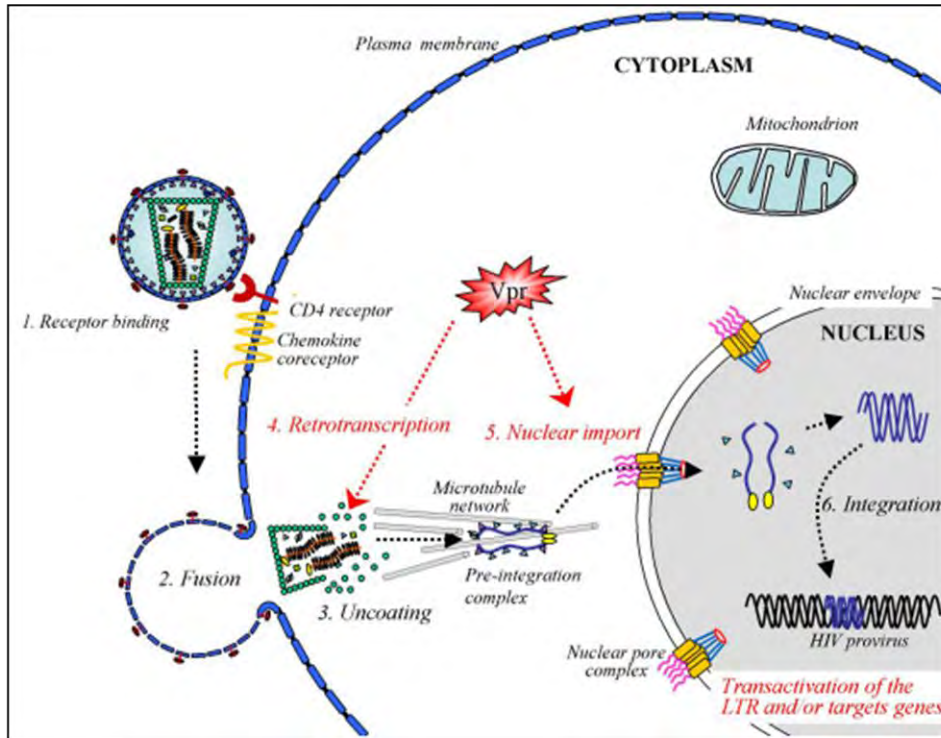


Figure 1.2 Schematic of early events in HIV-1 replication.

Interactions between the HIV-1 viral gp120 envelope proteins and cellular CD4 receptor and coreceptor promote fusion of the viral envelope with the target cell plasma membrane. Following fusion, delivery of the HIV-1 virion core into the cytoplasm results in un-coating of the viral genome and, subsequent formation of the reverse transcriptase complex. Viral cDNA bound to viral proteins (the pre-integration complex) migrates through the cell to the nucleus. After entering the nucleus, the viral integrase protein integrates the cDNA (viral genome) into the host chromatin. Image modified from Le Rouzic and Benichou, 2005, with permission (Open access).

inefficient, and requires an infectious particle release from the producer cell into the extracellular space where it is exposed to immune cells and antibodies generated by the host's adaptive immune system. By contrast, cell-to-cell

transfer occurs when an infected cell makes physical contact with a target cell either through formation of filopodia, cytonemes, or nanotubes, and transmits virus directly to the uninfected cell (50). Cell to cell transmission is suggested to be more efficient than virus to cell transfer as indicated by the results of one study that reported a 100-1,000 fold increase in transmission (70). Regardless, both transmission methods are mediated by HIV-1 Env glycoproteins which recognize and bind to the CD4 receptor on the surface of the target cell (57). Interaction between the CD4 receptor and the Env protein occurs in a cleft within the globular head of the gp120 subunit, initiating a conformational change in the molecule that unmask a CCR5 or CXCR4 co-receptor binding domain (48, 158). Reliance on CD4 and CCR5/CXCR4 receptors for infection limits the target population, at least initially, to cells of the immune system, specifically, CD4+ T-helper cells, macrophages and dendritic cells (48). Upon binding with the co-receptor, the transmembrane gp41 subunit of Env inserts a hydrophobic fusion peptide into the plasma membrane of the target cell. Structural rearrangements of a 6 heptad repeat bundle in gp41 serve to bring the viral envelope into close proximity with the cell membrane, and subsequently, fusion of the two membranes occurs (57).

The infection scheme detailed above, fusion of the viral particle to the plasma membrane, has until recently, been believed to be the method used by HIV-1 to access target cells, based on previous studies reporting that endocytosis of virions fails to elicit productive infection (264). Several lines of

evidence support this belief. For example, it has been shown that entry of HIV-1 into target cells is a pH independent process, suggesting that viral entry via acidified endosomes is not required (192). Additionally, cells in culture infected with HIV-1 virions are prone to fusion-from-without, a process that is the result of two cell membranes being fused together through a viral particle being held between them (58). Also, the use of membrane impermeable recombinant peptides of the gp41 heptad repeat, which block formation of the heptad bundle required to bring the two membranes into apposition, inhibit virus-cell fusion and prevent delivery of the viral core into the cytoplasm of target cells (193). Lastly, and most germane to this thesis, the well characterized enhancement of infectivity observed when Nef is expressed in donor cells is obviated when particles are pseudotyped with an envelope that directs entry to endosomes (61, 192, 238). However, recent evidence provided by the Melikyan group strongly argues for an endosomal method of virus entry (66, 193). Using a temperature block approach to retard endocytosis, they demonstrated that the HIV-1 envelope fuses with the plasma membrane without releasing contents of the viral particle into the cell, failing to productively infect the cell (66). In a subsequent report, they also demonstrated fusion of the viral envelope with endosomal membranes in a dynamin-dependent manner by imaging fluorescently labeled endosomes and viral Gag proteins (193). Lipid mixing at the endosome membrane was followed by deposition of the viral core into the cytoplasm, and resulted in productive infection (193). Interestingly, they also reported no difference in entry

of HIV-1 variants regardless of Nef expression status, consistent with previous reports of a lack of Nef-stimulated entry enhancement with virus pseudotyped to enter cells via endocytosis. However, as disease progression in infected individuals has been associated with a functional *nef* allele, it is unclear whether an endocytic route of entry is physiologically relevant or rather an interesting experimental finding.

To replicate within the infected cell, the positive sense RNA genome must be converted to cDNA, integrate into the host cell genome and access the host cell transcriptional machinery (82). During the process of particle assembly and maturation, the viral genome becomes condensed through interactions with multiple copies of the viral NC to form a ribonucleoprotein complex (RNP) (7). This RNP is then encapsulated by a protective shell generated from viral CA protein which is referred to as the viral core. The core, which is a lattice-like structure composed of approximately 1500 CA subunits arranged into 250 hexamers and 12 pentamers (33), disassociates in the cytoplasm allowing the RNP complex to traffic to the nucleus (7). The dissolution of the capsid core is thought to occur because of a reduced concentration of free CA molecules as the core transitions from the particle interior to the cytoplasm. The exact time and location of uncoating remains controversial (7). Several groups have reported that the viral core also contains measurable amounts of MA and Nef, which may be required to destabilize the core, facilitating uncoating, or assist in transporting

the core through the cytoplasm by interacting with host cell motor proteins (79, 151, 309).

Disassembly of the core, or uncoating, coincides with a rearrangement of the core components to form the structure referred to as the reverse transcription complex (RTC). The RTC traverses the length of the cell using the cellular microtubule network and dynein to migrate to the nucleus (191). During this transition, the process of reverse transcription, the conversion of the viral RNA genome to cDNA, is initiated prior to insertion of the cDNA genome in the host cell. These two steps, reverse transcription and integration, are accomplished by the actions of two viral enzymes (reverse transcriptase-RT and integrase-IN, respectively) that are contained within the RTC (7). Reverse transcription requires the priming of the RNA genome with a lysyl-tRNA provided by the producer cell (7). This process is believed to be initiated by exposure of the RTC to free deoxynucleotides in the cytoplasm (7). Following completion of reverse transcription, the DNA product (provirus) and associated proteins-now referred to as the pre-integration complex (PIC) - is imported into the nucleus and inserted into the host genome by the viral IN.

Although HIV-1 is able to infect resting T-cells, infection of activated T-cells is generally more efficient (142). Interestingly, Stevenson *et. al.* reported that infection of resting T-cells by HIV-1 results in production of viral cDNA that fails to integrate into the host cell genome (277). The extra-chromosomal

proviral cDNA is transcriptionally active, and able to produce Nef, which has been shown to lower the activation threshold of resting T-cells (17, 277). Thus early production of Nef proteins, even before integration of the viral genome, may play a significant role in enhancing replication.

Although beneficial to viral replication, activation of resting T-cells presents a risk for the virus. Activated and infected T-cells often die rapidly due to cytopathic effects induced by the virus or targeting by the immune system (205). However, some of these activated-infected CD4⁺ T-cells may survive the initial infection and revert back to a resting state (142, 274). As HIV-1 gene transcription requires recruitment of cellular transcriptional factors produced only during activation, the viral genomes in these infected cells become latent (274). Viral genomes present in latently infected cells have been shown to be transcriptionally silenced through epigenetic modification, and overcoming this silencing requires the cells become activated. (290).

Viral Infection - Late Stage

The late stage of HIV-1 replication starts with transcription of the integrated retroviral genome and ends with the release of infectious virus (Fig. 1.3). During this stage HIV-1 proteins are expressed from unspliced, singly or multiply spliced viral transcripts (26). Following translation, components of the virion migrate to the plasma membrane where they assemble into a progeny virus particle. Trafficking of viral components, assembly, and release of virus particles are dependent on interactions with the host-cell. These processes, as well as virion maturation, will be described in this section.

Transcription of viral proteins

Transcription of the HIV-1 genome is regulated by the presence of multiple transcription factor recognition start sites present in the 5' LTR of the integrated viral genome (142). Although the 5' LTR contains elements for multiple cellular transcription factors (NF κ B, NFAT, SP1, AP-1 etc), basal transcription from these factor-binding sites mostly fail to produce full-length transcripts, because RNA polymerase II pauses early in the process of elongation which results in truncated viral transcripts (254). Initial transcripts which are synthesized to full-length are spliced by the cellular mRNA processing machinery to produce the HIV-1 Tat, Rev and Nef mRNAs (26). Tat protein recognizes and binds to an

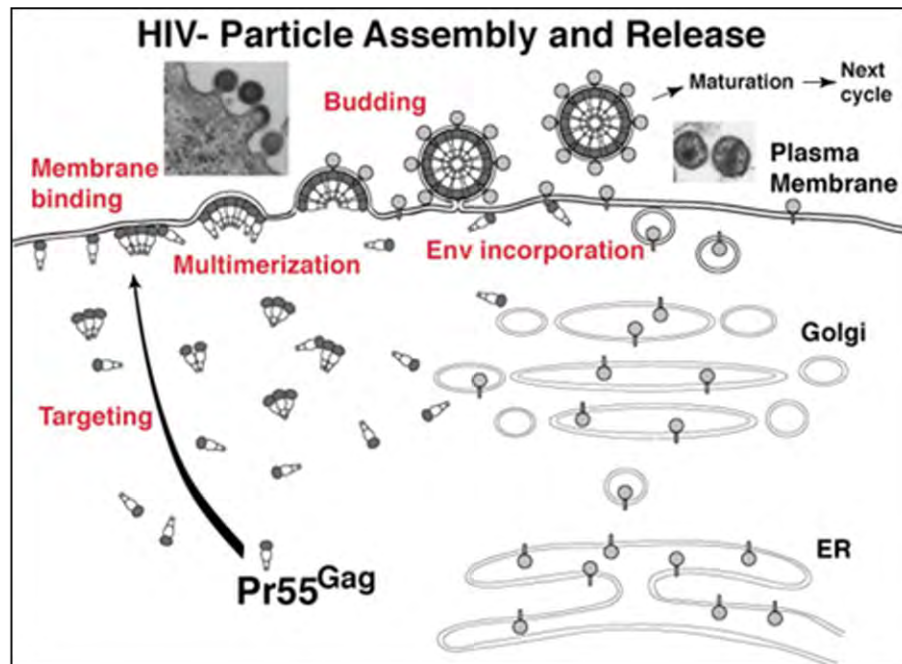


Figure 1.3 Schematic of HIV-1 Gag assembly and virus formation. Gag proteins associate into dimers and trimers in the cytoplasm, and assemble at lipid raft microdomains within the plasma membrane. The HIV-1 Env proteins traffick through the cellular secretory pathway to the plasma membrane, where they are incorporated in virus particles. The viral particles are released in an immature form. Following release, retroviral protease mediates cleavage of Gag into component subunits which are rearranged to generate the mature virion. Image taken from the NIH , NCI website (http://home.ncifcrf.gov/hivdrp/Freed_figure.html) no licensing permission required.

RNA stem-loop structure present in the viral mRNA (TAR) and recruits P-TEFb, a complex of Cyclin T-1 (CycT1) and Cyclin-dependent kinase 9 (CDK9), which hyper-phosphorylates RNA pol II to increase its processivity (142, 221, 254). Synthesis of additional full-length transcripts leads to increased production of Tat

mRNAs, generating a positive feedback loop and overall, an increase in full-length viral mRNA (254). This full length viral RNA can be spliced to generate the mRNA for selected HIV-1 proteins, or left unspliced or partially spliced to generate Gag, Gag-Pol and Env mRNA (26). Full length viral RNA is also required as genomes for the next round of virus particles. Export of Gag-Pol and Env mRNAs, as well as viral genomes, requires the activity of the highly conserved HIV-1 Rev protein.

The Rev protein binds to a cis-active RNA element termed the Rev-response element (RRE) within the *env* coding region of unspliced or partially spliced HIV-1 mRNAs, and directs their export from the nucleus in a CRM1-dependent manner (26). The requirement of mRNA encoding Gag, Gag-Pol and Env on HIV-1 Rev for nuclear export and translation has a direct effect on when viral proteins are translated (142). Similar to Tat mRNA, fully spliced RNA encoding Rev is one of the first species generated from the initial full length viral transcripts (26). Lacking an RRE itself, Rev mRNA is exported from the nucleus through interaction with the host cell mRNA splicing pathway components and subsequently translated in the cytoplasm (Fig. 1.4(172)). The presence of a nuclear localization signal in Rev targets the protein back to the nucleus (172). From there, it interacts with viral mRNAs containing an RRE to shuttle them out of the nucleus in a GTP-dependent fashion, for translation in the cytoplasm or via the endoplasmic reticulum (172). An important implication in this process is that through dependence on Rev-mediated mRNA nuclear export, HIV-1 is able to

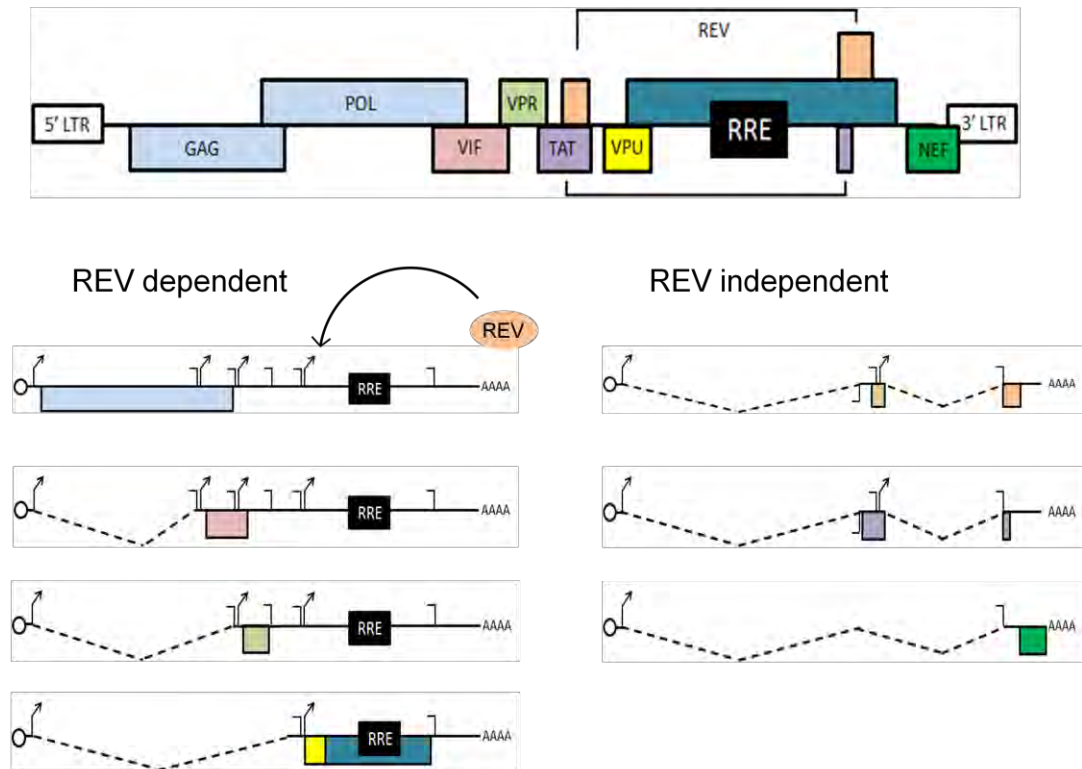


Figure 1.4 . Export of partially spliced and unspliced viral mRNA from the nucleus requires HIV-1 Rev. Rev-independent viral mRNAs utilize host cell splicing proteins for nuclear export, and are translated early in infection. Export of mRNAs containing a Rev Response Element (RRE) first require accumulation of HIV-1 Rev in the nucleus prior to export. Image adapted with permission from Bohne et al, 2006.

exert translational control of viral proteins that may trigger adaptive immune responses, and ensure that a minimal supply of viral proteins is available to form an infectious particle before stimulating the adaptive immune system (142).

It should also be noted that the accessory protein Nef is produced at the same time and same manner as Rev (26). Early expression of Nef prevents super-infection of the host cell by stimulating CD4 downregulation (19, 312). Nef is also important early in infection for immune evasion as Nef downregulates

Major Histocompatibility Complex Class I molecules from the surface, preventing the infected cell from displaying any HIV-1 antigens for immune recognition by the host (167, 170, 214). Additionally, through interactions with subunit proteins of the T-cell receptor, Nef is able to reduce the threshold of T-cell activation of infected cells (106, 207). Again, the findings of Stevenson et al indicated that the non-integrated cDNA of HIV-1 was transcriptionally active and suggest that early Nef expression is vital to lower the activation threshold of the infected cell leading to viral genome integration (277).

Trafficking of HIV-1 proteins

After exiting the nucleus, viral mRNA must be translated and viral component proteins must traffic to the site of particle assembly (Fig. 1.3). Transcripts containing the coding information for Env glycoproteins are translated at the rER as a polyprotein, where they are simultaneously glycosylated with the addition of mostly N-linked oligosaccharides (48). It is at this point that the HIV-1 Env gp160 (glycoprotein) assembles into trimers that traffick to the Golgi, where the oligosaccharides obtained in the rER are further modified in the trans-Golgi network by the addition of high molecular weight mannose side chains producing a shield which blocks Env from immune recognition (48). It is also in the trans-Golgi network that gp160 polyproteins are cleaved into gp41 (transmembrane domain) and gp120 (surface exposed) subunits by a cellular furin-like protease

(48). After proteolysis, gp41 and gp120 proteins associate via non-covalent interactions, and are shuttled to lipid rafts in the plasma membrane (48). HIV-1 Env proteins become incorporated into virus particles through interactions with the MA protein and the long cytoplasmic tail of gp41(73, 83).

All other viral components such as Gag are translated on soluble polysomes in the cytoplasm and traffick to the site of particle assembly (36, 48, 303). The mechanism by which soluble Gag trafficks to the plasma has yet to be clearly elucidated, as fluorescent microscopy and biochemical approaches have often provided conflicting results. Biochemical studies indicated a role for the cellular microtubule network, and the motor protein kinesin KIF-4 in the trafficking of Gag (285). Martinez, et. al. reported the co-immunoprecipitation of KIF-4 with HIV-1 Gag, and that the interaction required the C-terminal portion of KIF-4, previously shown to interact with MoMLV Gag in a similar fashion (187, 285). Subsequent analysis using RNAi directed against KIF4 confirmed its role in HIV-1 Gag trafficking as depletion of the kinesin resulted in perinuclear accumulation of Gag rather than plasma membrane localization (187). Movement of newly translated Gag from a perinuclear region to sites of particle assembly was confirmed by Perlman and Resh who followed Gag trafficking in several cell types using live-imaging techniques and reported that newly translated Gag trafficked from a perinuclear region to the plasma membrane forming a transient association with multivesicular bodies (MVB) during the process (227). This finding is consistent with other studies investigating HIV-1 Gag interactions with

viral genomes. These studies reported a perinuclear localized interaction between newly transcribed viral genomes and Gag, suggesting that HIV-1 Gag may associate with MVB prior to assembly at the PM (6, 129, 156). Interestingly, several of these investigations have reported that the interaction of MA-RNA plays a direct role in targeting Gag to the appropriate membrane; interaction between MA and the viral genome increases the affinity for MA with phosphatidylinositol 4,5 bis-phosphate in the PM (5, 129). This explanation may account for the reports of several labs that soluble Gag localizes to endosomal membranes which serve as either intermediary platforms or the sites of particle assembly, and traffick to the PM for release (72, 132, 134).

However, these results differ dramatically from labs who used real-time imaging of Gag-GFP constructs which demonstrate that Gag initially assembles at the plasma membrane from a cytoplasmic pool, but may then associate with internal membrane-bound structures such as endosomes (124, 135-137). A decrease in the availability of unbound vRNA may account for this difference, as Gag that is actively interacting with viral RNA would be less likely to diffuse from the assembly site. The observations of the real-time imaging studies would seem to indicate that the observed deposition of HIV-1 Gag on endosomal membranes results following saturation of the suitable interaction partners at the plasma membrane, either RNA scaffolds or lipid microdomains (124, 135-137). Of particular interest is a report by Jouvenet and Bieniasz, who confirmed Gag clustering at the PM before observing any localization of Gag-GFP to endosomes

(136). In addition, by treating the cells with nocodazole, they illustrated that trafficking along microtubules played no role in their system, further reinforcing the theory that Gag trafficks to the PM via passive diffusion through the cytoplasm (136). Taken together, the current literature suggests that newly translated Gag is maintained at a perinuclear location, perhaps via interaction with early endosomes, until it binds to viral genomic RNA. Monomers and dimers of Gag-RNA complexes travel to the cell periphery via passive diffusion and bind to the inner leaflet of the PM due to the higher affinity of the MA-RNA complex.

Viral particle assembly

HIV-1 viral particles assemble on the inner leaflet of the plasma membrane at specific microdomains known as lipid rafts (302). These rafts, also sometimes referred to as detergent-resistant membranes (DRM), are high in cholesterol, sphingomyelin and the phospholipid, phosphatidylinositol-4,5-bisphosphate (303). While the existence of lipid rafts has been controversial for some time (isolation of lipid rafts required extensive biochemical extraction which could be prone to generating artifacts), current analysis of the HIV-1 envelope indicates it shares many of the components of the DRM, including the presence of raft-associated proteins such as GM1, increased cholesterol and sphingomyelin relative to the content of the PM, and the exclusion of non-raft targeted molecules such as CD45 (36, 153, 210).

Interaction with the plasma membrane is driven by the MA subunit of Gag via attachment of a basic stretch of amino acids present in the N-terminus of MA (HBR) and acidic side chains of phospholipids (55). The HBR of MA has a specific and high affinity for the plasma membrane component phosphatidylinositol-(4,5)-bisphosphate (PI [4,5] P2), and Gag can be re-directed from the plasma membrane to intracellular membranes if the PM levels of PI [4,5] P2 are decreased (54, 55). For example, Ono's group observed accumulation of Gag on endosomal membranes by treating infected cells with 5-phosphatase IV which specifically reduced membrane PI [4,5] P2 levels (54). In contrast, higher levels of cholesterol have been reported to increase the affinity of MA for PI [4,5] P2 (5). Association of Gag with the plasma membrane is strengthened by the insertion of the myristic acid anchor of MA into the PM, which occurs after interaction between the MA HRP and the PM (259). The N-terminal myristic acid is sequestered into a cleft in monomeric MA, and becomes available for insertion into the membrane upon binding of MA to PI [4,5] P2 (55, 222, 259).

Formation of HIV-1 virus is a Gag-driven process; expression of Gag alone in cells is sufficient to generate non-infectious virus-like particles (95). Upon localization at the plasma membrane, neighboring Gag molecules begin to form higher order multimers. Multimerization of Gag is the result of interactions between the Gag subunit proteins and interactions with the viral genome (89, 113, 204). Following MA association with the PM, Gag forms trimers which associate into hexameric rings driven by interactions between the CA-CTD-SP1

regions of proximal Gag molecules (1, 67, 88). Additional higher order structure develops as the NC subunit interacts with the viral genome, which some suggest acts as a scaffold to drive Gag-multimerization (204, 220). At this time, the assembled Gag lattice assumes a dome-like structure, as CA-CA and NC-RNA-NC forces begin to pack Gag closer together, creating voids within the hexameric-Gag sheet (41). Crystallographic imaging of Gag displays a tapering molecule wider at MA and narrowing after the CA-CTD, which may account, in part, for the membrane curvature (89). Recent imaging studies using cryoelectron tomography (cET) with subtom averaging to analyze immature particles show that the interior of the budding dome is supported by a continuous lattice of Gag hexamers, which transition to an incomplete shell with irregularly spaced 'holes' following release (32). The authors of this report hypothesize that the irregularly spaced holes are necessary to support the curvature required for viral budding (32). A separate group reported that the Gag shell, though incomplete, forms a continuous lattice and attributes the membrane deformation that accompanies budding to the presence of Endosomal Sorting Complex Required for Transport (ESCRT) proteins which, when recruited by the p6 region of Gag, induced formation of the bud neck of the nascent viral particle (42).

It should be noted that while our focus has been on the activities of Gag with regard to particle assembly, the Gag-Pol polyprotein behaves very similarly with regard to trafficking, membrane binding and interactions with Gag, as it also contains MA, CA and NC domains, and thus is capable of forming interactions

with the PM and adjacent Gag molecules. However, as mentioned previously, the ratio of Gag to Gag-Pol is approximately 20:1, and this ratio has profound effects on the maturation of the viral particle (283). The transition from immature to mature HIV-1 virus is characterized by complete cleavage of the Gag polyprotein by the viral protease (PR) contained in the *pol* region (310). Only one subunit of PR is present in Gag-Pol, and it must dimerize with another PR molecule to become active (82). At this point, the importance of the 20:1 Gag:Gag-Pol ratio becomes apparent as interactions between PR monomers lead to dimerization and enzyme activation, thus resulting in cleavage of Gag prematurely (141). This observation has been verified in multiple studies examining not just the Gag:Gag-Pol ratio, but also mutations in Gag-Pol that affect Gag:Gag-Pol interactions and have deleterious effects on particle assembly and release, and both effects can be ameliorated by mutations that inactivate PR (109, 141, 244).

Formation of the fully infectious HIV-1 particle is contingent on the process of maturation, which is discussed in more detail below. Maturation occurs either concurrent with, or just after, release of the budding viral particle from the plasma membrane, which is regulated by cellular proteins known as the ESCRT machinery (283). Viruses that are defective for recruitment of ESCRT proteins fail to release from the host cell plasma membrane, and display defects in Gag cleavage associated with an intermediate stage of maturation (42, 98). The ESCRT machinery is recruited by interaction with the p6 domain of HIV-1 Gag

(186), and is responsible for localized membrane deformation (curvature) and the membrane scission event that liberates the nascent virus (313). Interestingly, a recent structural study using cryoelectron tomography (cET) has suggested that recruitment of the ESCRT machinery may also play an important role not only in particle release, but also in determining the amount of Gag present in the viral particle by the formation of a bud neck, which limits incorporation of additional Gag molecules (42). This finding implies that ESCRT recruitment can profoundly affect the process of particle assembly in addition to its well characterized role in stimulating release of viral particles (42).

Structure and contents of the Mature HIV-1 Virus

Maturation is the final step required for formation of an infectious viral particle and occurs either immediately after or concomitant with release. This process is characterized by a major re-arrangement of Gag proteins driven by Gag subunit interactions and the activity of the viral protease (PR) (283, 310). The immature particle consists of a lattice-like arrangement of Gag molecules beneath the viral envelope (formerly the inner leaflet of the host cell plasma membrane) centered on CA-CA and NC-RNA interactions (67, 68). This lattice is composed of hexameric subunits which comprise an incomplete Gag shell with the C-terminus of Gag (and Gag-Pol) molecules directed inward (67, 68). Activation of the viral PR results in an ordered cleavage of Gag molecules due to

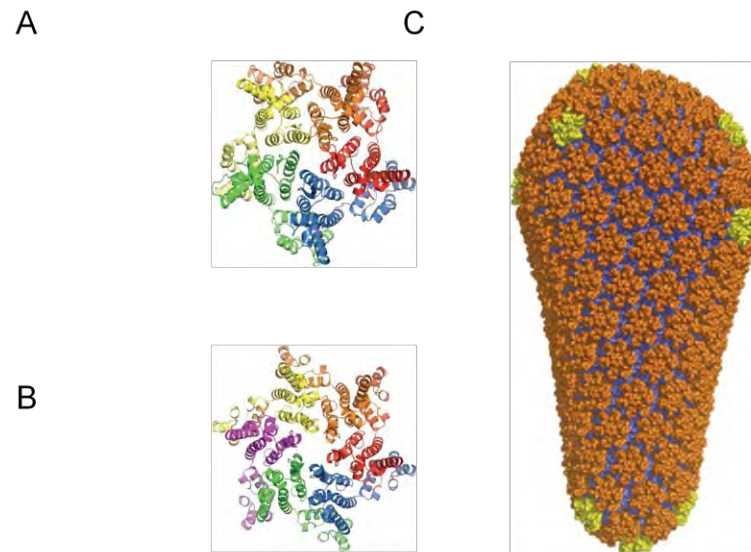


Figure 1.5 HIV-1 CA molecules form higher order multimers and assemble into fullerene cone. Cleaved HIV-1 CA molecules assemble into hexamers (A) and pentamers (B) mediated by interactions between helices of the CA-CTD. (C) Interaction of hexamers and pentamers mediated by CA-NTD results in the formation of the predicted fullerene cone composed of approximately 250 hexameric subunits with 12 pentameric defects (highlighted in yellow) Image reproduced with permission from Pornillios et al. 2011.

different affinities of PR and cleavage sites, and a subsequent cascade of protein-protein interactions ensues (67, 310). The RNA- NC complex is removed from the Gag lattice to form a condensed viral RNP (7). Cleavage of CA-SP1

from MA allows MA to remain bound to the viral envelope while releasing CA to form the conical viral core (89, 310).

The viral capsid or core (core will be used to avoid confusion with the CA protein) is an electron dense structure with the predicted form of a fullerene cone (90), made up of ~250 CA hexamers and 12 pentameric defects (5 at narrow tip, 7 at wider cap, Fig. 1.5)(88). Retroviral capsid proteins, which are structurally conserved despite significant sequence variability, contain two globular domains composed of 7-helices (NTD) and 5 helices (CTD) connected by a flexible linker (67). In HIV-1, interactions between adjacent NTD form either a hexameric or pentameric ring structure with six- or five-fold symmetry, while the CTD forms a 'belt' surrounding the subunit (87, 246). Non-covalent interactions between the CTD 'belts' of adjacent CA subunits act to stabilize the formation of the Gag lattice (246). A similar orientation of CA molecules within the context of the Gag polyprotein has been observed in imaging studies of immature virus particles, suggesting that the formation of the CA hexamers may be determined prior to release/maturation (87, 246).

At present, it is unclear whether core formation is the result of high local concentration of CA within the particle, or whether formation of the core is the result of CA hexamer multimerization following nucleation events. Several reports have provided evidence for either mechanism (12, 18, 34). For example, spontaneous formation of CA tubes that resemble authentic HIV-1 cores is

possible *in vitro* under the right conditions with concentrated CA monomers (12). A recent cET study by Briggs, *et. al.* quantified the number of Gag molecules in the purified immature particle at ~5,000, which by association suggests that the immature viral particle contains almost 2.5 times more CA than the amount present in the mature core. This estimate brings the internal viral CA concentration to ~40mM, a more than 4.0×10^4 increase in concentration from that used the *in vitro* assay (18, 34). However, the consistent observation of authentic viruses and VLP with multiple cores argues against a strictly concentration driven reaction as formation of the first core would substantially reduce the concentration of CA subunits within the virus (18, 34). It has been argued that the presence of multiple cores is indicative of growth from either one or multiple nucleation points (30). Using an *in vitro* fluorescent microscopy approach, one study reported that formation of CA tubes occurred both uni- and bi- directionally following a rate-limiting nucleation step (12). The finding is consistent with the work of Briggs, *et. al.*, who reported results of an *in vivo* study of viral core formation (30). Using cET, they found that the larger cone end of the viral core closely matched the curvature of the envelope in that location. They hypothesized that core formation initiated via a nucleation event at the narrow end, perhaps from the budding scar or a localized deposition of CA pentamers, and radiated to the far end of the particle, where elongation is halted by the viral envelope and the cone was capped (30).

In addition to the cleavage and re-arrangement of Gag, particle maturation has effects on the extracellular domain of the Env protein (73, 83, 84). Previous studies indicate an interaction between Gag (MA) and the transmembrane domain of the gp41 subunit of HIV-1 Env during particle formation. It has also been reported that point mutations in the MA core interfere with the ability of Env to associate with viral particles, but such interference could be abrogated by removal of the cytoplasmic tail of gp41, or the complete removal of *gag* (83, 84, 203, 253). Data from the Aiken laboratory suggests that the structural rearrangements of Gag occurring during particle maturation affect the display of surface epitopes of gp41 (139). Using a panel of fluorescently-tagged antibodies, they show that maturation of HIV-1 viruses can mask regions of gp41 that were otherwise surface-exposed, and thus recognized by their panel of antibodies (139). Collectively, these findings indicate that maturation of the viral particle can affect other aspects of the virion structure in addition to Gag re-arrangement.

In addition to incorporation of Env glycoproteins into viral particles, other HIV-1 proteins required for nascent infection are also packaged into virions, and in most cases, into mature viral cores. Based on previous studies which correlated the levels of specific viral proteins to Gag, recent work suggests there may be 700 molecules of Vpr, and 250 molecules of both HIV-1 integrase and reverse transcriptase (18, 33). The accessory protein Nef is also present in the mature viral particle (151). Nef associates with the membrane site of assembly through an N-terminal myristic anchor and is cleaved during maturation. What

possible role Nef plays during the subsequent round of infection is the subject of much debate, and will be considered in significant detail in Chapter III.

CHAPTER II

Rescue of HIV-1 Release by Targeting Widely Divergent NEDD4-type Ubiquitin Ligases and Isolated Catalytic HECT domains to Gag

SUMMARY

Retroviruses engage the ESCRT pathway through late assembly (L) domains in Gag to promote virus release. HIV-1 uses a PTAP motif as its primary L domain, which can interact with the ESCRT-I component Tsg101. In contrast, other retroviruses use PPxY-type L domains, which constitute ligands for NEDD4-type ubiquitin ligases. Surprisingly, although HIV-1 Gag lacks PPxY motifs, the release of HIV-1 L domain mutant viruses is potently enhanced by ectopic expression of NEDD4-2s, a native isoform of NEDD4-2 containing a truncated C2 domain. The basis of the unique potency of the NEDD4-2s isoform has remained to be determined. We show that the truncated C2 domain of NEDD4-2s functions as an autonomous Gag-targeting module that can be functionally replaced by the unrelated Gag-binding protein cyclophilin A (CypA). The residual C2 domain of NEDD4-2s was sufficient to transfer the ability to stimulate HIV-1 budding to other NEDD4 family members, including the yeast homologue Rsp5, and even to isolated catalytic HECT domains. The isolated catalytic domain of NEDD4-2s also efficiently promoted HIV-1 budding when targeted to Gag via CypA. We conclude that the regions typically required for

substrate recognition by HECT ubiquitin ligases are all dispensable to stimulate HIV-1 release, implying that the relevant target for ubiquitination is Gag itself or can be recognized by divergent isolated HECT domains. However, the ability to ubiquitinate Gag was not solely sufficient to stimulate HIV-1 budding. Rather, our results demonstrate that the preference of NEDD4.2s to synthesize polyubiquitin chains formed through K63-linkages is critical for the ubiquitin ligase-mediated virus release.

INTRODUCTION

Assembly of HIV-1 particles at the plasma membrane results in the formation of a budding virus composed of a spherical Gag shell surrounded by a viral envelope that is still contiguous with the plasma membrane (307). This structure must separate from the cell to initiate infection in a new target cell. Release is accomplished by recruitment of the host cell's Endosomal Sorting Complex Required for Transport (ESCRT) machinery (22, 85, 190, 199, 233, 307), which promotes the final membrane scission event that separates the viral envelope from the PM. The ESCRT machinery is used by the host cell in the formation of multivesicular bodies (MVB) in late endosomes, in cytokinesis to free the daughter cell and has been implicated in autophagy (43, 190, 197, 226, 251, 261). These processes all contain events of similar topology to virus budding, that is membrane curvature away from the cytoplasm followed by scission.

Access to the ESCRT machinery is mediated by interactions between ESCRT subunits and short motifs in retroviral Gag molecules known as Late (L)-domains (23, 69, 307). The first indication that HIV-1 contained a site for host cell protein recruitment came from deletions of the p6 region of Gag (98, 115), which, when expressed, forms particles at the PM that fail to release, and soon after, similar regions were found in other retroviruses (69). It has been observed that these regions can be moved to different areas of the Gag molecule, or used interchangeably between different viruses, suggesting that they behave as

recruitment domains for cellular factors (224). Analysis of the amino acid sequences of these regions has led to the identification of three common L-domain motifs, often found in combination, which each interact with a different cellular protein (69). The PS/TAP motif, the dominant L-domain in HIV-1 as well as in MLV and the Filovirus Ebola, recruits the ESCRT-I subunit Tsg101 (185, 269). The YPxL motif, an auxiliary L-domain in HIV-1 but the only one present in Equine Infectious Anemia Virus (EIAV), recruits the ESCRT accessory protein, ALIX. The third style of L-domain, not present in HIV-1 but found in both MLV and Ebola, is the PPxY motif which interacts with E3 ubiquitin ligases of the Nedd4 family (269). Each cellular host factor provides access to the ESCRT pathway in different fashions, but all result in the same process, scission of the plasma membrane and release of the virus.

The ESCRT pathway

The ESCRT machinery exists in all eukaryotes and consists of five heterooligomeric complexes, ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III and VPS4, as well as accessory proteins such as ALIX (Table 2.1) (117). ESCRT complexes are thought to be recruited sequentially to endosomal membranes, although there is evidence that upstream ESCRT complexes may act in parallel (120). ESCRT-0, ESCRT-I and ESCRT-II are recruited as pre-formed complexes, whereas the ESCRT-III complex transiently assembles on endosomes from

Table 2.1: ESCRT proteins involved in HIV-1 release

ESCRT Complex	Subunit	Required for HIV-1 release?	ESCRT related interaction partners	Role	Citation
ESCRT-0	HRS	No	Ubiquitin, STAM1/2, Tsg101	Interact with Ub	247
	STAM	Not determined	Ubiquitin, HRS	Interact with Ub	
ESCRT-I	Tsg101	Yes	Ubiquitin/Hrs/ ESCRT-I subunits	Interact with p6 PTAPP motif; interact with ubiquitin	91, 185
	Vps37	Yes	ESCRT-I subunits	required as ESCRT-I component	76,282
	Vps28	Yes	Eap45/ESCRT-I subunits	Structural component of ESCRT-I complex	184, 282
ESCRT-II	Mvb12a/b	No	ESCRT-I subunits	Structural component of ESCRT-I complex	196
	Eap20	No	ESCRT-II subunits	Structural component of ESCRT-II complex	162
	Eap30	No	CHMP6/ESCRT-II subunits	Interacts with ESCRT-III through Chmp 6	162
	Eap45	No	Vps28/ESCRT-II subunits	Interacts with ESCRT-I through Vps28	162
ESCRT-III	Chmp 3	No	CHMP2A/B, CHMP4	Formation of ESCRT-III, Membrane Scission	198, 301
	Chmp 6	No	Vps28/ESCRT-II subunits, CHMP4A/B Vps4	Formation of ESCRT-III, Membrane Scission	198
	Chmp 2	Yes	CHMP4A/B	Formation of ESCRT-III, Membrane Scission	43, 198, 282
	Chmp 4	Yes	ALX, CHMP6, Vps4	Formation of ESCRT-III, Membrane Scission	198
Accessory proteins	Alix	Sometimes	CHMP4 (A-D)	Interact with HIV-1 p6 and NC	184, 279
	Nedd4	Sometimes	Unknown	Interact with HIV-1 CA	56, 292, 308
	Vps4 A/B	Yes	CHMP2A/B	ESCRT complex recycling	91, 301
	LIF1	Yes	ESCRT-II subunits, VPS4A/B	ESCRT complex recycling	190

soluble cytosolic components (307). Mammalian ESCRT-III is formed by charged multivesicular body proteins (CHMPs), a family of structurally related but highly divergent α -helical proteins that has 12 known members in humans (307). A recent report by Wollert and Hurley demonstrated that the individual ESCRT complexes perform discrete functions (313). Using purified ESCRT components and giant unilamellar vesicles, they showed that 1) the ESCRT-0 complex functions to concentrate ubiquitinated cargo on endosomal membranes, 2) the ESCRT-I and ESCRT-II complexes together induced bud formation without themselves entering the lumen of the budding vesicle, and 3) the ESCRT-III complex mediates membrane scission from the cytosolic side of the bud (313). Rather than being consumed, ESCRT-III is disassembled and recycled by the VPS4-VTA1 complex, enabling further rounds of sorting (16). Thus, in the MVB pathway, all of the ESCRT complexes are required. Intriguingly, only certain

ESCRT complexes (Table 2.1) have been shown to be required for the budding and release of HIV-1 (190).

Although the ESCRT machinery was first characterized based on its requirement for MVB biogenesis, it has recently emerged that the ESCRT system is also involved in membrane remodeling and fission events that lead to midbody abscission during the final stages of cytokinesis (43, 44, 197). Additionally, the ESCRT machinery controls earlier stages of cell division. At least a subset of ESCRT proteins is required for the proper function of centrosomes, and it has been noted that certain ESCRT-III components localize to kinetochores (195). Interestingly, ESCRT-III and Vps4 homologs are also key components of the cell division system of certain archaea (173, 262). Since eukaryotes and archaea diverged at least a billion years ago, the promotion of cell division is likely to be the ancestral function of the ESCRT machinery.

ESCRT-I

The PTAP L domain of HIV-1 Gag binds directly to TSG101, the central component of ESCRT-I, a stable cytosolic heterotetramer formed by one copy each of TSG101, VPS28, VPS37 and MVB12 (117, 196). Human cells express four versions of VPS37 (VPS37A-VPS37D) and two versions of MVB12 (termed MVB12A and MVB12B), which are encoded by different genes (76, 196, 282). Thus, eight combinations of TSG101, VPS28, VPS37 and MVB12 are

theoretically possible, and all of these can indeed form stable ESCRT-I complexes in cells (196). The ESCRT-I heterotetramer consists of a core formed by a headpiece and a long stalk, and of conformationally dynamic TSG101 UEV and VPS28 C-terminal domains that are appended to opposite ends of the extended core (150).

The flexible attachment of the UEV domain of TSG101 to the ESCRT-I core is thought to facilitate binding of ligands, such as the PTAPP L domain in HIV-1 p6. In the endocytic pathway, the TSG101 UEV domain binds to PSxP motifs in the ESCRT-0 component HRS (27, 247), which has led to the proposal that HIV-1 Gag mimics the ability of HRS to recruit TSG101 (247). In support of this notion, an HRS fragment capable of recruiting TSG101 rescues virus-like particle production when fused to an active HIV-1 Gag construct that lacks its native PTAPP L domain (247).

The role of the TSG101 UEV domain in HIV-1 budding is limited to the recognition of the PTAP L domain since the N-terminal UEV domain is dispensable for the rescue of L domain defective HIV-1 when TSG101 is artificially recruited to assembly sites (185). However, under these artificial conditions, a C-terminal region of TSG101 that binds to VPS28 remains necessary to confer budding activity (186, 282). Interestingly, the VPS28 C-terminal domain, which is attached to the ESCRT-I core via a flexible tether (150), is sufficient to rescue budding when appended to an L domain defective

Gag construct, apparently by serving as an adaptor for the ESCRT-III component CHMP6 (234). TSG101 also interacts with VPS37 proteins, which again can provide L-domain function when fused directly to Gag (76, 282). Furthermore, binary TSG101-VPS37 complexes interact with MVB12 (196), which is thought to stabilize the extended stalk of the ESCRT-I core (150). Nevertheless, MVB12 does not appear to play a central role in HIV-1 release (196). However, the depletion of MVB12 proteins induces aberrant virion morphologies and inhibits infectivity (196).

ESCRT-II

Together with ESCRT-I, ESCRT-II mediates the deformation of endosomal membranes into buds during MVB biogenesis (9, 313). Yeast ESCRT-II binds to the ESCRT-I component Vps28 and to the ESCRT-III component Vps20 and thereby links ESCRT-I to ESCRT-III (119). Human ESCRT-I and ESCRT-II also interact, albeit in a different manner (162), and human ESCRT-II binds directly to the ESCRT-III component CHMP6 (162, 317). Together, these observations indicate that ESCRT-II also connects ESCRT-I to ESCRT-III in humans. Just as one might expect, human ESCRT-II transiently associates with endosomal membranes like its yeast counterpart and functions in receptor downregulation (162). However, human ESCRT-II is dispensable for

HIV-1 budding (162), indicating that HIV-1 p6-associated ESCRT-I recruits ESCRT-III membrane scission machinery in some other way.

ESCRT-III

The ESCRT-III complex is the main engine that carries out membrane scission in the ESCRT pathway (313, 314). The core ESCRT-III complex is formed by Vps20, SNF7, Vps24 and Vps2 in yeast and by their homologues CHMP6, CHMP4A-CHMP4C, CHMP3, and CHMP2A and CHMP2B in humans. As its yeast ortholog Vps20, human CHMP6 is N-terminally myristoylated and thought to nucleate the oligomerization of CHMP4 proteins on membranes (286, 287, 317). The remaining ESCRT-III core components CHMP3 and CHMP2 appear to function as termination factors that cap CHMP4 filaments and recruit VPS4 for ESCRT-III disassembly (286).

An essential role for the membrane fission activity of ESCRT-III in HIV-1 release is strongly implied by the fact that various dominant-negative versions of VPS4 (the ATPase that disassembles ESCRT-III) arrest the budding of HIV-1 and other retroviruses (91, 97, 268). HIV-1 budding is also strongly inhibited when both human VPS4 proteins are depleted simultaneously via siRNA (145). Moreover, individual ESCRT-III components, which are autoinhibited to prevent premature polymerization, can be activated to become extremely potent inhibitors of HIV-1 budding (10, 163, 184, 206, 273, 279, 301, 318). For

instance, CHMP6 fused to RFP strongly inhibits HIV-1 release in a dominant negative manner. It therefore comes as a surprise that CHMP6, the ESCRT-III subunit activated by ESCRT-II to trigger ESCRT-III assembly (287), is not required for HIV-1 budding (162). CHMP3 is also dispensable for HIV-1 budding, leaving only the core ESCRT-III components CHMP2 and CHMP4 as potential players in HIV-1 release. Indeed, a recent study demonstrates that the co-depletion of CHMP2 and CHMP4 family members profoundly impairs HIV-1 release, whereas other CHMPs are not required (198).

When overexpressed, human CHMP4A and CHMP4B polymerize into circular filaments that deform the plasma membrane into buds and tubules emanating from the cell surface (108). Filament formation by SNF7, the yeast homolog of the three human CHMP4 proteins, is required to sequester endosomal cargo (286) and appears to be the critical event during ESCRT-III catalyzed membrane scission (314). Indeed, when recruited via ESCRT-II and the nucleation factor Vps20 (CHMP6), SNF7 (CHMP4A-C) was sufficient to catalyze the scission of bud necks in an *in vitro* reaction (313). Thus, during HIV-1 assembly, the formation of circular CHMP4 polymers within Gag-induced bud necks could, in principle, lead to membrane constriction and drive virus release (226). CHMP4-mediated membrane constriction in concert with Gag may also explain the observation that HIV-1 virions are often released before the Gag shell is completely assembled (42). In one model (77), which is based on the dome shape of certain CHMP2/CHMP3 copolymers (164), the assembly of dome-

shaped CHMP4 polymers within HIV-1 buds would tighten the neck, owing to an affinity of CHMP4 for membranes, ultimately causing spontaneous fission.

However, the critical role of CHMP2 proteins, which recruit VPS4, also suggests an active participation of VPS4-VTA1 complex in HIV-1 release (198). Recent cell imaging studies reveal that ESCRT-III and VPS4 are rapidly and transiently recruited before HIV-1 particle release, supporting the notion that VPS4 has a direct role in budding (16, 138).

ALIX:

Although HIV-1 relies predominantly on a PTAPP type L domain to recruit the CHMP4 fission factor via ESCRT-I, it can also engage CHMP4 in an alternative manner. As noted earlier, HIV-1 p6 harbors a secondary L domain of the LYPx_nL type, which functions as a docking site for ALIX, another early-acting factor in the ESCRT pathway (184, 279, 301). ALIX is composed of a banana-shaped Bro1 domain, a V-shaped middle domain, and a presumably unstructured proline-rich C-terminal domain (PRD) that harbors binding sites for various interaction partners. Through its Bro1 domain, ALIX interacts with C-terminal residues of the human CHMP4 proteins (78, 184, 189, 279, 301). ALIX thus directly links HIV-1 Gag to ESCRT-III.

The central V domain of ALIX harbors the binding site for the LYPx_nL-type L domains in HIV-1 p6^{Gag} and EIAV p9^{Gag} (78, 169). Interestingly, the LYPx_nL

motif in HIV-1 p6^{Gag} (n=3) adopts a helical main-chain conformation when bound to ALIX, whereas the EIAV L domain (n=1) is bound in an extended conformation (321). Although the conserved residues in the LYPxnL-type L domains of HIV-1 and EIAV make equivalent interactions with ALIX, HIV-1 p6^{Gag} has a significantly lower affinity for ALIX than EIAV p9^{Gag} (321). Presumably, the difference in affinity is related to the fact that HIV-1 p6^{Gag} also has a docking site for ESCRT-I, whereas EIAV relies solely on ALIX for budding.

HIV-1 budding can be potently inhibited by dominant-negative ALIX fragments, as long as a V domain capable of mediating ALIX dimerization is maintained (202, 235, 279). Dominant-negative ALIX fragments also induce Gag processing defects resembling those seen in the absence of a functional L domain (202, 279). However, mutations in the ALIX binding site of HIV-1 p6^{Gag} generally have more modest effects on HIV-1 release and replication than mutations that disrupt the TSG101 binding site (78, 86). Also, HIV-1 p6^{Gag}-mediated budding was significantly less affected by ALIX depletion than EIAV p9^{Gag}-mediated budding (184). Overall, these findings are consistent with the notion that HIV-1 p6^{Gag} contains a bipartite L domain in which the TSG101 binding site predominates but does not account for all of the budding activity (182).

Remarkably, the release and infectivity of HIV-1 mutants that lack the PTAPP L domain can be dramatically stimulated by overexpressing ALIX (78,

291). This implies that ALIX, if expressed at sufficiently high levels, can essentially fully substitute for TSG101 in HIV-1 budding. As expected, the ALIX-mediated rescue is dependent on a V domain capable of binding to LYP_x_nL (78). Moreover, the presence of an intact LYP_x_nL motif in p6^{Gag} is required, confirming that this motif constitutes a genuine L domain that functions through ALIX (78, 291). The activity of ALIX also depends on the ability of its Bro1 domain to interact with CHMP4, implying that the recruitment of this fission factor is crucial for ALIX-mediated HIV-1 budding. In contrast, interaction sites for TSG101, endophilin, CIN85, CMS/CD2AP, and CEP55 in the PRD of ALIX are dispensable for its function in HIV-1 budding (43, 78, 291). Nevertheless, the very C-terminus of the PRD is essential (43, 78, 291), apparently because PRD-dependent ALIX multimerization is required to promote HIV-1 budding (43).

It has recently been revealed that HIV-1 Gag harbors a second binding site for ALIX in the NC domain (74, 243), in addition to the LYP_x_nL ALIX binding site in p6. ALIX interacts with HIV-1 NC via its N-terminal Bro1 domain (74, 243), and the widely divergent Bro1 domains of other human proteins share an affinity for HIV-1 NC, indicating that NC recognizes a structural feature of the Bro1 domain rather than a specific sequence (242). The ability of NC to interact with Bro1 domains depends on two conserved CCHC-type zinc finger motifs in NC that are also required for the selective packaging of genomic viral RNA during assembly (242, 243). Nevertheless, nucleic acid does not appear to be involved in the ALIX–NC interaction because the interaction was maintained in the

presence of nuclease (243). Interestingly, mutations that disrupt the ALIX binding site in NC cause defects in Gag processing and HIV-1 release resembling those typically seen with HIV-1 L domain mutants (243). Furthermore, electron microscopy analyses of Rous sarcoma virus and HIV-1 NC mutants have revealed budding defects similar to those of L-domain mutants (75, 168). Together, these observations suggest that NC may play a role in ESCRT engagement and virus release, in addition to its previously documented role in virus assembly (322).

NEDD4 E3-ubiquitin ligases:

The Nedd4 family of ubiquitin ligases serves as the ESCRT access point for viruses that contain the PPxY L-domain (24, 39, 100, 110, 183, 315). There are nine members of this family found in humans (122), and a number of them have been implicated in the release of a range of viruses, all in an ESCRT dependent manner (190). All family members share a common modular architecture with an N-terminal C2 domain, multiple WW domains, and a catalytic Homologous to E6-AP Carboxyl Terminal (HECT) domain (122). Recognition of the PPxY motif is determined by the ligase's WW domain, and a recent study has shown that affinity is determined by residues adjacent to the PPxY sequence (183). The C-terminal HECT domain is responsible for the addition of ubiquitin to substrate lysine residues, although in the case of viral budding the ubiquitination target has not yet been determined (56, 308, 320).

Ubiquitin-a surrogate Late-domain?

The importance for ubiquitin in viral particle release is well established, although the exact role that it plays remains controversial (219, 249). Almost twenty years ago, it was reported that the concentration of free ubiquitin in the viral particles of ALV were about 10-fold higher than the concentration of ubiquitin in the cytoplasm, suggesting specific incorporation of that molecule into virions (249). This finding (249) was soon followed by another reporting the presence of free ubiquitin in the particles of HIV and MLV, further reinforcing the idea that ubiquitin enrichment in virus particles was a conserved process (219). It has since been demonstrated that treatment of cells producing HIV-1 with proteasome inhibitors, which reduce cytoplasmic levels of free ubiquitin, resulted in a release defect typically observed with L-domain mutants (225, 265, 270). This observation clearly indicated a role for ubiquitin in the release of retroviral particles.

Not surprisingly, studies of retroviruses that utilize a PPxY motif to recruit members of the Nedd4 E3 ubiquitin ligase family have provided some additional insight into the relationship between ubiquitin and particle release. Over-expression of Nedd4 family members in cell lines transfected to express MLV, HTLV-1 or RSV all show an increase in the levels of Gag ubiquitination accompanied by a commensurate increase in particle release (24, 146, 269, 278,

315). A mutational analysis of HTLV-1, in which 4 Lys residues present in the MA subunit of that Gag molecule were mutated to Arg, illustrated that ubiquitination of Lys74 of MA was required for particle release (111). A similar result was obtained from a mutational analysis of HIV-1 Gag, in which the 17 Lys residues of SP1, NC, SP2 and p6 were changed to Arg, indicating that at least mono-ubiquitination of Gag was required for particle release (102, 127). Interestingly, an L-domain phenotype appeared only after all ubiquitin acceptor sites were mutated (101, 102), implying that while Gag-ubiquitination served a functional purpose, in the case of HIV-1, no specific site of ubiquitin attachment was required. It has been hypothesized that the addition of mono-ubiquitin to HIV-1 Gag may enhance recruitment of ESCRT proteins by providing additional interaction sites for Tsg101(245). Tsg101 can bind ubiquitin via an Ubiquitin E2 Variant domain while simultaneously interacting with the HIV-1-p6 PTAPP motif, thus increasing the affinity of Tsg101 for Ub-Gag (245). Other labs have found that late domain deficient versions of Equine Infectious Anemia Virus (EIAV) or Prototypic Foamy Virus could be rescued by the fusion of a mono-ubiquitin molecule to the C-terminal of both Gag molecules, indicating that ubiquitin itself could serve as a surrogate L-domain in this context (133, 320).

While the attachment of ubiquitin to Gag has been shown to be sufficient to drive recruitment of ESCRT proteins (133, 320), a question still remains as to whether retroviral Gag is the relevant substrate for ubiquitination. A recent report by Zhadina et al demonstrated that mutation of the sole ubiquitin acceptor site in

PFV Gag had no effect on the release of viral particles if the native Nedd4-recruitment site remained intact (320). Release of particles bearing this mutation still relied on an active E3 ubiquitin ligase, suggesting that ubiquitination of a separate protein may be necessary (320). A similar result was reported by Chung et al who demonstrated that in the presence of overexpressed Nedd4.2s, the ESCRT-I subunit, MVB12 was also ubiquitinated (56).

Participants in protein ubiquitination

Ubiquitination is a post-translational modification of a protein by the covalent attachment of one or more ubiquitin molecules (257). Substrate proteins may be ubiquitinated at their N-terminal amines or at specific amino acids such as cysteine, serine and lysine (257). By far the most common site of attachment is lysine with a covalent bond being formed between the C-terminal Glycine residue of ubiquitin and the ϵ -amide of the target Lysine (257). In addition to modifying target proteins with mono-ubiquitin, ubiquitin can also be attached to itself, forming chains built upon linkages with 7 of the available Lysine residues present in ubiquitin (228).

The covalent addition of ubiquitin to target proteins requires the function of a series of proteins that operate in a process commonly referred to as the ubiquitin cascade (257). This process is initiated by the formation of a thioester intermediate formed between ubiquitin and the E1 protein in a process that requires ATP (Fig. 2.1)(257). The 'charged' molecule is then passed from the E1

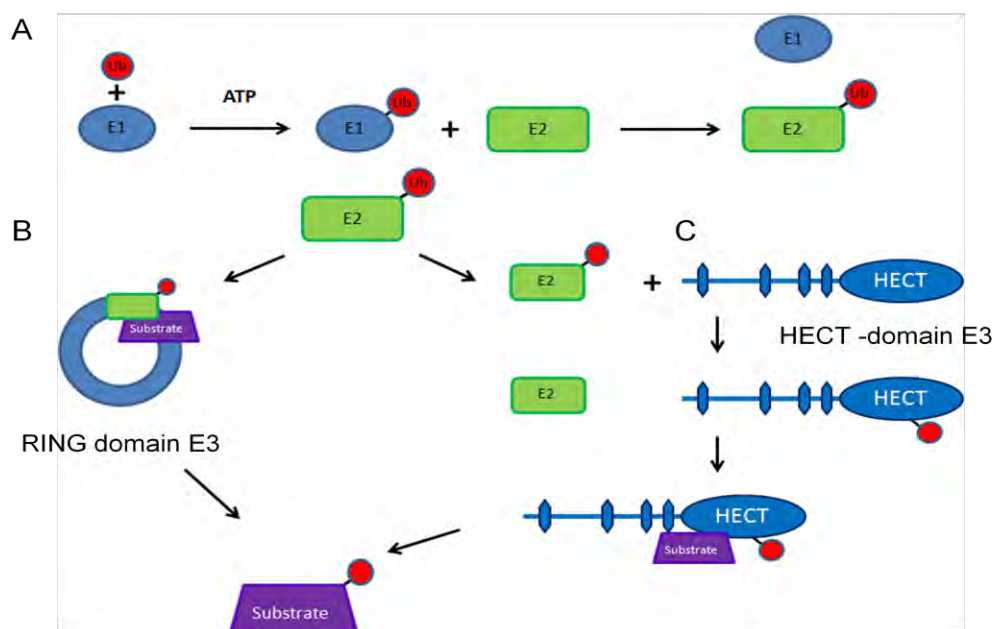


Figure 2.1 Schematic representation of protein ubiquitination by HECT and RING E3 ubiquitin ligases. Formation of thioester bond between E1 and ubiquitin requires the activity of ATP. Subsequent transfer of Ub to E2 is also through formation of a thioester intermediate. (B) RING domain E3 ubiquitin ligases form a scaffold between E2 and substrate to ubiquitinate substrate protein. (C) HECT domain E3 ubiquitin ligases form an additional thioester bond between Ub and E3 catalytic Cys prior to substrate ubiquitination.

to the E2 protein, through the formation of a second thioester intermediate (257). Attachment of ubiquitin to the target molecule requires the activity of the E3 ubiquitin ligase. Several hundred of E3 ubiquitin ligases have been found to be expressed in mammalian cells, where they participate in multiple processes such as protein turnover, receptor downregulation, and transcriptional regulators (257). Although there are hundreds of these enzymes, E3 ubiquitin ligases have been broadly grouped into two subsets based on the structures used to transfer ubiquitin from the charged Ub-E2 to the substrate.

Members of the RING family (Really New Interesting Gene) of E3 ubiquitin ligases serve as intermediates during the ubiquitination process (130). While these proteins are required for the formation of the amide bond between ubiquitin and the substrate, ubiquitin-substrate bond formation is the result of transfer from the charged E2-Ub to the substrate without the formation of an E3-Ub intermediate (130). This method of substrate ubiquitination differs greatly from that observed with the second group of E3s, those containing a HECT domain (130, 257).

HECT (Homologous to E6AP Carboxyl Terminus) domain E3 ubiquitin ligases direct the ubiquitination of target Lysines through the formation of a high energy thioester intermediate, directly interacting with ubiquitin at a Cys residue present in the enzyme's active site. Interestingly, it has been reported in the literature that specific E2 molecules are utilized by specific E3 ligases and that E2-E3 combinations may play a functional role in determining polyubiquitin chain linkages (154), although HECT E3 ligases have been shown to specify ubiquitin-linkage directly (147). Unlike RING containing E3 ubiquitin ligases, HECT domain containing E3s generally contain recognition sites within the enzyme for direct interaction with substrates (181). The type of substrate recognition site present in the enzyme has been used to organize these ligases into three distinct families (181). One family, the NEDD4 family of E3s, contains enzymes that possess multiple WW domains used to bind to substrates containing the PPxY recognition motif (122, 181). The second family contains two groups referred to

as the Large and Small HERC E3s; members of this group are designated by the presence of at least one RLD domain (Recognizer of Chromatin Condensation Like Domain) and based on cellular localization have been implicated in endosomal activity (112, 181, 257), although a role in innate immunity has also been reported (64). Finally, the family of HECT domain-containing ubiquitin ligases (SI) is comprised of all other HECT E3s (181). Members of this family are classified by the presence of multiple different substrate recognition domains, or in certain cases, no domain at all (257). For example, while HUWE1 ligase contains 5 different domains, the ubiquitin ligase E6AP contains none (257).

In addition to the varieties of substrate recognition domains, structural differences in the catalytic HECT domains themselves have been observed. The crystal structures of the HECT domains of E6AP, HUWE1 (SI family), Nedd4, WWP1 and ITCH (NEDD4 family) have all been reported allowing for their comparison (71, 114, 188, 223, 298). All 5 reported structures display overall similar tri-lobal arrangement with N-terminal and C-terminal lobes of the HECT-domain flanking the catalytic cysteine residue. However, the orientation of the lobes relative to each other differs between the two families; HECT domains of E6AP and HUWE1 have their lobes oriented in a T shape (114, 223), while the HECT domains of NEDD4 family members are oriented in more of an L shape (71, 166, 188, 298). This difference in HECT domain structure may account for the observed preferences in ubiquitin chain specificity, although it is possible that the chain-type specificity is dictated at the level of E2 interaction as well, and this

would make sense considering the catalytically inactive nature of the RING finger E3s (130). However, a recent study indicated that the C-lobe of HECT domain E3 ubiquitin ligases plays an important role in determining ubiquitin chain-type specificity (147).

Mono- and Polyubiquitin as recognition motifs

Recognition and interaction of ubiquitinated proteins of the cell are the result of hydrophobic interactions between ubiquitin and specific domains referred to here as ubiquitin binding motifs (241). The hydrophobic region of ubiquitin, termed the hydrophobic patch, is centered around three key residues, Leu 8, Ile44 and Val70, and mutation of these regions can result in variable recognition defects with cellular binding partners (166). Attachment of a single ubiquitin molecule to a substrate, monoubiquitination, is sufficient to target cellular receptors for internalization from the plasma membrane (272, 281). However, direction to other cellular pathways is based upon the recognition of polyubiquitin chains, formed by the attachment of one ubiquitin molecule to any of the seven Lys residues found in ubiquitin (13). Two of the most studied forms of polyubiquitin chains are those formed through linkages between Lys 48 or Lys 63 (respectively referred to as K48 or K63 chains)(13, 166).

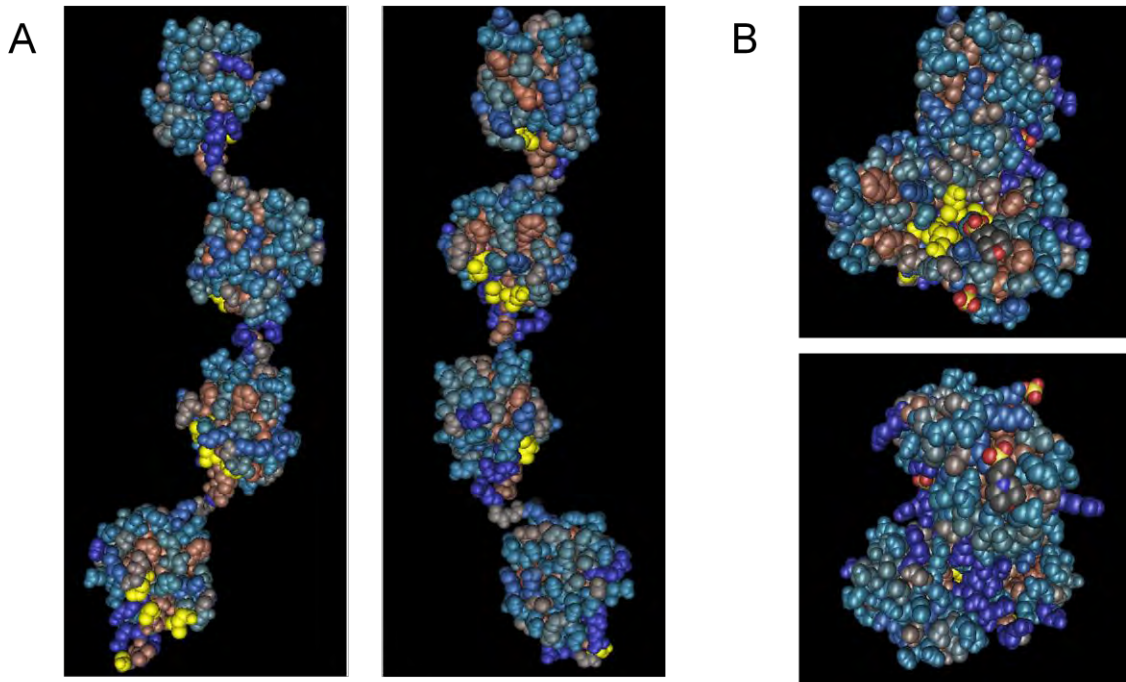


Figure 2.2 Ubiquitin Chain linkages impart secondary recognition patterns for ubiquitin recognition proteins. (A) Ubiquitin chains formed by linkages between K63 and ubiquitin leaves the entire hydrophobic pocket (highlighted in yellow) of ubiquitin accessible to ubiquitin interacting proteins. The orientation of the hydrophobic patches also allows for pattern recognition by certain UM containing proteins. (B) Formation of polyubiquitin chains through K48-linkages results in the formation of a more condensed molecule in which the hydrophobic patch is partially obscured by neighboring ubiquitin molecules. Images modified from structures deposited into PDB (K63-linked tetraubiquitin 3HM3, K48-linked tetraubiquitin 26OV). Original images from Datta et al., 2009. Reproduced with permission

Generation of either of these two polyubiquitin chains results in the formation of specific secondary structures (65) which serve as a different recognition motif for cellular proteins, and the formation of these chains is determined by at the level of the E3 ubiquitin ligase (147, 306) or at the level of E2 recruitment others (154). As Fig. 2.2 illustrates, the resulting structure of K48

linkages sequesters the hydrophobic patch of ubiquitin into a cleft formed between the two adjacent ubiquitin molecules (65). In contrast, the linear structure formed by K63-linked ubiquitin chains results in a repetitive structure of unobstructed hydrophobic patches (65). The more open structure of the K63-linked chain allows for recognition by molecules that contain only one ubiquitin interaction motif, such as Tsg101, as well as proteins that contain multiple sites capable of recognizing the specific pattern of hydrophobic patches (Fig. 2.2) (284, 295). Thus, it can be seen how the attachment of polyubiquitin chains formed through different linkages can add functional complexity to ubiquitin recognition and result in different downstream effects than monoubiquitin alone.

Research Aims

As mentioned previously, deletion or mutation of the endogenous PTAPP L-domain of HIV-1 results in a release defect characterized by viral particles that fail to mature and remain tethered to the plasma membrane (69). Particle release and infectivity could be rescued by overexpression of ALIX, which interacts with the less effective YPxL motif to recruit the ESCRT machinery (78, 291). Interestingly, two labs recently reported that overexpression of a Nedd4 isoform, NEDD4-2s, was able to potently rescue release of the same PTAPP deficient virus (HIV-1_{ΔPTAPP}) (56, 292). In addition, siRNA mediated knockdown of endogenous NEDD4-2s decreased the already limited release observed with this mutant, suggesting that NEDD4-2s accounted for the majority of released particles, and not ALIX (56). Interestingly, siRNA mediated knockdown of endogenous Tsg101 abrogated the rescue phenotype, although expression of dominant negative fragments of Tsg101 indicated that neither the UEV nor the PRD of the protein was required for NEDD4-2s stimulated rescue, implying that ubiquitin recognition or ALIX interaction was not a major contributor to this activity (56). It was determined that this rescue was dependent on both the N-terminal portion of NEDD4-2s and a catalytically active HECT domain (292). While most other Nedd4 family members contain a complete C2 domain, NEDD4-2s, a naturally occurring isoform of Nedd4.2, contains only a residual C2 domain due to alternative exon usage (123). The previous finding that this partial

C2 domain was required for the observed rescue activity of NEDD4.2s suggests that it may constitute a functional domain.

In light of these findings, we determined what roles the required partial C2 domain and HECT domain of NEDD4.2s play in the rescue of HIV-1_{ΔPTAPP} release. We hypothesized that the partial C2 domain constituted a Gag-targeting motif, and to test this we replaced it with a molecule known to interact with HIV-1 Gag, Cyclophilin A. We also sought to determine if the required HECT domain of NEDD4.2s could be replaced by other functional HECT domains that were either closely or distantly related. Finally, based on these findings, we investigated the role that the formation of K63-polyubiquitin chains played in rescuing the release of the L-domain-defective HIV-1 construct. The results of these experiments are presented here.

Results

Targeting of the catalytic HECT of NEDD4-2s to HIV-1 Gag is sufficient to rescue HIV-1_{ΔPTAPP} release

It was previously determined that the residual C2 domain of NEDD4-2s was required for the ability of NEDD4-2s to rescue HIV-1_{ΔPTAPP} release (292). We hypothesized that the partial C2 domain of NEDD4-2s constituted a Gag interaction motif, and to test this decided to replace it with a protein known to interact with Gag. For this purpose we chose the human protein Cyclophilin A (CypA), which has been shown to interact with the CA domain of HIV-1 Gag (81, 289). We replaced the partial C2 domain of NEDD4-2s, residues 1-31, with CypA followed by HA and FLAG tags, fused to the remaining region of NEDD4-2s (CypA-N_{Δ1-31}). The presence of the CypA molecule was expected to target the construct to HIV-1 Gag.

Consistent with previously published results (292), co-transfection of 293T cells with vectors encoding HIV-1_{ΔPTAPP} and FLAG-tagged NEDD4-2s robustly rescued particle release as measured by the amount of extracellular CA (Fig 2.3). NEDD4-2s also corrected the Gag cleavage defect of HIV-1_{ΔPTAPP}, which leads to the accumulation of CA-p2 at the expense of mature CA in virus-producing cells (Fig. 2.3). Although the mechanistic basis for this cleavage defect remains unknown, impaired processing at the CA-p2 site is considered a hallmark of late assembly defects. Also as expected, removal of the first 31

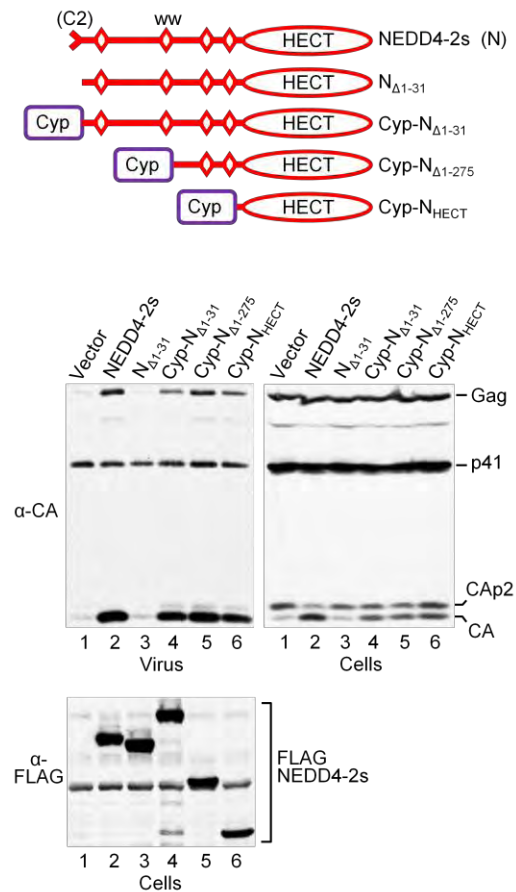


Figure 2.3. The isolated HECT domain of NEDD4.2s rescues HIV-1 Δ PTAPP when targeted to Gag. 293T cells were transfected with HXBH10 Δ PTAPP (1 μ g) and empty vector, or vectors (2 μ g each) expressing WT Nedd4.2s, a version lacking the residual C2 domain (N Δ 1-31), or versions that have portions of Nedd4.2s replaced by the HIV-1 Gag-binding protein CypA as illustrated. Virion pellets and the cell lysates were analyzed by Western blotting to detect Gag, Gag cleavage products, and FLAG-tagged proteins as indicated.

amino acids of NEDD4-2s, containing the partial C2 domain (N Δ 1-31), completely abrogated the rescue effect (Fig. 2.3 (292)). In contrast, expression of the protein containing CypA in place of the partial C2 domain (CypA-N Δ 1-31) with the HIV-1 Δ PTAPP construct was able to rescue viral particle production to levels comparable to that observed with WT NEDD4-2s (Fig. 2.3). In addition, CypA-

N_{Δ1-31} was able to correct the intracellular Gag cleavage defect of HIV-1_{ΔPTAPP} (Fig. 2.3). These results demonstrated that the CA-binding protein Cyclophilin A can be used in place of the native partial C2 domain of NEDD4-2s to stimulate release of HIV-1_{ΔPTAPP} particles.

The partial C2 domain of NEDD4-2s is separated from the catalytic HECT domain by a region that contains four WW domains that take part in substrate recognition (Fig. 2.3). To determine whether these domains played any role in the rescue of HIV-1_{ΔPTAPP}, we fused CypA followed by HA and FLAG epitopes to truncated version of NEDD4-2s that lacked the first two (CypA-N_{Δ1-275}) or all four (CypA-HECT) WW domains. As shown in Fig. 2.3, both chimeric constructs were highly active in the ΔPTAPP rescue assay. Both constructs stimulated the release of particle-associated CA, and exhibited modest effects on intracellular Gag cleavage. Of note, it was previously shown that truncated mutants that lack both the N-terminus and CypA displayed no activity in this assay (292). We thus conclude that the targeting of the isolated HECT domain of NEDD4-2s to Gag via CypA is sufficient to rescue release and correct HIV-1 budding defects.

Transfer of the residual C2 domain of NEDD4-2s confers the ability to rescue HIV-1 release to a yeast homologue

Previous results generated by our lab indicated that overexpression of Nedd4 ubiquitin ligase family members Nedd4, WWP1, WWP2 and ITCH had no significant effect on the release of HIV-1_{ΔPTAPP} (292). Since all of these proteins

possess an intact C2 domain, a variant of WWP1 was generated that lacks the exact portion of the C2 domain which is naturally absent from NEDD4-2s. The resulting WWP1s mutant remained inactive in the Δ PTAPP rescue assay, indicating that the robust activity of NEDD4-2s is not simply due to its naturally truncated C2 domain (292). To determine what region of NEDD4-2s accounts for its unique ability to rescue HIV-1 Δ PTAPP release, we generated FLAG-tagged chimeras between N-terminal portions of Nedd4.2.s and WWP1 as illustrated in Fig. 2.4. Interestingly, while full length WWP1s displayed no ability to rescue release of HIV-1 Δ PTAPP budding (292), a fusion protein containing the first 245 residues of NEDD4-2s in place of the endogenous region of the protein (N₁₋₂₄₅/WWP1), displayed a robust rescue phenotype with particulate CA levels at least as high as WT NEDD4-2s (Fig. 2.4). In addition, this chimera was also able to correct the characteristic Gag cleavage defect to levels similar to those observed with WT NEDD4-2s (Fig. 2.4). Quantitation of virion-associated CA in this experiment indicated that NEDD4-2s increased release 25-fold compared to empty vector, while the N₁₋₂₄₅/WWP1 construct demonstrated a 43-fold effect. Additional fusion proteins were generated to remove portions of NEDD4-2s to try and arrive at a minimal portion required for activity. The first 245 residues of NEDD4-2s contain the first WW domain as well as a long, non-interacting

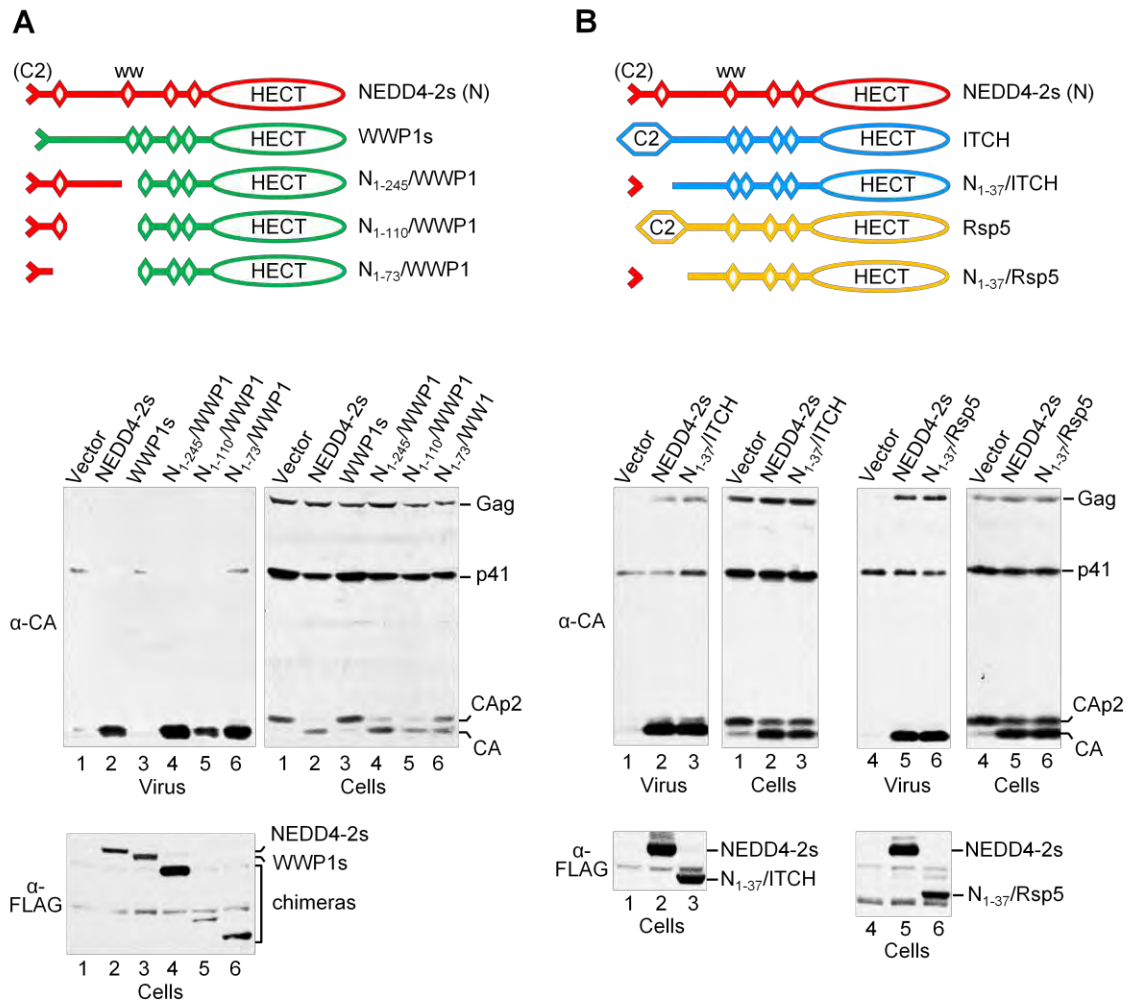


Figure 2.4. The residual C2 domain of NEDD4.2s is sufficient to transfer the ability to rescue HIV-1_{ΔPTAPP}. (A) The transfer of NEDD4.2s sequences upstream of the first WW domain to WWP1 is sufficient to confer activity in the Δ PTAPP rescue assay. (B) Human ITCH and yeast Rsp5 potently rescue HXBH10_{ΔPTAPP} when their C2 domain is replaced by the residual C2 domain of NEDD4.2s. 293T cells were transfected with HXBH10_{ΔPTAPP} (1 μ g) and vectors (2 μ g each) expressing the indicated parental or chimeric ubiquitin ligase constructs, or the empty vector. Virion pellets and the cell lysates were analyzed by Western blotting to detect Gag, Gag cleavage products, and FLAG-tagged proteins as indicated.

segment (Fig. 2.4). A construct that lacked this long intervening segment was generated (N₁₋₁₁₀/WWP1), and co-transfected with HIV-1_{ΔPTAPP}; this construct

was able to rescue release to levels 20-fold higher than vector, despite being poorly expressed (Fig. 2.4). Finally, a construct containing N-terminal portions of NEDD4-2s up to, but not including, first WW domain was created and stimulated a 40-fold increase in particulate CA, indicating that only sequences upstream of the NEDD4-2s WW domain were required for activity in this assay (Fig. 2.4).

The results above strongly suggested that the partial C2 domain of NEDD4-2s was specifically required for activity. To test this, we tried to confer the rescue ability of NEDD4-2s to the Nedd4 family member AIP4/ITCH by replacing its complete C2 domain with the first 37 amino acids of NEDD4-2s, which contain only the partial C2 domain of that molecule. As shown in Fig. 2.4, co-expression of this NEDD4-2s/ITCH chimera (N₁₋₃₇/ITCH) rescued the release of HIV-1_{ΔPTAPP} and corrected Gag cleavage to levels equal to that of WT NEDD4-2s. Although it has recently been reported that overexpression of WT ITCH is able to rescue release of a murine leukemia virus that lacks a functional Late domain (126), previous results from our lab have demonstrated that overexpression of WT ITCH shows no activity in the rescue of HIV-1_{ΔPTAPP} (292). Together, these findings indicate that the partial C2 domain of NEDD4-2s is both required and sufficient to transfer the ability to rescue HIV-1 budding to other, previously non-functional, ubiquitin ligases.

To determine the generality of these findings, we tested whether we could use the partial C2 domain of NEDD4-2s to target a yeast ubiquitin ligase to

rescue HIV-1 Δ PTAPP budding defects. The E3 ubiquitin ligase Rsp5 is the sole Nedd4 homologue encoded by *Saccharomyces cerevisiae*, and it contains the similar modular structure of an N-terminal C2 domain, three WW domains, and a C-terminal HECT domain. As illustrated in Fig. 2.4, we replaced the full-length C2 domain of Rsp5 with the N-terminal 37 amino acids of NEDD4-2s containing its partial C2 domain (N₁₋₃₇/Rsp5). When co-expressed in 293T cells with the vector encoding HIV-1 Δ PTAPP, the N₁₋₃₇/Rsp5 fusion protein displayed an almost identical ability to rescue particle release compared to WT NEDD4-2s, even though it was expressed at lower levels (Fig. 2.4). In contrast, the parental, full-length Rsp5 displayed only a minimal ability to rescue particle release and did not noticeably affect Gag cleavage defects (data not shown). These findings indicate that the residual C2 domain of NEDD4-2s is sufficient to confer the ability to function in HIV-1 budding to widely divergent Nedd4 family members from different species.

The residual C2 domain of NEDD4-2s confers the ability to associate with HIV-1 Gag

It was previously demonstrated that the residual C2 domain of NEDD4-2s is required for its ability to induce Gag ubiquitination, and for its incorporation into virus-like particles (VLP) (292). To examine those parameters, a Gag construct referred to as Z_{WT} was used. The Gag Z_{WT} construct contains a foreign

dimerization domain in place of HIV-1 NC-p6 and efficiently produces VLP in an ESCRT-independent manner (1). Furthermore, Z_{WT} Gag exhibits less baseline ubiquitination than authentic HIV-1 Gag. This construct was used to determine the levels of ubiquitin ligase incorporation into VLP as well as to determine the formation of Gag-ubiquitin conjugates, independent of VLP production.

Previous observations from our lab found that co-expression of Z_{WT} with FLAG-tagged NEDD4-2s resulted in the appearance of additional Gag species in Z_{WT} VLP that migrated slower than the Z_{WT} precursor, and it was determined that these slower migrating species represent Gag-ubiquitin conjugates (1, 292). These conjugates were not observed in Z_{WT} VLP that were obtained from samples co-expressed with either catalytically inactive FLAG-NEDD4-2s or active FLAG-WWP1s (292). Also, only WT NEDD4-2s was shown to incorporate into Z_{WT} VLP; no FLAG signal was observed with either inactive NEDD4-2s or WWP1s in Z_{WT} VLP (292). These findings were in agreement with a lack of activity for both of these constructs in the HIV-1_{ΔPTAPP} rescue assay. In contrast to this result, when Z_{WT} was cotransfected with FLAG-tagged versions of N₁₋₂₄₅/WWP1, N₁₋₁₁₀/WWP1, or N₁₋₇₃/WWP1- all of which rescued HIV-1_{ΔPTAPP} release- all were found to be incorporated into VLP (Fig. 2.5). In addition, all three constructs induced a similar pattern of Gag-ubiquitin conjugation to that observed with WT NEDD4-2s (Fig. 2.5). Results similar to those observed with the WWP1 chimeras were observed with the N₁₋₃₇/ITCH and N₁₋₃₇/Rsp5 chimeras

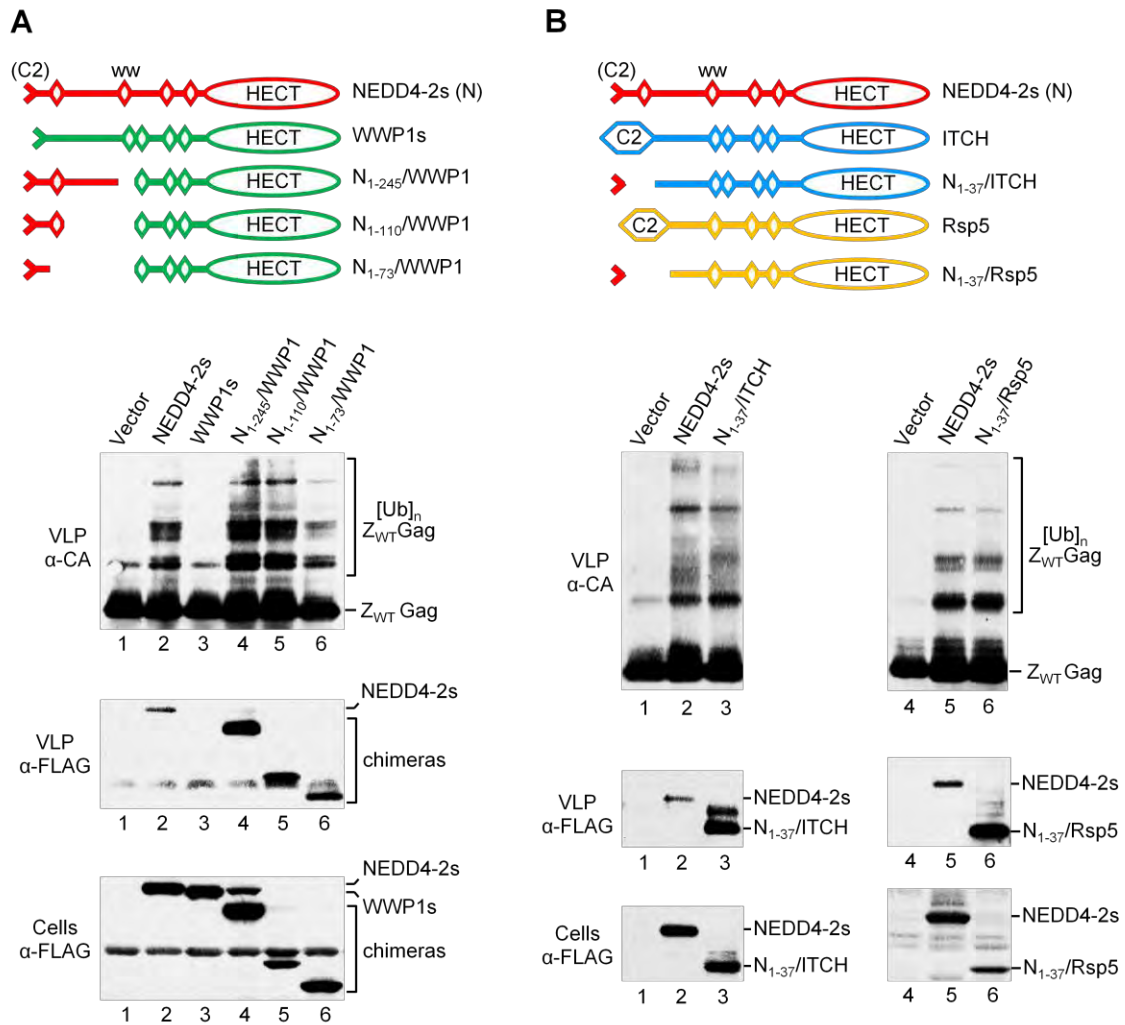


Figure 2.5. The residual C2 domain of NEDD4.2s functions as a Gag-targeting module. (A) NEDD4.2s sequences upstream of the first WW domain are sufficient to induced the ubiquitination of an L-domain-independent HIV-1 Gag construct (Z_{WT}) by WWP1, and the association of WWP1 with Z_{WT} VLP. **(B)** Human ITCH and yeast Rsp5 ubiquitinate Z_{WT} Gag and associate with VLP when their C2 domain is replaced by the NEDD4.2s residual C2 domain. 293T cells were transfected with the Z_{WT} Gag construct ($2\mu\text{g}$) and vectors ($2\mu\text{g}$ each) expressing the indicated parental or chimeric ubiquitin ligase constructs, or empty vector. VLP were analyzed by Western blotting with anti-CA to detect unmodified and ubiquitinated version of Z_{WT} Gag, and with anti-FLAG to detect the incorporation of ubiquitin ligase constructs into VLP. The cell lysates were also examined with anti-FLAG.

(Fig. 2.5). We found that both N₁₋₃₇/ITCH and N₁₋₃₇/Rsp5 induced very similar levels of Gag ubiquitination as NEDD4-2s (Fig. 2.5). Further, particularly with regard to N₁₋₃₇/Rsp5 construct, the pattern of Gag ubiquitination was virtually identical to that observed with NEDD4-2s (Fig. 2.5). In addition, both N₁₋₃₇/ITCH and N₁₋₃₇/Rsp5 were incorporated into VLP at levels even greater than those observed with NEDD4-2s (Fig. 2.5). Taken together, these results indicate that the residual C2 domain of NEDD4-2s is sufficient to transfer the ability to associate with HIV-1 Gag to other Nedd4 family members, which thereby gain access to VLP and the ability to ubiquitinate Gag.

Isolated HECT domains differ in their ability to rescue HIV-1 budding when fused to the residual C2 domain of NEDD4-2s

As described above, we found that the isolated HECT domain of NEDD4-2s is sufficient to rescue the release of HIV-1_{ΔPTAPP} particles if fused to a Gag interacting protein such as Cyclophilin A. Furthermore, our results suggested that the residual C2 domain of NEDD4-2s can serve as a Gag-binding module. We therefore asked whether the isolated HECT domains of other human HECT ubiquitin ligases can promote HIV-1_{ΔPTAPP} budding if fused to the residual C2 domain of NEDD4-2s. Among the HECT domains selected for this analysis were those belonging to Nedd4 family members SMURF1, HECW1 and HECW2, which were derived from different ancestral genes than NEDD4-2s, WWP1 or

ITCH (181). We also included the HECT domains of E6-AP and HERC6, which are members of the SI-HECT domain and HERC families, respectively (20). All selected HECT domains were directly fused to a FLAG-tagged construct containing the partial C2 domain of NEDD4-2s, and the fusion proteins were expressed in 293T cells with a plasmid encoding the PTAPP- deficient HIV-1 construct. Two of the HECT domain constructs (N₁₋₃₇/HECW1 and N₁₋₃₇/HECW2) displayed the ability to rescue particle release and to correct the Late-domain-associated Gag cleavage defect (Fig. 2.6). Release of particulate CA and intracellular conversion of CA-p2 to CA stimulated by both HECT E3 domains was similar to that observed with WT NEDD4-2s (Fig. 2.6). In contrast, constructs containing the HECT domains of SMURF1, E6-AP and HERC6 failed to rescue particle release to any noticeable extent, despite being expressed at comparable levels (Fig. 2.6).

As shown in Fig. 2.6, only the two constructs containing the HECT domains of HECW1 and HECW2 displayed the ability to induce formation of Z_{WT} Gag ubiquitin conjugates to levels similar to those observed with NEDD4-2s. In contrast, the constructs containing the HECT domains of SMURF1, E6-AP or HERC6 failed to induce any Gag ubiquitination. It is possible that these constructs were not catalytically active since no evidence of autoubiquitination was observed. Also, HERC6 is an interferon-induced protein that, based on similarity with human HERC5 and mouse HERC6, may be a ligase for ISG15 and not ubiquitin (64, 299). However, it did appear that the construct containing the

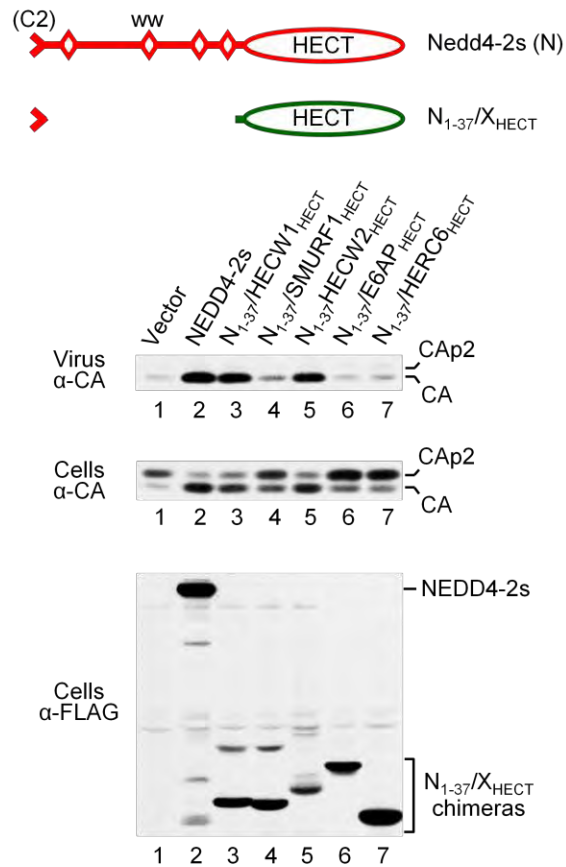


Figure 2.6. Rescue of HIV-1_{ΔPTAPP} by the isolated HECT domains fused to the NEDD4.2s residual C2 domain. 293T cells were transfected with HXBH10_{ΔPTAPP} (0.5 μ g) and empty vector, or vectors expressing NEDD4.2s (2 μ g) or the indicated fusion proteins (6 μ g each). Virion pellets and the cell lysates were analyzed by Western blotting to detect Gag products and FLAG-tagged proteins as indicated.

SMURF1 HECT domain was catalytically active, based on the presence of a slower migrating molecular weight band which was also observed in the constructs that were able to rescue HIV-1_{ΔPTAPP} release (Fig. 2.6).

To more directly determine whether the HECT domain constructs were capable of associating with HIV-1 Gag, we also examined their incorporation in

Z_{WT} VLP. The two active HECT domain constructs were readily detectable in VLP. In fact, relative to their expression levels, N₁₋₃₇/HECW1_{HECT} and N₁₋₃₇/HECW2_{HECT} were incorporated at 14- and 46-fold higher levels than NEDD4-2s, respectively (Fig. 2.7). In contrast, two of the three inactive constructs (N₁₋₃₇/SMURF1_{HECT} and N₁₋₃₇/E6-AP_{HECT}) were not incorporated, even though they were expressed as well or better than the constructs that were taken up into VLP (Fig. 2.7). The third inactive construct (N₁₋₃₇/HERC6_{HECT}) was incorporated at about 4-fold higher levels than NEDD4-2s, normalized for expression levels (Fig. 2.7). The N₁₋₃₇/HECW2_{HECT} construct exhibited the lowest level of expression but the highest level of VLP association, which suggested that the HECT domain of HECW2 in particular contributed to the association with HIV-1 Gag (Fig. 2.7). However, a version of N₁₋₃₇/HECW2_{HECT} that lacked the residual C2 domain of NEDD4-2s did not induce Gag ubiquitination, was not incorporated into VLP, and was inactive in the Δ P_{TAPP} rescue assay (Fig. 2.7)

Taken together, these results show that the residual C2 domain of NEDD4-2s is sufficient to target a subset of isolated HECT domains to HIV-1 Gag. They also show that the isolated HECT domain of NEDD4-2s is not unique in its ability to rescue HIV-1 budding when targeted to Gag. Rather, this ability is shared by the HECT domains of the two human Nedd4 family members that are overall most divergent from NEDD4-2s.

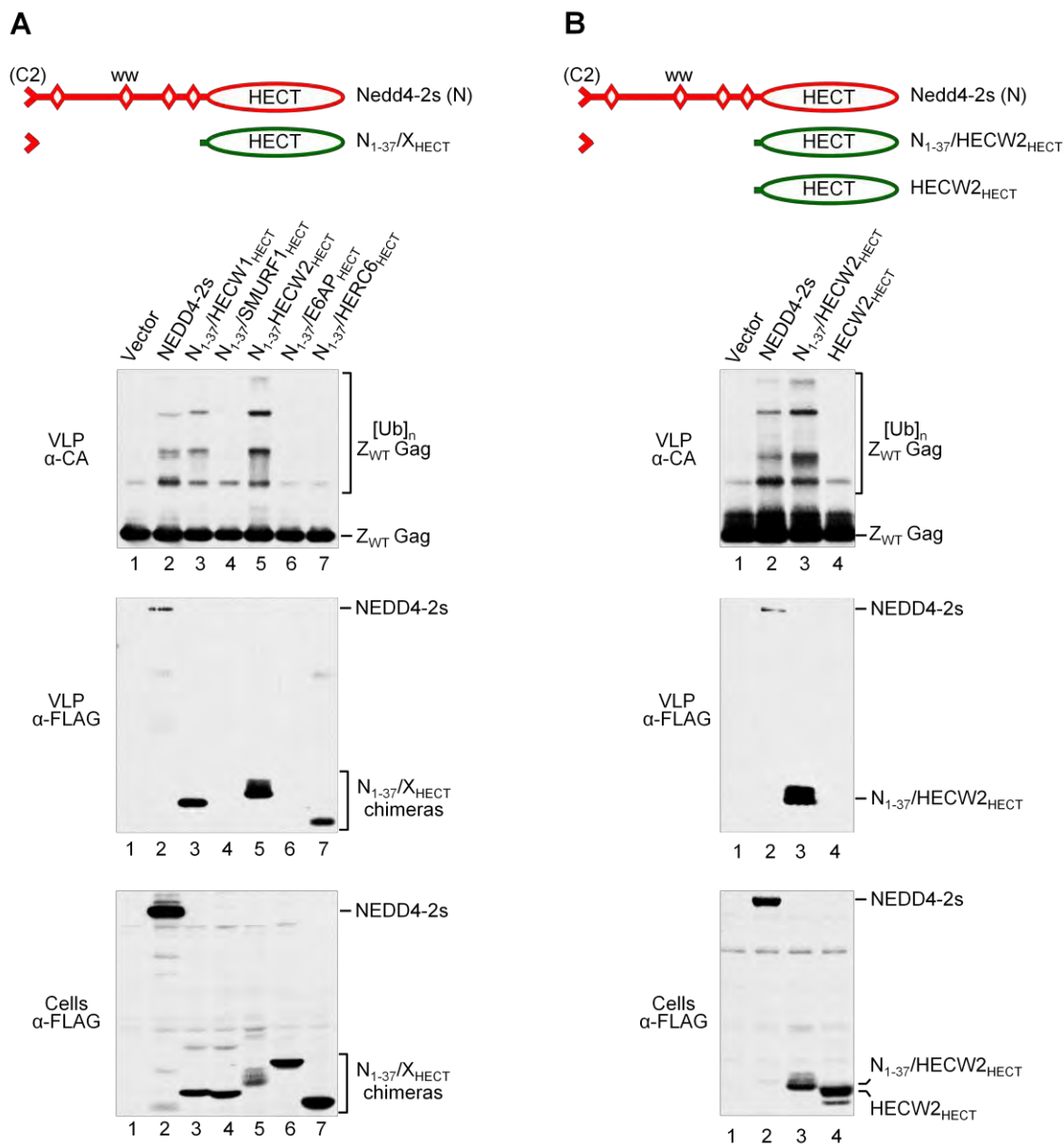


Figure 2.7. The NEDD4.2s residual C2 domain is sufficient to target some isolated HECT domains to Gag. (A) Residual C2 domain/HECT domain fusion proteins differ in their ability to induce the ubiquitination of Z_{WT} Gag, and to associate with Z_{WT} VLP. **(B)** The ubiquitination of Z_{WT} Gag by the isolated HECT domain of HECW2 and its association with Z_{WT} VLP were dependent on the presence of the NEDD4.2s residual C2 domain. 293T cells were transfected with the Z_{WT} Gag construct (2 μg) and empty vector, or the vectors expressing NEDD4.2s (2 μg) or the indicated HECT domain constructs (6 μg each).

Induction of Gag ubiquitination is not sufficient to rescue the release of HIV-1 Δ PTAP budding

The apparent inability of the N₁₋₃₇/SMURF1_{HECT} construct to associate with HIV-1 Gag offered a possible explanation for its failure to rescue HIV-1 Δ PTAPP budding and to ubiquitinate Z_{WT} Gag, despite being able to auto-ubiquitinate. We therefore examined the effect of targeting the SMURF1 HECT domain to HIV-1 Gag via CypA. As shown in Fig. 2.8, fusing the HECT domains of SMURF1, E6-AP and HERC6 directly to CypA did not confer the ability to rescue HIV-1 Δ PTAPP. In contrast, the equivalent CypA-N_{HECT} construct, which instead contains the HECT domain of NEDD4-2s, did rescue nearly as well as WT NEDD4-2s, as expected. Although all CypA-HECT domain fusion proteins were incorporated into Z_{WT} VLP, those containing the HECT domain of E6AP or HERC6 did not ubiquitinate Z_{WT} Gag, consistent with the possibility that these HECT domains are catalytically inactive in isolation (Fig. 2.8). Surprisingly, the CypA fusion proteins with the NEDD4-2s and SMURF1 HECT domains induced comparable level of Gag-ubiquitin conjugates (Fig. 2.8), even though only the former was active in the Δ PTAPP rescue assay (Fig. 2.8). Moreover, although the CypA fusion protein with the NEDD4-2s HECT domain (Cyp-N_{HECT}) and WT NEDD4-2s exhibited comparable activities in the Δ PTAP rescue assay (Fig. 2.8), the former induced a much lower level of Z_{WT} Gag ubiquitination (Fig 2.8). These results demonstrate that the mere ability to ubiquitinate Gag is not sufficient to rescue HIV-1 budding. Furthermore, if the ubiquitination of Gag is necessary to

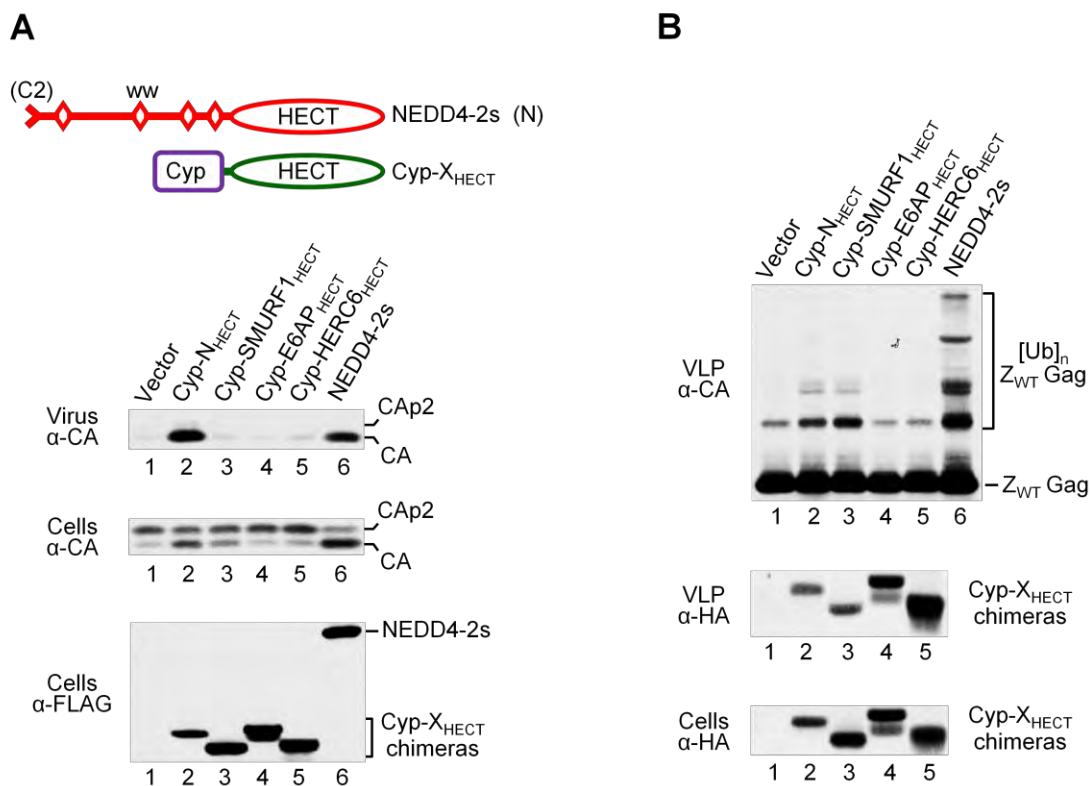


Figure 2.8. Rescue of HIV-1 release does not correlate with induction of Gag ubiquitination. (A) The isolated HECT domain of NEDD4.2s rescues HXBH10_{ΔPTAPP} when targeted to Gag via CypA (lane 2), whereas the isolated HECT domains of SMURF1, E6AP, or HERC6 do not (lanes 3-5). (B) The isolated HECT domains of NEDD4.2s (lane 2) and SMURF1 (lane 3) induced comparable levels of Gag ubiquitination when fused to CypA. 293T cells were transfected with HXBH10_{ΔPTAPP} (0.5 μg) or ZWT (2 μg) and empty vector, or vectors expressing the indicated CypA-HECT domain fusion proteins or NEDD4.2s (2 μg each). Gag proteins were detected with anti-CA, and the CypA-HECT domain fusion proteins were detected with anti-FLAG or anti-HA, as indicated.

promote budding, then only relatively low levels of Gag ubiquitination are required.

Rescue of HIV-1 budding correlates with ubiquitin chain-type specificity

The lack of correlation between the rescue of HIV-1 budding and the overall levels of Gag ubiquitination led us to investigate the possible role of the type of ubiquitin chain that can be formed. It was recently shown that ITCH, which potently rescues HIV-1 budding when targeted to Gag (Fig. 2.4), has a very high preference for the synthesis of K63-linked polyubiquitin chains (147). However, the replacement of the C-lobe of the HECT domain of ITCH with that of E6AP caused a complete switch to K48 chain type specificity (147). On the other hand, an ITCH-HUWE1 C-lobe chimera produced both relatively short K48 chains and even shorted K63 chains (147). HUWE1 is a large HECT domain ubiquitin ligase that does not belong to the Nedd4 family, but is relatively closely related in its HECT domain (257).

To examine the role of chain type specificity in the rescue of HIV-1 budding, we replaced the C lobe of N₁₋₂₄₅/ITCH with that of E6AP or HUWE1, as illustrated in Fig. 2.9. N₁₋₂₄₅/ITCH rescues HIV-1_{ΔPTAPP} as potently as N₁₋₃₇/ITCH, and thus was used here because HECT C lobe chimeras based on this construct were relatively stable (data not shown). The N₁₋₂₄₅/ITCH E6AP C lobe chimera appeared to have no effect on HIV-1 budding, but was too poorly expressed to yield reliable results (data not shown). In contrast, the N₁₋₂₄₅/ITCH HUWE1 C lobe chimera was expressed at higher levels. However, even at expression

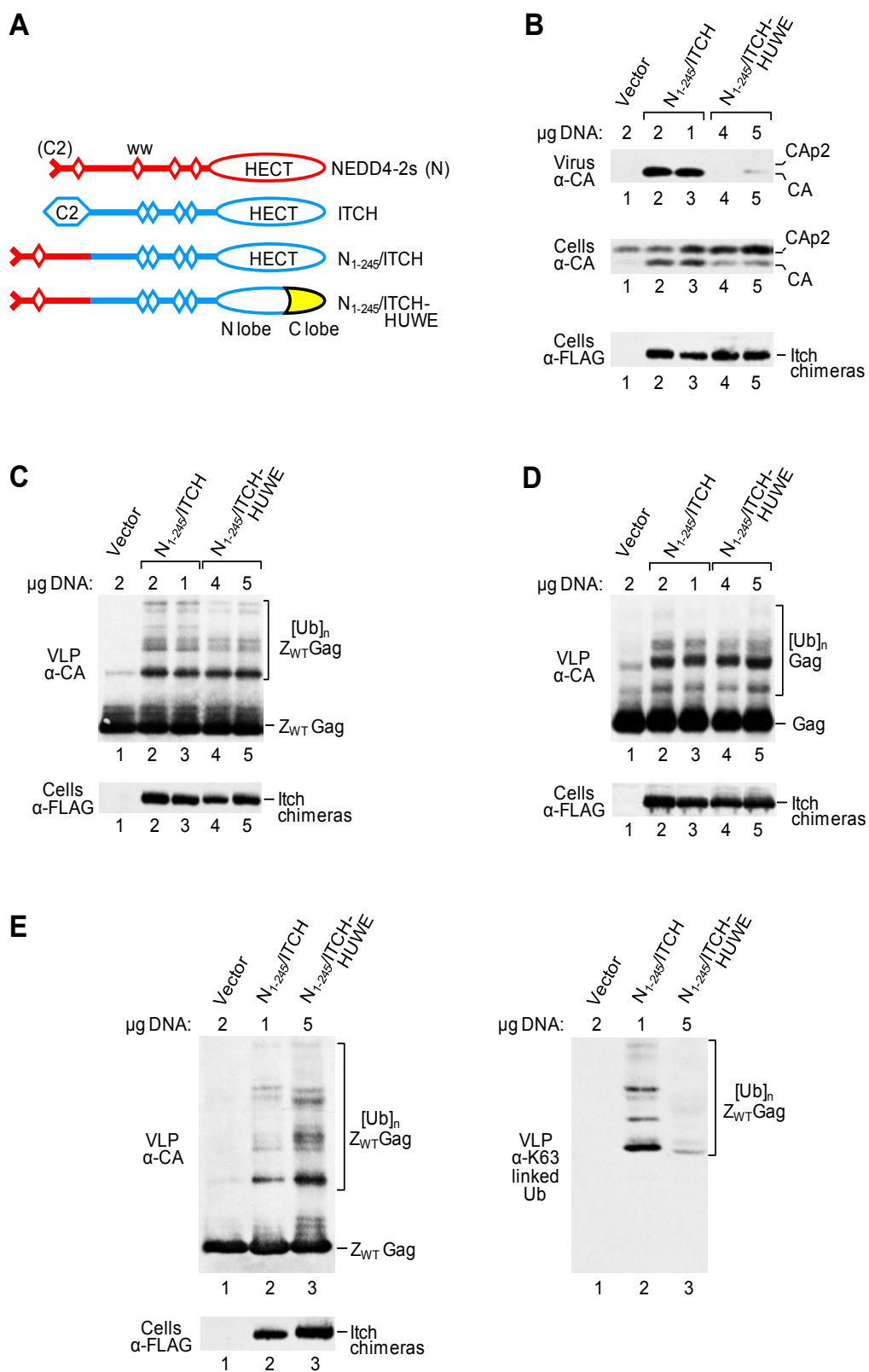


Figure 2.9. Rescue of HIV-1 release correlates with chain type specificity.

(A) Schematic illustration of chimeric ubiquitin ligase constructs. The C lobe of HUWE1 is indicated in yellow. **(B to D)** Replacement of the C lobe of the ITCH HECT domain by that of HUWE1 greatly impairs the rescue of HIV-1_{ΔPTAPP} by a NEDD4-2s/ITCH chimera (panel B), but not the overall ubiquitination of VLP-associated Z_{WT} Gag (**panel C**) or of virus particle-associated authentic Gag expressed by an HIV-1 provirus lacking protease (**panel D**). Expression vectors for HECT domain constructs were transfected in varying amounts as indicated to achieve comparable expression levels. **(E)** The parental N₁₋₂₄₅/ITCH construct, in contrast to the N₁₋₂₄₅/ITCH-HUWE1 HECT domain chimera, induces the attachment of K63-linked chains to Z_{WT} Gag. 293T cells were transfected with the Z_{WT} Gag construct and empty vector, or vectors expressing FLAG-tagged versions of the indicated HECT domain constructs. VLP samples were analyzed by Western blotting with anti-CA to detect unmodified and ubiquitinated versions of Z_{WT} Gag (left panel), and in parallel with Apu3 to detect K63-linked ubiquitin chains conjugated to Z_{WT} Gag (right panel). The cell lysates were also examined with anti-FLAG.

levels similar to or higher than those obtained with the parental N₁₋₂₄₅/ITCH construct, N₁₋₂₄₅/ITCH-HUWE1 had at most a small effect on the release of HIV-1_{ΔPTAPP}, whereas N₁₋₂₄₅/ITCH potently rescued budding (Fig. 2.9). Nevertheless, the parental N₁₋₂₄₅/ITCH construct and N₁₋₂₄₅/ITCH-HUWE1 induced comparable levels of ubiquitination both of Z_{WT} Gag (Fig. 2.9) and of authentic HIV-1 Gag expressed by a proviral protease mutant (Fig. 2.9).

Interestingly, the pattern of Z_{WT} Gag ubiquitination seen with N₁₋₂₄₅/ITCH-HUWE1 differed slightly from that obtained with N₁₋₂₄₅/ITCH (Fig 2.9), consistent with the possibility that these two proteins catalyze different chain linkages, as previously shown for ITCH-HUWE1 and ITCH in vitro (147). To examine this possibility directly, we made use of the linkage-specific antibodies Apu3 and HWA4C4, which exhibit high selectivity for K63-linked ubiquitin chains (209, 305).

In the experiment shown in Fig. 2.9, N₁₋₂₄₅/ITCH-HUWE1 was more highly expressed than N₁₋₂₄₅/ITCH, and induced higher overall levels of Z_{WT} Gag ubiquitination as detected by immunoblotting of Z_{WT} particles with anti-CA antibody (left panel). Nevertheless, when the same samples were examined by Western blotting with the K63-linkage specific Apu3 antibody, only the parental N₁₋₂₄₅/ITCH construct yielded three prominent bands (right panel). The mobility of these three bands was as expected for Z_{WT} Gag modified with di-, tri-, or tetra-ubiquitin chains. Equivalent results were obtained with HWA4C4, another K63-linkage specific antibody (data not shown).

In order to verify these findings, we attempted to return K63-linked chain specificity to the N₁₋₂₄₅/ITCH-HUWE1 construct. Previous work by Kim et al. reported that the chain-type specificity of HECT E3 ubiquitin ligases could be altered by exchanging portions of their C-lobes with that of another HECT domain (147). The C-terminal 57 amino acids of the ITCH HECT domain were fused in place of the equivalent region of the HECT domain of N₁₋₂₄₅/ITCH-HUWE1 – replacing the C-terminal 67 amino acids of that protein, generating the construct we refer to here as N₁₋₂₄₅/ITCH-HUWE-ITCH. While this construct was less well expressed than the parental N₁₋₂₄₅/ITCH protein, it was expressed at high enough levels for us to compare its activities to N₁₋₂₄₅/ITCH-HUWE1. As Fig. 2.10 demonstrates, replacement of the C-terminal portion of HUWE1 with that of ITCH resulted in release of HIV-1_{ΔPTAPP} particles at levels similar to those observed with N₁₋₂₄₅/ITCH. As before, rescue of HIV-1_{ΔPTAPP} by N₁₋₂₄₅/ITCH-

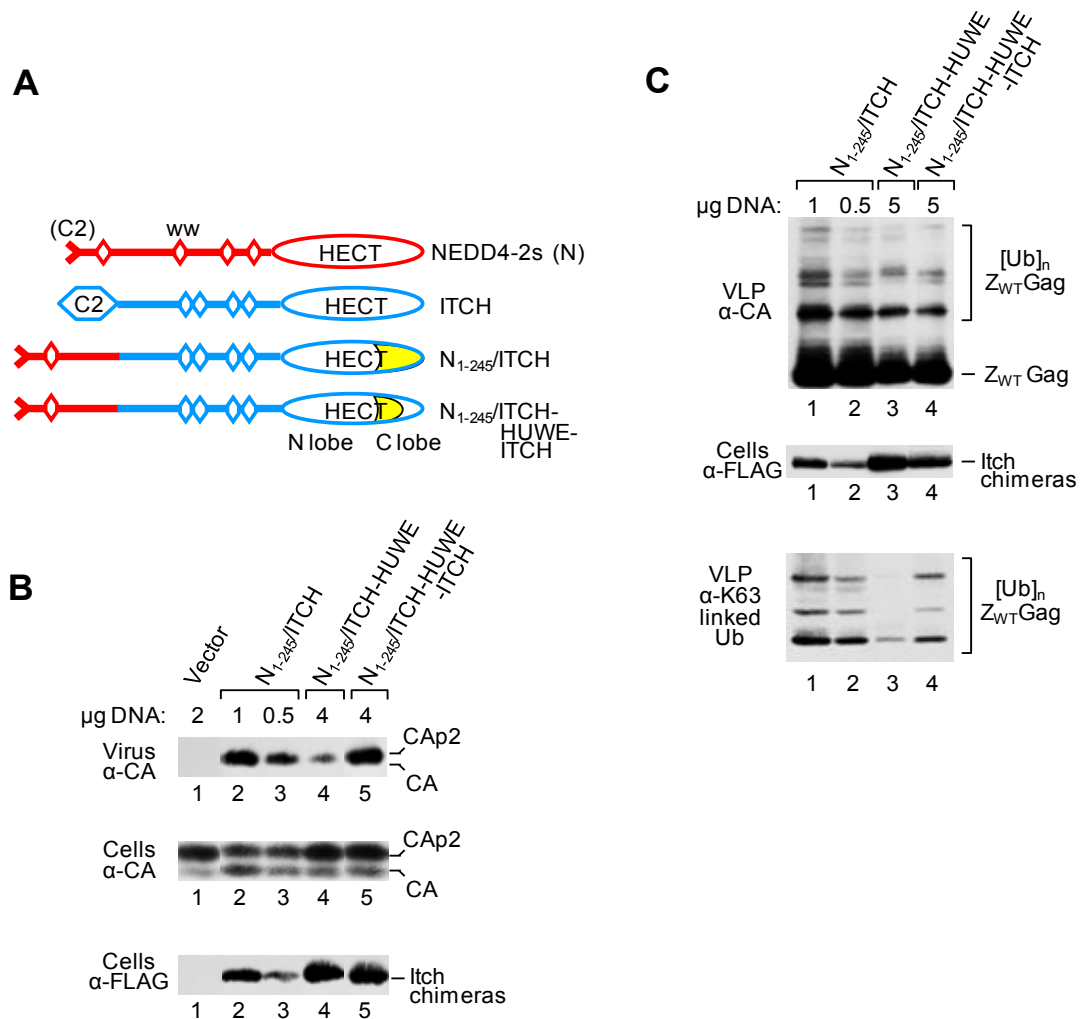


Figure 2.10. Rescue of HIV-1 release correlates with chain type specificity. (A) Schematic illustration of chimeric ubiquitin ligase constructs. Residues from HUWE1 are indicated in yellow. (B) Replacement of the C lobe of the ITCH HECT domain by that of HUWE1 greatly impairs the rescue of HIV-1_{ΔPTAPP} by a NEDD4-2s/ITCH chimera. Restoring the C-terminus of the ITCH C-lobe restores the ability to rescue HIV-1_{ΔPTAPP} release. (C) Restoration of release correlates with the restoration of K63 polyubiquitin chain specificity. 293T cells were transfected with HXBH10_{ΔPTAPP} or the Z_{WT} Gag construct and empty vector, or vectors expressing FLAG-tagged versions of the indicated HECT domain constructs. Gag proteins from released HXBH10_{ΔPTAPP} and VLP samples were analyzed by Western blotting with anti-CA to detect: Gag and Gag cleavage products (panel B) as well as unmodified and ubiquitinated versions of Z_{WT} Gag, and in parallel with Apu3 to detect K63-linked ubiquitin chains conjugated to Z_{WT} Gag (panel C). The cell lysates from both experiments were also examined with anti-FLAG to detect expression of HECT domain constructs.

HUWE1 showed hardly any effect, even when compared to a lower concentration of the parental construct. Analysis of the ubiquitination pattern of Z_{WT} particles by immunoblotting with the K63-specific Apc3 antibody demonstrated that the partial C-lobe of ITCH was sufficient to return K63-chain specificity to this chimera. We conclude that the chain type specificities of $N_{1-245}/ITCH$ and $N_{1-245}/ITCH-HUWE1$ in living cells differ considerably, and that the ability to catalyze K63-linked chains is of critical importance for the rescue of HIV-1 release.

Discussion

We show here that widely divergent human and yeast ubiquitin ligases of the Nedd4 family, and even a subset of isolated HECT domains, possess the intrinsic ability to function in HIV-1 release. The truncated C2 domain of NEDD4-2s provides a natural Gag-targeting module, which accounts for the unique ability of authentic NEDD4-2s to rescue HIV-1 budding defects. However, other Nedd4 family members, including yeast Rsp5, and in some cases even isolated catalytic HECT domains, acquire the same ability if targeted to HIV-1 Gag. A common property that is shared by widely divergent Nedd4 family members is the preferential catalysis of K63-linked ubiquitin chains, and at least in the case of yeast Rsp5, the isolated HECT domain is sufficient to synthesis such chains (147). Our data support a model in which the ability to conjugate K63 chains to a viral or cellular substrate in the immediate vicinity of the emerging bud is central to the ability to stimulate virus release.

In a previous study, the unique potency of NEDD4-2s in the Δ PPTAPP rescue assay did depend on its C2 domain being truncated, and was not shared by several other Nedd4 family members with intact C2 domains (292). One possible explanation for these observations was that the natural truncation of the C2 domain in NEDD4-2s relieves an auto-inhibition, which would be consistent with a study showing that catalytic activity of a subset of C2-WW-HECT E3s is

regulated through an inhibitory interaction between their C2 and HECT domains (311). On the other hand, the residual C2 domain of NEDD4-2s was essential for activity in the Δ PTAPP rescue assay (292). This finding raised the possibility that the C2 domain remnant of NEDD4-2s, which corresponds to β -strands 7 and 8 of the intact domain, constitutes a functional domain on its own that plays an active role in the rescue of HIV-1 budding (311). The present study supports this notion by demonstrating that the residual C2 domain of NEDD4-2s is sufficient to transfer the ability to rescue HIV-1 budding defects to other Nedd4 family ubiquitin ligases, and even to a subset of isolated HECT domains.

Previous results suggested that the C2 domain remnant of NEDD4-2s is required for the activity on the Δ PTAPP rescue assay, because it mediates the association of the ubiquitin ligase with HIV-1 Gag (292). In support of this concept, we now show that other Nedd4 family members, and some isolated HECT domains, associate with HIV-1 Gag if tagged with the residual C2 domain of NEDD4-2s. Additional strong support is provided by the fact that we were able to functionally replace the C2 domain remnant of NEDD4-2s with CypA, an entirely unrelated protein that has long been known to specifically interact with HIV-1 Gag (81, 289).

HECT E3s contain two broad functional regions: a large N-terminal region required for substrate recognition, and a C-terminal region (the HECT domain) which catalyzes the ubiquitination of bound substrates (304). Apart from the C2

domain, the N-terminal regions of Nedd4 family members harbor multiple WW domains, which were previously found dispensable for the rescue of HIV-1 budding by NEDD4-2s (292). In the present study, essentially the entire N-terminal substrate recognition portion of NEDD4-2s became dispensable in the presence of CypA, which served as a Gag-targeting module. The simplest interpretation of this result is that no substrate other than Gag needs to be recognized to stimulate virus release. However, if a transacting factor rather than Gag is the relevant substrate for ubiquitination as proposed (320), then the isolated catalytic HECT domain of NEDD4-2s must be sufficient to recognize that factor.

One potential transacting factor is ESCRT-I, because Sundquist and colleagues have demonstrated that the stimulation of HIV-1_{ΔPTAPP} release by NEDD4-2s depends on Tsg101/ESCRT-I (56). Furthermore, these authors showed that the NEDD4-2s overexpression induces the ubiquitination of ESCRT-I complexes, particularly of those that contain MVB12B. They also reported that a PPQY sequence in MVB12B, which constitutes a potential binding site for WW domains, contributes to the ubiquitination of MVB12B/ESCRT-I complexes by NEDD4-2s. Based on these results, it was suggested that NEDD4-2s-mediated ubiquitination may activate ESCRT-I to function in HIV-1 release (56). If this hypothesis is correct, then our observations imply that NEDD4-2s must remain capable of recognizing ESCRT-I as a substrate even in the absence of its N-terminal substrate recognition domain.

It has also been suggested that Nedd4 family E3s interact through their HECT domains with as yet unknown components of the ESCRT pathway, because several Nedd4 family members, and the isolated HECT domain of WWP1, localized to aberrant endosomal class E compartments induced by dominant-negative VPS4 (183). We have now observed that Rsp5, the single C2-WW-HECT E3 of *Saccharomyces cerevisiae*, can strongly stimulate HIV-1 Δ PTAPP release and Gag processing when its C2 domain is replaced. Thus, if an interaction with an ESCRT pathway component is required for activity in the Δ PTAPP rescue assay, such an interaction and the interfaces involved must be conserved between yeast and man. One reported interaction that potentially meets these criteria is that between Nedd4 and ALIX or their yeast homologues Rsp5 and Bro1 (212). Notably, the protein regions involved in the interaction appear conserved, because yeast Rsp5 co-immunoprecipitated with mammalian ALIX (212). However, the Nedd4-ALIX interaction may depend on the WW domains of Nedd4, which are dispensable for the ability of NEDD4-2s to rescue HIV-1 Δ PTAPP (212, 292).

In principle, ESCRT pathway components could also be recruited via ubiquitinated Gag, because the upstream ESCRT complexes each possess at least one component that binds ubiquitin (165, 251). For instance, the human ESCRT-I components Tsg101 and VPS37A contain ubiquitin-binding domains (120). It was also recently reported that the ESCRT-associated protein ALIX specifically binds to ubiquitin (133). However, at least the ubiquitin-binding

activity of Tsg101 is not required for the rescue of HIV-1 budding by NEDD4-2s (56). Also, there is evidence that the ubiquitination of Gag is dispensable, because the PPxY-dependent budding of a foamy virus Gag protein completely devoid of ubiquitin acceptors could be stimulated by catalytically active WWP1 (183). However, in the latter case, Gag-associated WWP1 could have served as an alternative ubiquitin acceptor, since the enzyme is capable of autoubiquitination.

K63-linked ubiquitin chains are required for the transport of at least some cargo into MVB (165), and have also been implicated in the function of PPxY-type L domains (280), which act by recruiting Nedd4 family members (23). Several Nedd4 family members have indeed been shown to preferentially synthesize K63-linked ubiquitin chains, including mammalian Nedd4.1 and ITCH (147, 148, 267), as well as yeast Rsp5 (147). In contrast, E6AP, another HECT domain E3, preferentially synthesizes K48-linked chains, which provide a signal for proteasomal degradation (143, 148). At least in the case of ITCH, chain type specificity is determined that the C lobe of the HECT domain (147). For instance, the replacement of the C lobe of ITCH with that of HUWE1 considerably reduces the preference for the synthesis of K63 chains. In the present study, we observed that an ITCH/HUWE1 C lobe chimera did not efficiently rescue HIV-1 budding. Interestingly, the C lobe chimera retained the ability to efficiently ubiquitinate Gag, but lacked the ability of the parental ITCH construct to induce the attachment of K63-linked ubiquitin chains to Gag. Taken together, these data

indicate that the ability to synthesize K63 chains is crucial for the stimulation of HIV-1 budding.

Structural studies indicate that the conformations of K63- and K48-linked chains are markedly distinct (65). Specifically, K63-linked di- or tetraubiquitin chains exhibit an extended conformation in which the functionally important surface hydrophobic residues are constitutively exposed, whereas K48-linked chains can adopt a closed conformation in which these hydrophobic surface residues are sequestered (65, 288, 295, 296). It is thus likely that linkage-specific conformations provide a basis for the recruitment of distinct cellular recognition factors. Interestingly, it has recently emerged that K63-linked ubiquitin chains serve as specific signals for the ESCRT-dependent sorting of cargo into MVBs. For instance, in the case of the yeast membrane protein Gap1, monoubiquitination is sufficient for its efficient endocytosis (166). However, the presence of short K63-linked chains is required for the entry of the Gap1 into the MVB pathway (166), suggesting preferential recognition of K63-linked chains by some component of the ESCRT machinery. The results presented here imply that HECT ubiquitin ligase-stimulated virus budding, which is also ESCRT-dependent (23, 49), is governed by the same type of ubiquitin modification.

UPDATE AND FUTURE RESEARCH

Since the publication of this manuscript, several studies have reported findings that are relevant to our results (252, 319). For example, using a retroviral Gag molecule (PFV) that was mutated to remove all potential ubiquitin acceptor sites, Zhadina and Bieniasz investigated the role of multiple NEDD4 E3 ubiquitin ligases in budding (319). As mentioned earlier, this construct, which contains an endogenous PPxY motif used to recruit NEDD4 family members, buds and releases efficiently when co-expressed with a broad range of NEDD4 ubiquitin ligases (319, 320). Interestingly, release of this construct required both the presence of the PPxY motif in the viral Gag molecule and a catalytically active HECT domain, in spite of the fact that the PFV Gag contained no ubiquitin acceptor sites, suggesting that either ubiquitination of some other ESCRT related protein was required or that the HECT domains themselves could be used as ESCRT recognition domains (319). This latter point was underscored by the correlation they observed between auto-ubiquitination of these HECT domains and release; those HECT domains which showed a high level of auto-ubiquitination also demonstrated an increase in particle release (319). Using RNAi they also demonstrated that release of PFV Gag under these circumstances required the presence of ALIX and Tsg101 (319). It has been shown previously that the UEV domain of Tsg101 is capable of binding to ubiquitin and recruiting ESCRT complexes to facilitate release (133, 245), and a similar interaction between ALIX and ubiquitin has been shown (133). In light of

the results we have obtained it is worth investigating whether the recruitment of Tsg101 and ALIX observed by Zhadina are related to the ability of the tested HECT domains to form K63-linked polyubiquitin chains.

A similar relationship between HECT domains and ALIX recruitment has also been reported by the Martin-Serrano group, which recently demonstrated that Arrestin-related proteins (ARTs) are involved in the release of viral particles that utilize PPxY L-domains, and thus recruit NEDD4 family members (252). Overexpression of ART proteins results in an inhibition of MLV particle release, a defect commonly observed when overexpressing ESCRT proteins (183, 252). Also, expression of dominant-negative VPS4, which also acts as an ESCRT pathway inhibitor, results in accumulation of ARTs at late endosomes, a phenotype often seen with ESCRT pathway subunit proteins (183, 252).

Using a yeast 2-hybrid (Y2H) approach they demonstrated interactions between Arrestin domain-containing proteins (ARRDC1-3) and NEDD4 ubiquitin ligases as well as ubiquitin, Tsg101 and ALIX (252). These proteins all contain either multiple PPxY motifs or in one case, ARRDC1, a Tsg101 binding site in addition to two NEDD4 binding sites (252). Predictably, the interactions between ARTs, NEDD4 family members and Tsg101 could be abrogated by removal of the C-terminal portion of the ARRDC1 protein, which contains the NEDD4 and Tsg101 recognition sequences (252). Interestingly, removal of these recognition sequences also prevents the interaction of ALIX with these proteins as

demonstrated by Y2H (252). As no LYPxnL sequence was observed in these proteins the lack of ALIX recruitment under these conditions suggests either the presence of a cryptic ALIX recruitment site, or that recruitment of ALIX is mediated by interaction with Tsg101 or NEDD4 family members (252). Using co-immunoprecipitation, the authors demonstrated an interaction between ARRDC1 and ALIX and mapped this interaction to the Proline Rich Repeat (PRR) of the ALIX protein using Y2H (252). Their data clearly provide additional evidence suggesting that NEDD4 recruitment of ESCRT is an ALIX mediated process.

Lastly, and perhaps most importantly, at the 2011 Cold Spring Harbor Conference on Ubiquitin a poster was presented by a member of Ron Kopito's group from Stanford University. In this poster, the author's described results obtained from investigating cellular proteins that bind specifically to K63-polyubiquitin chains. One of their principle findings was that ALIX was able to recognize and bind to K63-linked polyubiquitin chains through the V-domain of the ALIX protein (unpublished data). While this paper has yet to be published, and thus peer reviewed for content, this finding may indicate how PPxY-motif L-domains access the ESCRT pathway to promote viral release.

While recruitment of ALIX by K63-polyubiquitin chains formed by expression of NEDD4 family members answers the question of how these E3 ubiquitin ligases stimulate particle release, this idea remains unproven. A series of experiments would need to be undertaken to directly show that this suggested

recruitment is indeed the mechanism by which PPxY-containing viruses access ESCRT. Fortunately, the constructs that we have already used and published would be extremely beneficial in investigating this hypothesis. Using the previously described Z_{WT} Gag construct, which buds in an ESCRT independent manner while serving as a suitable substrate for ubiquitination (1), we would be able to definitively show recruitment of ALIX to HIV-1 Gag in response to K63-polyubiquitination by quantifying the amount of ALIX incorporated into these particles upon expression of our E3 ubiquitin ligase chimeras. Based on our previous results, we would also be able to use other HECT domain constructs that do not form K63 chains as controls. Also, this technique would allow us to map ALIX to determine the relevant domain required for interaction for both K63-recognition and ESCRT access. An additional line of investigation that has yet to be fully elucidated in the literature is whether retroviral Gag or the NEDD4 ubiquitin ligase itself is the relevant substrate for K63-polyubiquitination (56, 319, 320). Generation of chimeric E3-ubiquitin ligases capable of attaching K63-chains to substrates, but which are not NEDD4 family members, could be used to determine if ALIX recruitment is limited specifically to recognition of K63-chains or if it is mediated by the presence of the highly conserved HECT domains of these ligases.

Materials and Methods

Proviral constructs

HXBH10_{ΔPTAPP} is a mutant of HXBH10, a *vpu*-positive version of the infectious HXB2 proviral clone of HIV-1, with an in-frame deletion of codons 7 through 11 of p6. The Z_{WT} variant of HXBH10 encodes a chimeric Gag precursor that has NCp1p6 replaced by a leucine zipper dimerization domain.

Expression vectors

Plasmids expressing NEDD4-2s, N_{Δ1-31} (residues 32-834 of NEDD4-2s), or WWP1s (residues 110-922 of WWP1) with an N-terminal FLAG tag have been previously described, and are based on the mammalian expression vector pBJ5. Vectors expressing CypA-HA/FLAG-NEDD4-2s fusion proteins were created using an overlap extension technique. First, DNA fragments with overlapping ends were amplified in separate PCR reactions, using previously described vectors encoding CypA-HA and FLAG-tagged NEDD4-2s truncation mutants as templates. The two fragments were then recombined in a second PCR reaction as described, and inserted into pBJ5. The resulting CypA-N_{Δ1-31}, CypA-N_{Δ1-275}, and CypA-N_{ΔHECT} constructs encode CypA followed by HA and FLAG epitopes, which in turn are followed by NEDD4-2s residues 32-834, 276-834, and 432-843, respectively. Vectors expressing CypA-HA/FLAG fused to the isolated HECT

domains of SMURF1 (residues 366-757), E6AP (residues 462-852), or HERC6 (residues 637-1014) were made in an analogous manner using CypA-N_{Δ1-31} and cDNA clones encoding SMURF1 (KIAA1625), E6AP (BC009271), or HERC6 (BC042047) as templates. Overlap extension PCR was also used to generate pBJ5-based vectors expressing FLAG-tagged residues 1-73, 1-110, or 1-245 of NEDD4-2s fused to residues 383-922 of WWP1 (yielding N₁₋₇₃/WWP1, N₁₋₁₁₀/WWP1, and N₁₋₂₄₅/WWP1), using previously described vectors expressing FLAG-NEDD4-2s and FLAG-WWP1s as templates. Furthermore, over extension PCR was used to generate pBJ5-based vectors expressing FLAG-tagged residues 1-37 of NEDD4-2s fused to residues 142-862 of ITCH (yielding N_{Δ1-31}/ITCH), residues 138-809 of yeast Rsp5 (yielding N_{Δ1-31}/Rsp5), residues 366-757 of SMURF1 (yielding N_{Δ1-31}/SMURF1_{HECT}), residues 1207-1606 of HECW1 (yielding N_{Δ1-31}/HECW1_{HECT}), residues 1162-1572 of HECW2 (yielding N_{Δ1-31}/HECW2_{HECT}), residues 462-852 of E6AP (yielding N_{Δ1-31}/E6AP_{HECT}), or residues 637-1014 of HERC6 (N_{Δ1-31}/HERC6_{HECT}). The templates used included previously published plasmids and cDNA clones KIAA1625 (SMURF1), KIAA0322 (HECW1), KIAA1301 (HECW2), BC009271 (E6AP), and BC042047 (HERC6). The cDNA clones were provided by Kazusa DNA Research Institute or purchased from Open Biosystems. Of note, each HECT domain construct includes the N-terminal H1' helix of the HECT domain. An expression vector for full-length Rsp5 with an N-terminal FLAG tag was generated using standard PCR. Finally, overlap extension PCR was used to generate pBJ5-based vectors

expressing FLAG-tagged residues 1-245 of NEDD4-2s fused to residues 142-862 of WT ITCH (NM_031483), or to versions of ITCH in which the C lobe of the HECT domain has been replaced with to the corresponding region of E6AP or HUWE1.

Assays for viral particle production and Gag ubiquitination

293T cells (1.2×10^6) were seeded into T-25 tissue culture flasks 24 hrs prior to transfection. A calcium phosphate precipitation technique was used to transfect cell with HXBH10 Δ PTAPP (between 0.5 and 2 μ g) or Z_{WT} proviral DNA (2 μ g), along with expression vectors encoding the indicated E3 constructs (between 1 and 6 μ g) or empty vector. Total DNA transfected was normalized to 8 μ g by the addition of carrier DNA (pTZ18U). At 24 hrs post-transfection, cell culture supernatants were removed and clarified by low-speed centrifugation and passage through a 0.45 μ m filter. Clarified supernatants were layered on to 20% sucrose, and viral particles were separated using high-speed centrifugation (27,000 rpm, 2 h, 4°C). Cells were lysed using 1x RIPA buffer with protease inhibitors. Virus pellets and cell lysates were analyzed by SDS-PAGE and Western blotting. The anti-HIV CA antibody 183-H12-5C was used to detect Gag, Gag cleavage products, and Gag-ubiquitin conjugates. Ectopically expressed ubiquitin ligase constructs were detected using anti-FLAG (M2; Sigma) or anti-HA (HA.11; Covance). The K63-linkage specific antibodies Adu3

and HWA4C4 were purchased from Millipore. Western blots were quantified with the ImageJ software.

CHAPTER III

Analysis of Nef activity in the replication enhancement of HIV-1 in the MOLT3 System

Summary

The role of Nef in the virus life cycle is certainly of clinical importance, as the presence of a functional *nef* allele in the HIV-1 viral genome impacts disease progression in the infected individual. The effects of Nef on the infected cell, although broad in scope, pose multiple opportunities as therapeutic targets, particularly in light of the fact that most Nef effects are the result of interactions between the viral protein and host cell proteins. Such targets could potentially be more effective than current HAART targets, which are viral proteins and subject to high levels of mutation.

To further investigate the role of Nef in HIV-1 infection, we chose to use the MOLT3 cell line, in which we have observed a necessity for Nef for HIV-1 replication, despite modest levels of CD4 expression. Our results indicate that CD4 downregulation is not the major mechanism by which Nef enhances HIV-1 replication, contrary to published data. We also report that in the presence of a

CD4 variant incapable of downregulation, expression of Nef *in trans* is sufficient to restore replication of HIV-1 that lacks a functional *nef* allele. Rescue of HIV-1 Δ *nef* replication in the downregulation-resistant CD4 cell line was accomplished by Nef proteins from multiple HIV-1 groups and clades. We also report that in the parental MOLT3 cell line, the presence of *nef* in the viral genome leads to enhanced replication even after maximal stimulation with TNF α . Lastly, we report that enhanced replication stimulated by Nef is not dependent on levels of particle infectivity, as indicated by the delayed replication kinetics of HIV-1 genomes containing a portion of the Murine Leukemia Virus Glyco-Gag in place of Nef, which enhances HIV-1 particle infectivity to levels similar to WT Nef. While no specific mechanism for the increase in replication stimulated by Nef has been determined, the results of these experiments suggest that the MOLT3 cell culture system presents an opportunity to study how Nef positively impacts the replication of HIV-1.

Introduction

Produced early during viral infection, the lentiviral Nef protein is a crucial determinant of infection success. While an early study initially reported Nef to play a negative role in viral infection, (Nef – negative factor (51)) more recent research demonstrated just how vital this protein is regarding HIV-1 replication (144, 248). Although Nef is generally not required for replication of HIV-1 in most cultured cell lines (62), it plays a significant role in disease progression within the context of both HIV-1 and SIV infection in host organisms (144, 201, 248). Studies have shown that humans infected with HIV-1 variants that lack a functioning *nef* gene are protected from full progression to AIDS, and generally have relatively normal levels of circulating CD4+ cells (144, 248). Similarly, it has been observed that primates infected with SIV strains that have deleted or mutated *nef* alleles also fail to progress to a fully immuno-compromised state (201). It is clear that understanding the role that Nef plays during the course of infection is an important area of HIV-1 research.

In a perfectly permissive cell line, only a subset of genes contained in the HIV-1 genome would be required for productive rounds of infection, and in most cell lines this is certainly the case (62). The structural and enzymatic proteins encoded in the Gag and Gag-Pol transcripts provide the means of encapsulating and protecting the viral genome, as well as a means to copy and insert it into the

host cell genome, while the Env glycoprotein is sufficient to mediate viral entry into host cells (82). HIV-1, however, has devoted part of its limited genome to encode factors in addition to the viral structural proteins. In addition to the regulatory factors Tat and Rev (82) HIV-1 encodes genes for other proteins referred to as accessory factors. While these proteins may not be required for productive infection in certain cell culture lines, they perform essential functions in others, and more importantly, in the target cells of the host organism. These proteins are Vpr, Vif, Vpu and Nef.

The primary role for Vpr is to assist in the delivery of the retroviral genome into the nucleus of non-dividing cells, a necessary step for HIV which initially infects resting T-cells (103). Vif protects the viral genome from the innate immune effector APOBEC3G (A3G), a cytidine deaminase that is packaged into viral particles and hypermutates the genome by converting deoxycytosine nucleosides into deoxyuracil, thus significantly altering the viral genome (103, 179). Vif serves as an adaptor protein between APOBEC3G and a cellular E3-ubiquitin ligase, resulting in ubiquitination of A3G and proteasomal degradation (103, 179). Vpu assists in release of the viral particle from the surface of the cell by downregulating the molecule tetherin, another protein of the innate immune system (179). Vpu also plays a role in reducing the cell surface levels of CD4 by interacting with Env-CD4 dimers that form in the ER (179). These accessory proteins play important roles in the replication of HIV by altering the host cell to better suit the needs of the virus. In contrast, while expression of Nef in HIV-1

infected hosts has such a profound effect on disease progression (144, 248), expression of Nef in cultured cell lines appears to have only modest benefits to viral replication (62).

The lentiviral Nef proteins are typically between 26-34 kDa and in addition to a high degree of sequence conservation (229), they also share a common structure (See Fig. 3.1 and Fig 3.2). Nef proteins are co-translationally myristoylated at their N-terminus, have a unstructured loop connected to a conserved globular domain, and a variable-length C-terminus that also lacks a defined structure (Fig. 3.1) (92). The high degree of Nef sequence conservation correlates with functional conservation regardless of lentivirus strain or host organism (201). These activities can be assigned to several broad categories 1) evasion of the host adaptive immune response by downregulating MHC-I molecules (167, 170), 2) lowering the threshold for T-cell activation (215), 3) and an increase in individual particle infectivity and replication efficiency (53, 192, 201). As Nef lacks enzymatic activity, it brings about the changes in the host cell by acting as an adaptor, recognizing specific cellular proteins with one portion of the molecule and connecting to other cellular proteins such as enzymes and endosomal proteins (80, 161, 255).

Evasion of the host adaptive immune response

Nef is one of the first viral proteins to be expressed during infection, being transcribed early with the regulatory proteins Tat and Rev (26) and early

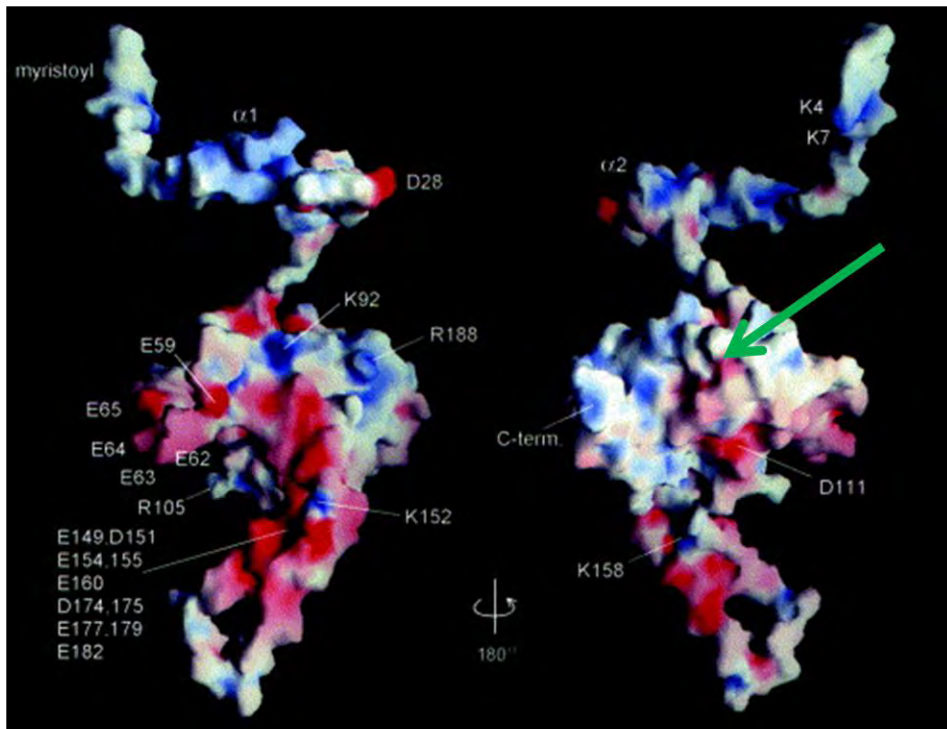


Figure 3.1 Three dimensional structure of nef. The three dimensional structure of nef was modeled from NMR and crystal structures of two nef fragments deposited in the protein data bank (1Q4A, 2NEF). Key amino acids previously shown to impact Nef activity are noted. Additionally, the hydrophobic patch/ dimerization domain is designated by green arrow. Image reproduced with permission from Geyer and Peterlin, 2001.

expression of this viral protein is beneficial for several key reasons. Initial Nef-induced downregulation of host cell surface CD4 protects against super-infection, ensuring that only one copy of the viral genome is active in the target cell (19, 312). Also, Nef induces downregulation of Major Histocompatibility Class-I (MHC-I) proteins from the host cell, while simultaneously directing intracellular MHC-I molecules in the trans-Golgi network for degradation via the

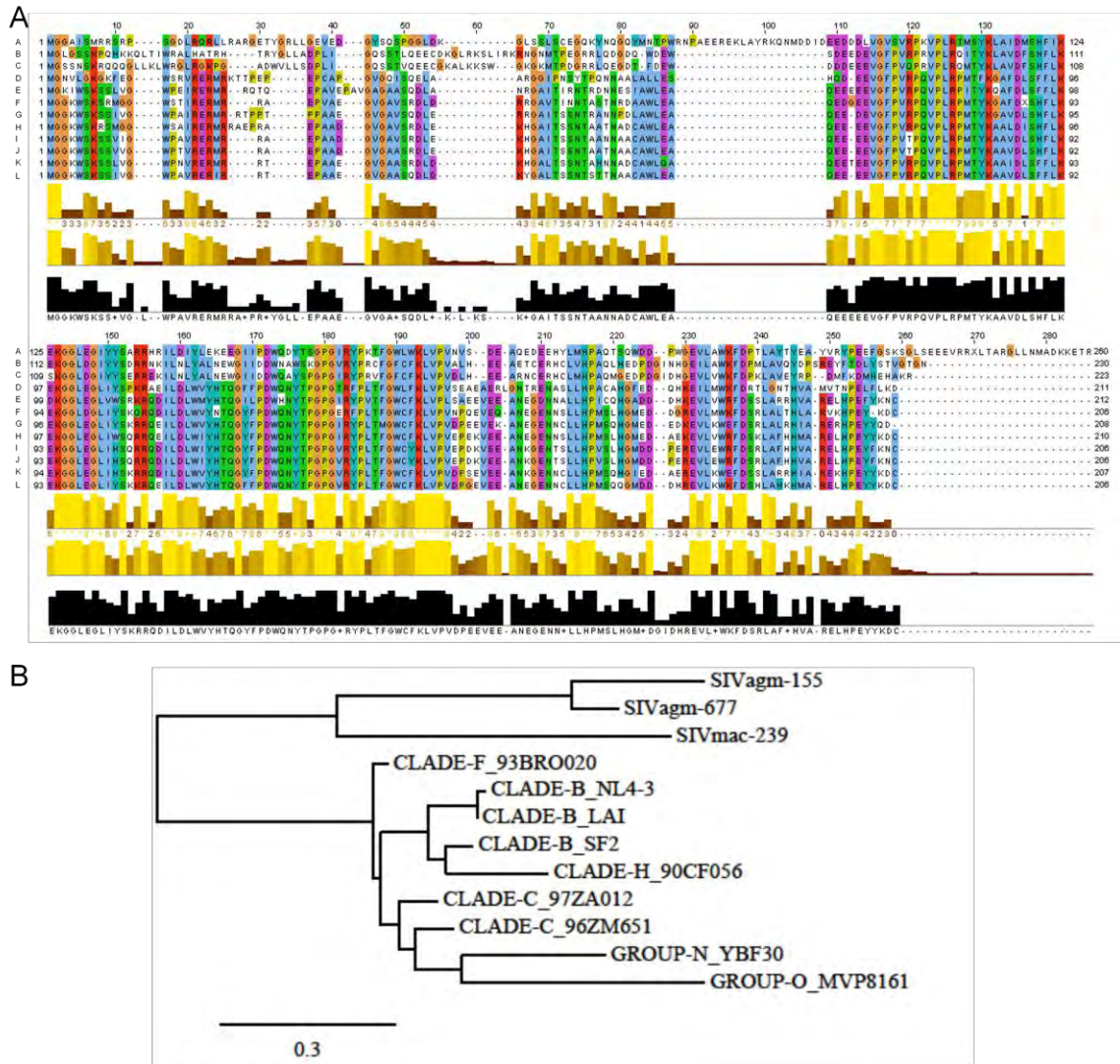


Figure 3.2 Sequence alignment of Nef proteins used in these experiments. (A) Nef proteins from various HIV-1 Groups and clades as well Nef proteins from SIV variants show significant conservation at the amino acid level. **(B)** Phylogenetic organization of Nef proteins used in this study. Nef proteins shown are A) SIVmac239 ,B) SIVagm155 ,C) SIVagm677, D) HIV-1 Group O, E) HIV-1 Group N, F) HIV-1 Group M, clade H, G) HIV-1 Group M, clade F, H) HIV-1 Group M, clade B (SF2), I) HIV-1 Group M, clade B (NL4-3), J) HIV-1 Group M, clade B (LAI), K) HIV-1 Group M, clade C (97ZA012) and L) HIV-1 Group M, clade C (96ZM651). Alignment performed using MUSCLE (v3.7) with highest accuracy, based on sequences from NCBI database. Phylogenetic tree designed using http://www.phylogeny.fr/version2_cgi/index.cgi.

endosomal/lysosomal pathway (80, 161). MHC-I molecules present peptides scavenged from the cytoplasm of cells and will present all peptides to cytotoxic

T-lymphocytes (CTL) for recognition (205). The CTL subset of T-cells is able to differentiate between peptides derived from the host cell and peptides that originate from pathogens, such as those that are scavenged from HIV-1 proteins. Recognition of a cell presenting a foreign antigen results in the destruction of the infected cell by CTL, and thus, the invading organism (205). By interfering with this surveillance system, HIV-1 genomes that encode Nef are able to avoid detection and propagate (205).

Nef disrupts MHC-I antigen presentation by recognizing a tyrosine-based sorting signal present in the cytoplasmic tail of the MHC-I proteins HLA-A and HLA-B. (167) HIV-1 Nef downregulates surface levels of MHC-I by forming a ternary complex between the MHC-I proteins HLA-A or HLA-B and the cellular adaptin protein AP-1 γ , which induces receptor internalization via a clathrin mediated mechanism (14, 128, 167, 176, 214, 275). In addition to reducing surface levels of MHC-I molecules, Nef is also able to induce MHC-I degradation while it trafficks through the secretory pathway by interacting with trans-Golgi network (TGN) coatomer β -COP and directing MHC-I to lysosomes for destruction (263). The timing of this interaction is important for two reasons: first, targeting MHC-I in the secretory pathway prevents any molecules loaded with viral antigens from being presented on the cell surface and second, early expression of Nef guarantees low levels of MHC-I when the full complement of HIV-1 proteins are present in the cytoplasm.

Lowering the threshold of T-cell activation

Although HIV-1 is capable of infecting resting CD4⁺ T-cells, viral replication is more efficient in activated cells (277). HIV-1 contains multiple binding sites for transcription factors in the 5' long terminal repeat (LTR) of its genome that are recognized by cellular transcription factors such as NFκB, NFAT, SP1 and AP1(142). However, high levels of these transcription factors are only produced by the cell in response to T-cell activation, indicating that translocation of the viral genome into the host cell nucleus itself is not sufficient to drive genome replication (142). A study by Stevenson *et. al.* demonstrated that infection of resting PBMCs results in delivery of viral cDNA into the nucleus of target cells, but that integration of the viral genome occurred only after activation of the target cell. The non-integrated viral genome is maintained in an extra-chromosomal state, although it is still transcriptionally active (277). Low levels of transcription from the non-integrated viral genome are able to produce Tat, Rev and Nef transcripts, in addition to small amounts of Gag. In this context, the early expression of Nef facilitates viral replication by lowering the threshold of T-cell activation through interactions with Src-family kinases (SFK) Fyn and Lck (105, 207), thus stimulating viral genome integration. These kinases, which are normally auto-inhibited (25), become active during T-cell receptor stimulation resulting in their phosphorylation, and initiation of a signaling cascade that results

in the production of NF κ B, NFAT, SP1 and AP1 transcription factors (205). HIV-1 Nef disrupts the auto-inhibition of these SFK molecules by displacing the inhibitory region with a higher affinity PxxP motif present in the retroviral protein (140). This displacement activates the kinase activity of the SFK molecule resulting in lowering the activation threshold of the T-cell and ultimately transcription of the viral genome. In addition to SFK-mediated activation, Nef has also been implicated in an interaction with p21-activated protein kinase 2 (PAK2), a downstream effector of T-cell receptor stimulation that plays an important role in rearrangement of the cytoskeleton (294) further impacting immune responses by interfering with the formation of immunological synapses between immune cells. This interaction has been shown to be genetically separable from that of the SFK effects and important in the previously observed replication enhancement often seen in Nef-positive viral isolates (215).

Nef-stimulated enhancement of particle infectivity and replication of HIV-1

Virus to Cell Transmission

One of the most intensely studied aspects of Nef function has to do with an observed enhancement of HIV-1 particle infectivity in viral isolates that contain an intact *nef* gene (53, 61, 160, 201, 264). This enhancement is dependent on the expression of Nef in the virus producer cell and its mechanism is a matter of some debate (53, 201, 264). Studies have shown that the enhancement occurs

sometime after fusion (192, 264) of the virus with the host cell, inviting the speculation that Nef by-passes a cytoskeletal barrier facilitating release of the viral core into the cytoplasm (250). This theory is bolstered by the finding that treatment of target cells with cytochalasin B, which depolymerizes the actin cytoskeleton, can substitute for a lack of Nef in producer cells (40). Others have speculated that Nef participates in the uncoating of the viral RTC which is suggested by the incorporation of Nef into viral particles and a reported interaction between Nef and the viral core (151). However, the results of a study by Zhou and Aiken showed that post-release incorporation of Nef into viral particles also enhanced infection, after the viral core had been formed, arguing against a need for Nef interaction with the viral core (325). This has led some to investigate the role that surface CD4 plays on Env incorporation into particles, finding that reduced CD4 at the cell surface correlated with an increase in gp120 in HIV-1 virions (61, 160, 208).

Several labs have investigated the activity of Nef on the downregulation of the CD4 receptor from the surface of the host cell and how this interaction relates to the observed infectivity phenotype (61, 160, 177, 208, 264). The presence of the CD4 glycoprotein on the surface of producer cells presents a problem for the virus, as expression of the gp120 HIV-1 envelope protein is capable of interacting with it during the process of particle assembly, negatively impacting future interactions between Env and CD4 on the target cell (48). HIV-1 has evolved several methods to circumvent this interaction, by downregulating CD4 from the

surface of the host cell, and directing its degradation as it trafficks through the secretory pathway (96, 231). Downregulation of CD4 from the cell surface is the result of the formation of a ternary complex between CD4, Nef and the μ -subunit of the clathrin adaptor protein AP-2 (46, 47, 60, 230), while an additional interaction between Nef and β -COP, mediated by cellular V-ATPase, has been shown to direct the internalized protein to the endosomal pathway for lysosomal degradation (63, 180, 231, 263). Mutational analysis of Nef has identified regions of Nef which are required for the specific interactions with host cell proteins. Notably, a dileucine motif (LL₁₆₄) found in the globular domain of Nef as well as an upstream Glu residue (E₁₆₀) and downstream diacidic region (DD₁₇₄), have all been shown to direct the interaction of Nef with AP-2 and V-ATPase (3, 46, 47, 94). Recruitment of Nef to β -COP is mediated by a diglutamate motif (EE₁₅₄) found upstream of the dileucine motif (231). Mutation of this β -COP interacting motif results in the internalization of CD4 from the cell surface, but these molecules are not degraded, and merely recycle back to the membrane (231).

Interaction of Nef with the CD4 cytoplasmic domain is mediated by several Nef residues including WL₅₈, D₁₀₈, and FPD₁₂₁; although the later region is thought to contain a putative dimerization domain which has been shown to be important for a number of Nef functions (Table 3.1). It has been suggested that residues comprising this region align to form a dimerization domain as well as an interaction site (8, 175), and Poe and Smithgall have presented a model of

Table 3.1 *nef* point mutants used in this study.

Mutation	Defect	Activity			Citation
		CD4 activity	MHC-I	Replication	
WT	none	+++	+++*	+++	80, 90, 161, 177
G ₇ A	Prevents interaction with PM	-	-	+ / ++	90, 177, 237
P _{69,72} A	Interaction with SFK	++	NT	+ / ++	155, 177, 215
K ₉₂ A	Unknown	+++*	+++*	+*	
E ₉₃ A	Unknown	+++*	+++*	+++*	
L ₁₀₀ A	Disrupts hydrophobic patch	+	+*	+++*	104
RR ₁₀₅ AA	Disrupts Nef dimerization, interaction with PAK1/2, CD4	+	++*	++	8, 177
D ₁₀₆ E	Disrupts Nef dimerization, interaction with hTE, PAK1/2	+	NT	+++	8, 59, 177
I ₁₀₉ A	Disrupts Nef dimerization domain, hydrophobic pocket	NT	+	+++	8
L ₁₁₂ A	Interaction with hTE, Dyn2, and PAK2	NT	NT	+++	175, 237
Y ₁₁₅ A	Disrupts Nef dimerization domain, hydrophobic pocket	NT	NT	+++	8
F ₁₂₁ A	Interaction with hTE, Dyn2	+	+*	+*	175, 237
D ₁₂₃ A	Dimerization, Interaction with hTE, Dyn2, and PAK2	+	+*	+*	175, 237
F ₁₂₁ A, D ₁₂₃ A	Dimerization, Interaction with hTE, Dyn2, and PAK2	+	NT	+*	175, 237
N ₁₂₆ A	Unknown	NT	NT	+++	
EE ₁₅₆ QQ	Prevents interaction with β -COP	+	+++*	+*	177, 231
E ₁₆₀ A	Disrupts interaction with AP-1 and AP-3	+++	NT	+++*	60, 177
LL ₁₆₄ AA	Removes interaction with AP1, AP2 and AP3	+	+++*	+*	6, 46, 177
DD ₁₇₃ AA	Removes interaction with AP1, AP2 and AP3	+	+++*	+*	3, 177
F ₁₉₁ I	Disrupts interaction with PAK2	+	NT	+*	215

Activities range from low (+) to high (+++), or no different from *nef*-deficient (-).

Abbreviations used in table. PM-plasma membrane, SFK-Src-family kinases, PAK2- p21-associated kinase, hTE-human thioesterase, Dyn2-dynamain2.

* Activities denoted by asterisks were performed by members of Gottlinger lab. MHC-I downregulation assay performed by M. Pizzato. CD4 downregulation and replication studies performed by E. Weiss.

dimerized Nef based on alignment of the crystal structure (240). However, it has also been reported that Nef proteins from different strains, including SIVs, are able to functionally dimerize while lacking structures similar to the HIV-1 Nef dimer interface (157). It was later reported that the dimerization of HIV-1 Nef is most likely an artifact that arises during preparation of the protein for analysis (29). Clearly, additional investigation into the role of these residues is required to fully understand their importance.

The importance of CD4 downregulation to the replication of HIV-1 is further illustrated by the functional redundancy exhibited by the virus to achieve this goal. In addition to Nef, HIV-1 disrupts surface levels of CD4 through the activities of the Vpu accessory protein (179), and through activity of the HIV-1 Env molecule itself (48), implying that reduction of CD4 at the cell surface is important enough for the virus to devote limited genome space to encode three separate methods of achieving this result.

Several labs have investigated the relationship between CD4 downregulation and viral particle infectivity, finding that increased levels of surface CD4 in producer cells do indeed correlate with a decrease infectivity of viral particles (61, 160, 177, 208). However, it should be noted that some of these results were obtained from studies in cultured cells of epithelial and myeloid lineage that were transfected with vectors expressing CD4 in addition to HIV-1 constructs (160, 208), which may have resulted in CD4 expression well beyond physiological levels. Conflicting results have been reported by groups using CD4+ T-cells derived from primary blood mononuclear cells (PBMCs), which can be considered a far more relevant system (61, 177). These experiments, which tested the infectivity of viral particles produced in non-transformed T-cells, demonstrate that some Nef mutants defective for CD4-downregulation retain the Nef-induced infectivity enhancement (61, 178, 208), although the authors of one study did suggest that late-cycle activities of Vpu may have been responsible for reduction of CD4 in their system (208).

Interestingly, one study showed a distribution of phenotypes from Nef mutants previously shown to be involved in CD4-downregulation at various stages ranging from almost completely WT to Nef-deficient (177). Finally, a separate study, also testing the infectivity of particles generated in PBMCs, reported no effect of Nef expression regarding HIV-1 Env incorporation or particle infectivity (208). It should also be noted that despite showing no significant difference in the infectivity of Nef+ viral particles produced in CD4+ PBMCs, both studies reported a significant difference in the replication of HIV-1 in the presence of Nef, although this may have been a result of using activated cells (177, 208).

Intriguingly, Nef is also able to increase the infectivity of viral particles from cells that do not express CD4 (192, 237, 264). HIV-1 particles generated in cells of epithelial lineage, such as HEK293 cells, show a modest increase in infectivity when a functional *nef* allele is present in the provirus or expressed *in trans* (4, 192, 237). This observation suggests that the presence of Nef in the producer cell leads to a fundamental difference in the composition of the particle, either at the level of assembly or protein content of the virus. One possible mechanism for this observed effect is downregulation of an as yet unidentified PM protein, a theory supported by a reported interaction between Nef and the GTPase dynamin, a participant in clathrin-mediated endocytosis (237). However, it is also possible that Dyn2 affects the lipid composition of the membrane by facilitating the turnover of non-raft membranes, enriching the raft content of the membrane which has been shown to enhance virion infectivity (37, 237). Several studies

have found a qualitative difference in the lipid composition of the viral envelope, with expression of Nef positively correlating with increased cholesterol levels in both viral envelope and host cell PM (36, 37, 323, 324). This observation may suggest a mechanism by which Nef can affect infectivity without altering viral protein content. Previous reports have indicated that reduction of cholesterol from both producer cells and virions has a negative effect on particle infectivity while increased envelope cholesterol levels seem to promote fusion with the target cell plasma membrane (323, 324). This observation may also explain the findings of Pizzato et al, who reported that receptor pseudotyped virus particles showed enhanced infectivity in the presence of Nef (238). Also, it has been demonstrated that Nef contains a cholesterol binding motif, and mutation of this region results in decreased particle infectivity, further illustrating the importance of the lipid composition of the viral envelope (323).

It should also be noted that Nef is packaged into viral particles, where it is cleaved by the viral protease (38, 151, 309). Imaging of HIV-1 cores produced in the presence or absence of Nef have shown no distinct morphological differences (79). Biochemical analyses suggest no significant difference in the levels of viral proteins outside of the presence of Nef itself (37, 151, 192). However, a report by Forshey et al indicated that Nef associated with the viral core, and suggested that Nef may serve to destabilize the core post-fusion (79). A separate study by Aiken et al showed that interaction with HIV-1 structural proteins was sufficient to induce Nef-associated infectivity enhancement (250)

perhaps indicating that effects on the viral core were responsible for Nef enhanced infectivity. However, a different group reported no difference in the levels of particle infectivity in producer cells expressing Nef, regardless of whether Nef was associated with the virions (159).

The role of Nef in Cell-to-Cell Transmission of virions

In addition to enhancing HIV-1 virion infectivity, it has been previously reported that the presence of an intact *nef* gene also has an effect on the replication of virus *in vitro* (177). Perhaps counter-intuitively, the enhanced effect of Nef on particle infectivity is separate from enhanced replication (177). For example, Nef mutants that have been shown to be defective in enhancing infectivity (removing the myristoyl anchor of Nef, or disrupting Nef's interaction with SFKs) can still display activity with regard to enhancing replication in activated PBMCs (177). This discrepancy most likely reflects a difference in transmission method with infectivity being considered the transmission of the viral genome via particle (virus-to-cell) compared to cell-to-cell transmission of the virus by cellular contact through formation of a virological synapse (VS) (50). However, both of these methods of transfer have clinical relevance as they account for the initial infection of the host (virus-to-cell) and dissemination of virus over the course of disease (cell-to-cell) (50).

One method by which Nef may enhance cell-cell transfer of virions is through actin rearrangement. Multiple studies have shown that Nef expression in

producer cells affects the cytoskeleton (106, 131, 213). Nef is able to affect actin rearrangement by activation of PAK2, replacing the activation of that protein by CDC42 and Rho GTPases (213). Actin restructuring by Nef has been implicated in formation of both immunological and virological synapses, which form the cell-to-cell contacts of APCs and immune cells (IS) and HIV-1 producer and target cells (VS) respectively (106, 213). In addition, expression of Nef has been correlated with an increase in filopodia production in T-cells (213). As filopodia are thought to be one of several methods by which HIV-1 infected cells interact with target cells (271), expression of Nef in producer cells may enhance viral replication by stimulating cell-to-cell contact to form a VS.

Another aspect of virological synapses that may be influenced by Nef expression is the concentration of HIV-1 Gag and Env proteins to sites of cell to cell contact (107, 131). This concentration may be the result of increased lipid raft clustering, due in part to direct activities of Nef which have been shown to increase the size of microdomains in infected cells (52). As both Gag and Env associate with lipid microdomains during particle assembly (21, 171), an increase in either the size or number of these domains may increase particle assembly kinetics. Alternately, clustering of viral components may facilitate infection of the target cell by concentrating virus to one area of the membrane. Nef-induced clustering of microdomains may also rely on actin remodeling (131), suggesting that the increase in cell-cell transfer of Nef-positive virions may depend on the cooperative effect of both functions.

Research Aims Chapter III

The results of an initial experiment investigating the replication efficiency of HIV-1 in the presence or absence of Nef in MOLT3 cells indicated an exquisite requirement for this lentiviral protein in replication. In light of the fact that nef expression typically has a limited effect in cultured cells (62), we chose to investigate the requirement for Nef expression in the MOLT3 cell line. Determination of CD4 expression levels on the cell surface showed that MOLT3 cells expressed only modest levels of CD4 in comparison to other cultured T-cell lines commonly used in HIV-1 research, in which Nef is dispensable for replication (62). Taken together, the modest levels of CD4 in MOLT3 cells and the requirement for Nef expression in HIV-1 replication suggested that this cell line was ideal to determine how Nef-induced CD4 downregulation impacted viral replication. To investigate this we first sought to observe the replication efficiency of HIV-1 viruses that contained mutations in the endogenous Nef protein known to interfere with CD4 downregulation. Results of these experiments indicated that HIV-1 was able to replicate in MOLT3 cells when a Nef-mutant defective for CD4 downregulation was expressed. In light of these results, we set out to determine how Nef expression enhanced replication of HIV-1 in the MOLT3 system. Toward that end we investigated the ability of Nef proteins from a variety of HIV-1 groups and clades to enhance replication in the MOLT3 system. We also sought to determine if the Nef-induced replication

enhancement could be attributed to any previously published interactions by testing the effects of an additional panel of Nef mutants on replication. Also investigated were the role that Nef may play in the regulation of lentiviral transcription and the relative contributions of virus to cell and cell to cell transmission in the observed phenotype.

Results

Nef mutants defective for CD4 down-regulation retain the ability to enhance replication of HIV-1 in MOLT3 cells

Having observed the profound effect of Nef on the replication of HIV in the MOLT3 cell line (data not shown), we set out to determine the mechanism used by Nef to achieve this result. It has previously been reported that one of the primary functions of Nef, downregulation of surface levels of CD4, serves to prevent interactions between CD4 and viral Env at the cell surface (61, 160, 208). Studies using transient transfection of 293T and HeLa cells with vectors expressing CD4 have shown that very high cell surface levels of the receptor impact particle infectivity, and this infectivity can be restored with Nef expression (61, 160, 208). However, as these cell types are not normal targets for HIV-1 infection, and the overexpression of CD4 may have been well beyond physiologically relevant levels, the results of these studies leave room for refinement. Our previous results demonstrating the complete reliance on Nef for the replication of HIV in the MOLT3 cell line despite modest CD4 surface levels suggested that these cells may provide a suitable model to test whether Nef-induced downregulation of CD4 is responsible for the observed enhancement of replication. In order to test this, we chose to infect these cells with HIV-1 that expressed Nef mutants known to be defective for CD4 downregulation.

Point mutations were made in the Nef coding region of the full-length NL4-3 proviral construct to mutate specific residues to Ala, and these constructs were transfected into 293T cells in order to produce infectious virus. Twenty-four hours after transfection, the supernatant was removed and the amount of released virus was quantified by p24 ELISA. MOLT3 cells were inoculated for 24 hrs with equivalent amount of virus as determined by ELISA, washed to remove unbound virus, and resuspended in media. Samples of supernatant were periodically removed over the course of the infection for analysis of released virus using the same p24 ELISA. As Fig. 3.3 indicates, infection of MOLT3 cells with 25 ng of WT NL4-3 input virus resulted in a 115-fold increase of particulate p24 14 days after the initial infection as compared to MOLT3 cells inoculated with Nef-deficient virus (FS). Interestingly, an enhancement of HIV-1 replication was observed by several Nef mutants known to be defective for various steps in the downregulation of CD4 (Table 3.1) For example, the viral genome expressing the Nef mutation EE_{155,156}QQ replicated almost as well as the HIV-1 virus encoding WT Nef. Previous findings indicate that these two Glu residues are responsible for the interaction of Nef with β -COP, which happens after clathrin-mediated CD4 internalization from the membrane, but prior to CD4 degradation (231). Thus this mutant most likely reduces the amount of surface CD4 to levels comparable to WT Nef. In contrast, mutations of the dileucine motif in Nef (LL_{164,165}AA) are known to interfere with CD4 internalization from the plasma membrane (46), as are mutations of a di-aspartate motif further upstream

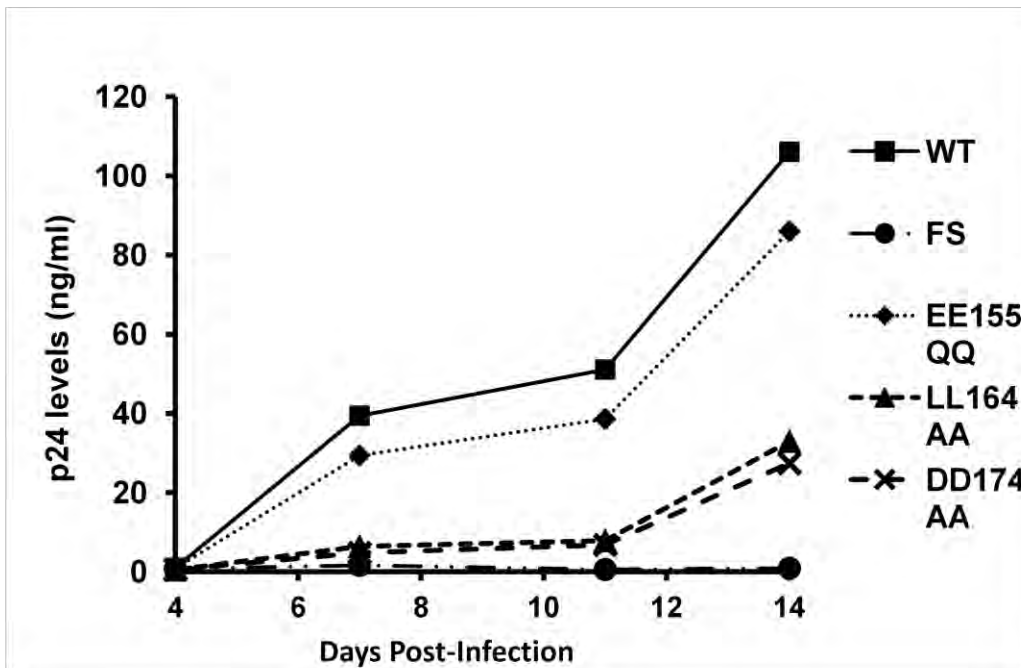


Figure 3.3. Nef –induced replication enhancement in MOLT3 cells is not dependent on residues required by Nef to downregulation CD4. Mutations in Nef that disrupt interactions between Nef and β -COP (EE₁₅₅QQ) or AP-1, AP-2, and AP-3 (LL₁₆₄AA and DD₁₇₄AA) retain the ability to enhance replication of HIV-1 in MOLT3 cells. MOLT3 cells infected with 10 ng of HIV-1 virus (NL4-3) expressing either WT Nef, NefFS, or Nef containing the indicated amino acid substitution. Input virus concentration determined by p24 ELISA of supernatants from 293T cells transfected with indicated proviral construct.

(DD_{174,175}AA) (3). It has been shown that both of these motifs are required for interaction with cellular clathrin adaptor protein molecules; AP-1, AP-2 and AP-3 in the case of the LL₁₆₄ motif and AP-2 in the case of DD₁₇₄ (46, 47, 60). Interaction with these adaptor proteins is required for both clathrin—mediated internalization and downregulation of surface CD4, therefore Nef proteins harboring mutations in these residues fail to reduce surface CD4 levels (46, 47, 60). As seen in Fig. 3.3, while mutation of these residues to Ala had a significant

impact on replication of HIV-1 in MOLT3 cells, both versions still displayed a clear Nef-related enhancement of virus replication, particularly compared to Nef-deficient virus (29-fold increase of released virus for LL_{164,165}AA and over 11-fold for DD_{174,175}AA compared to NefFS virus) in samples taken 14 days after infection. Thus, while downregulation of cell surface CD4 molecules may play a role in the replication of HIV-1 in MOLT3 cells, the inability to stimulate CD4 downregulation did not fully account for the Nef-enhanced phenotype observed. Alternatively, the inability of the LL_{164,165}AA and DD_{174,175}AA Nef mutants to interact with the adaptin complexes suggested a possible role for the endocytosis machinery in Nef-induced replication enhancement of HIV-1.

Replication enhancement of HIV-1 by Nef is observed in MOLT3 cells expressing a CD4 molecule that is not downregulated by Nef.

Based on the results of our mutational analysis, we decided to further investigate the role that Nef-induced CD4 downregulation plays in the observed replication enhancement in MOLT3 cells. To that end, we generated a cell line that stably expressed a version of CD4 that was incapable of being downregulated by Nef. Nef induced CD4 downregulation requires that the viral protein forms a ternary complex between Nef, the host cell AP-2 complexes and the cytoplasmic tail of CD4; this interaction between HIV-1 Nef and CD4 is mediated by the presence of a dileucine motif present in the cytoplasmic tail of

CD4 (2). Thus, removal of the cytoplasmic tail of CD4 results in the expression of CD4 at the plasma membrane that cannot be internalized and degraded via Nef (2, 104). Stable cell lines were made by infecting MOLT3 cells with virus containing either a retroviral genome encoding the truncated CD4 molecule or empty vector, and transformed cells were expanded under antibiotic selection; the MOLT3 cell line expressing the truncated CD4 molecule will be referred to as MOLT3CD4 Δ CT.

Detection of the surface CD4 levels of the transfected cells by FACS showed an increase in fluorescence by an order of magnitude in the MOLT3CD4 Δ CT cells, consistent with expression of additional CD4 from the integrated CD4 Δ CT vector. (Fig. 3.4, panel A. compare shadowed peak of parental MOLT3 cells to the dotted line of MOLT3CD4 Δ CT cells). These cells were then infected with 10 ng of NL4-3 WT or NL4-3 NefFS virus and supernatant samples were removed periodically for analysis of particulate p24. As demonstrated in Fig. 3.4, panel B, an almost 50-fold difference in the replication of HIV-1 WT vs. NefFS virus can be observed despite the fact that these cells express a CD4 molecule incapable of being downregulated by Nef. Also important to note is that the replication of HIV-1 NefFS was as poor in these cells as in the parental MOLT3 cell line, indicating that higher levels of CD4 expression did not increase overall permissiveness of these cells. These results indicate that the Nef-induced enhancement of replication characterized in MOLT3 cells is not strictly due to the ability of Nef to reduce surface levels of CD4.

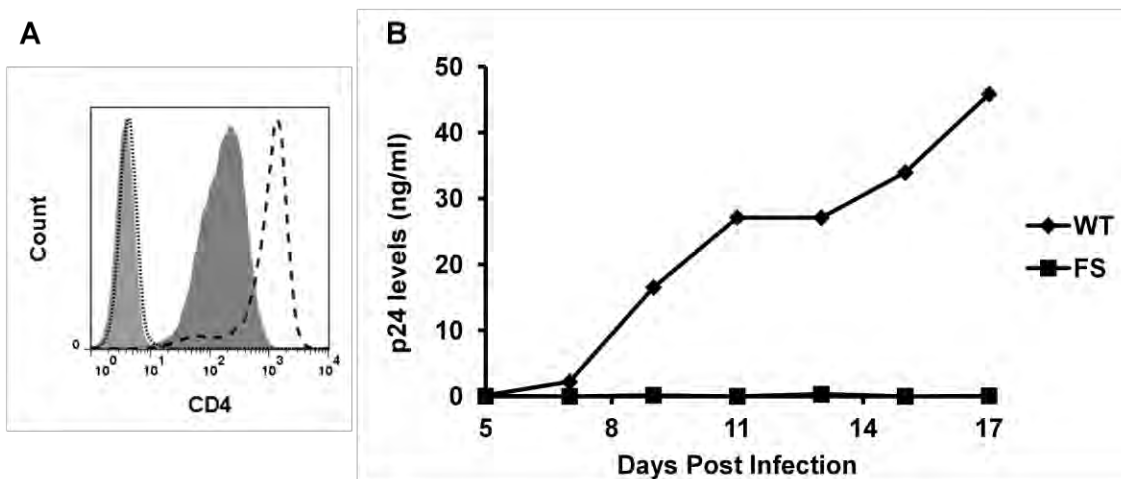


Figure 3.4. Expression of Nef is required for replication of HIV-1 in MOLT3 cells expressing a CD4 molecule resistant to downregulation. (A) MOLT3 CD4 Δ CT cells stably expressing a CD4 construct lacking the Nef-interacting cytoplasmic domain. FACS analysis of MOLT3 and MOLT3CD4 Δ CT cells for CD4 shows elevated levels of CD4 staining for MOLT3 cells expressing Δ CT construct. Isotype and CD4 peaks of MOLT3 cells presented as light and dark grey peaks, respectively. Isotype and CD4 curves for MOLT3CD Δ CT cells shown using solid and dashed black lines, resp. (B) Replication of HIV-1 in MOLT3 CD4 Δ CT cells retains requirement for Nef expression despite expressing a CD4 molecule incapable of being downregulated. Cells were infected with 10 ng of NL4-3 (WT) or NL4-3NefFS (FS). Input virus concentration determined by p24 ELISA of supernatants of 293T cells transfected with indicated proviral construct. Virus released from infected MOLT3CD4 Δ CT cells quantified with same ELISA.

Enhancement of viral infectivity can be returned to HIV-1 NefFS by providing Nef *in trans* in target cells.

We next attempted to determine if we could restore the WT replication phenotype to HIV-1 that lacked a Nef-coding region by expressing Nef *in trans* in target cells. There were multiple reasons to pursue this line of thought: 1) expression of Nef in target cells allowed us to determine the effect of 'adding back' Nef to the virus in a more controlled manner than transient transfection, 2)

positive results would enable us to test Nef molecules from multiple lentiviruses without having to clone them into a proviral HIV-1 vector, and 3) expression from a retroviral vector would allow us to express a Nef protein that could be detected by probing for a C-terminal epitope tag. To this end the MOLT3CD4 Δ CT cell line was transfected with a Murine Stem Cell Virus vector system (pMSCV) containing either empty retroviral vector or a vector containing the WT Nef protein from HIV-1_{LAI} (Nef_{LAI}) and an HA-tagged version (Nef_{LAI}-HA). As anticipated, determination of surface CD4 levels by FACS indicated that expression of Nef *in trans* did not decrease surface levels of CD4 Δ CT in these cells (Fig 3.5, panel A). MOLT3CD4 Δ CT cells stably expressing Nef_{LAI}, Nef_{LAI}-HA or empty vector were infected with 10 ng of HIV-1 lacking Nef (HIV-1 Δ *nef*) for 24 hrs, washed, and resuspended in fresh media. Over the following three weeks, supernatant samples were periodically removed for detection of p24 via ELISA. As Fig. 3.5, panel B indicates, infection of MOLT3CD4 Δ CT cells containing the empty retroviral vector (Vector) with NL4-3 lacking Nef resulted in a final p24 concentration of less than 1 ng/ml, similar to infection of the parental MOLT3 cells infected with the same virus (compare to Fig. 3.4). However, infection of MOLT3CD4 Δ CT cells that stably expressed Nef_{LAI} (Nef +) in the context of pMSCV vector resulted in a final p24 concentration that was 40-fold higher than empty vector (Fig. 3.5, panel B). In addition, comparison of released virus from one timepoint indicated that attachment of a C-terminal HA epitope to Nef_{LAI} did

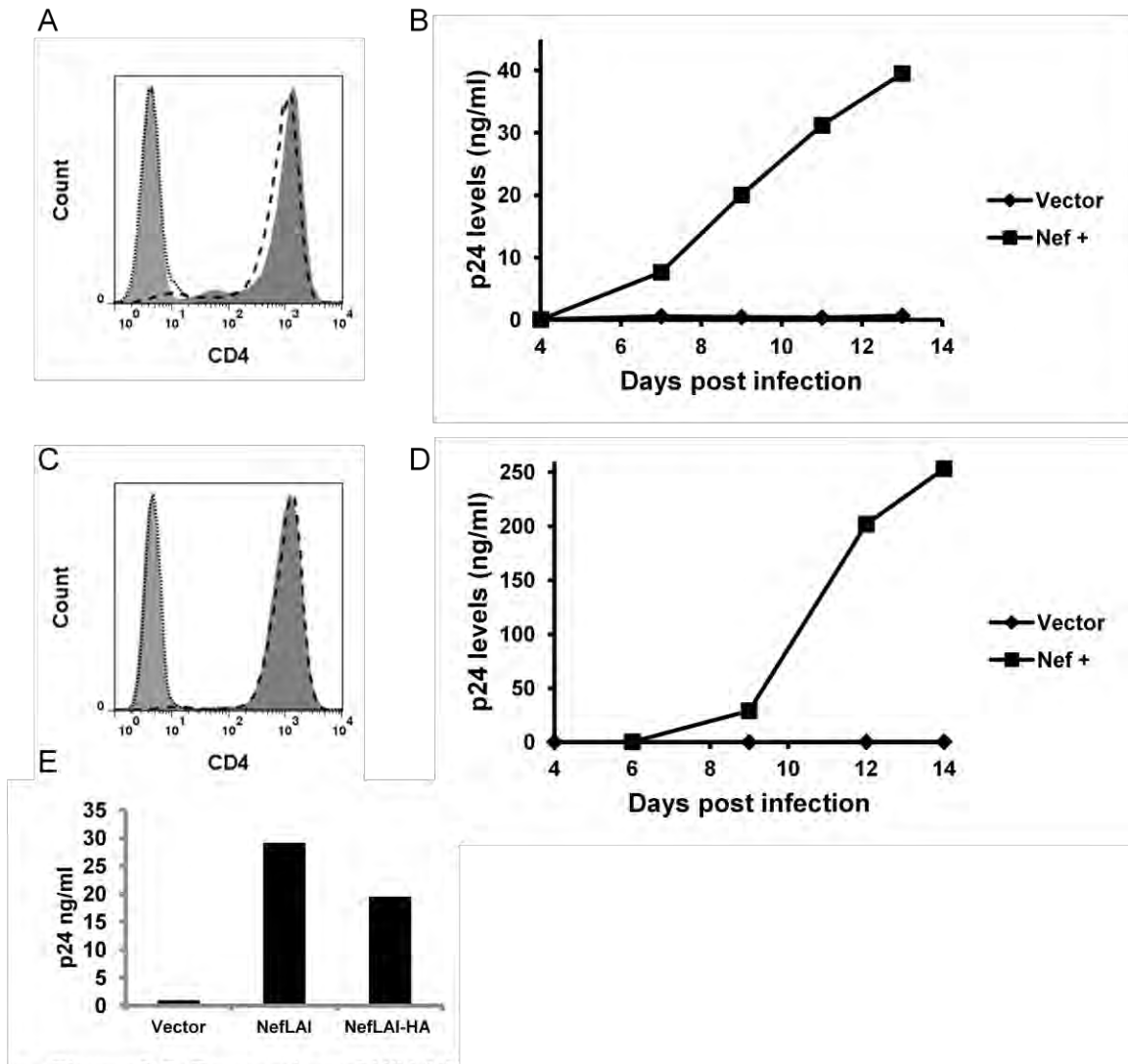


Figure 3.5. Expression of HIV-1 Nef in trans is sufficient to rescue replication defect of HIV-1 Nef FS in MOLT3-CD4 Δ CT cells. (A) Stable expression of NefLAI from pMSCV expression vector has no effect on CD4 surface expression. (B) Expression of NefLAI *in trans* from the pMSCV expression vector promotes replication of an HIV-1 provirus lacking *nef*. (C) Stable expression of NefLAI from pCX4 vector in trans does not downregulate CD4, but is sufficient to rescue replication of HIV-1 Δ *nef* (D). (E) Fusion of an HA tag to the C-terminus of Nef_{LAI} (Nef_{LAI}-HA) has only a modest impact on rescue of NL4-3 Δ *nef* replication compared to untagged protein. MOLT3CD4 Δ CT cells were transfected to stably express Nef_{LAI} from either the pMSCV or pCX4 expression vectors (Nef+), and respective empty vectors were transfected as controls (Vector). Stable cells were inoculated with 10 ng of NL4-3 Δ *nef*, and supernatant samples removed periodically for detection of released virus via p24 ELISA.

not severely restrict the ability of this protein to rescue replication of HIV-1 Δ *nef* virus in this assay (Fig. 3.5, panel E).

We next decided to test whether using a different expression system could improve the results we obtained in this experiment. Prior experience in our lab indicated that the pCX4 retroviral system would provide increased expression of Nef_{LAI} compared to that obtained with the pMSCV system (H.G. unpublished observation). Based on that observation, stable cell lines were made as described above instead using pCX4-based retroviral vectors in place of pMSCV-based ones. Again, following selection, MOLT3CD4 Δ CT cells expressing Nef_{LAI} or empty vector were analyzed for CD4 expression by FACS. As Fig. 3.5, panel C demonstrates, expression of Nef_{LAI} had no impact on the levels of cell surface CD4 compared to MOLT3CD4 Δ CT cells transfected with empty vector. These cell lines were then infected with equal titers (10 ng) of HIV-1 Δ *nef* virus and supernatant samples were removed periodically after infection. As shown in Fig. 3.5, panel D, expression of Nef_{LAI} from the pCX4 system in MOLT3CD4 Δ CT cells (Nef +) resulted in a robust rescue of HIV-1 Δ *nef* replication (250-fold) as compared to that observed from infection of cells containing empty vector (Vector). Together, the results of this experiment prove that Nef can dramatically enhance HIV-1 replication even under conditions where CD4 cannot be downregulated. They also indicate that addition of a small epitope, such as the HA tag, does not severely inhibit the activity of Nef-enhanced infectivity/replication.

Replication of Nef mutant viruses in MOLT3 cells

Due to its importance in disease progression (144, 248), investigation into the functions of Nef is one of the most intensely studied areas of HIV-1 research. Multiple labs have looked into interactions between Nef and cell proteins through mutational analysis of Nef and have published findings linking residues to specific functions (Table 3.1). In addition to these published mutations, we tested mutations in other residues that are conserved in Nefs from various HIV-1 groups and strains (Fig. 3.1). The indicated amino acid substitutions and residue numbering are based on the sequence for Nef_{NL4-3}. We initially chose to address mutations in Nef that have previously been implicated by other labs in specific interactions with host cell proteins (Table 3.1). In order to determine if any of these published interactions could be attributed to the enhanced replication phenotype we observed in the MOLT3 system, we introduced several of these mutations individually into the Nef coding region of the NL4-3 proviral clone. Infectious virions containing these mutations were generated by transfecting 293T cells with the specified proviral construct and equivalent p24 titers were used to infect MOLT3 cells. MOLT3 cells were infected with either 10 or 25 ng of input virus for 24 hrs, unbound virus was removed by washing the cells, and infected cells were resuspended in fresh media. Samples of supernatant were removed periodically for determination of released virus using the previously

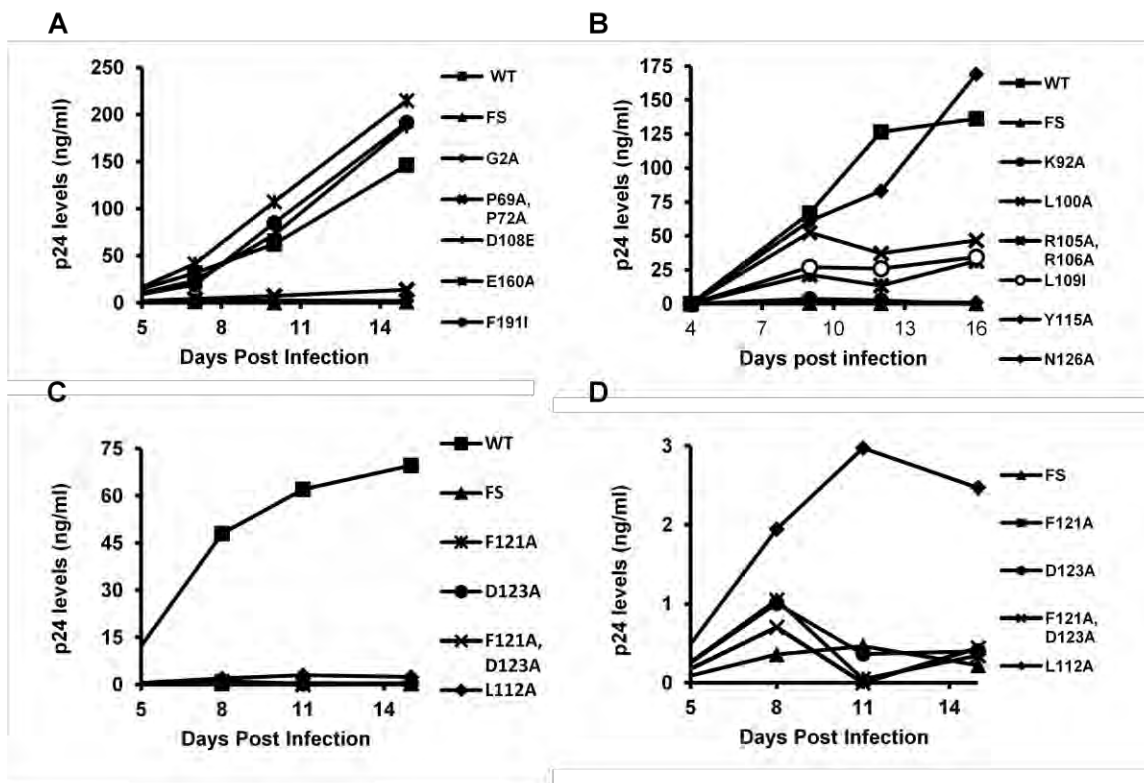


Figure 3.6. Replication of *nef* point mutant viruses in MOLT3 cells. MOLT3 cells were inoculated with wild-type HIV-1 (WT), HIV-1 Δ Nef (FS) or HIV-1 containing the indicated point mutation in HIV-1_{NL4-3} *nef*. Panel D is enlargement of same values from panel C. Inoculum virus concentration determined by p24 ELISA of supernatants from 293T cells transfected with indicated proviral construct.

mentioned p24 ELISA. Wild-type NL4-3 (WT) and NL4-3 Δ *nef* (FS) were used as positive and negative controls for these experiments.

Analysis of the replication efficiency of 18 of these mutants can be observed in Fig. 3.6. Mutation of the N-terminal Gly to Ala resulted in an almost complete loss of replication (Fig 3.6, panel A). This mutation removes the myristoylation site for Nef that serves to associate the viral protein with host cell membranes (237). It has been previously published that this mutation is

sufficient to abrogate most effects of Nef with regard to CD4 and MHC I downregulation and enhanced infectivity (178). We also observed a marked reduction in the replication efficiency of HIV-1 virus that contained the double Pro mutation PP_{69,72}AA (Fig. 3.6, panel A). These two residues are part of a highly conserved region in lentiviral Nefs (PxxPxxPxR) that have been indicated to interact with Src-family kinases such as Fyn and Lck, and actin remodeling effectors such as p21 associated kinase -2 (PAK2). Interaction of Nef with Fyn and Lck results in lowering of the activation requirements for T-cells (155, 215, 255). In contrast to results obtained with the PP_{69,72}AA mutant, another Nef mutant defective for interaction with PAK2, F₁₉₁I (215), replicated at levels similar to that observed in the presence of WT Nef (Fig. 3.6 panel A). A mutation of Nef which interferes with hTE interaction, and may be part of the Nef dimerization domain (D₁₀₈E), displayed no defect in replication in our assay (Fig. 3.6, panel A), in contradiction to other published results (59). Although the exact function of thioesterase recruitment in HIV-1 infection has yet to be fully elucidated, no effect from disrupting the dimerization domain was observed. Our data also indicate that in the MOLT3 system, the increase in replication induced by Nef does not rely on the ability of Nef to interact with AP-1 or AP-3 adaptor complexes. Our initial investigation of CD4 downregulation used the Nef dileucine mutant (LL_{164,165}AA) which is defective for interaction with AP-1, AP-2 and AP-3 adaptor proteins (230) (Fig. 3.3). However, mutation of the Glu at position 160 (E₁₆₀A) of Nef results in a mutant that is incapable of interacting with

AP-1 and AP-3 complexes, allowing Nef to interact only with AP-2 proteins (60). Analysis of the replication of HIV-1 virus containing the E₁₆₀A mutation, which replicated better even than WT HIV-1 (Fig. 3.6, panel A), suggests that the interaction with AP-1 or Ap-3 is not required for the Nef-induced increase in infectivity in this system (60).

Mutation of residues in the highly conserved FPD₁₂₁ motif (92, 175)(F₁₂₁A, D₁₂₃A, and FD_{121, 123}AA) led to dramatic decreases in replication (Fig.3.6 Panels C and D), and results of the ELISA used to quantify the p24 concentration indicated that these mutations released no more virus than HIV-1 that lacked the Nef protein entirely (Fig. 3.6, panel D). Crystal structures of the Nef protein have indicated that these residues are part of a domain which has been suggested to be part of the interface for Nef dimerization (157). It is also important to note that mutation of these residues has also been implicated in activity of Nef with regard to CD4 and MHCI downregulation, as well as being important for the interaction with dynamin2 (237) and hTE (157, 240). The Leu₁₁₂ residue, also previously indicated to play a role in dynamin2 interaction displayed a significant reduction in replication (Fig. 3.6, panel C and D), with supernatant p24 levels being only slightly higher than Nef-deficient virus (2.5 fold over Nef-deficient virus) (237, 240).

An additional panel of Nef proteins, containing mutations in residues localized to a putative dimerization domain (93, 157), was also tested using the

replication assay described above. The three constructs containing mutation at Leu 100 (L₁₀₀), the double Arg mutation at position 105 (RR₁₀₅AA), and the mutation of Leu 109 (L₁₀₉), all demonstrated similar levels of replication, with enhancements of 47-, 32- and 36-fold, respectively, over Nef-deficient virus (Fig. 3.6, panel B). Again, alignment of the Nef crystal structure has implicated these residues to be important for Nef dimerization (8, 157). Two additional mutations of highly conserved residues not reported previously in the literature demonstrated opposing effects on replication. Mutation of the Asn residue at position 126 (N₁₂₆), which is conserved across most Group M HIV-1 strains (Fig. 3.1), enhanced replication approximately 170-fold compared to cells infected with the NefFS virus (Fig. 3.6, panel B). This increase was in excess of the enhancement observed with WT Nef in this assay (137-fold over NefFS). Interestingly, a mutation of the Lys 92 (K₉₂A) residue, found in a similar position in almost all other Nef molecules (229), displayed an extreme defect in replication in comparison to WT Nef, and increased replication only 3-fold above HIV-1 NefFS (Fig. 3.6, panel B). This mutant was proven to be as active in the downregulation of both CD4 and MHC-I as WT Nef when assayed in 293T cells (data not shown). Additional replication assays performed to verify this result returned similarly low enhancement effects, with the maximum increase being 7-fold compared to NefFS replication (data not shown). This observation shows that CD4 downregulation and replication enhancement can be genetically separated.

The Nef proteins of HIV-1 virus from different Groups and Clades display a conserved ability to rescue replication of HIV-1 Nef-deficient virus when expressed *in trans* in target cells.

In order to determine whether the ability of Nef to enhance replication in this system was shared by all Nef molecules, we next chose to investigate the activity of Nef proteins from HIV-1 and SIV variants from a range of Groups and subtypes using our Nef *in trans* system. Based on genetic similarity, strains of HIV-1 that have been found circulating in the population have been organized into Groups and Clades (45). The most diverse Group is M, which is believed to have arisen from a single chimpanzee to human transmission from after which it diverged to generate the various Clades (45). HIV-1 variants found within each Group M clade also show regional bias i.e. Clade A variants predominate in West Africa, B variants in North America, etc (45). Like Group M viruses, Groups N and O are thought to have arisen from a separate zoonotic transmission between chimp and human (45), while P appear to have originated in gorillas (239).

Our previous Nef *in trans* experiment made use of the Nef protein from the HIV-1_{LAI} isolate, a member of Group M, clade B. In order to expand on our findings we tested Nef proteins from other Group M variants encompassing clades B, C, F, and H, as well as members of Group N and P (Fig. 3.7). Stable MOLTCD4ΔCT cell lines were generated to express the selected Nefs with a

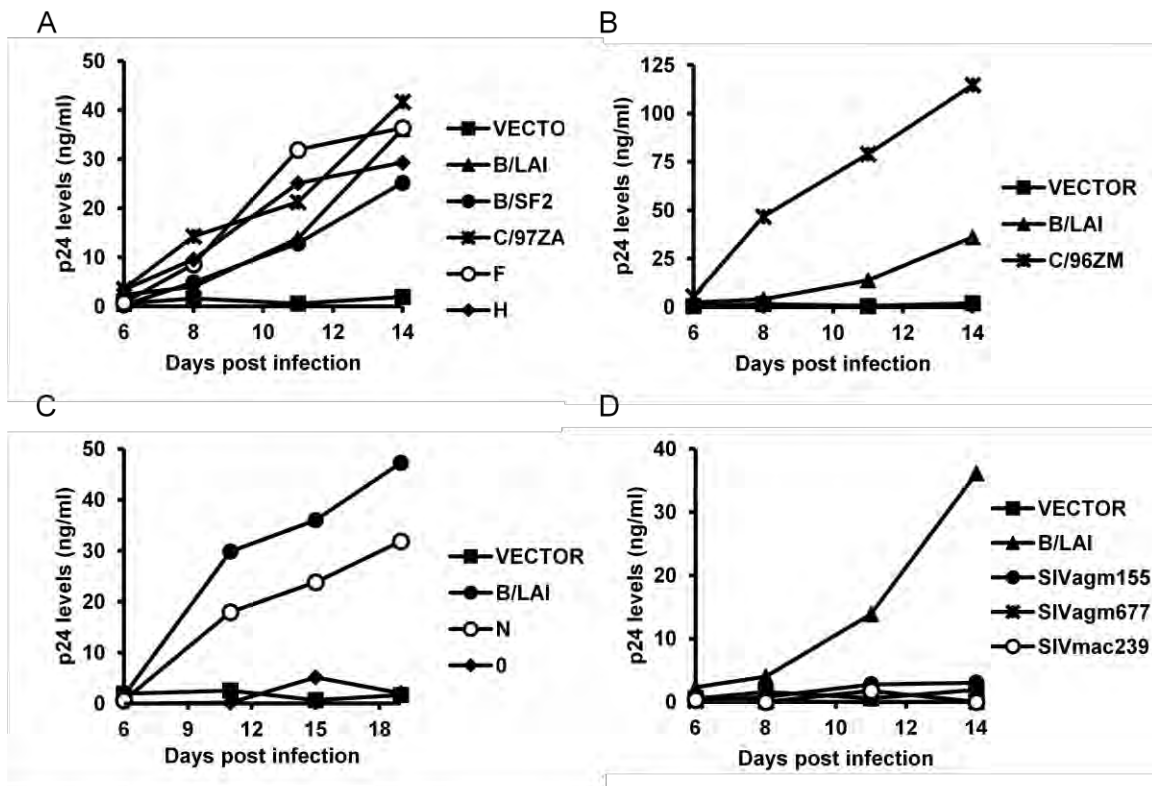


Figure 3.7. Stable expression of lentiviral nef proteins in trans show conserved ability to enhance replication of HIV-1NefFS in MOLT3CD4ΔCT cells. (A) Group M Nef proteins from clades B, C, F and H display similar levels of replication enhancement when stably expressed in MOLT3 CD4ΔCT cells infected with HIV-1Δnef. (B) Stable expression of Group M, clade C Nef from 96ZM651 displays particularly robust replication enhancement in comparison to both empty vector and the clade B member, LAI. (C) Stable expression of the Group N Nef is as capable of rescuing replication as clade B LAI, while Group O shows little effect on enhancing replication of HIV-1Δnef. (D) Stable expression of various SIV Nefs fails to enhance replication of HIV-1Δnef to any significant level above vector control. MOLT3 CD4ΔCT cells stably expressing the indicated lentiviral Nef protein (or empty vector) were inoculated with 25 ng of NL4-3Δnef. Input virus concentration determined by p24 ELISA of supernatants from 293T cells transfected with indicated proviral construct.

C-terminal HA tag so that expression levels could be determined. Expression levels of Nef proteins were similar as determined by Western blot of the epitope tag (results not shown). Analysis of cell surface CD4 by flowcytometry indicated that expression of various Nef proteins had no effect on the levels of CD4

present on the cell surface (Fig 3.8). Replication of 10 ng HIV-1 Δ *nef* input virus was followed as previously reported for the Nef *in trans* assay described above.

As Fig. 3.8, panel A indicates, expression of Nef from the clade B variant SF2 was able to restore replication to HIV-1 lacking an intact Nef gene to similar levels observed with the LAI construct (25-fold increase in cells expressing Nef_{SF2} and 36-fold increase in replication in Nef_{LAI} cells, compared to empty vector). It was also observed (Fig. 3.7, panel A) that replication of HIV-1 Δ *nef* virus in MOLT3CD4 Δ CT cells expressing Nef proteins from clades C, F, and H was as efficient as that observed with the HIV-1Nef_{LAI} construct (41-,36-, and 30-fold, respectively, over cells expressing empty vector), and in at least one case, Clade C 96ZM651, significantly exceeded that of Nef_{LAI} and resulted in a 115-fold increase in particulate p24 compared to empty vector (Fig. 3.7, panel B). As previously described, no substantial replication of HIV-1 Δ *nef* was observed in the MOLT3CD4 Δ CT cells expressing empty vector (Fig. 3.7, all panels).

Investigation of HIV-1 Nef proteins from other Groups showed mixed results; as Fig. 3.7, panel C demonstrates, expression of the Group N Nef resulted in rescue of replication just slightly less robust than that observed with the Group M variant LAI (32-fold increase over empty vector, compared to 50-fold increase from Nef_{LAI}). However, stable expression of the Group O Nef in the MOLT3CD4 Δ CT cell line failed to enhance replication to any greater levels than those observed from the cell line expressing the empty vector (Fig. 3.7, panel C).

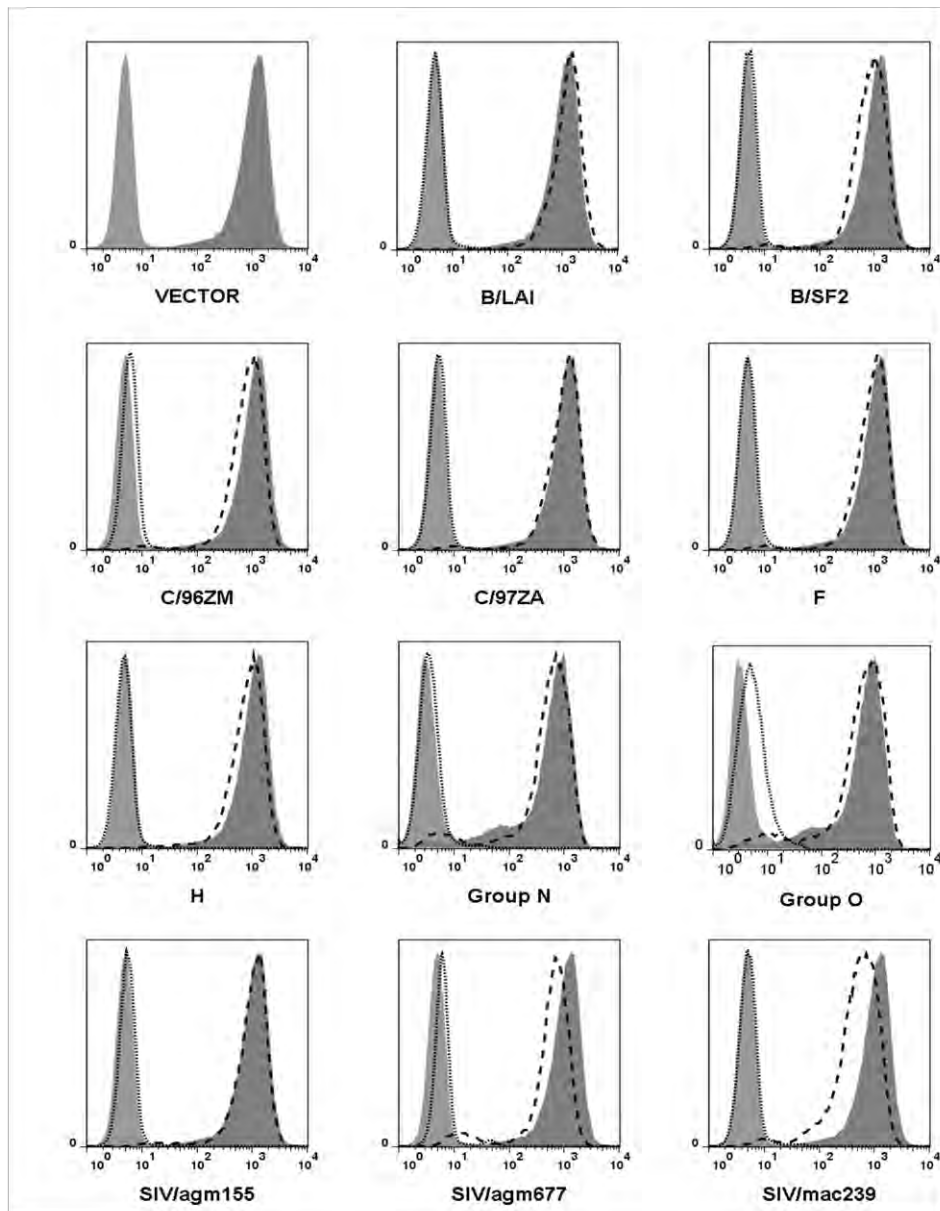


Figure 3.8. Stable expression of lentiviral Nef proteins in MOLT3-CD4 Δ CT cells display no or only modest effects in CD4 surface expression levels.

Determination of surface expression of the CD4 receptor of MOLT3CD4 Δ CT cell lines stably expressing the indicated HA-tagged Nef protein by FACS. Cells were stained with antibodies to detect CD4 and isotype controls were used to determine background. Isotype and CD4 peaks of parental MOLT3CD4 Δ CT cells shown in light grey and dark grey, respectively. Isotype and CD4 levels of MOLT3CD4 Δ CT cells stably expressing the indicated Nef protein shown as solid black or dashed black line, respectively.

A possible reason for the lack of replication in the Group O expressing cell line is the down regulation of the HIV-1 CXCR4 co-receptor, required for the replication by NL4-3. It has been reported that the Nef proteins of Simian Immunodeficiency Viruses (SIV) also downregulate HIV-1 co-receptors in addition to CD4 (297). Analysis of cell surface levels of all stable cell lines used in these experiments by flow cytometry indicated that while CD4 surface levels remained unchanged in all lines (Fig. 3.8), MOLT3CD4 Δ CT cells expressing the Nef protein of Group O did indeed significantly downregulate CXCR4 from the cell surface (Fig. 3.9).

We also opted to determine if the Nef proteins from selected SIV stains could be used in our Nef *in trans* assay to restore replication to Nef-deficient HIV-1. Cell lines were generated to stably express HA-tagged Nef proteins from two African Green Monkey variants (SIV_{agm155}, and SIV_{agm677}) as well as the Nef protein from SIV_{mac239} in CD4 Δ CT cells. Again, following selection, cell surface levels of CD4 were determined by FACS. Compared to the parental cell line, MOLT3CD4 Δ CT cells stably expressing Nef proteins from SIV_{agm155} and SIV_{agm677} displayed identical levels of surface CD4, while only a modest decrease in the surface levels of CD4 were observed in MOLT3CD4 Δ CT cells stably expressing Nef from SIV_{mac239} (Fig 3.8). These cell lines expressing the HA-tagged SIV Nefs, as well as empty vector (negative control) and HA-tagged Nef_{LAI} (positive control), were inoculated with 10 ng of HIV-1 Δ nef virus and replication was determined by quantifying particulate p24 in the supernatant over time. As Fig. 3.7, panel D demonstrates, none of the cell lines expressing SIV

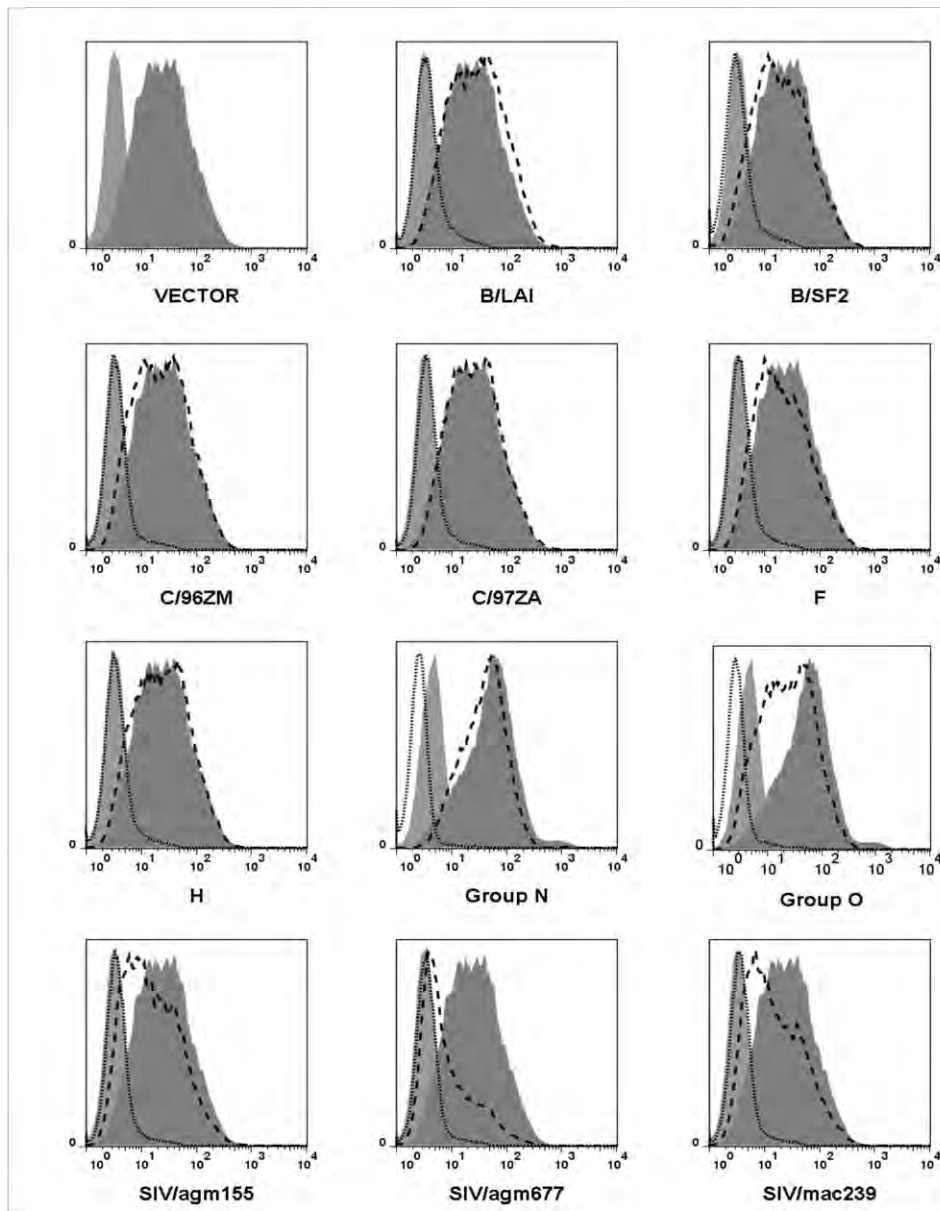


Figure 3.9. Stable expression of lentiviral nef proteins from SIV or HIV-1 strains closely related to SIV demonstrate decreased levels of CXCR4 in MOLT3-CD4 Δ CT. Determination of surface expression of the CXCR4 receptor of MOLT3CD4 Δ CT cell lines stably expressing the indicated HA-tagged Nef protein by FACS. Cells were stained with antibodies to detect CXCR4 and isotype controls were used to determine background. Isotype and CXCR4 peaks of MOLT3CD4 Δ CT cells shown in light grey and dark grey, respectively. Isotype and CXCR4 levels of MOLT3CD4 Δ CT cells stably expressing the indicated nef protein shown as solid black or dashed black line, respectively.

Nef proteins showed significant replication of HIV-1 Δ *nef*, although a very slight enhancement was observed in the cell line expressing Nef_{agm155} (a 3-fold increase over empty vector, panel D, Fig. 3.7). FACS analysis of these stable lines indicated that expression of the respective Nef proteins resulted in a significant downregulation of surface CXCR4, which could account for the low levels of replication (Fig. 3.9). However, no differences in cell surface levels of CD4 were observed in any of these stable cell lines. Thus, we can conclude that Nef proteins from Groups M, and N are able to restore replication enhancement to HIV-1 Δ *nef* in the MOLT3CD4 Δ CT cell line. Conversely, Nef proteins from more distantly related viruses, such as those from Group O and SIV strains, are unable to rescue HIV-1 Δ *nef* replication, although whether this deficiency is the result of an inherent Nef defect or CXCR4 down regulation is unclear.

MOLT3 cells maintain a reliance on Nef for replication regardless of cell activation

Expression of Nef in infected T-cells has been shown to lower the activation threshold for these cells and increase NF κ B-directed transcription from the retroviral 5'LTR (142). This effect is particularly pronounced in cells that have been infected prior to activation, where the presence of Nef can often lead to greater than 10-fold differences in replication (215). Conversely, there is little difference in replication observed if the T-cells are infected following activation,

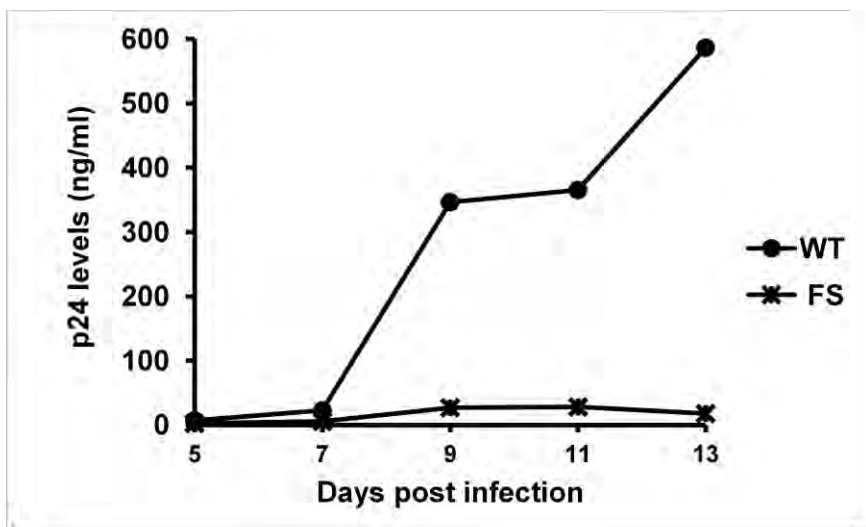


Figure 3.10. Nef-induced replication enhancement in MOLT3 cells persists even after T-cell stimulation. Replication of wild-type HIV-1 (WT) or HIV-1 Δ *nef* (FS) in MOLT3 cells stimulated with 100 ng/ml of TNF α 24 hrs prior to infection. MOLT3 cells were infected with 10 ng of input virus as determined by p24 ELISA of supernatants from 293T cells transfected with indicated proviral construct.

as this condition obviates the requirement for Nef in lowering the activation threshold of the host cell (215). In an effort to further elucidate the role Nef plays in the replication of HIV-1 in the MOLT3 cell line, we chose to test whether Nef offered any replication advantage to virus used to infect activated MOLT3 cells. Cells were activated by the addition of tumor necrosis factor- α (TNF α – 100 ng/ml) to cultured MOLT3 cells 24 hrs prior to infection (290). Treatment of T-cells with TNF α initiates a signal cascade that ultimately results in increased levels of nuclear NF κ B, which is a cellular transcription factor for inflammation related genes, as well as a transcription factor for HIV-1 (290). Twenty-four hours after TNF α treatment, MOLT3 cells were inoculated with equal titers of either WT or Nef-deficient HIV-1 (10 ng as determined by p24 ELISA) for 24 hrs,

washed and resuspended in fresh media containing an additional 100 ng/ml TNF α . Supernatant samples were removed periodically to assay viral replication using a p24 ELISA. Our results indicate that Nef continues to have a profound effect on replication even after T-cell activation in the MOLT3 system (Fig. 3.10). These results are contrary to published reports (215), and indicate that Nef plays a role in HIV-1 replication in the MOLT3 cell line even after activation of host T-cells.

Expression of Nef does not affect viral replication at the level of transcription

In an effort to further investigate the role of the endocytic pathway in Nef-induced replication enhancement, we decided to generate stable cell lines in which we separately knocked down expression of clathrin heavy chain protein, the enzyme dynamin2, and the μ -adaptin subunit of AP-2. Previous reports by our lab and others have implicated these proteins in Nef-associated functions such as increased infectivity and replication, and our hope was that an inducible siRNA-mediated knock-down of these proteins would provide insight into Nef's activity in the MOLT3 system (46, 47, 170, 230, 237). In order to accomplish this goal, we opted to use a lentiviral based vector (TRIPZ, Open Biosystems) that would stably integrate the shRNA element into the genome of infected MOLT3 cells, which could then be selected for by the addition of antibiotics. Interestingly,

we failed to observe any cells resistant to selection following infection with the TRIPZ system, despite the fact that this same virus preparation was able to transform MOLT4 cells, a cell line derived from the same donor as MOLT3. As the lentiviral system chosen to create these stable cells was derived from HIV, this observation suggested that MOLT3 cells may contain a restriction against lentiviral activity that may be alleviated by expression of Nef. We postulated that in the MOLT3 system, integrated viral genomes are transcriptionally silenced, and the presence of Nef may prevent this silencing from occurring.

We initially chose to test our hypothesis by infecting MOLT3 cells with VSV-G pseudotyped, envelope deficient HIV-1 containing either wt Nef or the Nef frameshift mutation. VSV-G pseudotyping of HIV-1 virus particles removes any effect of Nef infectivity enhancement on particles, and allowed us to ensure that target cells were infected equally (61). This was intended to limit the infection to a single round and allow us to observe the effects of Nef expression on transcription by measuring the levels of newly translated HIV-1 Gag proteins in infected cells. The results of our initial investigation showed no difference in the production of viral proteins in MOLT3 cells regardless of whether a functional *nef* gene was present in the viral genome (Fig. 3.11, panel A). Comparison of samples taken from the infections at two different timepoints indicated that the intracellular viral proteins could be “diluted out” by the addition of fresh media, indicating that active translation of the viral genome was no longer occurring (Fig. 3.11, panel B).

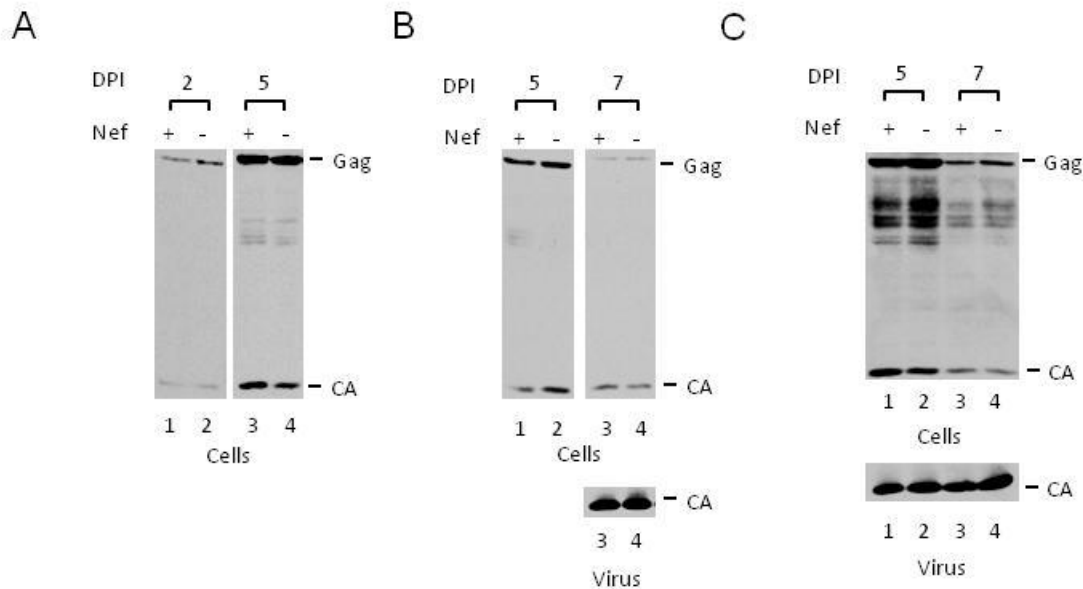


Figure 3.11 Expression of HIV-1 Gag from infected MOLT3 cells is transient and restricted to a short time after initial infection. (A) Nef does not increase expression of HIV-1 Gag in MOLT3 cells infected with VSV-G pseudotyped HIV-1 WT and HIV-1 Δnef virus. (B) Expression of HIV-1 Gag in MOLT3 cells declines or stops before the 5th day post-infection. No significant difference in the expression levels of Gag in cells infected with either WT HIV-1 or HIV-1 Δnef was observed. In addition, no difference in the release of viral particles was observed. (C) Expression of Nef had no effect on HIV-1 Gag expression levels when authentic HIV-1 Env bearing the D368R mutation was also expressed.

However, it has been reported that several Nef functions, e.g. increased infectivity, are only observed when the HIV-1 envelope is expressed (61, 178). It seemed possible that an interaction between the viral envelope protein and a component of the host cell produced a restriction that was alleviated by the expression of full-length Nef, so we chose to repeat this experiment with a different pair of proviral constructs. In order to ensure that we were observing single cycles of infectivity, we generated *nef*⁺ and *nef*⁻ HIV-1 vectors that

contained a mutation in the HIV-1 Env (D₃₆₈R) that blocks recognition of CD4 by HIV-1 Env (216). It was our belief that these constructs would accurately reflect the intracellular effect of Env expression, while simultaneously limiting HIV-1 replication to a single cycle. VSV-G pseudotyped, full-length and nef-deficient HIV-1 virus, containing the D₃₆₈R mutation in *env*, were generated in 293T cells and equal titers (100 ng p24) were used to infect MOLT3 cells. Samples of infected cells were removed and lysed for determination of Gag levels by Western blot. As can be seen in Fig. 3.11, panel C, comparison of Gag expression in the cells expressing Nef to cells infected with Nef-deficient virus display only a modest difference in the intracellular levels of Gag at the initial time point of 5 days post infection. This slight difference in Gag levels hardly accounted for the robust replication difference observed between Nef containing and Nef deficient HIV-1 in MOLT3 cells. It should also be noted that samples obtained two days later (7 days post infection) showed similar levels of intracellular Gag, regardless of Nef expression (Fig. 3.11, panel C).

This result further indicates that the presence of Nef offers no advantage at the level of viral protein production. In addition, a Western blot of released viral particles from these samples demonstrated no difference in p24 levels (Fig. 3.11, panel C) or envelope incorporation (results not shown). These results suggest that in the MOLT3 system, expression of viral proteins is limited to a short time just after infection, and after this time -approximately 2-4 days post-

infection, transcription of viral proteins is silenced. Expression of Nef from the provirus did not extend the window of viral protein expression.

Nef-induced enhancement of HIV-1 replication in the MOLT3 system does not positively correlate with an increase in viral particle infectivity.

Viruses come into contact with target cells in two different ways, either through diffusion of the virus through some extracellular medium (virus-cell transfer) or through directed contact between the producer and target cell (cell-cell transfer). Cell-to-cell transfer can occur following the formation of a so-called virological synapse, named for its resemblance to an immunological synapse, and benefits the virus by limiting diffusion of virions into the extracellular space, directing them instead to the membrane of the target cell (50). Several studies have reported that Nef plays a role in increasing cell-cell transfer of virus by clustering viral proteins and cellular receptors on the membranes of the respective cells through rearrangement of the actin cytoskeleton and lipid raft clustering (131, 276). In addition, several studies have indicated that expression of Nef in producer cells correlates with an increase in filopodia formation, as well as encouraging actin rearrangements that benefit viral delivery (107, 200, 213, 276).

In order to determine if the expression of Nef in the MOLT3 system enhanced replication by means of an increase in individual particle infectivity

(virus-to-cell) or instead enhanced transmission of virus from infected cell to target cell, we chose to compare the infectivity and replication of virus particles produced in the presence of HIV-1 Nef_{NL4-3} to that of HIV-1 produced in the presence of an active portion of the Murine Leukemia Virus (MLV) protein Glyco-Gag (236). MLV Glyco-Gag is produced occasionally during translation of the MLV Gag molecule due to transcription initiation from a cryptic start codon upstream of the normal start codon (236). In addition to enhancing infectivity of MLV particles, expression of this glycoprotein has also been shown to increase the infectivity of HIV-1 virions in a manner similar to Nef expression (236).

Infectious viruses expressing full-length HIV-1, HIV-1 Nef_{FS}, or HIV-1 in which the *nef* gene was replaced by a sequence encoding a short, active portion of Glyco-Gag were generated by transfection of HEK293T cells. Following p24 determination, this virus was then used to infect MOLT3 cells to determine the effects of Nef and Glyco-Gag expression on replication and infectivity. Virus used to determine particle infectivity was generated by infecting MOLT3 cells with either HIV-1 WT (10 ng of p24) or 100 ng of either HIV-1Nef_{FS} or HIV-1-Glyco-Gag. Due to the limited replication of both HIV-1 Nef_{FS} and HIV-1 Glyco-Gag (HG unpublished observation), increased virus titers were used to ensure production of enough output viruses to measure infectivity. Viruses produced in MOLT3 cells were then harvested, and their single cycle infectivity was determined using the TZMbl reporter cell line, which has been described previously (244). Briefly, a HeLa derived cell line, TZMbl cells stably express

CD4, CCR5 and CXCR4 receptors as well as β -galactosidase in a Tat dependent manner. As Fig. 3.12, panel A indicates, no significant difference in the infectivity of virus expressing either Nef or a portion of Glyco-Gag was observed, in line with previous reports (236). However, both of these viruses showed a dramatic increase in particle infectivity when compared to HIV-1 that lacked functional Nef. (The infectivity experiment detailed above was conducted by E. Popova)

We next sought to determine if the activity of Glyco-Gag could replace that of Nef with regard to enhancing HIV-1 replication in MOLT3 cells. Infectious HIV-1 virions expressing either: Nef, the NefFS mutant, or Glyco-Gag in place of Nef were generated in 293T cells as described above. Virus was quantified via p24 ELISA and MOLT3 cells were inoculated with HIV-1 encoding Nef (2 ng or 10 ng), HIV-1 Nef FS or HIV-1 Glyco-Gag (10 ng or 100 ng). At multiple timepoints following infection, supernatant samples were removed for determination of released virus as a measure of virus replication over time. As Fig. 3.12, panel B demonstrates, infection of MOLT3 cells with 10 ng of Nef expressing HIV-1 WT resulted in a 40 –fold increase in the levels of released virus compared to Nef-deficient virus 14 days after inoculation. Interestingly, infection of MOLT3 cells with virus expressing an active portion of Glyco-Gag failed to enhance replication of HIV-1 to any significant level when compared to Nef-deficient virus (Fig. 3.12, panel B). Infection with 100 ng of input HIV-1 Glyco-Gag demonstrated only a 3-fold increase over HIV-1 Δ *nef* (FS) (both 10 and 100 ng input, Fig. 3.12, panel B). In fact, a 50 fold difference in input virus between HIV-1 WT (2 ng) and HIV-1

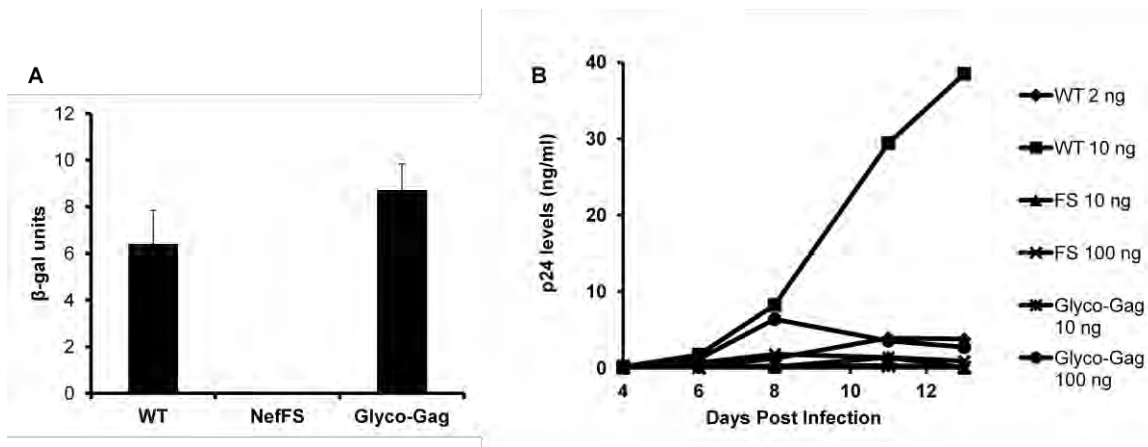


Figure 3.12. Nef-induced Enhancement of HIV-1 Replication in MOLT3 cells is Separate from Increases in Infectivity. (A) Infectivity of HIV-1 particles produced in MOLT3 cells in the presence of HIV-1 Nef_{NL4-3} (WT) or an active portion of the MLV Glyco-Gag protein are equally infectious ($p=0.09$) and more infectious than HIV-1 particles produced in MOLT3 cells lacking expression of either protein (NefFS). (B) Replication of HIV-1 in MOLT3 cells is rescued by expression of HIV-1_{NL4-3} Nef, but not by expression of an active portion of MLV Glyco-Gag. Infectivity of virions produced in MOLT3 cells expressing the indicated protein was determined by β -Gal production in TZM-bl reporter cells inoculated with 200 ng of virus. MOLT3 cells were infected with the indicated titer of HIV-1 virions produced in 293T cells.

Glyco-Gag (100 ng) resulted in higher levels of p24 in the HIV-1 WT sample than with the HIV-1 Glyco-Gag sample (Fig. 3.12, panel B). Together with the results of the infectivity assay, these results demonstrate that the effect that Nef has on enhancing the replication of HIV-1 in the MOLT3 cell system is not due to enhancement of individual particle infectivity.

Discussion:

The lentiviral Nef protein is a critical determinant in the progression of HIV-1 infected individuals to fully immune-compromised status (144), playing a number of roles in both transmission and replication (80, 161, 255). Due to the functional complexity of this viral protein, studies focused on understanding its contributions to particle infectivity and replication often conflict based on the nature of the system being used, i.e. cultured cell lines vs. PBMCs (61, 177, 208). In general, Nef is largely dispensable for replication of HIV-1 in cell lines, and replication in PBMC is only delayed if these cells are infected prior to stimulation. In contrast, we now report a cell culture system in which Nef is essentially required for HIV-1 replication.

It has been previously reported that the Nef-induced downregulation of cell surface CD4 plays a major role in determining particle infectivity in both virus-cell and cell-cell transmission, potentially by interacting with viral envelope at the cell surface, prior to particle release (61, 160, 177). This finding has been supported by the observation that Nef mutants unable to interact with CD4, or to direct it to the endosomal pathway for degradation, often display reduced infectiousness in reporter cell lines and modestly reduce replication (177). Our results suggest that downregulation of CD4 by Nef does not account for the reduced replication of HIV-1 observed in the absence of Nef. HIV-1 viral genomes containing

mutations in *nef* known to be required for CD4 downregulation remained replication competent and produced significantly higher amounts of virus compared to a Nef deficient mutant. In addition, expression of Nef in producer cells transfected to stably express high levels of CD4 resistant to Nef-induced downregulation was still required for HIV-1 replication, regardless of whether Nef was expressed from the provirus, or provided *in trans*. This rescue effect was able to be replicated by Nef proteins from a broad range of HIV-1 clades indicating that the replication enhancement is a conserved function of Nef. Interestingly, the activities of the Nefs used in this *in trans* assay seem to reflect with the world-wide spread of HIV-1 (45), with Group M Nefs displaying the greatest rescue effect and Group O being fairly modest in activity. The results obtained from expressing Nef proteins from SIV are far less conclusive and hampered by the downregulation of the co-receptor for the NL43 envelope, CXCR4. In order to determine if SIV Nefs are active in this assay additional CXCR4 will need to be expressed in these cells; this experiment has already been planned and will be undertaken soon. Alternatively, replication of a CCR5 tropic virus could be tested in this system provided that CCR5 levels are adequate.

Nef is a functionally complex molecule, possessing several reported effects on the host cell during particle formation and infection (80, 161, 255). We attempted to determine the relative contributions of several of these reported effects by analyzing the replication of viruses containing point mutations in Nef

that disrupted previously reported protein-protein interaction motifs. The results of our comprehensive, although not exhaustive, mutational analysis indicate that many of the reported interactions between Nef and host cell proteins do not play a significant role in Nef-induced replication enhancement. Nef mutants lacking residues known to be crucial for downregulation of MHC-I (14, 128, 214), interaction with PAK2 (215) or interaction with hTE (59) displayed no adverse effects on replication. Mutations of some residues found within the hydrophobic pocket/dimerization domain of Nef, which have been implicated in almost all Nef-related activities (8, 175), essentially abrogated the effect of expressing Nef and led to levels of replication similar to those obtained with an HIV-1 provirus lacking an intact *nef* gene. This result may indicate that an interaction between Nef and a host cell protein present in MOLT3 cells is required for the observed dependence of HIV-1 replication on Nef in this cell line, although we cannot definitively rule out a defect in Nef dimerization. While it has been proposed that residues which constitute this hydrophobic pocket also contain a significant portion of the Nef dimerization domain (8, 240), we do not attribute our observations to a dimerization defect for several reasons. First, additional mutants used in this assay are also part of the reported dimerization domain (RR₁₀₅ AA, Y₁₁₅A), and their activity in supporting HIV-1 replication was significantly higher than that observed for the F/D mutants (Fig. 3.8, panels B and C)(8, 240). Second, almost none of the Nef molecules expressed in cells have been shown to be present in dimers provided they retain their myristoyl anchor

(29). This suggests that if dimerization of Nef were required for activity, the greatest portion of Nef present in the infected cell is inactive (240). Lastly, Nef molecules from SIV and HIV have been shown to form heterodimers in solution despite lacking a structurally similar dimerization interface (157). In light of this information, we hypothesize that the F/D mutation results in a Nef protein incapable of binding with a MOLT3 protein.

In addition to the mutations disrupting the hydrophobic binding pocket, two additional point mutations in Nef also severely impacted replication in the MOLT3 cell line. The amino terminus of Nef is co-translationally modified by the addition of a myristic acid moiety which drives association of Nef with the plasma membrane (93). Mutation of the amino terminal Gly residue to Ala removes the site of myristoyl attachment (99), and the resulting Nef molecule is unable to interact with the membrane. As most of the interactions between Nef and the host cell are localized to membranes, removal of the membrane anchor of Nef is associated with phenotypes that mirror a complete lack of Nef (92, 93). We report similar effects with regard to viral replication in the MOLT3 system. Interestingly, Lundquist and colleagues reported near wildtype levels of replication in PBMCs that had been infected with HIV-1 with a similar mutation in Nef (177). This discrepancy could be explained by the fact that Lundquist used activated PBMCs, which have been shown to be less dependent on Nef for replication (177, 215). Whether the difference between the two studies reflects a difference in experimental methodologies or indicates a specific role for

membrane associated Nef in the MOLT3 system, will need to be determined. However, the near-absolute dependence of HIV-1 on Nef for replication in MOLT3 cells indicates that the MOLT3 system is significantly more robust, and likely to reflect differences in Nef activity accordingly.

A similar result was observed regarding the PP_{69,72}AA mutant. Again, Lundquist and colleagues reported no significant difference in the replication of an HIV-1 construct containing this mutation compared to HIV-1 Nef^{WT} in activated PBMCs (177). However, in our hands, infection of MOLT3 cells with provirus containing this mutation resulted in reduced replication efficiency when compared to HIV-1 encoding WT Nef. Our results show replication at levels more similar to virus lacking a functional *nef* allele (Fig. 3.8, panel A). The polyproline motif of Nef has been shown to be an essential factor for lowering the threshold of T-cell activation by interacting with Src-family kinases such as Fyn and Lck, as well as with p21-associated protein kinase (PAK2), and a separate role for downregulation of CD4 has been indicated (105, 155, 215). It is surprising that any mutation that affects the T-cell activation threshold is relevant in our replication studies, as MOLT3 cells infected with Nef mutants were not intentionally stimulated during the course of investigation. Based on previously published reports, it seems more likely that the results we obtained during testing of this mutant reflect an inability of Nef to affect actin rearrangement in non-activated cells (105-107, 213). A recent study by Haller et al. indicated a defect for actin rearrangement in Jurkat T-cells expressing Nefs in which either the

myristoylation site (G₂A) or the PxxP motif (PP_{69,72}AA) had been mutated (106). Using fluorescent microscopy they observed a ring of actin in TCR stimulated Jurkat T-cells that was disrupted upon expression of Nef and demonstrated that this defect was dependent on the actin polymerizing protein, N-WASP (105). The demonstrated defect of these two Nef mutants in promoting actin rearrangement may negatively impact the clustering of Gag and Env proteins at the virological synapse, a process previously shown to be actin-dependent (131). Disruption of VS formation would certainly explain the reduction in replication in the MOLT3 system. Whether the suspected decrease in clustering of Gag and Env disrupts formation of the virological synapse or the assembly of viral particles at this site will need to be further investigated.

Previous studies have reported that the presence of Nef provides the infected HIV-1 genome with a replication advantage in non-activated cells (215, 277). This advantage is directly related to the lowering of the activation threshold and results in increased expression of NFκB and other HIV-1 transcription factors, which in turn favor production of virus in these cells. However, the advantage provided by Nef is ameliorated if the T-cells are activated prior to infection as both Nef-expressing and Nef-deficient virus have access to sufficient transcription factors (215). We have found that HIV-1 containing a functional *nef* retains a profound replication advantage over Nef-deficient virus following infection of MOLT3 cells stimulated by the addition of TNFα (Fig. 3.10). This advantage is evident by the difference in replication kinetics observed

between stimulated MOLT3 cells infected with authentic HIV-1, HIV-1NefFS, or HIV-1 expressing Glyco-Gag in place of Nef (data not shown). This replication advantage is separate from any difference in particle infectivity, as we have demonstrated that Glyco-Gag increases HIV-1 particle infectivity at levels comparable to those observed with bona fide Nef, and yet offers almost no advantage to replication in MOLT3 cells.

In addition, we report that expression of HIV-1 in MOLT3 appears to be transient, and limited to a narrow timeframe of approximately 2-4 days immediately after infection; expression of Nef had no apparent effect on extending the period of viral expression. Comparison of intracellular Gag levels from cells infected with either Nef-expressing or Nef-deficient virus displayed similar levels of the Gag polyprotein when analyzed by Western blot (Fig. 3.11). Analysis of lysates from a later timepoint showed no increase in Gag expression. Rather, a clear decrease in signal was observed compared to samples taken at an earlier timepoint, suggesting that transcription of Gag had ceased. Taken together, we conclude that expression of HIV-1 in MOLT3 cells occurs for a limited time following infection, after which it is transcriptionally silenced by the host cell. Although transcription of viral proteins from non-integrated HIV-1 genomes has been reported (277), and integration was not confirmed for these experiments, we don't believe that an integration defect explains our results. While it has also been reported that expression of Nef increases the success of viral integration into host genomes (277), we failed to observe any differences in

the level of transcription, as measured by Gag expression, between WT and Nef-deficient strains

The results of our various replication studies are generally consistent with previously published results regarding an increase in replication efficiency by HIV-1 in the presence of Nef (177). However, in the MOLT3 system the requirement for Nef is far more pronounced. The difference in replication efficiency observed between authentic HIV-1 and HIV-1 expressing Glyco-Gag in place of Nef, despite the similar infectivity of individual particles, suggests Nef alone is able to enhance the spread of HIV-1 via cell-cell transmission.

Expression of Nef has previously been shown to increase cell-cell transmission by stimulating actin arrangement, clustering of lipid raft microdomains, as well as increasing the formation of filopodia and nanotubes (131, 200, 213). The results of our mutational analysis of Nef are compatible with a role for actin remodeling, raft clustering and formation of cellular processes in the MOLT3 system. As any of the Nef-associated activities listed above could individually explain our findings, additional investigation to determine the specific mechanism will need to be undertaken.

In light of the limited time frame of viral expression observed in this cell line, an increase in cell-cell transmission efficiency also provides a suitable explanation for the results we have observed. In our replication assays, MOLT3 cells infected with Nef-deficient virus often display their highest level of virus

release within 2-4 days after the initial infection, followed by a decrease in viral particle release over the duration of the respective experiments. This could indicate that Nef-deficient virus is less efficient at spreading from cell-cell than is WT, and that once this initial transmission opportunity is missed, not enough viral proteins are being produced for continued replication. As cell-to-cell transfer of HIV-1 is considered the primary mode of HIV-1 viral dissemination, we submit that the MOLT3 cell line provides an excellent system to study this phenomenon.

Future Directions

While the data we have obtained to date investigating the role of Nef in the replication of HIV-1 in the MOLT3 system are enough to suggest a possible mechanism, additional experiments will need to be undertaken to verify these theories. The expression of Nef from Group N and SIV viruses induced a reduction in the cell surface levels of the CXCR4 viral coreceptor, confounding our interpretation of their effects on replication. In order to get a clearer understanding of whether these Nefs are capable of supporting replication of Nef-deficient HIV-1 we intend to generate cell lines that stably express the specific Nef proteins as well stably express CXCR4, and then determine their activity in our Nef *in trans* system. It is our hope that increased levels of this receptor will enable us to obtain clearer results regarding the activity of these viral proteins.

In addition, we would like to better characterize the Nef K₉₂A mutation, which showed a significant defect in replication enhancement when compared to the Nef WT protein, despite showing WT levels of activity with regard to CD4 and MHC-I downregulation. At this time, no other publications have reported a function for this residue in any Nef-induced effect, e.g. enhanced replication, infectivity increase, or interaction with any cell protein. Interestingly, mutation of the neighboring residue in Nef (E₉₃) had shown a robust enhancement of replication in our system and exceeded even that of WT Nef (data not shown),

suggesting that the effect of the K₉₂A mutation is specific. Using flow cytometry and antibodies directed against CD4 and MHC-I molecules we have shown that this mutant is active in both regards (data not shown), again further indicating a defect in a specific function rather than a defect in activity due to improper folding or expression. We intend to investigate the activity of this Nef mutant in actin rearrangement using an assay similar to that published by Haller (106). Jurkat T-cells will be transfected with a GFP vector, as well as vectors expressing specific Nef proteins, and their adherence to fibronectin will be used as an indication of actin cytoskeletal stability. Further analysis of actin structure can be undertaken using the actin specific dye phalloidin. In addition to determining the activity of the K₉₂A mutation, we also plan on investigating the activities of both the membrane-binding defective G₂A mutant and the PP_{69,72}AA mutant in this assay.

In order to test whether the increase in replication efficiency in the MOLT3 system is dependent on an increase in cell to cell transfer two separate experimental methods can be undertaken. The first would separate the infected producer cells from the target cells by the insertion of a transwell insert that will allow for the free diffusion of viral particles while preventing contact between the two cell populations. Target cells can then be assayed for presence of viral genomes or viral protein expression (p24). The second method requires the gentle agitation of the infected cells to prevent the formation of cell to cell contacts, although what effect this method has on virus to cell transmission is questionable.

Lastly, the massive defect in replication enhancement observed in Nef mutants disrupting the hydrophobic pocket/dimerization domain suggest that interaction with a cellular protein may play a significant role in this process. In an effort to identify potential cell proteins involved in this process we have opted to immunoprecipitate HA-tagged Nef proteins from MOLT3 cells which stably express either WT Nef or Nef mutants that have been shown to abrogate the replication enhancement. Towards this end we have created cell lines expressing codon-optimized, HA-tagged versions of the Group M, Clade C Nef of 96ZM651, which displayed a robust effect in our Nef *in trans* assay system. The cell lines generated thus far include the WT version of the Nef 96ZM651, as well as a D₁₂₃A and K₉₂A version of this construct as potential negative controls, as well as cell lines containing the Groups N and O Nefs and the ARF6 protein as a non-Nef control. Cell proteins that interact with the respective Nefs will be determined by MudPit analysis of the complete precipitate.

Materials and Methods

Virus production and infections. Replication competent virus was generated by transfection of HEK293T cell with 2 µg of WT HIV-1 proviral vector using a calcium phosphate technique. Infectious particles of Nef-deficient HIV-1 (NefFS), HIV-1 encoding a portion of the MLV Glyco-Gag in place of Nef, or HIV-1 containing the indicated Nef mutant were generated similarly. Cells were washed six hours after transfection and fed with fresh DMEM+10% FBS. Twenty-four hours after transfection, supernatant was removed, cells were pelleted with low speed centrifugation, and supernatant was filtered through 2µm filter. Titer of released virus was determined using a p24 sandwich plate ELISA (prepared on site by CFAR). Equivalent levels of virus, as determined by ELISA were used to inoculate target cells. Twenty-four hours post-infection, infected cells were washed with RPMI +10% FBS and resuspended in fresh media. Samples of supernatant were removed from infections periodically, spun to remove suspended cells, and frozen for future quantification of virus release using p24 ELISA.

Generation of stable cell lines. The pCX4bsr-CD4ΔCT retroviral vector was generated by PCR cloning of CD4 from a previously described vector and inserting the sequence into the BamHI and NotI sites of pCX4bsr (Acc. No. AB086384). Infectious VSV-G pseudotyped virus was made in 293T cells with calcium phosphate technique using 2 µg of pCX4pur-CD4ΔCT as well as 2 µg

MLV Gag-pol, and 0.2 µg of VSV-G envelope. Twenty-four hrs after transfection, the supernatant was removed and cleared by low speed centrifugation (1,200 rpm, 5 mins, 4°C) followed by passage through a 0.2 µm syringe filter. Five-hundred microliters of cleared supernatant was used to infect 5.0×10^5 MOLT3 cells. Twenty-four hrs after infection, cells were washed to remove unbound virus and resuspended in RPMI + 10% FBS containing blasticidine (0.5 µg/ml) for selection of cells containing a successfully integrated gene.

Vectors used to stably express HA-tagged Nef proteins were cloned from their respective vectors by PCR using primers contain restriction sites for BamHI and EcoRI and inserted in the pCX4pur (Acc. No. AB08386) at the BamHI and EcoRI sites. The Nefs used in these experiments were: LAI and SF2 (subtype B, Acc. No. K02013 and K02007, respectively), 96ZM651 and 97ZA012 (subtype C, Acc. No. AF286224 and AF286227, resp.), 93BRO020 (subtype F, Acc. No. 005494) and 90CF056 (subtype H, Acc. No. 005496). Plasmids containing codon optimized, HA-tagged versions of Group N (YBF30, Acc. No. AJ006022) and Group O (MVP8161 Acc. No. AY536905) were ordered from Genewiz. These genes were used as templates for PCR and insertion into the same pCX4pur vector.

Generation of HIV-1 proviral constructs: Proviral vectors expressing full-length HIV-1 (NL4-3), HIV-1 NefFS (containing a FS mutation in the Nef gene resulting in a premature stop codon at residue 35 of Nef), and HIV-1ΔNef-Glyco-

Gag (containing the gene for an active portion of Glycosylated Gag from the MLV in place of the endogenous Nef gene) have been described previously (236). Full length proviral constructs containing Nef point mutations were generated from a series of Nef_{LAI} expression vectors previously made as follows. The portion of full length NL4-3 genome contained between the XhoI and NcoI restriction sites were inserted in the pTZ18U polylinker plasmid. The region of the NL4-3 Nef gene contained between the KpnI and BspEI was replaced by the corresponding region from the expression vector encoding the point mutation in HIV-1LAI Nef. Following sequence verification, the XhoI-NcoI fragment containing the NL4-3/LAI hybrid Nef was inserted back into full-length NL4-3. This strategy was used to generate full-length NL4-3-based vectors for all Nef point mutations.

Derivation of NL4-3 envelope mutants:

Vectors expressing the NL4-3 proviral clone of HIV-1 containing the D368R mutation in Env were generated using the Stratagene QuickChange XL Site-Directed Mutagenesis kit (Cat# 200516, Stratagene, La Jolla, CA) and the NL4-3 Env protein as a template. Following sequence verification, the region from NheI to BamHI of Env was transferred into the corresponding restriction sites of the full length plasmids encoding wild type NL4-3 and NL4-3 containing the Nef frame-shift previously described.

Flow Cytometry:

Cell surface expression of CD4 and CXCR4 was determined by staining target cells with anti-CD4 (BD Biosciences) or anti-CXCR4 (BD Biosciences). Isotype controls were used to determine cross-reactivity (IgG1a for CD4-BD Bio and IgG2k for CXCR4 – BD Bio). Cells were stained in 0.5% BSA in PBS at 4°C for 1 hr, washed and then stained with the fluorophore-conjugated secondary (PE-mouse and human Jackson) for one hour. Data was collected by UMASS Medical School FACS core, and analyzed using the FlowJo software suite (version 7.6.5).

Infectivity assay:

Infectious virions were generated by expressing proviral constructs encoding either NL4-3 WT, NL4-3 Nef FS, or NL4-3 Glyco-Gag in HEK293T cells using a calcium phosphate transfection technique. Viral titers were quantified by determination of soluble p24 in supernatants using sandwich ELISA to detect that protein. MOLT3 cells were inoculated with either 10 ng of supernatant from NL4-3 WT or 100 ng of supernatant from NL4-3 Nef FS or NL4-3 Glyco-Gag producing cells. Virus released from the MOLT3 cells was collected and p24 levels determined by ELISA. This virus was introduced to 5.0×10^4 TZM-bl cells, a HeLa derived reporter cell line that expresses CD4, CCR5 and CXCR4 and will express β -galactosidase upon infection with HIV-1 in a Tat-dependent manner.

Following infection, the cells were washed to remove unbound virus and treated with the protease inhibitor Saquinavir (1 $\mu\text{M}/\text{ml}$) to limit infection to a single round. Cells were lysed and the β -gal expression was quantified using an ONPG reporter system (Promega E2000). All sample conditions were done in triplicate and means \pm SEM are reported.

Chapter IV

General Discussion

In this thesis I have presented data from my research into the mechanism of NEDD4 stimulated release of HIV-1 particles. I have shown that NEDD4 activity is dependent on the presence of the partial C2 domain of the ubiquitin ligase which serves as a Gag targeting module sufficient to drive interaction of divergent HECT domains with viral particles. I have also demonstrated that the ability of NEDD4 family members to rescue release of HIV-1 particles requires the ability of these HECT domains to form K63-polyubiquitin chains. In addition, I have presented data from on-going research investigating the activities of Nef on HIV-1 replication in the MOLT3 system. To date, my findings demonstrate that Nef-induced downregulation of surface CD4 levels does not account for the enhancement of HIV-1 replication observed in the presence of Nef. I have also shown that a range of lentiviral Nef proteins retain the ability to enhance HIV-1 replication in this cell line, indicating the observed enhancement is a function conserved in Group M and N Nef proteins. In addition, I report the observation that equally infectious HIV-1 particles display different replication efficiencies in MOLT3 cells, demonstrating that these two functions of Nef are separable. This finding, coupled with the apparently narrow window of viral gene expression, suggests that the observed enhancement of HIV-1 replication induced by Nef

expression is due to an increase in cell-to cell-spread in culture early after the initial infection.

In the introduction I presented a brief overview of important events in the replication cycle of HIV-1 in an effort to impress upon the reader the manner in which this relatively small virus is able to manipulate its host cell to replicate. In the microcosm that is the host cell the virus will come into contact with all manner of proteins and pathways that may positively or negatively impact the ability of the virus to replicate. A successful virus must adapt to ensure that the majority of these interactions have a net positive effect on its own replication cycle. HIV-1 has gone to extraordinary lengths to achieve this, as evidenced by the reported activities of NEDD4 and Nef presented here.

Although addressed separately in this thesis, the roles of Nef and ESCRT in the formation and release of virus particles may overlap significantly to cooperatively enhance the assembly and infectivity of HIV-1 virions. In increasing the lipid raft content of the plasma membrane, either through increasing the number of lipid rafts or their size (37, 237, 323), Nef expression could have the added effect of increasing the efficiency of ESCRT-mediated particle release. The mechanism of ESCRT mediated membrane scission is still unknown (118), and although recruitment of enzymatically active VPS4 is required (116), the current literature suggests that its function is primarily to disassemble ESCRT-III complexes into component subunits (164). Release of

enveloped viral particles that are not reliant on ESCRT recruitment may provide some insight into ESCRT function. In contrast to HIV-1, release of Influenza virus is not dependent on the ESCRT machinery (35). A recent report by Rossman et al describing the budding of Influenza virus suggested that the M2 protein of Influenza was able to induce particle release by altering the line tension of the membrane from which it budded (256). In light of the findings of this group the question has risen as to whether membrane line tension plays a role in ESCRT-mediated membrane scission (118). In order to address this topic, an understanding of the chemistry and biophysics of lipid rafts must first be considered.

Lipid rafts are nano-scale membrane domains (10-200 nm) found dispersed throughout the plasma membrane (232). The existence of lipid rafts, or detergent-resistant microdomains (DRM), has remained somewhat controversial due to intensive purification strategies required to isolate them (303). However, more recent microscopic studies have provided evidence of their existence in living cells, and have also helped in further indentifying their components (232, 303).

Containing a higher concentration of cholesterol and sphingomyelin relative to the surrounding plasma membrane (232), lipid rafts are enriched in transmembrane proteins such as GM1, and tetraspanins (232, 303). Additionally, proteins such as actin and cofilin have also been shown to be

enriched on the cytosolic leaflet of these domains, indicating a specific connection with the cell cytoskeleton (232). Some researchers have questioned whether interaction with the cell cytoskeleton prevents aberrant formation of large clusters, and thus protects PM integrity (316). For example, in giant unilamellar vesicles (GUVs) containing phospholipid concentrations similar to those observed with authentic lipid rafts, clustering of raft-phospholipids can lead to spontaneous budding and fission of the membrane (15).

This spontaneous fission process is thought to be driven by the energetically favorable release of line tension that exists between the lipid raft and the surrounding membrane (15). As the existence of lipid rafts has become increasingly accepted, and the apparent importance of these domains in membrane mediated processes in the cell becomes clearer, biophysical theories have been generated to explain experimental observations. One such theory addresses the physical ordering of the lipids present in both rafts and the surrounding plasma membrane (174). The increased levels of cholesterol and sphingomyelin present in lipid rafts impart a more structured orientation of the lipid tails prompting these microdomains to be referred to as liquid ordered (Lo), while the unsaturated fatty acid chains of the surrounding membrane are termed liquid disordered (Ld) (232).

This difference in order also presents a difference in membrane thickness at these sites, with Lo regions being thicker than Ld regions due to the ability of

the tails of unsaturated fatty acids in the Ld phase to interact across the bilayer (232). Line tension between the two disparate domains is created by the interaction of the hydrophilic head groups of the Ld phase with the hydrophobic tails of the lipid rafts (Lo), with the resulting line tension being proportional to the circumference of the lipid raft. If the raft approaches an appreciable size, in the micrometer scale, it is more energetically favorable for the raft to deform and bud, reducing the line tension by decreasing the circumference of the raft(15, 300). It has been suggested that interaction of lipid rafts with the cytoskeleton helps protect against this spontaneous action by preventing clustering of rafts.

Interestingly, several biological functions are thought to make use of this difference in line tension in generating membrane bound vesicles in the cell. For example, it has been suggested that formation of transport vesicles in the trans-Golgi network is the result of protein mediated generation of line tension, and a similar process has been proposed to explain the activity of dynamin in endocytosis (258). A recent paper has suggested that dynamin serves to both constrict the membrane to form a bud neck while simultaneously inducing lipid domain segregation, resulting in the enrichment of Lo domains in the forming vesicle, and Ld regions in the bud neck (174). This induction of line tension between the vesicle and bud neck may cause scission of the membrane at the interphase between ordered and disordered regions, resulting in vesicle release (174). One wonders if a similar process can account for the ESCRT mediated scission of membranes observed in the release of HIV-1. In contrast to dynamin,

which supports the bud neck from the outside of the membrane (174), ESCRT-III proteins support the bud neck from the inside (77). Otherwise, the actions of these proteins appear quite similar, which is to support the Ld membrane during the process of membrane budding.

In addition to mediating membrane scission it has also been suggested that recruitment of ESCRT proteins may also play a significant role in deforming the plasma membrane, particularly at sites of negative membrane curvature (313). While an effect on membrane deformation has been shown in GUVs treated with purified ESCRT proteins (313), one wonders what role they play in the lipid raft rich membrane associated with HIV-1 nef expression. Research into the composition of the viral envelope indicates that it is enriched for lipid raft components (36, 324), suggesting also that this membrane composition is present during the process of particle assembly (303). This has also been demonstrated in studies reporting that Gag associates with and clusters lipid rafts during virus formation (210, 211, 217). Interestingly, it has recently been published that ESCRT-II also clusters lipid rafts prior to formation of MVB through recognition of ubiquitinated cargo (28). Thus, although HIV-1 does not utilize ESCRT-II proteins for release, a similar action is still required, in this case one accomplished by the viral protein, further illustrating the conserved relationship between lipid rafts and ESCRT-mediated activity.

Clustering of rafts large enough to support the formation of a single virus particle could generate significant line tension between the Lo and Ld regions of the membrane, encouraging membrane deformation and formation of the viral bud . This curvature is no doubt further enhanced by the presence of the Gag lattice itself, which also promotes positive membrane curvature of the membrane bud (31). Perhaps more important than this positive membrane curvature, is the zone of negative curvature referred to as the bud neck (313). Years of study into the role of ESCRT proteins in the formation and release of MVBs has resulted in the theory that the bud neck is formed through stepwise recruitment of ESCRT-0, ESCRT-I and ESCRT-II complexes, which localize to the bud neck and may act to impart the negative membrane curvature observed (313). However, this may not be the case. Again, recent studies into the activities of other vesicle-forming events in the cell provide an insight into potential alternative theories.

Mechanically formed vesicles generated by pulling a microbead bound to a cellular membrane results in the separation of the heterogeneous membrane into Lo and Ld regions, with the portion attached to the bead (the vesicle) composed predominantly of Lo lipids and the connecting membrane tube (bud neck) composed of Ld lipids (258). Interestingly, formation of a vesicle in the absence of segregation of Lo and Ld domains not only fails to release but will eventually resolve back into heterogeneous membrane (121, 152), suggesting that more than just mechanical manipulation of membranes is necessary for membrane scission and vesicle release. While similar studies have not been

reported for ESCRT-mediated vesicle formation, it is possible that ESCRT-III proteins perform a similar function and stabilize Gag-induced lipid segregation at the bud neck, rather than directly imposing the observed negative membrane curvature.

An additional consideration is the role that ESCRT protein recruitment may play not only in stimulating the release of virus particles, but also in determining the concentration of Gag present in the budding virus. Moloney Murine Leukemia Virus (MoMLV) lacking all three of its late domains was recently shown to be capable of low levels of release and replication in an ESCRT-independent manner (260). Indeed, despite a reliance on ESCRT proteins for release from most cell lines, infectious HIV-1 particles lacking a functional Late-domain are able to release from certain T-cell lines (i.e. Jurkat) although at lower levels than WT HIV-1 (118). Interestingly, the reliance on ESCRT mediated release by HIV-1 also seems to depend on the presence of cholesterol in the host cell membrane (118), as L-domain deficient HIV-1 was able to assemble and release from T-cells deprived of cholesterol by M β CD or statin treatment, but not from untreated cells (218). Thus, a decrease in lipid raft content in the plasma membrane also decreases retroviral reliance on the ESCRT machinery for release. These findings may suggest that the ability of retroviruses to recruit ESCRT proteins provides more than just a way for the virus to exit infected cells, and instead may indicate a functional role for ESCRT recruitment in particle assembly.

A series of cryoelectron tomography studies is illustrative in this regard. These studies, which were mentioned before in Chapter I, used cET to image immature HIV-1 viruses as they budded from the host cell membrane (42, 67, 68). One study in particular imaged budding of HIV-1 particles in MT-4 cells, from HIV-1 genomes expressed at physiological levels (42). The authors found that the overall structure of the mature HIV-1Gag lattice was formed during assembly and maintained during budding, indicating authentic viral particle formation (42). Interestingly, they also observed a subset of budding viruses that appeared to lack the density corresponding to the condensed NC-RNA core, which also contained a higher concentration of Gag compared to productively budding virus (42). The authors reasoned that this structure arose from prematurely activated viral protease, which is often observed in L-domain phenotypes (42). As the L-domain containing region of HIV-1 is present in the p6 fragment of Gag, downstream of NC, premature cleavage at the NC site removes the ability of Gag to recruit ESCRT proteins (68). Thus, the increase in nascent viral Gag content in this subset of budding virus may indicate that recruitment of the ESCRT proteins are important not just for release of the viral particle, but also for determining the number of HIV-1 Gag molecules present in the virus, possibly by formation of the structure referred to as the bud neck which is an area of negative membrane curvature localized the region between the positively curving virus bud and the surrounding plasma membrane.

The previous findings that Late-domain deficient HIV-1 and MoMLV virus can bud and release in the absence of ESCRT recruitment (260), albeit at significantly reduced levels, further suggest that ESCRT recruitment is not specifically required for virus release per se. Analysis of data from the Moloney Murine Leukemia Virus study may provide insight into understanding a potential second role for ESCRT recruitment. In this study, MoMLV was shown to assemble and release low levels of replication competent virus in the absence of all functional L-domains, and therefore ESCRT complex recruitment (260). Investigating the contents of the released viruses, the authors reported a significant increase in the levels of viral capsid present in the L-domain deficient viruses compared to WT virus capable of recruiting ESCRT proteins (260). These findings may indicate that more Gag molecules were incorporated into the particles released from the cells infected with MoMLV lacking a functional L-domain compared to WT MoMLV (260), and suggest that recruitment of ESCRT machinery may play a role in particle assembly by limiting the amount of Gag able to associate with the nascent virus. This theory is supported by the results of a live-imaging study of infected cells by Jouvenet et al. (138). Using GFP-Gag fusion proteins, the authors observed consistent recruitment of Gag to sites of particle assembly, followed by a short time during which no new Gag was recruited prior to release (138). Again, this may imply that formation of the bud neck may limit Gag recruitment to the nascent virus by sealing it off from the host cell cytoplasm. Assuming that formation of the bud neck limits HIV-1 virus

recruitment of Gag to some optimal level for the formation of infectious virus, one wonders whether formation of this bud neck is benefited by an increase in the lipid raft content of the membrane. Again, formation of large lipid rafts is believed to induce significant membrane curvature away from the cytoplasm in an effort to reduce line tension (31). Reduction in line tension, through formation of the budding membrane, would result in a smaller area of contact between the Ld and Lo domains (15), and the formation of bud neck-like structure. This structure, although likely larger than an authentic bud neck, would still have less surface area than the inner membrane of the budding virus and should in principle slow recruitment of Gag to the virus. Thus, it is conceivable that expression of Nef, which stimulates a commensurate increase in lipid raft content in the host cell membrane (323), may positively affect HIV-1 particle assembly by inducing the formation of a bud neck-like structure which limits Gag recruitment to an optimal level. Subsequent recruitment of ESCRT proteins may further narrow the bud neck and definitively stop Gag recruitment to virus assembly sites (138). Recruitment of ESCRT proteins to the bud neck may then stabilize the segregation of Lo and Ld regions stimulating membrane scission (77).

This idea is not specifically novel, as several labs have investigated the relative levels of Gag and Gag-subunit proteins in viruses produced in the presence or absence of Nef biochemically (37, 79, 250, 266). However, if there is a difference in the levels of Gag and Gag-pol proteins present in a optimally infectious virus particle compared to other particles, the difference is likely to be

slight. A number of reports have indicated that little or no difference exists in the protein content of particles generated under these conditions (37, 79, 250, 266). In addition, these studies have reported no difference in the association of Gag with lipid rafts regardless of Nef expression (37, 250, 266). However, these studies are limited by several fundamental flaws, not the least of which is a major logical tautology.

In an effort to determine the relative levels of Gag- and Gag-pol-related proteins present in particles generated in the presence or absence of Nef, several of these studies have equalized the amount of input virus based on the either the reverse transcriptase activity or concentration of p24 in the respective samples (4, 37, 192, 266). Not surprisingly, after equalizing the amount of p24 containing particles, no difference in the relative levels of viral proteins was observed when samples were separated via SDS-PAGE, in either released particles or in DRM (4, 37, 266). While these findings are important in demonstrating that there are no significant disparities in the composition of virions-meaning disproportionately more reverse transcriptase to nucleocapsid, for example- the method fails to take into account the overall amount of Gag and Gag-pol present in each particle. The recent imaging studies of budding particles are particularly illustrative in this regard, and suggest that the partial lattice of Gag observed in these structures may denote a specific concentration of Gag required for membrane deformation and thus affect the formation of an infectious particle (42, 68). Some undetermined minimal amount of Gag must be

necessary to induce membrane curvature, and likewise, to form the bud neck. Thus, viral particles containing too much or too little of Gag and Gag-pol in total may still show the same ratio of viral elements, but not the appropriate concentration for assembly of a virus with optimal infectivity. In this regard it may be beneficial to consider what role lipid rafts play in determining the overall concentration of Gag in the budding virus. However, due to the relationship between membrane lipid raft content and Gag assembly a different method of sample standardization will be required.

The current literature supports a model in which two copies of the retroviral genome are contained within the HIV-1 core (7). Assuming this to be accurate, then a strict quantification of HIV-1 genomes in a given preparation of virus should be sufficient to standardize samples prior to comparison of viral protein content. A previous study, investigating the relationship between Nef and virus content compared both the Gag levels and genome numbers in virus generated in the presence and absence of Nef, reported no difference in either component (266). Again, this group first standardized their samples by determining p24 levels in the virus samples prior to separation via SDS-PAGE (266). Interestingly, when analyzing the RNA content of these samples by hybridization with a probe against the HIV-1 genome, this group also reported no difference despite observing a significant disparity in probe signal at lower concentrations, with a greater signal coming from the Nef+ virus; no suitable explanation for this interpretation was presented in that manuscript (266). The

difference in genome content would have accounted for the modest effects (7-10 fold increase in the presence of Nef) in Nef-induced infectivity they reported after standardizing input samples for p24 level (266). A separate investigation by Aiken and Trono presented hybridization signals more closely matched in intensity than the previous study (266), but also a narrower range of genome dilution (0 ng, 10 ng and 25 ng of p24-standardized virus)(4). Certainly, a more exacting quantification of viral genomes, such as a Taqman assay, is necessary to determine what may be a very subtle difference in HIV-1 viral proteins. If a difference in the ratio of viral genomes to p24 is determined this would provide a suitable standard for the comparison virus produced in Nef expressing and Nef deficient cells, and allow for more direct comparison of the associated viral proteins.

Unfortunately, no single virus assay exists to specifically determine the components of a given particle using standard biochemical techniques. Lacking this type of assay the field is forced to study particles in the aggregate making assumptions of individual particles based on the observations of the whole viral population. In order to overcome this deficiency a larger number of parameters will need to be assessed to make comparisons between different populations. Absent a reliable standard, biochemically determining the effects of Nef on the assembly of viral particles will remain a question yet to be completely answered.

However, if Nef expression impacts particle assembly by acting like ESCRT proteins to form a bud neck to limit Gag recruitment, then data could conceivably be obtained using imaging strategies similar to those of Jouvenet et al (138). Total internal reflection microscopy (TIRF) would allow for the observation of GFP-Gag fusion assembly at the plasma membrane under a variety of experimental conditions. Using the fluorescence intensity of viral particles assembled in the absence of Nef as a baseline, the effects of Nef expression in the presence and absence of ESCRT proteins could easily be determined. An increase in the fluorescence signal above that observed from the baseline sample (i.e. assembly of "WT" GFP-Gag particles) would indicate an increase in GFP-Gag recruitment, while a decrease would suggest the opposite, reduced recruitment of Gag. Using these parameters the effects of Nef expression, ESCRT participation, and cholesterol on Gag recruitment could be directly determined. These experiments have the potential to add insight into finally determining the role that Nef expression may play in the assembly and release of infectious HIV-1 particles.

Appendix

Regulation of CHMP4/ESCRT-III Function in Human Immunodeficiency Virus Type I Budding by CC2D1A(293)

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Release of HIV-1 virions by the host cell requires the recruitment of proteins of the ESCRT pathway which promote scission of the viral envelope from the plasma membrane. Scission is accomplished through the recruitment and activation of the ESCRT-III subunit proteins known as CHMPs which associate with the inner leaflet of the plasma membrane at bud sites. A recent siRNA based study has found that release of HIV-1 particles relies predominantly on the actions of two ESCRT-III subunits, CHMP2 and CHMP4.

In this paper, our lab addressed the function of CC2D1A and CC2D1B proteins on release of HIV-1 through the regulation of the ESCRT-III subunit CHMP4B . Both proteins, CC2D1A and CC2D1B, were identified as potential targets of investigation based on microsequencing analysis of proteins that specifically interacted with CHMP4B. Subsequent experiments demonstrated that CCD21A overexpression inhibits HIV-1 release; more specifically, overexpression of this protein inhibited ALIX-mediated of HIV-1, a process known

to be reliant on interaction between ALIX and CHMP4B. In contrast, siRNA mediated knock down of CC2D1A and CC2D1B were shown to increase HIV-1 budding, as would be expected if CC2D1A/B played a negative role in CHMP4B regulation. Also, a series of immunoprecipitation experiments indicated that the interaction between CC2D1A and CHMP4B relies heavily on the presence of the DM14 domains of CC2D1A, specifically the third of four linear domains. In addition, it was shown that CC2D1A interacts with the N-terminal helical hairpin of the CHMP4B protein, and not the C-terminal site that has been shown to play a role in ALIX recruitment.

My contributions to the paper involved creating N-terminal truncation mutants for constructs based on the CHMP4B₁₋₁₅₃ core fragment. These constructs were used to investigate the minimal region of CHMP4B required to behave as an inhibitor to virus release. In addition, I performed an experiment designed to test whether co-expression of CCD21A with ALIX led to inhibition of particle release. As mentioned, ALIX-mediated release of the HIV-1_{ΔPTAPP} construct requires recruitment of CHMP4B by the Bro1 domain of ALIX. The data I generated indicated that expression of ALIX alone was sufficient to rescue release of HIV-1_{ΔPTAPP} particles, but co-expression of both ALIX and CC2D1A vectors failed to stimulate release of viral particles to any extent greater than that observed with empty vector alone. Lastly, I analyzed the release of HIV-1

particles from 293T cells that had been treated with siRNA directed against either CC2D1A or CC2D1B or both. This experiment, which was performed in tandem with the lead author of this paper and returned the same results, demonstrated that the knockdown of endogenous levels of CC2D1A or CC2D1B increased the release of HIV-1 particles. These results confirmed the previous findings in the paper that these proteins behave as regulators of CHMP4B activity in the release of HIV-1.

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