The Role of Adaptor Protein Complex-3 Delta-Mediated HIV-1 Gag Trafficking in HIV-1 Replication: A Dissertation

Adonia Lee Kim
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THE ROLE OF ADAPTOR PROTEIN COMPLEX-3 DELTA-MEDIATED HIV-1 GAG TRAFFICKING IN HIV-1 REPLICATION

A Dissertation Presented

By

ADONIA LEE KIM

Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

MAY 18, 2012

INTERDISCIPLINARY GRADUATE PROGRAM
THE ROLE OF ADAPTOR PROTEIN COMPLEX-3 DELTA-MEDIATED HIV-1 GAG TRAFFICKING IN HIV-1 REPLICATION

A Dissertation Presented
By
ADONIA LEE KIM

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Dean of the Graduate School of Biomedical Sciences

Interdisciplinary Graduate Program

MAY 18, 2012
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positive outlook on science and life.

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ABSTRACT

The process of HIV-1 particle production is a multi-step process directed by the viral structural protein Gag. As Gag is the only viral protein required to form virus-like particles, it presents a viable target for anti-viral therapeutics of which there are currently none. Although the functions of Gag during the particle assembly process have been well characterized, one of the least known parts of the assembly process is how Gag is targeted to the site of virus assembly.

Two main virus assembly sites have been identified in cells that support HIV-1 replication: the plasma membrane or multivesicular bodies (MVBs). However the mechanism by which Gag is targeted to either of these sites remains unknown. The δ subunit of Adaptor Protein Complex 3 has previously been identified as a cellular co-factor for HIV-1 Gag and was reported to mediate Gag trafficking to MVBs, providing a mechanism for Gag targeting to this assembly site. Additionally, AP-3δ was reported to be required for HIV-1 production, suggesting that Gag to MVB targeting is also required for HIV-1 production.

The work presented in this thesis further investigates the role of AP-3δ in Gag trafficking to MVBs and its role in HIV-1 production in previously unexplored host environments. Through the use of RNA interference-mediated depletion of AP-3δ, we determined that AP-3δ is dispensible for virus replication in infected HeLa cells, chronically infected HeLa-LAV cells and infected primary human monocyte-derived macrophages. We concomitantly disrupted AP-3 function by disrupting its association with membranes and observed no effect on virus production. Collectively, these results
demonstrate that AP-3δ is not required for HIV-1 replication. However, AP-3δ was demonstrated to be required for Gag targeting to MVBs thus presenting a new model for the function of AP-3δ in the context of HIV-1 replication.
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**LIST OF ABBREVIATIONS AND SYMBOLS**

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<th>Full Form</th>
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<tr>
<td>AGAP 1</td>
<td>ArfGAP with GTPase domain, ankyrin repeat and PH domain 1</td>
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<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
</tr>
<tr>
<td>AP</td>
<td>Adaptor Protein Complex</td>
</tr>
<tr>
<td>AP-3</td>
<td>Adaptor Protein Complex 3</td>
</tr>
<tr>
<td>AP-3δ</td>
<td>Delta Subunit of AP-3</td>
</tr>
<tr>
<td>ARF 1</td>
<td>Adenosine Diphosphate Ribosylation Factor 1</td>
</tr>
<tr>
<td>CA</td>
<td>HIV-1 capsid</td>
</tr>
<tr>
<td>Env</td>
<td>HIV-1 envelope</td>
</tr>
<tr>
<td>ESCRT</td>
<td>Endosomal Sorting Complex Required For Transport</td>
</tr>
<tr>
<td>Gag</td>
<td>Group Specific Antigen, HIV-1 Pr55(^{gag}) polyprotein</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
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<td>Guanosine diphosphate</td>
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<td>gRNA</td>
<td>genomic RNA</td>
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<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
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<td>HIV-1</td>
<td>Human Immunodeficiency Virus Type 1</td>
</tr>
<tr>
<td>IN</td>
<td>Integrase</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>kD</td>
<td>kilodalton</td>
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<tr>
<td>LAMP</td>
<td>Lysosome Associated Membrane Protein</td>
</tr>
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<td>LTR</td>
<td>Long Terminal Repeat</td>
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<td>MA</td>
<td>HIV-1 Matrix</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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<tr>
<td>MDM</td>
<td>Monocyte-derived macrophage</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity Of Infection</td>
</tr>
<tr>
<td>MVB</td>
<td>Multivesicular Bodies</td>
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<tr>
<td>NC</td>
<td>HIV-1 nucleocapsid</td>
</tr>
<tr>
<td>nt</td>
<td>Nucleotide</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PI(4,5)P₂</td>
<td>Phosphatidylinositol 4,5-Bisphosphate</td>
</tr>
<tr>
<td>PIC</td>
<td>Pre-Integration Complex</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma Membrane</td>
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<tr>
<td>RNAi</td>
<td>RNA interference</td>
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<td>RT</td>
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<td>RT-PCR</td>
<td>Reverse Transcription PCR</td>
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<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
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<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
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<tr>
<td>SNARE</td>
<td>Soluble N-ethylmaleimide-sensitive factor attachment protein receptor</td>
</tr>
<tr>
<td>TEM</td>
<td>Tetraspanin Enriched Microdomain</td>
</tr>
<tr>
<td>TGN</td>
<td>trans-Golgi network</td>
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<td>TM</td>
<td>Transmembrane</td>
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<td>VAMP7</td>
<td>Vesicle Associated Membrane Protein 7</td>
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<td>VCC</td>
<td>Virus Containing Compartments</td>
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<td>VSV-G</td>
<td>Vesicular Stomatitis Virus Glycoprotein</td>
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CHAPTER I: INTRODUCTION

1. Human Immunodeficiency Virus Type 1

1.1. Discovery and Global Impact of AIDS and HIV-1

Human Immunodeficiency Virus Type 1 (HIV-1), the causative agent of acquired immunodeficiency syndrome (AIDS) has been the subject of intense investigation since its discovery over 30 years ago. The first reported cases of AIDS in the United States (US) were published on June 5, 1981 by the Centers for Disease Control (CDC), establishing the beginning of the HIV/AIDS epidemic (1). The CDC report described the incidence of *Pneumocystis carinii* pneumonia (PCP) in five homosexual men between the ages of 29 and 36 who were also infected with cytomegalovirus (CMV) as a result of sexual transmission, and presented with candidiasis as a result of fungal infection. The incidence of PCP in these previously healthy patients was considered to be unusual, as PCP was typically only observed in severely immunocompromised individuals. The possibility was raised that these individuals exhibited cellular immune dysfunction due to a common but unidentified exposure thereby predisposing them to opportunistic infections such as PCP and candidiasis. Around this time, it was also reported that there was a population of young homosexual men in the US who were infected with CMV and suffered from an aggressive form of Kaposi’s Sarcoma, a typically benign but rare form of cancer that affects older individuals (181). As the incidence of these opportunistic infections and cancers increased, it became apparent that these were symptoms of an underlying novel disease that was later named AIDS. Although AIDS was initially
thought to afflict only homosexual men, this was dispelled by cases of AIDS in intravenous drug users, hemophiliacs, heterosexual men and women, infants and children.

Shortly after AIDS was identified, three independent groups reported the isolation of retroviruses specifically from AIDS or at-risk AIDS patients. In 1983, Luc Montagnier and colleagues were the first to isolate the novel virus, which was designated as lymphadenopathy-associated virus (LAV) (5). Levy and colleagues isolated a virus which was designated as AIDS-Related Virus (ARV) (93), and Gallo and colleagues designated the virus they isolated as human T-lymphotrophic virus type III (HTLV-III) (140). Among these three investigators, Gallo was the first to establish a causative link between the retrovirus, HTLV-III, and AIDS (50). This breakthrough led to the development of a commercially available enzyme-linked immunosorbent assay (ELISA) designed to detect HTLV-III antibodies in patient sera, providing a diagnostic tool for HIV infection (78).

As the novel retrovirus that was known to cause AIDS gained more attention from the scientific and global community, the need for a new name for the virus became evident, as there were three different names (i.e. LAV, HTLV-III, or ARV) associated with the virus. Therefore, the International Committee on the Taxonomy of Viruses assigned the virus a single name, Human Immunodeficiency Virus, now known as HIV-1 (28).

HIV-1 is transmitted from an infected to an uninfected individual through the exchange of bodily fluids, which primarily occurs as a result of unprotected sexual intercourse, blood exposure (i.e. intravenous drug use with contaminated needles, blood transfusions, organ transplantation), and perinatally (i.e. during pregnancy, at birth, or breastfeeding) (127). Of these modes of transmission, heterosexual intercourse remains
the most prevalent worldwide (156, 166). Once transmission occurs, HIV-1 targets the CD4-positive (CD4+) cells of the immune system, which include T-lymphocytes (T cells), macrophages, monocytes, and dendritic cells (29). The natural course of HIV-1 infection in untreated individuals results in a gradual decline in the number of CD4+ T cells over time, resulting in the progression to AIDS, and eventually, death. HIV-1 infection begins with the acute phase, which is characterized by exponential viral replication and massive depletion of CD4+ T cells, particularly in the gut associated lymphoid tissue (GALT) (15, 103). Over time, the immune system mounts a response through HIV-specific CD8+ cytotoxic T cell-mediated destruction of HIV-infected cells (13, 60) and anti-HIV neutralizing antibodies (65), resulting in the stabilization of the viral load. During this period, circulating CD4+ T cells are able to recover temporarily. The next stage is clinical latency, a period that can last between 2-10 years, and is characterized by low levels of viral replication and asymptomatic presentation (127). The inability of the immune system to control the virus however, promotes the gradual decline in CD4+ T cells, and leads to the diagnosis of AIDS when CD4+T cell counts fall below 200 cells/mL. At this stage, infected individuals are severely immunocompromised and become susceptible to opportunistic infections that are ultimately lethal. (128) (Figure 1-1).

To date, HIV has claimed the lives of more than 25 million people and infected an estimated 60 million people worldwide (165). In 2009, the global estimate of people living with HIV was reported to be 34 million, with a newly infected population of 2.7
Figure 1-1. The stages of HIV-1 infection.

In the acute stage, rapid viral replication reaches a peak viral load, followed by stabilization (red line, top), as cytotoxic CD8+T cells (blue line, bottom) and neutralizing anti-HIV antibodies develop (yellow line, bottom). Massive depletion of GALT (closed circles, bottom) and peripheral (green line, bottom) CD4+T cells occurs, with a gradual recovery of peripheral CD4+T cells due to immune system-mediated stabilization of viral replication. In the asymptomatic or chronic stage, viral load remains stable, as do CD8+CTLs, neutralizing antibodies, and peripheral CD4+T cells, while CD4+T cells in the GALT remain depleted. Risk of transmission is lower than in the acute stage, but viral diversity is increased. As viral diversity increase, viral replication increases, resulting in peripheral CD4+T cell decline and progression to AIDS, when CD4+T cell counts fall below 200 cells/mL (gray line). Adapted from Simon et al. (Lancet 2006).
million, as well as 1.8 million AIDS-associated deaths (166). The HIV/AIDS pandemic has been particularly devastating to the population of sub-Saharan Africa, where the number of people living with HIV is approximately 67% of the 2010 global estimate (166). However, the sub-Saharan African countries with the largest epidemics have demonstrated stabilization in the prevalence of HIV-1 infected individuals, a decline in the number of people newly infected with HIV, and a decline in the number of AIDS-related deaths over the past 10 years. This decline in incidence and death rates has been greatly attributed to the increased use and accessibility of anti-viral therapies (166). The development of effective therapeutics is a direct consequence of the significant scientific advancements that have been made in the field of HIV research over the past 30 years. However, without a cure or vaccine, the AIDS pandemic will continue to persist. Therefore, continued investigation of HIV and its pathogenesis is imperative to promote the scientific breakthroughs needed for the development of novel therapeutics and eventually a cure.
Figure 1-2. Global representation of the number of people infected with HIV in 2009. Adapted from The UNAIDS Report On The Global AIDS Epidemic:2010 UNAIDS Global Report.
1.2. HIV-1 Genome and Viral Proteins

Genome

HIV-1 is an enveloped positive single-stranded RNA virus belonging to the lentivirus family of retroviruses. It is classified as a retrovirus due to its use of reverse transcription to convert its RNA genome into DNA. Its further characterization as a lentivirus, derived from the Latin term “lente” meaning slow, is attributable to its long incubation period in the host and its ability to infect non-dividing cells. The HIV-1 genome is comprised of long terminal repeat regions (LTR) at its 5’ and 3’ termini ends and nine overlapping open reading frames: *gag, pol, env, tat, rev, vif, vpr, vpu, and nef* (Fig.1-3), which generate 15 viral proteins (46) as a result of alternative splicing, ribosomal frameshifting, and protease-mediated cleavage of polyprotein precursors.

Each virion contains two copies of positive-sense, single-stranded RNA of approximately 9.2 kilobases (kb) in length, referred to as genomic RNA (gRNA). Shortly after infection of the host cell, the single-stranded RNA genome is converted into double-stranded proviral DNA by reverse transcriptase. As a result of reverse transcription, a segment of each LTR is duplicated and added to each end of the proviral DNA, extending its total length to approximately 9.8 kb. The proviral DNA is integrated into the host genome and undergoes cellular RNA Polymerase II (Pol II)-mediated transcription, thereby generating gRNA that is capped and polyadenylated (24, 26). Genomic RNA contains many *cis*-acting elements that are required for different functions during viral replication, including: (1) the TAR element that is required for Tat-mediated
Figure 1-3. Schematic representation of the HIV-1 genome and the structure of a mature HIV-1 virion. Frankel and Young. Annu. Rev. Biochem. (1998)
transcription elongation; (2) the primer binding site that is required for tRNA$^{\text{Lys}}$ binding to initiate reverse transcription; the major splice donor sequence; (3) the packaging signal (Ψ) that is required for gRNA encapsidation into nascent virus particles; (4) the dimerization site that is required for the incorporation of dimerized gRNA; (5) the major splice donor site that is used to generate all spliced viral mRNAs; (6) the slippery sequence and the frameshift stimulatory signal that are required for the -1 ribosomal frameshift that occurs during the translation of Gag to generate the Gag-Pol precursor; (7) a central and 3’ polypurine tract that is required for plus-strand proviral DNA synthesis during reverse transcription; (8) the Rev-Response Element (RRE) that is required for Rev-mediated export of unspliced viral RNAs; (9) the splice acceptor sites that are required the generation of subgenomic viral mRNAs; and (10) the 3’ polyadenylation signal, which is required for polyadenylation of the 3’ end of gRNA (reviewed in (46)).

Viral RNAs are unspliced, partially spliced, or fully spliced, to generate the viral transcripts required for the synthesis of the nine viral genes. The nine viral gene products are organized into four groups: structural, enzymatic, regulatory, and accessory.

The Structural Proteins

The translation products Gag and Env comprise the structural proteins. The Gag (group-specific antigen) protein, encoded by the gag gene, directs particle production and provides the structural architecture for the virus. Gag is translated from gRNA on free cytoplasmic ribosomes, resulting in the synthesis of a 55 kD polyprotein precursor known as Pr55$^{\text{Gag}}$ (47). Gag is comprised of four domains: matrix (MA/p17), capsid (CA/p24),
nucleocapsid (NC/p7), p6, and the spacer peptides: SP1 and SP2. During the final stage of virion production, Gag undergoes protease-mediated cleavage at the junction of each domain resulting in individually processed mature forms of each domain. MA is positioned at the N-terminus of Gag and is required for targeting Gag and Gag-Pol to the plasma membrane through an N-terminal myristate and a highly basic region (HBR) located within the first 31 residues (91, 159). During virus assembly, MA has been implicated in facilitating the incorporation of Env into nascent virions (99). Within a mature virion, mature MA lines the inner surface of the virus particle and is in direct contact with the viral membrane. Following infection, mature MA is associated with the pre-integration complex (PIC) and contributes to PIC entry into the nucleus through its nuclear localization signal (NLS) (18, 168). The next domain of Gag is CA, which mediates Gag:Gag and Gag:Gag-Pol interactions required for virus assembly that are mediated by its C-terminal domain (51, 70). Within a mature virion, mature CA forms a conically shaped shell around gRNA and additional viral proteins (54). Following infection, mature CA undergoes an uncoating process required for infectivity that is mediated by the interaction between its N-terminal domain and the cellular factor cyclophilin A (96). The next domain is NC, which facilitates gRNA incorporation into virions through its two zinc-finger motifs and mediates Gag multimerization (30, 152). Within a mature virion, mature NC is tightly associated with gRNA inside the CA core (46). Following infection, mature NC acts as a nucleic acid chaperone in the post-entry steps of reverse transcription and integration, which enhances the efficiencies of these processes (19, 22, 64, 71). The final domain is p6, which mediates Vpr incorporation into
the nascent particle (129), and recruits the ESCRT (endosomal sorting complex required for transport)-associated components, Tsg101 and Alix, through interactions with its late domains, Pro-Thr-Ala-Pro (PTAP) and Tyr-Pro-Xₙ-Leu (YPXₙL), respectively, to facilitate the pinching off of fully assembled virus particles from the cell surface (161, 162).

The \textit{env} gene encodes the Env protein, which is required for virus entry into the target cell. Env is translated from partially spliced bicistronic \textit{vpu/env} mRNA in the rough endoplasmic reticulum (ER) and is synthesized as the precursor protein, gp160 (49). The gp160 precursor undergoes co-translational glycosylation and intramolecular di-sulfide bond formation, which promotes its trimerization. Following its modification in the ER, gp160 is transported to the Golgi apparatus, where it undergoes further modification of its glycosylated side chains and is cleaved by the cellular protease, furin or a furin-like enzyme, into gp120 and gp41 (176). Following cleavage, gp120 and gp41 form a non-covalent interaction and are trafficked together from the Golgi to the cell surface, through the secretory pathway, which promotes their incorporation into nascent virions (48). Viral attachment to the host cell is mediated by binding of surface protein, gp120, to the target cell receptor, CD4, triggering a conformational change in gp120 (14). This conformational change facilitates its interaction with the cellular co-receptor CCR5, or CXCR4 (35). Upon gp120 binding to CD4 and CCR5 or CXCR4, gp41 undergoes a conformational change, which brings the cellular viral membranes together until fusion of the two membranes occurs (36). Following fusion, the viral contents enter the cell and the HIV-1 replication cycle continues.
The Enzymatic Proteins

The pol gene, which encodes the enzymatic proteins: protease (PR), reverse transcriptase (RT), and integrase (IN). Similar to Gag, pol is synthesized as a precursor, Pr160\(^{\text{Gag-Pol}}\) or Gag-Pol, and is translated from gRNA. Unlike Gag, however, Gag-Pol is translated by a unique mechanism that is dictated by -1 ribosomal frameshifting (42). During Gag translation, the ribosome occasionally stalls after encountering the slippery sequence and the frameshift stimulatory signal that are located near the junction of the gag and pol genes. As a result, -1 frameshift occurs, which allows translation to proceed downstream of the gag termination codon, until the pol termination codon is reached. This mechanism for translation ensures that Gag-Pol is produced in a 1:20 ratio to Gag (48). Gag-Pol is incorporated into virions and during or shortly after virus release, PR cleaves itself from Gag-Pol and subsequently cleaves the junctions between the domains of Gag and Gag-Pol generating MA, CA, SP1, NC, SP2, p6 from Gag, and PR, RT, IN from Gag-Pol. This process results in a dramatic rearrangement of the virion interior and drives viral maturation, and is required for viral infectivity (84, 87). The RT enzyme is an RNA-dependent DNA polymerase that is required for the reverse transcription of single-stranded RNA genome into double-stranded proviral DNA. As a result of its lack of exonucleolytic proofreading activity, RT is highly error-prone, which enhances the genetic diversity of the virus and allows it to evade the cellular immune system (141). The IN enzyme catalyzes the integration of proviral DNA into the host genome by for integration into the host genome by processing its 3’ ends of proviral DNA to generate 5’ overhang (4). Following nuclear import of the PIC, IN catalyzes the staggered cleavage
of the host chromosomal DNA in a sequence-independent manner. The recessed 3’ ends of the proviral DNA are joined to the 5’ overhangs of the host DNA in a process known as strand transfer. Following integration, the cellular repair machinery fills in the gaps resulting from the strand transfer, which completes the integration process.

The Regulatory Proteins

The regulatory proteins Tat and Rev regulate viral gene expression at different stages and are required for viral replication. The Tat protein is encoded by two exons of the *tat* gene and is required for RNA Polymerase II (Pol II)-mediated transcription elongation, as its absence results in the synthesis of transcripts that are prematurely terminated. Upon binding cyclin T/CDK9 complex (positive transcription elongation factor b, pTEFb) through its activation domain, Tat undergoes a conformational change which increases its affinity for the TAR RNA element present within the first 59 nucleotides of all viral transcripts (77). After Tat binds to TAR, cyclin T/CDK9 are recruited to the Tat/TAR complex and CDK9 hyper-phosphorylates the C-terminal domain of Pol II, which promotes Pol II transcription elongation and results in the synthesis of complete transcripts (77).

The Rev protein is encoded by two exons of the *rev* gene. Rev contains an arginine-rich domain that is required for RNA binding and serves as nuclear localization signal (NLS) and is flanked by two oligomerization domains (97). It also contains a leucine-rich nuclear export signal (NES), which promotes its nuclear export (97). The primary function of Rev is to facilitate the nuclear export of unspliced and partially
unspliced viral RNAs, and this is mediated through its interaction with the RRE, which is present on these RNAs (98). As Rev contains both an NLS and an NES, it acts as a shuttling protein to continuously export viral RNAs and re-enter the nucleus to repeat the process. Nucleo-cytoplasmic export of Rev is facilitated by its use the CRM1 export pathway instead of the more widely used TAP (Tip-associated protein) mRNA export pathway, which is thought to allow unspliced RNAs to evade the splicing machinery (45).

The Accessory Proteins

The accessory proteins are Vif, Vpr, Vpu, and Nef, which were originally shown to be dispensible for HIV-1 replication in vitro, but their requirement for efficient virus replication in vivo was later established. The Vif protein is encoded by the vif gene and is incorporated into virus particles. Vif is not required for virus production, however, it plays a role in viral infectivity. The main function of Vif is to promote the degradation of the anti-viral cellular factor, APOBEC3G (apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G), a cytidine deaminase enzyme that interferes with HIV-1 reverse transcription by inhibiting tRNA\textsuperscript{Lys3} priming and by deaminating dC to dU in minus strand viral cDNA during reverse transcription (63, 101). Vif counteracts APOBEC3G by promotes its polyubiquitination which subsequently targets it to the proteasome for degradation (104).

The Vpr protein is encoded by the vpr gene and is incorporated into virus particles. Vpr contains two NLSs that mediate the nuclear import of the PIC by through
direct binding to the nuclear import factor, karyopherin α, which enhances its affinity for NLSs (139). Another function of Vpr is its ability to arrest cells in the G2 phase of the cell cycle, which is thought to increase HIV-1 LTR-driven transcription (56). Vpr has also been implicated in enhancing the fidelity of reverse transcription through its interaction with UNG2 (uracil DNA glycosylase), an enzyme that specifically removes the RNA base uracil from DNA as a result of its misincorporation or cytosine deamination (102).

Vpu protein is translated from a bicistronic vpu/env mRNA and directly targets the cytoplasmic tail of newly synthesized CD4 in the ER to the proteasome for degradation (153, 177). The involvement of Vpu in particle release has been demonstrated through the observation that Vpu-deficient viruses were retained at the plasma membrane in intracellular vesicles (86). This suggested that Vpu plays a role in virus release and prevents endocytosis of nascent virions from the plasma membrane (107). However the mechanism by which this occurred was not known until recent studies demonstrated that the release defect was caused by the cellular factor, Tetherin, a transmembrane protein which inserts its N-terminal extracellular domain into the membranes of nascent virions while remaining attached to the plasma membrane, thereby tethering the virion to the cell surface (108, 133). The inhibitory function of Tetherin is antagonized by Vpu, which targets Tetherin to the proteasome for its degradation (100).

The Nef protein is encoded by the nef gene and is incorporated into virus particles. Similar to Gag, Nef has a bipartite membrane signal comprised of on N-terminal myristate and an N-terminal patch of basic residues (172). One of the main
functions of Nef is to downregulate CD4 from the cell surface through its interaction with the di-leucine motif present in the cytoplasmic tail of CD4 and Adapter Protein Complex-2. This interaction induces the endocytosis of CD4 and Nef, and results in CD4 and Nef internalization in endosomes. In the endosome, Nef promotes the interaction between CD4 and the \( \beta \) subunit of the COP I vesicle coat protein, \( \beta \)-COP, which targets CD4 to the lysosome for degradation (137). In addition to its role in CD4 downregulation, Nef also downregulates MHC (major histocompatibility complex) class I, which further demonstrates its function as an immune modulator (27).

1.3. The HIV-1 Replication Cycle

The HIV-1 replication cycle is a complex, multi-part process that begins with viral infection of the target cell and ends with the production of mature, infectious virus particles (reviewed in (46, 47)) (Fig. 1-4). The early stage of the viral life cycle begins with viral attachment to the target cell through an interaction between gp120 and the cellular receptor CD4 and the chemokine co-receptors CCR5 or CXCR4. Upon CD4 and co-receptor binding, gp120 and gp41 undergo a conformational change resulting in viral and cellular membrane fusion. Following fusion the viral contents are released into the cytoplasm of the infected cell. The CA core undergoes a process of uncoating that liberates its internal contents: genomic RNA, NC, RT, IN, and Vpr. Uncoating promotes the formation of the reverse transcription complex (RTC), in which gRNA is reverse transcribed by RT into double-stranded proviral DNA. Completion of reverse transcription leads to the formation of the pre-integration complex (PIC), a nucleoprotein
Figure 1-4. The HIV-1 replication cycle. The HIV-1 replication cycle is divided into early (pre-integration) and late stages (post-integration). The early stage is comprised of viral attachment, fusion, viral entry, uncoating, reverse transcription of the viral genomic RNA into proviral DNA, import of the proviral DNA and associated viral proteins into the nucleus, and integration of the proviral DNA into the host genome. The late stage is comprised of viral gene transcription, nuclear export of viral RNAs, viral RNA translation, virus assembly, budding, viral egress from the target cell, and viral maturation. D’Souza and Summers. Nat. Rev. Microbiol. (2005)
complex comprised of proviral DNA, MA, RT, IN, and Vpr (160). The PIC translocates from the cytoplasm into the nucleus through the nuclear localization signals (NLS) present in MA and IN, as well as Vpr-mediated enhancement of NLS binding to the nuclear import factor, karyopherin α (139). Following nuclear entry, the proviral DNA is integrated into the host genome by IN and undergoes Pol-II-mediated transcription, which requires Tat for transcription elongation. RRE-containing unspliced and partially spliced viral RNAs are exported by Rev into the cytoplasm through the CRM1 pathway, and fully spliced viral RNAs are exported through the TAP pathway. Following nuclear export, viral RNAs are translated, and the viral components required for infectious particle production localize to the site of virus assembly and are incorporated into progeny virions. Gag dictates these late events in the viral life cycle through the function of its domains. MA targets and binds Gag to the plasma membrane, where assembly occurs, and also facilitates Env incorporation into the virion. CA, SP2, and NC mediate Gag-Gag interactions which are required to form the particle and facilitates Gag-Pol incorporation. NC incorporates gRNA into the particle by binding to the Ψ of gRNA. The p6 domain incorporates Vpr into the nascent virion and also recruits the ESCRT machinery, which is required for virus budding and pinching off from the membrane. Either during or immediately after virus release, PR cleaves the polyproteins, resulting in viral maturation and a dramatic rearrangement of the viral interior. Following maturation, virus particles are competent to infect and replicate in new target cells, thus completing the viral replication cycle.
The resulting virus is roughly spherical in shape and is approximately 130 nm in diameter (21). At the center of the virion is a gRNA dimer, surrounded by NC, RT, IN, and Vpr, which are all contained within a conically-shaped protein core comprised of CA monomers. PR is present in the space between the core and an outer layer comprised of MA monomers. The layer of MA monomers directly interacts with the lipid bilayer, the outermost layer of the virus, which is acquired after viral budding from the host cell membrane. The virion surface is studded with trimeric glycoprotein spikes comprised of gp120 and gp41.

2. HIV-1 Particle Production

2.1. Role of Gag

Successful completion of the HIV-1 replication cycle is marked by the production of infectious virus particles, which ensures the continued propagation of the virus. The viral structural protein, Gag, drives virus production and is necessary and sufficient for this process. Although Gag relies on host factors for virus production, it is the only viral protein required for this process, as its expression alone results in the formation of virus-like particles (55, 155). Gag mediates virus production through a complex series of steps directed by its subdomains that involve Gag targeting to the site of assembly, Gag binding to cellular membranes, Gag multimerization, Gag-mediated virus budding and release (170) (Fig. 1-5).

The MA domain targets and binds Gag to membranes through a bipartite signal consisting of an N-terminal myristate, which is added during translation, and a highly
Figure 1-5. Schematic representation of HIV-1 Gag and HIV-1 Gag domains.
(A) Organization of the domains within the Gag polyprotein. (B) Domains of Gag and their key features. *Freed, Virology. (1998)*
basic region (HBR) spanning residues 14-31 (27). Inhibition of MA-mediated membrane binding of Gag through disruption of the myristate modification and mutation of the HBR has been shown to also inhibit virus production (122, 182, 183). The CA and NC domains promote the Gag-Gag interactions that contribute to the overall architecture of the virus particle, which contains approximately 5000 copies of Gag (16). NC additionally promotes multimerization through its interaction with gRNA, which serves as a scaffold for Gag multimerization (88). In addition, Gag multimerization has been suggested to enhance Gag binding to membranes by promoting the exposure of myristate on MA (66, 163). During the viral assembly process, Gag-Pol, gRNA, Env (gp120/gp41), Vif, Vpr, and Nef, are incorporated into the developing virus particle (46). Finally, the C-terminal p6 domain recruits the ESCRT (endosomal sorting complex required for transport) through its interaction with the ESCRT components Tsg101 and ALIX, initiating the final stage of viral budding and release (161, 162).

2.2. Sites of Virus Production

Plasma membrane

The plasma membrane has been demonstrated to be the predominant site of virus production in all cell types. Although the cell interior is comprised of a multitude of organelles and compartments bound by membranes of various compositions, Gag has developed the capability of navigating the cell interior from the site of its translation and specifically bind to the plasma membrane through its N-terminal myrsitate and HBR. Recent studies have shown that plasma membrane specificity is mediated by the
interaction of the basic residues within the HBR of MA and phosphatidylinositol (4, 5) bisphosphate (PI(4,5)P2), an acidic phospholipid that is enriched in the inner leaflet of the plasma membrane (117). Mutating the basic residues of the HBR results in reduced Gag-membrane binding as well as aberrant localization to intracellular compartments. Myristate exposure is triggered by Gag multimerization, but it is also triggered by the interaction of the HBR with PI(4,5)P2, which enhances Gag binding to the plasma membrane (27). Depletion of PI(4,5)P2 through the overexpression of polyphosphoinositide 5-phosphatase IV (5ptaseIV) or a constitutively active Arf6 mutant, Arf6/Q67L, disrupts Gag association with the plasma membrane and instead results in the targeting of Gag to multivesicular bodies (118). In the presence of the PI(4,5)P2 inhibitors, particle production is severely compromised, indicating that Gag localization to the plasma membrane is essential for virus production.

In addition to PI(4,5)P2, lipid rafts and tetraspanin-enriched microdomains (TEM) have also been demonstrated to promote virus assembly at the plasma membrane (116). Lipid rafts are microdomains enriched in cholesterol and glycosphingolipids and contain specific markers that are found on the membranes of virus particles, suggesting that virus production takes place at these sites (2, 111). In addition, Gag has been shown to colocalize and co-fractionate with lipid raft markers providing further evidence for the involvement of lipid rafts in virus assembly (69, 111, 121). Furthermore, Gag and Env have been shown to associate with detergent resistant membranes, which are thought to be derivatives of lipid rafts (11).
Tetraspanins represent a large family of proteins comprised of four transmembrane domains and are expressed on the cell surface (94). These transmembrane proteins can form heterogeneous clusters at the plasma membrane known as TEMs (94). TEM involvement in virus assembly has been demonstrated by microscopy studies in which Gag localized to TEMs comprised of the tetraspanins CD9, CD63, CD81, and CD82 (116). In addition, Env co-localized with Gag at these TEMs providing evidence that virus assembly takes place at these microdomains (116). Furthermore, tetraspanins are incorporated into the membranes of virions (25, 79, 110, 132) and have been observed at plasma membrane-derived intracellular virus-containing compartments (34), strongly suggesting that TEMs are sites of virus assembly.

**Multivesicular bodies**

Multivesicular bodies (MVB) are membrane-bound organelles that are derived from early endosomes and contain a number of intraluminal vesicles (ILV) as a result of inward budding from their limiting (outer) membrane (138). The process of inward budding and ILV formation is mediated by the ESCRT complexes and their related components (178). Proteins that reside within the ILVs of MVBs are typically mono-ubiquitinated and are destined for lysosomal degradation. The delivery of MVB-containing cargo to lysosomes is mediated by the fusion of the two compartments (138). In addition to fusion-mediated delivery to lysosomes, MVBs can also deliver their contents to the extracellular space as exosomes, upon fusion with the plasma membrane (167). Virus assembly has been demonstrated to occur in MVBs in multiple cell types,
but is more apparent in macrophages. Early electron microscopy (EM) studies demonstrated that in macrophages, HIV-1 production predominantly occurs in intracellular compartments identified as MVBs, according to their co-localization with MVB markers CD63 and LAMP-1 (110, 120, 126, 132, 144). In addition, MVB-derived viruses are infectious, suggesting that MVBs are productive sites for virus assembly and budding in macrophages (110, 132). Viral egress from these compartments has been suggested to occur through exocytosis, which has been supported by EM studies as well as the demonstration that extracellular virions immunoprecipitate with MVB marker-specific antibodies (110, 132). The discovery that HIV-1 uses the ESCRT machinery to facilitate its budding and release has also provided support for the proposal that MVBs are *bona fide* sites for virus production, due to the link between MVB biogenesis and the function of the ESCRT machinery (53). Virus production has also been demonstrated to occur in the MVBs of non-macrophage cell types such as T-cells, HeLa cells, and COS cells previously understood to promote virus production only at the plasma membrane (53, 115, 154). In addition, MVB-derived viruses in these cell types were shown to be infectious (61). Collectively, these results demonstrate that MVBs are sites of virus assembly, however, it is controversial whether they are productive sites for HIV-1 replication.

3. Adaptor Protein Complex-3

3.1. Adaptor Protein Complexes
Adaptor Protein Complexes (AP) coat vesicles and mediate the transport of proteins from a donor membrane to a target membrane. To date, five APs have been identified: AP-1, AP-2, AP-3, AP-4, and AP-5 (68). The first four AP complexes are ubiquitously expressed and have been well characterized (12, 106), while AP-5 was recently identified and awaits further characterization. APs are made up of two large ~100-160 kD subunits (γ/β1, α/β2, δ/β3, ε/β-4), one medium ~50 kD subunit (µ1-4), and one small ~20 kD subunit (σ1-4) (reviewed in (12, 106)). Each subunit or adaptin has a separate function. The β1–3 subunits contain a clathrin-binding motif known as the clathrin box, which facilitates clathrin recruitment. β4 lacks a clathrin box and therefore does not interact with clathrin. The other large subunit of each AP is responsible for binding the complex to the target membrane. The µ subunit is responsible for selecting cargo through its recognition and interaction with tyrosine-based sorting signals (YXXΦ, X is any amino acid, and Φ is any amino acid with a bulky hydrophobic side chain) or dileucine motifs (D/EXXXLL/I, where the first amino acid can be D or E, and X is any amino acid, and the last amino acid can be L or I) that are present in the cytoplasmic domains of the cargo. The σ subunit is responsible for stabilization of the complex. The APs assemble into a structure that has been reported to resemble the head of “Mickey Mouse,” due to its brick-like “head,” from which two “ears” protrude, and are connected by flexible hinges. The “head” region is comprised of the amino-terminal domains of the two large subunits, medium subunit, and small subunit, and the two “ears” are comprised of the carboxy-terminal domains of the large subunits (67, 130, 145).
3.2 Identification of AP-3

AP-3 was initially identified as a result of two studies (157, 158) conducted with proteins that were identified by two different groups: β-neuronal adaptin-like protein (β-NAP) (109) and p47 (136), which shared ~30% homology with the β and µ subunits of AP-1 and AP-2, respectively (32, 109, 136, 157, 158). The existence of p47 in non-neuronal (p47A) and neuronal (p47B) forms prompted a search for a non-neuronal form of β-NAP, which was identified through a search of the human expressed sequence tag (EST) database. β-NAP and p47 complexed with one another and could be recruited from the cytosol onto membranes in vitro. This complex immunoprecipitated with two other proteins: a ~160 kD protein, p160, and a ~25 kD protein, p25, suggesting that the complex was a heterotetramer (158). As predicted, p160 and p25 were found to be homologous with the γ/α and σ1/σ2 subunits of AP-1 and AP-2, respectively. These findings ultimately led to the identification of a third novel adaptor protein complex, which was aptly named AP-3. Non-neuronal and neuronal β-NAPs were renamed β3A and β3B, respectively; p47A and p47B were renamed µ3A and µ3B; p160 was named δ; and p25, which was identified as two isoforms was named σ3A and σ3B, although both forms are ubiquitously expressed (32, 158). The identified AP-3 complex was the neuronal form, while subsequent studies identified the ubiquitous form of the complex (32). The ubiquitous and neuronal forms of AP-3 have been designated as AP-3A and AP-3B, respectively, in this thesis, only the ubiquitous form will be addressed and will be referred to as AP-3.
3.3 Function and Regulation

Function

The cellular function of AP-3 is to transport transmembrane proteins to lysosomes or lysosome-related organelles from early endosomes (130) or the trans-Golgi network (32, 157, 158) (Fig.1-6). There are two distinct lysosomal trafficking pathways, and both are used by AP-3. In the direct pathway, AP-3 traffics newly synthesized LMPs from the trans-Golgi network (TGN) to the late endosome/MVB, which subsequently fuses with the lysosome for delivery of the LMPs. In the indirect pathway, newly synthesized LMPs are first transported directly to the plasma membrane and are subsequently endocytosed and are localized to the early sorting endosome (73). AP-3 traffics the LMPs from the early endosomes to MVBs, and the LMPs are delivered to the lysosome following its fusion with the MVB. The \( \mu_3 \) subunit selects AP-3-directed cargo through its recognition of tyrosine motifs or dileucine motifs within the cytoplasmic tails of the cargo (106).

The current understanding of AP-3 function in trafficking specific lysosomal membrane proteins to lysosomes has been made possible through AP-3 subunit deletion studies in yeast, and the existence of naturally occurring AP-3 subunit mutations in Drosophila, murines, and humans (145). In Drosophila, four mutants: garnet, ruby, carmine, and orange, which exhibit altered eye-color pigment have naturally occurring mutations in the genes that encode the \( \delta \), \( \beta_3 \), \( \mu_3 \), and \( \sigma_3 \) subunits, respectively (145). Further investigation of the garnet mutant revealed a reduction in the number of red and brown pigment-containing pigment granules of the eye (124). This suggested a role for AP-3 in the biogenesis of pigment granules, which are lysosome-related organelles.
Further insight into AP-3 function has been attributed to studies of the mocha mouse, a murine mutant that does not express the δ subunit due to a null mutation in the δ-encoding gene (83). As a consequence, the remaining subunits are degraded and therefore the mice lack the entire complex (131). These observations demonstrated that the subunits only exist in the context of the complex. As a consequence of AP-3 deficiency, mocha mice exhibit decreased levels of renal lysosomal enzymes in the urine, hypopigmentation of the eye and coat, prolonged bleeding times, as well as neurological defects (83). These symptoms are attributable to defects in protein trafficking to lysosomes, melanosomes, platelet dense granules, and synaptic vesicles, with the latter four compartments representing LROs.

In humans, a mutation in the β3A subunit causes a rare disorder known as Hermansky-Pudlak Syndrome Type 2, which results in hypopigmentation of the eye, hair, and skin, platelet dysfunction, and immunodeficiency resulting from neutropenia and cytotoxic T-lymphocyte dysfunction (72, 174). Cellular studies have also shown that the absence of AP-3 results in the re-routing of the most abundant lysosomal membrane proteins: lysosome associated membrane proteins 1 and 2 (LAMP-1 and LAMP-2), lysosomal integral membrane protein 2 (LIMP-2), and tetrapanin CD63 to the PM (150). Taken together, these observations have strongly implicated AP-3 function in the trafficking of cargo destined for lysosomes and lysosome-related organelles.

Although AP-3 is often referred to as a clathrin adaptor, the role of clathrin in AP-3 function is controversial. Initial studies that led to the discovery of AP-3 demonstrated by EM that AP-3 coated vesicles were thinner than that of clathrin-coated vesicles and
Figure 1-6. AP-3 trafficking pathways. (A) Schematic representation of AP-3 complex. (B) AP-3 trafficking occurs from the trans-Golgi network (TGN) or early endosome (EE) to the multivesicular body (MVB)
did not co-localize with clathrin. In addition, the AP-3-coated vesicles did not copurify with clathrin (157, 158). Additional studies have demonstrated that inactivation or depletion of clathrin does not affect AP-3-dependent protein transport or AP-3 association with membranes (169, 175). Immuno-colocalization studies of AP-3 and clathrin have produced mixed results (31, 131, 157). Nevertheless, the β3 subunit contains a clathrin-binding motif, LLDLD, known as the clathrin box, and has been shown to bind clathrin in vitro (31, 164). AP-3 has also been demonstrated to recruit clathrin onto synthetic liposomes (31, 41). However, protein sorting in AP-3 deficient cells has been shown to be partially rescued by a β3 mutant lacking the clathrin box, further providing evidence for clathrin-independent AP-3 function (131). These collective observations have made it difficult to assess the role of clathrin in AP-3 function.

**Regulation**

AP-3 recruitment to membranes is regulated by its interaction with the small GTP-binding protein, ADP-ribosylation factor 1 (ARF1). ARF1 can exist either in an active state or inactive state, and this active or inactive state is dictated by ARF1 binding to GTP or GDP, respectively. In the active GTP-bound state, ARF1 binds to membranes where it actively recruits coat components, such as AP-3 (123), which promotes vesicle budding. In the inactive GDP-bound state, ARF1 dissociates from membranes. ARF1 membrane association or dissociation is dictated by its myristoylated N-terminal amphipathic helix, which acts as a molecular switch. When ARF1 is GDP-bound, the helix is folded into the protein, thus preventing membrane association. Conversely, when
ARF1 is GTP-bound, the myristoylated N-terminal helix becomes exposed and is anchored into the membrane (3). The factors that mediate ARF1 association with GTP or GDP are guanine nucleotide exchange factors (GEFs) and GTP-ase activating proteins (GAPs), respectively (39). GEFs exchange GDP for GTP, resulting in ARF1 activation. In contrast, GAPs promote ARF1 inactivation by inducing ARF1-mediated GTPase activity, resulting in the hydrolysis of GTP to GDP.

As ARF1 functions at multiple sites and recruits as many as seven different vesicle coat proteins onto membranes, the specificity of recruitment for each coat component is thought to be mediated by ARF GAPs (112). The ARF GAP that regulates ARF1-mediated recruitment of AP-3 to membranes is the ubiquitously expressed ARF GAP with GTPase domain ankyrin repeat and PH domain 1 (AGAP1) (112, 114). AGAP1 is comprised of a GTP-binding protein-like domain (GLD); a split pleckstrin homology domain (PH), which mediates binding to the phospholipids; an ARF GAP domain; and ankyrin repeats, which mediate protein-protein interactions (95, 114, 179). The interaction between AGAP1 and AP-3 is mediated by the PH domain of AGAP1 and the AP-3 hemi-complex of δ and σ (112). Overexpression of AGAP1 has been shown to disrupt AP-3 membrane association, which alters AP-3-mediated trafficking of LAMP-1, providing further evidence that AGAP1 plays a role in AP-3 function (112).

3.4. Role of AP-3δ in HIV-1 Replication

A recent study suggested that AP-3 plays a critical role in HIV-1 assembly and intracellular Gag trafficking (40). The N-terminal helix of the MA domain of Gag was
shown to bind directly to the hinge domain of AP-3δ. When the MA:δ interaction was disrupted by depleting endogenous AP-3δ by RNA interference or overexpressing a truncated AP-3 dominant-negative mutant, HIV-1 production was severely compromised. Immunofluorescence studies revealed that disrupting the MA:AP-3δ interaction by overexpressing the dominant-negative AP-3 mutant resulted in the failure of Gag to localize with LEs/MVBs. Together, these results suggested that AP-3δ-mediated Gag trafficking to LEs/MVBs was required for subsequent Gag targeting to the plasma membrane in order for productive particle production to occur. In infected primary human dendritic cells, virus particles have been reported to accumulate in AP-3δ and CD81-enriched compartments and AP-3δ depletion results in a reduction in virus production and an increase in intracellular Gag levels (52), suggesting that AP-3δ plays a role in HIV-1 replication in this cell type in a non-MVB compartment.
CHAPTER II: MATERIALS AND METHODS

2.1. Cell culture

293T, HeLa, HeLa-LAV and TZM-bl cell lines

293T and HeLa cell lines were obtained from the American Tissue Culture Collection. TZM-bl is a HeLa-derived cell line that expresses CD4, CCR5, and CXCR4 and contains stably integraded copies of the β-galactosidase and luciferase genes, which are under the transcriptional control of the HIV-1 LTR. This cell line was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc. The HeLa-LAV cell line is a HeLa derivative that expresses CD4 and CXCR4, is chronically infected with the HIV-1 BRU (LAV) strain, and expresses all viral proteins and produces infectious particles. This cell line was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Dr. Joerg Berg and Dr. Matthias Wabl. Cells were grown in 100 mm tissue culture plates and maintained with Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 3.7 g/L sodium bicarbonate, 2.0 g/L HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 25000 units/L penicillin, and 25000 ug/L streptomycin at 37°C, 5% CO2.

Primary human monocyte-derived macrophages (MDM)

Elutriated primary human monocytes were obtained through the University of Massachusetts Medical School CFAR Virology Core. Cells were seeded at a density of
1.25 \times 10^6 \text{ cells per well in 6-well tissue culture plates and differentiated with Media A [DMEM (GIBCO) supplemented with 10\% pooled human male AB serum (SeraCare), 2 mM L-glutamine (Invitrogen), and 10 ng/mL macrophage colony stimulating factor (MCSF) (R&D Systems)] at 37°C, 5\% CO}_2. \text{ Four days post cell seeding, and one day prior to transfection, cells were supplemented with 1 mL per well of macrophage media (Media A with no MCSF) and maintained at 37°C, 5\% CO}_2.

2.2. Virus Stock Preparation

VSV-G/NL4-3Δenv and NL4-3(YU2) virus stocks were produced in 293T cells transfected with 10 \mu g pHEF-VSV-G and 10 \mu g of pNL4-3Δenv or 20 \mu g pNL4-3(YU2) using the calcium phosphate transfection method, respectively. One day prior to transfection, cells were seeded in 100 mm tissue culture plates with antibiotic-free media (10\% FBS in DMEM), to yield a cell density of 70-75\% confluence on the day of transfection. For each transfection reaction, 20 \mu g of the indicated DNA was combined with 124 mL 2 M calcium chloride and brought to a final volume of 1 mL with sterile water, followed by the drop-wise addition of 1 mL 2X HEPES Buffered Saline [50 mM HEPES pH 7.05, 10 mM KCl, 12 mM Glucose, 250 mM NaCl, 1.5 mM Na\_2HPO\_4] and subsequent incubation for 1-2 minutes at room temperature. For each plate, cell culture media was replaced with 8 mL fresh antibiotic-free media, with the addition of 5 \mu L of 25 mM chloroquine. Transfection reactions were added to cells and cells were incubated at 37°C, 5\% CO}_2. Approximately 14-16 hours post transfection, cell culture media was removed, cells were washed twice with 1X Phosphate Buffered Saline, pH 7.2 (PBS),
PBS was removed, and cells were re-fed with 6 mL of fresh antibiotic-free media. At 48 hours post transfection, cell supernatants were collected and clarified by centrifugation at 1200 rpm for 5 minutes at room temperature. Supernatants were aliquoted into cryovials and stored in liquid nitrogen.

2.3. Virus Titration and Infectivity Assay

Virus Titration

TZM-bl cells were seeded at a density of 2.0 X 10⁴ cells per well in a 48-well tissue culture plate with antibiotic-free media (10% FBS in DMEM) one day prior to infection. Virus was thawed at 37°C and diluted 1:10 in antibiotic-free media in a total volume of 300 mL, followed by four 300 µL 10-fold serial dilutions. Cell culture media was removed, and cells were infected in duplicate with 100 µL of undiluted virus, serially diluted virus, or antibiotic-free media alone (negative control). At 3 hours post-infection, 0.5 mL of fresh antibiotic-free media was added to cells. At 48 days post-infection, cell culture supernatants were removed, cells were washed twice with 1X PBS and fixed with 0.5% gluteraldehyde in 1X PBS for 15 minutes at 4°C. Cells were subsequently washed twice with 1X PBS and were stained immediately or stored at 4°C in 1X PBS. Following fixation, infected TZM-bl cells were stained with 200 µL staining solution [0.5 mg/mL 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-Gal) in 1X PBS containing 3 mM potassium ferrocyanide, 3 mM potassium ferricyanide, and 1 mM magnesium chloride] and incubated overnight at room temperature. Staining solution was removed and cells were washed twice with 1X PBS. Cells were visualized by light microscopy and infected,
blue cells were counted to determine the titer of the virus stock in infectious units (IU) per µL.

*Infectivity Assay*

For the infectivity assay, MDM-derived NL4-3(YU2) and HeLa-LAV-derived viruses were used to infect TZM-bl cells as described in the virus titration protocol, with undiluted virus and one 10-fold serial dilution. TZM-bl cells were infected with equal reverse-transcriptase activity units in a total volume of 300 uL. The assay was continued using the virus titration protocol.

**2.4. Cell Supernatant and Cell Harvesting**

Cell supernatants were removed from tissue culture wells and transferred to 1.5 mL microfuge tubes. Due to the non-uniform evaporation of supernatants from well to well during incubation, the volume of each supernatant was measured and the supernatant volumes were normalized with antibiotic-free media for HeLa cell supernatants and macrophage media for MDM supernatants. Cell supernatants were clarified by centrifugation at 3000 rpm for 3 minutes at room temperature, followed by filtration through 0.45 µm PVDF filters. A portion (270 µL) of each supernatant was lysed with 30 µL 0.5% Triton X-100 lysis buffer: 0.5% Triton X-100 in 0.25 M Tris, pH 7.4 and kept on ice. Another portion (900 uL) of each supernatant was transferred to 1.5 mL screw-cap microfuge tubes and centrifuged at 25000 x g for 2 hours at 4°C to pellet the virus particles, followed by removal of the supernatant. Cells were washed twice with 1X PBS,
scraped and collected into 1.5 mL microfuge tubes. Cells were pelleted by centrifugation at 14000 rpm for 1 minute at room temperature followed by removal of the supernatant. Uninfected cells were harvested using the same protocol.

2.5. Reverse Transcriptase Activity Assay

In a round-bottomed 96-well plate, 10 µL of lysed supernatants were added to wells containing 10 µL of solution A [100 mM Tris-HCl pH 7.9, 300 mM potassium chloride, 10 mM dithiothreitol, 0.1% Nonidet P-40 (Igepal CA-630) (Sigma Aldrich)] and incubated at 37°C for 15 minutes. Following incubation, 25 µL of solution B [50 mM Tris-HCl pH 7.9, 50 mM dithiothreitol, and 15 mM magnesium chloride] containing 10 µCi [³H]-dTTP/mL, 10 µg/mL polyadenylic acid (poly rA), and 5 µg/mL oligo-dT₁₈ was added to wells, and the reactions were incubated overnight at 37°C. Supernatants were assayed in triplicate. The following day, reactions were pipetted onto circular 2.3 cm DE81 ion exchange filters (Whatman) and air-dried. Filters were washed twice with 2X SSC buffer [300 mM sodium chloride, 30 mM sodium citrate] for 5 minutes per wash at room temperature, and once with ethanol for 30 seconds at room temperature. Filters were air-dried and placed into scintillation vials, and 5 mL scintillation fluid (EcoScint H, National Diagnostics) was added to each vial. Samples were analyzed in a liquid scintillation counter programmed to detect tritium for 1 minute per scintillation vial.

2.6. β-galactosidase activity assay
Infected TZM-bl cell lysates (15 μg total protein) were incubated with 1.5 μL 100 mM MgCl₂, 33 µL ONPG (ortho-nitrophenyl-β-D-galactopyranoside) (Sigma), 100.5 µL 0.1 M Na₂HPO₄/NaH₂PO₄, pH 7.5, for 1 hour at 37°C. After incubation, 100 µL of each reaction was added to 900 µL dH₂O and spectrophotometrically analyzed at an absorbance of 420 nm.

2.7. SDS-PAGE and Western blot analysis of viral and cellular lysates

Cell pellets were lysed with 0.5% Triton X-100 lysis buffer on ice for 30 minutes and clarified by centrifugation at 14000 rpm for 10 minutes at 4°C, followed by transfer of clarified cell lysates to new tubes. Total protein concentration in the cell lysates was determined by Bradford protein assay. A total protein amount of 10 or 15 μg for each cell lysate was aliquoted into new tubes with 5X SDS loading buffer [62.5 mM Tris-HCl pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate, 5% β-mercaptoethanol, 0.0625% bromophenol blue], volumes were normalized with water, and samples were boiled for 4 minutes. Pelleted virus particles were lysed with 10 µL 0.5% Triton X-100 lysis buffer on ice. A volume of 2.5 mL of 5X SDS loading buffer was added to each lysate and samples were boiled for 4 minutes. Cellular and viral lysates were analyzed by 6%, 8%, 10%, or 12% SDS-PAGE, for the downstream detection of AP-3δ, HA-AGAP1 and HA-AGAP1[R599K], cell-associated Gag, or virus-associated Gag, respectively. Gels were electrophoresed with Tris-Glycine-SDS-PAGE Buffer [25 mM Tris, 192 mM glycine, 0.1% SDS] (National Diagnostics) followed by transfer onto Immobilon-P PVDF membranes with transfer buffer [10% methanol in Tris-Glycine-SDS-PAGE Buffer] for
50-55 minutes at 100 mA, using the semi-dry transfer method. Membranes were blocked in blocking solution: 10% dry milk in 1X TBS-T [0.1M Tris pH 7.4, 0.1M Tris pH 8.0, 0.5% Tween-20] for at least 1 hour at room temperature with gentle agitation. Blots were incubated overnight at 4°C in primary antibody, with gentle agitation. The following day, blots were washed 3 times with 1X TBS-T for 5 minutes per wash. Blots were incubated for 1 hour at room temperature in horseradish peroxidase (HRP)-conjugated secondary antibody diluted in 2 mL of blocking solution, with gentle agitation. Blots were washed 3 times with 1X TBS-T for 5 minutes per wash, incubated for 1 minute in ECL reagent (GE Healthcare Life Sciences), followed by exposure to Hyperfilm (GE Healthcare Life Sciences). The following antibodies were used: mouse monoclonal anti-adaptin δ (BD Transduction Laboratories), mouse monoclonal anti-p24 (NIH AIDS Research and Reference Reagent Program), mouse monoclonal anti-HA.11 (Covance), goat polyclonal anti-hRIP (Santa Cruz Biotechnology), rabbit polyclonal anti-CRM1 (Santa Cruz Biotechnology), and rabbit polyclonal anti-Sam68 (Santa Cruz Biotechnology).

2.8. siRNA Transfection

*HeLa, HeLa-LAV, and TZM-bl cell lines*

One day prior to transfection, cells were seeded at a density of 1.25 X 10^5 cells/well in 6-well plates with antibiotic-free media (10% FBS in DMEM) to achieve a cell density of 40-50% confluence on the day of transfection. Cells were transfected using Oligofectamine (Invitrogen) transfection reagent per the manufacturer’s protocol for transfection in a 6-well tissue culture plate format. For each transfection reaction, 4 uL of
Oligofectamine was diluted in 11 uL OPTIMEM-I (Invitrogen) reduced serum media and incubated for 10 minutes at room temperature. siRNA was diluted in a final volume of 185 µL with OPTIMEM-I and combined with the 15 µL oligofectamine/OPTIMEM-I mixture, followed by incubation at room temperature for 20 minutes. Cell culture media was removed, cells were washed once with DMEM, DMEM was removed, and the cells were fed with 0.8 mL/well of fresh DMEM. Transfection reactions were added to cells, followed by incubation at 37°C, 5% CO₂. Four hours post transfection, 0.5 mL of 30% FBS in DMEM was added to each well. Cells were maintained at 37°C, 5% CO₂ for the duration of the transfection.

**MDM**

Cells were cultured and differentiated as described in 2.1. siRNA transfection was performed using Lipofectamine 2000 (Invitrogen). For each transfection, 5 µL Lipofectamine 2000 was diluted in 495 µL OPTIMEM-I and incubated at room temperature for 5 minutes. siRNA was diluted in a final volume of 500 µL with OPTIMEM-I and combined with the Lipofectamine 2000/OPTIMEM-I mixture, followed by incubation at room temperature for 20 minutes. Conditioned cell culture media was removed from cells and mixed with fresh macrophage media at a 1:1 ratio to generate 50/50 macrophage media. Prior to the addition of the transfection reactions, cells were washed twice with 1X PBS, followed by PBS removal with a serological pipette, due to cell sensitivity to aspiration. Transfection reactions were added to cells, followed by incubation at 37°C, 5% CO₂. Four hours post transfection, transfection reactions were removed and cells were washed twice with 1X PBS. PBS was removed and 1.5 mL per
well of 50/50 macrophage media was added to the cells. Cells were maintained at 37°C, 5% CO₂ for the duration of the transfection.

2.9. siRNA duplexes

_Nonsilencing siRNA_

The proprietary AllStars Negative Control siRNA (siANC) was obtained from Qiagen and has no known homology to any mammalian gene.

_Ap-3δ-specific siRNA_

AP-3δ 1-4 siRNAs [siAP-3δ(1)-(4)] and siAP-3δ5 were previously published in (40) and (20), respectively. These twenty-one nucleotide siRNA duplexes with 3’dTdT overhangs were manufactured by Qiagen. The target region of AP-3δ, sense, and anti-sense sequences in the direction of 5’ to 3’ are included for each AP-3δ siRNA.

**siAP-3δ(1) target region: nt 176-194**

sense: UCUGCAAGCUGACGUUAUUTT; anti-sense: AAAUACGUCAGCUUGCAGATT

**siAP-3δ(2) target region: nt 489-507**

sense: GAAGAAGGCUGUCUGUAUCTT; anti-sense: AAAUACGUCAGCUUGCAGATT

**siAP-3δ(3) target region: nt 2438-2456**

sense: GCGAGAAACUGCCUAUUCATT; anti-sense: UGAAUAGGAGUUUCUGCGTT

**siAP-3δ(4) target region: nt 2493-2511**

sense: GAAGGACGUUCCCAUGGUATT; anti-sense: UACCAUGGGAACGUCCUUCTT

**siAP-3δ(5) target region: nt 3159–3177**

sense: CCCUGUCCUUCAUUGCCAATT; anti-sense: TTGGCAATGAAGGACAGGGTT
**hRIP-specific siRNA**

This twenty-one nucleotide siRNA duplex with 3’dTdT overhangs was published in (151), and manufactured by Qiagen. The target region of hRIP, sense, and anti-sense sequences in the direction of 5’ to 3’ are included.

sihRIP target sequence: nt 647-665

sense: GCCAAAGUCGUGGCAUCAGTT; anti-sense: CUGAUGCCACGACUUUGGCTT

**2.10. DNA Transfection**

One day prior to transfection, cells were seeded at a density of 1.5 X 10^5 cells/well in 6-well plates with antibiotic-free media (10% FBS in DMEM) to achieve a cell density of 60-70% confluence on the day of transfection. Cells were transfected using FugeneHD (Roche) transfection reagent per the manufacturer’s protocol for transfection in a 6-well tissue culture plate format. For each transfection, 2 µg of DNA was diluted in 100 mL of OPTIMEM-I followed by the addition of 3 mL of FugeneHD. Reactions were incubated at room temperature for 15 minutes, followed by the addition of the reactions to the cells. Cells were kept at 37°C, 5% CO2 for the duration of the experiment. For virus experiments, cells were transfected with 0.5 µg pHA-AGAP1stop, pHA-AGAP1, or pHA-AGAP1[R599K] + 1.5 µg pUC118, or 1.0 µg pHA-AGAP1stop, pHA-AGAP1, or pHA-AGAP1[R599K] + 1.0 µg pUC118. For immunofluorescence experiments, cells were transfected with 2 µg of pHA-AGAP1stop, pHA-AGAP1, or pHA-AGAP1[R599K]. For co-transfection experiments, cells were transfected with 2 µg of
pHA-AGAP1stop, pHA-AGAP1, or pHA-AGAP1[R599K] using the FugeneHD transfection method. At 24 hours post-transfection, cells were trypsinized and reseeded onto glass coverslips, followed by transfection with Attractene (Qiagen) transfection reagent, per the manufacturer’s protocol for transfection in a 6-well tissue culture plate format. For each transfection, 1.2 µg pCO-Gag-GFP or pCO-Gag_K29E/K31E-GFP was diluted in DMEM followed by the addition of 4.5 µL Attractene (Qiagen) per transfection. The reactions were incubated for 15 minutes at room temperature and were added to the cells. Cells were incubated at 37°C, 5% CO₂ for the duration of the experiment.

2.11. Plasmids

pHEF-VSV-G: the expression vector for the glycoprotein of vesicular stomatitis virus (VSV-G), was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: pHEF-VSVG from Dr. Lung-Ji Chang

pNL4-3: the chimeric proviral clone encoding a the NY5 and LAV HIV strains, was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: pNL4-3 from Dr. Malcolm Martin

pNL4-3Δenv: a pNL4-3 derivative that contains two tandem premature stop codons in env, as a result of a single nucleotide insertion in the Nde I site of env. pNL4-3Δenv was generated using the QuikChange site-directed mutagenesis kit (Stratagene), pNL4-3 as the template, and the following oligos:

Forward: 5’ CAGATGCTAAAGCATAATGATACAGAGGTAC 3’
reverse: 5’ GTACCTCTGTATCATTATGCTTTAGCATCTG 3’

**pNL4-3(YU2):** a pNL4-3 derivative that encodes the env gene of the YU2 strain in place of NL4-3 env. (received from Dr. Joseph Sodroski)

**pEGFP-CO-Gag:** an expression vector that generates codon-optimized NL4-3 Gag with an N-terminal EGFP tag. (received from Dr. Heinrich Gottlinger)

**pEGFP-CO-GagK29E/K31E:** a pEGFP-CO-Gag derivative that contains two K to E amino acid substitutions at positions 29 and 31 (numbering based on G at position 1, after M is removed during N-terminal myristylation), which have been shown to target Gag to MVBs (120). This expression vector generates codon-optimized NL4-3 GagK29E/K31E with an N-terminal EGFP tag. This construct was generated using the QuikChange site-directed mutagenesis kit (Stratagene), pEGFP-CO-Gag as the template, and the following oligos:

forward: 5’ GGCAAGAAGCAGTACGAGCTGGAGCACATCGTGTGGGCC 3’
reverse: 5’ GGCCCACACGATGTGCTCCAGCTCGTACTGCTTCTTGCC 3’

**pHA-AGAP1:** an expression vector that generates wild-type AGAP1 with an N-terminal HA tag. AGAP1 was cloned by PCR into the Xho I and Not I sites of pCMV-HA (Clontech) using pCI-FLAG-AGAP1 (received from Dr. Paul Randazzo) as the template and the following oligos:

forward: 5’ CCCGGGGAATTCGGAACGAGCTGGACAGCTAGGCCACACCTGGCT3’
reverse: 5’ CCCGGGGCCGCGGCTGAGATGTGCTGACCCCTCCACTGGCT3’

**pHA-AGAP1[R599K]:** an expression vector that generates mutant AGAP1 with an N-terminal HA tag that lacks GAP activity as a result of an R to K amino acid
substitution at position 599. AGAP1[R599K] was cloned by PCR into the Xho I and Not I sites of pCMV-HA (Clontech) using pCI-FLAG-[R599K]AGAP1 (received from Dr. Paul Randazzo) as the template and the following oligos:

forward: 5’ CCCGGGGAATTCGGAACTACCAGCAGCAGCTGGCCAACTCGGCT3’
reverse: 5’ CCCGGGGCGGCCGCTCAGATGATGGTGGGCACCCTCCACTGCT3’

**pHA-AGAP1[stop]:** is a pHA-AGAP1 derivative that encodes but does not express AGAP1 due to the introduction of two premature stop codons in the HA coding region. This construct was generated using the QuikChange II site-directed mutagenesis kit (Stratagene) using pHA-AGAP1 as the template and the following oligos:

forward: 5’ CCCACCATGTACCCATAGTGATGTTCCAGATTACGC 3’
reverse: 5’ GCGTAATCTGGAACATCACTATGGGTACATGGTGGG 3’

**pUC118:** cloning vector obtained from the ATCC

### 2.11. Indirect Immunofluorescence

Cells seeded on glass coverslips were washed twice with 1X PBS, followed by fixation in ice cold methanol for 5 minutes at -20°C. Cells were washed once with 1X PBS for 5 minutes at room temperature and stored in 70% ethanol overnight. On the day of staining, cells were washed 3 times with 1X PBS for 5 minutes per wash and incubated in blocking solution (1% (w/v) bovine serum album (BSA) in 1X PBS) for 1 hour at room temperature. Cells were incubated overnight with gentle agitation in primary antibody diluted in blocking solution at 4°C. The following day, cells were washed 3
times in 0.1% BSA in 1X PBS for 5 minutes per wash, followed by incubation with Alexa Fluor 488- and/or Alexa Fluor 594-conjugated secondary antibodies diluted in 1.5 mL of 1% BSA in 1X PBS for 1 hour at room temperature protected from light. Cells were washed three times with 1X PBS for 5 minutes per wash, with the addition of DAPI diluted 1:10000 (from a 1 µg/µL stock) during the second 1X PBS wash. Following the third 1X PBS wash, cells were washed with water for 5-10 minutes. Cells were mounted onto glass slides, with ProLong Gold Antifade mounting medium (Invitrogen). Slides were dried overnight at room temperature. Cells were viewed with a Zeiss Axioplan 2 wide-field epifluorescence microscope equipped with a 63X Plan Apochromat objective. Images were acquired and analyzed using OpenLab 2.2.5. software. For the detection of endogenous AP-3δ, HA, and CD63 in HeLa cells, goat polyclonal anti-AP-3δ primary antibody (Santa Cruz Biotechnology, SCB) with AlexaFluor (AF) 488 or 594-conjugated donkey anti-goat secondary antibody; rabbit polyclonal anti-HA primary antibody (Santa Cruz Biotechnology) with AF594-conjugated chicken anti-rabbit secondary antibody; and mouse-monoclonal anti-CD63 primary antibody (SCB) with AF594-conjugated goat anti-mouse secondary antibody were used, respectively. For the detection of AP-3δ, HIV-1 Gag, and CD63 in HeLa-LAV cells, mouse monoclonal anti-AP-3δ primary antibody (BD Transduction Laboratories) with AF546-conjugated goat anti-mouse secondary antibody; rabbit polyclonal anti-p24 primary antibody (Advanced Biotechnologies) with AF488-conjugated goat anti-rabbit; and mouse-monoclonal anti-CD63 primary antibody (SCB) with AF546-conjugated goat anti-mouse secondary antibody was used. All AF-conjugated secondary antibodies were from Invitrogen.
3.1. Introduction

The highly coordinated process of HIV-1 particle production is directed by the viral structural protein Gag that is both necessary and sufficient for particle production as evidenced by its ability to form virus-like particles in the absence of other viral components (55, 155). Particle production involves a series of steps that include Gag targeting to membranes that serve as viral assembly sites, Gag binding to membranes, Gag-multimerization, and Gag-mediated viral budding and release (170). Gag mediates each of these steps through the functions of its distinct domains: MA, CA, NC, and p6 (47, 57). The MA domain targets and binds Gag to membranes, the CA and NC domains facilitate Gag multimerization (119), and the p6 domain mediates viral budding and release through its recruitment and interaction with the endosome sorting complex required for transport (ESCRT) machinery (161, 162). Although significant progress has been made in understanding the role of Gag in the steps of particle production, the mechanism by which Gag is targeted to sites of virus assembly is unknown.

Gag is targeted to two sites of virus assembly in T-cells, macrophages, and cell lines (ie. HeLa and 293T): the plasma membrane (PM) (43, 59, 81, 118, 121, 173) and multivesicular bodies (MVBs) (59, 61, 115, 120, 126, 132, 134, 144, 154). However, in T-cells and experimental cell lines, the plasma membrane (PM) is the predominant site of virus assembly whereas the MVB serves as the predominant site of virus assembly in macrophages (120). Ultimately, Gag must be targeted to the PM as assembled virus
particles must traverse the PM to exit the cell. Furthermore, abolishment of Gag targeting to the PM has been demonstrated to significantly inhibit virus production (118). The necessity for Gag targeting to and subsequent virus assembly in MVBs is less clear, despite evidence to support a productive role for virus assembly in these compartments. Studies have demonstrated that viruses derived from the MVBs of chronically infected T-cells, macrophages, and HIV-1 expressing 293T cells are infectious (61, 132). In addition, treatment of chronically infected T-cells and HIV-1-expressing 293T cells with ionomycin, a trigger of MVB-mediated exocytosis, stimulates virus release (61). Exocytosis of MVB-containing virus particles has also been observed in infected macrophages (110, 132), suggesting that exocytosis may be the principle mechanism of particle release for virions that assemble into MVBs. In addition, live imaging of newly synthesized Gag demonstrated that Gag traffics along an intracellular route that begins at the perinucleus, proceeds to MVBs, and ends at the PM, suggesting that Gag trafficking to MVBs occurs prior to its localization at the PM (134). Furthermore, the ESCRT machinery is typically associated with MVBs as a result of its function in MVB biogenesis (reviewed in (62)). Therefore, Gag may be targeted to MVBs to recruit the ESCRT machinery required for virus budding and release. Taken together, these results raise the possibility that MVBs are productive sites for virus assembly and suggest that Gag trafficking to MVBs serves as an intermediate step in subsequent localization to the PM. However, the specific mechanism by which Gag traffics to MVBs remains unclear.

One possible mechanism of Gag trafficking to MVBs was revealed in a study of Adaptor Protein Complex 3 (AP-3), a cellular binding partner for Gag that was
implicated in mediating Gag trafficking to MVBs (40). AP-3 is a heterotetrameric protein complex that mediates vesicular transport of lysosomal membrane proteins to MVBs (6, 147). AP-3 is comprised of two large subunits: β and δ, one medium subunit, μ, and one small subunit, σ (32, 158). The N-terminal H1 helix of the MA domain of Gag was demonstrated to specifically bind the δ subunit of AP-3 (AP-3δ), and disruption of the AP-3δ:MA interaction was reported to inhibit Gag trafficking to MVBs and negatively effect virus production (40). These results suggest that Gag trafficking to MVBs is necessary for productive for viral replication and is dependent on AP-3δ for this process. However, it is unknown whether AP-3δ plays a similar role in infected primary macrophages, which are natural targets of HIV and promote virus assembly primarily at MVBs. In addition, the role of AP-3δ function in virally infected cells that promote virus assembly primarily at the plasma membrane remains to be determined.

Here, we investigate the role of AP-3δ in HIV-1 production in infected HeLa cells, chronically infected HeLa-LAV cells, and primary human monocyte-derived macrophages (MDMs). We also investigate the role of the regulator of AP-3 function, AGAP1, in HIV-1 production in infected HeLa cells. Our results demonstrate that RNAi-mediated depletion of endogenous AP-3δ does not effect HIV-1 production in infected HeLa, HeLa-LAV, and primary human MDMs nor does it affect viral infectivity or cell permissiveness to viral infection. Curiously, through an individual test of a panel of AP-3δ-specific siRNAs we observed inhibition of virus production with two of the five siRNAs tested. These siRNAs share the same sequences with siRNAs that have been used in two studies that demonstrated severe inhibition on virus production in cells
treated with these siRNAs. Finally, we demonstrated that specific regulation of AP-3 by AGAP1 does not alter HIV-1 production, further suggesting that AP-3 is not required for HIV-1 production.
3.2. Results

3.2.1. Depletion of endogenous AP-3δ has no discernible effect on HIV-1 production

Previous studies have demonstrated that AP-3δ plays a significant role in HIV-1 production in transfected HeLa cells (20, 40), however, the role of AP-3δ in the production of virus particles in infected cells is unknown. In order to investigate the role of AP-3δ in the more biologically relevant context of host cell infection, HeLa cells were treated with transfection reagent alone [mock], a non-silencing control siRNA (siANC), and an AP-3δ-specific siRNA (siAP-3δ(1)), for 48 hours when near complete depletion of AP-3δ was observed (Fig. A2-1). Cells were subsequently infected with VSV-G-pseudotyped HIV-1 NL4-3Δenv (VSV-G/NL4-3Δenv) virus for 48 hours followed by harvesting of the cell supernatants and cells. The cell supernatants were assayed for the presence of virus particles, using a standard enzymatic reverse-transcriptase (RT) activity assay (90), and cell and viral lysates were assayed for protein expression by SDS-PAGE and Western blot. Nearly undetectable levels of AP-3δ were observed HeLa cells treated with siAP-3δ(1) whereas basal levels of AP-3δ were observed in the mock and siANC-treated cells (Fig. 3-1B). Depletion of AP-3δ did not affect the level of intracellular Gag expression nor did it affect extracellular p24 expression (Fig. 3-1B). Supernatants from siAP-3δ(1)-treated cells retained full RT activity when compared to both mock and siANC-treated cells (Fig. 3-1A). As a positive control for virus inhibition, cells were treated with hRIP-specific siRNA to demonstrate that the experimental conditions would allow for the detection of inhibited virus production (Fig. A2-2A). Collectively, these
Figure 3-1. Effect of AP-3δ depletion on HIV-1 production in infected HeLa cells.
HeLa cells were transfected with reagent alone (Mock), 50 nM non-silencing siRNA [siANC], and 50 nM AP-3δ specific siRNA (siAP-3δ(1)). At 48 hours post transfection, cells were infected with VSV-G/NL4-3Δenv (MOI = 0.25). At 48 hours post infection, cell supernatants and cells were harvested. (A) Cell supernatants were analyzed for HIV-1 production using a standard enzymatic reverse transcriptase (RT) activity assay. Bars represent the mean percentages of mock ± SEM for three independent experiments performed in duplicate. The RT activities (counts per minute/10µL of supernatant) of the cell supernatants derived from mock-treated cells were: 48653.94, 54598.62, and 62509.72. (B) Cell and viral lysates were analyzed by SDS-PAGE and Western blot with anti-AP-3δ, anti-p24, and anti-Sam68 antibodies.
results demonstrate that AP-3δ is not required for virus production in HeLa cells.

To further evaluate the role of AP-3δ in virus production we next examined whether AP-3δ depletion had an effect on HIV-1 production in cells chronically infected with HIV-1 BRU (LAV) that expresses CD4, CCR5, and CXCR4, and supports the production of wild-type infectious virus particles at high levels (9). HeLa-LAV cells were transfected with transfection reagent alone (mock), siANC, or siAP-3δ(1) for 72 hours, followed by supernatant removal and re-feeding of the cells. At 12 hours post-feeding, cell supernatants and cells were harvested. Similar to the HeLa cells, siAP-3δ(1) treatment efficiently and specifically depleted AP-3δ in HeLa-LAV cells and additionally had no effect on intracellular Gag expression (Fig. 3-2B). No discernible difference in supernatant RT activity (Fig. 3-2A) and p24 expression in viral lysates (Fig. 3-2B) between mock, siANC, and siAP-3δ-treated cells was observed. These results demonstrate that AP-3δ is also not required for virus production in chronically infected HeLa-LAV cells.

We next investigated whether AP-3δ depletion had an effect on virus production in a natural target of HIV-1 infection, namely, primary human monocyte-derived macrophages (MDM). In addition to their role as natural targets of HIV-1 infection, MDMs promote virus particle production in multivesicular bodies. Monocytes obtained from donor blood were differentiated into MDM for five days and were treated with transfection reagent alone (mock), siANC, and siAP-3δ(1). At 48 hours post-transfection, the MDMs were infected by spinoculation with HIV-1 NL4-3(YU2), a chimeric
Figure 3-2. Effect of AP-3δ depletion on HIV-1 production in chronically infected HeLa-LAV cells. HeLa-LAV cells were transfected with reagent alone (Mock), 100 nM siANC, and 100 nM siAP-3δ. At 72 hours post transfection, cell culture media was changed. At 12 hours post-media change, cell supernatants and cells were harvested. (C) Cell culture supernatants were analyzed for HIV-1 production by RT activity assay. Bars represent the mean percentages of mock ± SEM for three independent experiments. The RT activities (counts per minute/10µL of supernatant) of the cell supernatants derived from mock-treated cells were: 22869.78, 24675.79, and 36179.89. (D) Cell and viral lysates were analyzed by SDS-PAGE and Western blot with anti-AP-3δ, anti-p24, and anti-Sam68 antibodies.
Figure 3-3. Effect of AP-3δ depletion on HIV-1 production in primary human monocyte-derived macrophages (MDM). Primary human monocyte-derived macrophages (MDMs) differentiated for five days followed by transfection with reagent alone (Mock), 10 nM siANC, and 10 nM siAP-3δ. At 48 hours post transfection, cells were infected with NL4-3(YU2) (MOI = 1.0). At five days post infection, cell supernatants and cells were harvested. (E) Cell culture supernatants were analyzed for HIV-1 production by RT activity assay. Bars represent the mean percentages of Mock ± SEM for three independent experiments performed in duplicate. The RT activities (counts per minute/10µL of supernatant) of the cell supernatants derived from mock-treated cells were: 7341.34, 25015.44, and 37211.05. (F) Cell and viral lysates were analyzed by SDS-PAGE and Western blot with anti-AP-3δ, anti-p24, and anti-CRM1 antibodies.
virus that expresses the envelope of the macrophage-tropic YU2 strain in an NL4-3 background. At 24 hours post-infection, cell supernatants were removed and cells were washed and re-fed with fresh media. At five days post-infection, cell supernatants and cells were harvested and analyzed for virus production and protein expression. Efficient and specific depletion of AP-3δ was observed in the siAP-3δ(1)-treated MDM lysates, whereas intracellular Gag expression was unaffected (Fig. 3-3B). No discernible difference in RT activity (Fig. 3-3A) and p24 expression in viral lysates (Fig. 3-3B) between mock, siANC, and siAP-3δ-treated cells was detected. As a positive control for virus inhibition, cells were treated with hRIP-specific siRNA to demonstrate significant inhibition of virus production under similar experimental conditions (Fig. A2-2B). These results indicate that AP-3δ is not required for virus production in primary human MDMs.

3.2.2. Virus particles produced from AP-3δ depleted cells are infectious

Although our results suggest that virus production is unaffected by the depletion of AP-3δ in infected cells, it is unclear whether the particles produced are infectious. In order to determine the infectivity of the viral particles, TZM-bl indicator cells were incubated with the cell supernatants from infected AP-3δ-depleted cells and then assayed for productive infection. TZM-bl is a HeLa-derived cell line that stably expresses high levels of CD4, CCR5, and CXCR4 and has been engineered to stably express the β-galactosidase (β-gal) and firefly luciferase genes under the transcriptional control of the
Figure 3-4. Effect of AP-3δ depletion on HIV-1 infectivity. TZM-bl cells were infected with virus derived from Mock, siANC, or siAP-3δ-treated HeLa-LAV cells or NL4-3(YU2)-infected MDMs. At 48 hours post infection, cells were fixed and stained. (A) Bars represent the viral titer as mean of percentages of Mock ± SEM for three experiments, assayed in duplicate. Viral titers (in infectious units/µL) for mock-treated HeLa-LAV cell supernatants were: 4.40, 8.00, and 11.35. (B) Bars represent the viral titer as mean of percentages of mock ± SEM for two independent experiments assayed in duplicate. Viral titers (in infectious units/µL) for mock-treated MDM cell supernatants were: 48.00 and 65.50.
HIV-1 long terminal repeat (LTR) (171). Productive infection of TZM-bl cells induces the expression of β-gal and luciferase, which can be readily detected by direct quantification methods. Cells were separately infected with equivalent RT units of virus derived from mock, siANC, and siAP-3δ(1)-transfected HeLa-LAV and NL4-3(YU2)-infected MDM cells. At 48 hours post-infection, cells were fixed and treated with X-gal, a β-gal substrate that results in the blue staining of cells that express β-gal, an indicator of productive infection. Direct quantification of the infected cells demonstrated comparable levels of infection by the cell supernatants derived from mock, siANC, and siAP-3δ-transfected HeLa-LAV (Fig. 3-4A) and MDMs (Fig. 3-4B), suggesting that virus particles produced from AP-3δ-depleted cells retain wild-type infectivity.

3.2.3. AP-3δ-depleted cells are permissive to HIV-1 infection

We next investigated the role of AP-3δ-depletion on cell permissiveness to HIV-1 infection. TZM-bl cells were treated with reagent alone (mock), siANC, or siAP-3δ(1) and infected with VSV-G/NL4-3Δenv for 48 hours. The permissiveness of cells to infection was assayed by measuring the levels of β-gal activity in the cell lysates. Efficient and specific AP-3δ depletion was observed in siAP-3δ(1)-treated cell lysates whereas intracellular Gag expression was unaffected (Fig. 3-5B). In addition, the levels of β-gal activity were comparable across the mock, siANC, and siAP-3δ(1)-treated cell lysates (3-5A), demonstrating that cell permissiveness to HIV-1 infection is not affected by AP-3δ depletion.
**Figure 3-5. Effect of AP-3δ depletion on permissiveness to HIV-1 infection.** TZM-bl cells were transfected with reagent alone (Mock), siANC (50 nM), and siAP-3δ (50 nM). At 48 hours post-transfection, cells were infected with VSV-G/NL4-3Δenv (MOI = 0.25). At 48 hours post-infection, cell supernatants and cells were harvested. (A) Cell lysates were assayed for β-galactosidase activity. Bars represent β-galactosidase activity as mean percentages of Mock ± SEM for a duplicate experiment, assayed in duplicate. (B) Cell lysates were analyzed by Western blotting with anti-AP-3δ, anti-p24, and anti-Sam68 antibodies.
3.2.4. Evaluation of the effects of siRNA-mediated AP-3δ depletion on HIV-1 production using a panel of AP-3δ-specific siRNAs

To address the discrepancy regarding the effect of AP-3δ depletion on HIV-1 production between our results and previously published results (20, 40), we systematically examined a panel of five individual AP-3δ-specific siRNAs that share the target sequences with those previously studied. The silencing efficiency of each AP-3δ-specific siRNA was first investigated by transfection of HeLa cells with siANC or each of the five siRNAs, siAP-3δ(1)-(5). AP-3δ depletion was observed in all siAP-3δ(1)-(5)-treated cells with similar efficiencies (Fig. A2-3). We next tested the effect of siAP-3δ(1)-(5)-mediated AP-3δ depletion on HIV-1 production in chronically infected HeLa-LAV cells and infected HeLa and MDM cells. HeLa-LAV and MDM cells were treated with siANC or individually with siAP-3δ(1)-(5). HeLa-derived cell supernatants treated with siANC siAP-3δ(1), and siAP-3δ(4) exhibited full levels of comparable RT activity (Fig. 3-6A) whereas those treated with siAP-3δ(2), siAP-3δ(3), and siAP-3δ(5) exhibited ~63%, 52%, and 88% reduction in RT activity, respectively, when compared to siANC-treated cell supernatants (Fig. 3-6A). The observed level of AP-3δ depletion was similar for siAP-3δ(1)-(4), however siAP-3δ(5)-mediated AP-3δ depletion was slightly less efficient (Fig. 3-6B). In addition, the levels of intracellular Gag expression in siAP-3δ(2), siAP-3δ(3), and siAP-3δ(5)-treated HeLa lysates were reduced in comparison to that of siAP-3δ(1) and siAP-3δ(4)-treated cells (Fig. 3-6B). In HeLa-LAV cells, AP-3δ was efficiently depleted with siAP-3δ(1)-(5) to similar degrees
Figure 3-6. Effect of AP-3δ depletion on HIV-1 production using a panel of AP-3δ-specific siRNAs. HeLa cells were transfected with 50 nM of siANC or each of siAP-3δ(1)-(5) followed by infection with VSV-G/NL4-3Δenv (MOI = 0.25). At 48 hours post-transfection, cell supernatants and cells were harvested. (A) Cell supernatants were
analyzed for virus production by RT activity assay. Bars represent mean RT activity as mean percentages of siANC ± SEM for a single experiment, performed in duplicate. The RT activity (counts per minute/10µL of supernatant) of the cell supernatant derived from siANC-treated cells was 7958.85. (B) Cell lysates were analyzed for protein expression by SDS-PAGE and Western blot with anti-AP-3δ, anti-p24, and anti-Sam68 antibodies. HeLa-LAV cells were transfected with 100 nM of siANC or each of siAP-3δ(1)-(5) for 72 hours, followed by media change. At 12 hours post-media change, cell supernatants and cells were harvested. (C) Cell supernatants were assayed for virus production by RT activity assay. Bars represent mean RT activity as mean percentages of siANC ± SEM for three independent experiments. The RT activities (counts per minute/10µL of supernatant) of the cell supernatants derived from siANC-treated cells were: 23172.36, 31170.03, and 32625.70. (D) Cell lysates were analyzed for protein expression by SDS-PAGE and Western blot with anti-AP-3δ, anti-p24, and anti-Sam68 antibodies. MDM cells were differentiated for five days, followed by transfection with 10 nM of siANC or each of siAP-3δ(1)-(5) for 48 hours. Cells were infected with NL4-3(YU2) (MOI = 1.0) and cell supernatants and cells were harvested five days post-infection. (E) Cell supernatants were assayed for virus production by RT activity assay. Bars represent RT activity as mean percentages of siANC ± SEM for three independent experiments performed in duplicate. The RT activities (counts per minute/10µL of supernatant) of the cell supernatants derived from siANC-treated cells were: 16594.69, 20322.83, and 26710.42. (F) Cell lysates were analyzed for protein expression by SDS-PAGE and Western blot with anti-AP-3δ, anti-p24, and anti-CRM1 antibodies.
(Fig. 3-6D), and consistent levels of RT activity were observed in all treated cells (Fig. 3-6C). In MDM cells, AP-3δ was efficiently depleted with treatment of siAP-3δ(1)-(4), whereas treatment with siAP-3δ(5) was less efficient by comparison (Fig. 3-6F). RT activity of the MDM supernatants was comparable for mock, siANC, and siAP-3δ(1)-(5) (Fig. 3-6E). These results indicate that virus production is inhibited in an AP-3δ-depleted manner but only in the setting of select AP-3δ-targeting siRNAs.

Previous studies have demonstrated that the specificity of gene silencing by siRNA is reduced with increasing siRNA concentration, resulting in off-target effects (75, 135). In order to explore this possibility, we measured the level of virus production in cells treated with increasing concentrations of each siRNA. First, HeLa cells were treated with three 10-fold dilutions of each siRNA and assayed for AP-3δ expression. A dose-dependent depletion of AP-3δ was observed for each AP-3δ-specific siRNA, with siAP-3δ(5) being the least effective in depleting AP-3δ. (Fig. A2-4). To determine the individual effects of siAP-3δ(1)-(5)-mediated AP-3δ depletion on virus production, HeLa cells were mock-treated or treated with increasing concentrations of siANC, or each siAP-3δ(1)-(5). Cells were then infected with VSV-G/NL4-3Δenv, and cell supernatants and cells were harvested and analyzed. No significant differences in AP-3δ-depletion efficiency were observed in siAP-3δ(1)-(5)-treated cells, however the level of intracellular Gag expression in siAP-3δ(5)-treated cells was reduced (Fig. 3-7B). RT activity was minimally affected by siRNA concentration in siANC, siAP-3δ(1), and siAP-3δ(3)-treated cell supernatants, moderately affected in siAP-3δ(4)-treated cell
Figure 3-7. Effect of AP-3δ-specific siRNA concentration on HIV-1 production. HeLa cells were transfected with reagent alone (Mock), siANC (1 nM, 10 nM, 100 nM), and AP-3δ specific siRNAs: siAP-3δ(1)-(5) (1 nM, 10 nM, 100 nM). At 48 hours post transfection, cells were infected with VSV-G/NL4-3Δenv (MOI = 0.25). At 48 hours post infection, cell culture supernatants and cells were harvested. (A) Cell culture supernatants were analyzed for HIV-1 production by RT activity assay. Bars represent RT activity as mean percentages of mock ± SEM. Data shown is representative of two independent experiments performed in duplicate. The RT activities (counts per minute/10μL of supernatant) of the cell supernatants derived from mock-treated cells were: 5055.01 and 14761.50. (B) Cell and viral lysates were analyzed by SDS-PAGE and Western blot with anti-AP-3δ, anti-p24, and anti-Sam68 antibodies.
supernatants and significantly affected in siAP-3δ(2) and siAP-3δ(5)-treated cell supernatants (Fig. 3-7A). The most pronounced inhibition of viral production was observed in siAP-3δ(5)-treated cell supernatants, where over 70% reduction in virus production was observed at the highest siRNA concentration tested. A negative dose-dependence of p24 expression in viral lysates from siAP-3δ(2) and siAP-3δ(5)-treated cells was also observed (Fig. 3-7B). A negative dose-dependence of p24 expression in viral lysates from siAP-3δ(2) and siAP-3δ(5)-treated cells was also observed (Fig. 3-7B), and directly corresponded with RT activity.

3.2.5. Disruption of AP-3 function by overexpressing AGAP1 does not affect HIV-1 production

In addition to using an RNAi-based approach to study the role of AP-3 function in HIV-1 production, we also disrupted AP-3 function through the overexpression of its regulator, AGAP1 (Arf-GAP with GTP-ase domain, ankryin repeat, and pleckstrin homology domain 1). AGAP1 is an ADP ribosylation factor GTPase activating protein (ARF GAP) that specifically regulates ARF1 (ADP-ribosylation factor 1)-dependent recruitment of AP-3 to membranes by modulating the active state of ARF1 (112, 113). ARF1 is active and membrane associated when bound to GTP. Conversely, ARF1 is inactive and non-membrane associated when bound to GDP (38, 39). AGAP1 induces ARF1-mediated hydrolysis of GTP to GDP, resulting in ARF1 inactivation. Therefore, overexpression of AGAP1 increases the intracellular population of inactive GDP-bound ARF1, resulting in the inhibition of AP-3 recruitment to membranes and disruption of
Figure 3-8. Effect of wild-type or mutant AGAP1 overexpression on HIV-1 production. HeLa cells were transfected with 0.5 µg or 1.0 µg of pHA-AGAP1[stop], pHA-AGAP1, or pHA-AGAP[R599K]. At 24 hours post-transfection, cells were infected with VSVG/NL4-3Δenv (MOI=0.25). At 48 hours post-infection, cell supernatants and cells were harvested. Cell culture supernatants were analyzed for HIV-1 production by RT activity assay (A and C). Bars represent RT activity as mean percentages of mock ± SEM. Data shown is representative of two independent experiments performed in duplicate (A) or three experiments (C). The RT activities (counts per minute/10µL of supernatant) of the cell supernatants derived from cells transfected with pHA-
AGAP1[stop] were: 335.00 and 483.33 for 0.5 µg and 445.00 and 489.67 for 1.0 µg in (A). The RT activities (counts per minute/10µL of supernatant) of the cell supernatants derived from cells transfected with pHMA-AGAP1[stop] in (C) were: 45811.04, 55815.72, and 57879.72 for 0.5 µg and 52238.07, 79850.12, and 76441.11 for 1.0 µg. (B and D) Cell and viral lysates were analyzed by SDS-PAGE and Western blot with anti-HA, anti-p24, and anti-Sam68 antibodies.
AP-3 function. Conversely, overexpressing a GAP-inactive AGAP1 mutant does not affect AP-3 membrane association (112). To determine whether overexpression of AGAP1 affected virus production, HeLa cells were transfected with increasing concentrations of control vector pHAGAP1[stop], wild-type expression vector pHAGAP1, or GAP-inactive mutant expression vector pHAGAP1[R599K]. At 24 hours post-transfection, cells were infected with VSV-G/NL4-3Δenv and cell supernatants and cells were harvested and analyzed 48 hours post-infection. Wild-type and mutant AGAP1 expression increased in a dose-dependent manner while intracellular Gag levels remained constant (Figure 3-9B). We observed no significant differences in RT activity in cells transfected with pHAGAP1[stop], pHAGAP1 or pHAGAP1[599K] with either DNA concentration tested (Fig. 3-8A). The effect of wild-type or mutant AGAP1 overexpression on AP-3 membrane binding was confirmed by indirect immunofluorescence (Chapter IV, Fig. 4-4B). These results indicate that overexpression of wild-type and mutant AGAP1 has no effect on virus production, suggesting that AP-3 membrane association and function do not play a significant role in virus production.
3.3. Discussion

In this study, we investigated the role of AP-3 in HIV-1 replication through two different approaches aimed at disrupting AP-3 function. In one approach, AP-3δ was depleted in infected HeLa, HeLa-LAV, and primary monocyte-derived macrophages. In another approach, AGAP1 was overexpressed in infected HeLa cells to inhibit AP-3 association with membranes. We demonstrated using both strategies that AP-3 is not required for HIV-1 production, contrary to previous reports.

We initially performed AP-3δ depletion studies using a single AP-3δ-specific siRNA to determine whether AP-3δ was required for virus production. We were able to efficiently and reproducibly deplete endogenous AP-3δ in a variety of cell types thereby permitting us to investigate virus production in an AP-3δ-deficient cellular environment. Unexpectedly, we observed that AP-3δ depletion had no effect on virus production in all cells tested including a natural target of HIV-1 (MDM) and chronically infected host (HeLa-LAV). HeLa cells were first tested to clarify previous reports implicating AP-3δ as an essential host factor for virus production in these cells. Our results contradict those studies and raise the possibility that HIV-1 replicates in an AP-3δ-independent manner. Concern over the sensitivity of our viral output measurements was addressed through hRIP depletion studies resulting in the expected inhibition of virus production (180). We next depleted AP-3δ in chronically infected HeLa-LAV cells, which enabled us to investigate the effect of AP-3δ depletion in cells where infection was already established. In this system, AP-3δ depletion again had no discernible effect on virus production. After
examining the role of AP-3δ in virus production in cells that promote virus production primarily at the plasma membrane, we wanted to determine whether AP-3δ would be required for virus production in primary human MDMs, which promote virus assembly primarily at MVBs. As AP-3δ has been implicated in mediating Gag trafficking to MVBs, MDMs provide the ideal experimental system to study this particular role of AP-3δ. Surprisingly, AP-3δ was also found to dispensible for virus production in MDMs despite the prevalence of virus assembly at MVBs in these cells. Our results therefore bring into question whether AP-3δ truly mediates Gag trafficking to MVBs. Additionally, if AP-3δ does specifically mediate Gag trafficking to MVBs, our results question the overall significance of Gag trafficking to MVBs in virus production.

To further demonstrate whether AP-3 was required for virus production, AP-3 membrane association was disrupted by overexpressing AGAP1. Inhibition of AP-3 membrane association requires the inactivation of ARF1, which can be induced with brefeldin A, a fungal metabolite that targets and inactivates ARF proteins (37, 143). A previous study using brefeldin A demonstrated that AP-3-membrane dissociation had no effect on virus production (80). However, the effect of brefeldin A cannot be solely attributed to AP-3, since ARF1 interacts with multiple coat proteins that would also be affected by brefeldin A treatment. Therefore, our studies exploiting disruption of AP-3 recruitment to membranes through AGAP1 overexpression provides specificity to our approach (112-114). We demonstrated that overexpression of wild-type AGAP1 or GAP-inactive mutant AGAP1[R599K] had no effect on virus production, suggesting that AP-3 membrane association and consequently its function, is not required for virus production.
Further investigation would be required to determine whether AP-3 mediates Gag targeting to MVBs, as we would expect Gag targeting to MVBs to be severely compromised in the setting of overexpressed AGAP1.

Although our studies suggest that AP-3 is not required for HIV-1 production, we acknowledge that our findings directly dispute previous studies reported by Dong et al. and Camus et al. (20, 40). Based on our examination of a panel of unique AP-3δ-specific siRNAs, we suggest that the negative effect on virus production observed in previous studies are not attributed to AP-3δ depletion but are more likely a consequence of siRNA off-target effects. Two of the five siRNAs, siAP-3δ(2) and siAP-3δ(5), significantly inhibited virus production in an siRNA dose-dependent manner compared to three of the five siRNAs which exhibited little to no effect on virus production. The more potent inhibitor of virus production was siAP-3δ(5), which represents the one siRNA used in the Camus et al. study. siAP-3δ(2) represents one of the four pooled siRNAs used in the Dong et al. study and is therefore most likely responsible for the reported inhibitory phenotype seen with the siRNA pool; the three other siRNAs constituting the remainder of the pool had no effect in virus production. Considering that only one AP-3 silencing siRNA demonstrated a significant reduction in virus production, it is highly likely that an off-target effects of siAP-3δ(2) or siAP-3δ(5) are the primary mediators of viral inhibition rather than their on-target effect of AP-3 depletion. A dose-dependence in inhibition of viral production was observed in the context of siAP-3δ(2) and siAP-3δ(5) consistent with previous reports suggesting that off-target gene silencing by non-specific siRNAs occur in a dose-dependent manner. Alternatively, silencing of a second or
multitude of genes may act synergistically with AP-3 depletion to negatively affect productive HIV-1 replication. The observation that the off-target siRNAs were representative of the siRNAs used in both studies provides an explanation for the disparity between our results and the results of the two studies. Furthermore, our results highlight the importance of screening individual siRNAs designed against the same target to validate a phenotype with the associated depletion of a target.
CHAPTER IV: THE ROLE OF AP-3 IN HIV-1 GAG TRAFFICKING TO
MULTIVESICULAR BODIES

4.1. Introduction

HIV-1 particle production begins with the targeting of the structural protein Gag to membranes, which serve as the sites of virus assembly. Gag is synthesized as a polyprotein precursor comprised of four domains: matrix (MA), capsid (CA), nucleocapsid (NC), and p6. During its translation on free cytoplasmic ribosomes, the MA domain of Gag is modified by the enzyme N-myristoyl transferase, resulting in the myristoylation of its N-terminus. The N-terminal myristate in addition to a highly basic region between residues 14 and 31 of MA, are required for Gag binding to membranes, as abrogation of myristoylation by mutating the N-terminal glycine residue, or mutating of the basic residues of the HBR leads to defects in Gag membrane binding and subsequent inhibition of particle production (17, 58, 122, 182). These findings implicate Gag binding to membranes as a requirement for subsequent virus assembly and particle production.

Although the plasma membrane is considered to be the predominant site for Gag binding and virus assembly in most cell types (81, 173), Gag has also been demonstrated to associate with the limiting (outer) membrane of intracellular compartments known as multivesicular bodies (MVBs) (115, 154). In addition, virus budding into MVBs has been demonstrated to produce infectious particles (61, 132). However, the significance of the observed Gag:MVB association and its role in virus production has been a subject of debate. Studies have shown that targeting Gag to MVBs by introducing point mutations in MA, replacing the MA domain with membrane binding domains that have an affinity for endosomal membranes, or depleting cells of PI(4,5)P2 results in inhibited particle
production, suggesting that Gag targeting to MVBs may not be a productive process for viral replication. In addition, Gag association with MVBs has been shown to occur as a result of its endocytosis from the plasma membrane, which can be abolished without affecting virus production by inhibiting endocytosis or expressing Vpu (81, 107).

Evidence for a productive role in Gag targeting to MVBs has been demonstrated in a study of Adaptor Protein Complex 3 (AP-3), which is currently the only Gag-interacting cellular factor reported to be specifically involved in trafficking Gag to MVBs (40). We previously demonstrated that AP-3 does not play a role in virus production, which strongly suggests that AP-3δ-mediated Gag trafficking to MVBs is not essential for virus production (Chapter III). In this study, we investigated whether AP-3 plays a role in Gag trafficking to MVBs in HeLa cells, by observing Gag localization in AP-3δ-depleted cells, or in the presence of overexpressed AGAP1, the regulator of AP-3. In addition, we examined the effect of AP-3δ depletion on the localization of a mutant form of Gag (Gag_{K29E/K31E}) that is specifically targeted to MVBs (120). Our results show that disruption of AP-3 function, either by depleting AP-3δ or by overexpressing AGAP1 inhibited Gag trafficking to MVBs. However, Gag_{K29E/K31E} trafficking to MVBs was unaltered in the absence of AP-3δ. Collectively, these results indicate that AP-3 mediates wild-type Gag targeting to MVBs, but does not play a role in Gag_{K29E/K31E} targeting to MVBs, suggesting that wild-type and mutant Gag traffic to MVBs by different mechanisms.
4.2. Results

4.2.1. HIV-1 Gag trafficking to MVBs is abrogated in the absence of AP-3δ

Disruption of the AP-3δ:Gag interaction by the overexpression of a dominant-negative AP-3δ mutant has previously been shown to inhibit Gag association with MVBs (40). However, Gag localization to MVBs has not been investigated in the absence of AP-3δ. In order to investigate the role of AP-3δ expression on Gag trafficking, we expressed a codon-optimized GFP-Gag in HeLa cells previously transfected with reagent alone (mock), siANC, or siAP-3δ(1). We first determined whether GFP-Gag co-localized with MVBs by staining mock-treated, GFP-Gag-expressing cells for endogenous MVB-marker CD63 analyzed intracellular localization indirect immunofluorescence microscopy. We observed that GFP-Gag co-localized with CD63 and exhibited a punctate distribution that was concentrated near the perinucleus and exhibited an outward radiation (Fig. 4-1A). Mock, siANC, and siAP-3δ-treated, GFP-Gag-expressing cells were then stained for endogenous AP-3δ, to examine the intracellular localization of Gag in its presence or absence. In mock and siANC-treated cells, GFP-Gag exhibited similar localization patterns seen in the mock-treated GFP-Gag-expressing cells stained for CD63 (Fig. 4-1B, top and middle rows, respectively), suggesting that GFP-Gag is associated with MVBs in the presence of AP-3δ.
Figure 4-1. Effect of AP-3δ depletion on intracellular Gag localization in HeLa cells.
HeLa cells were transfected with reagent alone (mock), siANC, or siAP-3δ(1). At 48 hours post-transfection, cells were transfected with pEGFP-CO-Gag and fixed 24 hours post-transfection. (A) Mock-treated GFP-Gag (green)-expressing cells were co-stained for endogenous CD63 (red) and the nucleus (blue) using an anti-CD63 antibody with an Alexa Fluor (AF) 594-conjugated secondary antibody and DAPI, respectively. (B) Mock (top row), siANC (middle row), and siAP-3δ(1) (bottom row)-treated GFP-Gag (green)-expressing cells were co-stained for endogenous AP-3δ (red) and the nucleus (blue), using an anti-AP-3δ antibody with an AF594-conjugated secondary antibody and DAPI, respectively. Cells were analyzed by fluorescence microscopy.
In contrast, in siAP-3δ(1)-treated cells GFP-Gag exhibited a diffuse distribution throughout the cytoplasm that extended to the cell periphery, with a slight accumulation at the cell periphery (Fig. 4-1B, bottom row). This indicated that GFP-Gag was not associated with intracellular membranes, and suggests that Gag trafficking to MVBs was inhibited in the absence of AP-3δ.

To confirm these results, we next depleted AP-3δ using the panel of five siRNAs that were used in Chapter III to determine their effect on Gag to MVB trafficking. Gag-GFP was expressed in cells previously treated with siANC or each of siAP-3δ(1)-(5), followed by staining for endogenous AP-3δ and analysis by indirect immunofluorescence microscopy. In siANC-treated cells, GFP-Gag exhibited a punctate intracellular distribution with some co-localization with AP-3δ, while in siAP-3δ(1)-(5)-treated cells, GFP-Gag exhibited a diffuse pattern with no apparent intracellular accumulation (Fig. 4-2, rows 2-6). These results indicate that although siAP-3δ(1)-(5) exhibited differential effects on virus production, they exhibited the same effect on Gag localization due to their ability to downregulate AP-3δ. These results also suggest that the change in intracellular Gag localization is not attributed to the negative effects on virus production previously observed in siAP-3δ(2) and siAP-3δ(5)-treated cells (Chapter III).

We also investigated whether AP-3δ-depletion would affect Gag trafficking to MVBs in the chronically infected cell line, HeLa-LAV. HeLa-LAV cells were transfected with reagent alone (mock), siANC, or siAP-3δ(2). Mock-transfected cells were co-stained for Gag and endogenous CD63 and analyzed by indirect immunofluorescence
Figure 4-2. Effect of AP-3δ depletion on HIV-1 Gag localization using a panel of AP-3δ-specific siRNAs. HeLa cells were transfected with reagent alone (mock), siANC, or individually with siAP-3δ (1)-(5). At 48 hours post-transfection, cells were transfected with pEGFP-CO-Gag and fixed 24 hours post-transfection. GFP-Gag (green)-expressing cells were co-stained for endogenous AP-3δ (red) and the nucleus (blue), using an anti-AP-3δ antibody with an AF594-conjugated secondary antibody and DAPI, respectively. Cells were analyzed by fluorescence microscopy.
Figure 4-3. Effect of AP-3δ depletion on intracellular Gag localization in HeLa-LAV cells. HeLa-LAV cells were transfected with reagent alone (Mock), siANC, or siAP-3δ(2). At 96 hours post-transfection, cells were fixed with methanol. (A) Mock-treated cells were co-stained for Gag (green), endogenous CD63 (red), and the nucleus (blue), using an anti-p24 primary antibody with an AF488-conjugated secondary antibody, an anti-CD63 primary antibody with an AF546-conjugated anti-mouse secondary antibody, and DAPI, respectively. (B) Mock, siANC, and siAP-3δ(2)-treated cells co-stained for Gag (green), endogenous AP-3δ (red), and the nucleus (blue), using an anti-p24 primary antibody with an AF488-conjugated secondary antibody, an anti-AP-3δ primary antibody with an AF546-conjugated anti-mouse secondary antibody, and DAPI, respectively. Cells were analyzed by fluorescence microscopy.
microscopy. Gag co-localized with CD63 and exhibited a punctate, cytoplasmic distribution that was similar to what was observed in mock-treated, GFP-Gag-expressed HeLa cells (Fig. 4-3A), indicating that Gag was associated with MVBs. Mock, siANC, and siAP-3δ(2)-transfected cells were co-stained for Gag and endogenous AP-3δ, and analyzed by indirect immunofluorescence microscopy. Gag exhibited a punctate distribution in mock and siANC-treated cells, and exhibited some co-localization with AP-3δ (Fig. 4-3B, top and middle rows, respectively). However, in siAP-3δ(2)-treated cells, Gag exhibited a diffuse distribution throughout the cell with no apparent intracellular accumulation (Fig. 4-3B, bottom row), indicating that Gag association with MVBs was inhibited in the absence of AP-3δ. These results demonstrate that Gag localization to MVBs in chronically-infected HeLa-LAV cells is dependent on the AP-3δ and parallel what we observed for GFP-Gag in HeLa cells.

4.2.2. Overexpression of AGAP1 inhibits Gag trafficking to intracellular compartments

We also investigated whether disruption of AP-3 membrane association by overexpressing AGAP1, would have an effect on Gag trafficking to MVBs. GFP-Gag was expressed in HeLa cells previously transfected with control vector pHA-AGAP1[stop], pHA-AGAP1, or pHA-AGAP1[R599K], which lacks GAP activity and does not disrupt AP-3 function (112). To observe the effect of wild-type and mutant HA-AGAP1 on endogenous AP-3δ localization, GFP-Gag-expressing cells transfected with pHA-AGAP1 or pHA-AGAP1[R599K] were stained for endogenous AP-3δ and
Figure 4-4. Effect of the overexpression of wild-type or mutant AGAP1 on Gag localization. (A) HeLa cells were transfected with pHA-AGAP1, or pHA-AGAP1[R599K] for 48 hours and were fixed 24 hours post-transfection. Cells were co-stained for wild-type or mutant HA-tagged AGAP1 (red), endogenous AP-3δ (green), and the nucleus (blue), using an anti-HA primary antibody with an AF594-conjugated secondary antibody, an anti-AP-3δ primary antibody with an AF488-conjugated secondary antibody, and a DAPI-staining kit.
secondary antibody, and DAPI, respectively. (B) HeLa cells were transfected with pHA-AGAP1[stop] (top row), pHA-AGAPI (middle row), or pHA-AGAPI[R599K] (bottom row). At 48 hours post-transfection, cells were transfected with pEGFP-CO-Gag and fixed 24 hours post-transfection. GFP-Gag (green)-expressing cells were co-stained for wild-type or mutant HA-tagged AGAPI (red) and the nucleus (blue), using a rabbit anti-HA primary antibody with an AF594-conjugated secondary, and DAPI, respectively. Cells were analyzed by fluorescence microscopy.
analyzed by indirect immunofluorescence microscopy. As expected, AP-3δ exhibited a
diffuse intracellular distribution indicative of membrane dissociation in the presence of
HA-AGAP1, but in the absence of HA-AGAP1 exhibited a punctate intracellular
distribution indicative of membrane association (Fig. 4-3A, top row). However, AP-3δ
distribution was unaffected by the overexpression of HA-AGAP1[R599K], which lacks
GAP activity (Fig. 4-3A, bottom row). These results indicate that the overexpression of wild-type HA-AGAP1 specifically inhibits AP-3 intracellular membrane association, and
is dependent on its GAP activity, which is consistent with previous reports (112, 113).

We next analyzed GFP-Gag localization in cells transfected with pHA-AGAP1[stop],
pHA-AGAP1, or pHA-AGAP1[599K] and stained for HA, by indirect
immunofluorescence microscopy. In HA-AGAP1[stop] and HA-AGAP1[R599K]-
expressing cells, GFP-Gag exhibited a punctate intracellular distribution (Fig. 4-4B, top
and bottom rows, respectively) resembling the intracellular distribution of CD63-stained,
mock-treated cells from our earlier experiments (Fig. 4-1A). However, in cells that
overexpressed HA-AGAP1, GFP-Gag exhibited a diffuse distribution throughout the
cytoplasm, with minimal accumulation at the cell periphery (Fig. 4-4B, middle row). This
observed Gag distribution was very similar to what we observed in AP-3δ-depleted cells
(Fig. 4-1B), demonstrating that disruption of AP-3 function by overexpressing wild-type
but not mutant AGAP1 inhibits Gag trafficking to intracellular compartments that are
presumably MVBs, and corresponds with our previous observations in AP-3δ-depleted
cells.
4.2.3. HIV-1 Gag\textsubscript{K29E/K31E} trafficking to MVBs is AP-3\(\delta\)-independent

We next investigated whether AP-3\(\delta\) was required for mutant Gag (Gag\textsubscript{K29E/K31E}) trafficking to MVBs. This Gag mutant associates with MVBs as a result of two K to E amino acid substitutions at residues 29 and 31 of MA (120) and is downstream of the AP-3\(\delta\) binding site. GFP-Gag\textsubscript{K29E/K31E} was expressed in HeLa cells previously transfected with reagent alone (mock), siANC, or siAP-3\(\delta\)(1). To determine whether GFP-Gag\textsubscript{K29E/K31E} co-localizes with MVBs, mock-transfected cells were stained for CD63 and analyzed by indirect immunofluorescence microscopy. We observed that GFP-Gag\textsubscript{K29E/K31E} was concentrated at the perinuclear region and co-localized with MVBs that appeared larger than what we previously observed in HeLa and HeLa-LAV cells (Fig. 4-4A). We next examined the localization of Gag\textsubscript{K29E/K31E}-GFP in mock, siANC, or siAP-3\(\delta\)(1)-treated cells stained for endogenous AP-3\(\delta\). Gag\textsubscript{K29E/K31E}-GFP exhibited punctate clusters around the perinucleus presumably associated with MVBs all cells treated (Fig. 4-4B, top, middle, and bottom rows, respectively), indicating that Gag localization to these compartments is not dependent on AP-3\(\delta\).
Figure 4-5. Effect of AP-3δ depletion on mutant Gag<sub>K29E/K31E</sub> localization.

HeLa cells were transfected with reagent alone (Mock), siANC, or siAP-3δ(1). At 48 hours post-transfection, cells transfected with pEGFP-CO-Gag<sub>K29E/K31E</sub> and fixed 24 hours post-transfection. (A) Mock-treated GFP-Gag<sub>K29E/K31E</sub> (green)-expressing cells were co-stained for endogenous CD63 (red) and the nucleus (blue) using an anti-CD63 primary antibody with an AF594-conjugated secondary antibody and DAPI, respectively. (B) Mock (top row), siANC (middle row), and siAP-3δ(1) (bottom row)-treated GFP-Gag<sub>K29E/K31E</sub> (green)-expressing cells were co-stained for endogenous AP-3δ (red) and the nucleus (blue), using an anti-AP-3δ antibody with an AF594-conjugated secondary antibody and DAPI, respectively. Cells were analyzed by fluorescence microscopy.
4.3 Discussion

In this study, we used previously unexplored strategies to investigate whether AP-3 plays a role in Gag trafficking to MVBs. We examined wild-type Gag localization in AP-3δ-depleted cells and in AGAP1-overexpressing cells, which disrupted AP-3 membrane binding. In addition, the localization of a Gag mutant with mutations in MA that targets Gag specifically to MVBs was examined in AP-3δ-depleted cells. Through these studies, we demonstrated that AP-3 is involved in Gag trafficking to MVBs.

AP-3δ, which directly interacts with the MA domain of Gag, has previously been implicated in targeting Gag to MVBs (40). This was demonstrated by overexpressing an AP-3δ dominant-negative (DN) mutant thought to disrupt the AP-3δ:Gag interaction (40). However, this approach relied on the assumption that the only function of the AP-3δ DN mutant in the presence of Gag would be to inhibit the AP-3δ:Gag interaction. It is possible that the AP-3δ DN mutant itself mislocalized Gag, or prevented the interaction between Gag and another cellular factor that potentially mediated Gag trafficking to MVBs. Therefore, we used a direct approach to study the role of AP-3δ in Gag trafficking to MVBs by examining Gag localization in AP-3δ-depleted cells. AP-3δ-deficiency resulted in a diffuse cytoplasmic distribution of Gag with no apparent intracellular accumulation. In contrast, Gag distribution was punctate and co-localized with MVB-marker CD63 in the presence of AP-3δ. Additionally, this effect on Gag localization was observed in cells that were individually treated with the five different AP-3δ-specific siRNAs, siAP-3δ(1)-(5), previously described in Chapter III. The
demonstration that treatment with each of the five AP-3δ-specific siRNAs resulted in similar Gag localization phenotypes strongly implicates AP-3δ in mediating Gag localization to MVBs. While we previously observed off-target effects on virus production associated with siAP-3δ(2) and siAP-3δ(5) treatment, these siRNAs affected Gag localization similarly to siAP-3δ(1), (3), and (4), suggesting that intracellular redistribution of Gag does not contribute to the reduction in virus production associated with siAP-3δ(2) and siAP-3δ(5) treatment. We observed a similar effect on Gag localization in the absence of AP-3δ in chronically infected HeLa-LAV cells, demonstrating that the observed change in Gag distribution is attributable to AP-3δ depletion. The similar intracellular distributions of GFP-Gag and virally-expressed Gag also demonstrate that Gag adopts the same localization pattern independent of its mode of expression.

AP-3-mediated protein transport to MVBs is initiated by its recruitment to membranes, which gives rise to the formation of AP-3-coated vesicles on which protein cargo is bound and trafficked to MVBs. Therefore, disrupting AP-3 recruitment to membranes inhibits all downstream functions of AP-3 and prevents AP-3-mediated trafficking of protein cargo to MVBs. We used a specific approach to inhibit AP-3 membrane binding by overexpressing its regulator, AGAP1, which disrupted AP-3 membrane association and Gag localization to MVBs. Gag localization to MVBs occurred in cells expressing the GAP-inactive AGAP1 mutant, demonstrating the specificity of the observed Gag localization phenotype associated with AGAP1 overexpression. The observed change in Gag localization in the presence of
overexpressed AGAP1 was similarly observed in AP-3δ cells, suggesting that Gag utilizes AP-3-mediated transport for its localization to MVBs.

A previously characterized Gag mutant, Gag_{K29E/K31E}, has been reported to specifically traffic to MVBs by an unknown mechanism (120). Based on our findings that AP-3 mediates wild-type Gag trafficking to MVBs, we hypothesized that AP-3 also mediated the trafficking of Gag_{K29E/K31E} to MVBs. However Gag_{K29E/K31E} localization to MVBs was unaltered in AP-3δ-depleted cells, suggesting that Gag_{K29E/K31E} traffics to MVBs by an AP-3δ-independent mechanism. This could be explained by the differences in distribution observed between the mutant and wild-type Gag. Gag_{K29E/K31E} associated with MVBs in perinuclear clusters that were larger than the MVB compartments observed in wild-type Gag-expressing cells. Additionally, the effect on the morphology of the MVBs appears to be mediated by Gag_{K29E/K31E}, as neighboring cells that did not express Gag_{K29E/K31E} exhibited normal MVB distribution and morphology. Wild-type Gag exhibited a punctate distribution that appeared to concentrate near perinuclear regions and gradually spread out towards the cell periphery, suggesting directionality or movement. In contrast, Gag_{K29E/K31E} was localized mainly at the perinuclear region, suggesting that Gag and the MVBs may be static. As expression of Gag_{K29E/K31E} in the context of proviral clone pNL4-3 has been reported to inhibit virus production in HeLa cells, the Gag_{K29E/K31E}-associated decline in virus production may be explained by the possible immobilization of Gag_{K29E/K31E}-associated MVBs. This could suggest that Gag-associated MVBs require exocytosis to deliver their viral contents to the extracellular space, which appears to be inhibited in the presence of the K to E mutations in MA. The
demonstration that Gag targeting to MVBs can occur by an AP-3-independent mechanism suggests that other determinants are involved in mediating Gag targeting to MVBs. It is possible that the K to E substitutions disrupted the MA:AP-3δ interaction for which Gag compensated by using another mechanism to traffic to MVBs.

Although our studies demonstrate that wild-type Gag is dependent on AP-3 for its association with MVBs, it is unclear whether Gag is directly trafficked to the MVBs or localizes to MVBs as a result of endocytosis. Determining the origin of Gag that associates with MVBs would aid in understanding the overall significance of this association in viral replication. Direct Gag targeting to MVBs could demonstrate that this process contributes to virus production, while indirect Gag targeting to MVBs as a result of its endocytosis could demonstrate that this process does not play a significant role in virus production. As there are two mechanisms by which AP-3 mediates direct and indirect protein trafficking to MVBs (73), further investigation to delineate the specific trafficking pathway would be required to determine the origin of Gag in these compartments.
CHAPTER V: SUMMARY AND GENERAL DISCUSSION

Summary of Results

The work presented in this thesis demonstrated that AP-3 is not required for HIV-1 replication, but is required for trafficking Gag to multivesicular bodies, indicating that AP-3δ-mediated Gag trafficking to MVBs does not play a productive role in HIV-1 replication. We demonstrated through efficient and reproducible depletion of endogenous AP-3δ in infected HeLa, HeLa-LAV, and primary MDM cells that AP-3δ expression is not required for HIV-1 replication. AP-3δ depletion also did not affect infectious particle production or permissiveness to infection. The inhibition of AP-3 membrane association by overexpression of AGAP1 was also demonstrated to have no effect on HIV-1 replication in infected HeLa cells. An examination of the individual effects of five different AP-3δ-specific siRNAs on virus replication revealed the existence of two different phenotypes associated with siRNA treatment, either having no effect on or severely inhibiting virus production. Further investigation demonstrated that the inhibitory effect on virus production by two siRNAs could not be solely attributed to AP-3δ depletion, as the remaining three siRNAs depleted AP-3δ with the same efficiency without significantly affecting virus production. These results provided validation for previous results, which demonstrated that AP-3δ is not required for HIV-1 production. In addition, we demonstrated that Gag trafficking to MVBs was inhibited in the absence of AP-3δ or when AGAP1-mediated AP-3 membrane association was disrupted. However,
this was not apparent for mutant Gag\textsubscript{K29E/K31E}, as its trafficking to MVBs was not affected by AP-3δ depletion.

**General Discussion**

*Gag Trafficking to Multivesicular Bodies and HIV-1 Replication*

Our studies demonstrate that Gag is trafficked to MVBs in an AP-3δ-dependent manner that is dispensable for HIV-1 replication. While the observation that demonstrated that AP-3δ is required for localization of Gag to MVBs is in support of the original study by Dong *et al.*, our conclusion that AP-3δ is not required for HIV-1 replication directly contradicts their assertions regarding the role of AP-3δ in HIV-1 replication. Dong *et al.* proposed a model in which AP-3δ-mediated Gag trafficking to MVBs provides Gag with access to the ESCRT machinery, which it redirects to the plasma membrane by an unknown mechanism. The observations that virus particle production was severely compromised by the depletion of AP-3δ or the disruption of the AP-3δ:MA interaction suggest that Gag trafficking to MVBs and its subsequent targeting to the plasma membrane is the primary pathway used by Gag during the process of particle production. However, this model does not fit with our results as well as those from previous studies. The rationale for the association of Gag with MVBs to acquire the ESCRT machinery assumes that MVBs are the only sites where Gag can acquire ESCRT. Although ESCRT is primarily known for its role in MVB biogenesis and sorting mono-ubiquitinated cargo into MVBs for subsequent cargo degradation, it has been implicated in additional biological processes including cytokinetic abscission, autophagy, mRNA
transport, and RNAi-mediated mRNA silencing (149). Thus, these multiple functions would require ESCRT to be localized at multiple intracellular sites and could be acquired by Gag in a non-MVB context. To address the assertion that AP-3δ-mediated Gag trafficking to MVBs is required for virus production has been demonstrated by our work, which indicates that AP-3δ is not required for virus production. Several lines of evidence also demonstrate that virus production occurs without the need for Gag association with MVBs. Although a live imaging study of newly synthesized Gag demonstrated that it trafficked to MVBs prior to its localization to the plasma membrane (134), a similar study demonstrated that newly synthesized Gag trafficked directly to the plasma membrane without associating with MVBs (148). A temporal study of Gag distribution also demonstrated that Gag trafficked directly to the plasma membrane, which promoted robust virus production (81). Additionally, this study demonstrated that Gag localization to MVBs occurred at later time points as a result of its endocytosis from the plasma membrane. Furthermore, inhibiting endocytosis abrogated Gag localization to MVBs, while having no effect on virus production (81). In the presence of Vpu, which prevents the endocytosis of virus particles through its antagonization of Tetherin, Gag localization to MVBs is absent, but occurs in the absence of Vpu (107). These results strongly suggest that Gag targeting to MVBs is not a productive pathway for HIV-1 replication, and appears to be an artifact of endocytosis. In some cases, Gag targeting to MVBs has been demonstrated to be inhibitory for virus production. Studies in which Gag was targeted to MVBs by altering its affinity for the plasma membrane inhibits virus particle production in 293T cells, HeLa cells, and macrophages (81). Collectively, these results strongly
suggest that Gag trafficking to MVBs is not a productive pathway for HIV-1 replication and in can actually be detrimental for virus replication, therefore challenging the implication that Gag trafficking to MVBs plays a critical role in viral replication. In addition, these results also indicate that AP-3δ does not play a productive role in viral replication.

**siRNA Off-Target Effects**

The use of siRNAs to specifically silence a particular gene of interest has proven to be a powerful tool in understanding the role of expressed genes in biological processes. However, siRNAs have also been demonstrated to concomitantly induce specific and non-specific gene silencing resulting in off-target related phenotypes (75, 135). Through a study of five different AP-3δ-specific siRNAs, we identified three siRNAs that exhibited no effect on virus production and two siRNAs that exhibited inhibitory effects on virus production. All five siRNAs efficiently depleted AP-3δ, suggesting that the inhibitory effect on virus production could not be solely attributed to AP-3δ depletion. The inhibitory effect of the two siRNAs was more pronounced with a dose at which off-target effects are frequently observed (81). The two siRNAs, siAP-3δ(2) and siAP-3δ(5), shared the same target sequence as the siRNAs used in the Dong et al. and Camus et al. studies, respectively. These results strongly suggest that the virus inhibition observed in the two studies was not a result of AP-3δ depletion alone, but was mediated by off-target effects of the particular siRNAs used in their studies. Further analysis of the target regions of all five siRNAs by a BLAST (basic local alignment search tool) search
revealed that the target sequences of siAP-3δ(1), siAP-3δ(4), and siAP-3δ(3), which did not exhibit significant inhibitory effects on virus production, shared 15/19 nucleotide (nt) homology to 0, 0, or 1 cDNA (homeobox B8, HOXB8), respectively. In contrast, siAP-3δ(2) and siAP-3δ(5) each shared 15/19 nucleotide (nt) homology with two (plant homeodomain finger protein 2 (PHF2) and POM121) or three (solute carrier family 38, member 9 (SLC38A9), GATA5, and Epstein-Barr Virus Induced 3 (EBI3)/IL-27B) other cDNAs, respectively (Figure 5-1). Stringent siRNA design typically eliminates putative target regions that share 15/19 nt sequence homology with any other target, therefore it is unusual that these siRNAs were selected for use in experimentation. These observations further support the possibility that siAP-3δ(2) and siAP-3δ(5) exhibit off-target silencing effects. It is evident through our study that stringent siRNA design and validation of siRNA-associated phenotypes should have been performed to avoid the incorrect assessment that AP-3δ plays a significant role in virus production.

The site of virus assembly in macrophages: revisited

The primary function of macrophages is to phagocytose surrounding pathogens, cells, and debris, and as a result they require an active endosomal-lysosomal sorting system (7). As AP-3δ functions in the endosomal-lysosomal system and was reported to play a role in HIV-1 replication by directing Gag to MVBs, we hypothesized that AP-3δ-mediated Gag trafficking to MVBs would play a significant role in HIV-1 replication in macrophages. During the course of our study, AP-3δ was reported to be required for mediating Gag trafficking to a tetraspanin-rich compartment in dendritic cells (DC) viral
Figure 5-1. AP-3δ-specific siRNA target sequence BLAST results. Bars represent the number of genes with 15 of 19 nucleotide homology to the AP-3δ-specific siRNA target sequences tested, as determined by basic local alignment search tool (BLAST).
replication, which provided some support for our hypothesis. Therefore, we were surprised at our finding that virus production in macrophages was not affected by AP-3δ depletion. The differences in function between DCs and macrophages may account for the differences in phenotypes observed, however without having studied the role of AP-3δ in these cells we cannot make a direct comparison between our results and the results of the DC study.

However, our results in macrophages could be explained by recent studies that have suggested that the intracellular virus assembly compartments have been misidentified as MVBs and are actually deep invaginations of the plasma membrane (34, 173). This was demonstrated through ultrastructural studies of infected macrophages that were stained with the membrane-impermeant dye, ruthenium red (RR) (34, 173). Apparently intracellular VCCs were positively-stained with RR (RR+) suggesting that they were connected to the plasma membrane. In addition, the RR+ VCCs were devoid of endosomal markers, suggesting that the VCCs were not of endosomal origin but did co-stain with the plasma membrane marker CD44. Furthermore, extracellular virions derived from infected macrophages precipitated with CD44, indicating that CD44 was incorporated into viral membranes as a result of budding from the plasma membrane (173). A study that used ion-abrasion scanning electron microscopy demonstrated that the VCCs are connected to the plasma membrane by tubules that allow sequestered viruses to access the extracellular space (8). The authors of this study suggested that the use of sectioned specimens with conventional transmission scanning electron microscopy provides an incomplete picture, which could have contributed to the misidentification of
intracellular compartments as MVBs instead of invaginations of the plasma membrane. Rapid movement of Gag from VCCs connected to the cell:cell synapse in the presence of uninfected T-cells has been observed in live-cell imaging studies of infected macrophages demonstrating that Gag may traffic along these plasma membrane-connected tubules (59). Interestingly, an ultrastructural study comparing *in vitro* versus *in vivo* infected macrophages revealed a striking difference in viral assembly sites. In *in vitro* infected macrophages virus particles were often observed in MVBs, however in *in vivo* infected macrophages (ie. macrophages isolated from HIV-1 infected individuals) the vast majority of virus assembly occurred at the plasma membrane and was rarely observed in MVBs (125). Taken together, these results demonstrate the possibility that in macrophages, the MVB is not the intracellular site once thought to promote virus assembly, which would obviate AP-3δ-mediated Gag trafficking to this compartment.

**A model for AP-3-mediated Gag Trafficking To MVBs and its role in virus replication**

AP-3 mediates the trafficking of proteins that are destined for lysosomes by initially transporting protein cargo to MVBs, which deliver the cargo to lysosomes upon the fusion of the two compartments (33, 89). Protein cargo typically binds to the μ subunit of AP-3 or the AP-3 σ-δ hemicomplex through tyrosine or dileucine motifs, respectively (74, 76). Therefore, it is interesting that Gag interacts with the hinge region of AP-3δ, a region of the subunit that is not typically used for cargo recognition. In addition, although Gag encodes a dileucine motif and tyrosine motifs, they are not present within the AP-3δ binding site of MA. As it would seem nonsensical for Gag to be
a willing participant in a degradative pathway to the lysosome, it is possible that binding AP-3δ non-canonically would aid in the circumvention of its delivery to lysosomes. To date, only two other proteins known to bind the hinge region of AP-3δ have been identified. One of the proteins is the glycoprotein of the Vesicular Stomatitis Virus (VSV-G), which is atypically trafficked by AP-3 directly to the plasma membrane from the trans-Golgi network (TGN) through the secretory pathway. The other protein, cellular SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) VAMP7 (vesicle associated membrane protein 7), undergoes AP-3-mediated transport along the canonical trafficking pathway from early endosomes to MVBs. However, in addition to fusing with lysosomes, VAMP7-associated MVBs also undergo exocytosis via VAMP7-mediated fusion with the plasma membrane, subsequently releasing the MVB contents into the extracellular space as exosomes (reviewed in (23). By binding non-canonically to the AP-3δ hinge region, VAMP7 does not compete with standard cargo, thus enabling AP-3 to bind more than one cargo at a time (85). A recent study has reported that the HIV-1 Nef protein is released from cells in exosomes (92), providing evidence that this mode of release is used by another viral protein. Virus containing MVBs have been shown to fuse with lysosomes and the plasma membrane (125), suggesting that Gag does not discriminate between these MVBs. In addition, although exocytosed assembled virus particles could contribute to productive HIV-1 replication, our data suggests that this is not the primary route by which Gag traffics to the plasma membrane.

Based on these observations, previous studies suggesting that Gag or virions localize to the MVB as a result of endocytosis, and our demonstration that AP-3δ
mediates Gag trafficking to MVBs but is not required for HIV-1 replication, we have developed a model to describe the intracellular routes of Gag and their roles in HIV-1 replication. In this model (Fig. 5-2), Gag undergoes AP-3δ-mediated trafficking to MVBs directly or as a result of endocytosis and is either delivered to the lysosome for degradation or released into the extracellular space via exocytosis. Gag is also targeted to the plasma membrane by an AP-3 independent mechanism, which is the predominant pathway for viral assembly. In the absence of AP-3δ, Gag trafficking to MVBs is inhibited, while its trafficking to the plasma membrane remains intact. As a result, Gag that would have been trafficked by AP-3 to MVBs is available to traffic to the plasma membrane where it promotes virus assembly. Therefore, no discernible difference in virus production would be observed between AP-3δ expressing and non-expressing cells.

**Future Directions**

Work presented in this thesis aimed to extend previous work on the role of AP-3 in HIV-1 replication. While we demonstrated that AP-3δ was not required for HIV-1 replication in the cells tested, there are still areas that warrant further investigation. As we established that AP-3δ mediates Gag trafficking to MVBs in HeLa and chronically infected HeLa-LAV cells, we do not yet know the role of AP-3δ on the intracellular
Figure 5-2. A model for AP-3-dependent Gag trafficking and HIV-1 production. (Left) Gag undergoes AP-3δ-mediated trafficking to MVBs directly or as a result of endocytosis and is subsequently delivered to the lysosome for degradation, or is released into the extracellular space via exocytosis. Upon MVB fusion with the plasma membrane, internalized virus particles are released into the extracellular space. Gag that is unassociated with AP-3δ traffics directly to the plasma membrane, the predominant site of virus assembly and release. (Right) In AP-3δ-deficient cells, Gag is exclusively trafficked directly to the plasma membrane resulting in virus assembly and release.
localization of Gag in MDMs. The intracellular virus assembly site in MDMs is suggested to be plasma membrane-derived instead of endosomal. Therefore, it would be even more interesting to observe the intracellular site that AP-3δ traffics Gag, if it does indeed play a role in Gag trafficking in this cell type. Given that virus assembly has been observed at tetraspanin-rich domains in dendritic cells and AP-3δ has been implicated in trafficking Gag to these sites (52), it is possible that a similar site is used by Gag in MDMs.

As the role of AP-3δ in cells that predominantly promote virus assembly at the plasma membrane has been limited to investigation in HeLa and 293T cells, future investigation in T-cells would provide insight into whether AP-3δ plays a functional role in these cells, which are the major target of HIV-1. As virus assembly has been observed in the MVBs of T-cell lines (61), which would require Gag targeting to these intracellular compartments, it is possible that AP-3δ is responsible for this localization. In addition, determining the role of AP-3δ function on HIV-1 replication would provide much needed aid in clarifying whether Gag targeting to MVBs is truly a significant contributor to viral replication, a subject that has caused much controversy in this area of HIV-1 research.
APPENDIX I: DEVELOPMENT OF AN MS2-BASED REPORTER CONSTRUCT TO VISUALIZE HIV-1 GENOMIC RNA TRAFFICKING IN LIVING CELLS

Introduction

HIV-1 genomic RNA is an essential component of HIV-1 virus particles and plays a number of essential roles in the viral life cycle. During the late stage of viral replication, gRNA is localized to virus assembly and is incorporated into nascent virions by the NC domain of Gag (10). This NC:gRNA is mediated by the two zinc-fingers of the NC domain and the gRNA packaging signal, which is only present in gRNA and provides specificity for the viral incorporation of this particular RNA. Although the determinants for gRNA packaging have been well characterized, the intracellular route taken by gRNA is not well understood.

As gRNA trafficking is likely to be a dynamic process, live-cell imaging techniques would be required to study the movement of gRNA. One method to detect RNA in living cells exploits the elements of the bacteriophage, MS2, to selectively tag the RNA of interest with a GFP-tagged protein (reviewed in (142, 146)). The MS2 coat protein binds with high affinity and specificity to a 19 nt stem loop in the genomic RNA of MS2 and directs its packaging into phage particles, which is analogous to NC binding to the packaging signal of the gRNA of HIV-1. These stem loops can be cloned in tandem into cDNA that encodes the RNA of interest, thus providing binding sites for the MS2 coat protein to bind. Fusion of the MS2 with the fluorescent protein, GFP, allows for the tracking of the MS2-GFP-bound RNA of interest in living cells.
Subgenomic HIV-1 constructs have been developed and used as a surrogate for *bona fide* gRNA (82, 105), however, a full-length gRNA reporter has not been developed. In this study, we have constructed a gRNA reporter from pNL4-3 that contains 24 tandem MS2 binding sites within the integrase open-reading frame and characterized its ability to produce virus particles, incorporation into nascent virions, and intracellular localization.
Figure A1-1. Schematic representation of the MS2 Reporter System. Tandem MS2 binding sites integrated into the RNA of interest are bound by the MS2-GFP fusion protein, which binds to each stem loop as a dimer. GFP fused to the MS2 protein allows for the visualization of the MS2-GFP-bound RNA in living cells.
**Materials and Methods**

**Constructs**

**pNL4-3-Rd4:** a pNL4-3 derivative with ablated BamHI and SphI sites in *gag* and *env* which were reintroduced into the integrase open reading frame. This construct was generated by four successive rounds of site-directed mutagenesis, using the QuikChange site-directed mutagenesis kit (Stratagene). For the first round, pNL4-3 was used as the template and the resulting PCR product was used as the template for the next round, and so on, until the fourth round was completed. The following oligos were used:

(ALK51+/- were used to ablate the SphI site at nt 1447 of pNL4-3)

ALK51+: 5’ GATTGCATCCAGTCCACGCAGGGCCTATTG3’

ALK51-: 5’ CAATAGGCCCTGCGTGGACTGGATGCAATC3’

(ALK52+/- were used to ablate the BamHI site at nt 8465 of pNL4-3)

ALK52+: 5’ CGATTAGTGAACGCATTCTTAGCACTTATC3’

ALK52-: 5’ GATAAGTGCTAAGAATGCGTTCACTAATCG3’

(ALK53+/- were used to generate a BamHI site at nt 4370 of pNL4-3)

ALK53+: 5’ GCTAAAAGGGATCCCATGCATGGAC3’

ALK53-: 5’ GTCCATGCATGGGATCCCCTTTAGC3’

(ALK54.1+/- were used to generate a SphI site at nt 4764 of pNL4-3)

ALK54.1+: 5’ GACAGCAGTACAAATGGCATGCTTCATCCAC3’

ALK54.1-: 5’ GTGGATGAAGCATGCCCATTTTGACTGCTGTC3’

**pNL4-3-MS2-6:** a pNL4-3 derivative with 6xMS2 binding sites isolated from pSL-MS2-
6X cloned into the BamHI and SphI sites of pNL4-3-Rd4

**pNL4-3-MS2-12**: a pNL4-3 derivative with 12xMS2 binding sites isolated from pSL-MS2-12X cloned into the BamHI and SphI sites of pNL4-3-Rd4

**pNL4-3-MS2-24**: a pNL4-3 derivative with 24xMS2 binding sites isolated from pSL-MS2-24X cloned into the BamHI and SphI sites of pNL4-3-Rd4

**pSL-MS2-6X**: an expression vector containing 6 tandem MS2 binding sites (received from Dr. Robert Singer)

**pSL-MS2-12X**: an expression vector containing 12 tandem MS2 binding sites (received from Dr. Robert Singer)

**pSL-MS2-24X**: an expression vector containing 24 tandem MS2 binding sites (received from Dr. Robert Singer)

**Fluorescence In Situ Hybridization**

Cells mounted onto coverslips were fixed with 2% paraformaldehyde/5mM MgCl₂ followed by incubation in hybridization solution: 30% formamide, 0.2X saline sodium citrate, 1mg/mL bovine serum albumin, 10% dextran sulfate, and 0.1 mg/mL herring sperm DNA for 1 hour at 37°C. Coverslips were incubated with a Cy-3 labeled *gag-pol* DNA probe in the hybridization solution for 1 hour at 37°C. Coverslips were drained and incubated twice in hybridization solution for 30 minutes at 37°C. Coverslips were washed in 20% formamide/2XSSC for 30 minutes at room temperature, once in 20% formamide/1XSSC for 15 minutes at room temperature, and twice with 1XSSC for 15 minutes at room temperature. Cells were stained with DAPI (1:10000) of a 1 µg/µL
stock and coverslips were mounted onto glass slides using Prolong Gold Anti-fade reagent (Invitrogen). Cells were visualized using a Zeiss Axioplan 2 fluorescence microscope equipped with a 63× plan Apochromat objective. Images were acquired and analyzed using Open Lab 2.2.5 software.

**RT-PCR**

RNA was purified from 1 mL of cell supernatants using the QIAamp UltraSens Virus Kit (Qiagen) following the manufacturer’s protocol. The RNA was treated with RQ1 RNase-free DNase (Promega) prior to RT-PCR. First-strand cDNA synthesis was performed using the SuperScript II RT Kit (Invitrogen) according to the manufacturer’s protocol, the DNase-treated RNA and reverse primer ALK-RT1(-). Following first-strand synthesis, PCR was performed using the cDNA from the first-strand reaction and the following oligos: ALK-RT1(-) and ALK-RT(+).

ALK-RT1(-): 5’CTTTAGTTTTGTATGTCCTGTTG3’

ALK-RT1(+): 5’GTGCTGGATCAGAAAGTAC3’
Results

Cloning Strategy

In order to specifically tag gRNA while keeping the required viral genes intact, we chose to insert the MS2 binding sites into the integrase open reading frame. Typically, 24 MS2 binding sites are inserted into the cDNA of interest to provide more sites for MS2-GFP to bind and thus maximize signal intensity. The addition of 24 MS2 binding sites in gRNA would increase its overall length and possibly prevent its incorporation into virions. Therefore, generated three different constructs by inserting either 6, 12, or 24 MS2 binding sites into the integrase open reading frame. In order to insert the binding sites, we first introduced silent mutations in the original BamHI and SphI sites in pNL4-3 to destroy the sites. Next, we introduced the BamHI and SphI sites into the desired regions of the integrase open reading frame. These mutations required four different rounds of site-directed mutagenesis by PCR, and therefore this construct was referred to as pNL4-3-Rd4. The 6, 12, and 24 binding sites were cloned into the BamHI and SphI sites in pNL4-3-Rd4 to generate pNL4-3-MS2-6 (pN-M-6), pNL4-3-MS2-12 (pN-M-12), and pNL4-3-MS2-24 (pN-M-24), respectively. A schematic detailing the cloning strategy is shown in Figure A1-1.

MS2-containing gRNAs produce virus particles

To determine whether the generated clones could produce virus particles, pNL4-3-Rd4, pN-M-6, pN-M-12, and pN-M-24 were transfected into HeLa cells. Cell
Figure A1-2. Schematic representation of the cloning strategy used to generate the MS2 reporter constructs. The original BamHI and SphI sites in pNL4-3 were ablated and new BamHI and SphI sites were generated in the integrase open reading frame.
Figure A1-3. NL43-MS2 constructs produce virus. HeLa cells were transfected with 2 μg of pNL43-Rd4, pNL43-MS2-6, pNL43-MS2-12, or pNL43-MS2-24. At 48 hours post-transfection, cell supernatants and cells were harvested. (A) Cell supernatants were assayed for virus production by RT activity. Bars represent RT activity. (B) Cell lysates were assayed for Gag expression by SDS-PAGE and Western blot using an anti-p24 antibody.
supernatants and cells were assayed for virus production and protein expression, respectively. Interestingly, RT activity inversely correlated with the number of binding sites present in the constructs (Fig. A1-2A), which was also reflected by intracellular Gag expression (Fig. A1-2B). Although the constructs produced virus particles, the overall production was low in comparison to NL4-3-Rd4.

**MS2 gRNA is incorporated into virions**

We next determined whether the MS2-containing gRNA constructs could be packaged into virus particles. HeLa cells were transfected with pNL4-3, pNL4-3-Rd4, pN-M-6, pN-M-12, and pN-M-24, followed by collection of the cell supernatants. RNA was purified from the cell supernatants and subjected to reverse-transcription (RT)-PCR using specific primers. The resulting cDNAs were resolved by ethidium bromide-stained agarose gel electrophoresis. The cDNAs that represent gRNA purified from virus particles were present for all samples tested, but only in the samples that were treated with reverse-transcriptase during the first strand reaction (Fig. A1-4B). Furthermore, each fragment migrated to the predicted size (Fig. A1-4A, B). These results demonstrate that the MS2-binding site containing gRNAs were incorporated into virus particles.

**MS-2 gRNA localization is unaltered by the presence of MS2 binding sites**

To determine whether MS2-site containing gRNA localization is altered by the presence of the MS2 binding sites, HeLa cells were transfected with pNL4-3, pNL4-3-
Figure A1-4. NL4-3-MS2 reporter RNAs are packaged into virus particles.

(A) Schematic representation of RT-PCR strategy and expected fragment sizes. (B) HeLa cells were transfected with 3 µg of pNL43, pNL43-Rd4, pNL43-MS2-6, pNL43-MS2-12, or pNL43-MS2-24. At 48 hours post-transfection, cell supernatants were harvested and subjected to RNA purification. Purified RNA was subjected to reverse-transcription PCR. RT-PCR products (DNA) were resolved by gel electrophoresis and visualized by ethidium bromide staining.
Figure A1-5. NL4-3-MS2 reporter RNA localization. HeLa cells were transfected with 2 ug of pNL4-3, pNL4-3-Rd4, pNL4-3-MS2-6, and pNL4-3-MS2-12 for 48 hours, followed by fixation. Cells were stained by fluorescence in situ hybridization using a Cy3-conjugated gag-pol probe and analyzed by fluorescence microscopy.
Rd4, pN-M-6, and pN-M-12 for 48 hours and fixed with paraformaldehyde. Cells were stained for RNA by fluorescence in situ hybridization using a Cy3-conjugated \textit{gag-pol} DNA probe and were analyzed by fluorescence microscopy. MS2-site-containing gRNAs exhibited similar localization patterns as NL4-3 and NL4-3-Rd4 gRNAs, which demonstrates that the MS2 binding sites do not alter the intracellular localization of these gRNAs (Fig. A1-5).

**A1.3 Discussion**

In this study, we generated three pNL4-3 constructs, pNL43-MS2-6, pNL43-MS2-12, and pNL43-MS2-24, which contain 6, 12, or 24 MS2 binding sites in the integrase open reading frame, in order to specifically visualize genomic RNA in downstream applications. We demonstrated that the constructs could make virus particles, the resulting gRNA could be packaged into virus particles, and the resulting gRNA exhibited the same intracellular localization pattern as wild-type gRNA.

Although the virus particles could be generated by the expression of the constructs, the overall level of virus production was very low compared to the positive control, pNL4-3-Rd4. It is possible that ablation of the integrase open reading frame has contributed to the low replication efficiency of these constructs. However, it has been demonstrated that integrase-deleted HIV-1 molecular clones can be complemented \textit{in trans} by expressing a Vpr-IN fusion protein (44). Future studies in which Vpr-IN is expressed in conjunction with the NL4-3-MS2 constructs that we generated would be warranted to determine whether virus production could be improved. In addition, further
studies could be performed to determine whether the incorporation of Vpr-IN into NL4-3-MS2 virus particles could support integration, would enable future studies to be conducted by infection rather than transfection.

The other component of the MS2 reporter system is the MS2-GFP fusion protein. We were unable to successfully express the fusion protein with high efficiency by transient transfection. Attempts to generate a stable cell line were also unsuccessful, as the fusion protein exhibited cytotoxicity. Therefore, further optimization would be required in order to carry out downstream experiments involving the visualization of gRNA trafficking in living cells.
Figure A2-1. Optimization of AP-3δ-specific siRNA-mediated depletion of endogenous AP-3δ in HeLa cells. (A) HeLa cells were either not transfected (NT) or transfected with siAP-3δ(2) (10, 50, 100 nM) and harvested at 24, 48, and 72 hours post-transfection. Cell lysates were analyzed for protein expression by SDS-PAGE and Western blot, using anti-AP-3δ and anti-Sam68 antibodies. (B) HeLa cells were either not transfected (NT) or transfected with 0-100 nM of siAP-3δ(1) and harvested 48 hours post-transfection. Cell lysates were analyzed for protein expression by SDS-PAGE and Western blot, using anti-AP-3δ and anti-Sam68 antibodies.
Figure A2-2. Effect of hRIP depletion on HIV-1 production. (A) HeLa cells were transfected with reagent alone (Mock) or 50 nM hRIP-specific siRNA (sihRIP) for 48 hours and infected with VSV-G/NL4-3Δenv (MOI = 0.25). At 48 hours post-infection, cell supernatants were analyzed by RT activity assay and cell lysates were analyzed by Western blot using anti-AP-3δ and anti-hRIP antibodies. (B) MDM cells were transfected with reagent alone (Mock) or 50 nM hRIP-specific siRNA (sihRIP) for 48 hours and infected with VSV-G/NL4-3Δenv (MOI = 1.0. At 5 days post-infection, cell supernatants were analyzed by RT activity assay and cell lysates were analyzed by SDS-PAGE and Western blot using anti-AP-3δ and anti-hRIP antibodies.
Figure A2-3. Depletion of AP-3δ with a panel of AP-3δ-specific siRNAs. HeLa cells were transfected with reagent alone (Mock, M) or 50 nM individually with siAP-3δ(1)-(5). At 48 hours post-transfection, cells were harvested. Cell lysates were analyzed for protein expression by SDS-PAGE and Western blot using anti-AP-3δ and Sam68 antibodies.
Figure A2-4. Effect of siRNA concentration on AP-3δ expression. HeLa cells were transfected with 1 nM, 10 nM, and 100 nM of siANC or individually with siAP-3δ(1)-(5). At 48 hours post-transfection, cells were harvested and subsequently lysed. Cell lysates were analyzed by SDS-PAGE and Western blot with anti-AP-3δ, and Sam68 antibodies.
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