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I, Vishwanutha Shamshabad, hereby submit this original work as a part of the requirements for the degree of Master of Science in Immunology.

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Significance of Tubular Recycling Endosomes in Envelope Glycoprotein Trafficking and Particle Incorporation in HIV-1 Clade B Primary Isolates

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Significance of Tubular Recycling Endosomes in Envelope Glycoprotein Trafficking and Particle Incorporation in HIV-1 Clade B Primary Isolates

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By

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Abstract

Human Immunodeficiency virus (HIV-1) was discovered in 1983 from the lymph node sample of a patient at the Pasteur Institute and subsequently was found to be strongly linked to onset of AIDS. HIV-1 and its pathogenesis has been extensively studied now for decades. Many crucial components of the virus such as the viral replication machinery, proteins involved in replication and assembly, fusion of viral and host cells during infection, and many other aspects of the viral lifecycle have been defined. The HIV-1 envelope glycoprotein (Env) is an essential component of the virus, present on the lipid envelope of the virus and involved in cell membrane fusion and entry. An aspect of HIV-1 Env biology that is not well understood is how it interacts with host cell machinery to reach the site of particle assembly on the plasma membrane. This study investigates the involvement of tubular recycling endosome (TRE), a component of the cellular recycling machinery that is involved in transporting internalized cargo, in the trafficking of Env following endocytosis from the plasma membrane. In particular, this study addresses the interactions of primary isolates of HIV-1 Clade B Env with the TRE, using immunofluorescence staining and imaging of TRE markers. This study also focuses on the role of Phosphatidic Acid (PA) in modulating Env interaction with the TRE, and its regulation through phospholipase D (PLD). The effect of disruption of PA production through inhibition of PLD on Env-TRE colocalization was critically assessed using live cell and pulse-chase imaging along with monitoring of TRE markers. The effects of inhibition on infectivity and particle incorporation were evaluated after PLD inhibition. This study confirmed that primary isolate Env associates strongly with the TRE, and that inhibition of TRE formation through PLD inhibition disrupts Env trafficking. Findings here build on those with laboratory isolates of HIV-1 and provide a new potential therapeutic target to inhibit replication of the virus.

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List of Abbreviations

WHO World Health Organization

HIV Human Immunodeficiency Virus

AIDS Acquired Immunodeficiency Syndrome

ER Endoplasmic Reticulum

PM Plasma Membrane

Env Envelope Glycoprotein

CT Cytoplasmic Tail

FIPs Family Interacting Proteins

TRE Tubular Recycling Endosomes

CME Clathrin Mediated Endocytosis

ERC Endosomal Recycling Compartment

MICALL1 Molecules Interacting with Cas-like L1 protein

EHD1 Eps-15 Homology Domain

PLD Phospholipase D

PA Phosphatidic Acid

Chapter 1: Introduction

AIDS or Acquired Immunodeficiency Syndrome was first reported in 1981 when there was a rise in homosexual men succumbing to unusual infections and malignancies[1]. According to the World Health Organization (WHO), there are 40 million people living with AIDS worldwide. Human Immunodeficiency Virus or HIV is the causative agent for AIDS and is classified under *Retroviridae* family and *Lentivirus* genus. Through rapid isolation of virus from patients and determination of molecular characteristics of HIV genome helped determine the virus origin[2]. HIV is classified into HIV-1 and HIV-2 based on their genetic differences[3]. HIV-1 was discovered to have arisen in African nonhuman primate species, with likely transmission to humans from the closely related virus SIVcpz in chimpanzees, while HIV-2 is closely related to SIVsm found in sooty mangabeys[1]. HIV-1 is divided into four groups namely M, N, O and P that contribute to 95% of infections[4]. The M group contributes to 70% of infections worldwide and comprises of 9 different clades or subtypes and the main ones being C, B and A[5]. It is believed that HIV infection and onset of the AIDS pandemic originated due to cross-species infections between humans and primates[6].

Individuals who are initially infected with HIV develop acute HIV infection also known as primary HIV infection. Following primary infection, patients tend to have detectable plasma viremia for one week to three months and develop an immune response towards HIV[7]. HIV transmission occurs via exchange of bodily fluids through blood transfusions, sexual intercourse, drug injections, and unsterile medical practices. Blood tests are the most common way to diagnose HIV. HIV tests primarily include antibody/antigen tests and nucleic acid tests. People infected with HIV over time develop significant depletion of CD4+ T cells, and plasma viremia is variable but may increase in late-stage disease in untreated individuals. There is also observable loss in T-cell

subsets such as Th-17 cells[8]. Development of a HIV vaccine is challenging due to the high diversity amongst HIV clades and presence of clades, sub-clades and isolates that infect people based on geographical location which demands the production of a vaccine with high degree of diversity and cross-reactivity[9]. HIV exhibits high mutation rates as well as recombination rates, leading to extreme variability and the ability to escape from immune responses, posing a major challenge for development of a preventive vaccine[10]. The reverse transcriptase (RT) of HIV is error-prone, contributing to overall genetic variability. Heavy glycosylation of Env poses another barrier to effective vaccine development, shielding the virus from effective neutralizing antibody recognition[11]. Moreover, the ability of HIV to integrate its genome into the host cell as an essential part of its lifecycle, and the early establishment of latent viral reservoirs following infection, poses additional barriers to immune control and cure[12,13]. HIV therapeutics have achieved many milestones including the current treatment methods that include a combination of inhibitory drugs used as Highly Active Antiretroviral Therapy (HAART)[14,15]. HAART consists of combination of drugs that focus on inhibiting the enzymes essential for initial infection such as reverse transcriptase, integrase or protease[16]. Despite great advances in therapeutic approaches that save lives and can lead to near-normal lifespans for persons living with HIV, highly effective antiretroviral therapy fails to eradicate the virus in infected people.

HIV Structure and Pathogenesis:

HIV virions are roughly spherical in shape with an approximate 100nm in diameter with a lipid bilayer as thick as 4nm[17]. They are primarily comprised of two copies of the single-stranded 9.8 kb positive-sense RNA genome which are enclosed inside a capsid[18] as denoted in **Figure 1**. There are three polyproteins produced by the virus- Gag, Pol and Env which are produced by the major genes *gag*, *pol* and *env* that promote interaction between the virus and target human cells[18]. Gag

forms the major structural core of the virus and directs the virus assembly process, while Pol is cleaved into the major enzymatic machinery of the virus (protease, RT, and integrase). Env is a trimeric glycoprotein that is assembled onto the surface of the virion and the subject of this thesis work. The rest of the genome produces regulatory proteins – Tat, Rev and the accessory proteins – Vif, Vpr, Vpu and Nef[19].

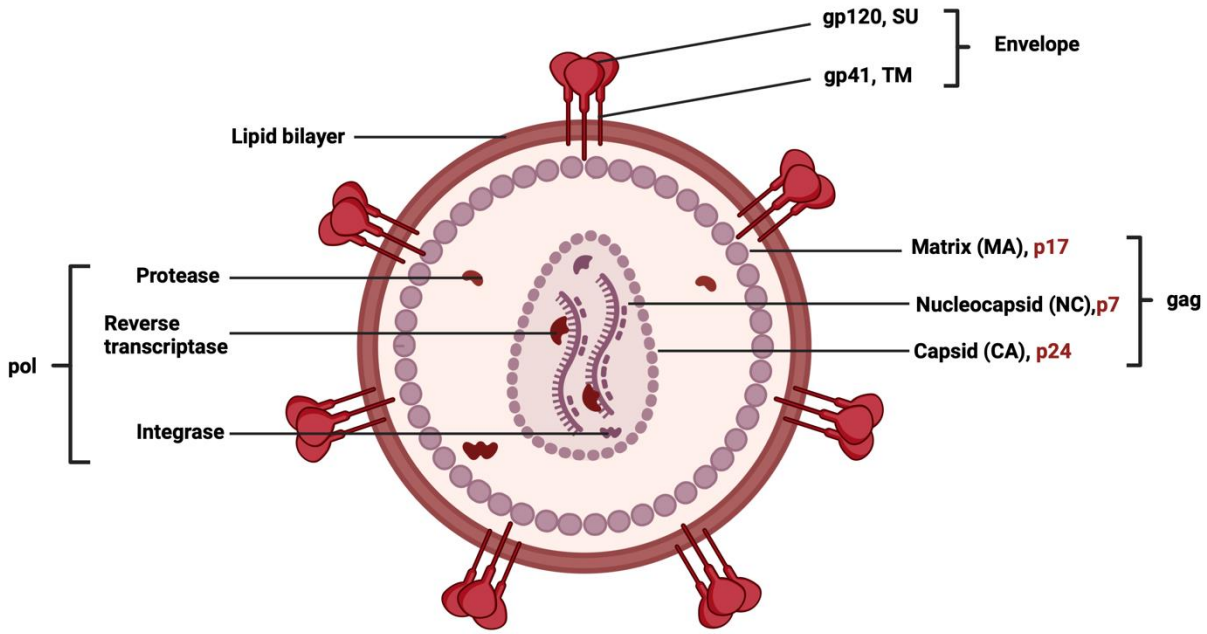


Figure 1: *Structure of HIV-1 virion. Reverse transcriptase, Integrase and Protease are produced by pol gene; gag gene produces outer coat proteins, and env gene produces the envelope glycoprotein (Generated using BioRender)*

Env is a heterotrimer comprising of two subunits gp120, a surface glycoprotein subunit, and gp41, a transmembrane subunit[20]. HIV interacts with host receptors CD4 receptor and the coreceptors CXCR4 and CCR5. gp120 initiates fusion by binding to CD4 receptor, triggering a conformational change that facilitates coreceptor binding. Following binding of gp120 to the co-receptors CXCR4 or CCR5, conformational changes are induced in the gp41 subunit triggering membrane fusion[21].

Lentiviruses are characterized by the presence of a large cytoplasmic tail (CT) in the Env. HIV Env variants in infected individuals exhibit specific tropism for either CCR5 or CXCR4 coreceptor, designations previously known as M-, T- and dual tropic variants. M-tropic variants interact through CCR5 to infect primary T lymphocytes, with a subset capable of infecting macrophages and monocytes. T-tropic variants infect T lymphocytes. A more precise classification is R5 (CCR5-tropic) vs X4 (CXCR4-tropic) variants.[22] Other minor co-receptors such as CCR1, CCR3, CCR8, CCR2b to mention a few have been found to be involved in infections in dendritic cells and macrophages[23,24].

HIV infects immune cells by the process of fusion of host and viral cell membranes triggered by the interactions of Env with the CD4 receptor and co-receptors CXCR4 or CCR5. Other cells that are also affected by HIV include macrophages, with some evidence for rare infection of monocytes, dendritic cells even in microglia. Env consists of heavily glycosylated heterotrimers comprising of the gp120 and gp41 subunits that interact with the receptors and co-receptors on the target cells. The viral core is released into the cell cytoplasm post fusion. The viral genome is converted from RNA to DNA via the reverse transcription process, with the genomic viral RNA serving as the template and the host $Trna^{Lys3}$ as the primer. The DNA copy of the viral genome is termed the provirus and integrates into the host cell genome.[25,26] The integrase enzyme is responsible for catalyzing the integration of the viral DNA into the host cell genome. Integrase remains bound to the LTR present on the viral DNA, forming a pre-integration complex (PIC)[27]. The viral DNA undergoes 3' OH-processing involving the removal of the dinucleotides from the 3' end allowing the hydroxyl groups to interact with the 5'- phosphate groups of the target DNA, integrating the viral RNA into the host genome[28,29]. The proviral genome subsequently undergoes transcription and translation using the host's replication mechanism. Tat and Rev accessory

proteins are produced through fully spliced transcripts,[30,31] HIV Env is produced in a similar manner through singly spliced RNA export that acts as the mRNA template. Unspliced RNA is translated to produce *gag* and *pol* proteins[32]. 5' to 3' transcription of the *gag* reading frame encodes for outer core membrane (MA, p17), capsid protein (CA, p24) and nucleocapsid (NC, p7). During assembly, the protease cleaves Gag to generate these new sets of proteins MA, NC, CA and spacer peptides SP1 and SP2[33]. Transcription of Pol reading frame encodes for enzymes protease (PR, p12), integrase (IN) and reverse transcriptase (RT, p51)[34]. The gag-pol precursor translates to form the enzymes necessary for viral multiplication. The gag-pol polyprotein complex is later cleaved during maturation by the protease[33]. Envelope glycoprotein (Env) is projected on the virion surface as spike proteins promoting host-viral cell interactions. Viral proteins are assembled through the assistance of Gag, as uncleaved Gag promotes assembly such as gRNA packaging and directing interactions with the plasma membrane[35]. The Gag protein initially forms an immature Gag lattice, while cleavage of the lattice by HIV-1 protease results in formation of a mature Gag lattice. The reassembly of Gag forms a mature virion comprising of the necessary machinery for production of infectious virus after release of the mature virions[36]. Assembly and Env incorporation onto newly formed virions is promoted by the stable interaction of $p55^{gag}$ and the gp41 subunit of env glycoprotein[37]. Env- $p55^{gag}$ interactions are proven to be stable in the presence of detergents[38] and any disruption in the matrix domain of $p55^{gag}$ lowers env incorporation[39,40].

Envelope Glycoprotein (Env):

Env is the only complex that projects itself on the surface of the HIV virion and is responsible for membrane fusion between host and the virus during infection. Env is comprised of heavily glycosylated heterotrimer subunits gp120 and gp41. Envelope is initially available as gp160 precursor that is present at the endoplasmic reticulum (ER). Due to presence of a stop transfer

signal in the gp41CT, gp160 is not fully released into the ER lumen. Gp160 is then glycosylated with oligosaccharides which continue to be modified as Env traffics to the Golgi complex. Cleavage of the precursor into the surface glycoprotein gp120 and transmembrane glycoprotein gp41 occurs through furin or furin-like proteases[41]. These subunits remained linked to each other non-covalently as heterotrimeric Env on the viral membrane. The Env heterotrimer is then transported to the Plasma membrane which is rapidly endocytosed through clathrin-dependent endocytosis[42.] Rapid endocytosis of envelope from the surface contributes to low surface envelope levels likely aiding in evading host immune responses. The gp120 subunit of Env glycoprotein has five variable regions and five constant domains, and conserved cysteine residues that assist in maintaining the Env tertiary structure. The constant regions of the gp120 subunit are responsible for binding of Env to CD4 receptor[43]. Binding of gp120 to the CD4 receptor results in conformational changes in gp41 and thus results in the exposure of the fusion peptide. Fusion peptide guides the formation of a fusion pore that promotes membrane destabilization and fusion of host and viral cell membranes. gp41 is comprised of an extracellular domain or ectodomain, a TMD and the CT. Env CT contains multiple dileucine motifs one of which is the clathrin binding motif YXXΦ motif that assists in directing endocytosis through interactions with clathrin adaptor protein complexes such as AP2[44]. Four models have been proposed to help explain how Env is incorporated into budding particles: i) incorporation of PM proteins including Env randomly ii) specific incorporation guided by protein-protein interactions with Env iii) Gag-Env co-targeting to the same region on the PM and iv) indirect incorporation assisted by bridging factors[21]. YXXΦ motifs such as Y^{712}SPL and Y^{768}HRL are also responsible for high affinity binding to the μ2/AP2 for Env endocytosis through CME[45]. Env CT is required for particle incorporation and contains various motifs which are believed to be involved in this process. The YW^{802} motif is known to

assist the Env trimer in interacting with the Gag polyprotein complex for particle incorporation[46]. Rab11 Family Interacting Proteins (FIPs) are molecules binding to the Rab11 family to assist in trafficking and recycling processes[47]. Studies have shown that Rab11-FIP1C is involved in the trafficking of Env to the plasma membrane which was observed by performing shRNA treatment to deplete the known FIPs showing that there was depletion in particle Env levels as well as decreased gp120 and gp41 levels[48]. FIP1C is also involved in the trafficking on full-length Env to the plasma membrane which was observed through the redistribution of FIP1C from the perinuclear compartment, that strongly colocalizes with Rab11[49], in presence of cells expressing Env when transfected with NL43 provirus[48]. Studies have shown that the motif YW_{795} acts as a crucial CT component and mutations in this motif disrupt the FIP1C-GFP trafficking to the surface from the perinuclear location, suggesting that Rab14 and FIP1C might direct Env trafficking to the particle budding sites. One pathway that Env is believed to potentially utilize for trafficking is the use of Tubular Recycling Endosomes (TREs)

Tubular Recycling Endosome (TREs):

The tubular recycling endosome (TRE) is a tubular network of membranes that are involved in cargo segregation, in particular cargo that is internalized through either clathrin mediated endocytosis (CME) or clathrin independent endocytosis (CIE)[50]. Env is believed to be internalized through CME. Internalization of plasma membrane proteins or lipids is believed to be regulated by this tubular network that radiates from the perinuclear endosomal recycling compartment (ERC). Internalized cargo is trafficked to a special organelle called the Early Endosomes (EE) or the Sorting Endosomes (SE). The EE/SE is a compartment that allows trafficking of the internalized cargo to the late endosomes (LE) and then to the lysosomes for degradation or they get recycled back to the PM[51]. Internalized cargo can be recycled to the PM through “slow

recycling" and "fast recycling" processes. In the fast-recycling process, the cargo is recycled to the PM from the EE. "Slow recycling" process involves the ERC, believed to be located in close proximity to the MTOC and Golgi Complex, where the cargo is trafficked to from the EE[52]. Known markers for TREs include Eps-15 Homology Domain (EHD1), Molecule Interacting with CasL like L1 protein (MICAL-L1)[53] and Rab-GTP binding proteins Rab8 and Rab10[54]. MICAL-L1 is from the family of MICAL proteins that is known to interact with CasL and colocalize in the perinuclear area[55]. MICAL-L1 and EHD1 interact through the first NPF motif present in MICAL-L1 which is also known to contribute to membrane remodeling[56]. MICAL-L1 can associate with the TRE independent of EHD1. Moreover, depletion of MICAL-L1 has also led to the loss of EHD1 recruitment to the tubules. MICAL-L1 depletion impedes the uptake of transferrin and β1 integrin, which are internalized during clathrin-dependent and -independent pathways, respectively[57]. Orthologs of EHD, such as EHD1 are responsible for regulating the exit of cargo from the ERC to the PM[58]. MICAL-L1 has a C-terminal coiled-coil (CC) domain that is an essential domain necessary for localization with the TRE. Any mutation in this domain, specifically in the 700-730 amino acid region causes a significant decrease in colocalization with the TRE. [57] The CC domain also acts as a marker as it selectively binds to the crucial lipid component Phosphatidic Acid (PA) of the TRE[59]. Rab-GTPases are monomeric binding proteins that are proven to be involved in endocytic transport and colocalize at different areas of the endosomes to promote transport. Rab 4, Rab 5, Rab 11, Rab10, Rab8 and Rab35 have been identified as proteins that are involved in regulating endocytic trafficking[54]. Studies indicate that Rab10 knockout caused the disruption of TRE tubules but did not alter the level of expression of both Rab8 and MICAL-L1. Rab10 is also essential for tubulation, as it recruits KIF13A/B and facilitates tubulation through the KIF13 motor activity[60]. Rab10 has also been observed to

colocalize with long TREs which were rich in both phosphatidylinositol-(4,5)-bisphosphate (PI(4,5)P2) as well as PA but did not show high colocalization near the plasma membrane and the perinuclear regions[61]. Studies have also shown that HIV Gag is targeted to the plasma membrane through the specific interactions with PI(4,5)P2, providing a potential connection between Gag binding and the recent finding that Env is located on the TRE [62].

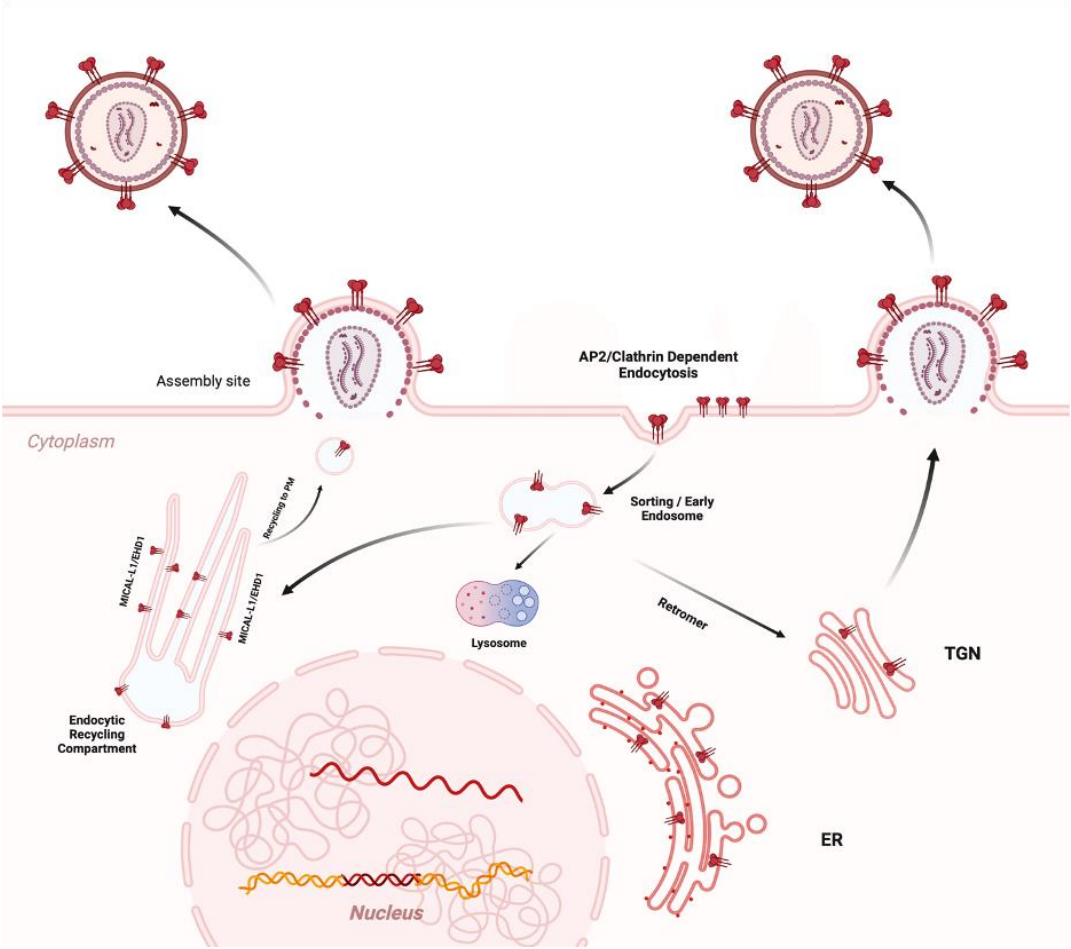


Figure 2: *Pathways available for Env trafficking. Env undergoes rapid internalization (AP2/CME) after undergoing cleavage and can follow multiple pathways for intracellular trafficking. Pathways includes utilizing the TRE to traffick to perinuclear ERC and recycled to PM through Gag microdomain association; trafficking to SE/EE and then to lysosomes for degradation and retromer-mediated trafficking to trans-Golgi network (Generated by BioRender)*

Phosphatidic Acid – crucial TRE component:

Phosphatidic acid (PA) is known to play a crucial role in diverse cellular mechanisms including cytoskeleton remodeling, receptor endocytosis, exocytosis, cell death, membrane trafficking as well as organelle homeostasis[63]. PA is produced through three pathways a) conversion of phosphatidylcholine (PC) to PA through the lipase activity of the phospholipase D (PLD) b) conversion of lysophosphatidic acid (LPA) into PA through the activity of LPA acyltransferase c) conversion of diacylglycerol (DAG) to PA by the kinase activity of DAG kinase[59]. Endosomal systems are known to be enriched in lipids such as $PI(4,5)P_2$ which is essential for the trafficking machinery that mediated through the interactions with the pleckstrin homology (PH) domain. Mammalian PLD has two isoforms, PLD1 and PLD2, with molecular weights 120-kDa and 106-kDa respectively[63,64] which have a similar structure consisting of PH domains, terminal phox consensus sequence (PX) domain helping bind to the PIPs. PLD1 colocalizes with organelles such as Golgi complex and late endosomes, as well as on secretory granules such as lysosomes where PLD1 is activated to generate PA[65]. PLD1 is activated through signals from the proteins such as ARF, Rho and Rac as well as protein kinase C (PKC)[66]. Disruption of PLD2 or examination of cells that had reduced PLD2 showed accumulation of transferrin receptor in both the perinuclear regions when compared to control cells. Cells treated with siRNA against PLD2 showed reduction in transferrin recycling, as well as slower internalization indicating that PLD is crucial for endocytic recycling of cargo[67].

Hypothesis and Specific Aims:

Recent studies conducted by the Spearman Lab have shown colocalization of Env with TRE markers MICAL-L1, EHD1 and Rab10. These studies used either the clade B Env isolate JR-FL or the lab-adapted NL4-3 Env. Moreover, the disruption of the TRE by shRNA-mediated depletion of MICAL-L1 or EHD1 led to a dramatic reduction in Env incorporation in H9, Jurkat, and CEM T cell lines as well as in primary macrophages. This new finding suggests strongly that TRE trafficking of Env is a required step for incorporation into particles. This led to the development of my first specific aim for my project: To determine if HIV-1 Clade B primary isolates of Env interact with the TRE and utilize this pathway for trafficking and incorporation into particles.

The Caplan laboratory has shown that PA depletion through inhibition of Phospholipase D disrupts the TRE, producing striking alterations in localization of MICAL-L1 and Syndecan2. This was confirmed when delayed recycling of transferrin was observed in PLD inhibitor treated cells when compared to control[57]. Studies conducted by our lab have also shown that there was strong colocalization between the TREs expressing the PI(4,5)P2 biomarker PLCδ1-PH and Env. This contributed to my second aim: To determine if PLD inhibition through chemical inhibitors and its effect on the TRE leads to defective Env trafficking and particle incorporation into virions.

Chapter 2: Materials and Methods

Cell lines

HeLa, 293T, H9 cells were obtained from the American Type Cell Culture (ATCC; CCL-2, CRL-3216, HTB-176). TZM-bl cells were obtained from the NIH AIDS reagent program, Division of AIDS, NIAID, NIH. HeLa, TZM-bl, and 293T cells were maintained in Dulbecco's modified Eagle Medium (DMEM) (ThermoFisher Scientific) supplemented with 10% FBS (Fetal Bovine

Serum), 2mM L-glutamine, 100 IU penicillin, and 100μg/mL streptomycin. H9 cells were maintained in RPMI 1640 (Roswell Park Memory Institute) containing 10% FBS (Fetal Bovine Serum), 2mM L-glutamine 100 IU penicillin, and 100μg/mL streptomycin.

Preparation of fluorescent tagged constructs of TRE markers

TRE markers used for both fixed cell and live cell imaging were MICALL-1, EHD1 and Rab10. Restriction digestion of EHD1-GFP, MICALL1-GFP and C1 – mCherry plasmid using 3μL of a standardized 1mg/mL concentrated stock, 2μL of CutSmart Buffer (New England Biolabs) with 0.5μL of restriction enzymes XhoI and EcoRI (New England Biolabs) each. The digested products were run on a 1% agarose gel at 500mAmps, 120V for 1 hour. The DNA products MICALL1, EHD1 and the C1- mCherry plasmid were extracted from the gel under Ultraviolet light into separate microcentrifuge tubes. The gel products were then extracted using the QIAquick Gel Extraction Kit (QIAGEN)and the concentration was measured. Separate ligation reactions were performed for 2 hours using standard ligation protocol with the C1-mCherry plasmid, EHD1 and C1-mCherry plasmid, MICALL1 maintaining the concentrations of vector to product at 1:3. The ligated product is then transformed to JM109 (Promega) bacterial cell line following standard Transformation protocol. Transformed culture is then plated onto a Kanamycin selection plate and incubated for 24 hours at 37^0C. Colonies were picked, grown in LB broth overnight on a rotary shaker. Mini prep was performed using the QIAprep Plasmid Miniprep kit (QIAGEN). The results were sent for sequencing to the CCHMC DNA Core. Cultures with the sequence of interest were inoculated in 500mL of LB broth overnight at 37^0C on a continuous rotary shaker. Maxiprep was performed using the QIAfilter Plasmid Maxiprep kit to extract the purified DNA of interest which was later normalized to a concentration of 1mg/mL.

Immunofluorescence microscopy

For imaging fixed samples, HeLa cells were plated in a 35mm^2 poly-d lysine treated dishes (MatTek) overnight and then transfected with HIV-1 primary isolate envelope at a concentration of 250ng and TRE markers (MICALL1/EHD1/Rab10) at a concentration of 250ng for single staining experiment using jetPRIME (Polyplus). For triple staining experiments, 200 ng of HIV-1 Clade B primary isolate Env was used and 75ng of Rab10GFP, MICALL1 mCherry and EHD1 were utilized for transfections. Cells were washed with PBS and then fixed for 15 minutes in an incubator with 4% paraformaldehyde in PBS prewarmed to 37^{0}C. Following fixation, cells were washed with 100mM glycine solution in PBS. Cells were then permeabilized by incubating the dishes with PBS containing 0.025% Triton X-100 (Sigma Aldrich) for 10-15 minutes and then blocked with Dako protein block (Agilent Technologies) for 30 minutes at room temperature. Blocking solution is washed out with 0.025% Triton X-100 solution and stained overnight with the primary antibody diluted in Dako antibody diluent (Agilent Technologies). Primary antibodies and dilutions used were as follows: 2F5 (1:1000), EHD1 (1:500), MICALL1 (1:300). For live cell experiments, cells were transfected with fluorescent tagged constructs of TRE markers, MICALL-GFP and Rab10-GFP, using jetPRIME (Polyplus). 20-24 hours later, the Matek dishes were serum starved with the DMEM serum free media. Live cell imaging was performed under 5% CO_2 at 37^{0}C chamber on the Deltavision Elite live cell deconvolution imaging system. Phospholipase D inhibitor CAY10593 and CAY10594 (Cayman Chemicals) at a 20μM concentration was added together directly to the middle of the supernatant while imaging at a rate of 15 seconds for 40 minutes. The inhibitors were diluted in Fluorobrite media with Prolong Long Live Antifade (1:100) to prevent loss of signal through photobleaching. Washout was performed by adding fresh DMEM serum-containing media to the supernatant and performing live cell imaging under the same

conditions. For infected cells, infect cells with VSVG pseudotyped NL43 virus and incubate for 24 hours.

Pulse Chase Imaging

For pulse chase imaging experiments, HeLa cells were plated in 35mm^2 poly-d lysine treated dishes (MatTek) overnight and then transfected with 75 ng of pcDNA5/TO JRFLoptgp160-FAP and 125ng of fluorescently tagged TRE markers, Rab10-GFP and MICALL1-GFP, using jetPRIME (Polyplus). 20-24 hours later, live cell imaging was performed under 5% CO_2 at 37^{0}C chamber on the Deltavision Elite live cell deconvolution imaging system. 20μL of FAP reagent αRED-np001 (Spectragenetics) was directly added to the supernatant as images were acquired. Pulse chase imaging was done for 12 minutes for control cells by choosing 5 z-stacks for the cells selected and images at time intervals of 15 seconds. For PLD treated cells, cells were serum starved for 1 hour with DMEM Serum free media and pulsed with 20μL of pcDNA5/TO JRFLoptgp160-FAP in Fluorobrite media (with Prolong Live Antifade diluted at 1:100) for 10 minutes. Cocktail mix of PLD inhibitors CAY10593 and CAY10594 (Cayman Chemicals) at 20μM concentration was added to the middle of the supernatant as Pulse chase was performed for 40 minutes at 15 second intervals.

Virus Harvest

H9 cells obtained from were plated in a 12-well tissue culture treated plate at a density of 4 million cells per well in RPMI 1640 (Roswell Park Memory Institute) containing 10% FBS (Fetal Bovine Serum), 2mM L-glutamine, 100 IU penicillin, and 100μg/mL streptomycin and incubated for 24 hours. In BSL2+, cells were infected with 150 ng of VSVG pseudotyped NL43 virus. The cells from the 12-well plate were transferred to microcentrifuge tubes, centrifuged at 400g for 3-5

minutes and pellet was resuspended in 1mL PBS and centrifuged under same conditions. Different concentrations of the PLD inhibitor were used to test differences in effects. The pellet was resuspended in 10mL of RPMI 1640 media with the inhibitor in 25mL Tissue Culture Flasks and incubated for 72 hours before harvest.

After 72 hours, H9 cells were resuspended and transferred to 14mL Corning Tubes. Centrifuge these tubes at 600g for 5 minutes at room temperature and collect 400µL of the supernatant to be used for TCID50 and store at -80^{0}C. Aliquot 20µL of the supernatant into 180µL of ELISA Sample Diluent at 1:10 final concentration to be used for p24 ELISA (supernatants). Use 0.45µm PES Syringe filters (Thermofisher Scientific) and syringe and Ultracentrifuge tubes (Beckman Coulter). Filter the supernatant without disturbing the cell pellet and without foaming. Utilizing a 20% sucrose solution build a continuous sucrose gradient and perform ultracentrifugation at 35000rpm for 2 hours at 4^{0}C (Beckman Coulter, SW 41 Swinging Bucket Rotor). Collect cell pellets after ultracentrifugation, resuspended in 45µL RIPA and performed end dilutions at 1:100 in ELISA sample diluent (viral lysates).

Cell pellets remaining in the Corning tubes were resuspended in 500µL PBS, centrifuged at 0.1g for 5 minutes at 4^{0}C and pellet was resuspended in 100µL of RIPA for lysis. Cell pellets were centrifuged at 17000rpm for 30 minutes at 4^{0}C, supernatant is collected and diluted at 1:100 in ELISA sample diluent for p24 ELISA (cell lysates).

All plates and flasks containing the virus were decontaminated with Betadine solution and discarded appropriately according to BSL2+ safety protocols.

Enzyme-Linked Immunosorbent Assay (ELISA)

To measure the amount of virus harvested in the samples, 96-well flat-bottom Maxisorp plates were coated overnight with 100µL of CA183 antibody at room temperature. The plate was washed with PBS + 0.2% Tween20 twice. The plate is then blocked with 5% FBS for one hour at 37^0C. Samples and p24 standards are then diluted in ELISA sample diluent in concentrations of interest and plated in a 96-well round bottom plate before transferring it onto the blocked flat-bottom ELISA plate. Wash the ELISA plate 4 times with the PBS + 0.2% Tween20 and transfer 100µL of the sample onto the ELISA plate. Incubate for 2 hours at 37^0C. Wash the plate four times with the PBS + 0.2% Tween20 solution and add 100µL of the primary antibody HIV-Ig and leave at 4^0C overnight. Wash the plate and incubate with 100µL of secondary antibody Antihuman IgG HIV-HRP (Invitrogen) at 37^0C for one hour. Add 100µL 1:1 diluted TMB + Peroxide solution. Leave for a few minutes and extinguish the reaction by adding 100µL of 4N H_2SO_4 solution. Read the plate on EMax Plus Microplate reader. Note the concentrations of the samples for western blot.

$TCID_{50}$

TZM-bl cells maintained in DMEM are plated on a 96-well tissue culture treated flat bottom plate at a cell density of 1.5 million cells per plate and incubated overnight at 37^0C.

In BSL2+, 120µL of DMEM +Dextran solution (40µL of Dextran for every 10mL DMEM serum containing media) is transferred onto a 96-well round bottom plate. 30µL of virus harvested from our PLD inhibitor treated samples was then suspended in this media and serial dilutions were performed, with constant pipetting, leaving the last column blank. 100µL of the virus was transferred to the TZM-bl plate and incubated for 48 hours. 100µL of the media in the wells of

TZM-bl plate was transferred to a reservoir and discarded and 100µL of Brightlite Luciferase substrate was added to all wells of the plate and incubated for few minutes at room temperature. 150µL of this sample-substrate mix was transferred to a black plate and read immediately to measure virus titers produced.

All sample plates and reservoirs and anything in contact with the virus were decontaminated using Betadine solution and discarded appropriately according to BSL2+ safety protocols.

Western Blots

To test the effects of PLD inhibition on Envelope incorporation, we performed a Western blot on the cellular and viral lysates. Samples were loaded after normalizing the amounts of p24 determined by the p24 ELISA. Samples were diluted in RIPA buffer (containing Protease inhibitor at 1:100) to which NuPage Sample Loading Buffer was added (Invitrogen) at 1X final concentration. The samples were incubated at 92^0C for 7 minutes and loaded onto NuPage 4 – 12% Bis - Tris gel (Invitrogen) with SDS running buffer under 500A, 120V for 2 hours. The gel is then transferred onto a Nitrocellulose Membrane Filter Paper Sandwich (Invitrogen) using Transfer buffer at constant voltage 18V, 2.5A for 80 minutes. Membrane was blocked with Intercept Blocking buffer (LiCOR Biosciences) with 0.15% Tween20 for 30 minutes and all antibodies were diluted in Intercept Antibody diluent with 0.15% Tween20 (LiCOR Biosciences) at the following dilutions: gp120 was detected with human antibody 2G12 (Polymun) (1:1000), gp41 was detected with 2F5 (Polymun) (1:1000), p24 was detected with CA183 (1:1000), actin was detected with mouse anti-actin (ThermoFisher Scientific) (1:3000), MICALL1 was detected using mouse anti-MICALL1 (Novusbio) (1:500), EHD1 was detected with rabbit anti-EHD1 (Sigma Aldrich) (1:500). Primary antibodies were detected against appropriate LICOR IRDyes (LiCOR

Biosciences) at 1:10000 dilution in Intercept Antibody diluent (LiCOR Biosciences) with 0.15% Tween20.

Statistical Analysis

Colocalization in images taken between Env and TRE were quantified using Pearson's and Mander's coefficients generated by Volocity software package. Colocalization graphs were generated using GraphPad Prism.

Chapter 3: Results

HIV-1 Clade B Primary isolates Envelope Glycoprotein utilizes Tubular Recycling Endosomes for Trafficking:

To examine whether the Env of HIV-1 Clade B primary isolates utilize the TRE for trafficking, we utilized a fluorescently tagged construct expressing the TRE marker Rab10. Three primary isolates of HIV- 1 Clade B (Rejo, WITO and RHPA) were initially employed to test the hypothesis that HIV-1 Clade B primary isolates utilize the TRE for intracellular trafficking. HeLa cells were plated and transfected with the Env of the above primary isolates and GFP tagged Rab10 using jetPRIME (Polyplus). Cells were incubated and fixed 24 hours post transfection using the TRE staining protocol utilizing warm 4% PFA and 0.025% TritonX-100. Throughout multiple experiments (n=4s), we observed remarkable colocalization between the Env of HIV-1 Clade B primary isolates and Rab10-marked TREs (**Figure 3**), which was subsequently quantified using Pearson's coefficient and Mander's coefficients. Pearson's correlation coefficient measures pixel-to-pixel colocalization and is independent of signal noise since the values are generated through subtraction

of mean intensity. Mander's colocalization coefficients measures the cooccurrence of pixels and is independent of signal proportionality. M1 is the fraction of red channel that overlaps with the green channel and M2 is the fraction of green channel that overlaps with the red channel[68]. For **Figure 3**, colocalization values for JRFL were Pearson's: 0.343; M1: 0.512 and M2: 0.956. Colocalization values for Rejo were Pearson's: 0.629; M1: 0.666 and M2: 0.977. Colocalization values for WITO were Pearson's: 0.760; M1: 0.760 and M2: 0.966.

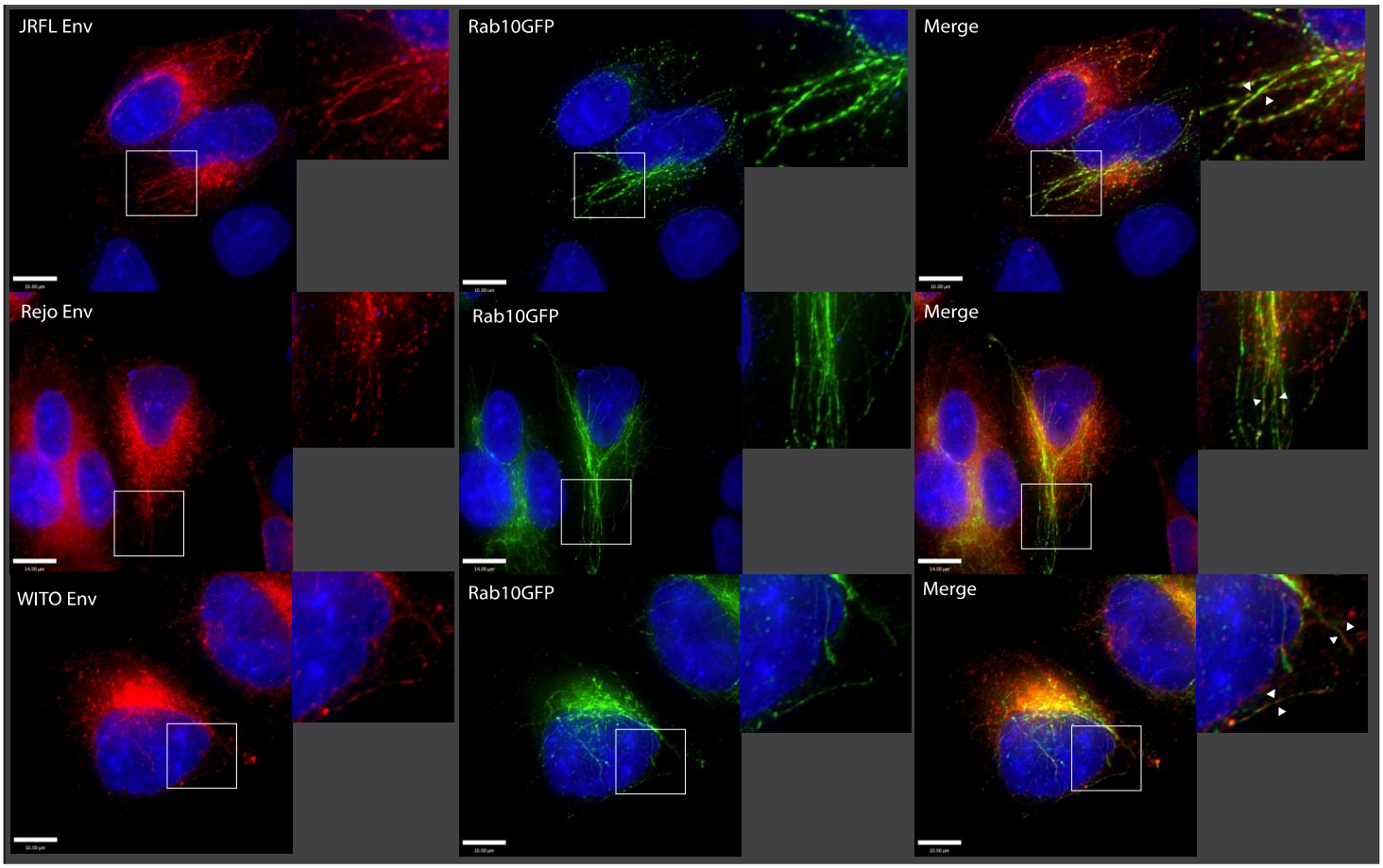
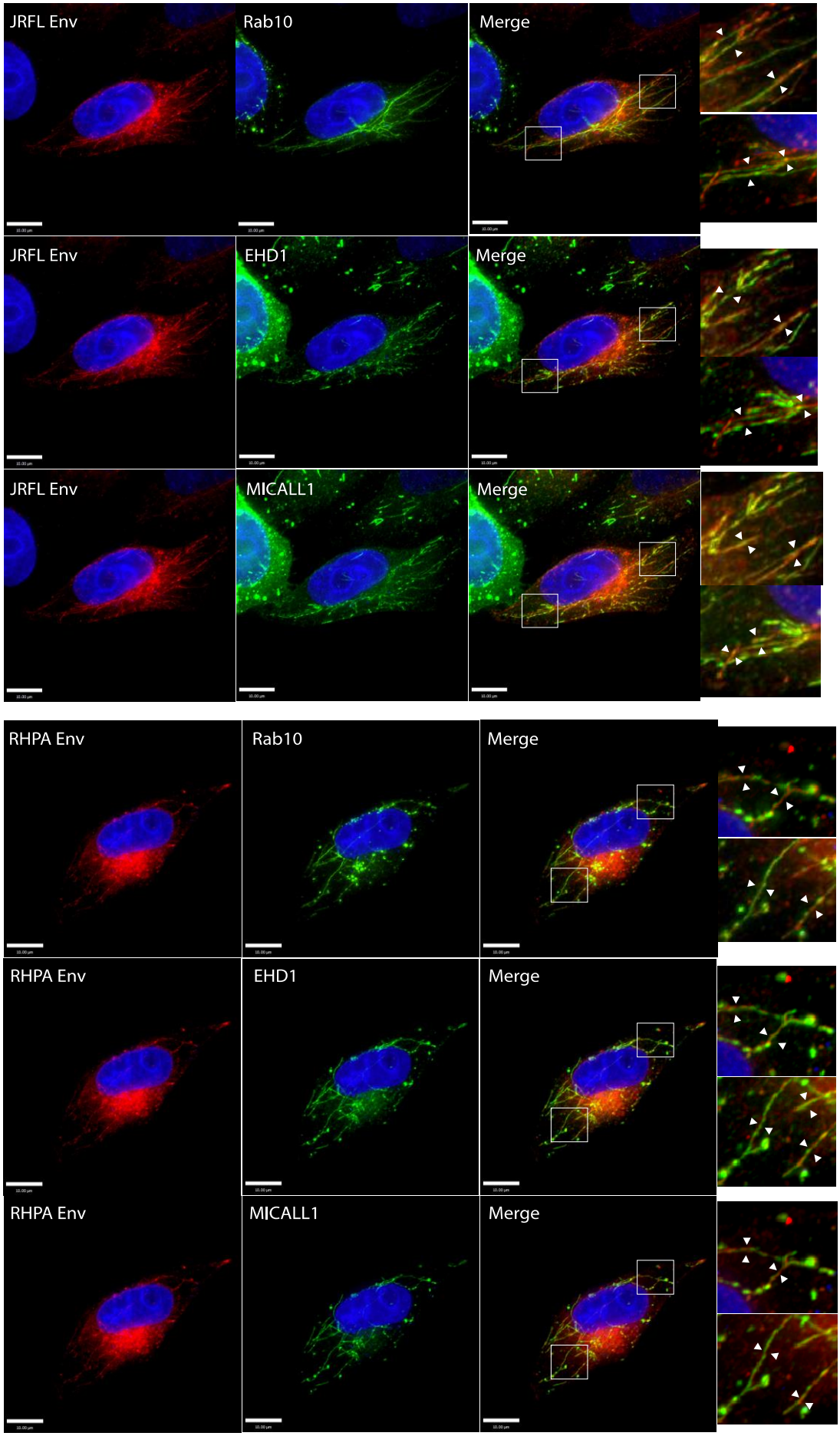


Figure 3: *HIV-1 Clade B Primary Isolate Env (Rejo, WITO and JRFL) displaying significant colocalization with TRE marked by Rab10-GFP. Env is stained with 2F5 (1:1000).*

Next, we examined the TRE markers MICAL-L1 and EHD1 by constructing fluorescent tagged constructs, MICAL-L1-mCherry and EHD1-GFP, and utilizing them to observe colocalization. A triple staining experiment was performed using HeLa cells co-transfected with HIV-1 Clade B primary isolate Env - JRFL, RHPA or 6535 together with TRE markers MICALL1-mCherry, Rab10-GFP and EHD1. A significant degree of colocalization was observed between HIV-1 primary isolate Env and all three TRE markers as expected (**Figure 4**), and colocalization was quantified by calculating Pearson's coefficient and Manders coefficients.

JRFL displayed colocalization values ranging from 0.45 to 0.75, RHPA ranged from 0.43 to 0.67, and 6535 exhibited colocalization between 0.42 and 0.77 across all three markers, as indicated by Pearson's coefficient. Mander's coefficient for JRFL ranged from 0.65 to 0.79 for M1 and 0.8 to 0.95 for M2. RHPA had M1 values ranging from 0.52 to 0.75 and M2 values ranging from 0.95 to 0.98. Whereas 6535 had M1 values ranging from 0.59 to 0.89 and M2 values ranging from 0.79 to 0.9. The images in **Figure 4** are representative of the images captured in multiple experiments. **Figure 5** represents colocalization graphs denoting the colocalization quantification of images through Pearson's and Mander's coefficients in these experiments.

After scrutinizing results from multiple experiments (n=3), we concluded that HIV-1 Clade B primary isolate Env proteins colocalize with the TRE, strongly suggesting that primary isolates utilize the TRE for intracellular trafficking.


JRFL Env
Rab10
Merge
JRFL Env
EHD1
Merge
JRFL Env
MICALL1
Merge
RHPA Env
Rab10
Merge
RHPA Env
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RHPA Env
MICALL1
Merge

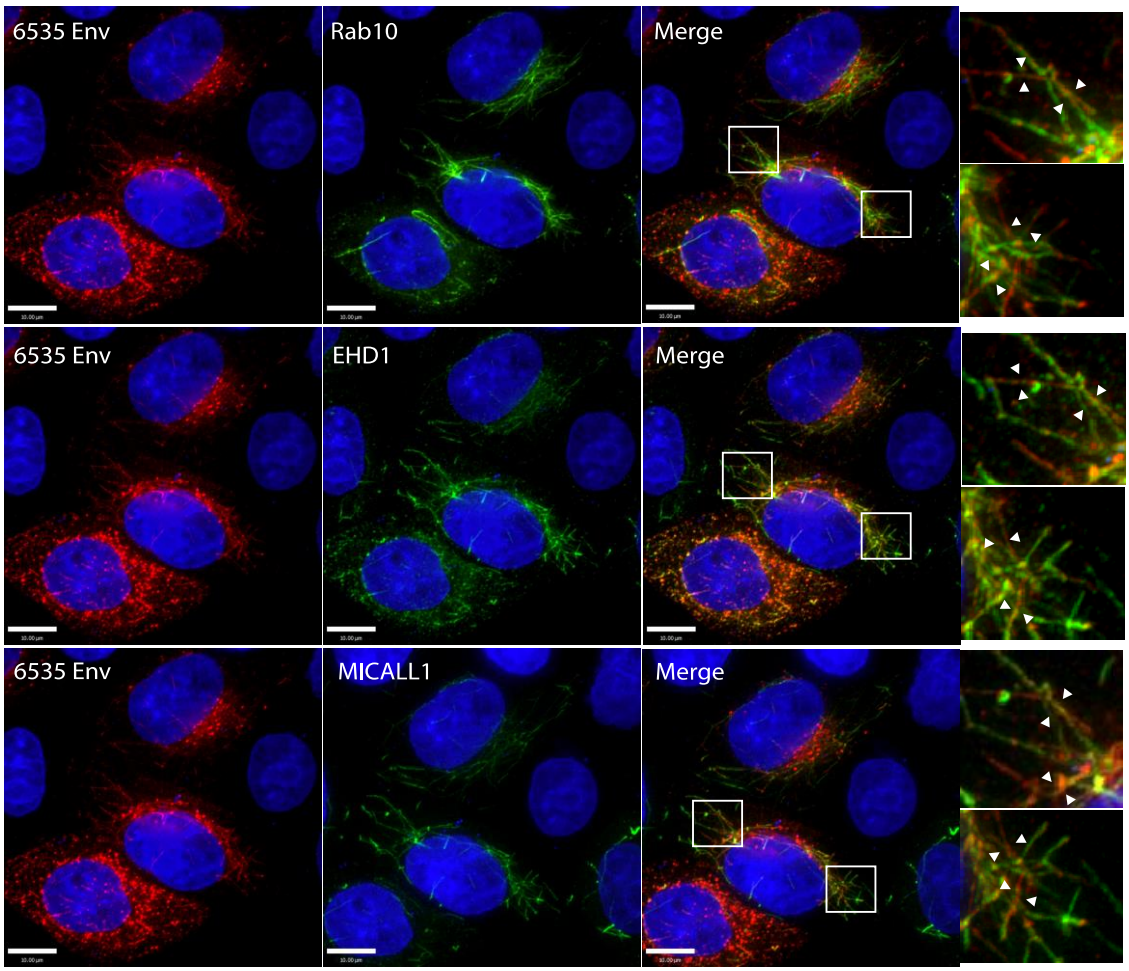


Figure 4: *HIV-1 Primary Isolate Env (JRFL, RHPA and 6535) displaying significant colocalization with TREs marked by Rab10-GFP, MICAL-L1-mCherry and EHD1 in HeLa cells. Env is stained with 2F5 (1:1000) for JRFL and RHPA isolates, PGT121 (1:1000) for 6535. EHD1 is stained with Anti-EHD1 (1:500).*

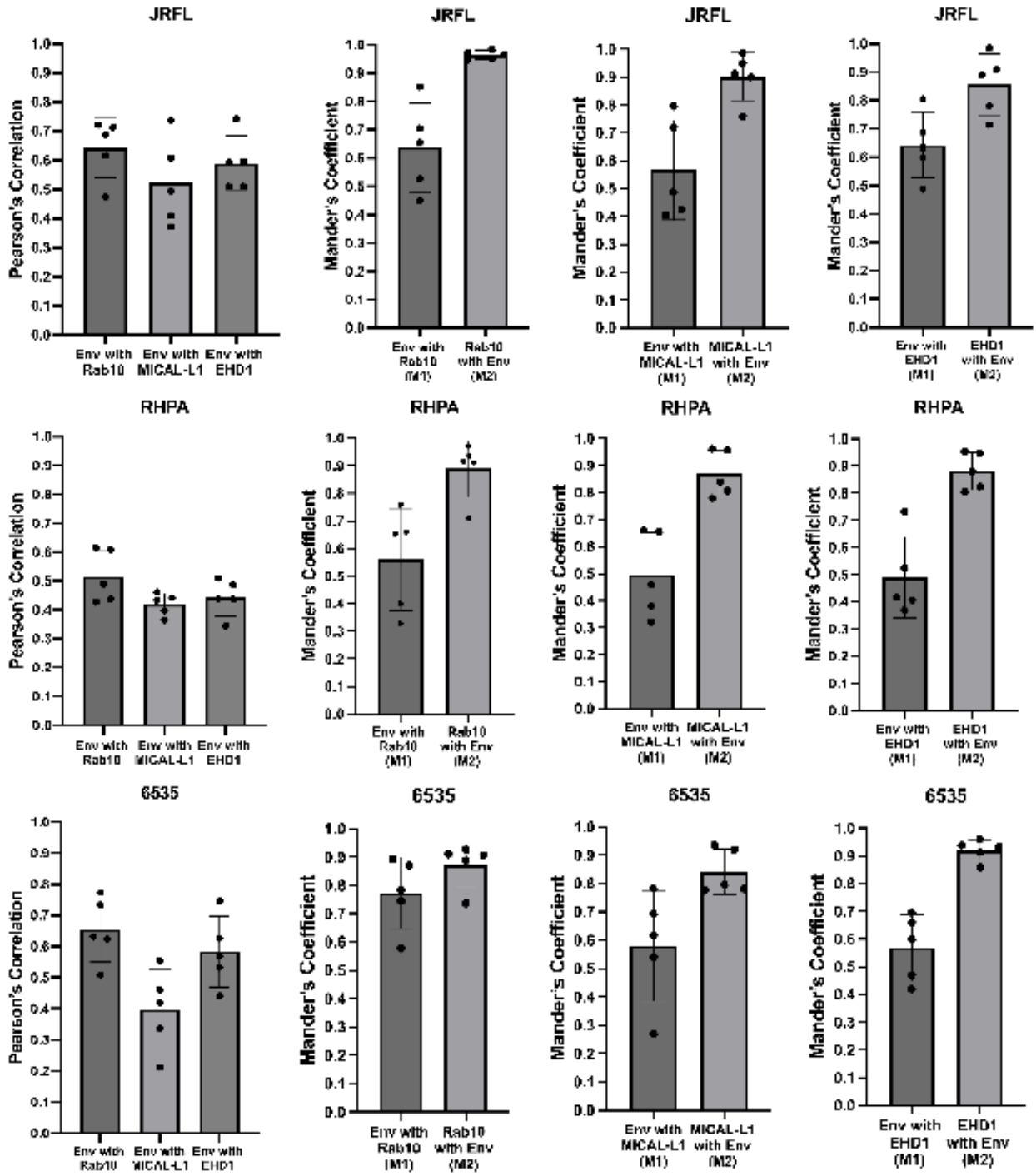


Figure 5*: Colocalization graphs denoting Pearson's Correlation and Mander's Colocalization Coefficients, M1 and M2, images quantified for colocalization between HIV-1 Primary isolates (JRFL, RHPA and 6535) with TRE marked with Rab10, MICAL-L1 and EHD1.*

Phospholipase D inhibition disrupts formation of the Tubular Recycling Endosome:

Prior studies have shown that PA is a crucial lipid component for the formation of TRE and is required for the recruitment of vital TRE components such as MICALL1 and Syndapin-2 through PA interaction[59]. PA is produced through the lipase activity of PLD To test whether the inhibition of phospholipase D (PLD) affects TRE formation, we utilized two chemical inhibitors, CAY10593 and CAY10594 (Cayman Chemicals) that specifically inhibit PLD isoforms PLD1 and PLD2, respectively. The exact mechanism of action of these compounds has not been studied yet but it is known that these compounds act on the catalytic domain of the PLD isoforms[69]. Studies have shown that DAG inhibition also caused the depletion of PA but the effects of PLD depletion were more pronounced probably due to the indirect effects of the inhibitors on PI(4,5)P2 levels and maybe other phosphoinositides[67].

The experimental design included plating HeLa cells and transfecting them with the fluorescent tagged TRE markers of Rab10-GFP and MICAL-L1-GFP. The cells were serum starved for 1 hour in serum free DMEM. The inhibitors were used at a concentration of 20μM each to inhibit both PLD1 and PLD2. Cell viability was not affected during fixed cell imaging process post PLD inhibition. Fixed cell imaging was performed using standard TRE staining procedures at specific timepoints following the addition of inhibitor. Live cell imaging was also performed after adding the PLD inhibitor cocktail mix, with a duration of 40 minutes and acquiring images at 15 second intervals. As demonstrated through multiple images (n=3) and live cell imaging videos (n=4), prolonged PLD inhibition led to the disruption of tubules in HeLa cells, further confirming that PA is a crucial lipid component for the formation of TREs (**Figure 6**). The washout of these inhibitors was then performed using DMEM serum containing media, in order to determine if the formation

of TRE was restored along with Env localization to the TRE. These experiments were performed under the same conditions, imaging for 40 minutes at 15 second intervals. We observed regeneration of tubules after the washout of inhibitors over time (**Figure 6**), indicating that the inhibitors have temporary effects on tubule inhibition and that the TRE can be restored following removal of the inhibitor.

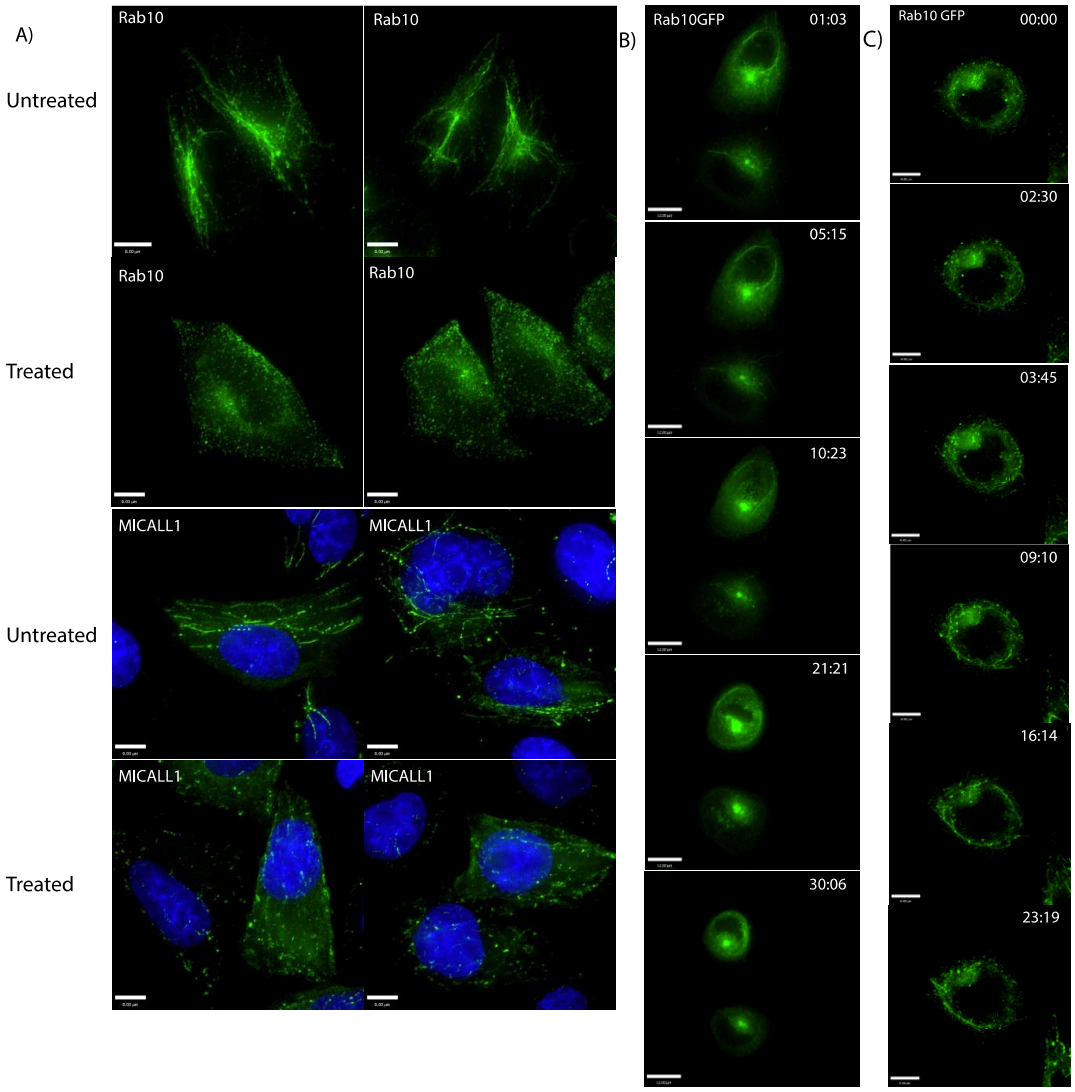


Figure 6: *A) HeLa cells displaying inhibition of tubules marked with MICALL1 and Rab10 – fixed cell imaging. B) Live cell imaging video of inhibition of TREs marked with Rab10. C) Washout of PLD inhibitor with DMEM Complete media displayed regeneration of tubules marked with Rab10 overtime as shown through live cell imaging.*

Phospholipase D inhibition disrupts Envelope glycoprotein trafficking:

Based on our immunofluorescence imaging observations, we next aimed to define the effects of PLD inhibition on Env trafficking. HeLa cells were plated and were transfected with MICAL-L1-GFP or Rab10-GFP to observe the TRE and with pcDNA5TO-opt-JRFLgp160alphaFAP, a plasmid expressing a Fluorogen Activated Peptide (FAP)-tagged Env that allows visualization of dynamic Env trafficking events. Using a cell-impermeable fluorescent dye that reacts with the FAP tag, we were able to pulse-label Env on the surface in order to follow its subsequent internalization and trafficking. Utilizing this technique, we observed that surface Env was rapidly delivered onto the TRE marked with MICALL1 (**Figure 7**). Delivery of Surface Env was observed to occur within 3 minutes of addition of the Fap Env reagent. This denotes that Env present on cell surface undergoes rapid internalization and colocalizes with the TRE (marked with MICAL-L1 GFP) suggesting that internalized Env potentially utilizes TRE for intracellular trafficking.

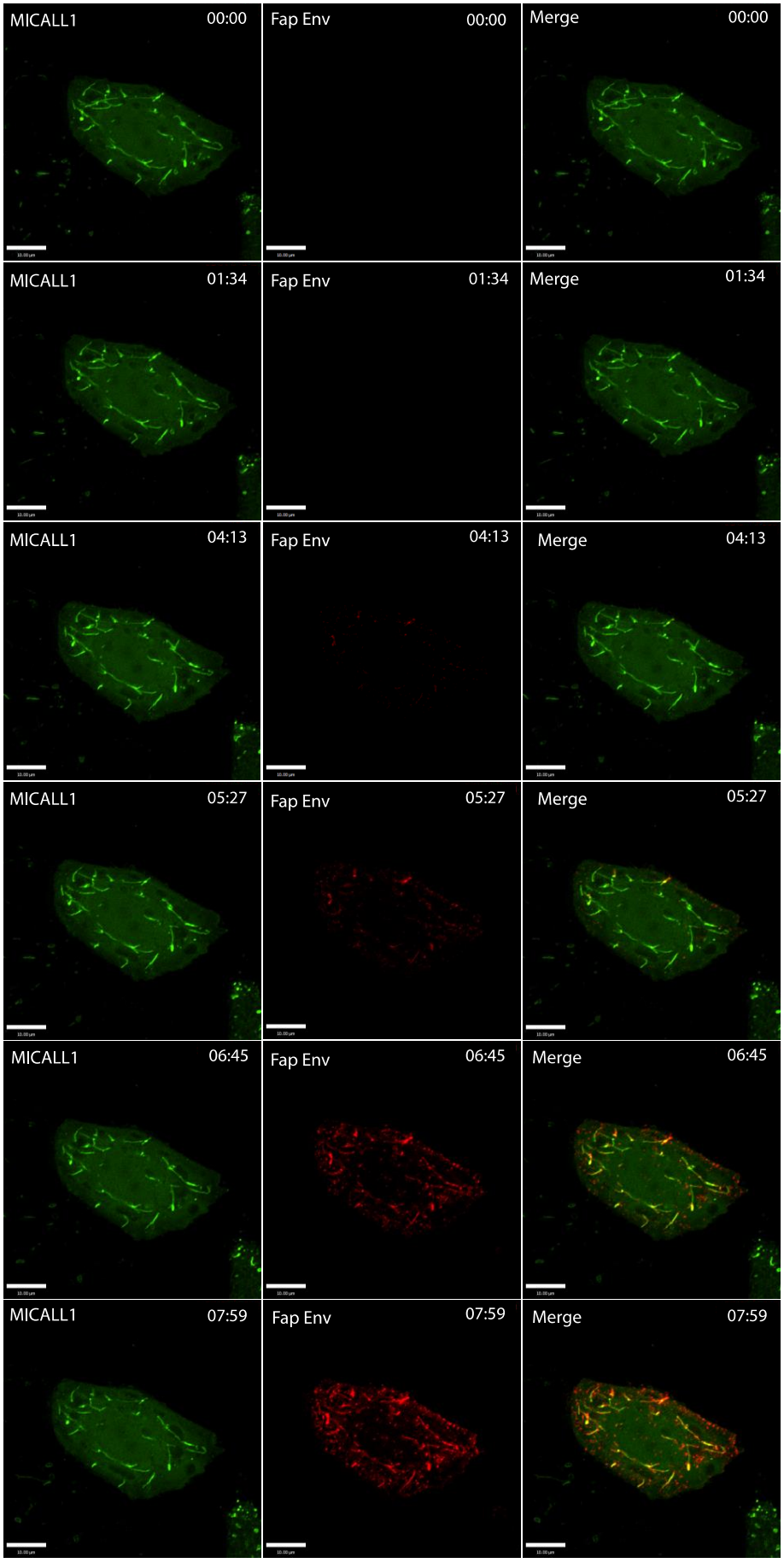
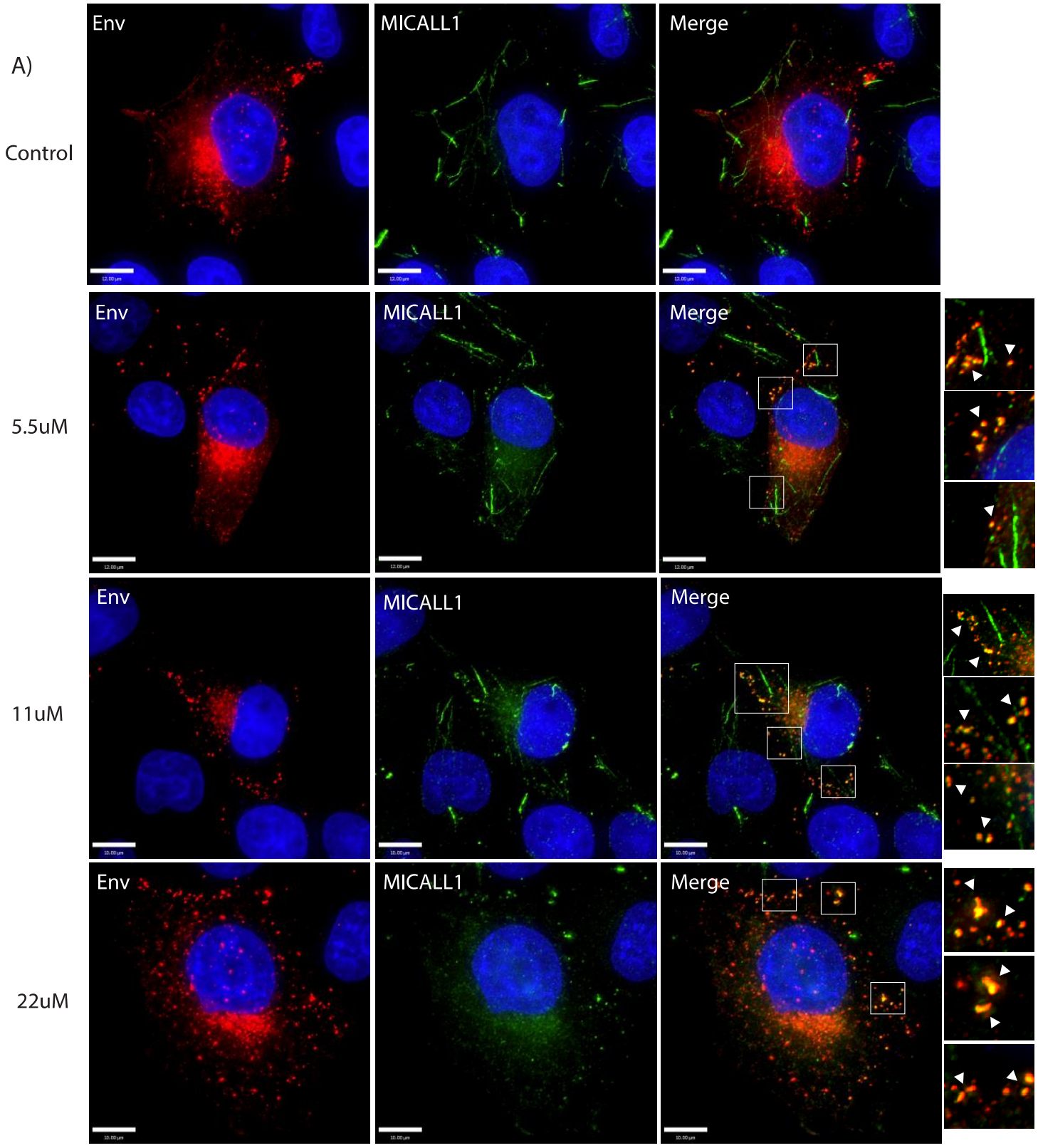


Figure 7: *Surface Env is rapidly delivered onto TRE marked with MICAL-L1 GFP in HeLa cells as observed through Pulse Chase Imaging. Images were taken for 10 minutes at 20 second intervals – where surface Env internalization was observed within minutes reagent addition*

We next sought to evaluate the trafficking of Env to the TRE following infection of cells with HIV-1. HeLa cells were infected with 50ng of VSV-G-pseudotyped NL4-3 virus and incubated for 48-hours. Cells were then serum starved and treated with PLD inhibitors at different concentrations for 1 hour. Inhibitor concentrations used were 22μM, 11μM and 5.5μM. The cells were then fixed and stained for Env and endogenous MICAL-L1. We observed that PLD inhibitors at higher concentrations successfully inhibited TRE formation in infected HeLa cells. Remarkably, Env was no longer found on tubular membranes following inhibitor treatment, but instead formed distinct puncta which colocalized with endogenous MICAL-L1. These results suggest that MICAL-L1 and Env remain associated even in the absence of intact recycling endosomes. To further examine this, we performed live cell imaging, and again observed that Env colocalized with MICAL-L1 positive tubules that were disrupted following inhibitor treatment into distinct puncta (**Figure 8**).


A)
Control
5.5uM
11uM
22uM
Env
MICALL1
Merge

B)

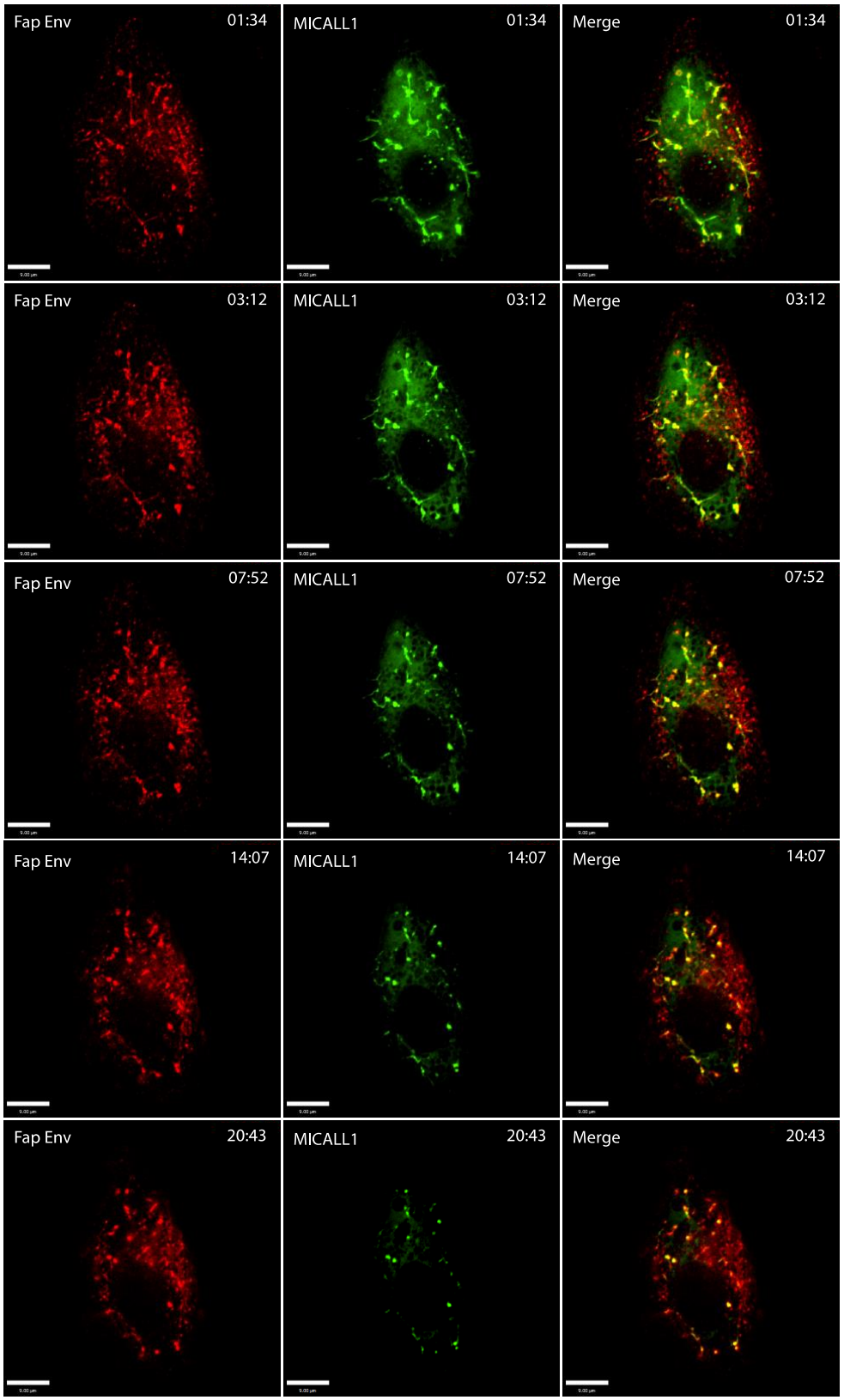


Figure 8: *A) Infected HeLa cells treated with different concentrations of PLD inhibitor and stained for Env (2F5- 1:1000) and endogenous MICAL-L1 (Anti-MICALL1 – 1:300) displayed punctate morphology for Env post-inhibition and colocalization between Env and MICALL1.*

B) Live cell imaging displays inhibition of MICAL-L1 marked tubules and formation of punctate Env structures.

We quantified the effect on levels of MICAL-L1 and EHD1 post PLD inhibition by western blot. A notable observation was that post-infection of H9 cells and treatment with varying concentrations of PLD inhibitor, the cellular levels of MICAL-L1 and EHD1 remained consistent, regardless of the inhibitor concentration. Next, we measured the effect of TRE inhibition on Env particle incorporation and infectivity. These experiments utilized the H9 T cell line, which is known to be a cell line that depends on Env CT-dependent trafficking for particle incorporation, similar to authentic primary T cells. H9 cells were infected with VSV-G-pseudotyped NL4-3 virus and treated with PLD inhibitors for 48 hours before harvesting for supernatants and lysates. Inhibitor concentrations used here were 5μM, 10μM and 15μM (as determined by performing kill curves). Preliminary experiments did not reveal a decrease in Env incorporation into particles by western blotting. However, this experiment requires repeats with careful attention to the timing of inhibitor addition as related to virus harvest from the supernatants (**Figure 9**). The infectivity of the released virus was measured using the TZM-bl luciferase reporter cell line. We observed a marked decrease in infectivity that correlated with increasing PLD inhibitor concentrations, as compared to control. This suggests that increasing PLD inhibitor concentration might have an effect on particle infectivity as observed through multiple infectivity assays but is hypothesized to have a minimal observable effect on Env incorporation when treated with the inhibitors. Further studies need to be conducted to observe effects of PLD inhibition on Env particle incorporation and if PLD inhibition alone effects Env trafficking or other crucial components that are responsible for HIV infectivity.

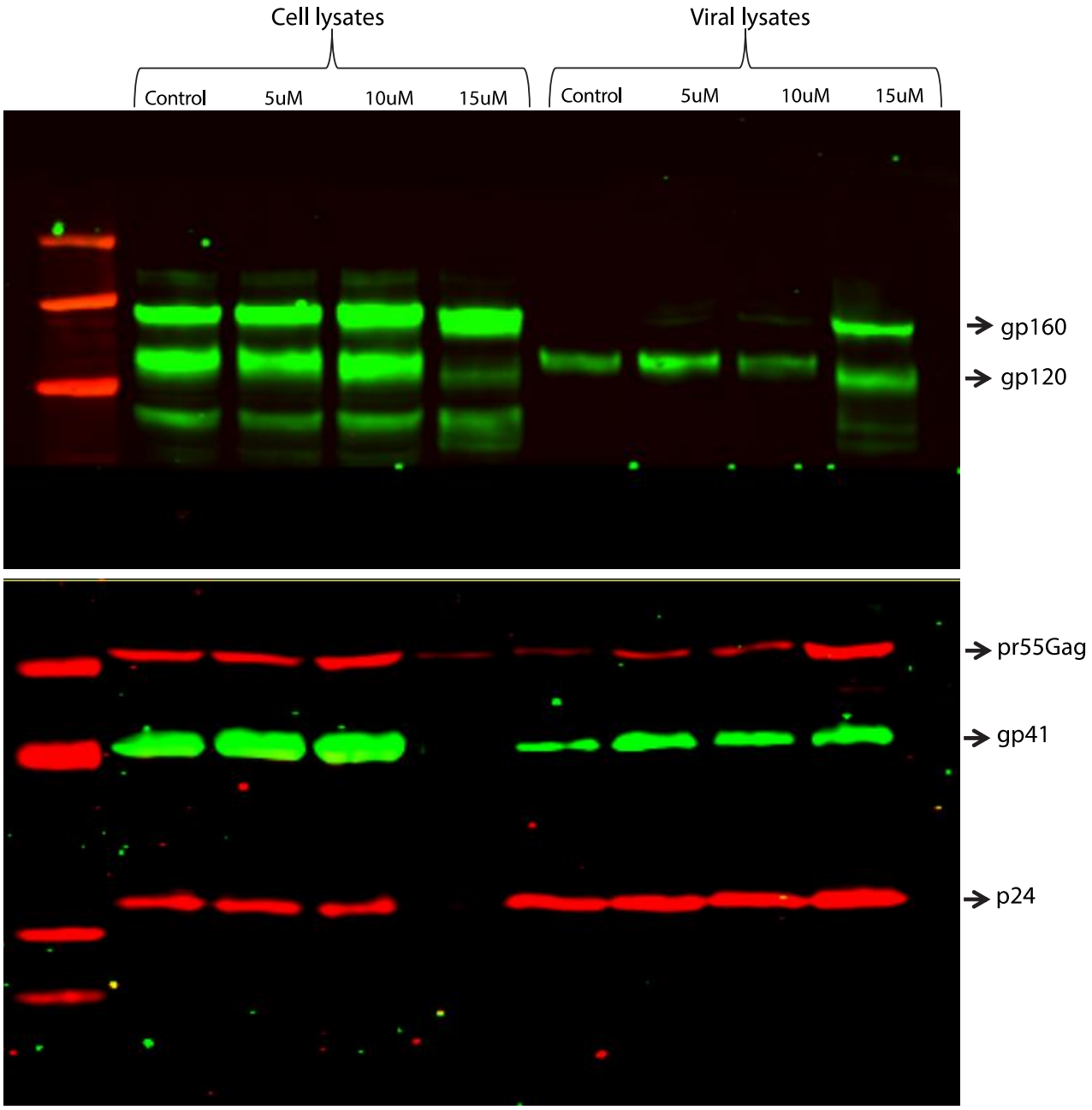


Figure 9: *Western blot performed on H9 cells to measure the effects of PLD inhibition on Env particle incorporation. Reduced shedding of gp160 was observed prominently in viral lysates treated with highest concentration of the inhibitor (15μM). No observable discrepancies in cell lysates.*

Discussion:

HIV-1 infects immune cells such as CD4 T cells and monocytes/macrophages. Cell tropism is determined by the interaction of surface glycoprotein Env with host cell co-receptors. Env is responsible for the fusion of host and viral cell membranes[21]. Lentiviruses such as HIV are characterized by their long CT, with an HIV-1 CT of 150 amino acids in length. The HIV-1 Env CT includes dileucine and tyrosine motifs that mediate clathrin-mediated endocytosis of Env, mediating its rapid internalization from the host cell surface. FIP1C and Rab proteins were identified to be responsible for directing trafficking of internalized Env to compartments near the PM for incorporation[46]. Further studies conducted on Env trafficking pathways have shown that cargo sorting occurs in the ERC prior to reaching the assembly site[66].

Internalization and trafficking of Env leads to its localization on endosomes, including the perinuclear ERC, before recycling back to the PM. Studies here follow up on a model for Env trafficking in which components of the TRE mediate Env transport back to the plasma membrane. Previous studies conducted by our lab have shown that Env associates with the TRE[70]. Live cell pulse-label imaging studies indicated that Env on the PM is rapidly delivered to the TRE, where it strongly colocalizes with PIP2, MICAL-L1, EHD1, and Rab10. siRNA knockdown of TRE components significantly reduces Env incorporation into particles, suggesting that recycling from the TRE is an important step in delivering Env to the particle assembly site on the PM. HIV-1 Clade B strains were used to perform these experiments, which did not address the potential of other clades utilizing the TRE pathway versus alternative pathways for Env incorporation.

High mutation rates in HIV-1 lead to tremendous genetic variability, resulting in diverse quasispecies of virus within individual, and in the circulation of distinct subtypes or clades of virus

in geographic regions across the globe. These subtypes or clades have varied genetic sequences for the production of Env and Gag thus accounting to differences in therapy-associated resistance, infectivity and transmissibility[71]. An additional distinction of viruses used in this study involves laboratory isolates vs. primary isolates. Primary isolates are those viruses found in HIV infected people, and when utilized in the laboratory are most frequently passaged minimally in primary cells[72,73]. Clade B was chosen for this study due to its high prevalence in North America as well as Western and Central Europe. Our central question for the first portion of this study was whether primary isolate, clade B Env localizes with the TRE, as had been previously only for a limited number of laboratory strains.

During the initial stages of the experiment, we utilized a prominent TRE marker Rab10 to observe whether Env colocalizes with the TRE. Fixed cell immunofluorescence imaging experiments displayed remarkable colocalization of primary isolates Rejo, WITO and RHPA with TRE marked with Rab10 as concluded by multiple experiments and quantified using Pearson's and Mander's coefficient. Throughout multiple experiments, we observed remarkable colocalization of this panel of clade B Env with TRE markers, indicating that trafficking through the TRE is a conserved aspect of the HIV lifecycle and is not an artifact of laboratory-based evolution of virus. One primary isolate, QH0692, did not display apparent colocalization with TRE and will require further study. This could be due to differences in genetic makeup in certain domains of the cytoplasmic tail. Another question raised by our study is whether the TRE may be a site of Gag-Env interaction. While we did not directly address this, the potential of both viral components to come together on PIP2-enriched TRE membranes is under further study in the laboratory.

The TRE can be defined as a tubular network which plays a crucial role in transport of cargo internalized through CME or CIE from the PM to the endosomal structures and the ERC and

trafficked back to the PM. MICAL-L1 and EHD1 interact with each other, and siRNA knockdown studies of MICAL-L1 have shown the depletion of MICAL-L1 to have an effect on tubular localization of EHD1. MICAL-L1 knockdown caused disassociation of EHD1 with the tubular membranes. Depletion of TRE components such as MICAL-L1 has been proven to effect endocytic recycling where MICAL-L1 knockdown caused the internalized Transferrin to accumulate in the ERC when compared to mock-treated cells that only retained 20% of their internalized transferrin[55].

Previous studies from the Caplan lab have investigated the importance of PA in TRE development. PA is produced through two intracellular pathways involving PLD and DAG as the initial components. PA depletion through depletion of PLD and DAG kinase (involved in DAG production) have shown similar results where they caused tubule degeneration. In the present study, we investigated whether the depletion of PLD through chemical inhibition effects TRE development. Live cell imaging experiments in this study confirmed that PLD depletion over time caused loss of TREs rich in PA and marked with Rab10 and MICAL-L1. Washout of the PLD inhibitors following disruption of the TRE revealed regeneration of these lost tubules marked with MICAL-L1 and Rab10, indicating that the inhibitor has temporary effects and does preclude tubule regeneration after washout. We therefore utilized PLD inhibition as a tool to further evaluate the role of the TRE in Env trafficking and incorporation into particles.

The Caplan lab has shown that PLD inhibition caused effects on recycling of cargo internalized by CME, such as reduction of transferrin recycling. To observe whether the inhibitor has similar effects on Env trafficking, we used different concentrations of inhibitors to treat cells infected with HIV, and defined the resulting effects on Env trafficking. Remarkably, we observed disassociation of Env from TRE marked with MICAL-L1 in these infected HeLa cells treated at higher

concentrations of the inhibitor. Env formed distinct puncta after treatment, while retaining colocalization with MICAL-L1. This could mean that PLD inhibition does affect Env trafficking and its association with the TRE but does not cause any disassociation of Env with associated proteins including MICAL-L1 that are recruited to the TRE. Similar results were observed when HeLa cells were co-transfected with pcDNA5TO-opt-JRFLgp160alphaFAP which is the Fap tagged construct of Env and MICAL-L1 GFP. In conclusion, we did observe that PLD inhibition disrupts the TRE and alters the subcellular localization of Env.

In preliminary studies, we evaluated the infectivity and Env particle incorporation in H9 cells following treatment with PLD inhibitors. Remarkably, we found that particle infectivity was significantly diminished following this treatment. However, we were unable to document loss of Env incorporation from released particles by western blot, perhaps due to the timing of inhibitor addition vs. particle formation in these studies. Further investigation is required to understand why we observe a significant decrease in infectivity despite seeing no major differences in Env incorporation. We plan to advance our current findings by determining if PLD inhibitor washout causes the Env to reassociate with the tubular structures, and potentially restores Env trafficking and particle incorporation.

The present study substantiates the importance of intracellular trafficking pathways and in particular the TRE in trafficking of Env. We observed that HIV-1 Clade B primary isolate Env, despite the genetic variation, utilizes these tubules for trafficking. This could help us further investigate common targets that assist in decreasing Env incorporation and reduced infectivity in other HIV-1 subtypes and even in other cell types such as macrophages and T cells. The composition of the TRE includes lipid components such as PA and PIP2 and a series of associated proteins involved in sorting of cellular cargo. One of the future goals of this project is to define

which TRE components are specifically required for Env trafficking, providing targets for interventions to disrupt Env trafficking and inhibit infectious particle production. Further work will also examine the utilization of the TRE for intracellular transport in primary CD4+ T cells and macrophages. In conclusion, this study establishes the trafficking of primary isolate Env to the TRE, and presents disruption of the TRE as a potential means of inhibiting Env trafficking and particle infectivity, setting the stage for additional exploration of the role of the TRE in HIV replication.

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