Characterization of HIV-1 capsid-binding host proteins during infection

by

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Abstract

The human immunodeficiency virus type 1 (HIV-1) capsid is an assembly of over 1,500 capsid (CA) monomers that encapsulates the viral RNA genome. After infection of the cell, this structure dissociates in a process termed uncoating to reveal the reverse transcribed genome that must be integrated into the host cell genome for productive infection. The HIV-1 capsid interacts with a number of host proteins during capsid trafficking and nuclear import in the cell. This study characterizes the importance of cellular localization of capsid-binding host proteins that have been implicated in HIV-1 infection. First, the microtubule motor protein kinesin, a mediator of capsid trafficking during disassembly, was depleted in cells, which was associated with inhibition of HIV-1 infectivity. An inhibitable, fluorescently-tagged kinesin construct was successfully introduced into these kinesin-depleted cells. Second, the re-localization of cleavage and polyadenylation factor 6 (CPSF6) to the cytoplasm impacts HIV-1 capsid trafficking and infectivity. As cyclophilin A (CypA) binding was shown to prevent cytoplasmic CPSF6 binding to HIV-1 capsid, localization of CypA was evaluated and found to differ between HIV-1 target CD4+ T cells and macrophages, which correlates to disparate findings on capsid uncoating dynamics during infection of these cells.

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"Life is a dynamic process. Logically, the elements of a process can be only elementary processes, and not elementary particles or any other static units. Cell life, accordingly, can never be defined in terms of a static inventory of compounds, however detailed, but only in terms of their interactions"[1]

First and foremost, I would like to extend a world of gratitude to the lab of Zandrea Ambrose. Zhou Zhong taught me everything that I now know about microscopy, including how to combat the circadian rhythm disruption that comes from sitting in a dark room all day. Douglas K Fischer patiently answered approximately 10,000 questions from me daily, very few of which had to do with HIV. And, of course, Zandrea Ambrose herself for making all of this possible.

None of this would have been possible without the support of my mother, who devoted her life to creating a better life for me, and of my stepfather, who chose to think of me as his own. Closer to my home here at Pitt, my dog Samson and my partner Frances Morin gave me a reason not to work overnight and sometimes even forced me to rest.

Lastly, I'd like to honor the millions of people who have succumbed to AIDS-related complications to this date. Especially Al, who spent so much of his time on Earth with myself and my older sister. I would also like specifically express my appreciation for Randy Shilts and the recently deceased Larry Kramer, both of whom played indispensable role in my choosing a career in HIV research.

1.0 Introduction

1.1 History of HIV-1

1.1.1 Epidemiology

On June 5th, 1981, an unprecedented five cases of the exceedingly rare *Pneumocystis jirovecci* pneumonia (PJP), then misclassified as *P. carinii* pneumonia (PCP), were reported among men who have sex with men (MSM) in Los Angeles, California[2]. At this same time, Sandy Ford of the Center for Disease Control (CDC) Parasitic Diseases Division, the nation's sole distributor of pentamidine isethionate, noted a concerning increase in demand for the orphan drug typically used to treat scarce, imported cases of African trypanosomiasis[3]. More concerning was the fact that these requests were coming from physicians treating PJP in adult male patients with no history of international travel. She ratcheted this information up the administrative ladder at the CDC, and in doing so unknowingly became the first person to alert the federal government to a then unappreciated incoming public health crisis. When New York City clinicians then reported what appeared to be clusters of Kaposi's Sarcoma, a similarly rare cancer with no known infectious etiology[4], and more PJP in people who inject drugs (PWID)[5], the stage was set for the recognition of an extraordinary emergent infectious disease. Together, these findings ultimately represented the discovery of what we now call acquired immunodeficiency syndrome (AIDS): a pandemic the likes of which the modern world had never before encountered.

In 2019, there were 1.7 million new documented cases of HIV-1 globally[6]. This represented a 23% decrease from 2010, but was still three times higher than the UNAIDS goal for

the end of the decade. More than half of these new infections occurred within well-defined risk groups and among their sexual partners, but distribution within these populations varies widely between regions. Nearly half of all new infections in Eastern Europe and central Asia were among PWID, but this risk group represented only 15% of new infections in western Europe and North America[6].

1.1.2 Virology

1.1.2.1 Discovery of HIV and development of antiretroviral therapy

Deeper investigation into AIDS became possible with the isolation of its causative agent, a novel retrovirus later named human immunodeficiency virus (HIV), by the research groups of Luc Montagnier of the French Pasteur Institute and Robert Gallo of the US National Cancer Institute (NCI) in 1983[7, 8]. The earliest pathogenic human retroviruses had been discovered not long before HIV; it was Robert Gallo himself that first reported the existence of human T-lymphotropic virus (HTLV-I) in 1980, just three years prior[9]. Owing to this short record and the unique replication cycle of retroviruses, described in section **1.3**, effective drugs to combat HIV infection were not readily available. Further complicating matters, the few human retroviruses known to the scientific community by 1983 caused cancer, not immunodeficiency. It was not until 1987 that the U.S. Food and Drug Administration approved zidovudine (azydothimidine, or AZT), the world's first ever drug for the treatment of retroviruses in humans[10]. HIV proved adept at escaping inhibition by monotherapies like AZT, so as new antiretroviral drugs (ARVs) were developed they were combined in a "cocktail" called combination antiretroviral therapy (ART)[11-13]. It was this introduction of ART in the mid-1990s that shifted a diagnosis of HIV from being a certain death sentence to a manageable condition.

1.1.2.2 Origin of HIV

Despite not being designated as a novel virus until the 1980s, phylogenetic analyses posit that the ape precursor to HIV spilled over into humans between 1915 and 1941[14]. The discovery of well-preserved, paraffin-embedded tissue in the Democratic Republic of the Congo allowed for genetic comparisons of HIV in samples from 1959 and 1960, well before recognition of AIDS in the United States. These experiments revealed that HIV sequences diverged considerably between samples collected in the mid-twentieth century, suggesting a significant history of evolution by that time[15]. Multiple cross-species transmissions of Simian Immunodeficiency Virus (SIV) to humans and between non-human primates have occurred[16], but a human exposure to chimpanzee SIV (SIV_{cpz}) from bushmeat practices in Cameroon resulted in the predominant HIV-1 group M at the root of the AIDS epidemic[17, 18]. A separate spillover of sooty mangabey SIV (SIV_{sm}) to humans resulted in the development of HIV-2, which remains largely restricted to west Africa, is more clinically mild, and will not be discussed here[19, 20].

1.2 HIV-1 Pathogenesis

1.2.1 Transmission

HIV-1 is transmitted through mucosal membrane contact with infected sexual fluids, direct introduction of virus into the bloodstream (namely by percutaneous inoculation), and vertically either through the placenta, exposure to maternal sexual fluids and/or blood during delivery, or consumption of infected breastmilk. In contrast to early conceptualizations of AIDS as a "gay cancer," 48% of new HIV-1 infections are now among women globally, with the proportion in

sub-Saharan Africa being as high as 59%[21]. In the United States, ~85% of these new HIV-1 infections among women are attributed to penile-vaginal intercourse[22]. Sexually transmitted diseases that result in genital ulceration or inflammation increase both transmissibility of and susceptibility to HIV-1[23, 24]. Mucosal membrane transmission occurs disproportionately among MSM due to a lack of self-lubrication of the anus. Investigations into condom adherence among MSM has thusly revealed proper lubricant selection and usage to be more important than condom thickness in preventing condom failure during anal sex[25].

Following initial introduction of HIV-1 to abrasions in the mucosal epithelium, the time between infection of the first cell to detection of virus in the bloodstream is referred to as the eclipse phase. This period can last anywhere from 7-21 days[26], after which HIV-1 will have established a systemic, self-propagating infection. HIV-1 first infects resting CD4+ T cells in the sub-mucosa[27, 28]. When these infected founder cells expand and migrate to the draining lymph nodes, where circulating T cells mature as part of the adaptive immune response, the virus is disseminated to a high-density population of target cells[29]. Once HIV-infected T cells are activated in the lymph node, they are directed throughout the body, allowing the virus to circulate widely[29]. Dendritic cells (DCs) near the point of viral entry are also capable of endocytosing viral particles and delivering them to lymph nodes[30]. It remains unclear whether DCs themselves are productively infected, but DCs exposed to HIV-1 efficiently infect CD4+ T cells in co-culture, making them important in at least the enhancement of infection[31]. Macrophages are additional HIV-1 target cells present in the genital mucosa[32], but little is known about their role in early infection. As with DCs, HIV-infected macrophages are capable of transmitting virus to T cells through cell-to-cell contacts during normal immunological interactions[33].

Once within the bloodstream, HIV-1 enters a target cell by binding of the viral glycoprotein gp120 to the host cell surface CD4 receptor in conjunction with a coreceptor, usually CCR5 and/or CXCR4. CD4+ T cells are frequently referred to as "helper" T cells due to their roles in activating multiple arms of the host immune response[34]. As such, the host immune system is profoundly impaired in their absence[35]. Even prior to cell death, HIV-infected CD4+ T cells are highly dysfunctional[36]. 90% of viruses isolated immediately following primary infection are CCR5-tropic[37], while the gradual rise in the proportion of R4-tropic virus over time is associated with declining numbers of CD4+ T cells and progression to disease[38]. As CCR5 is expressed predominantly on memory CD4+ T cells but CCR5 and CXCR4 are both present on naïve T cells, this co-receptor adaptation allows for viral infection of the naïve subset[39]. Alongside this switch in co-receptor usage, the virus evolves the ability to infect cells expressing relatively lower levels of CD4, like macrophages[40]. Unlike CD4+ T cells, little cytopathic effect is observed in HIV-1 infection of macrophages[41], allowing them to sustain production of virus over long periods of time[42].

1.2.1.1 Prevention

In the same way that ARVs must be combined for successful retroviral suppression, retroviral prevention should incorporate both sociobehavioral and biochemical methods[43]. Behavioral adaptations to limit the transmission of HIV-1 include but are not limited to the consistent usage of condoms, a reduction in the number of sexual partners, and a decrease in the incidence of "high-risk" sexual activities. These changes were demonstrated to significantly abate local rates of HIV-1 transmission as early as 1986[44]. In Thailand's 100% Condom Programme, when usage of condoms among sex workers rose from 10% in 1989 to > 90% in 2003, there was a corresponding 50% decrease in the incidence of HIV-1 among the group[45]. On the contrary,

PWID are still woefully underserved in terms of HIV-1 prevention. The most significant danger of injecting drug use in terms of HIV-1 transmission is the re-use of contaminated equipment between PWID. The establishment of needle and syringe exchange programs that provide sterile equipment is globally associated with a decrease in rates of transmission, but these programs remain extremely controversial[46].

The benefits of prophylactic ARVs were first observed in preventing transmission of SIV in macaques[47]. Since then, this approach has been extended to HIV-1 uninfected people in high risk groups. Prior to migration of infected cells to lymph nodes, HIV-1 replicates locally and at low levels in an "intraepithelial pocket" of target cells within the sub-mucosa at the site of entry[48, 49]. It is this brief hiatus that is targeted by preexposure prophylaxis (PrEP). ARVs utilized in PrEP protocols have largely been drawn from those developed for the treatment of HIV-1 infection, as desirable drug characteristics overlap between the two. This dual-potential for the use of novel ARVs in PrEP is especially important for therapeutics targeting pre-integration events of the HIV-1 replication cycle, which could hypothetically prevent the expansion of infected founder cells and the establishment of a latent reservoir, as discussed in **1.3**.

1.2.2 Clinical disease

1.2.2.1 Acute stage

Soon after infection, patients experience a self-limiting period of non-specific, flu-like symptoms. Viremia peaks during this introductory illness, then falls to a set-point as symptoms diminish. Despite this high viremia representing an ideal opportunity for the detection of circulating HIV-1, screening is rarely performed at this point in the absence of risk factors. While viremia can be a predictor of time from infection to development of AIDS[50, 51], peak and set point

viral concentration in the blood paint an incomplete picture and there is not a distinctly reliable correlation[52]. Following the resolution of this early illness, host CD4+ T cells will decline in the absence ART.

1.2.2.2 Chronic stage

The descent from initial HIV-1 infection to AIDS is highly variable but generally slow. The average time from infection to development of disease is approximately ten years without therapy[53], but "rapid progressors" develop disease within 2-3 years of exposure and "long-term non-progressors" can maintain normal CD4+ T cell counts for over twenty years[54]. This lengthy incubation period led to the early misconception that the virus lies latent for long periods following infection, when in reality HIV-1 replicates and depletes CD4+ T cells continuously prior to the development of symptoms[54, 55]. During sub-clinical infection, rates of viral replication are controlled by a silent immunological arms race wherein the immune system produces cellular and humoral responses to inhibit the virus and HIV-1 mutates rapidly to escape them[56]. During this clinical latency, HIV-1 establishes a reservoir of cells with the potential to continue producing infectious viral particles for long periods of time, as detailed in **1.3.5**.

1.2.2.3 AIDS

AIDS-defining illnesses typically begin to appear when patient CD4+ T cell counts fall below 200 cells/μL. Since the early identification of PJP and Kaposi's sarcoma in infected persons, opportunistic infections with *Toxoplasma gondii*, *Mycobacterium tuberculosis* (often disseminated), cytomegalovirus, *Cryptococcus neoformans*, and scores of others are now likewise considered hallmarks of the disease. These diagnoses are more easily prevented in the era of ART as people can live for decades with HIV-1 without ever progressing to AIDS, but chronic

complications are associated with long-term infection. Current research on long-term complications of HIV-1 infection includes investigation into an increased risk of chronic hepatitis, neurodegeneration, and cardiovascular disease among infected persons[57].

1.3 HIV-1 Biology

The inner workings of retroviruses first began to be elucidated in the 1960s. When Baltimore and Temin independently described an RNA-dependent DNA polymerase in oncogenic RNA viruses[58, 59], it was an upheaval of the contemporary understanding of genetics. Prior to this, the central dogma of molecular biology had posited that genetic information was transferred strictly in one direction from deoxyribonucleic acid (DNA), to ribonucleic acid (RNA), and then to operative protein[60]. Conflictingly, Baltimore and Temin demonstrated that both Rous sarcoma virus and Rauscher murine leukemia virus converted their RNA genomes into DNA genomes within the host cell during infection using an enzyme known now as reverse transcriptase (RT).

1.3.1 Entry

To gain access to the interior of the host cell, the enveloped HIV-1 particle must first make contact with the host cell surface[61]. This interface is comprised of the HIV-1 Env glycoprotein trimer binding to host cell receptors and co-receptors[62]. The outer gp120 subunit of Env binds to cellular CD4 and induces a conformational change that then allows for gp120 binding to co-receptors CCR5 and/or CXCR4[63, 64]. Once bound to CD4 through the gp120 peptide, the gp41 fusion peptide of Env pierces the host cell membrane to tether the viral particle to the target cell.

This juncture induces a conformational change in gp41 that allows for the formation of a gp41 six-bundle helix, which physically brings the viral membrane and host plasma membrane into close enough proximity to fashion a fusion pore[65]. At this point the viral membrane fuses with the host cell membrane, allowing for the deposition of the viral capsid into the cytoplasm[66].

1.3.2 Trafficking

1.3.2.1 The host cytoskeletal architecture

Popular illustrations of the cell depict the cytoplasm as a wide, open expanse occasionally interrupted by organelles. In reality, it is crowded and dense[67]. Because of this, only small cargo, such as individual proteins, can diffuse unassisted throughout the cell. Larger cargo, like vesicles, organelles, the HIV-1 capsid and that of other viruses, must hijack the host cell highway system of the cytoskeleton to make their way towards the interior of the cell[68-70].

The cytoskeleton is a dynamic network of structures that provide the cell with physical rigidity, facilitate intracellular trafficking, and mediate cellular stability and translocation during division. This network is composed of microtubules, actin filaments, and intermediate filaments. While movement of the HIV-1 capsid along actin filaments has been observed, actin-mediated trafficking appears to occur only in unpredictable, short bursts[71, 72]. Rather, the HIV-1 capsid travels consistently and over long distances along microtubules during cytoplasmic transit to the nucleus[71, 72].

1.3.2.2 Motor proteins and viral transit

Disruption of microtubule function with nocodazole treatment impairs HIV-1 trafficking and results in an accumulation of HIV-1 capsids at the cell periphery[72-74]. While microtubules

provide the scaffolding for intracellular movement, motor proteins must "walk" along them to carry cargo. Motor proteins dynein and kinesin facilitate microtubule-mediated trafficking of cargo to (retrograde) and from (anterograde) the microtubule organizing center (MTOC) abutting the outer nuclear membrane, respectively[75]. The earliest live cell observations of HIV-1 trafficking noted a "distinct back and forth behavior" wherein the capsid containing a fluorescently labeled protein would unpredictably switch between inward and outward movement during overall inward movement towards the nucleus[71]. Later studies confirmed that unidirectional post-entry movement of the HIV-1 capsid depends on the activity of both dynein and kinesin[76].

Other viruses also utilize motor proteins of opposing directionality to facilitate transit in one overall direction. The herpes simplex virus type I (HSV-I) capsid has been shown to bind kinesin and dynein simultaneously[77], which then mediate its disassembly through a physical "tug-of-war" mechanism[78]. Kinesin interactions with the HSV-1 capsid are also required for disruption of the organization of nuclear pore complexes (NPCs) to accommodate nuclear docking[79]. For adenoviruses, kinesin binding to capsid is required for final disassembly of the capsid at the NPC[79]. Similarly, kinesin heavy chain (KIF5B) interaction with the HIV-1 capsid has been shown to re-localize Nup358, a nucleoporin in the NPC that is required for nuclear entry[80], but much of the requirement for KIF5B in HIV-1 infection remains unknown. Methods used to investigate these mechanisms in DNA viruses have been made possible by an intricate composition of their capsid structures. Because the HIV-1 capsid is relatively fragile and does not tolerate mutations[81, 82], these methods are not readily transferrable and novel strategies are needed

Capsid uncoating is a spatiotemporally dependent process, meaning that uncoating occurring either too swiftly or too slowly is unfavorable to infection[83]. As the capsid itself can be recognized by host innate immune effectors, so this perfect "sweet-spot" time and location for

HIV-1 uncoating is critical for limiting these interactions[84-86]. Production of viral DNA is similarly dependent on uncoating kinetics, as CA mutants that alter core stability also impair rates of reverse transcription[81, 82, 87]. The kinetics of uncoating are influenced not only by the surrounding cellular environment, discussed in **1.4.3**, but also by structural changes within the capsid. Indeed, inhibiting reverse transcription precludes uncoating and results in an accumulation of intact capsids at or around the nuclear membrane[88].

1.3.3 Nuclear import

While some viruses require the breakdown of the nuclear membrane seen in an actively dividing cell to enter the nucleus, HIV-1 and other lentiviruses can infect non-dividing cells in a manner mediated by capsid[89, 90]. Historically, it was thought that the ~60 nm diameter of the intact HIV-1 capsid required full dissociation of the structure prior to transit of the viral DNA genome through a ~30 nm nuclear pore[91, 92]. In line with this, decades of studies reflected a wholly cytoplasmic model of uncoating. Recently, mounting evidence contradicts this long-held dogma, and suggests that uncoating of the HIV-1 capsid completes within the host cell nucleus[93-96]. The proposition that an intact or mostly intact conical capsid may be present at or even transported through the nuclear pore represents a remarkable paradigm shift within the field, but the exact nature of HIV-1 capsid uncoating is still unclear.

The most thoroughly described nucleoporins that mediate HIV-1 nuclear import are the cytoplasmic leaflet Nup358[97] (also referred to as RANBP2) and intra-nuclear Nup153[98], both of which bind directly to HIV-1 capsid. Depletion of transportin-3 (TNPO3) restricts HIV-1 nuclear import, though this is likely an indirect influence owing to its interaction with second host factor CPSF6, as discussed in **1.4.3.2**. Interestingly, the requirement of Nup358, Nup153, and TNPO3

for HIV-1 nuclear import can all be subverted by a mutation in capsid[99]. This suggests the existence of at least one redundant, unidentified pathway for HIV-1 nuclear import. Other nucleoporins, such as Nup214 and Nup98, have been implicated in the regulation of HIV-1 nuclear import but their precise role has yet to be described[97, 100].

1.3.4 Reverse transcription

The retroviral RNA genome is converted into double-stranded DNA by reverse transcription, which is catalyzed by reverse transcriptase (RT). Encapsulation of RT and the viral genome within the capsid allows for the spatial apposition necessary to keep the enzyme near its genomic substrate[101]. Reverse transcription begins within an intact capsid and partly mediates uncoating by applying pressure to the capsid lattice during the accumulation of rigid DNA[87, 102]. Early products of reverse transcription can be detected in vitro even when only disassembled patches of hexameric capsid lattice are present, indicating that the capsid shell itself is vital for initiation of reverse transcription[103].

RT both synthesizes DNA from the viral RNA template and then degrades that same RNA from the ensuing RNA:DNA duplex[104]. Two copies of the HIV-1 RNA genome are contained within one capsid, and RT can switch templates during reverse transcription. HIV-1 sequences vary widely within individuals[105]. When distinct genomes are incorporated within the same capsid, template-switching during reverse transcription can produce chimeric, recombinant viruses with new capabilities of evading the host immune response[106]. In addition to the relatively high rate of mutation brought on by the infidelity of RT, host cytidine deaminases in the APOBEC3 family induce hypermutations during this process[107-110].

1.3.5 Integration

In the second characteristic step of the retroviral replication cycle, the reverse transcribed HIV-1 genome is integrated into the host cell genome, permitting persistence of this provirus for the lifespan of the cell. Similar to reverse transcription, integration of the DNA HIV-1 genome is accomplished by the viral enzyme integrase (IN). Ordinarily, HIV-1 IN processes both ends of the viral genome and preferentially integrates it in gene-dense sites of active transcription within the host genome[111]. Positioning of these integration events is facilitated by host proteins lens epithelium-derived growth factor / transcriptional coactivator p75 (LEDGF/p75)[112, 113] and CPSF6[114]. As current research into a functional cure for HIV-1 includes targeting of proviruses, understanding integration sites is critical.

Cells containing proviral sequences retain the ability to produce infectious viral particles upon induction and are collectively referred to as the latent reservoir[115, 116]. This reservoir is largely comprised of memory CD4+ T cells, and normal activation of these cells can reverse said latency[117]. Less than 1% of these proviruses can be stimulated to produce infectious viral particles under optimal conditions in vitro[118], but the potential for latently infected cells to re-activate requires people living with HIV-1 to adhere to ART permanently.

1.3.6 Assembly, budding, and maturation

Integration of the reverse transcribed HIV-1 DNA genome allows the proviral sequence to be insidiously transcribed and translated by the host cell replication machinery[119, 120]. HIV-1 gene expression is regulated, however, by the two viral proteins Tat and Rev. Retroviruses contain their own promoter sequence, a long terminal repeat (LTR), but Tat is required for LTR-driven

transcription[121, 122]. Tat binding to the transactivation-responsive region (TAR) sequence within the LTR RNA[123] recruits cellular P-TEFb, part of the host cell transcription complex, to nascent transcripts as an activator of elongation[124, 125]. In the absence of this interaction, transcription is non-processive. HIV-1 transcripts can be multiply or singly spliced or unspliced[126]. Unspliced viral transcripts are exported by a mechanism facilitated by the viral protein Rev[127].

Egress of the HIV-1 particle is coordinated by the Gag polyprotein, which consists of the structural proteins CA, nucleocapsid (NC), and matrix (MA). The *pol* gene, encoding the viral enzymes RT, IN, and protease (PR), is translated following a frameshift as the larger Gag-Pol polyprotein[128]. Assembly of the HIV-1 particle takes place at the host membrane, where direct interactions between Gag, other viral proteins, and the viral genome bring virion contents together. During budding from the cell, PR cleaves the HIV-1 polyproteins to produce an infectious, mature particle. This processing of the Gag polyprotein allows for the formation of the HIV-1 capsid by its constituent CA protein.

1.4 The HIV-1 Capsid

1.4.1 Capsid structure

The HIV-1 capsid is comprised of only the capsid (CA) subunit protein (**Fig 1**). Approximately 1,500 monomers coalesce into an enclosed lattice of roughly 250 hexamers and 12 pentamers to encapsulate two copies of the viral RNA genome, unique retroviral proteins, such as RT and IN, and a select few host proteins[129, 130]. The differential placement of these pentamers on opposite ends of the capsid is responsible for the asymmetrical, “fullerene cone” shape of the

structure[131]. Each hexamer contains a pore for the import of external materials, most notably dNTPs necessary for reverse transcription[132].

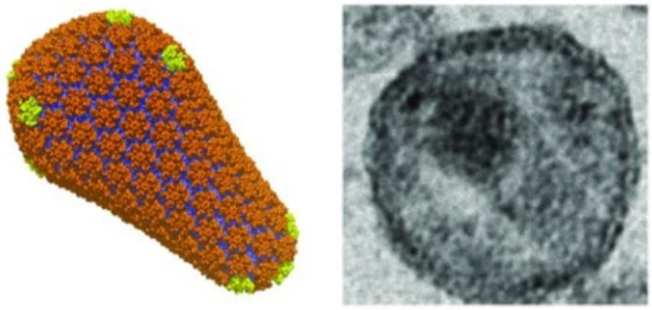

Figure 1: Structure of HIV-1 Capsid. ~250 CA hexamers (orange) come together with 12 pentamers (yellow) in a lattice to form the distinct chrysalis-shaped HIV-1 capsid. Reprinted with permission from Novikova, M., Zhang, Y., Freed, E.O. et al. Multiple Roles of HIV-1 Capsid during the Virus Replication Cycle. *Virol. Sin*. 34, 119–134 (2019).

1.4.2 Capsid function

The HIV-1 capsid impacts all steps of the post-entry replication cycle. Owing to the unique replication cycle of retroviruses, multiple components necessary for HIV-1 replication are not found within the target cell. Because retroviruses need to bring in their own replication machinery, these materials must be kept from diffusing aimlessly into the cytoplasm. The capsid shell, for example, provides the physical constraints necessary to keep RT in close proximity to its viral genome substrate[101]. The physical barrier provided by the capsid additionally protects the recognizably "non-self" contents within from detection by cellular immune sensors[133, 134]. Reverse transcription and uncoating are intrinsically tied, with the initiation of reverse transcription facilitating capsid disassembly[135, 136] and the capsid lattice itself serving as an initiator of reverse transcription[103]. Lastly, capsid is the major viral determinant of nuclear import.[137]

1.4.3 Host factor involvement in HIV-1 capsid-dependent processes

1.4.3.1 CypA

CypA is a host peptidylprolyl isomerase first implicated in HIV-1 infection nearly thirty years ago[138, 139]. Despite this early discovery, the exact role of CypA in HIV-1 infection has remained elusive in large part due to the cell-type dependence of experimental results regarding the protein. On the CA monomer, CypA catalyzes the isomerization of the peptide bond joining residues G89 and P90[139, 140]. Single amino acid substitutions G89V and P90A mutants abolish CypA binding and, in turn, diminish infectivity in most cells[141-143]. CypA was first identified as a target of the existing immunosuppressant drug cyclosporine A (CsA)[144], which can be used to inhibit CypA interaction with HIV-1 capsid in infected cells[138]. CypA has been shown to impact all post-entry events of HIV-1 replication, but results concerning interactions between CypA and the HIV-1 capsid have been conflicting. CypA has been demonstrated to both stabilize capsids[145] and destabilize CA tubes in vitro[146]. Because the presence of CypA in target cells, not producer cells, determines its impact on infectivity, it is thought that differential levels of CypA expression between cell types could be to blame for the protein's divergent influence[147, 148]. In support of this idea, decreasing the level of CypA present in in vitro assays can lead to CypA stabilization of HIV-1 CA assemblies[146]. The impact of CypA on nuclear import is of major consequence given recent results suggesting that an intact or mostly intact HIV-1 conical capsid could be traversing the nuclear pore. HIV-1 relies on specific nuclear pore proteins, most notably Nup358 and Nup153, to facilitate nuclear entry. The disruption of CypA binding to capsid undermines the dependence of HIV-1 on both Nup358[149] and Nup153[98], allowing nuclear entry to proceed through a presently unknown pathway independent of both.

CypA interactions with the capsid affect intracellular innate immune recognition of HIV-1. Infectivity of CypA-independent CA mutants G89V and P90A HIV-1 are not inhibited by myxovirus resistance protein B (MxB), which is encoded by an IFN-stimulated gene that restricts nuclear entry of WT HIV-1[85, 86]. HIV-1 also exploits CypA to protect the capsid from recognition by tripartite motif containing protein 5 (TRIM5α), which restricts infection[150]. Binding of CypA to the HIV-1 capsid sterically hinders binding of TRIM5α, and depletion of TRIM5α is sufficient to rescue the reduced infectivity of G89V and P90A HIV-1 in primary CD4+ T cells [151, 152].

1.4.3.2 CPSF6

CPSF6 is a mediator of mRNA processing found predominantly within the cell nucleus under normal conditions[153]. This localization is due to binding of CPSF6 to the nucleocytoplasmic shuttle protein TNPO3 through its C-terminal arginine and serine-rich (R/S) domain[154, 155]. CPSF6 binds directly to and disrupts CA tubes in vitro[73, 99, 156, 157]. A truncated form of CPSF6 at amino acid 358 that lacks the R/S domain, CPSF6-358, diminishes binding to TNPO3[99]. It was demonstrated that in the absence of TNPO3 binding, CPSF6-358 was distributed throughout the cytoplasm and inhibited HIV-1 infectivity by blocking nuclear entry[99]. Production of HIV-1 in cells expressing CPSF6-358 selected for the CPSF6-independent N74D CA mutant[99], which infected cells independently of Nup358 and Nup153[98, 149, 158]. N74D HIV-1 infection is normal in HeLa cells but impaired in macrophages[158] Dependence of HIV-1 infection on TNPO3 has been noted in multiple instances, but CPSF6-independent capsid mutant N74D is insensitive to TNPO3 depletion[99], suggesting that the impact of TNPO3 on infectivity is indirect and requires CPSF6. Depletion of CPSF6 does not inhibit HIV-1 infectivity in most cells but does decrease the amount of CA that co-localizes with nuclear viral DNA and shifts integration into heterochromatin[114, 159, 160].

1.4.3.3 The CypA-CPSF6 connection

Our lab has shown that cells expressing full-length CPSF6 contain perinuclear CPSF6 puncta within the cytoplasm[73]. During HIV-1 infection, these CPSF6 puncta form higher-order complexes around the HIV-1 capsid and co-traffic with it along microtubules[73]. Loss of capsid binding to CypA with either G89V and P90A capsid mutants or CsA treatment increases both the size and number of these CPSF6 complexes, suggesting that CypA interactions with the capsid prevent CPSF6 binding[73]. Similar results were observed for CPSF6-358[157].

1.4.3.4 Motor protein complexes

Upon fusion with the host cell membrane, the HIV-1 capsid travels along microtubules to the nucleus[71, 72]. Depletion of microtubules and inhibition of microtubule-mediated trafficking with nocodazole treatment both perturb HIV-1 infectivity[76, 161]. Motor proteins kinesin and dynein are responsible for movement of cargo along microtubules to and from the MTOC abutting the nucleus, respectively[75]. Counterintuitively, both kinesin and dynein are required for successful HIV-1 uncoating, which occurs as the capsid moves unidirectionally towards the cell interior[76, 162]. More recently, dynein adaptor protein bicaudal D2 (BICD2) and kinesin-1 adaptor fasiculation and elongation factor ζ (FEZ1) have been implicated in promoting HIV-1 microtubule trafficking in a motor protein-dependent manner[163, 164].

1.4.4 Public health significance: Capsid as a drug target

While the advent of ART has greatly improved the prognosis of people living with HIV-1 in terms of both chronic viremia and progression to AIDS[165], the timely discovery of new

potential targets for HIV-1 treatment is essential to competing with the rapid pace of antiretroviral resistance[166].

As detailed herein, the HIV-1 capsid influences all post-entry events of the HIV-1 replication cycle. The capsid protects its contents from detection by immune sensors[133, 134], prevents unique retroviral material from diffusing away from the virus[101], is essential for the initiation of reverse transcription[103], and is the major determinant of nuclear import[137]. Targeting events prior to nuclear entry for inhibition of HIV-1 could prevent integration and, in turn, the establishment of a latent reservoir. Unlike enzymatic targets, which have proven to be capable of adapting functionality when circumventing ARV activity, the capsid is extremely intolerant of even single amino acid substitutions[81, 82]. The number of different viral processes influenced by capsid function and the fragility of the capsid-encoding sequence make it an increasingly desirable target for the development of new antiretroviral compounds as complications from conventional drugs continue to arise[167].

Capsid inhibitors in development aim to disrupt capsid stability directly or indirectly by inhibiting binding to essential host proteins. Many promising small molecules specifically interfere with interactions between the HIV-1 capsid and CPSF6. Within the laboratory, small molecule PF-3450074 (PF74) occupies the HIV-1 capsid binding site to CPSF6 and Nup153 and interferes with uncoating[168-170]. Previously, the largest barrier to the translation of capsid inhibitors like PF74 to clinical application has been prohibitive toxicity and bioavailability. GS-6207, a small molecule that contains the PF74 scaffold, has demonstrated potent efficacy in a phase I clinical trial as a long-acting therapeutic in the treatment of HIV-1[171]. As CPSF6 has proven to be a viable mediator of capsid inhibition, insights into how CPSF6 interacts with the capsid can theoretically aid in the drug development process. Defining interactions between the capsid and KIF5B could likewise

disrupt binding and allow for investigation into mechanisms that inhibit the arrival of the HIV-1 PIC at the nuclear membrane.

2.0 Statement of Project and Specific Aims

Upon fusion with the host cell membrane, the HIV-1 particle deposits its capsid within the cytoplasm. Transit of the HIV-1 capsid towards the nucleus is facilitated by motor proteins dynein and kinesin, both of which are required for successful capsid uncoating[76]. During infection, kinesin heavy chain KIF5B interacts with nuclear pore complex (NPC) components critical for HIV-1 nuclear entry in a capsid-dependent manner[80]. Despite canonically mediating transport of cargo away from the nucleus, kinesin has been implicated as a regulator of both capsid dissociation and nuclear entry during overall inward movement of other viruses[172]. Depletion of KIF5B is known to abrogate HIV-1 infection, but the mechanism by which this occurs remains unclear.

While trafficking along microtubules to the cell nucleus, the HIV-1 capsid interacts with cytoplasmic host proteins as it disassembles, including CPSF6[73, 99]. Point and truncation mutants that disrupt CPSF6 binding to TNPO3 increase cytoplasmic expression of the protein and inhibit HIV-1 nuclear entry[99, 155]. Our lab has shown that CypA[138] influences cytoplasmic CPSF6 interactions with the HIV-1 capsid[73, 157]. Because HIV-1 uncoating is a spatiotemporally dependent process critical to productive infection, the cellular staging of interactions between the HIV-1 capsid, CypA, and CPSF6 is of great import but remains to be clearly defined.

AIM 1: Examine requirement for KIF5B in HIV-1 capsid microtubule trafficking and nuclear import. To determine whether motor proteins that canonically traffic cargo away from the cell interior are involved in HIV-1 capsid trafficking to the nucleus, we will evaluate the effect of a fluorescently-tagged, inhibitable kinesin family member, KIF5C, on HIV-1 microtubule trafficking[173]. Expression of this construct in infected cells will allow for the visualization of HIV-1 trafficking in live cell microscopy. Furthermore, inhibition of the motor activity of this

construct during live cell analysis will allow for real time visualization of the role of KIF5 in HIV-1 trafficking.

AIM 2: Visualize the localization of HIV-1 CA-binding host proteins within cells and quantify their impacts on infectivity. Fluorescently tagged full-length, point, or truncation mutants of CPSF6 will be expressed in cells using a lentiviral vector. These cells will allow for the visualization of CPSF6 localization using confocal microscopy and for the examination of WT and N74D HIV-1 infectivity using a luciferase reporter virus. Fluorescent antibody staining will then be employed to visualize the distribution of CypA, endogenous CPSF6, and microtubules in HeLa cells, SupT1 CD4+ T cells, and primary human macrophages.

3.0 Materials and Methods

3.1 Cells

HeLa cells and HEK293T cells were cultured in Dulbecco's modified Eagle medium (DMEM; Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (PSG; Thermo Fisher Scientific). SupT1 cells were cultured in RPMI 1640 medium (Thermo Fisher Scientific) with 10% FBS, and PSG. Human peripheral blood mononuclear cells (PBMC) were isolated from leukapheresis obtained from the Central Blood Bank (Pittsburgh, PA) using Ficoll-Paque Plus (GE Healthcare) density gradient centrifugation. CD14+ monocytes were isolated from PBMC using human anti-CD14 magnetic beads with LS Columns (Miltenyi Biotec). CD14+ monocytes were differentiated into monocyte-derived macrophages (MDMs) in RPMI 1640 medium supplemented with 10% FBS, PSG, and 50 ng/ml recombinant granulocyte–macrophage colony-stimulating factor (R&D Systems) for 7 days. All cells were incubated at 37° C with 5% CO_2.

3.2 Viruses

WT and N74D replication-defective HIV-1_{NL4-3} encoding luciferase in place of *nef* and pseudotyped with VSV-G were produced by transfection of HEK 293T cells with pNLdE-luc, pL-VSV-G, and pVpr-pcs-mRuby3-IN at a weight ratio of 5:5:1. Replication-defective lentiviruses encoding fluorescently-tagged CPSF6 were produced by transfection of HEK 293T with lentiviral

plasmid pSICO encoding CPSF6 fused to iRFP670 (iRFP), a packaging plasmid, and pL-VSV-G at a weight ratio of 4:3:1. Both transfections were performed with Lipofectamine 2000 (Thermo Fisher Scientific) according to manufacturer protocol. Transfectant media was removed after 8h, at which point samples were incubated in fresh media for 48h. Media was then spun down at 300 g x 10min to remove cell debris, and resulting supernatant was processed through a 0.45 μM filter for collection of virus. Viruses were quantified by p24 ELISA (Xpress Bio).

3.3 siRNA Transfection

HeLa cells were incubated overnight in a 24-well dish. 300 ng of either KIF5B siRNA or control RLuc siRNA (Millipore-Sigma, EHU060991 and EHURLUC MISSION® esiRNA) were transfected into the cells using Oligofectamine™ Stealth™/siRNA Transfection (Thermo Fisher Scientific). After incubating at 37° C for 4h, reactions were quenched via the addition of DMEM containing 30% FBS. HeLa cells treated with no siRNA were treated with Oligofectamine™ Transfection Reagent, kept in serum-free media, and quenched with 30% FBS DMEM at same time points as siRNA-treated cells. This procedure was repeated the following day, then cells were incubated for 72h prior to assessment of knock down (KD) via western blot.

3.4 Western Blot

Untreated and siRNA-treated HeLa cells were centrifuged at 300 x g for 10min and frozen at -80° C. Pellets were lysed in RIPA buffer (Boston BioProducts) and lysates were clarified by

centrifugation at 17,000 x g for 15min. An equal number of lysed cells or total lysate protein were added to 4X XT Sample Buffer (Bio-Rad) with 5% β-mercaptoethanol (β-ME) and boiled at 100° C for 5min. Samples and Spectra™ Multicolor Broad Range Protein Ladder (Thermo Fisher Scientific) were run on a 10% Criterion™ Tris-HCL Protein Gel (Bio-Rad) at 150V for 1h in Tris-Glycine buffer containing 1% SDS (Bio-Rad). Proteins were transferred to nitrocellulose using a semi-dry transfer apparatus (Fisher-Scientific) for a total of ~1400 mAh/cm^2 overnight. Membranes were blocked for 1h at room temperature in 5% milk in PBS containing 0.1% Tween-20 (PBST) before incubating in primary rabbit anti-KIF5B (Abcam, ab167429) or mouse anti-β-actin (Sigma-Aldrich, A5441) for 1h. The membrane was washed three times in 5% milk in PBST for 15min each before secondary anti-mouse IgG or anti-rabbit IgG conjugated with horseradish peroxidase (Cell Signaling, #7076S and Sigma-Aldrich, AP132P) was added for 45min. The membrane was washed three times in PBST for 15min each, processed in SuperSignal West Pico Chemiluminescent substrate (Thermo Fisher Scientific) for 5 min, and exposed on Amersham Hyperfilm (GE). Knockdown was quantified by comparing average mean gray value between lanes on a film scan converted to 8-bit using ImageJ.

3.5 Plasmid DNA Transfection

KIF5B-KD, RLuc siRNA-treated, or untreated HeLa cells were plated in DMEM containing 10% FBS without antibiotics in a 24-well dish. They were transfected 16h later with 0.8μg plasmid encoding DmrB-KIF5C-mNeon at a 1:2 ratio with Lipofectamine 2000 Transfection Reagent. Opti-MEM I Reduced Serum Media containing this transfectant mixture

was applied to samples and incubated for 6h. After 72h, transfection efficiency was measured by flow cytometry.

3.6 Flow Cytometry

Cells were trypsinized at 37° C for 5min. This reaction was quenched with an equal volume of 2% fetal bovine serum (FBS) in PBS. This cell mixture was fixed in a final concentration of 2% PFA. Samples were then assessed on a BD Accuri™ C6 Plus Flow Cytometer. Dead cells and cell debris were gated out using a FSC/SSC dot plot, and fluorescence in the FITC channel was measured to assess mNeon expression in live cells. This mNeon expression was then compared between DmrB-KIF5C-mNeon transfected cells and un-transfected cells using FlowJo.

3.7 Luciferase Assay

In Aim 1, HeLa cells were seeded in a 24-well dish and transduced with equal p24 amounts of WT or N74D HIV-1, both encoding luciferase, the following day. In Aim 2, untreated HeLa cells or siRNA-treated HeLa cells were transduced with equal p24 amounts WT HIV-1 encoding luciferase. Virus media was removed 2hr post-infection, and cells were incubated in fresh media for 48h. Cells were lysed and assessed for luciferase production (Promega) on a BioTek Synergy 2 Multi-Mode Microplate Reader by measuring luminescence.

3.8 Immunofluorescence Sample Preparation

HeLa cells or macrophages were plated within the glass insert of Mat-Tek dishes overnight. The next day, samples were fixed with 2% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) and then permeabilized with 1% Triton-X 100 in PBS for 15min each. Fixed and permeabilized samples were blocked in either 20% bovine serum albumin (BSA) or 5% normal donkey serum (NDS) diluted in PBS supplemented with 0.5% BSA (PBB) for 45min. Primary antibodies for mouse anti-CypA (AbCam, ab58144), rabbit anti-CPSF6 (Novus Biologicals, NBP1-851676), and/or rabbit anti-α tubulin (acetyl-K40) (AbCam, ab179484) in PBB were added to samples for 1h. Samples were washed five times in PBB. Cells were incubated in secondary antibody solutions in PBB for 1h in the dark and again washed five times with PBB. Finally, cells were stained with Hoescht at a concentration of 1:2000 for 15min, washed three times with PBS to remove any residual serum, and mounted with gelvatol to a coverslip.

3.9 Immunofluorescence Microscopy

A Nikon Ti inverted confocal microscope was used to acquire 3D stacks images of fixed samples with a 100X 1.49 NA oil-immersion objective. LU-NV laser launch (Nikon) was used to emit lasers at 405 nm, 488 nm, 561 nm, and 640 nm. Fields of view were randomly chosen by quick scanning in the Hoechst channel. ND Acquisition in Elements (Nikon) was applied to collect 3D multi-channel imaging (1024 x 1024 pixels) with 2X line averaging. Images of 488 nm and 561 nm channels were acquired by GaAsP detectors (Nikon). 3D stacks were acquired with 0.125-

0.250 μm step intervals to cover the entire cell volume (6-10 μm) with a motorized piezo Z stage (Nikon).

4.0 Results

The work described below is comprised of unpublished results (Aim 1) and data adapted from the following publication (Aim 2):

Zhong Z, Ning J, Boggs EA, Jang S, Wallace C, Telmer C, Bruchez MP, Ahn J, Engelman AN, Zhang P, Watkins SC, Ambrose Z. Cytoplasmic CPSF6 regulates HIV-1 capsid trafficking and infection in a cyclophilin A-dependent manner, mBio 2021, 12:e3142-20.

All work was performed by me, with the exception of the SupT1 cell imaging in Figure 8.

4.1 AIM 1: Examine requirement for KIF5B in HIV-1 capsid microtubule trafficking and nuclear import

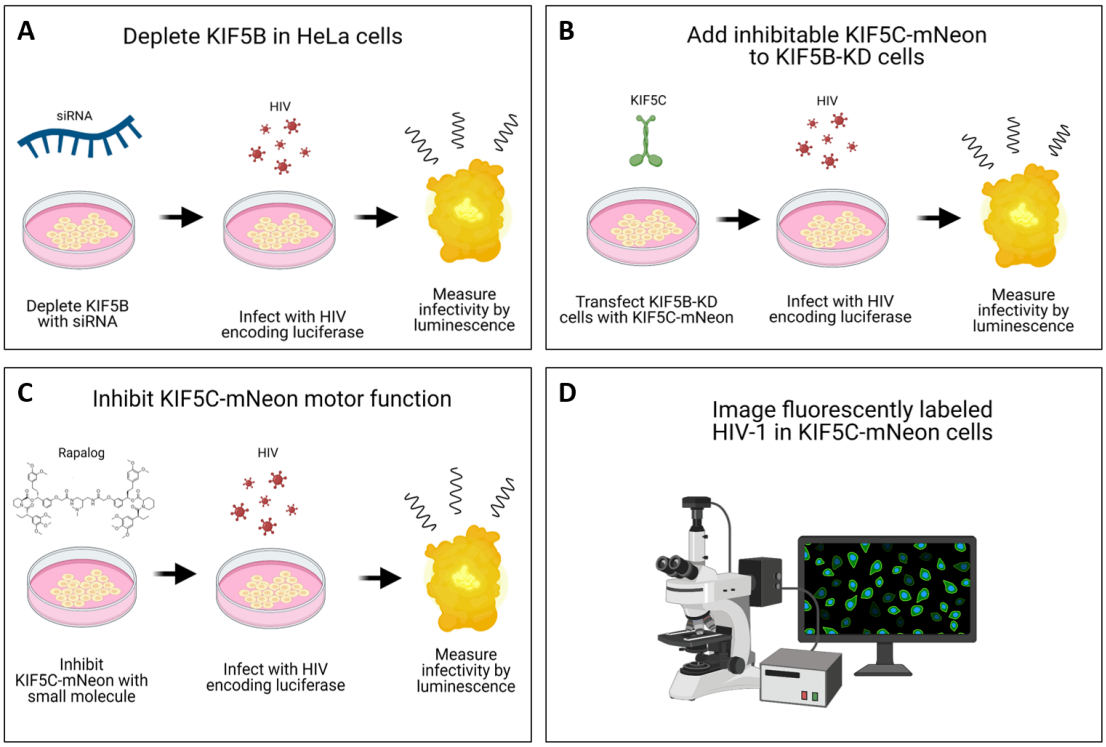

Figure 2: Aim 1 Experimental Design. A) KIF5B will be depleted from HeLa cells and HIV-1 infection will be assessed. B) A plasmid encoding inhibitable KIF5C-mNeon will be transfected into cells. C) KIF5C-mNeon motor activity in transfected cells will be inhibited by treatment with a small molecule. (D) HIV-1 trafficking will be evaluated using live cell microscopy in these different conditions. Created with BioRender.com.

Trafficking of the HIV-1 capsid to the host cell nucleus requires dynein and kinesin, motor proteins of opposing directionality[76]. KIF5B has been shown to be involved in both capsid uncoating[76] and nuclear docking[80], but its exact role in HIV-1 trafficking remains unclear. To investigate the requirement for KIF5 in HIV-1 trafficking and infection, we chose to express fluorescently labeled KIF5C containing the DmrB domain, which becomes homodimerized within minutes after exposure to a rapamycin analog (DmrB-KIF5C-mNeon)[173]. Dimerization of the

DmrB domain leads to inhibition of kinesin movement on microtubules, which would allow evaluation of its effect on HIV-1 capsid microtubule trafficking. KIF5C is a kinesin heavy chain paralogue typically found in neurons, which was utilized as KIF5B was not found to be inhibitable[173]. As KIF5B is the predominant kinesin motor component in HeLa cells and has been more extensively studied in HIV-1 infection, the ability of the HIV-1 capsid to utilize KIF5C would first need to be assessed. A set of experiments were designed to deplete KIF5B in HeLa cells and replace with DmrB-KIF5C-mNeon to determine its effect on HIV-1 infection and trafficking (**Fig 2**).

First, KIF5B expression was depleted by transfecting different amounts of KIF5B-targeting siRNA (150, 200, 250, and 300 ng) into HeLa cells. To detect potential non-specific silencing and to control for any impact of the transfection process on KIF5B expression, a separate population of cells were transfected in parallel with siRNA targeting Renilla luciferase (RLuc), which is not expressed in mammalian cells or by our luciferase reporter HIV-1. Cells were collected at 24h, 48h, and 72h post-transfection and assayed for KIF5B expression by western blot. β-actin was included as a control to ensure that equal cell lysates were loaded in each lane. As no reduction in KIF5B expression was detected after a single siRNA transfection (data not shown), HeLa cells were transfected with 300ng KIF5B siRNA twice, 24h apart. KIF5B depletion was observed at 72h post-second transfection by KIF5-targeting siRNA and not by the control siRNA (**Fig 3A, B**).

To assess whether KIF5B depletion inhibited HIV-1 infectivity, KIF5B knock down (KD), control siRNA-treated, or untreated HeLa cells were infected with 10ng equivalent of HIV-1 encoding a luciferase reporter. After 72h, cells were lysed and examined for luciferase expression.

KIF5B KD significantly inhibited HIV-1 infectivity, whereas control siRNA did not (**Fig 3C**). Our results are consistent with those previously reported[76].

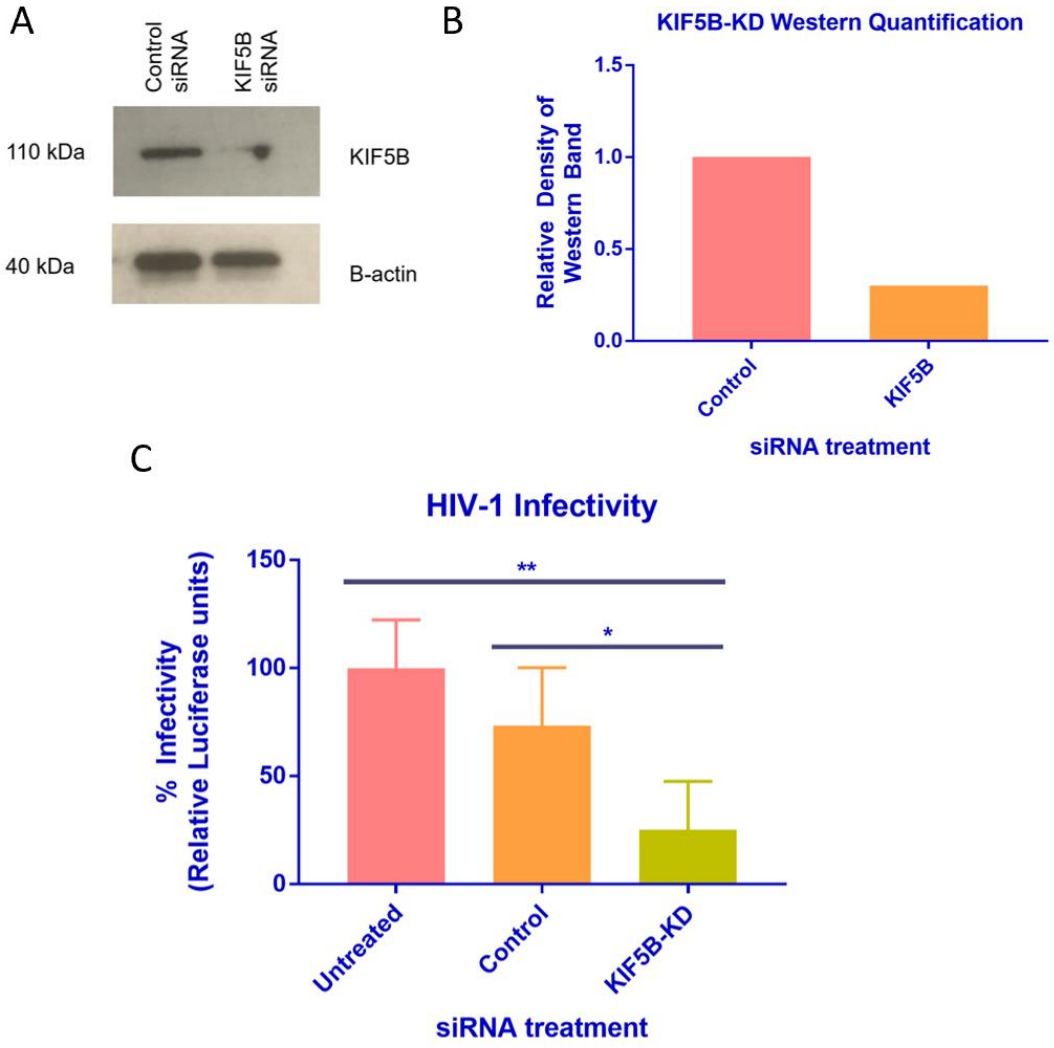

Figure 3: Depletion of KIF5B Inhibits HIV-1 Infectivity. A) KIF5B depletion by siRNA transfection in HeLa cells was confirmed by western Blot. β-actin is included as a loading control. B) Quantification in western blot results are shown. C) HeLa cells treated with no siRNA, control siRNA, or KIF5B siRNA were infected with WT HIV-1 that expresses luciferase. Infection was measured by detection of luciferase (relative light units). Error bars represent standard deviations in duplicate infections. Statistics performed by GraphPad PRISM with unpaired t-tests between

columns, where P values <0.05 were deemed statistically significant. * designates P = 0.01 to 0.05 and ** designates P = 0.01 to 0.001.

To investigate whether inhibitable KIF5C-mNeon could rescue infection in cells depleted of KIF5B, a plasmid encoding DmrB-KIF5C-mNeon was transfected into untreated HeLa cells, control siRNA-treated cells, and KIF5B KD cells. The fluorescent mNeon reporter can be measured via flow cytometry to assess transfection efficiency. Flow cytometry revealed that cells successfully expressed mNeon at 72h after transfection (**Fig 4**). Surprisingly, inhibitable KIF5C-mNeon expression resulted in no HIV-1 infection regardless of KIF5B depletion after multiple attempts (data not shown). Ongoing studies are underway to determine how KIF5C-mNeon overexpression in HeLa inhibits HIV-1.

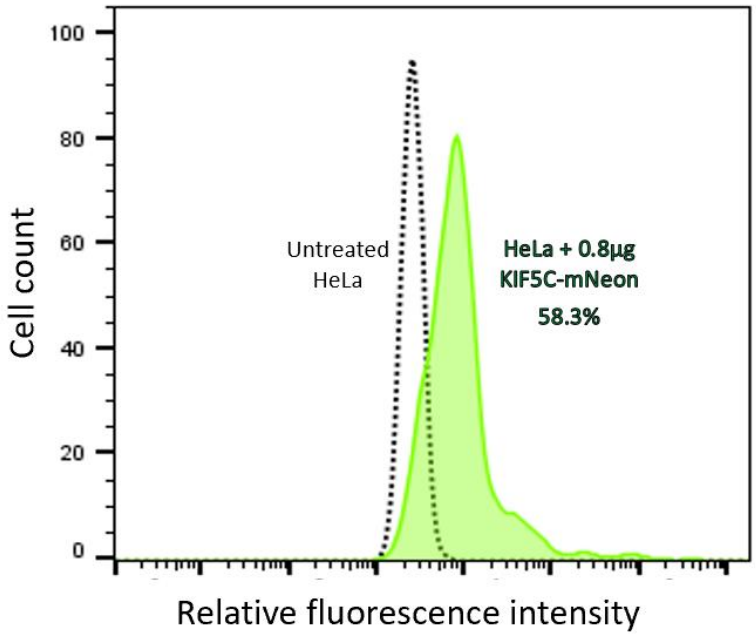

Figure 4: Transfection of KIF5C-mNeon into HeLa Cells. Relative mNeon intensity was detected and compared in HeLa cells containing no fluorescent reporter and HeLa cells transfected with 0.8µg of DmrB-KIF5C-mNeon.

4.2 AIM 2: Visualize the localization of HIV-1 capsid-binding host proteins within cells and quantify their impacts on infectivity

Our lab previously identified the presence of full-length CPSF6 puncta within the host cell cytoplasm and observed that these puncta co-traffic along microtubules with the WT HIV-1 capsid, but not the CPSF6-independent N74D mutant[73]. To further investigate interactions between the HIV-1 capsid and cytoplasmic CPSF6, point and truncation mutants known to shift cellular distribution of CPSF6 were produced. Alteration of four critical, positively-charged amino acids (K547, R549, R559 and R561) within the RS domain to negatively-charged glutamic acid (CPSF6-4Glu) and truncation of CPSF6 at residue 358 (CPSF6-358) upstream of the RS domain (**Fig 5**) lead to reduced TNPO3 interaction, decreased nuclear localization of CPSF6 and restricted WT HIV-1 infectivity at the step of nuclear entry[99, 155]. We previously demonstrated that CPSF6-358 restricted HIV-1 nuclear entry by a reduction in the accumulation of 2-LTR circles, an abortive product of unintegrated viral DNA, within the nucleus[99]. Expression of iRFP670 tagged CPSF6-4Glu or CPSF6-358 in HeLa cells shifted expression of fluorescently labeled CPSF6 to the cytoplasm (**Fig 6A**). Restriction of infectivity corresponded to a roughly proportional degree of cytoplasmic CPSF6 expression (**Fig 6B, 6C**). N74D HIV-1, which does not bind to CPSF6, was not restricted by any mutant (**Fig 6C**). Alteration of WT HIV-1 complex trafficking increased with greater cytoplasmic localization (data not shown)[73].

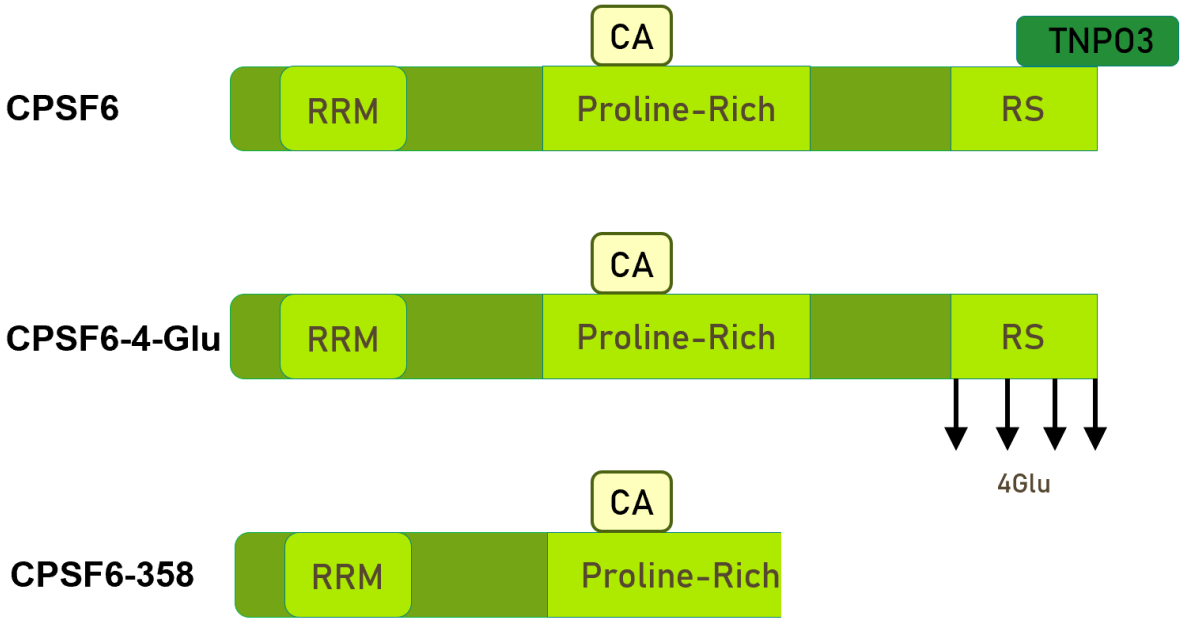

Figure 5: Locations of CPSF6 Mutations. The CPSF6 arginine/serine (RS)-rich domain is the binding domain for the karyopherin TNPO3. CPSF6-4-Glu has 4 amino acid substitutions in the RS domain, while CPSF6-358 has a truncation upstream of the RS domain. These mutant CPSF6 proteins have diminished binding to TNPO3. The proline-rich domain includes the binding domain for HIV-1 capsid, which is maintained by all mutants.

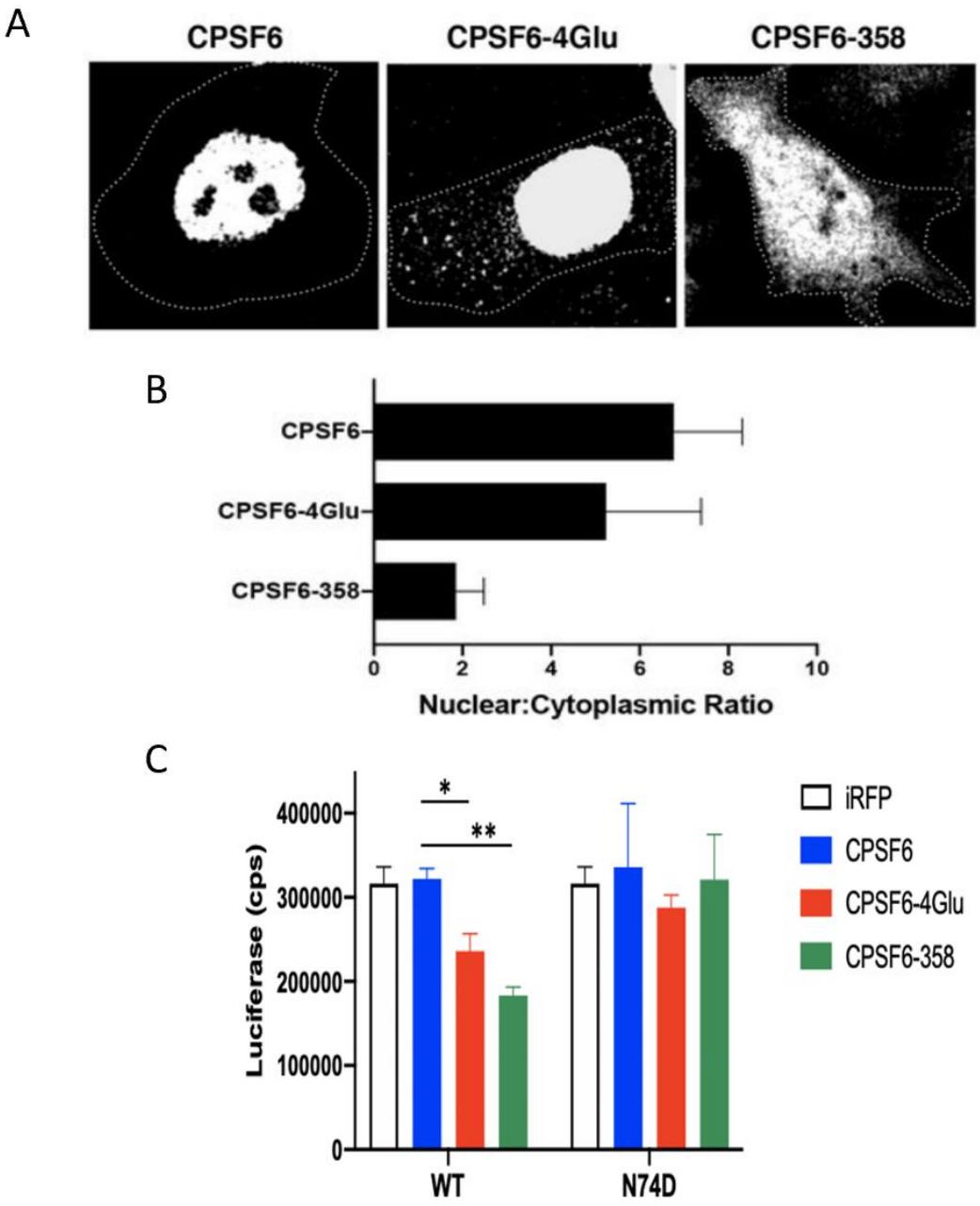

Figure 6: Localizations and Infectivity of CPSF6 Mutations. A) CPSF6 constructs were fused to iRFP and expressed in HeLa cells. Mutations in or truncations of the CPSF6 RS domain produce a phenotype with more cytoplasmic expression. B) The ratio of nuclear to cytoplasmic expression of fluorescently tagged CPSF6 was quantified. C) HeLa cells expressing fluorescently tagged CPSF6 or CPSF6 mutants were infected with WT HIV-1 or N74D HIV-1 that express luciferase. Infection was measured by detection of luciferase (relative light units). Error

bars represent standard deviations in duplicate infections. Unpaired t-tests were performed to compare infection in cells expressing different CPSF6 constructs. * designates p = 0.01 to 0.05 and ** designates p = 0.01 to 0.001.

Full-length CPSF6 binds to WT HIV-1 capsid but not to that of the N74D mutant during infection [73, 157]. Our laboratory showed that when CypA binding to capsid is inhibited by treatment of infected cells with CsA, both the frequency and volume of these CPSF6-capsid complexes increase in a dose-dependent fashion[73, 157]. CsA treatment of cells infected with CypA-independent G89V HIV-1 produced no difference in CPSF6 complexes[73]. Accordingly, fewer puncta were seen in cells infected with HIV-1 labeled with CypA-DsRed, a CypA construct that oligomerizes upon the capsid[73]. In vitro CA tubular assemblies incubated with fluorescently labeled CypA bound less CPSF6 than CA tubes incubated in a control buffer (data not shown) [73]. Incubation with CsA prevented CypA binding to CA tubes, which resulted in more CPSF6 binding. Altogether, these results indicate that CypA binding to HIV-1 capsid prevents CPSF6 from binding to capsid.

To further examine the interplay between cytoplasmic CPSF6 and CypA during infection, we sought to illustrate the cellular distribution of CypA. As expected, endogenous CPSF6 was expressed predominantly in the nucleus (**Fig 7**). Surprisingly, endogenous CypA, which is abundantly expressed in cells, was excluded from both the nucleus and the adjacent perinuclear regions in HeLa cells (**Fig 7**). A similar pattern of CypA expression was observed SupT1 CD4+ T cells, a more relevant cell line for HIV-1 infection (**Fig 8**). This physiologic reason for this expression pattern is unknown. We hypothesized that the perinuclear region that lacked CypA localization may correspond to the MTOC. Therefore, CypA and tubulin expression were evaluated in HeLa and SupT1 cells. As expected, the perinuclear region of CypA exclusion showed high tubulin expression in both cell types, suggesting it is indeed the MTOC (**Fig 8**).

We similarly visualized localization of endogenous CypA in primary human macrophages, another HIV-1 target cell. The nuclear exclusion of CypA encountered in HeLa cells and SupT1 CD4+ T cells was not recapitulated in MDM, in which CypA was expressed in the nucleus, distributed evenly throughout the cytoplasm, and concentrated in patches at the cell periphery (**Fig 9**). These cell-type dependent results on CypA localization correlate to recently observed findings on differential capsid uncoating dynamics between HeLa cells, CD4+ T cells, and macrophages.

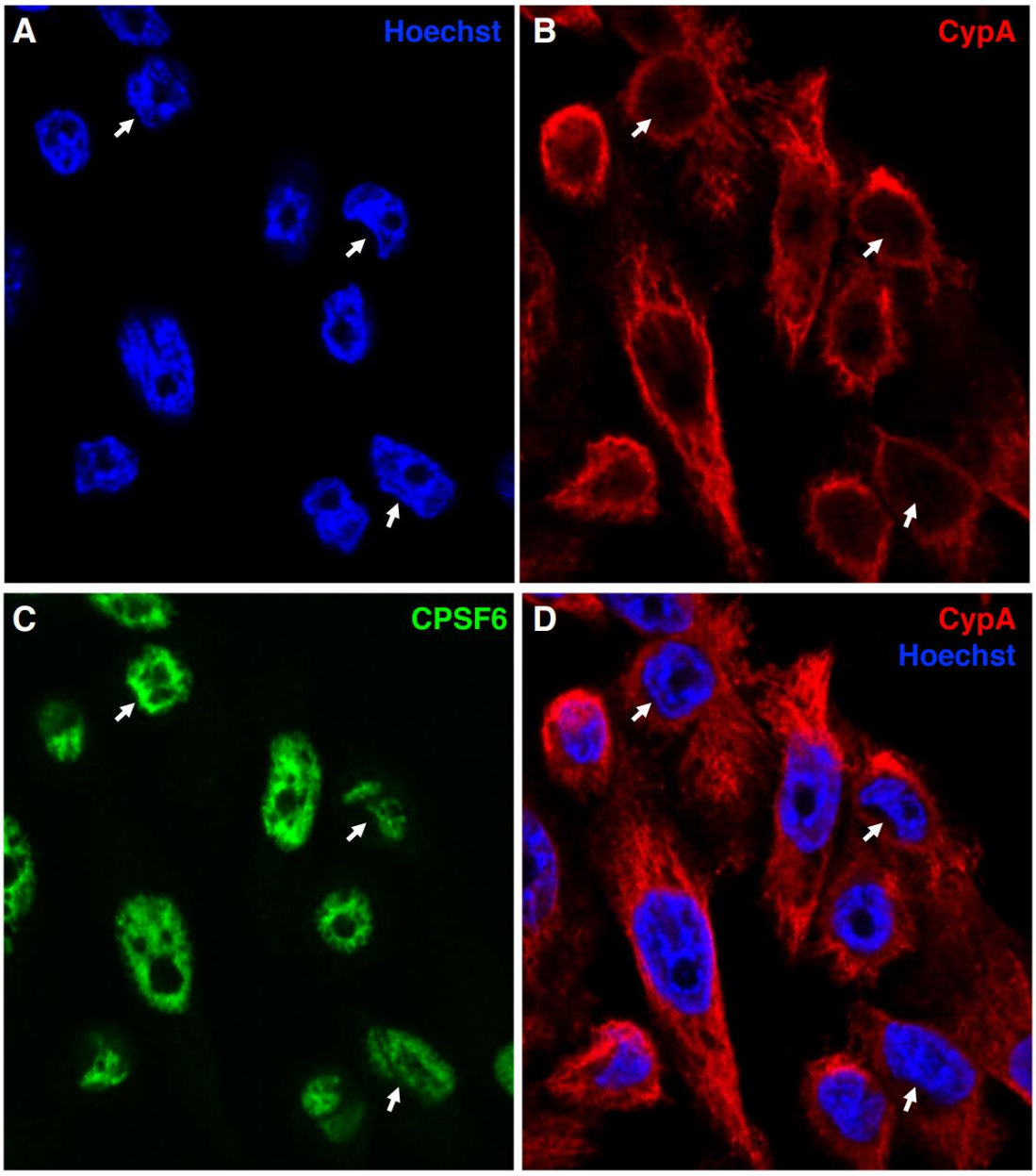

Figure 7: Localization of CypA in HeLa. HeLa cells were stained with A) nuclear marker Hoechst (blue) and with antibodies to B) CypA (red) and C) CPSF6 (green). D) Mid-section images reveal a lack of CypA expression in the nucleus and perinuclear region as indicated by white arrows.

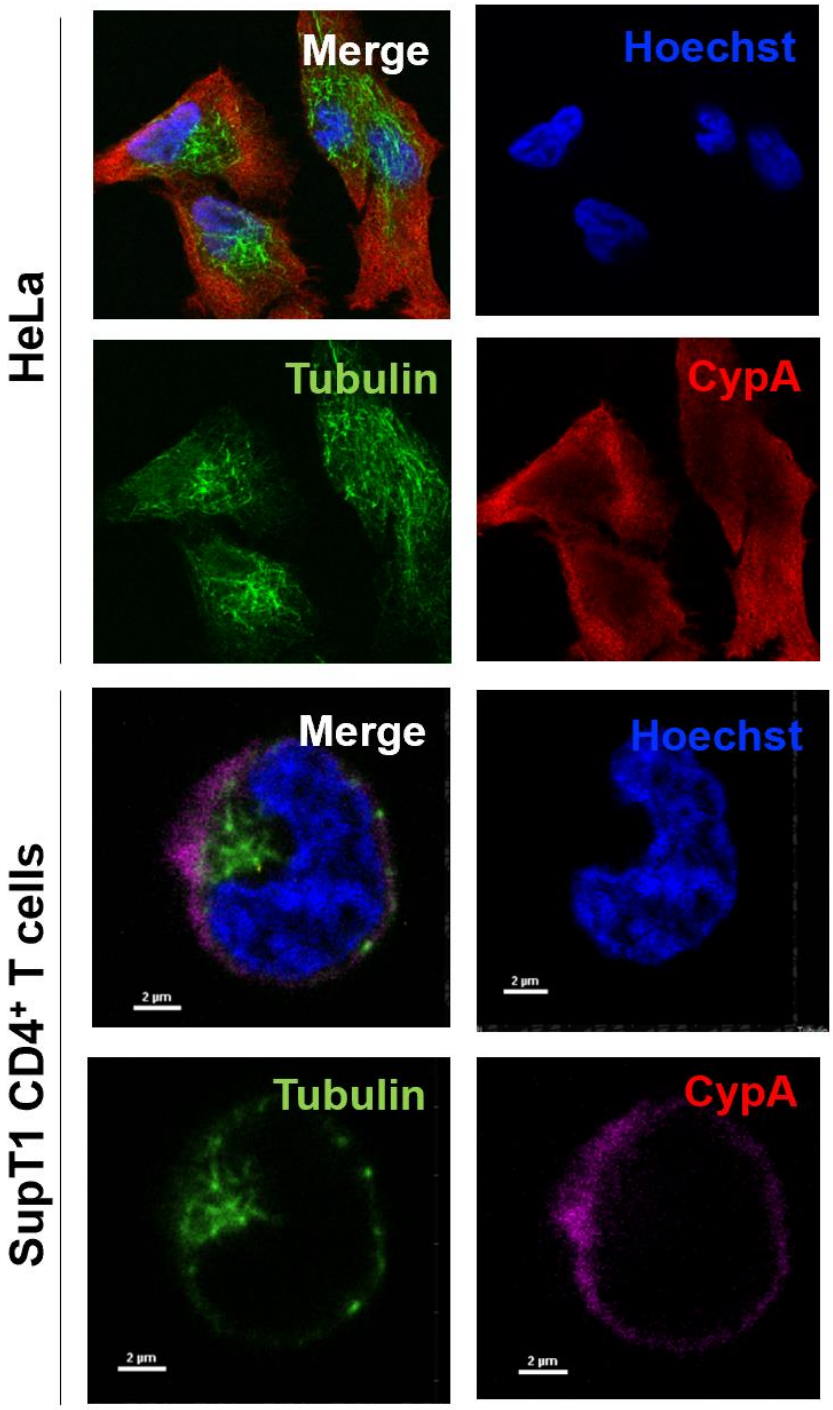

Figure 8: Perinuclear Exclusion of CypA in HeLa and SupT1 Cells. HeLa and SupT1 CD4+ T cells were stained with Hoechst and antibodies for CypA (red) and tubulin (green).

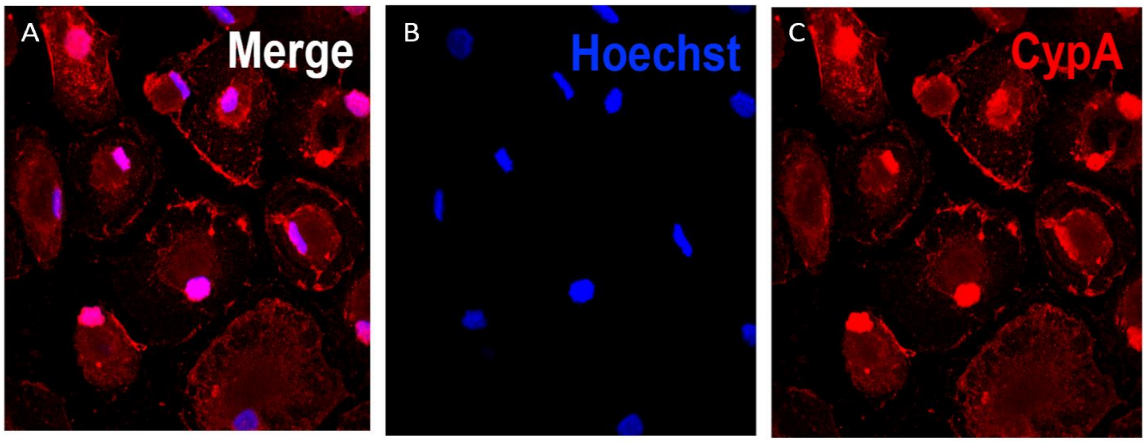

Figure 9: Localization of CypA in Macrophages. Primary human MDMs were fluorescently stained with Hoechst (blue) and an antibody recognizing CypA (red).

5.0 Discussion

The first human ARV, AZT, was licensed for the treatment of HIV-1 just 34 years ago. By 2019, 67% of people living with HIV were receiving ART[6]. This rapid expansion in access to ARVs has been accompanied by a correspondingly rapid increase in the rates of drug resistance due to improper adherence. Variants of HIV-1 resistant to first-line drugs are widespread enough that up to 26% of people living with HIV-1 initiating ART for the first time exhibited pre-treatment resistance in 2019[174]. Because the delicate HIV-1 capsid is intolerant to many point mutations, it could represent a novel opportunity for the development of drugs that limit the infectious potential of escape mutants. A capsid inhibitor, GS-6207, was recently shown to inhibit capsid binding to both CPSF6 and Nup153, produced escape mutants either unfit for infection or sensitive to other ARVs, and significantly reduced viremia in recipients when administered as a once-monthly injection in a phase I clinical trial[171]. None of the mutations associated with escape from GS-6207 function were identified in ART-naïve people living with HIV-1[175]. As suboptimal adherence to ART regimens that rely on daily dosing is associated with a decrease in ART efficacy and increased development of ARV resistance, long-acting agents like GS-6207 hold additional promise [176]. Furthermore, as capsid plays an indispensable role in all steps of the HIV-1 replication cycle prior to gene expression, capsid inhibitors targeting post-entry events could potentially prevent establishment of the HIV-1 reservoir that requires life-long adherence to ART to prevent AIDS.

Depletion of KIF5B has been shown to inhibit HIV-1 infection[76], but little is known about the mechanism by which this occurs. The reliance of other viruses on KIF5B is due to impact of the motor protein on both capsid disassembly and docking of capsid at nuclear pores required for

viral nuclear entry[77, 79]. Here we sought to assess the viability of employing an inhibitable, fluorescent construct containing KIF5C, a kinesin heavy chain isoform typically found in neuronal cells, for directly studying the role of KIF5 in HIV-1 capsid trafficking. Application of a rapamycin analog to single molecule samples containing processive DmrB-KIF5C-mNeon immediately inhibits motility along microtubules[173]. Perhaps more importantly, this cessation of motor activity can be observed in real time upon small molecule treatment of samples being examined for normal secretory pathway trafficking during live cell imaging[173]. The results herein illustrate a successful depletion of KIF5B from HeLa cells with a resultant inhibition of HIV-1 infectivity, and confirm that DmrB-KIF5C-mNeon can successfully be transfected into KIF5B-KD cells. To our knowledge, further development of a system in which this construct is functionally expressed in HIV-1 target cells would represent the first application of an inhibitable motor protein to the study of viral trafficking.

Unfortunately, DmrB-KIFC-mNeon inhibited HIV-1 infection regardless of KIF5B KD. This may be due to overexpression of KIF5 driving cellular trafficking predominantly toward the cell periphery, including incoming HIV-1 capsid via VSV-G pseudotyping. To explore this possibility, experiments could examine the localization of kinesin-trafficked cargo, such as endosomes, within cells expressing DmrB-KIF5C-mNeon. Once working, future studies employing DmrB-KIF5C-mNeon could investigate the relationship between the kinesin heavy chain, the HIV-1 capsid, and the impact of this interaction on the vital stages of uncoating and nuclear entry in the HIV-1 life cycle.

During motor protein-mediated transit along microtubules, the HIV-1 capsid co-traffics with host protein CPSF6[73]. Truncation or point mutants of CPSF6 within or upstream of its nuclear localization signal increased expression in the cytoplasm relative to full-length CPSF6,

which was directly correlated with a reduction in WT but not CPSF6-independent N74D HIV-1 infectivity. Binding of cytoplasmic CPSF6 mutants to the HIV-1 capsid causes premature capsid permeabilization[157], and we have shown that CypA binding to capsid prevents this interaction in vitro[73]. In HeLa cells and CD4+ T cells, CypA was found to be excluded from both the nucleus and perinuclear MTOC, while full-length CPSF6 was predominantly nuclear. However, in primary macrophages CypA was found throughout both the nucleus and cytoplasm as well as in patches at the cellular periphery.

Increased nuclear CypA expression in primary macrophages but not HeLa or CD4+ T cells corresponds to differences that have been observed in HIV-1 capsid trafficking and infectivity. For example, co-localization of CA staining with nuclear HIV-1 PICs is markedly more pronounced in MDMs than HeLa cells[160, 177]. The presence of CypA in the nucleus of MDMs may make it so that CypA protects the capsid from CPSF6 binding-induced disruption for longer periods, allowing for the detection of more CA within the nucleus. Others have recently found that binding of CypA to capsid hinders binding of capsid-destabilizing factor TRIM5α in primary CD4+ T cells and MDMs, which thusly inhibits TRIM5α-mediated triggering of type I interferon responses[151, 152]. Interactions between CypA and cytoplasmic host factors could influence the integrity of the core upon arrival at the nuclear membrane and, in turn, could have significant implications for a recently proposed model of nuclear uncoating[93-96]. While intact HIV-1 capsids have recently been visualized inside NPCs in a CD4+ T cell line[96], it is not clear if some permeabilization or loss of CA can occur in the cytoplasm. Uncoating of the HIV-1 capsid within the host cell nucleus would require the careful import of an intact or nearly-intact capsid through the NPC. The spatial staging of interactions between the HIV-1 capsid, restrictive host factors, and protective host factors preceding nuclear docking is of utmost import in this framework. Future studies into nuclear

uncoating should incorporate this meticulously orchestrated network of protein-protein interactions when investigating cell type dependence.

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