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The Role of Human Ubc9 During the Human Immunodeficiency Virus Replication Cycle

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THE ROLE OF HUMAN UBC9 DURING THE HUMAN IMMUNODEFICIENCY VIRUS REPLICATION CYCLE

by

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THE ROLE OF HUMAN UBC9 DURING THE HUMAN IMMUNODEFICIENCY VIRUS REPLICATION CYCLE

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Human immunodeficiency virus type 1 (HIV-1) is a retrovirus and the causative agent of the acquired immune deficiency syndrome (AIDS) pandemic. The retrovirus replication cycle is divided into early infectious events, which involve the infection and integration of the viral DNA into target cell chromosomes; and late events, which involve the expression of viral genes and assembly of infectious virions. To complete the replication cycle, HIV-1 utilizes various cellular pathways.

We identified the Ubc9 E2 SUMO conjugating enzyme as a HIV-1 Gag interaction partner. When this interaction was disrupted in HIV-1 producer cells by Ubc9 siRNA, the virus that was released from the cells was approximately 10-fold less infectious than virus released from control cells. The decreased virion infectivity was found to be due to decreased levels of intracellular mature Env, which in turn decreased the amount of Env packaged into assembling virions. Surprisingly, the defect in mature Env levels in Ubc9 knockdown cell was dependent upon Gag expression. The mechanism of decreased mature Env in Ubc9 knockdown cells was examined and we found that gp120/gp41 appeared to be preferentially degraded at a post gp160 cleavage step, but before transport/association with the plasma membrane and lipid rafts. The intracellular gp120 levels were restored when cells were treated with lysosome inhibitors,

which suggested that the decreased intracellular Env stability is due to increased lysosomal degradation; however, inhibiting Env degradation did not restore Env packaging into assembled virions. Since lysosomal degradation is linked with autophagy, we examined autophagy in Ubc9 knockdown cells and found that autophagy was increased approximately 3-fold compared to control cells. Autophagy inhibitors were unable to block autophagy, and we were unable to determine if increased lysosomal degradation of gp120 was occurring through an autophagy dependent or independent mechanism.

We have also examined the potential role of Ubc9 during the HIV-1 early infectious events. Decreased LTR driven gene expression was detected following the infection of Ubc9 knockdown cells with VSV-G pseudotyped HIV-1 virions. These results suggested that Ubc9 plays a functional role during the early events of the HIV-1 replication cycle at a post-entry step.

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List of Abbreviations

3-MA: 3-methyladenine

AIDS: Acquired immunodeficiency syndrome

ABCE1: ATP-binding cellular protein

AIP1/ALIX 1: ALG-2-interacting protein 1

AMPK: AMP-activated protein kinase

AP: adaptor complex

APOBEC3G: apoplipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G

ARF: ADP-ribosylation factor

Atg: autophagy-related

ATP: adenosine triphosphate

BCA: bicinchoninic acid

bp: base pair

BSA: bovine serum albumin

CA: Capsid, p24

c-Jun: cellular Jun

CPZ: chlorpromazine

CT: cytoplasmic tail

C-term: carboxy terminus

Ctr. RNA: non-silencing control RNA

DRM: detergent resistant membranes

DSM: detergent soluble membranes

DTT: dithiothreitol

eE2F: eukaryotic elongation factor 2F

Endo H_f: Endoglycosidase Hf

Env: Envelope glycoprotein

ER: endoplasmic reticulum

ERAD: endoplasmic reticulum associated degradation

ERK: extra-cellular-signal-regulated kinase, also known as MAPK

ESCRT: endosomal sorting complex required for transport

FBS: fetal bovine serum

GFP: green fluorescent protein

GGA: Golgi-localizing, gamma-adaptin ear homology domain

GPC: Gag perinuclear clusters

HIF1/2: hypoxia inducible factor 1 and 2

HIV-1: Human Immunodeficiency Virus, Type 1

HRP: horseradish peroxidase

hrs: hours

I-domain: interaction domain

IN: Integrase

kDa: kilodalton

KIF: Kinesin superfamily

LC3: microtubule-associated protein 1 light chain 3

L-domain: late domain

LE: late endosome

LR: lipid rafts

LTR: long terminal repeat

MA: Matrix, p17

MAbs: monoclonal antibodies

MDM2: murine double minute 2

M-domain: membrane binding

MEK: MAPK/ERK kinase, also known as MAPKK

MHC-I: major histocompatibility complex class I

MoMuLV: Moloney murine leukemia virus

M-PMV: Mason-Pfizer monkey virus

mTOR: mammalian target of rapamycin

MVB: multivesicular bodies

MW: molecular weight

NC: Nucleocapsid, p7

Nef: Negative Factor

NSF: N-ethylmaleimide-sensitive factor

N-term: amino terminus

ORF: open reading frame

P: pulse

PAbs: polyclonal antibodies

PBS: primer binding site

PBS: phosphate buffered saline

PI3K: phosphoinositide 3-kinase

PIC: pre-integration complex

PKA: protein kinase A

PKB: protein kinase B

PKCδ: protein kinase Cδ

PLAGL2: pleomorphic adenoma gene like-2

PM: plasma membrane

PNGase F: N-Glycosidase F

PPT: polypurine tract

PR: Protease

PSB: protein sample buffer

Ptdlns3P: phosphatidylinositol-3-phosphate, also known as PI3P

Ptdlns(4,5)P_2: phophatylinositol 4,5-bisphosphate, also known as PIP_2

Rab9/11: ras-related proteins in brain

RER: rough endoplasmic reticulum

REV: regulator of expression of virion proteins

ROS: reactive oxygen species

RRE: Rev response element

RT: Reverse Transcriptase

RTC: reverse transcription complex

RT PCR: reverse transcription polymerase chain reaction

SA: splice acceptor site

SD: splice donor site

SDS: sodium dodecyl sulfate

SDS PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

Smad: homologues of the drosophila protein mother against decapentaplegic

SNAP: α-soluble NSF attachment protein

SNARE: soluble N-ethylmaleimide-sensitive factor attachment protein receptor

SOCS1: suppressor of cytokine signaling

SP1: spacer peptide 1

SP2: spacer peptide 2

Strv: starvation treatment

SU: surface subunit of Envelope (gp120)

SUMO: small ubiquitin-like modifier

TAR: trans-activating response element

TAT: *trans*-activator of transcription

TfR: Transferrin receptor

TG2: tissue transglutaminase

TGF-β: Transforming growth factor-beta

TGN: trans-Golgi network

Tip47: tail interacting protein of 47 kDa

TM: transmembrane subunit of Envelope (gp41)

TSG101: tumor susceptibility gene 101

TX-100: Triton X-100

Ubc9 RNAi: Ubc9 specific siRNA

Un/Untrans: untransfected

UPR: unfolded protein response

V3: variable loop 3 in gp120

vDNA: viral DNA

VLP: viral like particle

Vpr: Viral Protein R

Vps: vacuolar protein-sorting

Vpu: Viral Protein U

vRNA: viral RNA

XBP-1: X-box binding protein 1

X-gal: 5-bromo-4-chloro-3-indolyl-beta-D-glactopyranoside

Chapter I

Introduction and literature review

Human immunodeficiency virus

Human immunodeficiency virus type-1 (HIV-1) is the etiological agent of acquired immune deficiency syndrome (AIDS), a disease that progressively depletes the immune system over a long period of time, due to the infection and death of the host's primary lymphocytes, leading to complications with opportunistic infections or malignancies that result in death. The World Health Organization and the United Nations Program on HIV/AIDS estimate that there are 33.3 million people worldwide infected with HIV. Since the start of the pandemic, it is estimated that 60 million people have been infected, with approximately 25 million deaths related to AIDS. While progress has been made in the fight against the AIDS epidemic through the implementation of educational programs and an increased accessibility of anti-retroviral drugs, there was still an estimated 2.6 million new infections in 2009 (362).

HIV-1 is a human retrovirus and is a member of the *Retroviridae* family. It is classified as a lentivirus (genera) based on the organization of the viral genome and particle morphology. Retroviruses are enveloped, plus-stranded RNA viruses that replicate via a DNA intermediate. This replication strategy is a defining characteristic of the *Retroviridae* family. The viral genome is packaged into virion particles as a dimer of plus-strand, linear RNA (vRNA). Upon receptor-mediated attachment and entry into a target cell, the core of the virus in released into the cytoplasm where the vRNA is reverse transcribed by viral proteins into linear DNA (vDNA). The vDNA is then shuttled to the nucleus where it is covalently integrated into the host cell genome by the viral integrase,

aided by host proteins. The host transcription/translation machinery expresses the viral genes. Taking advantage of the various cellular pathways, the viral proteins assemble and release new virions (reviewed in 71, 112, 114).

Virion structure

The mature HIV-1 virion is a spherical particle with a centrally located cone-shaped core. The virions are heterogeneous in size with a mean diameter of approximately 135-145 nm (42, 120). The core is also heterologous in size and shape with an average length and diameter of approximatley103 nm and 52 nm respectively (377) (Fig. 1a). The core encapsulates the viral ribonucleoprotein complex consisting of the vRNA coated with the viral nucleocapsid protein, the replication enzymes reverse transcriptase and integrase, protease, Nef, and Vpr (107, 191, 377). The virion is coated with a host derived lipid envelope. The viral envelope is derived from the plasma membrane of the virus producer cell, however the viral envelope is enriched in cholesterol, sphingomyelins, glycphingolipids, saturated phosphoglycerolipids and phosphoinositides, ceramide, GM3, phosphatidylinositol 4,5-bisphosphate [PI(4,5)P(2)], and is more highly ordered compared to the plasma membrane of the viral producing cells (7, 52, 225). The mature viral glycoproteins, homotrimers of gp120/gp41 heterodimers, are inserted into the viral envelope and protrude from the surface of the virus along with other host derived membrane proteins (194, 195, 277, 317, 327). It is estimated that fewer that 10 glycoprotein timers are present on a virion (62, 408). The matrix proteins are located directly beneath the lipid membrane. A number of host proteins are both selectively packaged and randomly packaged into virions. The location of these proteins within the virion are unknown, but it is likely that the majority are either

associated with the outside of the capsid core, or in the intravirion space between the core and the matrix shell (2, 63, 377) (Fig. 1b).

Viral genome and transcriptional regulation

The HIV-1 is classified as a complex retrovirus because it encodes a number of regulatory, and accessory proteins. The HIV-1 genome contains 9 open reading frames that encode for a number of structural and non-structural proteins (Fig. 2). Identical viral long terminal repeats (LTRs) flank the viral genome and encode the *cis*-acting elements involved in regulating viral gene expression. The 5'-LTR contains the majority of the host transcription factor binding sites that regulate the transcriptional activation of RNA Polymerase II. These are primarily located within the 5'-U3 region. The transcription start site located at the boundary between U3-Repeat (R) regions (reviewed in 170). The R region also contains the transactivating responsive sequence (TAR) that enhances transcription from the viral promoter through interactions with the viral Tat accessory protein (reviewed in 300). The 3'-LTR contains the cis-acting sequences that are responsible for the 3'-end processing and polyadenylation of the vRNA and are located at the boundary between the 5'-R-U5 regions (72). Approximately half of the vRNA is spliced within the nucleus by the host RNA splicing machinery creating more than 40 different mRNA species. The majority of these alternatively spliced vRNAs are used in the expression of Env and viral accessory proteins (160, 301, 345). The unspliced and partially spliced vRNAs are exported to the cytoplasm through the interactions of the viral accessory protein Rev and the cis acting sequence Rev response element (RRE) in the Env gene (reviewed in 295), and are used for Gag and Gag-Pol expression or packaging into assembling virions.

Viral proteins and function

Gag

The *gag* gene encodes the major viral structural protein Gag. Gag is translated from full-length unspliced vRNA on cytoplasmic ribosomes. The HIV-1 Gag protein is a 55 kDa polyprotein precursor that encodes all the necessary functional domains/motifs to drive the assembly and release of virus like particles (VLPs) in the absence of all other viral proteins. The Gag protein undergoes a viral protease-dependent maturation event where Gag is systematically cleaved into the mature, infectious virion proteins during, or shortly after release from the cell. The Gag polyprotein is cleaved into: matrix (MA), capsid (CA), spacer peptide 2 (SP2), nucleocapsid (NC), spacer peptide 1 (SP1), and p6. The functional domains within Gag are located through the Gag protein and have various functional roles during assembly. The membrane binding-domain (M-domain) is located in the N-terminus of MA, and functions by regulating the targeting and binding of Gag to specific host cell membranes. The interaction domain (I-domain) sequences are found within the CA and NC domains and function in Gag-Gag interactions and multimerization that leads to the assembly of immature virions. The late domain(s) (L-domain) is located within p6 and facilitates the budding and release of newly assembled virions by interacting with components of the endosomal sorting complex required for transport (ESCORT) pathway (reviewed in 363, 375). Sequences within p6 are also responsible for the packaging of the viral accessory protein Vpr (189). The Gag protein is responsible for the binding and packaging of the full-length genomic vRNA through interactions with the Psi packaging sequences in the vRNA and zinc-finger motifs within NC (Gag function reviewed in 113, 352, 387).

Replication enzymes (Protease, Reverse transcriptase, and Integrase)

The *pol* gene encodes for three essential viral enzymes protease, reverse transcriptase, and integrase. The *pol* proteins are expressed as a Gag-Pol fusion polyprotein through a -1 frame shift that occurs near the c-terminus of Gag and occurs approximately 5-10% of the time (161). The *pol* encoded proteins are packaged into assembling virions through interactions within the I-domains within the Gag sequences of Gag-Pol. The viral protease (PR) is encoded in the 5' end of the *pol* gene. PR is a small (10 kDa) homodimeric aspartic protease and it is essential for virion infectivity (188). The mechanism of protease auto-activation is not well understood, however dimerization is essential for the creation of the active site. The viral Protease is activated during or shortly after virion budding and is responsible for the cleavage maturation of Gag and Gag-Pol into the various infectious proteins found within the mature virion. PR-dependent maturation of Gag is accompanied with a morphological change in the virion. Nef is also cleaved by PR and may play a role in virion infectivity in T-cells (46, 283). PR is one of the targets of anti-retroviral drug treatment in patients (reviewed in 380).

Reverse Transcriptase (RT) is the second protein encoded in *pol*. Reverse Transcriptase is the viral RNA-dependent DNA polymerase. The functional RT is a heterodimers of two subunits (p66/51), which differ only in the presence or absence of the RNase H domain. Interestingly, the two subunits fold into distinctly different structures, with only the p66 contributing to the enzymatic activity (150, 206). The p51 subunit appears to play a structural role and may be involved with binding of the $tRNA_3^{lys}$ primer. RT initiates the synthesis of minus-strand DNA using $tRNA_3^{lys}$ that is base paired to the primer binding sequence within the vRNA. After the RT synthesizes

vDNA, the RNase H domain degrades the vRNA template. As RT reaches the end of the 5'-vRNA, the RT complex transfers to the 3' end of the vRNA where the R regions base pair and minus-strand vDNA synthesis continues. The RNase H resistant, polypurine tract is then used by the RT complex as a primer to extend the 3'-plus-strand vDNA. The remaining RNA is degraded by RNase H, and the plus-strand vDNA is transferred to the 5'-end of the vDNA where it base pairs with complementary sequences and continues vDNA synthesis (Fig. 3) (reviewed in 71). RT has low intrinsic fidelity that leads to nucleotide misincorporation during the vDNA synthesis. These nucleotide misincorporations lead to high genomic variability among viruses. The viral genome also undergoes multiple recombination/cross over events during reverse transcription, increasing genetic variability (212). Because RT is unique to the viral genome, it is also a major target for anti-retroviral drug treatments (reviewed in 324, 366).

Integrase is the last protein encoded by *pol*. The integrase functions to assist the nuclear import of the pre-integration complex (PIC), and to covalently insert the vDNA into the host chromosome. The Integrase protein accomplishes this through two different catalytic activities carried out by two different integrase complexes. Integrase forms dimers on the ends of the vDNA at each LTR. The dimer processes the 3'-end of the LTRs through an endonucleotide cleavage and removal of a dinucleotide from the 3'-end from each of the LTRs. The ends of the vDNA are brought together and the integrase dimers form a tetramer, the functional integration complex (61). The tetrameric integrase then facilitates the vDNA strand transfer and integration into the host chromosome. Weak palindromic consensus sequences have been identified at integrations sites

suggesting that there are preferred integration sites within the host genome. Integrase is also a target for anti-retroviral drug treatment (132, 393) Integrase reviewed in 71, 82).

Envelope glycoprotein

The *env* gene encodes the viral glycoprotein (Env) and is responsible for virion attachment and entry into a target. Env translated from a singly spliced vRNA in the endoplasmic reticulum (ER) as a precursor protein (gp160). The gp160 is highly N-glycosylated and forms timers in the ER. As gp160 trafficks out of the ER and through the Golgi complex, the glycans are modified into complex sugars and the gp160 trimers are cleaved by Furin in the trans-Golgi complex (TGN) into the mature fusogenic forms (trimers of gp120 and gp41 heterodimers). The mature Env trafficks through the cellular secretory system to the plasma membrane (PM) where it is incorporated into the assembling virions, or endocytosed and recycled back to the TGN, or degraded in the lysosome. The gp120 surface subunit (SU) functions by binding and attaching the virion to the surface of a target cell through specific interactions with the viral receptor (CD4) and co-receptor(s) (CXCR4 and CCCR5). The interaction of the co-receptor of the variable loop 3 (V3 loop) of gp120 directs cell tropism. T-tropic viruses that infect T-cells utilize CCCR4, while M-tropic viruses that infect primary macrophage utilize CCCR5 as a co-receptor. The gp120 subunit is highly variable among isolates in both sequence and amino acids length, leading to variations in stability, receptor binding affinity, and antibody neutralization. The gp120 is non-covalently bound to gp41, and is often shed from the cell. The gp41 transmembrane subunit (TM) is anchored in lipid membranes through a transmembrane domain and contains a long C-terminal cytoplasmic tail (CT) that mediates packaging into the assembling virion and limits cell surface

expression through endocytosis. The gp41 mediates the fusion of the viral lipid envelope through interactions with the target cell PM and N-terminal fusogenic peptide leading to the release of the viral core into the cytoplasm (reviewed in 58, 116). Env functions in viral replication are also targets for anti-retroviral drug treatment (356).

Accessory proteins (Vif, Vpu, Vpr, Nef, Tat, and Rev)

The *vif* gene encodes a small 23 kDa multifunction protein that is associated with the nucleus, host cell membranes, and is essential for viral replication in primary T-cells (reviewed in 71). Virion infectivity factor (Vif) is translated from a singly spiced vRNA and is packaged into assembling virions through interactions with Gag at levels similar to the Pol products (39, 50, 109, 219). Vif forms multimers and has been implicated to be involved in virion assembly by increasing Env incorporation into virions by regulating PR activation, Gag processing, core stability, and morphology. However the mechanisms of these functions is unknown (35, 148, 268, 337, 396). Vif is most well known for its function in preventing the incorporation of the host restriction factor apoplipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G (APOBEC3G or A3G) and APOBEC3F (A3F) by targeting them for degradation through the ubiquitin dependent proteasomal pathway (247, 399). When packaged into virions, A3G and A3F inhibit viral reverse transcription and introduce G to A hypermutations into the viral genome during the early steps of infection of a target cell (reviewed in 127, 235).

The *vpu* gene encodes a small 16 kDa protein that functions to down regulate host factors from the cell surface. Vpu is translated from a singly spliced vRNA, and is a type-1 integral membrane protein that forms multimers (232). Vpu functions to down regulate the expression of cellular CD4, which is thought to increase Env incorporation

due to decreased premature Env-CD4 interactions within the cell as Env trafficks to assembly sites at the plasma membrane. Vpu interacts with the CT of CD4 in the ER and targets it for degradation through the ubiquitin dependent proteasomal pathway (reviewed in 235). Vpu has also been demonstrated to increase virion release from the cell, however the mechanism was not understood until recently (130, 184, 348, 397). It was recently discovered that Vpu binds the host protein tetherin, a newly identified host restriction factor that blocks the release of virions from the plasma membrane and targets it for degradation in the lysosome. The degradation mechanism is not fully elucidated, but proteasomal degradation has also been shown to be involved (94, 158, 237).

The *vpr* gene encodes a small 15 kDa multifunctional protein that plays important roles in a number of steps of the HIV-1 life cycle. Vpr is translated from a singly spliced vRNA. Vpr is packaged into the assembling virions through interactions with Gag, at approximately a 1-10 ratio (73). Vpr is encapsulated inside the mature viral core and is part of the pre-integration complex (PIC). One of its functions is to facilitate the nuclear import of the PIC into the nucleus of non-dividing cells (296, 297). Vpr appears to use various cellular mechanisms to localize to the nucleus, however it appears to do so in a unique manner that is independent of the classical NLS and M9 nuclear import pathways (162, 164). Vpr has been demonstrated to interact with importin-α, which was suggested to mediate the nuclear import of Vpr (179). It has also been shown that Vpr interacts directly with nuclear pore proteins, however mutations that disrupt those interactions block perinuclear localization, but did not block import into the nucleus (367). Vpr has also been implicated in upregulation of LTR driven transcription, immune dysfunction, apoptosis, neurotoxicity, and G2 cycle arrest (5, 9, 293, 372, reviewed in 187, 235).

The *nef* gene encodes a small 27 kDa myristoylated multifunctional protein that is translated from a doubly spliced vRNA. The HIV-1 negative factor (NEF) has been jokingly referred to as "not enough function", as it has been found to interact with a number of host factors and plays various roles during multiple steps of the viral replication cycle. Nef localizes primarily to the paranuclear regions and to the PM. Nef has many functions and has been demonstrated to play a critical role in HIV-1 pathogenesis. Individuals infected with Nef deleted HIV-1 progress at a much slower rate than those that are infected with viruses that encode a functional *nef* gene, however the mechanisms involved are not well understood (reviewed in 108, 129). Nef also functions to down regulate the surface expression of CD4 and major histocompatibility complex-I (MHC-I). Nef has been shown to interact with the CT of CD4 causing its clathrin dependent endocytosis and degradation in the lysosome (57, 291, 292). The mechanism resulting in the down regulation of surface MHC-I by Nef is unclear and there was evidence to suggest that Nef increased endocytosis from the plasma membrane and blocked the transport of MHC-I from the TGN to the PM (133, 205, 227, 332, 351). Nef has also found to mediate cell signaling and activation (102, 208, 229, 288).

The *tat* gene encodes a 15 kDa protein that is essential for HIV-1 replication (81). The viral *trans*-activator of transcription (Tat) is a multifunctional protein and primarily located in the nucleus, but can also be secreted from the cell (53). Tat forms homodimers (111), and binds the *trans*-activating response element (TAR) stem-loop structure within the 5'-vRNA and enhances LTR-directed translation by hundreds to thousands fold (140, 256, 314, 339). Tat interacts with various host factors in the RNA Polymerase II complex and increases vRNA transcription by enhancing both initiation and elongation of

host transcriptional complexes (reviewed in 40). Tat has also been shown to be involved in the upregulation of host genes, apoptosis, and neurotoxicity (reviewed in 300).

The *rev* gene encodes a 19 kDa phosphoprotein that is essential for expression of the viral structural proteins. The viral regulator of expression of virion proteins (Rev) is absolutely required for the cytoplasmic export and accumulation of full-length unspliced and singly spliced vRNA. Monomers of Rev are imported into the nucleus and bind the rev response element (RRE), a *cis*-acting sequence that forms stem loop (236, 238). It then multimerizes, forming a large RNA-protein complex containing Rev and various host proteins, then shuttles out of the nucleus along with the vRNA (reviewed in 295). The RRE is located in the *env* intron, and is present in full-length and singly spliced vRNA (238). Rev has also been demonstrated to increase the half-life and the translation of RRE containing RNAs (13, 78, 103, 234)

HIV-1 life cycle

The HIV-1 life cycle is a multi-step process that requires approximately 24 hours to complete one replication cycle (defined as the time it takes to infect a new target cell and the subsequent production of new virions). The average generation time in infected individuals (defined as the time from release of an infectious virion, infection of a new target cell and subsequent release of the next generation of virus particles) is approximately 2.6 days (145). The HIV-1 life cycle is divided into two multi-step events, early and late events. Early events encompass the step from virus attachment through integration of the provirus, while late events involve the transcription/translation of viral genes that culminates in the assembly and release of the newly infectious virions.

Early events

Early infectious events of the HIV-1 life cycle begin by the attachment of the virus to the target cell (helper T-cells and macrophage) through interactions between the host CD4 and viral Env subunit gp120. After attachment and interaction with the host CD4, gp120 undergoes subtle conformational changes that allow the V3 loop of gp120 to engage the viral co-receptors (host CXCR4 and CCR5). This is followed by large conformational change in gp120 and gp41, where the fusogenic peptide in gp41 is inserted into the target cell membrane. Through further conformational changes, gp41 brings the host and viral lipid membranes into close proximity where they undergo lipid mixing and fusion of the two membranes. Interestingly, DC-sign and $\alpha 4\beta 7$, expressed on the surface of dendritic and natural killer cells respectively, can also bind HIV-1 gp120 and are involved in trans-infection of target cells in the lymph tissue of an infected individual (68, 123). HIV-1 Env mediated fusion takes approximately one hour to complete (121, 154). The fusion of the virus at the plasma membrane releases the viral core into the cytoplasm where it undergoes rearrangement to form the reverse transcription complex (RTC). The RTC is transported to the cytoplasmic side of the nucleus where the viral RNA reverse transcription into viral DNA is completed (vDNA). The RTC undergoes another rearrangement to become the PIC and is transported into the nucleus (reviewed in 373). In the nucleus, the vDNA is intergraded into the host chromosome directed by the viral integrase and cellular factors. Integrated proviruses can be detected within 3 hours post infection (43). During these early events, the virus can both utilize, and evade, host proteins in order to its replication cycle (reviewed in 202, 262, 349, 373).

Late events

The viral genes are expressed from the integrated provirus using the host transcriptional and translational machinery. HIV-1 gene expression is biphasic (Rev dependent and Rev-independent). The double spliced accessory proteins (Tat, Rev, and Nef) are the first proteins to be expressed from a newly integrated provirus. The expression of Tat greatly increases the LTR-directed expression by biding the TAR region of the viral RNA and increasing both the initiation and elongation complexes of host RNA Pol II. As Rev levels increase in the cell, Rev binds the RRE in full-length and singly spiced vRNAs and shuttles them to the cytoplasm for translation before they can further spliced in the nucleus by the host splicing factors (295). The predominant viral structural proteins are expressed from the full-length and singly spliced vRNAs.

Gag (Pr55) and Gag-Pol (Pr160) are translated in the cytoplasm from full-length unspliced vRNA and traffics through a poorly defined route to the site of assembly on the PM. Gag is co-translationally modified at the N-terminal glycine residue and is essential for the correct targeting of Gag to the PM (44, 131, 265, 407). Gag trafficking to the PM occurs quickly after translation, and can be detected at the PM within 5 minutes after Gag synthesis (276). The myristate is sequestered inside the MA domain within Gag and only becomes exposed when phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) binds a highly basic region within MA. The exposure of the myristate has been hypothesized to target Gag to lipid rafts within minutes after binding the PM (217, 276, 316). Lipid rafts are microdomains that have been shown to be important microdomains in the plasma membrane that facilitates virion assembly and release, however tetraspanin enriched membranes have also been implicated to be the site of virion assembly (reviewed in 273,

369, 370). The intracellular route that Gag takes to the plasma membrane is not well defined, however a number of cellular proteins have been identified to play important roles in Gag intracellular stability and trafficking to the correct assembly site.

As Gag is trafficked through the cytoplasm to the site of assembly, it functions to specifically bind and package the viral genome through the zinc-finger motifs in NC and interactions with the host protein Staufen (255)

Once trafficked to the PM, Gag forms high-order multimers through interactions between the I-domains of Gag and Gag-Pol proteins at the plasma membranes (85, 149, 153, 200). Multimerization with Gag-Pol ensures the packaging of the replicative enzymes into the assembling virions and initiates the capsid assembly process. After the viral and cellular factors are correctly trafficked to the PM and virion assembly is initiated through Gag multimerization, the ribonucleic protein complex is retained at the assembly site as the nascent virion is assembled and released from the cell surface through a process called budding. The budding and release of progeny virions occurs simultaneously with capsid assembly and utilize the host vacuolar protein sorting pathway through direct interactions between tumor susceptibility gene 101 (TSG101) or ALIX and the L-domain within p6 of Gag (122, 347). During or shortly after release, the immature virion core containing approximately 2100 Gag proteins (42) undergoes viral protease-dependent cleavage maturation where the Gag proteins are cleaved into their infectious virion associated forms to facilitate infection of new target cells (Gag function reviewed in 113, 352, 387).

During the virion assembly process, Gag must also interact with the mature viral Env glycoprotein and package it into the assembling virions. The Env gp160 is highly

glycosylated with N-linked high mannose sugars, interacts with ER chaperones and quickly folds, forms disulfide bonds, and becomes competent to bind CD4 within 15 minutes after synthesis. Trimers of gp160 are assembled approximately 30 minutes after synthesis, and are trafficked to the Golgi apparatus within 80 minutes of synthesis (48, 98, 104, 204, 279). As gp160 trafficks through the Golgi, the glycans are extensively modified and gp160 is cleaved by a host protease into its mature forms (gp120 and gp41) (23, 106, 281, 321, 343). Env continues through the secretory pathway where it is inserted into the plasma membrane at approximately 2 hours after synthesis. If Env is not packaged into assembling virions, it is quickly endocytosed through interactions with AP-1 or AP-2 with the endocytosis signals in the CT of gp41 in a clathrin dependent manner, and can be recycled back to the trans-Golgi network through interactions with Tip47, or degraded in the lysosome (29, 30, 49, 271, 385).

The mechanism of Env packaging is not well understood and published data indicates that sequences with the MA domain of Gag interact with sequences within the CT of Env to facilitate the active packaging of Env into the assembling virions (reviewed in 167). Interestingly, many of the host factors that have been identified to be involved in intracellular Gag trafficking are also involved with intracellular trafficking of Env. Microscopy studies that showed virion components localizing and assembling into vesicles that contained cellular markers for late endosomes/MVBs led many in the field to hypothesize that virion assembly occurred in, or was initiated at the membranes of endocytic vesicles (266, 275, 287). This also led people to hypothesize that Gag may first interact with Env that is localized in the endosomal pathway and this interaction leads to selective packaging of Env into assembling virions because both proteins had

been previously demonstrated to utilize the endocytic pathway during virion assembly. Strengthening this hypothesis, a gp41 tail interacting protein with the molecular weight of 47 kDa (Tip47) was identified as a linker protein that physically linked the MA domain of Gag with the CT of Env. Over expression of Tip47 led to increased Env packaging into virions, while the knockdown of Tip47 expression with siRNA led to decreased Env packaging that resulted in decreased virion infectivity (20, 224).

Interestingly, a few years later, published data indicated that the intracellular vesicles that were visualized and hypothesized to be sites of assembly were not late endosomes, but were deep invaginations of the plasma membrane that were also enriched in CD63, a commonly cellular used marker for late endosomes. Because of the sectioning technique and the enrichment of CD63, the virion assembly sites were mistakenly identified as late endosome/multivesicular bodies (MVBs)(84, 176, 378). While these data refute the hypothesis that HIV-1 assembles in/at late endosomes, it did not offer any insights as to where Gag and Env first interact which then led to packaging.

While the accessory proteins are not essential for virion assembly, they do have important functions that optimize virion production and infectivity. During assembly, Nef and Vpu function to down regulate the cellular expression of CD4. Down regulation of CD4 was hypothesized to decrease premature CD4/Env interactions, which optimize Env trafficking through the secretory pathway to the assembly site. It was also hypothesized to increase virion release from virus producing cells by inhibiting unwanted virion attachment to the producer cell through interaction between surface CD4 and newly synthesizes virions. While Nef has been attributed many functions, one of the important functions is to decrease the cell surface expression of MHC-I. The down

regulation of MHC-I is hypothesized to be a mechanism to avoid immune detection and clearance of virus producing cells by cytotoxic T-cells in an infected individual. Vif and Vpu function to down regulate host restriction factors and reduce their detrimental affect on virion assembly and subsequent replication. Vif degrades and prevents the packaging of APOBEC host proteins, while Vpu degrades cellular tetherin and prevents it from blocking virion release. The combined functions of the 15 proteins expressed by HIV-1 work efficiently together in a consorted effort to recapitulate the HIV-1 lifecycle while the infected individual's immune system tries to block their function and clear the infection from the body.

Host factors that are involved in virion assembly

As detailed above, the HIV-1 virion assembly pathway is a complex process that requires the intricate trafficking of viral and cellular proteins that culminates in the assembly and release of infectious virions. Viral proteins optimize the assembly process through the utilization of host factors. Identifying and understanding important virus:host interactions could potentially identify new pathways that could be pursued to develop new antivirals that interrupt important virus/host interactions. Because Gag plays such an essential role in the assembly pathway, it has been extensively studied in efforts to identify cellular pathways that are important in facilitating Gag function.

A number of host factors have been identified to be functionally important in trafficking Gag to the assembly site on the PM. Members of the kinesin motor family (KIF4A, KIF3A and KIF3C) have been implicated in intracellular Gag trafficking and virion assembly (15, 41, 242). Kinesin proteins function to regulate the movement of various intracellular components along the microtubule network. KIF4A was

demonstrated to decrease Gag stability and VLP release when KIF4A expression was knocked down with siRNA. It was found that in KIF4A knockdown cells, Gag trafficking to the PM was blocked and Gag accumulated at large perinuclear sites named Gag perinuclear clusters (GPC). This data suggested that KIF4A was involved in trafficking Gag from this intermediate, intracellular trafficking site to the PM. Knockdown of KIF3A and KIF3C by siRNA inhibited the release of VLPs and infectious virus respectively, but the mechanism was not further characterized.

The expression of suppressor of cytokine signaling-1 (SOCS1), an E3 ubiquitin ligase, was found to be upregulated in T-cells during HIV-1 infection. It has also been shown to function in Gag stability and trafficking through the cytoplasm. SOCS1 was found to directly bind the MA domain of Gag and colocalize with Gag at perinuclear punctate sites. The overexpression of SOCS1 increased Gag stability and viral assembly and release, while depletion of SOCS1 by siRNA caused decreased Gag stability and viral assembly and release. It was also observed that in SOCS1 knockdown cells, Gag formed large perinuclear clusters, similar to GPCs and displayed decreased stability. However, these perinuclear clusters co-localized with lysosome markers and Gag stability was restored with the use of lysosome inhibitors indicating that they are functionally different than the previously identified GPCs. This data suggested that SOCS1 is involved in Gag trafficking and stability by trafficking Gag to the PM and preventing its degradation in the lysosome (315).

The E3 ubiquitin ligase hPOSH, has been shown to be important for targeting Gag from the TGN to the PM. The hPOSH was identified in a siRNA screen directed against components of the ubiquitin pathway in an effort to identify proteins that were important

for ubiquitin dependent HIV-1 virion release (331). Interestingly, hPOSH was found to localize to the cytoplasmic side of the TGN and depletion of endogenous hPOSH by siRNA decreased Gag localization at the plasma membrane and increased localization to the TGN. It was found that the E3 ligase activity was important for the altered trafficking of Gag, however they were not able to detect differences in the ubiquitination state of Gag in the presence or absence of hPOSH, which led to the hypothesis that hPOSH functions by indirectly affecting Gag trafficking to the PM, by directly affecting the trafficking of vesicles that contained or were associated with Gag (8).

Interesting, many components involved in proteins sorting though the secretory and endocytic pathway are involved in proper Gag trafficking to the assembly site. Members of the Ras superfamily that are responsible for the regulation of vesicular transport pathways have been identified to be important in Gag trafficking and in virion assembly and release. Rab9 is a GTPase that facilitates vesicular trafficking from late endosomes to the TGN through interactions with p40 (90). Rab11A is GTPase that facilitates vesicular trafficking from the TGN to the PM (60). Rab9 and Rab11A knockdown with siRNA resulted in significantly decreased HIV-1 replication. It was also observed that Gag displayed increased localization to CD63+ late endosomes in Rab9 knockdown cells, which suggested that the decreased replication in Rab9 knockdown cells was due to decreased virion assembly and release due to increased sequestration of Gag in late endosomes caused by altered trafficking of Gag (260).

Various subunits of the Adaptor Complexes AP-1 (51), AP-2 (19), and AP-3 (93) have all been implicated in facilitating intracellular Gag trafficking. The AP-1 complex is required for the bi-directional proteins sorting and trafficking between the TGN and

endosomes (270). The AP-1 complex has also been implicated in protein trafficking from the TGN, or early endosomes to late endosomes or lysosomes (286, 306). The μ1A subunit of the AP-1 complex was found to interact with the MA domain within Gag and depletion of μ1A expression by siRNA significantly reduced virion release, however no differences in virion infectivity were observed. Interestingly, over expression of μ1A led to increased virion release (51).

The μ2 subunit of the AP-2 adaptor complex, which directs clathrin dependent endocytosis, has been reported to interact with Gag at the MA-CA interface. It was found that virion release was increased when a trans-dominant negative μ2 subunit was overexpressed, however those virions were less infectious due to decreased Env packaging. Gag trafficking to the PM was found to altered in the presence of the trans-dominant negative μ2. Gag appeared to target to fewer, but larger clusters at the periphery of the PM compared to cells without the trans-dominant negative μ2, which showed small but distinct areas of Gag localization on the PM (19). Interestingly, clathrin has recently been shown to interact with the IN domain in Gag-Pol and specifically packaged into virions. Knockdown of clathrin by shRNA led to decreased specific virion infectivity, however the mechanism is not known (298).

The AP-3 δ subunit was found to interact with the N-terminus of the MA domain in Gag. The function of AP-3 is not well defined. It is involved in protein sorting and trafficking to the lysosome, however whether it functions in the sorting from TGN to the lysosomes, or from endosomes to lysosomes is controversial (286). Knockdown of the AP-3 complex using siRNAs specific for the various subunits of AP-3 showed that the AP-3 complex is important for Gag trafficking through the cell. Gag showed altered

trafficking to the PM, late endosome/MVBs, and diffuse intracellular staining patterns in AP-3 knockdown cells or when a trans-dominant negative AP-3 δ subunit was over expressed in HIV-1 producing cells (93).

The Golgi-localized γ–ear containing Arf binding proteins (GGA) and ADP ribosylation factors (ARF) are involved in protein sorting and tracking from the TGN and endosomes. GGA proteins are recruited to membranes and function through interactions with various members of the ARF protein family. Knockdown of GGA2 or GGA3 in virus producing cell increased the amount of virion release, while overexpression of GGA1, GGA2, or GGA3 decreased Gag binding to the membrane and virion release, which suggest that the GGA proteins are negative regulators of HIV-1 assembly. Interestingly, it was also found that over expression of GGA1 causes defects in gp160 processing and led to decreased levels of intracellular gp120 and increased levels of intracellular gp160. Knockdown of Arf 1,3,5 and 6 with siRNA, or overexpression of trans-dominant negative forms of Arf 1,3 and 5 led to decreased virion assembly and release (174).

The soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) machinery has been shown to be important for HIV-1 virion production (173, 253). SNARE machinery is involved in the catalysis of fusion events between intracellular vesicles/membranes and are required at each step of the endocytic and exocytic trafficking pathways (383). Knockdown of N-ethylmaleimide-sensitive factor (NSF) and α-soluble NSF attachment protein (SNAP), or overexpression of a NSF trans-dominant negative mutant led to decreased virion production and correlated with decreased Gag localization at the plasma membrane. Disruption of the SNARE

machinery also blocked Env trafficking and cleavage, which resulted in decreased virion infectivity (173). The mechanism of how the SNARE machinery stimulates virion production is not fully understood.

Once trafficked to the assembly site, Gag initiates the assembly of the virion capsid through multimerization. The ATP-binding cassette protein family E (ABCE1), has been shown to interact with Gag through the basic residues in the NC domain and play an important role in virion assembly. Mutations that inhibited interactions with ABCE1 were blocked in the early stages of immature capsid assembly, thus preventing virion assembly. ABCE1 was hypothesized to function as a Gag chaperone that mediated Gag multimerization and subsequent virion assembly (218).

CD81 has also been implicated as playing an important role during virion assembly. CD81 is a tetraspanin that is part of the tetraspanin-enriched microdomain in the PM and can also be found in late endosomes/MVBs (101). CD81 is highly enriched at assembly sites and virological synapses (169). Gag and CD81 have been shown to form intracellular complexes, and when CD81 expression on the cell surface was down regulated, either by shRNA or incubation with CD81 antibodies, virion release was decreased. Interestingly, this release was accompanied with a change in Gag staining patterns on the PM (less clustered puncta) and an increase in virion infectivity.

The budding and release of progeny virions occurs simultaneously with capsid assembly and utilize the host vacuolar protein sorting pathway through direct interactions between TSG101 or ALIX and the L-domain within p6 of Gag. TSG101 and ALIX are members of the ESCRT-1 complex and have been shown to be essential in virus budding from the cell surface (122, 347). TSG101 and ALIX have also been shown to interact

with the components of the ESCRT-3 complex, which was also found to be essential in facilitating budding from the plasma membrane (reviewed in 66, 294, 340).

The host protein Staufen has been implicated to play a role in the selective packaging of vRNA during the assembly pathway (56, 255). It has been found that Staufen selectively binds the genomic vRNA and interacts with Gag cytoplasmic complex through the NC domain. Staufen knockdown with siRNA led to decreased specific virion infectivity by approximately 50% compared to virions produced in normal cells (56, 255). This finding was supported by, a recently published report indicating that Gag/RNA interactions occur in the cytoplasm and involves Gag dimers or low-order multimers (200).

Ubc9, SUMOylation, and Retroviruses

Several laboratories including ours have published data that has demonstrated that the host SUMOylation pathway plays an important role during the viral life cycle. Ubc9 was first identified as a retrovirus interacting partner in yeast two-hybrid screen using a HeLa cDNA library and the full-length Gag protein (Pr78) of Mason-Pfizer monkey virus (MPMV) as the bait protein. It was demonstrated that Ubc9 and Pr78 interacted through sequences within the capsid (p27) domain of Gag (376). Even though a functional role for Ubc9 in the MPMV replication cycle was not elucidated, our laboratory has expanded the studies to HIV-1. We demonstrated that Ubc9 and HIV-1 Gag (Pr55) interacted in vitro and in vivo and mapped the interaction to the NC-p6 domain within Gag. We discovered that in Ubc9 knockdown cells, the amount of mature virions released into the medium was unchanged, which indicated that the general functions of Gag were not disrupted, however specific infectivity was decreased approximately 10-fold compared to

virions produced in control cells. We used immunoblotting to assay the virion composition and found that the defective virions contained less mature Env (gp120/gp41). Pseudotyping experiments with VSV-G restored virion infectivity, indicating that the defect was primarily due to deficiencies in Env packaging and entry of target cells, and not due to disruptions in the function of the HIV-1 core or replicative enzymes during early infection events. Env production was examined in the Ubc9 knockdown cells and it revealed that intracellular mature Env levels were decreased. Unexpectedly, the mechanism of decreased intracellular Env levels was found to be dependent upon Gag expression, which suggested that the interaction between Gag and Ubc9 somehow stabilized intracellular Env levels. Pulse chase data suggested that the decreased levels of intracellular Env was likely due to decreased stability of mature Env in Ubc9 knockdown cells. We also found that the overexpression of the trans-dominant negative, catalytically inactive Ubc9 mutant (C93A) did not negatively affect virion infectivity, which suggested that the SUMO conjugating activity was not involved with Ubc9 dependent decreased infectivity. We published this data in 2009 in the Journal of Virology (159).

During this time, Gurer et al. published data demonstrating that HIV-1 p6 interacted with Ubc9 and SUMO-1. They showed that the major Ubc9 binding site was at or near the lysine at position 27 (K27). A GFP-p6 fusion protein could be SUMOylated at K27 at low levels in vivo if SUMO-1 was overexpressed. SUMOylation of GFP-p6 was prevented when the lysine at position 27 was mutated to arginine (K27R). The K27R mutation in the context of NL4-3 appeared to be unaffected during virion assembly, however they showed that if SUMO-1 was overexpressed in HIV-1 producer

cells, the completion of reverse transcription was inhibited in target cells. Interestingly, unconjugated SUMO-1 was found in the virion when overexpressed in producer cells, however they were not able to detect SUMOylated viral proteins in the virion. The K27R mutant relieved the block in target cells when SUMO-1 was overexpressed in the producer cells, however the mechanism of how the overexpression of SUMO-1 led to changes in reverse transcription are not fully understood (137). The phenotype that we observed was different from that of Gurer et al. This may be due to differences in experimental procedures, or as we had hypothesized previously, only a small amount of Gag may be needed to interact with Ubc9 to carry out its functional role during HIV-1 assembly (159). This interaction may be occurring through a second binding site within Gag, as K27R does not completely inhibit Ubc9 interactions (unpublished data).

Another group, Martinez et al. published data demonstrating that when the expression of KIF4, a microtubule motor protein, was knocked down using siRNA, Gag stability and trafficking to the plasma membrane was disrupted. Gag trafficking appeared to be blocked at the early intermediate step at an intracellular site where they characterized as GPCs. Using a Myc-tagged Ubc9 construct from our laboratory, they showed that Myc-Ubc9 and SUMO-1 were co-localized to the GPCs with Gag in KIF4 knockdown cells (242).

Very recently, a French group reported that HIV-1 Integrase is SUMOylated *in vitro* and *in vivo* when SUMO-1, SUMO-2, or SUMO-3 were overexpressed in cells expressing HIV-1 Integrase. Mutations of three lysines at position 46, 136, and 244 lead to decreased SUMOylation of Integrase and was found to correlate to defects in provirus

integration, suggesting that Ubc9 and SUMOylation are involved in the early events during HIV-1 infection (401).

Interestingly, Ubc9 and SUMOylation have also been found to be important during the early infectious events of a murine retrovirus. Yueh et al. reported that Moloney murine leukemia virus (MuLV) capsid (p30) protein interacted with Ubc9 and PIASy (E3-SUMO ligase). Mutations of lysine residues near the Ubc9 binding domain blocked p30 SUMOylation and correlated with a defect in replication at an early infectious step after reverse transcription of the vRNA, before targeting to the nucleus (400). This phenotype is different from what Gurer et al. and Zamborlini et al. described, which indicated that different viruses may utilize Ubc9 and SUMOylation differently.

Ubc9 and SUMOylation

Ubc9 is a small protein of approximately 18 kDa, it is constitutively expressed in every cell type, and is the only E2 small ubiquitin-like modifier (SUMO) conjugase identified to date (192). Ubc9 plays an essential role in regulating various aspects of cellular function and replication through SUMO-dependent and SUMO-independent mechanisms (142, 261). The SUMOylation pathway involves a series of enzymatic steps that is analogous to the ubiquitin pathway that results in the covalent attachment of SUMO to target proteins. Ubc9 has considerable sequence similarity and adopts a similarly folded structure as an E2 ubiquitin conjugase (358). Ubc9 is responsible for target recognition of proteins to be SUMOylated, but it is through the interaction with the E3 SUMO ligases that facilitates target specificity in the covalent attachment of SUMO to target proteins.

SUMOylation occurs through an isopeptide bond between the C-terminal glycine of SUMO and the lysine side chain of the target protein in an ATP dependent manner. *In vitro*, Ubc9 is able to covalently attach SUMO to the target protein in the absence of an E3 SUMO ligase (88, 248, 272). The cysteine at position 93 is essential in the enzymatic activity of Ubc9 and the amino acids that surround the active site have been found to bind directly to the putative SUMOylation motif (22, 322, 355). The putative SUMOylation motif is ψKXE/D, where ψ is a hydrophobic amino acid and X is any amino acid (309). However this motif alone is not the only contributing factor in SUMOylation, non-canonical SUMOylation motifs have also been reported, and it is not known how the non-consensus sites are recognized by Ubc9 (67 reviewed in 124, 141, 166).

Interestingly, Ubc9 itself has been found to be SUMOylated at a non-consensus site that modulates Ubc9 target interactions and SUMOylation by causing a conformational change in Ubc9, which creates a new binding surface for altered interactions with target proteins (186). The regulation of SUMOylation of target proteins has also been previously hypothesized to involve the phosphorylation, ubiquitination, and/or acetylation state of the target protein (Ubc9 and SUMOylation reviewed in 124, 141, 166, 325, 384, 405). Ubc9 was often found to be involved in the SUMOylation of a number of cellular proteins, but Ubc9 can also function in SUMO-independent mechanisms. Lu/BCAM (75), androgen receptor (54), MEKK1, type I TNF-alpha receptor (318), GLUT4 (220), RNA helicase A (11), glucocorticoid receptors (181), Vsx-1 (199), SOX4 (282), and HHV-6 IE2 (357) have been demonstrated to require Ubc9 binding for their function and correct intracellular targeting, but are not targets for SUMO modification.

Ubc9 and SUMOylation in autophagy regulation

Autophagy is a degradation/recycling pathway that turns over long-lived protein, damaged organelles, and cellular cytoplasmic factors. Old or damaged organelles and the surrounding cytoplasm are engulfed in a double, or multi-membrane vesicle known as the autophagosome, which fuses with lysosomes to form autophagolysosomes. The contents of the autophagolysosome are degraded and recycled for anabolic cellular metabolism. Autophagy is highly regulated, but can be quickly upregulated over basal levels in response to cellular stress, starvation, activation of the cell signaling pathways, or by infection of a pathogen. The formation of an autophagic vacuole requires a sequential cascade of various protein-protein interactions and protein-lipid interactions leading to the formation of the double membraned autophagic vacuole. The upregulation of autophagy is commonly initiated by cellular stress through inhibition of the mTOR kinase activities, however other pathways have been demonstrated to regulate autophagy. Changes in the levels of reactive oxygen species (17, 33, 38, 92, 228, 328, 365), increase in free intracellular Ca^{2+} (89, 151), altered cell signaling through the MEK/ERK pathways (198, 364, 371), activation of phosphorylation pathways (PKA, PKB, PKC, and AMPK) (12, 45, 77, 165, 209, 214, 249, 344), or toll-like receptor signaling (196, 197, 278, 305, 359) have all been demonstrated to be involved in the upregulation of autophagy. Interestingly many of the alternative activation pathways converge at some point with the SUMOylation pathways, either directly or indirectly (1, 6, 24, 37, 136, 165, 180, 198, 223, 226, 353, 365). Even though SUMOylation of various proteins within these regulation pathways have been demonstrated (HIF-1 and HIF-2, MDM2, P53, tissue transglutaminase 2, eE2F, MEK, c-Jun, Smad4, transforming growth factor–

beta), the SUMOylation of these proteins have not been analyzed with regards to their roles during the regulation of autophagy (6, 24, 37, 59, 180, 198, 223, 310, 353, 365). Given the far- reaching functions of Ubc9 and SUMOylation in cellular function, it is likely that Ubc9 and SUMOylation play important roles in autophagy regulation. To our knowledge, there are no reports demonstrating that Ubc9 or SUMOylation have an effect on the proteins that are involved with autophagic vesicle elongation or fusion with the lysosome (Atg3, Atg4, Atg5, Atg7, Atg9, Atg12, Atg16, Atg18, or LC3) (autophagy reviewed in 95, 230, 231, 252, 289).

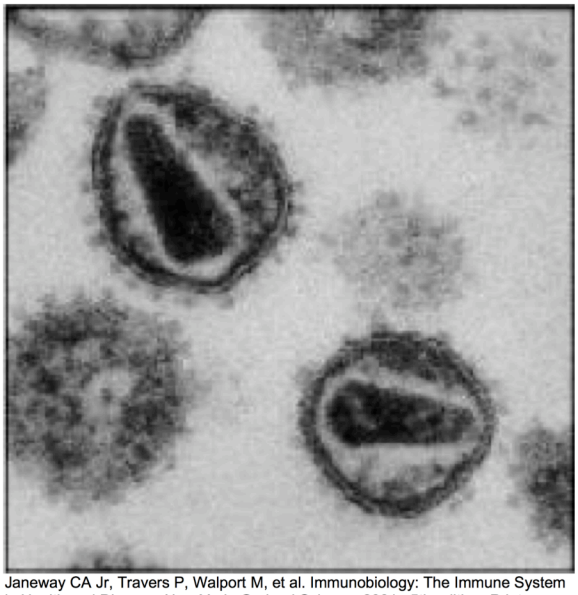
Research aims

The aim of this research is to expand the functional studies, which began with identification of Ubc9 as a binding partner of M-PMV Gag, to HIV-1 and examine if Ubc9 plays a functional role in the HIV-1 life cycle. In this dissertation we demonstrated that Ubc9 is a binding partner of HIV-1 Gag and that Ubc9 is important during the late events of the virus life cycle. We demonstrated that Ubc9 plays an important role during the replicative HIV-1 life cycle. Using siRNA specific for Ubc9, we found that knockdown of Ubc9 in HIV-1 producer cells led to decreased specific infectivity of the released virions. The decreased infectivity was due to a decrease in intracellular gp120 levels. The lower levels of gp120 were demonstrated to be due to enhanced selective degradation of intracellular gp120 in the lysosome, which occurred in a Gag dependent manner. It was also discovered that autophagy was upregulated in Ubc9 knockdown cells. Whether an induction of autophagy directly leads to an increase in lysosomal degradation of gp120 needs to be further elucidated.

Figures and Figure legends

Figure 1

a



Janeway CA Jr, Travers P, Walport M, et al. Immunobiology: The Immune System in Health and Disease. New York: Garland Science; 2001.. 5th edition. Print

b

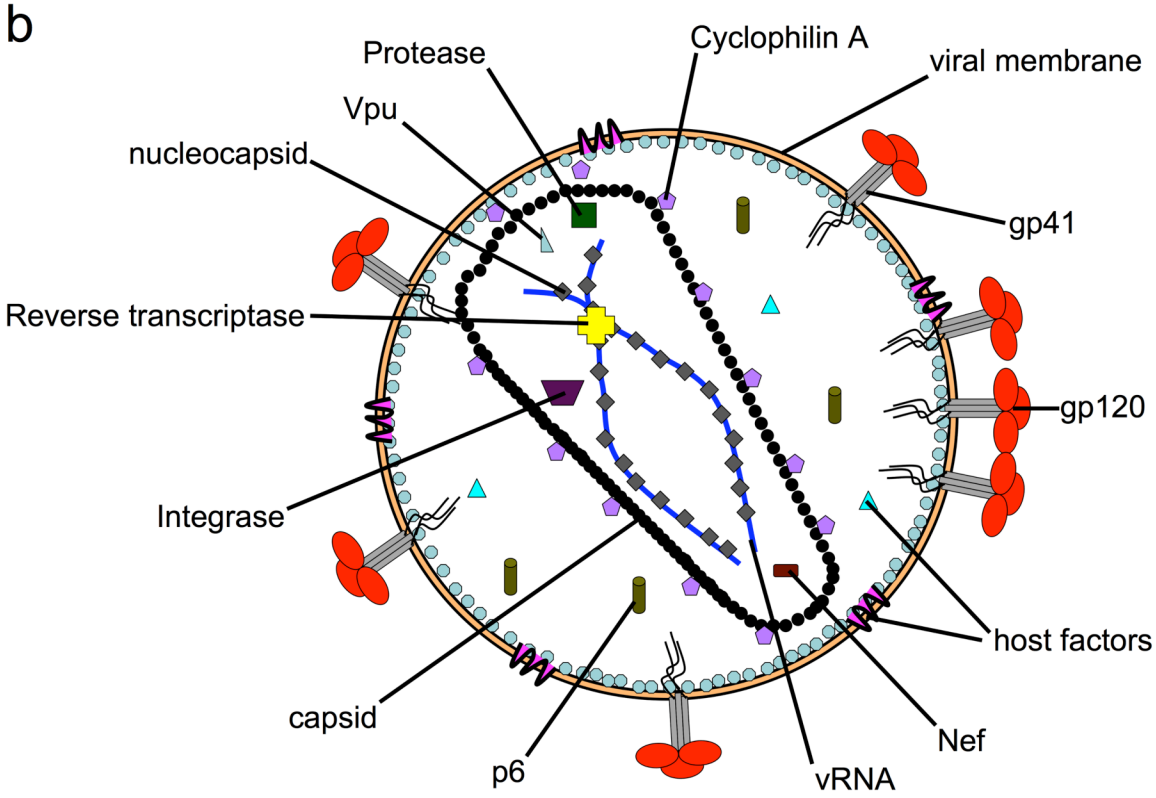
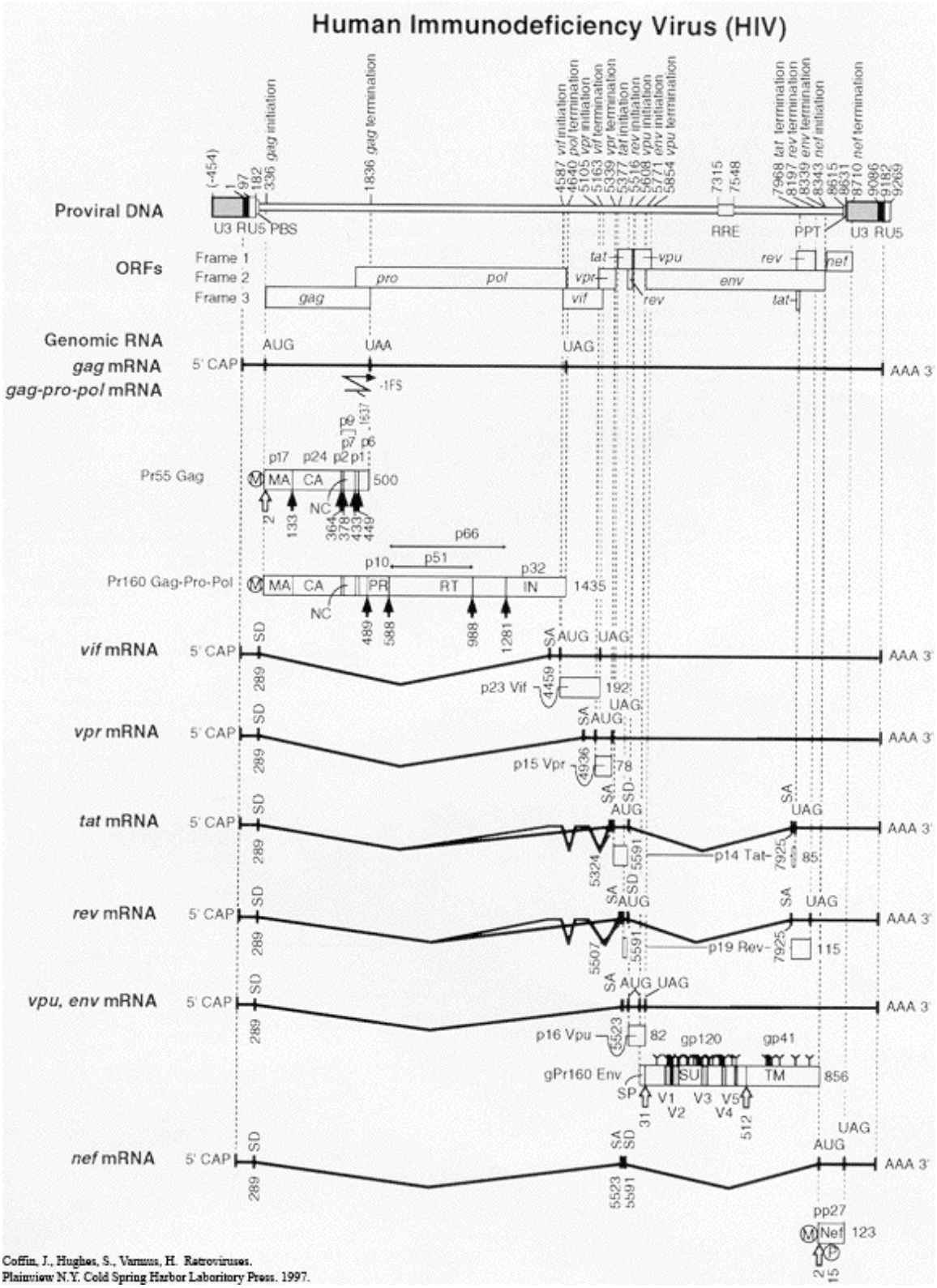


Figure 1. HIV-1 virion morphology. (a) Thin-section transmission electron microscopy of HIV-1 virions. The cone-shaped core, lipid envelope, and virion glycoproteins are visible (259). (b) Schematic of known protein localization of various viral and host components within the virion.

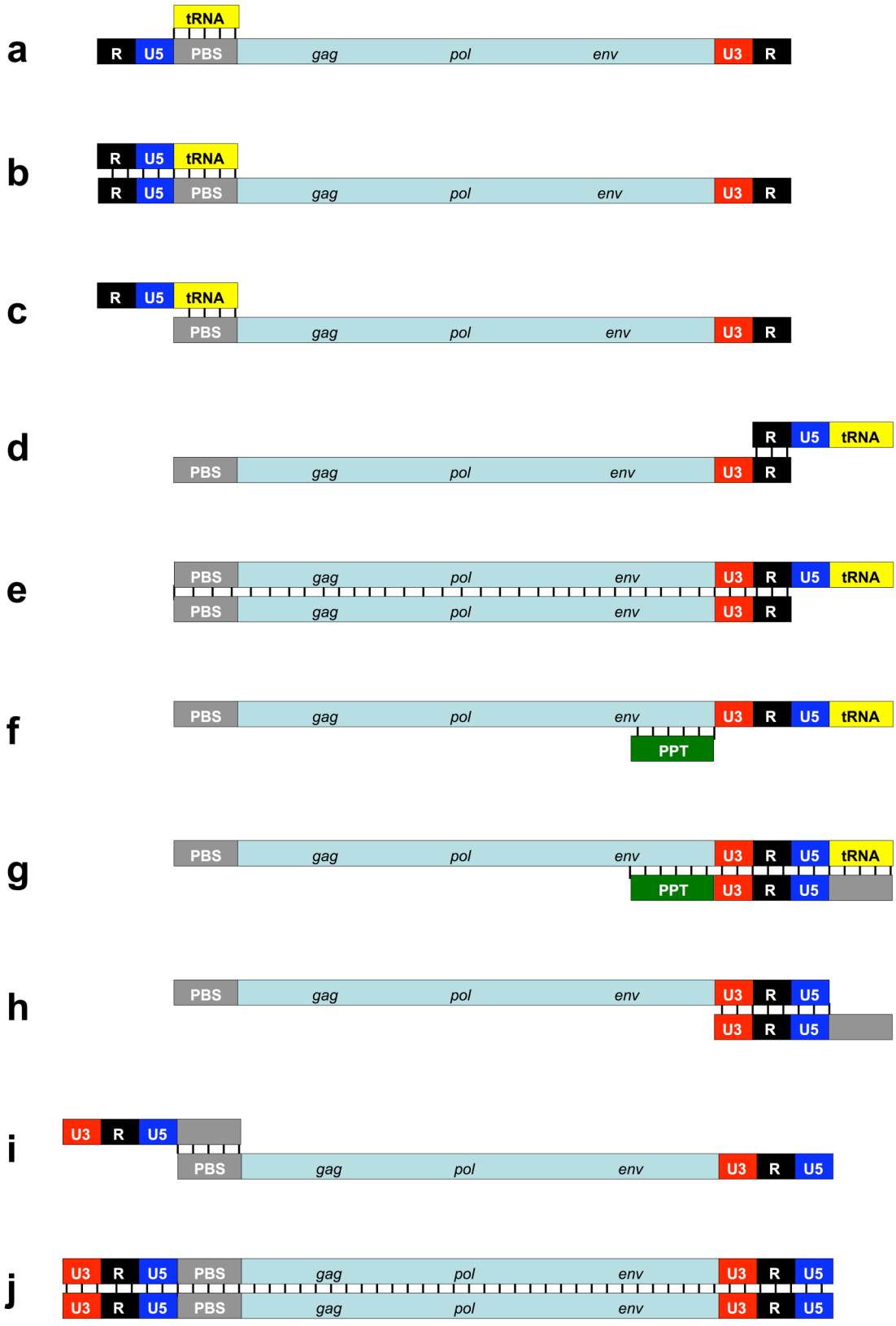
Figure 2



Coffin, J., Hughes, S., Varmus, H. Retroviruses.
Plainview N.Y. Cold Spring Harbor Laboritory Press. 1997.

Figure 2. HIV-1 Genome, genomic landmarks, and viral transcripts. Proviral DNA genomic map was designed from the modification of HXB2CG proviral sequences (GenBank: AF033819.3)(71, 304). Transcriptional start site and open reading frame (ORF) within the proviral sequence and nucleotide positions are labeled. The transcriptional start and stop sites are located at the boundary between the 5'-U3-R and 3'-R-U5 regions respectively. The primer biding site (PBS), Rev response element (RRE), and the polypurine tract (PPT) are identified. The predominant splice donor (SD) and splice acceptor (SA) sites utilized by the host splicing machinery are labeled. The full-length genomic vRNA and predominant spliced vRNA transcripts and the ORFs encoding Env and accessory proteins are shown. The vRNAs contain a 5'-7-methylguanylate cap and 3'-polyadenylated tail. The site of the -1 frame shift that produces Gag-Pol is identified as a zigzag arrow on the full-length genomic vRNA. Viral protease-dependent cleavage sites with Gag and Gag-Pol are identified as black arrows. White arrows identify cellular host protease-dependent cleavage sites within Gag, Gag-Pol, and Env. Post-translational modification of viral proteins are identified with symbols; myristoylation is identified as a circled upper-case M, phosphorylation is identified as a circled upper-case P, and glycosylation is depicted as upper-case Y.

Figure 3


a
tRNA
R
U5
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gag
pol
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U3
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b
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Figure 3. Reverse transcription of the vRNA to vDNA. (a) The $tRNA_3^{lys}$ binds the vRNA in through complementary sequences within the PBS. (b) RT uses the tRNA primer to initiate the synthesis of minus-strand DNA (first strong stop DNA). (c) The RNase H activity of the p66 subunit of RT degrades the vRNA from the vRNA/DNA hybrid. (d) The RT/first strong stop complex is transferred to the 3'-end of the vRNA (first strand transfer) where it base pairs through complimentary sequences with in the terminal repeat region (R-region). (e) Complementary vDNA is synthesized. (f) The vRNA template is degraded, with the exception of a RNase H resistant, PPT. (g) The PPT is used by the RT complex as a primer to extend the 3'-plus-strand vDNA. (h) The remaining RNA is degraded by RNase H. (i) The plus-strand vDNA is transferred to the 5'-end of the vDNA (second strand transfer) where it base pairs with complementary sequences within the PBS. (j) The vDNA synthesis is continued, completing the synthesis of vDNA. Genomic sequences are not drawn to scale.

Chapter II

Human Ubc9 contributes to the production of fully infectious HIV-1 virions

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Abstract

Ubc9 was identified as a cellular protein that interacts with the Gag protein of Mason-Pfizer monkey virus. We show here that Ubc9 also interacts with the HIV-1 Gag protein and their interaction is important for virus replication. Gag was found to co-localize with Ubc9 predominantly at perinuclear puncta. While cells in which Ubc9 expression was suppressed with RNAi produced normal numbers of virions, these particles were 8-10 fold less infectious than those produced in the presence of Ubc9. The nature of this defect was assayed for the dependence on Ubc9 during viral assembly, trafficking, and Env incorporation. The Gag-mediated assembly of virus particles and protease-mediated processing of Gag and Gag-Pol were unchanged in the absence of Ubc9. However, the stability of the cell associated Env glycoprotein was decreased and Env incorporation into released virions was altered. Interestingly, overexpression of the Ubc9 trans-dominant negative mutant C93A, which is a defective E2-SUMO-1 conjugase, suggests that this activity may not be required for interaction with Gag, virion assembly, or infectivity. This finding demonstrates that Ubc9 plays an important role in the production of infectious HIV-1 virions.

Introduction

Production of infectious HIV-1 particles is a coordinated series of poorly defined events wherein the virus takes advantage of cellular components to target its structural proteins and genomic RNAs to common sites on cellular membranes. This co-localization drives the assembly of immature capsids, packaging of the diploid viral genomic RNA, incorporation of viral glycoproteins and virion-associated proteins, and viral budding/release. The major structural component of the immature HIV-1 core is the Gag polyprotein. Gag is synthesized on cytoplasmic ribosomes as a 55 kDa polyprotein, $Pr55_{Gag}$, and is co- or post-translationally myristylated. Expression of Gag alone is sufficient for assembly and release of non-infectious virus-like particles. However, infectivity of HIV-1 can only be attained by inclusion of the receptor-binding Env glycoproteins (gp120 and gp41) and the *pol*-encoded enzymes (protease, PR; reverse transcriptase, RT; and integrase, IN). PR is essential for the cleavage of the Gag polyprotein into the structural proteins, NH_2 - MA (p17, the membrane-associated, matrix protein), CA (p24, the major capsid protein), SP1 (spacer peptide 1, also known as p2), p7 (NC, the nucleocapsid protein), SP2 (spacer peptide 2, also known as p1), and p6-COOH (113, 387), to form the mature, infectious virion. The cleavage of the precursor polyprotein has been the target of the highly effective protease inhibitor class of antiretrovirals.

Gag-mediated packaging of viral enzymes, structural proteins (e.g. Gag-Pol polyprotein, mature Env glycoproteins, Vpu, Vpr, Vif), the viral RNAs, and cellular components (e.g. Cyclophilin A (110) and Lysyl-tRNA) are necessary for the production of infectious particles (113, 118, 387). Since these molecules are synthesized in different

cellular compartments, it is imperative that cellular pathways traffic these components to common assembly sites on infected cell membranes. Cell fractionation, immunohistochemistry, electron and fluorescent microscopy studies have shown that Gag assembles and buds almost exclusively from the plasma membrane in primary CD4+ T cells and in most cell lines such as HeLa, 293 and COS-1 cells (143, 147, 275, 360). However, a significant but minor portion of Pr55 in T-cells can also be found associated with late endosomes (LE) (134). These observations led to the postulation that most of $Pr55_{Gag}$ buds primarily from the plasma membrane, but a portion of the polyprotein may fail to bud and cycles off the plasma membrane into the LE (139). Alternatively, it has been suggested that Gag is initially targeted to the LE/multivesicular bodies (MVB) compartments and is subsequently transported to the plasma membrane (134, 264, 290, 303, 336). Consistent with the latter hypothesis, further studies have shown that Gag co-localizes with LE/MVB marker proteins in infected/transfected cells and is packaged into released virions (193, 287). Studies have shown that the membranes of these intracellular structures are contiguous with the plasma membrane (84, 176, 378) and HIV-1 may utilize these tetraspanin enriched microdomains as assembly sites (84, 168). The current model is that HIV-1 assembles and buds from specialized regions of the plasma membrane in most, if not all cell types.

The intracellular trafficking pathways of the viral structural proteins must converge at common sites to allow the requisite assembly interactions to occur. However, the intracellular location of these interactions and the temporal or spatial regulation of these events are not well defined. Gag and Gag-Pol proteins are transported through the cytoplasm to cellular membranes by poorly understood processes that likely

involve components of the cellular cytoskeletal system. The viral glycoproteins are synthesized on rough endoplasmic reticulum (RER) and are co-translationally translocated into the ER lumen as gp160 precursor proteins. The gp160 molecules are heavily N and O-linked glycosylated concomitant with trimerization prior to being transported to the Golgi apparatus for oligosaccharide maturation. During transport from the cis-medial Golgi to the cell surface, gp160 is likely cleaved by Furin in the medial trans Golgi (138, 254), yielding the mature surface (SU, gp120) and transmembrane (TM, gp41) glycoproteins. However, other proteases may also be involved (155, 269, 392).

It is hypothesized that Env trimers interact with Gag at budding sites, yet biochemical and genetic evidence suggest that the initial Gag-Env interactions, whether direct or indirect, occur prior to their arrival at the final assembly sites (221, 222, 323, 335). In fact, Tip47, a tail interacting protein involved in retrograde transport of cellular factors from endosomes to the trans-golgi network (TGN), was found to be required for Env packaging. This model suggests that during retrograde transport to the TGN, Env comes into contact with Gag on endosomes with Tip47 directly facilitating Gag and Env interaction. This complex is then targeted to the final assembly site on the plasma membrane (224).

Coordinated trafficking of Gag, Gag-Pol, and Env requires interactions with cellular proteins. A number of host factors such as, AP-1 (51), AP-2 (19), AP-3 (93), Rab9 (260), POSH (8), PI(4,5)P_2 (274), Tip47 (224), SOCS1 (315), Arf proteins (174), and KIF4 (241) are found to interact directly with HIV-1 structural proteins, promoting proper intracellular trafficking and driving virion assembly. Ubc9, an E2 SUMO conjugating enzyme (333), was found to interact with several different retroviral proteins,

but its function during virus replication is unclear. Ubc9 was shown to interact with the full-length Gag protein of Mason-Pfizer monkey virus (M-PMV) near the nuclear membrane, suggestive of a possible function for Ubc9 during virus assembly (376). In contrast, Ubc9 was subsequently shown to interact with and SUMOylate the CA protein of Moloney murine leukemia virus (MoMuLV) (400). Unlike the observations made with M-PMV, an interaction between the full-length MLV Gag protein and Ubc9 was not observed in the yeast two-hybrid assay. Biochemical and genetic analyses suggested the MLV CA-Ubc9 interaction is required during the early stages of replication, post-reverse transcription.

Finally, Ubc9 was shown to interact with the HIV-1 $p6^{gag}$ protein in yeast and SUMOylate a p6-GFP fusion protein in mammalian cells. Overexpression of SUMO-1 resulted in the packaging of free SUMO-1 into HIV-1 virions and was shown to cause a 5-fold decrease in infectivity by these virions when compared to virions produced in control cells. It was found that the defect in infectivity of wild-type (wt) HIV-1 virions produced in the presence of overexpressed SUMO-1 occurred during a post-entry step (137), and suggests that SUMOylation of p6 negatively regulates HIV-1 infectivity during an early infectious event. Interestingly, mutation of the SUMOylation site, in the context of an infectious provirus, had no apparent effect on virus replication in normal cells. However, unlike wt HIV-1, the SUMOylation mutant did not exhibit a defect in virion infectivity when SUMO-1 was overexpressed in producer cells. Thus, the role of SUMOylation on p6 function is still not clear.

Recently it was reported that GagGFP co-localizes with myc-Ubc9 at Gag perinuclear clusters (GPCs) in the absence of KIF4 (241), a kinesin family motor protein.

Blocking KIF4 function resulted in Gag accumulation at GPCs, inhibiting Gag trafficking, stability, and subsequent release. However, neither an interaction between Ubc9 and full-length Pr55 nor SUMOylation of Pr55 by Ubc9 during assembly was examined in the previous report. In this study, we further investigated the role of Ubc9 during HIV-1 replication. We demonstrated that; a) Ubc9 interacts with Pr55, b) Ubc9 and C93A Ubc9, a trans-dominant negative mutant (354), co-localize with Gag at perinuclear sites, c) Ubc9 is crucial during the late stages of replication to produce infectious particles, and d) the E2 SUMO conjugation activity of Ubc9 does not appear to be a prerequisite for its effect during the late stages of the HIV-1 life cycle.

Materials and Methods

Cell lines and bacterial strains. TZM-bl indicator cells were obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: TZM-bl from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc. HeLa and 293T cells were obtained from the American Type Culture Collection. *Escherichia coli* BL21 (DE3) cells were used for expression of GST and GST-Ubc9 (376).

Plasmids. The infectious HIV-1 proviral clone pNL4-3, and non-infectious clone pNL4-3-ΔE-EGFP were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: pNL4-3 from Dr. Malcolm Martin (4) and pNL4-3-ΔE-EGFP (Cat# 11100) from Drs. Haili Zhang, Yan Zhou, and Robert Siliciano (403). The pLTR Env_{NL4-3} construct encoding HIV-1 Envelope was created by introducing an *EcoRI* site pNL4-3 at position 823. The 4920 bp *EcoRI* (nt 823 and 5743) fragment was removed and the remaining vector was religated. The plasmids pCMVGAGPOL-RRE-R and pCMVrev, encoding HIV-1 Gag and Rev respectively, were a kind gift from Dr.

David Rekosh (University of Virginia, Charlottesville, VA) (213, 342). The *in vitro* Gag expression plasmids pTM/55BAM, pGPG1-39G1, pGPG1-15G1, pGPG1-41G1, pGPG1-17G1, pGPG1-24G1 were a kind gift from Dr. Paul Spearman (Emory University, Atlanta, GA) (341). The plasmid pHyg-VSV-G encoding VSV-G was a kind gift from Dr. Asit Pattnaik (University of Nebraska-Lincoln, Lincoln, NE).

Plasmids pET41c+ (Novagen), pET Ubc9, pCMV-Tag3A, and pCMV Myc-Ubc9 have been previously described (376). Myc-C93A was created by PCR based site directed mutagenesis of Myc-Ubc9 by mutating nucleotides 276 and 277 to cytosine and guanine respectively in the Ubc9 ORF. The plasmid pEGFP-SUMO-1 encoding an EGFP-SUMO-1 fusion protein was a kind gift from Dr. Yongsok Kim (182)

RNA interference (RNAi) and DNA transfections. Synthetic, double-stranded siRNAs with 3' dTdT overhangs, corresponding to nucleotides 86-104 of the Ubc9 open reading frame were purchased from Dharmacon and are similar to those previously described (216). Fluorescent, non-silencing 21 base pair double-stranded RNA (Qiagen) was used as a control RNA (Ctr. RNA). 293T cells were transfected twice with RNAs, at 0 and 24 hrs, using Lipofectamine 2000 reagent (Invitrogen) as per manufacturer's protocol. After an additional 24 hrs, cells were transfected with either plasmid pNL4-3, pLTR Env_{NL4-3}, pNL4-3 ΔEnv-EGFP, or pNL4-3-ΔE-EGFP with pHyg-VSV-G. Plasmids pCMVGAGPOL-RRE-R and pCMVrev were co-transfected into HeLa cells using Fugene 6 (Roche) as per manufacturer's suggestions.

GST pull-down assays. pTM/55BAM, pGPG1-39G1, pGPG1-15G1, pGPG1-41G1, pGPG1-17G1, and pGPG1-24G1 were used to express [^{35}S] methionine-cysteine radiolabled wt Gag and Gag deletion mutants using the TNT Coupled Reticulocyte

Lysate System (Promega). GST-Ubc9 (pET Ubc9) and GST (pET41c+) were expressed in and purified from *E.coli* as previously described. Radiolabeled Gag or Gag deletion mutants were incubated with equimolar concentrations of GST or GST-Ubc9, immobilized on glutathione beads and pull-down assays were carried out as previously described (376).

Viral infectivity assays. Virus released into the culture media from transfected cells was quantified by determining the amount of released p24 by immunoblotting. TZM-bl indicator cells were infected with equivalent amounts of viruses (normalized to p24 content). The number of infected cells in this single round infectivity assay was determined by staining for β–galactosidase activity at 36 hrs post infection as previously described (183).

Antibodies. Anti-Ubc9 (H-81) rabbit polyclonal antibodies (pAb), anti-Actin goat pAb, anti-Cyclophilin A pAb, HIV-1 anti-gp41 (10E9) monoclonal antibodies (mAb), and HRP-conjugated chicken anti-goat pAb were purchased from Santa Cruz Biotechnology, Inc. The following rabbit, goat, and mouse anti-HIV-1 antibodies: anti-p24 mAb 24-2, gp41 mAb 2F5, HIV-1_{sf2} p24 pAb, HIV-1 gp160/120 goat pAb H3T, HIV-1_{HXB2} Vif pAb, HIV-1_{NL4-3} Vpu pAb, and Vpr pAb were obtained from the NIH AIDS research and reference reagent program. Anti-SUMO-1 #12783 rabbit polyclonal antibodies, a kind gift from Dr. Van Wilson (Texas A&M University System Health Science Center, College Station, TX), were previously described (313). HRP-conjugated goat anti-rabbit and HRP-conjugated sheep anti-human antibodies were purchased from Amersham Pharmacia Biotech. Cy5 conjugated donkey anti-mouse and Cy2 conjugated donkey anti-rabbit sera were purchased from Jackson Immunoresearch Laboratories. Pooled

AIDS patient serum and pooled normal human serum were obtained from a patient cohort.

Immunoblotting analysis. Media from transfected cells were harvested and virions were purified from medium by pelleting through a 20% sucrose cushion (34). Pelleted virions and cells were solublized directly in 2X protein sample buffer (PSB), separated by SDS-PAGE and analyzed by immunoblotting. For immunoblot detection of SUMOylated proteins, transfected cells were lysed as previously described (16) with SUMO lysis buffer containing 20mM Iodoacetamide and NEM. Lysates were separated by SDS-PAGE, transferred to a PVDF membrane (GE Healthcare), and analyzed by immunoblotting.

Viral RNA extraction and real time PCR. Viral RNA (vRNA) was extracted from equal numbers of virions (normalized by p24 content) and reverse transcribed (34). Viral cDNA was quantified by real time PCR (iCyclerIQ, Bio Rad) using previously described primers (74).

Cell viability assay. Viability of Ubc9 siRNA transfected cells and control cells was assayed using BD ApoAlert Annexin V & Apo 2.7-PE kit (BD Biosciences) as per manufacturer's protocol. Annexin-EGFP V staining and Propidium Iodide uptake was measured using BD FACSCalibur (BD Biosciences).

Confocal microscopy. Transfected 293T cells were cultured on sterile cover slips treated with poly-L-lysine in 35mm culture dishes. Cell were fixed at room temperature in 4% paraformaldehyde in PBS for 20 minutes and permeabilized with 0.2% TX-100 in PBS for 5 minutes. Fixed cells were processed and stained as previously described (34). Cover slips were mounted on slides and analyzed by confocal microscopy (Olympus

FV500 and upright BX Olympus florescence microscope). Proteins in transfected cells were detected using the following antibodies: Pr55, anti-p24 mAb, Env, anti-gp41 mAb 10E9, Ubc9, anti-Ubc9 pAbs. Fluorescent Cy5-conjugated donkey anti-mouse and Cy2-conjugated donkey anti-rabbit antibodies were used as secondary antibodies. Anti-Ubc9 antibodies were not able to detect endogenous Ubc9 in this assay. Consequently, Cy2 background antibody staining and any potential low level, endogenous Ubc9 staining signals were reduced to undetectable levels; such that, the fluorescent signals detected were due to exogenous protein expression only. 0.3-um Z-sections were analyzed using Flowview imaging software (Olympus).

Metabolic labeling and immunoprecipitation. Transfected 293T cells were pulse labeled for 45 min with 300 μCi [^{35}S]methionine-cysteine (>1000Ci/mmol, NEN). The labeling media were removed and the cells were chased for 2 and 4 hours in normal growth media. The cell culture supernatants were then collected, clarified by centrifugation (13,800 xg/1 min), and adjusted to 1X lysis buffer (320). Cells were lysed with 1X lysis buffer containing protease inhibitors (Roche) and centrifuged to remove cellular debris. Viral proteins were immunoprecipitated (IP) with a pooled patient serum, separated by SDS-PAGE, and visualized by fluorography. For co-immunoprecipitation experiments, transfected HeLa cells were lysed with GTN buffer containing 0.1% BSA (376). Cell lysates were centrifuged to remove cellular debris, Ubc9-Gag complexes were immunoprecipitated with anti-Ubc9 antibodies that recognizes both endogenous Ubc9 and Myc-Ubc9, and the complexes were detected by immunoblotting using anti-p24 sera.

Quantitation of gp160 processing efficiency. Autoradiograms were scanned using a Fluor-S™ system. The accompanying Discovery Series Quantity One™ software (BioRad) was used to quantitate the intensities of the bands corresponding to gp160 and gp120 from the scanned images. The molar equivalence of each protein was calculated by dividing the band intensity by the number of methionines for each protein (gp160 and gp120 contain 14 and 9 methionines, respectively). To calculate the percent of gp160 cleaved into gp120 in Env-only expressing cells, the molar equivalence of gp120 was divided by the total molar equivalence of Env (gp120 and gp160).

Results

HIV-1 Gag interacts with hUbc9. While we have shown that Ubc9 interacts with the full-length Gag protein of M-PMV, others have not detected an interaction with the full-length Gag proteins from other retroviruses. However, a Gag cleavage product, p6, was identified as a Ubc9 binding partner and target for SUMOylation (137). Furthermore, recent work shows that HIV-1 GagGFP is targeted to a vesicle containing Ubc9 and SUMO-1 early in the assembly pathway, suggesting that Ubc9 may play a role during the assembly process (241). To test if an interaction between Ubc9 and HIV-1 Pr55 could be detected *in vitro*, Pr55 was translated *in vitro* in the presence of [^{35}S] methionine. GST-tagged-Ubc9 was expressed in *E. coli* and purified using glutathione-agarose beads. The Gst-Ubc9 bound beads were mixed with *in vitro* translated Pr55. Bound proteins were eluted and examined by SDS-PAGE and fluorography (Fig. 1a). While GST control beads showed no detectable binding to Pr55, the GST-Ubc9 beads efficiently bound Pr55 under conditions previously used to detect Ubc9-M-PMV Gag interactions. The 35 kDa band that was pulled down with Gst-Ubc9 is a peptide produced during the *in vitro*

transcription reaction from the use of an internal ATG as the translation start site. This peptide could contain the Gag/Ubc9 binding site. Gst-Ubc9 pull-down assays using a panel of Gag deletion mutants (Fig. 1b) showed that the Ubc9 binding site on Pr55 is located within the carboxy-terminal NC-p2-p6 region, since only clones pGPG1-39G1 and pGPG1-15G1 retained the ability to be pulled down by Gst-Ubc9 (Fig. 1c). These results were consistent with previous observations that the p6 domain of Pr55 contains a Ubc9 binding site (137) and demonstrated that the MA-CA-p1 regions of Pr55 do not contain any additional major Ubc9 binding sites.

Co-immunoprecipitations confirmed Ubc9 and Gag interact *in vivo* (Fig. 2a). pNL4-3 or Pr55 was expressed in HeLa cells alone or with Myc-tagged Ubc9. At 48 hrs post-transfection, cells were lysed using the same buffer as used in the GST pulldowns and incubated with either polyclonal anti-Ubc9 antibodies or with preimmune rabbit sera. Immunoblots using anti-p24 sera indicated that while the control preimmune sera did not precipitate Pr55, anti-Ubc9 antibodies co-immunoprecipitated Pr55. The overexpression of Myc-tagged Ubc9 only increased the amount of Pr55 pulled down by about 30%. The small increase in the amount of Gag pulled down when Ubc9 was overexpressed was not unexpected as Ubc9 interactions with target proteins were shown to be transient and weak (215).

To further substantiate Ubc9-Pr55 interactions in cells, immunofluorescence assays were utilized to examine Ubc9 and Gag subcellular localization. 293T cells were transfected with either Myc-Ubc9 expression plasmid alone (Fig 2b-d) or co-transfected with pNL4-3 and Myc-Ubc9 (Fig. 2e-j). Cells were processed 48 hrs post transfection and examined by confocal microscopy. Myc-Ubc9 staining occurred in the nucleus,

throughout the cytoplasm, in areas near the plasma membrane [Fig. 2b-d, e, and h (white arrows)], and as distinct foci juxtaposed to the nuclear membrane [Fig. 2b-d, e and h (yellow arrows)]. In cells co-transfected with Myc-Ubc9 and pNL4-3 (Fig. 2e-j), HIV-1 Gag stained the cytoplasm, plasma membrane, and at perinuclear foci (Fig. 2f). HIV-1 Gag and Ubc9 co-localized in HIV-1 expressing cells (Fig. 2g) in a similar pattern that was observed with M-PMV Gag and Ubc9 in infected cells (376) and is similar to what was observed recently for HIV-1 GagGFP (241). Gag was also observed to co-localize with Ubc9 in the cytoplasm near the plasma membrane and at perinuclear foci (Fig. 2g). In an effort to identify the Gag-Ubc9 co-localization site and determine whether Ubc9 and Env also co-localize, we stained for both Env and Ubc9. Env and Ubc9 did not seem to co-localize (Fig. 2h-j) and certainly not at the same punctate perinuclear site observed between Gag and Ubc9 (yellow and white arrows). Furthermore, these results suggest that the Ubc9/Gag foci observed in this study are not in the ER, golgi, or post-golgi vesicles, all cellular organelles that Env is known to occupy. These results are similar to what Martinez *et al.* reported, where they describe a similar subcellular site containing Gag, Ubc9, and SUMO-1 (44),

Ubc9 is important for the production of infectious HIV-1 particles. Having determined that Ubc9 interacts with the full-length HIV-1 Gag protein, we sought to further elucidate the role of Ubc9 in the production of replication competent HIV-1 particles by ascertaining whether infectious virions could be produced in the absence of Ubc9 expression. To achieve this, 293T cells were transfected twice with either a non-silencing control RNA (Ctr. RNA) or a Ubc9-specific silencing RNA (Ubc9 RNAi) at 24 hr intervals followed by transfection with wt HIV-1 proviral DNA (pNL4-3) after an

additional 24 hrs. The effects of Ubc9 RNAi transfection on Ubc9 expression, cell viability, virus production, and viral infectivity were assayed at 72 hrs post-initial RNA transfection. As shown in figure 3a, Ubc9 expression was reduced to almost undetectable levels in cells transfected with Ubc9 RNAi as compared to cells transfected with the Ctr. RNA. The levels of controls, p53 and Actin, remained constant in all samples, demonstrating the specificity of Ubc9 silencing. Because Ubc9 is required to sustain cellular replication (142), we examined cell viabilities at 72 h post-transfection when media containing virions were collected. Flow cytometry quantifying propidium iodide incorporation, Annexen V staining, and trypan blue exclusion (data not shown) assays demonstrated that greater than 97% of control RNA and Ubc9 RNAi transfected cells were viable when the virions were harvested (Fig. 3b).

To examine whether Ubc9 is important during virus assembly and release, cell-free virions released into culture supernatants from transfected cells were pelleted, lysed, and analyzed by Western blots. We found that Ubc9 expression is not required for virus assembly, budding, or Pr55 processing since equivalent amounts of p24 were released from cells as pelletable virus particles in the presence or absence of Ubc9 expression (Fig. 3c). These virions also contained equivalent amounts of reverse transcriptase (data not shown). The effect of Ubc9 depletion on virion infectivity was analyzed by using equivalent amounts of virions (normalized to p24 content) released from these cells in single-round infectivity assays using TZM-bl reporter cells to quantify the number of infectious events relative to virions produced in the presence of Ubc9. Virions released from cells that did not express detectable levels of Ubc9 were on average 10-fold less infectious than those virions released from cells expressing normal levels of Ubc9 (Fig.

3d). The average viral titer for cells transfected with no RNA, Ctr. RNA, and Ubc9 RNAi were 3750, 4083, and 367, respectively. These results are the first to demonstrate that while Ubc9 has no effect on Gag synthesis, processing, or assembly into virions, its expression in the producer cells is important for the generation of infectious HIV-1 virions.

Ubc9 is important for the correct production and packaging of viral components. We initially anticipated that Ubc9 must be packaged into HIV-1 virions to achieve full infectivity. However, this scenario appears unlikely since we and others (63) were unable to detect Ubc9 or SUMO-1 in HIV-1 particles released from cells even when myc-Ubc9 was overexpressed in the producer cells (data not shown). Our results (Fig 3c) demonstrated that the interaction between Gag and Ubc9 is not required for Gag release and processing, but is important for infectivity (Fig 3d). Therefore, Ubc9 must influence Gag-mediated biosynthesis, the packaging of other viral components, or the inclusion of cell-derived viral inhibitors. The deficiency in assembly of released virions was identified by comparing the composition of replication defective virions harvested from Ubc9 knock-down cells to fully-infectious HIV-1 from normal and control RNA transfected cells.

As shown, Ubc9 expression was not required for packaging of the vRNA (Fig. 4a), the HIV-1 accessory proteins Vpr, Vpu, and Vif, or the cellular protein Cyclophilin A (Fig.4b). In contrast, virions produced in the absence of Ubc9 expression contained significantly lower amounts of Env glycoproteins (gp120, and gp41) than virions produced from cells transfected with pNL4-3 alone or with pNL4-3 and control RNA (Fig. 4c). Env production was assayed in these producer cells and it was found that cells

transfected with pNL4-3 and Ubc9 RNAi contained similar amounts of the Env precursor protein gp160, but exhibited a decrease in gp120 and gp41 levels when compared to those that were transfected with pNL4-3 alone or with pNL4-3 and Ctr. RNA (Fig. 4d).

The changes observed in gp120 expression and packaging were unexpected and suggested that the decrease in virion infection may be due to the inability of the virions to attach and enter a nascent cell; however, the SUMOylation pathway has also been implicated during early infectious events of HIV-1 (137) and MLV (400). Gurer *et al*. had previously shown that overexpression of SUMO-1 in producer cells leads to a decrease in HIV-1 virion infectivity and was attributed to a defect during an early step of reverse transcription of the viral genome. Similarly, Yueh *et al*. reported that point mutations of lysines (K202 and K220) that block the SUMOylation of the capsid protein of MLV also resulted in a defect at an early infectious event, albeit at a post reverse transcription step.

To examine the possibility that the decrease in virion infectivity could be attributed to defects that occurred during post-entry events, we assayed the ability of VSV-G pseudotyped HIV-1 cores to complete early infection events and nascent viral gene expression. These experiments allowed us to examine early events independently from any potential defects in entry caused by the decreased Env packaging in the absence of Ubc9 expression. Virion infectivity was carried out as before using TZM-bl indicator cells. As shown in figure 4e, the decreased expression of Ubc9 by RNAi had no affect on the infectivity of the pseudotyped HIV-1 cores. This data indicated the defect observed with virions produced in the absence of Ubc9 expression is not due to a defect during

early post-entry events, but is likely the result of the virions' inability to enter a susceptible cell as a result of the decreased amount of Env packaged.

Ubc9 contributes to viral glycoprotein stability in the presence of Gag expression. Immunoblot data that examined viral proteins at steady state levels showed a significant decrease in the amount of gp120/gp41 in the transfected cells and in the amount of Env packaged into the virions. The decrease in gp120 in the producer cells suggested that this could be due to a defect in gp160 maturation, a decrease in stability of gp120, or a fewer number of mature virions being endocytosed in the absence of Ubc9 expression. A defect in gp160 maturation was less likely, as immunoblot data (Fig. 4d) did not show the increase in gp160 expression levels at steady state, which was expected if gp160 was not undergoing cleavage during maturation. Therefore, pulse chase experiments were carried out to further examine these possibilities and to access Env stability in the absence of Ubc9 expression. Equivalent amounts of Env and Gag proteins were synthesized in all samples (Fig. 5a). However, a 90% reduction of Ubc9 expression resulted in an approximately 2-fold decrease in the amount of cell-associated gp120 (Fig. 5a and c). This reduction in cell-associated gp120 was not due to a defect in gp160 processing or an increased release of gp120 into the media (Fig. 5a and b). When pulse-labeled Gag proteins were chased, the levels of cell-associated Pr55 decreased as virus-associated p24 increased at the same rates regardless of the Ubc9 expression levels. The amount of cell associated p25/p24 did not change relative to the control cells, indicating that the decrease in cell-associated gp120 is not due to decreased endocytosis of mature virions.

The amount of gp120 packaged into particles released from Ubc9 RNAi treated cells was also approximately 2-fold less than the particles released from control cells.

This is consistent with what was observed in the amounts of cell-associated gp120. The small amount of gp120 that was detected in Ubc9 RNAi transfected cells and released into the media was probably from cells that were not transfected with the Ubc9 RNAi. These data suggest that Ubc9 plays an important role on gp120 levels in the virus producing cells, either directly or indirectly, resulting in decreased packaging of gp120 into the released virions.

Ubc9 has been reported to interact with a variety of cellular proteins affecting subcellular location, function, and stability (250). Some of these proteins share similar biosynthetic pathways as HIV-1 Env and are found in organelles known to be utilized by Env during the late virion assembly pathway (75, 325, 389). Because Ubc9 has been shown to act directly upon cellular glycoproteins utilizing the secretory pathway, pulse chase experiments that assayed Env stability were done in the absence of Gag expression to rule out the possibility that Ubc9 was directly acting upon Env. This was carried out by using a pNL4-3 that was modified to express Env glycoproteins in the absence of Pr55 by deleting the *gag* and *pol* genes. 293T cells were transfected with this construct (LTR Env_{NL4-3}) either alone or with the Ctr. RNA or Ubc9 siRNA. Env processing kinetics and stability were examined by pulse-chase experiments at 24 hrs post transfection. Surprisingly, in the absence of gag, pretreatment of cells with Ubc9 RNAi had no effect on the amount of gp160 that was synthesized and processed or on the kinetics and efficiency of processing gp160 into gp120 and gp41 (Fig. 6). We concluded from these experiments that Ubc9, in the absence of Gag, does not play a significant role either in gp160 cleavage, trafficking through the secretory pathway to the TGN, or in gp120 stability. Together these results demonstrate that Ubc9 functions during the virus

assembly process, which is required for full infectivity, and suggest that Ubc9 is involved in the processing and packaging of mature Env glycoproteins.

Ubc9 functions independently of its E2 SUMO conjugase activity. Ubc9 is the only E2 SUMO-conjugating enzyme identified (124) and many of the Ubc9 interacting partners are substrates for SUMOylation. However, there is an increasing number of reports which demonstrate that Ubc9 also functions in a SUMO conjugase independent manner (11, 55, 181, 199, 282, 318, 357). To determine whether Ubc9 SUMO conjugase activity is involved or required for its interaction with Gag and if it affects viral infectivity, we tested the effect of the overexpression of a trans-dominant negative mutant, C93A, that has no E2 SUMO conjugase activity (354). 293T cells were co-transfected with equal amounts of EGFP-SUMO-1 and pCMV-Tag3A (empty vector), EGFP-SUMO-1 and Myc-C93A, or with pCMV-Tag3A alone to confirm that Myc-C93A acts as a trans-dominant negative. Endogenous SUMO-1 is the limiting factor for SUMOylation (87), thus overexpression of exogenous EGFP-SUMO-1 proteins greatly increases the amount of SUMOylated proteins detected by immunoblot. A 4-fold overexpression of Myc-C93A over endogenous Ubc9 lead to approximately a 70% decrease in the global amount of EGFP-SUMOylated proteins. Quantitation of the 80 kDa band demonstrated a 90% decrease when compared to the control (Fig. 7a). This result suggests that overexpression of Myc-C93A can inhibit SUMOylation effectively.

To examine if the SUMOylation activity of Ubc9 is important for HIV-1 infectivity, 293T cells were co-transfected with pNL4-3 and either pCMV-Tag3A (Fig. 7b, set 1), Myc-Ubc9 (Fig. 7b, set 2), or Myc-C93A (Fig. 7b, set 3). As expected, immunoblot analysis of whole cell lysates and virions pelleted from the medium of

transfected cells shows that the overexpression of either Ubc9 or C93A had no effect on virion budding, maturation, or release (Fig. 7b). We then further examined whether the virions produced in the presence of C93A mutant displayed decreased infectivity. As in previous experiments, the released virions were normalized for p24 content and equal amounts of virions were used to infect TZM-bl indicator cells. In contrast to the loss of virion infectivity observed in the absence of Ubc9, overexpression of C93A and inhibition of SUMOylation in the producer cells had little effect on virion infectivity when compared to control samples (Fig. 7c). In addition, Gag trafficked normally to perinuclear sites and co-localized with C93A Ubc9 (yellow and white arrows)(Fig. 7d). Collectively, these results suggest that Ubc9 may function independently of its E2 SUMO conjugating activity during the late stages of the HIV-1 life cycle.

Discussion

In this study we have shown that Ubc9 interacts with the full-length HIV-1 Gag protein and have demonstrated for the first time that Ubc9 plays a role during the late stages of virus replication and its expression in HIV-1 producing cells is important for conferring full infectivity to the nascent virus stock. Our results indicate that Ubc9 influences the stability and incorporation of mature Env into budding particles and may function during the HIV-1 assembly process in a SUMO-independent manner. These conclusions are supported by the following: *i*) Ubc9-Gst fusion proteins can interact with *in vitro* translated Gag through its NC-p6 domain; *ii*) Gag-Ubc9 complexes can be co-immunoprecipitated from cell lysates; *iii*) Gag, Ubc9, and trans-dominant negative C93A co-localize to discrete locations in the cytoplasm; *iv*) overexpression of Ubc9 or C93A does not affect virion infection, *v*) Ubc9 silencing leads to impaired virion infectivity; *vi*)

Ubc9 silencing has no effect on Gag assembly, budding, and processing or on Gag-mediated packing of the viral RNAs, Vif, Vpr, Vpu, or the cellular protein Cyclophilin A; *vii*) Ubc9 is important for proper Env stability and incorporation of mature Env glycoproteins into virion particles when Env is expressed in the context of wt HIV-1 replication; and *viii*) Ubc9 and Env do not appear to co-localize at the distinct perinuclear foci that contain Ubc9 and Gag.

While most studies that have characterized Env processing and stability were carried out in Env-only expression systems, our data suggested that in the context of wt HIV-1, Gag and Ubc9 are important for Env stability and incorporation into nascent virions. It was unexpected that the knockdown of a Gag interacting partner would cause a change in Env stability and packaging, but it was even more so to find that this phenotype was directly tied to the presence or absence of Gag expression. While many have speculated that Env trimers interact with Gag at budding sites, biochemical and genetic evidence suggests that the initial Gag-Env interactions, whether direct or indirect, occur prior to their arrival at the assembly sites (86, 221, 222, 224, 280).

The mechanism of how Gag influences gp120 stability in the absence of Ubc9 has not been elucidated, but our data suggests the Gag/Ubc9 complex could interact/affect Env after it has passed through the trans-golgi, possibly in secretory vesicles, plasma membrane, or recycling endosomes. Our data suggest that Ubc9 is not involved directly with Env processing, as gp160 processing and gp120 stability are normal in the absence of Gag and Ubc9 and Env and Ubc9 do not strongly co-localize at the same subcellular compartments. Although the majority of Ubc9/Gag co-localization is at perinuclear foci, which may be the Ubc9/SUMO-1 positive cellular structures where GPCs transiently

traffic through during an early stage of assembly (44), co-localization was observed in areas adjacent to plasma membrane with both Ubc9 and C93A as well. It is possible that this area is a functional subcellular site of interaction between Ubc9 and Gag. The close proximity of this co-localization site to the plasma membrane and the physical distance from the distinct perinuclear foci containing Gag and Ubc9, suggests that Ubc9 may also function at a later stage of assembly. A stage where Gag has already trafficked through assembly intermediates and has been targeted to and is in close proximity to the plasma membrane, possibly at virus assembly sites.

The trafficking of Gag to the sites of Ubc9 colocalization is most likely very transient as overexpression of Myc-Ubc9 greatly increases the amount of Gag localization to perinuclear sites containing Ubc9, but does not lead to a change in the amount of Gag released into the media as virions. If Gag trafficking to sites of Ubc9 colocalization were not transient or Gag trafficking to assembly sites slowed, we would have expected a difference between the amount of virions released from cells when Ubc9 or C93A were overexpressed. However, this phenotype was not observed suggesting very transient Gag trafficking through the sites of Ubc9 colocalization. The full extent of how Ubc9 functions during the HIV-1 life cycle is still not well understood and it may be involved with various steps during assembly.

Ubc9 is an E2 SUMO conjugase that is part of the cytoplasmic portion of the nuclear pore complex (402). It appears to localize to distinct, yet undefined perinuclear regions, the secretory pathway, and is also free in the cytoplasm (241, 325, 376, 389). Ubc9, through its SUMOylation activity, is involved in the targeting of transcription factors, cellular receptors, and viral proteins (reviewed in 124, 146, 250)). However,

there are a number of reports in the literature demonstrating Ubc9 functions in a SUMO independent manner. Androgen receptor (55), MEKK1, type I TNF-alpha receptor (318), GLUT4 (220), RNA helicase A (11), glucocorticoid receptors (181), Vsx-1 (199), SOX4 (282), and HHV-6 IE2 (357) require Ubc9 binding for their function and correct intracellular targeting, but they are not targets for SUMO-1 modification.

Our experiments with the dominant negative C93A support a model where Ubc9 functions as an essential binding partner of Gag in the late HIV-1 life cycle, but may do so independently of its SUMOylation conjugation function. If SUMOylation was important during the late stages of the HIV-1 life cycle, we would have expected to see a difference in virion infectivity with the trans-dominant C93A mutant as compared to virions produced in control cells, yet little difference was observed. This model is supported by data reported by Gurer *et al*. where the mutant HIV-1_{K27R} displayed no defects in virion assembly, even though it contained a mutated version of an *in vivo* SUMOylation site within p6. We have attempted to detect SUMOylated Gag or other viral proteins in both cell lysates and released virions under steady state levels and when Ubc9 and SUMO-1 have been overexpressed, but have been unsuccessful. It is interesting to note that p6 (K27R) showed diminished Ubc9 binding in yeast (137), but the mutation did not abolish its interaction with Ubc9 completely. Because of this, we carried out GST-Ubc9 pull down experiments with K27R in the context of Pr55 and found that p6 (K27R) can still interact with Ubc9 *in vitro* (data not shown). This result suggests that there is more than one Ubc9 binding site within Gag or mutation of lysine 27 in p6 is not enough to completely abolish Ubc9 binding.

In this study we mainly focused on the effects of Ubc9 on the late events of the viral life cycle in producer cells. Even though Ubc9 is not packaged into virions under steady state levels or when Ubc9 is overexpressed (data not shown), it has been reported that SUMO-1 plays a role during the early infectious events of HIV-1 (137, 400) and may regulate various steps within the retrovirus life cycle. To further understand mechanisms of how Ubc9 is functioning in the HIV-1 life cycle, studies following Gag and Env trafficking in the absence of Ubc9 are currently underway. We are also investigating whether Ubc9 directly plays a role during the early infectious events of HIV-1 in target cells after entry. Our data shows that Ubc9 plays an important role during HIV-1 replication and we have identified another protein/pathway that could potentially be developed into novel anti-retrovirals.

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Figures and Figure Legends

Figure 1

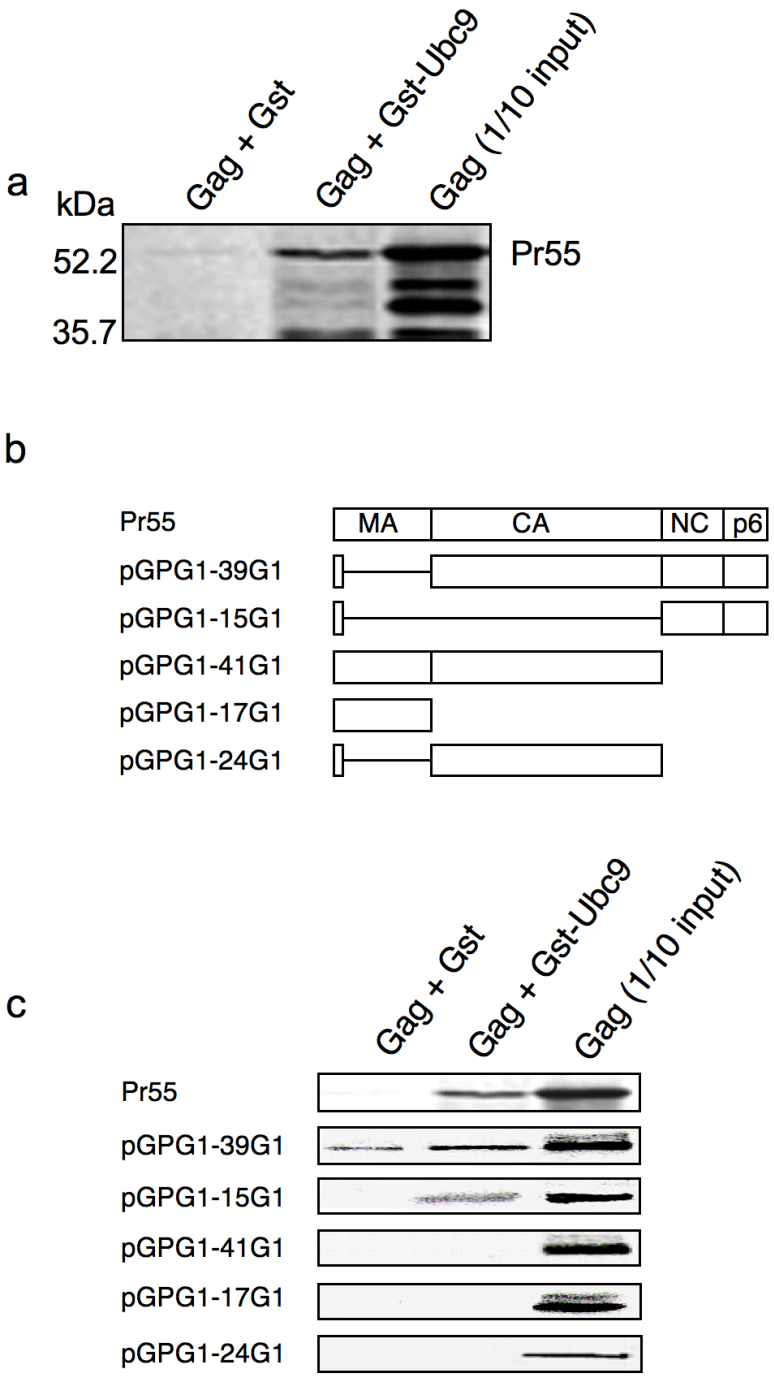


Figure 1: HIV-1 Gag proteins interact with Ubc9 *in vitro*. (a) GST-Ubc9 pull-down assay. [^{35}S] Methionine-labeled, *in vitro* translated Pr55 was incubated in GTN buffer with GST-bound glutathione beads or GST–Ubc9 bound beads. As a loading control, 1/10 of the amount of Pr55 added to the GST pull down reactions was loaded directly. Bound proteins were eluted with PSB and separated by SDS–PAGE. The radio-labeled proteins were visualized by phosphor-imaging. (b) Mapping the Ubc9 binding domain within HIV-1 Gag. (c) GST-Ubc9 pull-down assays using wt Gag and the Gag deletion mutants were done as described above.

Figure 2

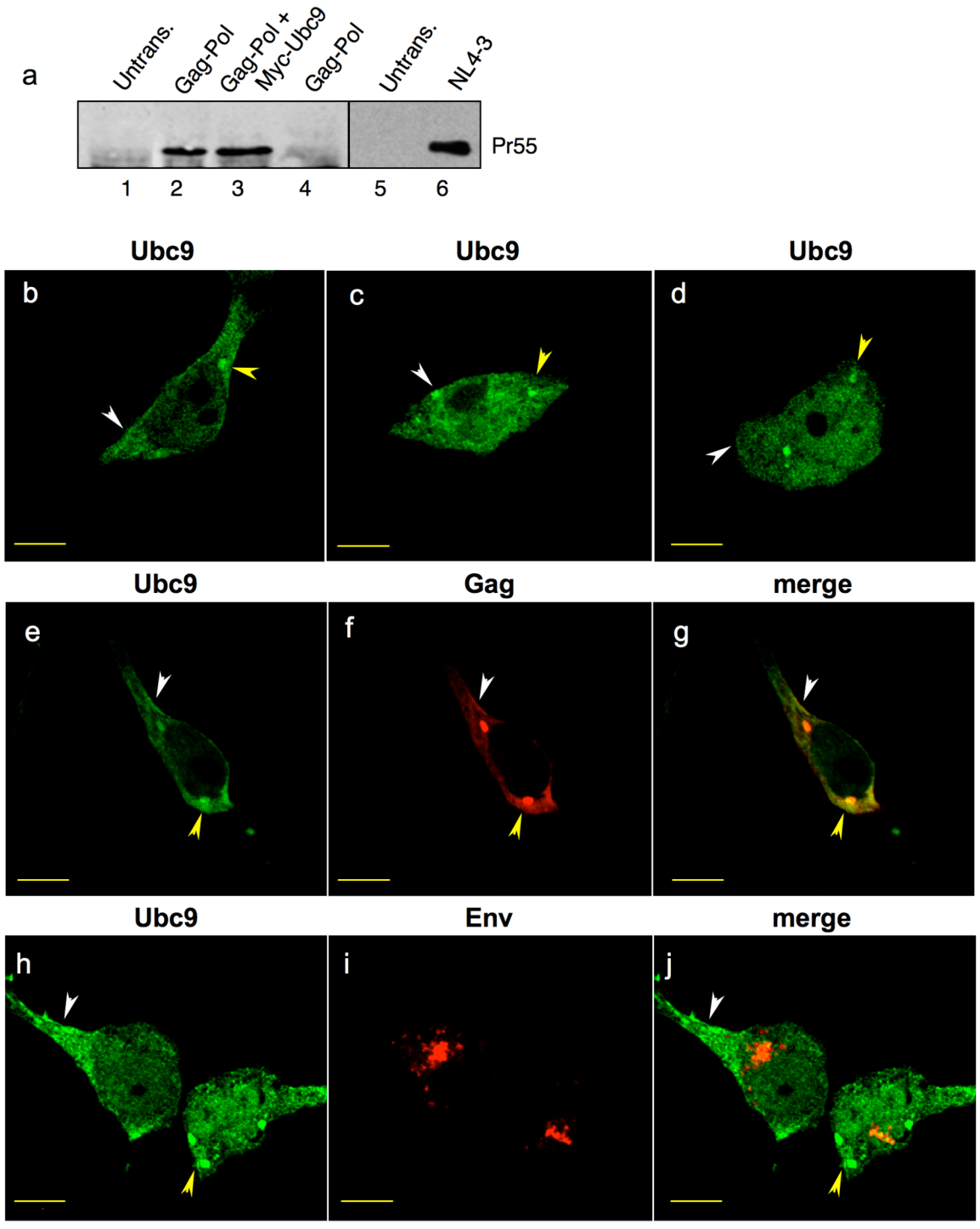

a
Untrans.
Gag-Pol
Gag-Pol + Myc-Ubc9
Gag-Pol
Untrans.
NL4-3
Pr55
1 2 3 4 5 6
Ubc9
Ubc9
Ubc9
b
c
d
Ubc9
Gag
merge
e
f
g
Ubc9
Env
merge
h
i
j

Figure 2: HIV-1 Gag proteins interact with Ubc9 *in vivo*. (a) Co-immunoprecipitation of endogenous Ubc9 and HIV-1 Gag. HeLa cells were either mock transfected (lane 1 and 5); co-transfected with pCMVGAGPOL-RRE-R and pCMVrev (lane 2 and lane 4); co-transfected with pCMVGAGPOL-RRE-R, pCMVrev and pCMV Myc-Ubc9 (lane 3); or transfected with pNL4-3 (lane 6). Cells were lysed with GTN buffer and immunoprecipitated (IP) with anti-Ubc9 (lane 1, 2, 3, 5 and 6) or preimmune sera (lane 4), and analyzed by immunoblotting using anti-p24 antibodies. (b-g) Gag and Ubc9 co-localize at distinct perinuclear puncta and areas near the plasma membrane. 293T cells were transfected with Myc-Ubc9 (b-d) or co-transfected with pNL4-3 and Myc-Ubc9 expression plasmid (e-j). Cells were fixed and processed for immunofluorescence with (b-d) anti-Ubc9 pAb, (e-g) anti-p24 mAb and anti-Ubc9 pAb (h-j), anti-Ubc9 pAb and 10E9 anti-Env mAb. Cy5-conjugated donkey anti-mouse and Cy2-conjugated donkey anti-rabbit antibodies were used as secondary antibodies. Co-localization was examined by confocal microscopy using 0.3-um optical-sections. Representative medial sections are shown. Yellow arrows indicate Ubc9 and Gag co-localization at perinuclear puncta. White arrows indicate areas of Ubc9 and Gag co-localization near the plasma membrane. Scale bar represents 10 μm.

Figure 3

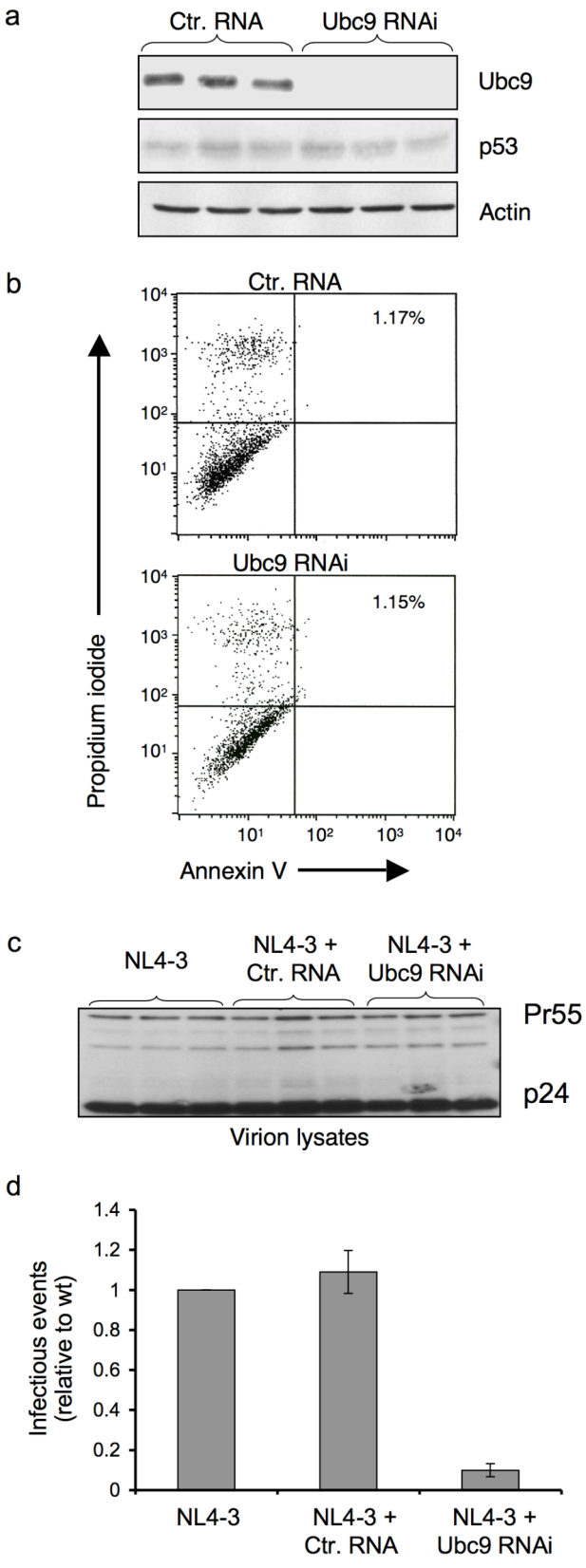

a
Ctr. RNA
Ubc9 RNAi
Ubc9
p53
Actin
b
Ctr. RNA
1.17%
Ubc9 RNAi
1.15%
Propidium iodide
Annexin V
c
NL4-3
NL4-3 + Ctr. RNA
NL4-3 + Ubc9 RNAi
Pr55
p24
Virion lysates
d
Infectious events (relative to wt)
NL4-3
NL4-3 + Ctr. RNA
NL4-3 + Ubc9 RNAi

Figure 3: Ubc9 is important for the production of infectious HIV-1 virions. (a) Ubc9 expression can be suppressed by RNA silencing. 293T cells were transfected with either control RNA (Ctr. RNA) or Ubc9 siRNA. A second transfection with RNAs was performed 24 hrs later. Cells were lysed in PSB, separated by SDS PAGE, and protein expression levels were analyzed by immunoblotting using antibodies against Ubc9, Actin, and p53 at 72 hrs post-initial siRNA transfection. (b) Suppression of Ubc9 does not affect cell viability. Viability of Ctr. RNA and/or Ubc9 RNAi transfected 293T cells was analyzed using the BD ApoAlert Annexin V & Apo 2.7-PE kit following the manufacturer's suggested protocol. The percentages of cells undergoing apoptosis were measured by flow cytometry. (c) Ubc9 is not required for budding. 293T cells were either left untreated or transfected twice with Ubc9 siRNA or Ctr. RNA. Wild-type HIV-1 proviral DNA (pNL4-3) was transfected 24 hrs following the second siRNA transfection. Twenty-four hrs later (72 hrs post initial siRNA transfection), culture medium was collected and clarified. Virions in the culture medium were pelleted through 20% sucrose cushion and lysed with PSB. Equal volumes of the lysates were separated by SDS-PAGE, and p24 levels were measured by immunoblotting using anti-p24 antibodies. (d) Ubc9 in virus producing cell is required for HIV-1 infectivity. Equivalent amount of virus (normalized to pelletable p24) present in the medium from transfected 293T cells was used to infect TZM-BL indicator cells for 36 hrs. The numbers of infectious events were analyzed counting the number of cells that were positive for X-gal staining.

Figure 4

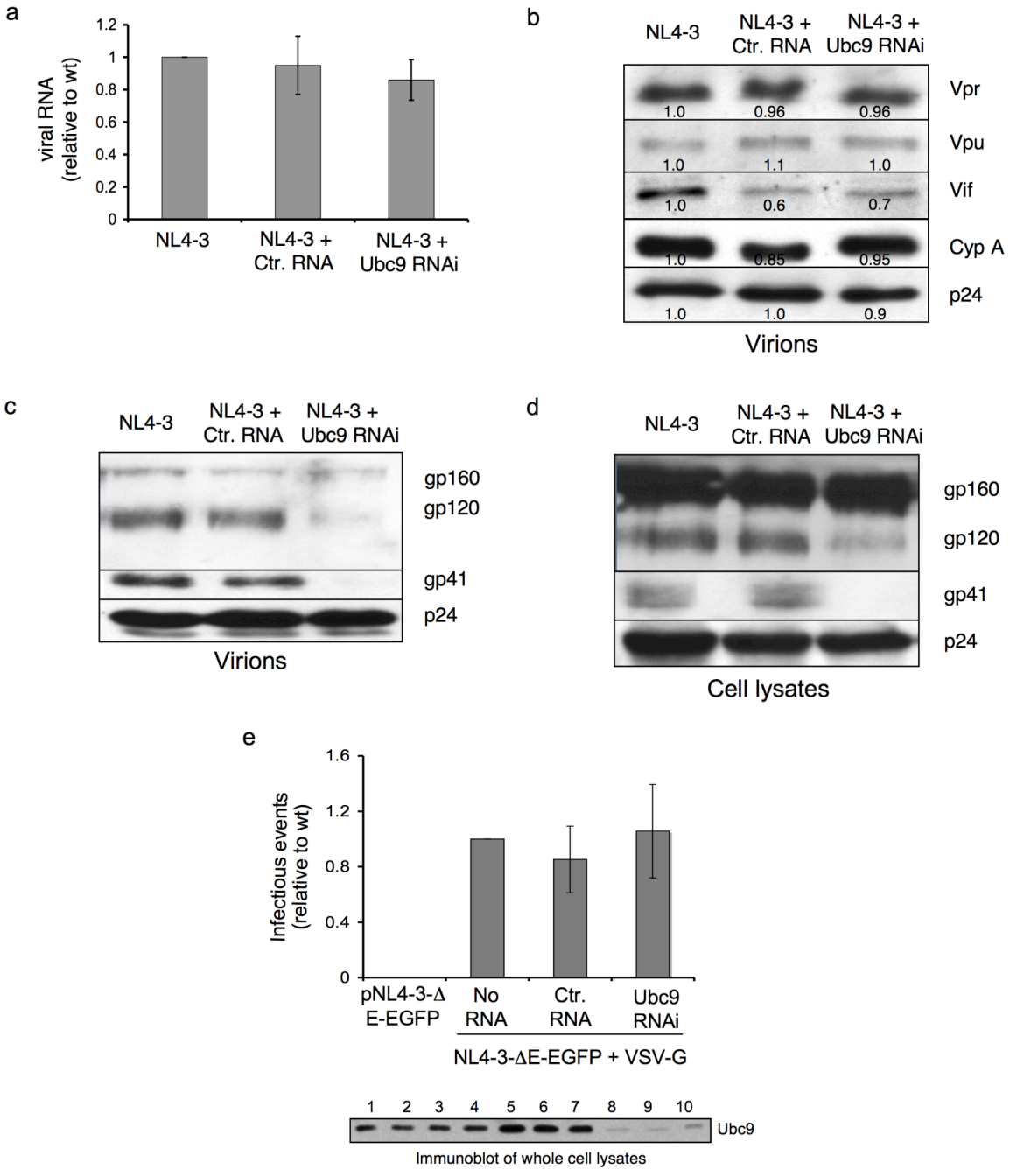

a
viral RNA (relative to wt)
1.2
1
0.8
0.6
0.4
0.2
0
NL4-3
NL4-3 + Ctr. RNA
NL4-3 + Ubc9 RNAi
b
NL4-3
NL4-3 + Ctr. RNA
NL4-3 + Ubc9 RNAi
Vpr
1.0
0.96
0.96
Vpu
1.0
1.1
1.0
Vif
1.0
0.6
0.7
Cyp A
1.0
0.85
0.95
p24
1.0
1.0
0.9
Virions
c
NL4-3
NL4-3 + Ctr. RNA
NL4-3 + Ubc9 RNAi
gp160
gp120
gp41
p24
Virions
d
NL4-3
NL4-3 + Ctr. RNA
NL4-3 + Ubc9 RNAi
gp160
gp120
gp41
p24
Cell lysates
e
Infectious events (relative to wt)
1.6
1.2
0.8
0.4
0
pNL4-3-Δ E-EGFP
No RNA
Ctr. RNA
Ubc9 RNAi
NL4-3-ΔE-EGFP + VSV-G
1 2 3 4 5 6 7 8 9 10
Ubc9
Immunoblot of whole cell lysates

Figure 4: Biochemical composition of viral particles. The relative levels of viral RNAs and proteins packaged into equivalent amounts of defective and infectious virions (normalized to p24 content) were analyzed. (a) Viral RNA Packaging. RNA from pelleted virions was extracted, reverse transcribed using oligo (dT)12-18, and quantified by real-time PCR using vRNA specific primers. Real-time PCR data from three independent experiments is shown as the amount of vRNA packaged into virions relative to that of vRNA packaged into virions produced from cells transfected with pNL4-3 only. (b) Packaging of cellular and HIV-1 regulatory proteins. Levels of cellular (Cyclophilin A) and viral regulatory proteins (Vif, Vpr, Vpu) packaged into virions were analyzed by immunoblotting. The bands were quantified using The Discovery Series Quantity One software. (c) Glycoprotein packaging into virions. Envelope packaging into virions was analyzed by immunoblotting using polyclonal anti-HIV-1 and monoclonal anti-gp41 antibodies. (d) Glycoprotein production in cell lysates. Envelope production inside transfected cells was analyzed by immunoblotting using polyclonal anti-HIV-1 antibodies. (e) Decreases in virion infectivity are not due to defects in the early post-entry events. Cells were transfected with RNAs as in previous experiments, followed by DNA transfections with pNL4-3-ΔE-EGFP and pHyg-VSV-G or pNL4-3 ΔEnv alone. Media containing pseudotyped virions were harvested and clarified 24 hrs post-DNA transfections. Virion infectivity was carried out as before using TZM-bl target cells.

Figure 5

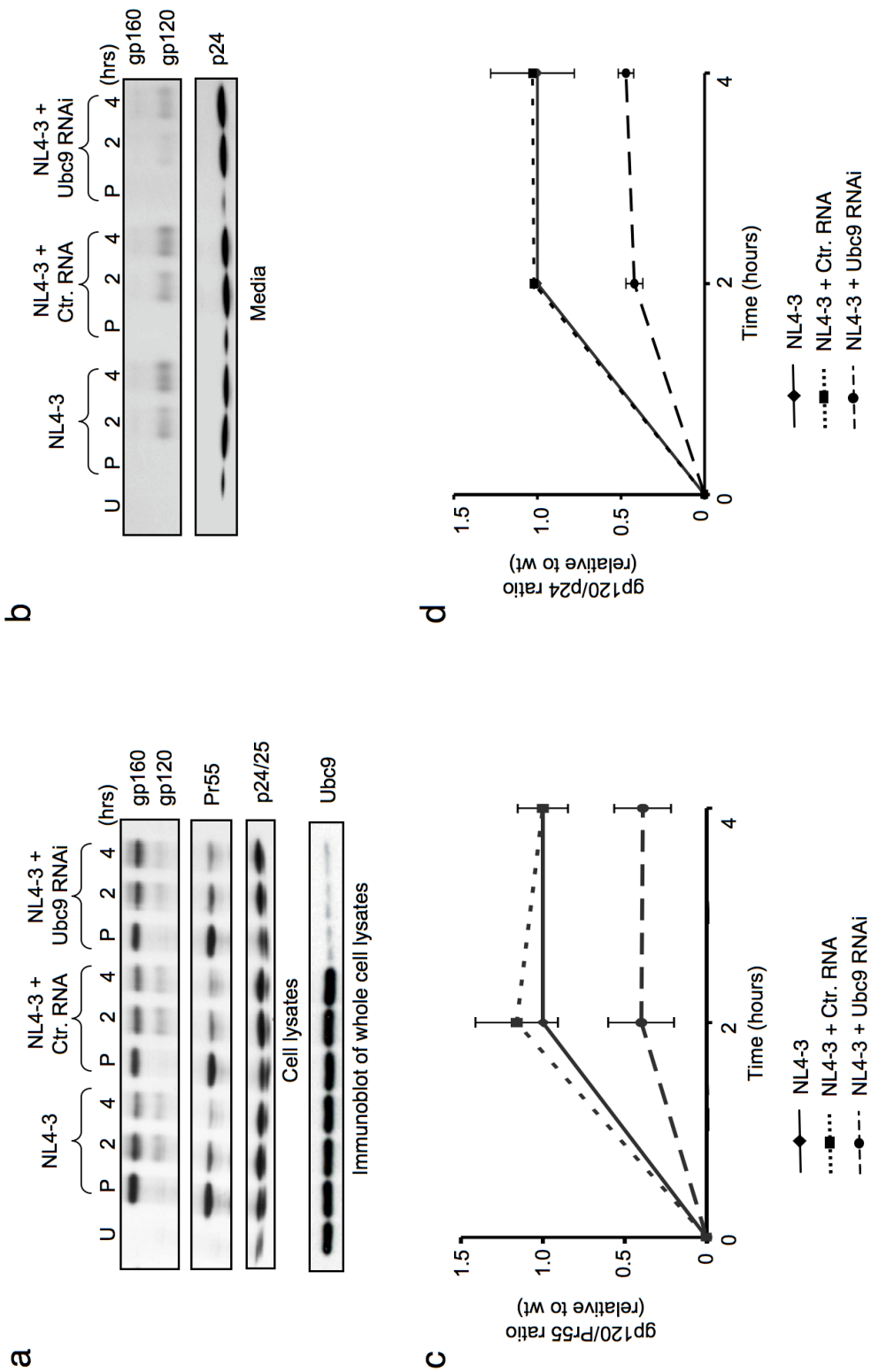
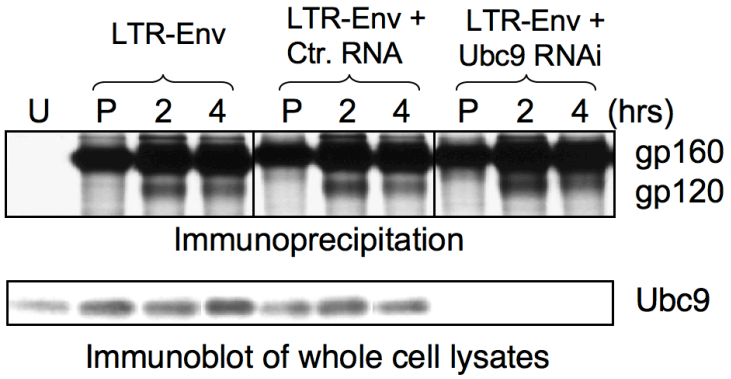

a
NL4-3
NL4-3 + Ctr. RNA
NL4-3 + Ubc9 RNAi
U P 2 4 P 2 4 P 2 4 (hrs)
gp160
gp120
Pr55
p24/25
Cell lysates
Ubc9
Immunoblot of whole cell lysates
b
NL4-3
NL4-3 + Ctr. RNA
NL4-3 + Ubc9 RNAi
U P 2 4 P 2 4 P 2 4 (hrs)
gp160
gp120
p24
Media
c
gp120/Pr55 ratio (relative to wt)
1.5
1.0
0.5
0
0
2
4
Time (hours)
NL4-3
NL4-3 + Ctr. RNA
NL4-3 + Ubc9 RNAi
d
gp120/p24 ratio (relative to wt)
1.5
1.0
0.5
0
0
2
4
Time (hours)
NL4-3
NL4-3 + Ctr. RNA
NL4-3 + Ubc9 RNAi

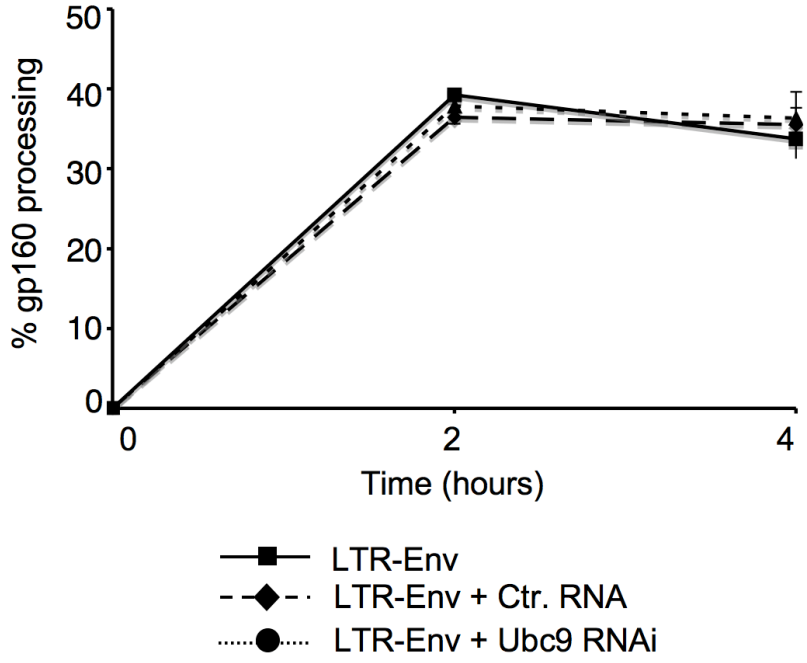
Figure 5: Gag and Env processing in the absence of Ubc9. (a and b) Viral protein assembly kinetics. 293T cells were transfected with NL4-3, NL4-3 + Ctr. RNA, NL4-3 + Ubc9 RNAi, or left untransfected (U). Cells were pulsed (P) with [^{35}S] methionine for 45 min and then chased for 2 and 4 hrs. HIV-1 proteins in the cell lysates (a) and the culture supernatants (b) were immunoprecipitated with pooled AIDS patient serum, separated by SDS-PAGE, and detected by fluorography. The viral protein bands from multiple pulse chase experiments were quantified and analyzed using The Discovery Series Quantity One software. (c) Ratio of cellular gp120/Pr55. (d) Ratio of gp120/p24 released into the media.

Figure 6

a



b



c

Gag	Ubc9	Env	Env processing
+	+	+	+
–	+	+	+
–	–	+	+
+	–	+	–

Figure 6: Env processing in the absence of Gag and Ubc9 expression. (a) Ubc9 does not directly affect Env processing. 293T cells were transfected with LTR-Env, LTR-Env + Ctr. RNA, LTR-Env + Ubc9 RNAi, or left untransfected (U). Cells were pulsed (P) with [^{35}S] methionine for 45 min and then chased for 2 and 4 hrs. HIV-1 proteins were immunoprecipitated from cell lysates with AIDS patient serum. (b) Immunoprecipitated gp160 and gp120 bands were quantitated using The Discovery Series Quantity One software and the % of gp160 processed was calculated at 2 and 4 hours. (c) Table summarizing proteins involved in Env processing defect.

Figure 7

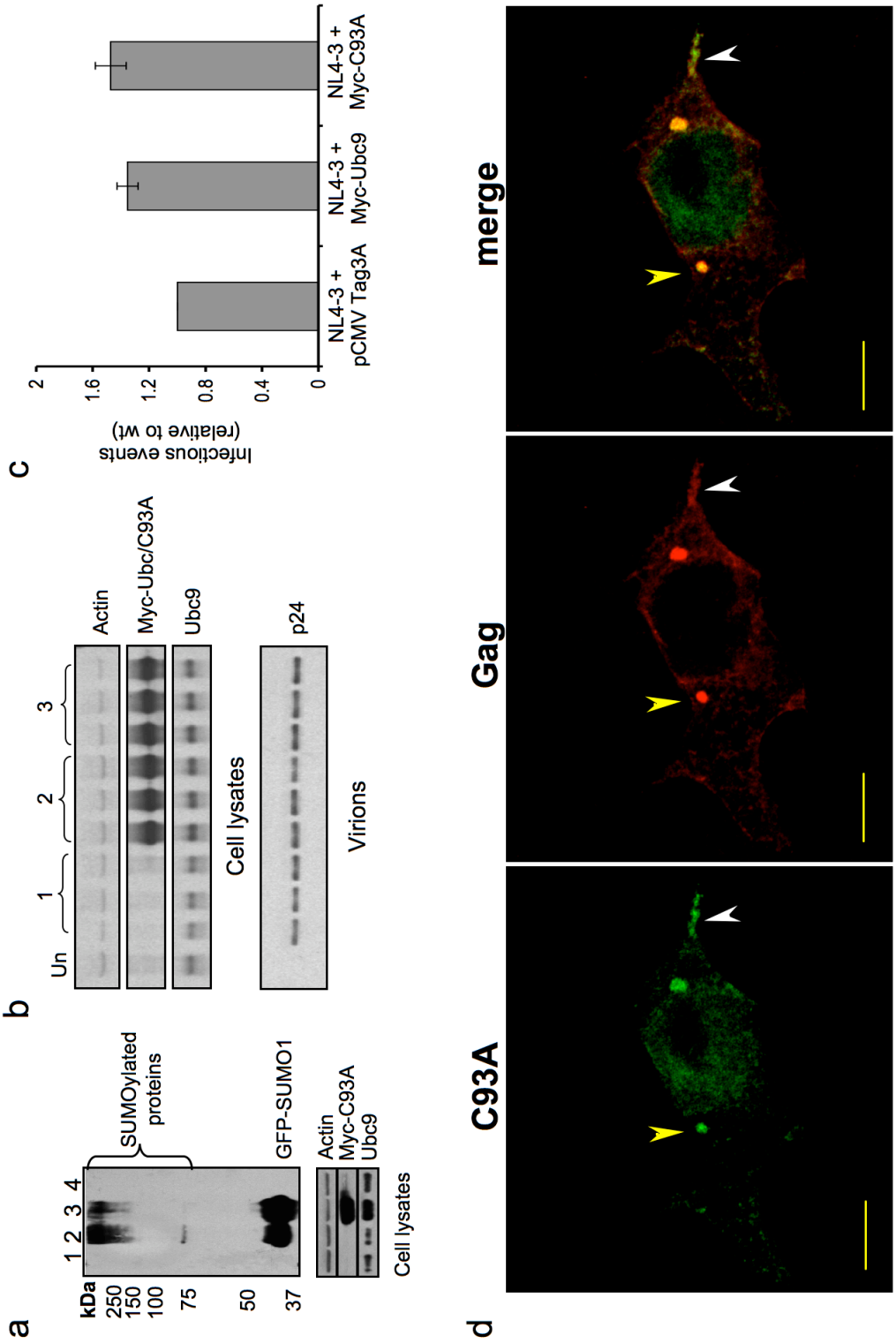

a
kDa
250
150
100
75
50
37
1 2 3 4
SUMOylated proteins
GFP-SUMO1
Actin
Myc-C93A
Ubc9
Cell lysates
b
Un
1
2
3
Actin
Myc-Ubc/C93A
Ubc9
Cell lysates
p24
Virions
c
Infectious events (relative to wt)
2
1.6
1.2
0.8
0.4
0
NL4-3 + pCMV Tag3A
NL4-3 + Myc-Ubc9
NL4-3 + Myc-C93A
d
C93A
Gag
merge

Figure 7: Ubc9 may function independently of its E2 SUMO conjugating activity during HIV-1 assembly. (a) Myc-C93A is a trans-dominant negative active site mutant and suppressed SUMOylation of cellular proteins. 293T cells were co-transfected with pEGFP-SUMO-1 and either pCMV-Tag3A (lane 2) or Myc-C93A (lane 3), with pCMV-Tag3A alone (lane 4), or left untransfected (lane 1). Cells were washed with PBS, lysed in SUMO lysis buffer, and boiled for 10 minutes. Lysates were assayed by immunoblotting using antibodies against SUMO-1 and Ubc9. (b) Overexpression of Myc-Ubc9 or Myc-C93A does not affect virion budding. 293T cells were co-transfected with pNL4-3 and either PCMV-Tag3A (sample set 1), Myc-Ubc9 (sample set 2), or Myc-C93A (sample set 3). Media was harvested and virions were pelleted through a 20% sucrose cushion. Virion budding and release was assayed by immunoblotting using antibodies against p24. Transfected cells were lysed directly in PSB and protein expression was analyzed by immunoblotting using antibodies against Ubc9 and Actin. (c) Overexpression of Myc-Ubc9 or Myc-C93A does not affect HIV-1 infectivity. Medium from transfected cells was harvested and equal amounts of virus were used to infect TZM-bl indicator cells as in previous experiments. (d) HIV-1 Gag and Myc-C93A does not affect Gag trafficking or co-localization with Ubc9 at perinuclear sites. Transfected 293T cells were fixed and processed for immunofluorescence analysis by confocal microscopy, using anti-Ubc9 pAb (1) and anti-p24 mAb (2). Cy5-conjugated donkey anti-mouse and Cy2-conjugated donkey anti-rabbit antibodies were used as secondary antibodies. Co-localization was analyzed using 0.3-um optical-sections. Representative medial sections are shown. Yellow arrows and white arrows indicate

areas of Ubc9 and Gag co-localization at perinuclear puncta and near the plasma membrane respectively. Scale bar represents 10 μm.

Chapter III

Human Ubc9 is involved in intracellular HIV-1 Env stability after trafficking out of the trans-Golgi network in a Gag dependent manner.

Unpublished data

Abstract

We have previously shown that Ubc9 is important for the production of fully infectious HIV-1 virions. We demonstrated that Ubc9 was a Gag interaction partner and Ubc9 expression was important for the stability of mature intracellular Env levels and packaging into the released virions. We have continued to characterize the defect in the intracellular mature Env levels in Ubc9 knockdown cells to further understand how Ubc9 and Gag contribute to intracellular Env stability and packaging into the assembled virions. We found that gp160 stability in the endoplasmic reticulum (ER) was unaffected and that gp160 trafficked to the trans-Golgi network (TGN) in Ubc9 knockdown cells normally. We examined the stability of a gp160 cleavage mutant (MUT 511) and found that it was unaffected in Ubc9 knockdown cells. These results combined with data that showed gp160 stability was not affected when cleavage and trafficking from the TGN was blocked, indicated that the decreased intracellular mature Env levels in Ubc9 knockdown cells were due to selective degradation of mature Env gp120 after it is trafficked out of the TGN and cleavage from gp160. Decreased levels of Gag and mature Env were found to be associated with the plasma membrane and lipid rafts, which suggest that mature Env was degraded before insertion into the plasma membrane and association with lipid rafts. We also demonstrated that the defect in virion infectivity due to the knockdown of Ubc9 expression was not isolate specific. Virions produced from Ubc9 knockdown cells transfected with the HIV-1 pSG3.1 proviral clone also exhibited decreased infectivity.

Introduction

Assembly of infectious HIV-1 virions requires a variety of viral and host factors that function in a coordinated manner to co-traffic the essential viral and cellular factors to the site of assembly on the plasma membrane (PM), and in some cases exclude the factors, such as tetherin and APOBEC3G from the assembling virions (64, 263). A large number of host factors have been identified to interact with viral proteins to direct their correct trafficking to the assembly site. These include KIF3A (15), KIF3C (41), KIF4 (242), POSH (8), SOCS1 (315), Rab9 and Rab11a (260), AP-1 (51, 271), AP-2 (19, 30), AP-3 (93), Tip47 (20, 29, 224), Staufen (56), GGA and ARF (174), SNARE proteins (173), ABCE1 (218), CD81 (135), phosphatidylinositol (4,5) bisphosphate (274), lipid rafts, (276), and Clathrin (298). Once correctly trafficked to the PM, the viral and cellular factors are retained at the assembly site as the nascent virion is assembled and released from the cell surface through a process called budding. The budding and release of progeny virions utilize the host vacuolar protein sorting pathway through direct interactions with TSG101 (122) or AIP1 (347). During or shortly after release, the immature virion core undergoes viral protease-dependent cleavage maturation where the Gag proteins are cleaved and the virions mature into their infectious forms (assembly reviewed in 71).

Gag (Pr55) is the predominant structural protein of HIV-1, which can drive the assembly of virus like particle in the absence of other viral proteins because the functional domains necessary for assembly are encoded within the Gag polyprotein precursor. Gag is subsequently cleaved into matrix (MA), capsid (CA), nucleocapsid (NC), p6, and two small spacer peptides (SP1 and SP2). The domains needed for proper

viral assembly include: the M-domain in MA, it is responsible for Gag targeting and binding to specific host cell membranes; the I-domain sequences within CA and NC which are involved in Gag multimerization, and the L-domain within p6 which directs budding and release of newly assembled virions. Gag is translated on free ribosomes in the cytoplasm. It then traffics to the site of assembly on the PM, multimerizes, and initiates the budding process as the immature capsid assembles. Gag also specifically binds and packages the viral genome through the zinc-finger motifs in NC and is responsible for packaging the viral protease and replication enzymes: Protease, Reverse Transcriptase, and Integrase. These enzymes are initially expressed as a Gag-Pol fusion protein and packaged through Gag-Gag interactions mediated by the I-domain of the Gag precursor Pr55 and Pr160. The replication enzymes are cleaved into the mature forms along with Pr55 during virion maturation events (Gag function reviewed in 113, 352, 387).

The Envelope glycoprotein is another essential viral structural protein that is packaged into the assembling virions, and is responsible for virion binding and entry into a target cell. Envelope is expressed as an immature precursor protein (gp160) within the lumen of the endoplasmic reticulum (ER). The gp160 is highly glycosylated, forms trimers within the ER, and is then trafficked to the Golgi complex. As gp160 traffics through the Golgi, the sugar moieties are extensively modified and gp160 undergoes proteolytic cleavage into gp120 and gp41. Cleavage of gp160 is believed to be mediated by Furin, a host encoded protease located in the trans-Golgi network (TGN). After cleavage, the mature fusion competent Env, a trimer of gp120 and gp41 heterodimers is then formed and traffics through the unregulated secretory pathway to the PM where it is

packaged into assembly virions. Alternatively, the Env in the PM can be endocytosed and recycled back the TGN (29, 99, 271). Currently, the mechanism leading to Env packaging is still not fully understood. However, there is evidence to suggest that under normal conditions, sequences within the cytoplasmic tail (CT) of Env interact with the MA domain within Gag, resulting in the specific packaging of the mature fusion competent Env (reviewed in 58, 167).

We have previously reported that the host factor Ubc9 interacts with Gag, and plays an important role in the HIV-1 assembly pathway (159). Ubc9 is an E2 SUMO conjugase which normally interacts with target proteins which are modified post-translationally by the addition of SUMO (SUMOylation reviewed in 124, 405). SUMOylation generally leads to altered protein function. However, a growing list of proteins were found to interact with Ubc9 without being targeted for SUMOylation, even though the physical interaction with Ubc9 altered the function of these interacting proteins (11, 54, 136, 199, 220, 282, 318, 357). Ubc9 and SUMOylation have been shown to play important roles in the replication cycles of both DNA and RNA viruses, and some of these viruses deregulate the host SUMOylation pathway to enhance their replication (31, 32, 388). We previously reported that Ubc9 is a HIV-1 Gag interaction partner and when the endogenous Ubc9 expression was knocked down in HIV-1 producer cells, it resulted in the production of virions with decreased infectivity. The defect in infectivity was found to be due to decreased levels of mature intracellular Env which resulted in a reduced amount of Env being packaged into the released virions. Interestingly, the decreased intracellular Env levels in Ubc9 knockdown cells were dependent upon Gag expression, as intracellular Env levels were not affected in the

absence of Gag and Ubc9 expression. Experiments with the trans-dominant negative Ubc9 (C93A), that lacks SUMO conjugase activity, suggested that the enzymatic activity of Ubc9 did not play a role in Gag dependent reduction in intracellular Env levels in Ubc9 knockdown cells. However, Zamborlini et al. recently demonstrated that Ubc9 may be important during HIV-1 infection as they have found that HIV Integrase is SUMOylated at a low level by SUMO-2. They were also able to detect a small amount of SUMOylated Gag-Pol cleavage products in cells over expressing SUMO-2, indicating that Gag-Pol may be SUMOylated in producer cells, however, SUMOylated viral proteins were undetectable in the virion (401).

In this study, we continued to examine the role of Ubc9 during HIV-1 assembly in the context of a fully infectious provirus, specifically determining how the knockdown of a Gag interacting partner can lead to the decrease in mature intracellular Env, resulting in less infectious virions. We also examined which step(s) in the assembly pathway might be affected by a knockdown of Ubc9 thus leading to a reduction in Env. We report here that in Ubc9 knockdown cells, the decreased intracellular mature Env is not due to an increase in gp160 instability in the ER, or a defect in subsequent trafficking of gp160 to the TGN compartment. We found that mature Env gp120 is selectively targeted for degradation after cleavage, maturation and transport out of the TGN. Less Gag and mature Env were found to be associated with the PM and lipid rafts, which suggested that in Ubc9 knockdown cells, less Gag and Env are associated with microdomains known to be important for virion assembly, and that the degradation of mature Env likely occurred before trafficking to the PM.

Materials and Methods

Cell culture and transfection. 293T cells were obtained from the American Type Culture Collection, they were cultured and transfected as previously reported (159). TZM-bl indicator cells were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: TZM-bl from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc.

Plasmids. The infectious HIV-1 proviral clone pNL4-3 and psG3.1 were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: pNL4-3 from Dr. Malcolm Martin (4) and pSG3.1 from Drs. Sajal Ghosh, Beatrice Hahn, and George Shaw (125). The cleavage defective Envelope mutant (pNL4-3 MUT 511) was a kind gift from Dr. Valerie Bosch and has been previously described (36).

HIV-1 infectivity assays. Virion infectivity assays were carried out as previously described (159). Briefly, medium from transfected 293T cells containing infectious HIV-1 virions were used to infect TZM-bl cells for approximately 36 hours. TZM-bl cells were assayed for HIV-1 infection by staining for β-galactosidase activity and counting positive cells. Infectivity was normalized to pelletable p24 levels in the media as determined by immunoblot.

Metabolic labeling, immunoprecipitation, and Endoglycosidase treatments. Metabolic labeling experiments were carried out as previously reported with differences in labeling times with [^{35}S] methionine/cysteine (>1,000 Ci/nMol; NEN) (159). Briefly, transfected and untransfected 293T cells were pulse labeled for 30 minutes, 1 hour, or 4 hours with 300 μCi, 600 μCi, or 2.0 mCi respectively. For pulse chase experiments, labeling media were removed and chased for 2 and 4 hours in complete culture media.

The culture media was removed, clarified by centrifugation (13,000 RPM for 2 min), and adjusted to 1x lysis buffer (320). Cells were lysed with 1X lysis buffer containing Halt protease inhibitors cocktail (Pierce) and clarified by centrifugation. Viral proteins were immunoprecipitated using AIDS patient sera and ultralink A/G beads (Thermo Scientific). The beads were washed 4X with lysis buffer and the viral proteins were released from the beads by boiling for 5 minutes in 0.04% SDS and 200mM 2-mercapitoethanol (319). The solublized proteins were split into two equal volumes and treated with Protein N-Glycosidase F (PNGase F) or Endoglycosidase H_f (Endo H_f)(NEB) per manufacturers suggestions for 3.5 hours, or left untreated. All samples were adjusted to 1X protein sample buffer (PSB), separated by SDS-PAGE, and visualized by phosphor imaging. Band intensities were quantified using Discovery Series Quantity One software (Bio-Rad).

Unfolded protein response. Activation of the unfolded protein response was analyzed by XBP-I splicing as previously described with slight modifications (163). Briefly, transfected and untransfected 293T cells were treated with 5mM dithiothreitol (DTT) for 3 hours or left untreated. Total RNA was extracted from 293T cells using the RNeasy mini kit (Qiagen) and treated with DNase I amplification grade (Invitrogen) per manufacturer's protocol. Three micrograms of RNA was reverse transcribed using oligo (dT) and SuperScript III (Invitrogen) per manufacturer's protocol. XBP-1 cDNA was amplified using standard PCR techniques with primers that flank the splice site (5'-CCTTGTTGAGAACCAGG-3' and 5'-CTAAGACTAGGGGCTTGGTA-3').

Lipid raft/detergent resistant membrane isolation. Lipid raft isolations were preformed as previously described with slight modifications (25). Transfected 293T cells

were radiolabeled for 4 hours with 2.0 mCi [^{35}S] methionine/cysteine. The labeling media was removed and the cells were lysed on ice with 500 µl of TNE buffer (10 mM Tris [pH 7.5], 100 mM NaCl, 10 mM EGTA) containing 0.5% Triton X-100 (TX-100) for 30 min. Lysates were collected and homogenized with 10 strokes through a 25-G needle and clarified by low speed centrifugation at 10,000 RPMs at 4°C for 10 minutes. Post nuclear lysates were adjusted to 60% sucrose by adding 1.5 ml of 80% sucrose TNE (w/v). The lysates were layered over 500 µl of 80% sucrose TNE, followed by 2 ml of 50% sucrose TNE, 6ml of 38% sucrose TNE, and 1.5 ml of 10% sucrose TNE. The sucrose gradients were centrifuged at 100,000 X g at 4°C for at least 18 hrs in a Beckman SW41 rotor. 11 fractions were collected using a piston gradient fractionator (Biocomp). Densities of each fraction were determined by the refractive index of each sample. Fractions were adjusted to 1X lysis buffer and viral proteins were immunoprecipitated with pooled patient serum, separated by SDS-PAGE, and visualized by phosphor imaging. Cellular proteins were precipitated from each fraction by methanol/chloroform/water precipitation (381) and then analyzed by immunoblotting to identify which sucrose fractions contained lipid rafts (LR), detergent resistant membranes (DRM), and/or detergent soluble membranes (DSM).

Immunoblotting. Samples were lysed in lysis buffer as described above and normalized by BCA assays (Thermo), or samples were directly solublized in 2x PSB. Proteins were separated by SDS PAGE, transferred to nitrocellulose (GE Water & Process Technologies), and analyzed by immunoblotting as previously described (159) or with a LI-COR Odyssey infrared imaging system.

Antibodies. Anti-Ubc9 (N-15) goat polyclonal antibodies (PAb), anti-Actin goat PAb, anti-Flotillin (K-19) goat PAb, anti-E-Cadherin (H-108) rabbit PAb, anti-TGN38 (H-300) rabbit PAb, HIV-1 anti-gp120 (1994) monoclonal antibodies (MAb), HIV-1 anti-gp41 (10E9) MAb, horseradish peroxidase (HRP)-conjugated chicken anti-goat, and HRP-conjugated donkey anti-mouse PAbs were purchased from Santa Cruz Biotechnology, Inc. HIV-1 anti-gp120 goat PAb was purchased from Affinity BioReagents. Anti-human transferrin receptor monoclonal antibodies were purchased from Invitrogen. Rabbit anti-p17 PAbs was obtained from the NIH AIDS Research and Reference Reagent Program. Alexa Fluor 488 conjugated donkey anti-mouse, Alexa Fluor 488 conjugated donkey anti-rabbit, Alexa Fluor 647 conjugated donkey anti-sheep, Alexa Fluor 647 conjugated donkey anti-mouse, and Alexa Fluor 647 conjugated donkey anti-rabbit PAbs were purchased from Molecular Probes. IRDye 800CW conjugated donkey anti-goat and IRDye 680LT conjugated donkey anti-rabbit PAbs were purchased from LI-COR. Pooled AIDS patient sera were obtained from a patient cohort.

Confocal. 293T cells were cultured on sterile, poly-L-lysine coated glass coverslips and transfected as previously reported (159). 24 hours post transfection with NL4-3, medium was removed, and the cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 20 minutes at room temperature. The coverslips were washed with PBS, and when necessary, permeabilized with 0.2% TX-100 in PBS for 5 minutes at room temperature. The coverslips were washed again in PBS followed by blocking for 1 hour with 2% bovine serum albumin (BSA) in PBS. Cover slips were incubated with primary antibodies in 2% BSA for 2 hours at room temperature followed by 3X washes with 2% BSA for 10 minutes. Cover slips were incubated with secondary antibodies in 2% BSA

in PBS for 45 minutes at room temp followed by 2X washes in 2% BSA for 10 minutes, and a final wash in PBS. Cover slips were mounted to slides and analyzed by confocal microscopy (Olympus FV500 with an upright BX Olympus fluorescence microscope). Cellular and viral proteins were detected using antibodies against Gag (PAb anti-p17), Env (MAb anti-gp120 and MAb anti-gp41), and TGN (PAb anti-TGN38). Alexa Fluor 488 conjugated donkey anti-mouse, Alexa Fluor 488 conjugated donkey anti-rabbit, Alexa Fluor 647 conjugated donkey anti-sheep, Alexa Fluor 647 conjugated donkey anti-mouse, and Alexa Fluor 647 conjugated donkey anti-rabbit PAbs were used as secondary antibodies. The 0.3 μm optical z-sections were analyzed with Fluoview imaging software (Olympus).

Results

Effect of Ubc9 on viral infectivity is not unique to a specific isolate of HIV-1. We have previously demonstrated that in the context of NL4-3, an infectious proviral clone, the endogenous Ubc9 expression in HIV-1 producer cells was important for the production of fully infectious HIV-1 virions. Knockdown of Ubc9 expression in producer cells using Ubc9 specific RNAi techniques reduced the specific infectivity of the released virions approximately 10-fold as compared to virions produced in the presence of Ubc9. Pseudotyping experiments using VSV-G demonstrated that the decreased infectivity of virions produced in Ubc9 knockdown cells was likely due to a reduced level of mature Env gp120 packaged into the virions, and the reduction was reflected in a decrease in the intracellular Env level in the virus producing cells (159). To confirm that the effect of Ubc9 was not HIV-1 strain specific we have repeated the virion infectivity assay using a different HIV-1 strain, SG3.1. The SG3.1 Gag and Env are 6% and 16% divergent at the

amino acid level when compared with NL4-3 Gag and Env respectively. The pSG3.1 provirus lacks Vpu, however it can produce highly infectious/pathogenic virions, and can readily replicate in chimpanzees and SCID-hu mice (125). To assess the infectivity of SG3.1 virions produced in the presence or absence of Ubc9 expression, 293T cells were transfected with pSG3.1 alone, or in combination with control siRNA or Ubc9 siRNA. In the absence of Ubc9, SG3.1 virions did not exhibit any significant defects in the amount of virions released into the media, as quantified by the amount of pelletable p24 in the media with immunoblotting (Fig. 1a). However, the specific infectivity of viruses produced from cells in which Ubc9 expression was knocked down by Ubc9 siRNA was decreased by more than 60% (Fig. 1b). This data confirmed that Ubc9 expression in HIV-1 SG3.1 producing cells is important for the production of fully infectious HIV-1 virions, and that the decrease in HIV-1 infectivity in the absence of Ubc9 expression is not specific to the HIV-1 NL4-3 isolate.

No evidence for endoplasmic reticulum associated degradation (ERAD), or activation of the unfolded protein response (UPR) as a factor in gp120 stability in the absence of Ubc9 expression. The decrease in intracellular mature Env (gp120 and gp41) in Ubc9 knocked down cells could be caused by a number of mechanisms. Decreased intracellular gp120 levels could be due to: a defect in intracellular gp160 stability; a defect in gp160 trafficking to the TGN and maturation into gp120 and gp41; gp120 might be mistargeted for degradation as it traffics out of the TGN before reaching the plasma membrane assembly site; or gp120 may be incorrectly endocytosed from the plasma membrane and then degraded. We have previously hypothesized that the decrease in intracellular mature Env levels was not likely due to an overall decrease in

stability of gp160 prior to cleavage into gp120 and gp41. This was based on data from pulse chase experiments where the levels of intracellular gp160 decreased over time at similar rates regardless of whether the cells were transfected with Ubc9 siRNA, control RNA, or no RNA (159). However it could not be ruled out that the decrease in intracellular gp120 was not due to a defect in the endoplasmic reticulum (ER) leading to an increase in gp160 degradation.

To examine if gp160 stability was affected in the ER in Ubc9 knockdown cells, we examined two major ER degradation pathways that could lead to gp160 degradation. The ERAD is an ER based pathway that can degrade gp160, but under certain conditions it can cause gp160 to migrate at a molecular weight (MW) similar to gp120 and affect the quantitation of the gp160 and gp120 levels experimentally (76, 163). Therefore, the effect of Ubc9 knockdown on gp160 ERAD was further examined by assaying the MW of viral glycoproteins after treatment with PNGase F (Fig. 2a), which hydrolizes nearly all forms of N-linked glycans between the asparagines and N-acetyl-D-glucosamine (100). As expected, the viral glycoprotein was expressed as gp160 during the pulse-labeling period. During the chase, gp120 could be detected in cells transfected with NL4-3 alone, or in combination with control RNA. However, cells transfected with Ubc9 siRNA contained less gp120. When the lysates from the pulse period were treated with PNGase F, the band corresponding with glycosylated gp160 disappeared and a band with a MW of approximately 90 kDa appeared. The 90 kDa band correlates with the expected MW of deglycosylated gp160. When the chase samples were treated with PNGase F, the gp160 band shifted down as expected to approximately 90 kDa. The band corresponding to gp120 disappeared, and a band with a MW of approximately 60 kDa appeared, which

correlates to the expected weight of deglycosylated gp120. The 60 kDa band could be seen throughout the chase period when samples were treated with PNGase F, and it was much less evident in cells transfected with Ubc9 siRNA as expected. A band with a MW of approximately 30 kDa also appeared in these samples, which correlates with the expected size of deglycosylated gp41. The presence of the 60 kDa band in Ubc9 siRNA transfect cells indicated that the band around 120 kDa was in fact gp120, and not a partially deglycosylated form of gp160 undergoing ERAD. More importantly, gp160 was not preferentially degraded in Ubc9 knockdown cells compared to control cells. Extreme over exposure of the gels did not show any unique or excess banding between 90 and 60 kDa, ruling out the possibility that gp160 was undergoing degradation (data not shown).

With no evidence for ERAD gp160 degradation in Ubc9 knocked down cells, we examined if another ER degradation pathway, the unfolded protein response (UPR), was triggered and involved in the decrease of intracellular gp120 through degradation of gp160. The UPR is triggered when improperly folded proteins accumulate in the ER, and UPR activation is a marker for overall ER stress and cellular dysfunction. Upon UPR activation the X-box binding protein 1 (XBP-1) mRNA is alternatively spliced, leading to a frame-shift that increases the transcriptional activity of XBP-1, resulting in the upregulation of chaperones in the ER (UPR reviewed in 329). To examine ER stress and activation of UPR, XBP-1 splicing in Ubc9 knocked down cells was determined using total cellular RNA extracted from transfected 293T cells, either treated or untreated with dithiothreitol (DTT), an UPR activating agent. A 450 bp unspliced XBP-1 mRNA product was detected in untreated cells and upon addition of DTT, a 26 bp smaller spliced

XBP-1 mRNA PCR product was detected, indicating that the UPR was activated (Fig 2b). However, neither HIV-1 gene expression nor knockdown of Ubc9 by siRNA activated the UPR. Replicate plates transfected with control RNA, Ubc9 siRNA, or left untransfected were lysed and immunoblotted for Ubc9 and actin levels to confirm knockdown of Ubc9 (Fig. 2c). Taken together, the decrease in intracellular gp120 levels did not appear to be due to gp160 instability in the ER as an outcome of Ubc9 knockdown.

Gp160 was trafficked to the trans-Golgi network normally in the absence of Ubc9 expression. With no evidence implicating that the decreased intracellular gp120 levels were due to ER dysfunction and associated gp160 degradation in the Ubc9 knockdown cells, we next determined whether there was a defect in gp160 trafficking to the TGN, and whether cleavage and maturation could be involved in the decreased levels of intracellular gp120. To determine if Ubc9 knockdown caused defects in gp160 TGN trafficking, we tracked Env movement through the secretory pathway of Ubc9 knockdown cell and control cells by pulse chase analysis following Endo H_f treatments (Fig. 3a). HIV-1 gp160 is extensively modified with N and O-linked oligosaccharides in the ER, which are highly sensitive to removal by Endo H_f, an endoglycosidase which primarily cleaves high-mannose sugars (233). As Env proteins trimerize in the ER and traffic through the TGN, the polyprotein oligosaccharides are substantially modified and the susceptibility to Endo H_f cleavage decreases. As gp160 traffics through the TGN it is cleaved into its mature forms (gp120 and gp41) (23, 321, 343 reviewed in 71). During the pulse period (P), only gp160 could be detected, in control cells that were transfected with NL4-3 proviral DNA alone (Fig. 4a, left panel), in cells co-transfected with NL4-3

and control RNA (middle panel), or in cells co-transfected with NL4-3 and Ubc9 siRNA (right panel). Cells co-transfected with control or Ubc9 siRNA consistently produced on average 5-6% less gp160 than control cells. Treatment of the pulse samples with Endo H_f led to the disappearance of the band of 160 kDa (gp160) and the appearance of a band with an approximate MW of 90 kDa. The 90 kDa band correlated with the expected MW of gp160 with its N-linked glycans removed by Endo H_f (gp160s). Susceptibility to Endo H_f indicated that during the pulse period, all the gp160 was located in the ER because it has only been glycosylated with high-mannose sugars. As expected, during the chase period, gp160 levels decreased, and gp120 appeared along with a very diffuse band with an approximate MW of 170-180 kDa. This diffuse band correlated to a population of gp160 that had trafficked to the Golgi and had been modified with more complex sugars, giving it a larger molecular weight as has been reported previously (119, 138). When the chase samples were treated with Endo H_f, gp160, gp120, and the 170-180 kDa bands disappeared, and bands with approximate MW of 130, 90, and a smear around 80 kDa appeared. The band at around 130 kDa is consistent with the migration pattern of a partially Endo H_f resistant gp160 (gp160r), the population of gp160 that would have trafficked out of the ER with its high mannose sugars modified to more resistant complex sugars in the Golgi complex. The band at approximately 90 kDa is a population of gp160 that still resides in the ER (gp160s). The band/smear around 80 kDa is consistent with forms of gp120 that have undergone extensive glycosylation modifications in the Golgi and were partially resistant to Endo H_f (gp120r) (211).

Upon transfection with siRNA to knockdown Ubc9, or with control RNA, levels of partially Endo H_f resistant forms of gp160 (gp160r) could be detected beginning at

two hours. Detection of similar amounts of gp160r in cells where Ubc9 expression was knocked down indicated that gp160 was able to trimerize and traffic out of the ER (98, 386). Mature Env gp120 and gp120r were detected at similar levels in cells transfected with NL4-3, or in combination with control RNA. In contrast, gp120 and gp120r were on average reduced by 50-60% in cells transfected with Ubc9 siRNA (Fig. 3a). Similar levels of gp160 were found to traffic out of the ER to the Golgi complex and total gp160 decreased at similar rates during the chase times, suggesting that slower gp160 trafficking and cleavage in the TGN could not account for the lower levels of intracellular gp120. As expected, the lower intracellular gp120 in the absence of Ubc9 expression resulted in less gp120 packaged into the virion (Fig. 3a, lower panels).

Normal Env trafficking to the TGN in the absence of Ubc9 expression was further confirmed using confocal microscopy. TGN staining by TNG38 antibodies (red) appeared as cytoplasmic puncta that grouped together near the periphery of the nucleus regardless of whether the cells were transfected with RNA and/or NL4-3, or left untransfected (Fig. 4a-c, e, h, and k). Cells transfected with NL4-3 alone (d-f), or in combination with control RNA (g-i) or Ubc9 siRNA (j-l) showed typical intracellular HIV-1 Env (green) patterns, which are known to be associated with the secretory and endocytosis pathways. When the signals for Env and TGN38 from the same optical z-section were merged, distinct areas of co-localization could be seen in the cytoplasm (Fig. 2 f, i, and l), which confirmed that Env trafficked to the TGN even in Ubc9 knockdown cells. Taken together, the knockdown of Ubc9 expression did not affect gp160 oligomerization, trafficking out of the ER to the TGN, oligosaccharide modification in the TGN, or gp160 maturation.

Gp120 and not gp160 was specifically degraded in the absence of Ubc9. Our data so far has suggested that the reduced level of intracellular gp120 in Ubc9 knocked down cells was not due to defects associated with gp160 functions, and the expression of both Ubc9 and Gag were required for normal levels of intracellular gp120. To examine whether gp120 was specifically degraded after gp160 cleavage in Ubc9 knockdown cells, we examined the stability of an Env cleavage mutant (MUT 511) in the presence and absence of Ubc9, in the context of a complete provirus. MUT 511 has a point mutation at position 511 (Arg to Ser) in gp160, which blocks its cleavage into gp120 and gp41. Mut 511 is not efficiently packaged into virions, but still traffics to the plasma membrane (36, 97). If gp120 is specifically targeted for degradation, MUT 511 stability should be unchanged as it trafficks through the secretory pathway to the plasma membrane. Transfected 293T cells were again assayed by pulse chase experiments to assess MUT 511 stability in Ubc9 knockdown cells and control cells. Cells were pulse chased and treated with Endo H_f as in previous experiments to track MUT 511 stability and trafficking through the TGN (Fig 5a). As expected, only gp160 was observed, and it was not processed into gp120 and gp41 because of the mutation. During the pulse period, gp160 resided in the ER and was completely Endo H_f sensitive as observed earlier (Fig. 3a). At the two-hour chase time a band with an approximate MW of 180 kDa appeared and persisted through the 4-hour chase period. Endo H_f treatment resulted in the appearance of band of 90 kDa (gp160s), a partially resistant Endo H_f gp160 (gp160r) as observed earlier (Fig. 3a), confirming that MUT 511 was trafficking through the secretory pathway even in Ubc9 knockdown cells. Intracellular Env levels remained stable during the chase in cells that were transfected with either Ubc9 siRNA or control

RNA, indicating that the decrease in gp160 observed over time in previous experiments was due to gp160 maturation into gp120/gp41 and not due to degradation. Taken together our results suggest that the decreased intracellular gp120 in Ubc9 knockdown cells was due to specific degradation of gp120.

Ubc9 knockdown did not affect Furin activity or endogenous E-cadherin stability. To further confirm that the increase in gp120 degradation in Ubc9 knockdown cells is specific to mature Env, and not due to a more generalized decrease in overall stability of proteins trafficking through the secretory pathway, we assayed the maturation and stability of endogenous cellular E-cadherin. E-cadherin is a cellular, type-1 transmembrane adhesion factor and its biosynthetic pathway is very similar to that of HIV-1 Env. E-cadherin is translated in the ER as a glycosylated inactive proprotein that undergoes a post-translational maturation step. E-cadherin is cleaved into its functional subunits as it moves through the secretory pathway, presumably by Furin in the TNG, as it traffics to the plasma membrane. Equal amounts of whole cell lysates from Ubc9 or control siRNA transfected, and untransfected 293T cells were analyzed for E-cadherin expression. Pro E-cadherin (135kDa) and mature E-cadherin (120 kDa) can be detected in whole cell lysates, with cells transfected with Ubc9 siRNA containing on average 19-23% less total E-cadherin (immature and mature forms) compared to control cells. The lower E-cadherin expression was not unexpected as previous studies have shown that the over expression of Ubc9 led to an increase in miR-200b, which targets and causes the down regulation of E-cadherin transcriptional repressors, ZEB-1 and ZEB-2 (65, 190, 284, 409). Even though less total E-cadherin was expressed in the Ubc9 knocked down cells, the mature E-cadherin represented 43% of total cellular E-cadherin when

normalized to the levels of actin regardless of Ubc9 expression (Fig. 6). Our results indicate that in Ubc9 knockdown cells, there was no evidence of a general decrease in stability of proteins such as E-cadherin, while trafficking through the secretory pathway, and that only gp120 was affected. In addition Furin activity was not affected since pro E-cadherin was properly cleaved by Furin, suggesting that the lower levels of gp120 in Ubc9 knockdown cells is not due to a defect in cleavage of gp160 by Furin.

Degradation of intracellular mature Env in the absence of Ubc9 required trafficking out of the TGN. Taken together, our data indicates that in Ubc9 knockdown cells, Env trafficked to the TGN as expected and that gp120 was specifically degraded after cleavage. To examine if gp120 degradation requires trafficking out of the TGN, we examined Env stability and trafficking through the secretory pathway when cultured at 20°C. Incubating cells at 20°C inhibits the budding of immature secretory vesicles from the TGN, effectively blocking transport out of the TGN and leads to an accumulation of proteins in the TGN (244). For this study, we carried out pulse chase experiments as before. After metabolically labeling at 37°C, cells were shifted to 20°C and chased for 2 and 4 hours. We found that when transfected cells were shifted to/and incubated at 20°C, gp160 cleavage was significantly inhibited and remained stable during the chase period. Interestingly, an extremely faint Endo H_f resistant band with a MW of approximate 140-150 kDa (gp160r*) was detected during the 2-hour chase time on over-exposed images, and the intensity of this band increased to become more easily detectable at the 4 hours. This Endo H_f resistant band could represent a fraction gp160 that has trafficked to the TGN and undergone aberrant glycosylation modifications because of the altered trafficking kinetics through the secretory pathway when cultured at 20°C. Gag cleavage

and virion assembly was also slowed significantly as Pr55 was more stable over time and less p24/p25 was cell associated (Fig. 7). This data supports previous data indicating that gp120 was specifically degraded and that degradation of gp120 occurred after Env trafficked out of the TGN as it migrated through the secretory pathway.

Degradation of gp120 occurred before transport to the plasma membrane and the lipid rafts. Our results have so far indicated that gp160 stability, trafficking from the ER to the TGN and cleavage were normal, and the degradation of gp120 occurred after transport out of the TGN in Ubc9 knockdown cells. In a normal cell, after Env cleavage maturation occurs, the trimmer consisting of the gp120 and gp41 is trafficked to the PM. The mature Env is then either packaged into assembling virions, or it is quickly endocytosed by the cellular endocytosis machinery through interactions with adaptor protein complexes, primarily through AP-2 binding (21, 30, 271). It is possible that in the absence of Ubc9, gp120 stability is affected prior to reaching the PM or may be affected only after endocytosis. To further delineate when the degradation of gp120 occurred in the ubc9 knockdown cells, we conducted experiments to examine the amount of gp120 associated with the PM and lipid rafts. To examine the levels of mature Env (gp120) associated with the PM, Ubc9 or control siRNA transfected 293T cells were metabolically labeled and chased for 1.5 and 2.5 hours (Fig. 8a). During the pulse, very little Env proteins appeared to be associated with the PM fraction in any samples. At 1.5 and 2.5 hours, gp160 and gp120 can be readily detected in the PM fraction of cells transfected with NL4-3 alone, or with NL4-3 and control RNA. Cells transfected with NL4-3 and Ubc9 siRNA showed a decrease of 83% in the levels of gp120 associated with the PM fraction as compared to the control cells (Fig. 8b). This result suggests that the

effect on gp120 stability in the absence of Ubc9 likely occurred before insertion into the PM and that a more thorough examination of the PM and PM microdomains would be needed to further confirm this hypothesis.

To further examine the localization of HIV-1 Gag and Env at the surface of the cell, immunofluorescent confocal microscopy was utilized. 293T cells were grown on coverslips, transfected as in previous experiments, fixed, and examined using polyclonal Abs against HIV-1 p17 and in combination with either monoclonal Abs against HIV-1 gp120 (Fig. 9a-c) or gp41 (Fig. 9d-f). Cells transfected with NL4-3 alone, or in combination with control RNA, showed that both Env (red) and Gag (green) can be detected in transfected cells, and showed co-localization in areas along the periphery of the cell (Fig.9a,b,d, and e). Cells transfected with NL4-3 and Ubc9 siRNA showed indistinguishable Gag staining from the control cells, however, less Env was detected at the cell surface and less co-localization between Gag and Env was observed (Fig. 9c and 9f). This result supports the preliminary PM isolation experiment. Together, they suggested that in Ubc9 knockdown cells, less Env is present at the plasma membrane and, gp120 stability was affected prior to its insertion into the PM.

To more extensively examine the amount of Gag and Env associated with the PM during the assembly process we analyzed their association with lipid rafts (LR). Multiple lines of evidence have implicated that lipid rafts are important microdomains of the PM that facilitate virion assembly. To examine viral protein association to lipid rafts in Ubc9 knockdown cells, metabolic labeling combined with lipid raft floatation experiments were utilized. Transfected 293T cells were metabolically labeled and cell lysates were fractioned, and analyzed for the presence or absence of viral and cellular proteins.

Flotillin-1 is a cellular LR protein marker and was only detected at the top of the gradient in fractions 1 and 2. Transferrin receptor (TfR), a protein excluded from detergent resistant membranes (DRM) and lipid rafts, was only detected in the bottom of the gradient in fractions 9, 10, and 11, indicating that there was a good separation between LR, DRM, and detergent soluble membranes (DSM) (Fig. 10a). The densities of each of the fractions across all samples were assayed and showed that the gradients were consistent and reliably reproduced among samples (Fig. 10b). DSM fractions from transfected cells were combined and immunoblotted for Ubc9 expression. (Fig. 10c). The majority of Gag and Env were detected in the DSM factions, with very small amount found in the DRM and LR fractions regardless whether the cells were co-transfected with control RNA, Ubc9 siRNA, or with NL4-3 alone (Fig. 10d-f). In cells transfected with NL4-3, or in combination with control RNA, gp160, gp120, and Gag were detected in the DSM and LR fractions. Viral proteins associated with DRM decreased as the sucrose density decreased towards the top of the gradient, then increased again when the fractions were enriched with LR. The ratios of Pr55 with gp160 and/or gp120 in LR fractions were similar between control cells. Interestingly, cells co-transfected with NL4-3 and Ubc9 siRNA showed altered levels of viral proteins associated with the DSM and LR factions. Ubc9 siRNA transfected cells showed about a 5-fold reduction in the amount of gp120 associated with LR fractions. However, similar LR Pr55/gp160 ratio was found when compared to control cells, suggesting that the amount of gp160 associated with the LR was not affected by Ubc9 expression levels even though there was a reduction in gp120 level (Fig. 10g). The percentage of Gag associated with LR was also examined, as previous reports have suggested that Env trafficking to LR is dependent upon Gag co-

trafficking to LR (26, 285). We found that in the absence of Ubc9 expression, Pr55 association with LR was decreased by almost 50% compared to the control cells (Fig. 10h). In summary, our data suggests that Gag and Env trafficking and association with lipid rafts in the absence of Ubc9 is altered, and that gp120 stability is affected prior to the trafficking of gp120 to the PM and to the lipid raft microdomain in Ubc9 knockdown cells.

Discussion

We have previously demonstrated that when endogenous Ubc9 expression was knocked down by Ubc9 specific siRNA in HIV-1 producer cells, HIV-1 specific infectivity was reduced 10-fold when compared to control cells. The defect in infectivity was found to be a defect in mature, intracellular Env production and subsequently reduced Env packaging into assembling virions. In this study, we have continued to examine HIV-1 assembly in Ubc9 knockdown cells in an effort to further characterize the decrease in intracellular Env levels, and to further understand how Ubc9 and Gag contribute to mature, intracellular Env stability and packaging into assembling virions. Our data indicated that in Ubc9 knockdown cells, there was no increase in ERAD or activation of UPR, and Env stability and trafficking from the ER to the TGN appeared to proceed normally. We also found that in Ubc9 knockdown cells, gp160 trafficked as expected to the Golgi apparatus, suggesting that glycosylation, oligomerization, transport out of the ER, and modification of the high mannose carbohydrates to complex carbohydrates occurred as in control cells expressing Ubc9. However, we found that mature Env was being selectively targeted for degraded after transport out of the TGN in Ubc9 knockdown cells. Examination of the surface expression of viral proteins by

various approaches demonstrated that in Ubc9 knockdown cells, Gag and Env displayed altered trafficking and association with the cell surface and PM microdomains, suggesting that the defect in Env stability occurred after being transported out of the ER, but before trafficking to the PM (Fig. 11).

HIV-1 assembly predominantly takes place at the plasma membrane (156, 175), at sites where viral and host derived factors co-localize and interact to assemble infectious viral particles. There are a number of intracellular sites where important viral and host factors interact during the assembly of an infectious virion (126). However, the timing and location of where most these proteins interact during the viral assembly pathway are still not well understood. One essential gap is when and where Gag and Env first interact during the assembly process to facilitate Env packaging into the released virions. Various mechanisms have been proposed for Env packaging, however specific interactions, either directly or indirectly, between amino acids within MA and the cytoplasmic tail of Env results in the selective packaging of the viral Env seems most likely to occur under normal conditions (mechanisms reviewed in 58, 167). Confocal microscopy data examining the site of colocalization between Gag and Env suggested that Gag and Env may interact at the PM, as they primarily co-localize at the PM (143, 224). However various lines of evidence have suggested that the interaction may occur before virion assembly at the PM, either through direct or indirect interactions, resulting in the active incorporation of mature Env into the assembling virions (80, 86, 221, 222, 224, 280, 323). Regardless of where Gag and Env first interact, there was evidence to suggest that both Gag and Env can influence the stability and trafficking of each other (99, 143, 171, 203, 210, 221, 222, 374).

Based on our results, there are three potential mechanisms that may explain how interrupting the interaction between Gag and the cellular host factor Ubc9 may lead to gp120/gp41 instability and its decreased packaging into the assembling virions (Fig. 11). First is the altered trafficking of Gag, either at an early or late stage of assembly. In Ubc9 knockdown cells, Gag displayed altered trafficking and association with lipid rafts, a microdomain important during the very late stages of virion assembly (reviewed in 273, 370). It is possible that other earlier stages of Gag trafficking may have also been disrupted in Ubc9 knockdown cells. Ubc9 has been identified as a host factor localized to the Gag perinuclear clusters (GPC) which were hypothesized to be early intermediate Gag trafficking sites (242). In Ubc9 knockdown cells, Gag may not be targeted correctly through the cytoplasm if Ubc9 is not present at the GPC. It is also possible that Ubc9 interacts with Gag at a later stage of trafficking, as we have also observed that Gag co-localized with Ubc9 in close proximity to the PM, which suggests that this interaction is important during the later stages of virion assembly (159). If Env and Gag first interact and influence each other at an intracellular site, at or near the TGN or recycling endosomes (8, 20, 224, 323), an alteration of Gag trafficking through the interruption of Gag-Ubc9 binding, Gag and Env interactions and co-trafficking to the site of assembly may also be disrupted. This disruption could lead to Env being mistrafficked/sorted and targeted for degradation instead of trafficking to the normal virion assembly site (Fig. 11 green arrow).

The second potential mechanism to explain these observations is that if the site of interaction between Gag and Env is at the site of assembly on the plasma membrane, altering Gag trafficking to the site of assembly by Ubc9 knockdown may in turn alter the

ability of Gag to stabilize Env on the PM (99, 210). Without Gag dependent Env stabilization at the PM, Env would be quickly endocytosed and might then be targeted for degradation instead of recycling to the TGN (Fig. 11 violet arrows). Interestingly, Ubc9 and SUMOylation have been previously shown to be involved in the endocytic pathway by modulating the endocytosis of cellular proteins at the plasma membrane (251, 395). Lastly, it is possible that by interrupting Gag and Ubc9 interactions, it may have altered the trafficking or function of yet other unknown cellular factor(s) that are involved in Env stability and packaging into assembling virions, since the host factors and pathways used during assembly are not fully understood.

Our study cannot rule out any of the proposed models, but our results seem to favor the first model, where Gag undergoes aberrant trafficking in Ubc9 knockdown cells, which then interrupts interactions with Env at an intracellular site. The altered Gag/Env interaction, directly or indirectly causes the mistargeting of the Env containing secretory vesicles for degradation before trafficking to the PM. This is supported by reports implicating that Ubc9 involvement in TGN protein sorting, leading to aberrant trafficking within the cell. Studies with GLUT4, an insulin-regulated glucose transporter, have shown that Ubc9 plays a role in regulating GLUT4 stability and trafficking. In Ubc9 knockdown cells, GLUT4 levels decreased by approximately 50% and this was found to be due to an increase in its degradation. It was hypothesized by Liu et al. that Ubc9 may function by interacting with Arf-binding proteins (GGA), which function in sorting proteins that move through the secretory pathway and are involved with the formation of GLUT4 storage vesicles. Alternatively, Ubc9 may function to negatively regulate the trafficking of secretory vesicles from the TGN to sites of degradation (220).

Interestingly, GGA and Arf proteins have been shown to be important host factors that regulate HIV-1 assembly and release, and GGA2 appeared to play a role in Env maturation, however the mechanism of how it is involved is not understood (174).

It has been previously reported that interactions between cellular factor $p14^{ARF}$ and Ubc9 enhanced the SUMOylation of $p14^{ARF}$ binding partners. This demonstrated that cellular factors, that are Ubc9 binding partners, may act as a "tether" to retain the SUMO-Ubc9 complex in close proximity to other unmodified target proteins, which increases the efficiently by which they are SUMOylated (308). A similar model has been proposed for dynamin and SUMOylation mediated endocytosis (251) At this point it cannot be ruled out that SUMOylation mediated by Ubc9 may also be involved. We had previously reported that over expression of the catalytically inactive trans-dominant negative Ubc9 mutant (C93A) did not lead to a decrease in infectivity of the HIV-1 virion produced. However a total block in SUMOylation was unlikely (159), and it is possible that only a very low level of SUMOylation was needed during the assembly process. Recently, Zamborlini et al. demonstrated that HIV-1 Gag-Pol cleavage intermediates are SUMOylated with SUMO-2 in a cell line that over expressed SUMO-2. However, they could not detect SUMO-2 associated with the virion, suggesting that SUMOylation may even play a role during assembly but its effect my be very transient and not detectable in the mature virion (401).

If Ubc9 is involved with Gag and Env interactions, directly or indirectly, it is most likely dependent upon sequences in matrix and the cytoplasmic tail of gp41, as these domains have been previously reported to be important for Gag dependent Env packaging into the assembling virions (115, 117, 257, 258). It has been previously

hypothesized by Salzwedel et al. that the CT of mature Env may undergo a conformational change after cleavage to allow it to be preferentially packaged over gp160, presumably through interaction with MA (321). A similar mechanism in Ubc9 knockdown cells involving the CT may be involved with the selective degradation of mature Env when both mature Env, and uncleaved gp160 are both trafficking through the secretory pathway, yet only mature Env is degraded. Experiments are underway to further clarify the potential role of endocytosis or altered TGN sorting/trafficking as mechanisms for the increase in degradation of mature Env in Ubc9 knockdown cells.

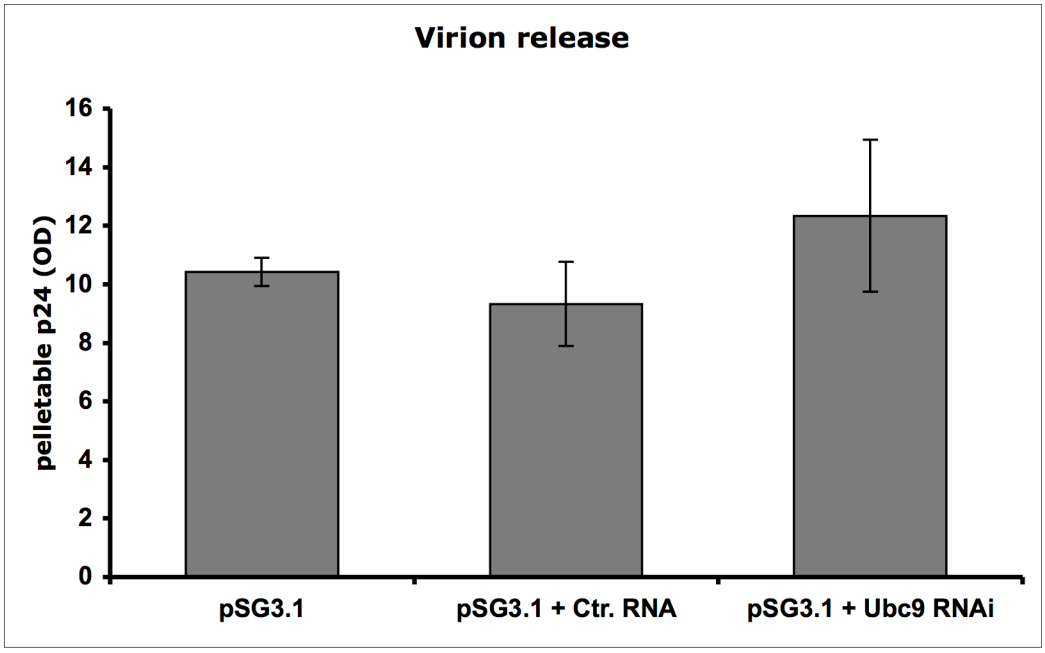
Acknowledgments

We thank members of the Wood lab for thoughtful discussions. We also thank Terri Fangman at the University of Nebraska Microscopy Core Facility for her help with confocal imaging. Dr. Valerie Bosch for pNL4-3 MUT 511 construct. This study was supported in part by PHS grants P30 RR031151 and T32 AI060547 to CW. C.R.B is a NIH Ruth L. Kirschstein Fellow.

Figures and Figure Legends

Figure 1

a



b

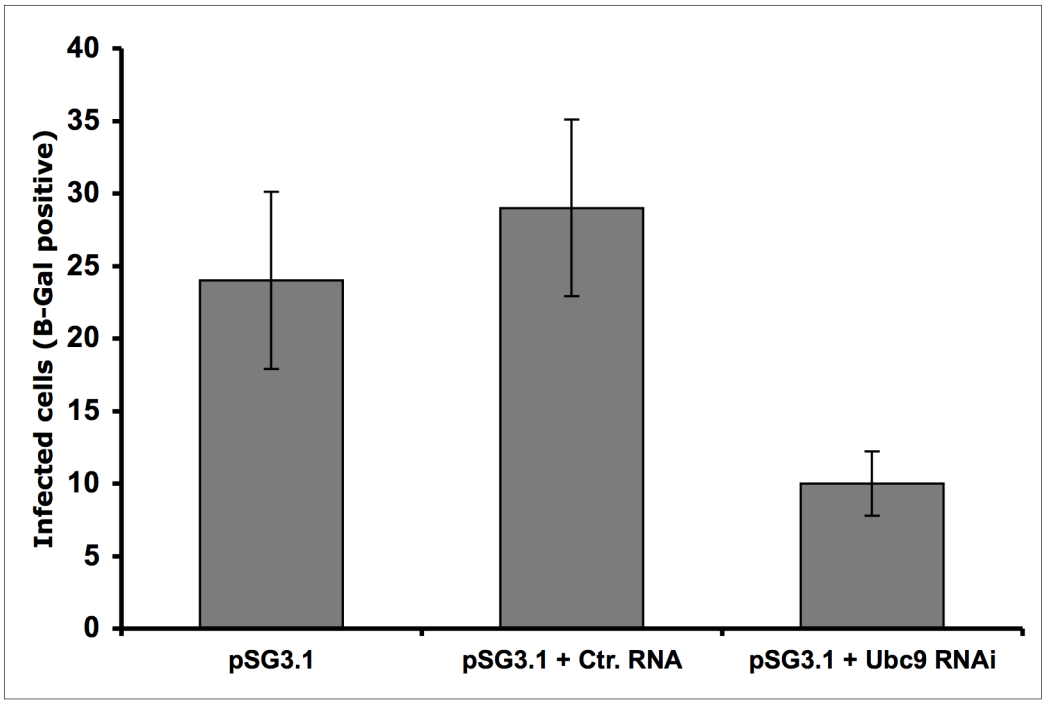


Figure 1. HIV-1 molecular clone pSG3.1 assembly and virion infectivity is reduced by Ubc9 knockdown in producer cells. (a) Ubc9 is not required for virion budding and release. 293T cells were transfected with pSG3.1 alone, or in combination with Ctr. RNA or Ubc9 siRNA. 24 hour after DNA transfection, medium was collected, clarified, and virions were pelleted through a 20% sucrose cushion. Pelleted virions were lysed directly in PSB, separated by SDS PAGE, transferred to nitrocellulose, and assayed by immunoblotting using anti-p24 PAb. p24 was quantified using the LI-COR Odyssey infrared imaging system using 680LT conjugated donkey anti-rabbit PAbs as secondary antibodies. (b) Specific infectivity of virions produced in the presence or knockdown of Ubc9 expression. Clarified medium from transfected 293T cells were used to infected TZM-bl indicator cells and were assayed 36 hours post infection for β-galactosidase activity. Infectivity was normalized by p24. Infections were carried out in triplicate.

Figure 2

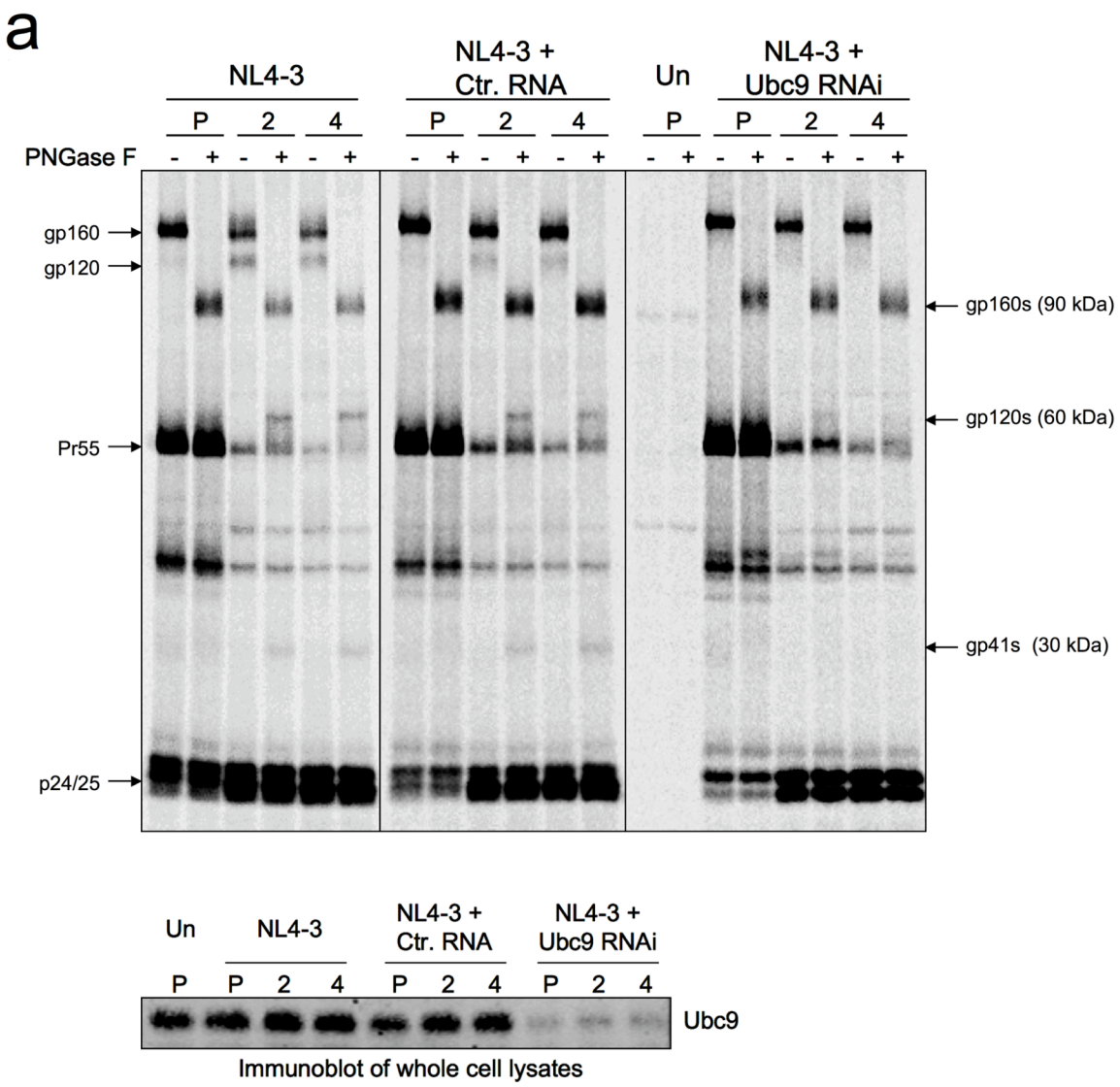

a
NL4-3
NL4-3 + Ctr. RNA
Un
NL4-3 + Ubc9 RNAi
P 2 4
PNGase F
- + - + - +
gp160
gp120
Pr55
p24/25
gp160s (90 kDa)
gp120s (60 kDa)
gp41s (30 kDa)
Un
NL4-3
NL4-3 + Ctr. RNA
NL4-3 + Ubc9 RNAi
P P 2 4 P 2 4 P 2 4
Ubc9
Immunoblot of whole cell lysates

Figure 2 (continued)

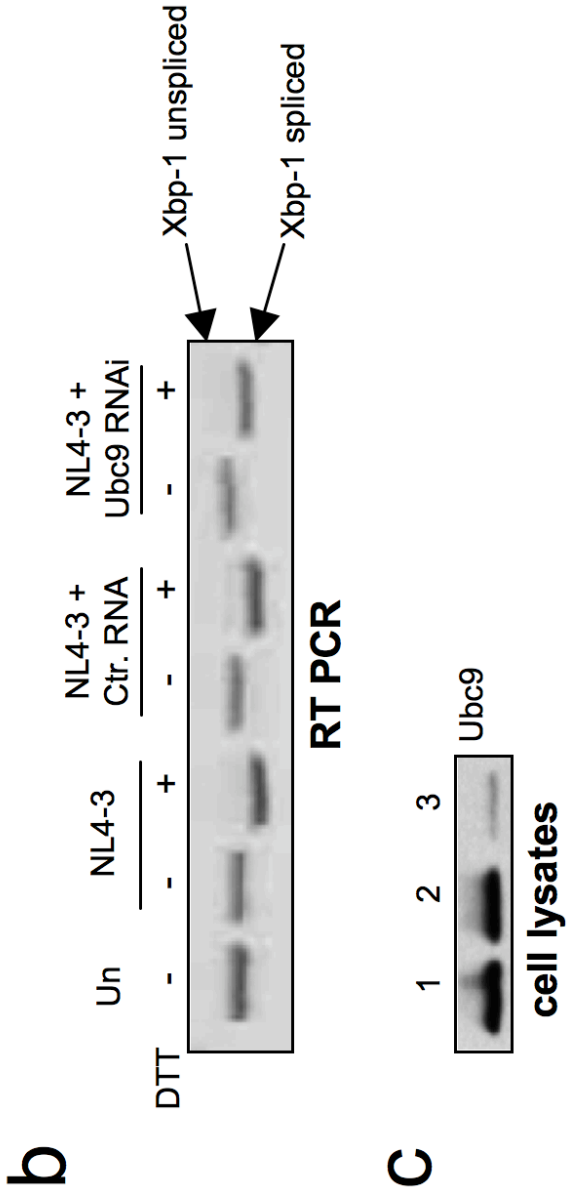


Figure 2: The decrease in HIV-1 Env stability in of Ubc9 knockdown cells is not due to degradation pathways associated with endoplasmic reticulum stress responses. (a) Molecular weights of deglycosylated Env proteins in the presence or near absence of Ubc9 expression. 293T cells were transfected with pNL4-3 alone, or in combination with either non-silencing RNA (Ctr. RNA) or siRNA directed against Ubc9 (Ubc9 RNAi), or left untransfected (Un). Cells were pulse (P) labeled with [^{35}S] methionine/cysteine for 1 hour and chased for 2 and 4 hours. Cell associated viral proteins were solublized and immunoprecipitated with pooled AIDS patient sera, split equally, and incubated for 3.5 hours at 37° C in the presence, or absence of N-glycosidase F (PNGase F). Samples were separated by SDS PAGE and visualized by phosphorimaging using The Discovery Series Quantity One software. A representative, over-exposed gel is shown so that partially Endo H_f resistant Env can be more easily visualized. The identity and position of PNGase F untreated viral proteins are labeled on the left of the gel. Deglycosylated, PNGase F sensitive, viral proteins are labeled on the right and are denoted with a (s) to identify the position of gp160s, gp120s, and gp41s in the gel after PNGase F treatment. (b) Unfolded protein response activation state in the presence or knockdown of Ubc9 expression. Total RNA was extracted from transfected 293t cells that were either treated with 5mM DTT for 3 hours to induce UPR, or left untreated. Total mRNA was reverse transcribed with oligo (dT) followed by PCR amplification of XBP-1 cDNA using primers that flank an alternative splicing site with in the XBP-1 mRNA. (c) Immunoblot of Ubc9 expression in whole cell lysates. Untransfected (lane 1), Ctr. RNA (lane 2), and Ubc9 RNAi (lane3)

Figure 3

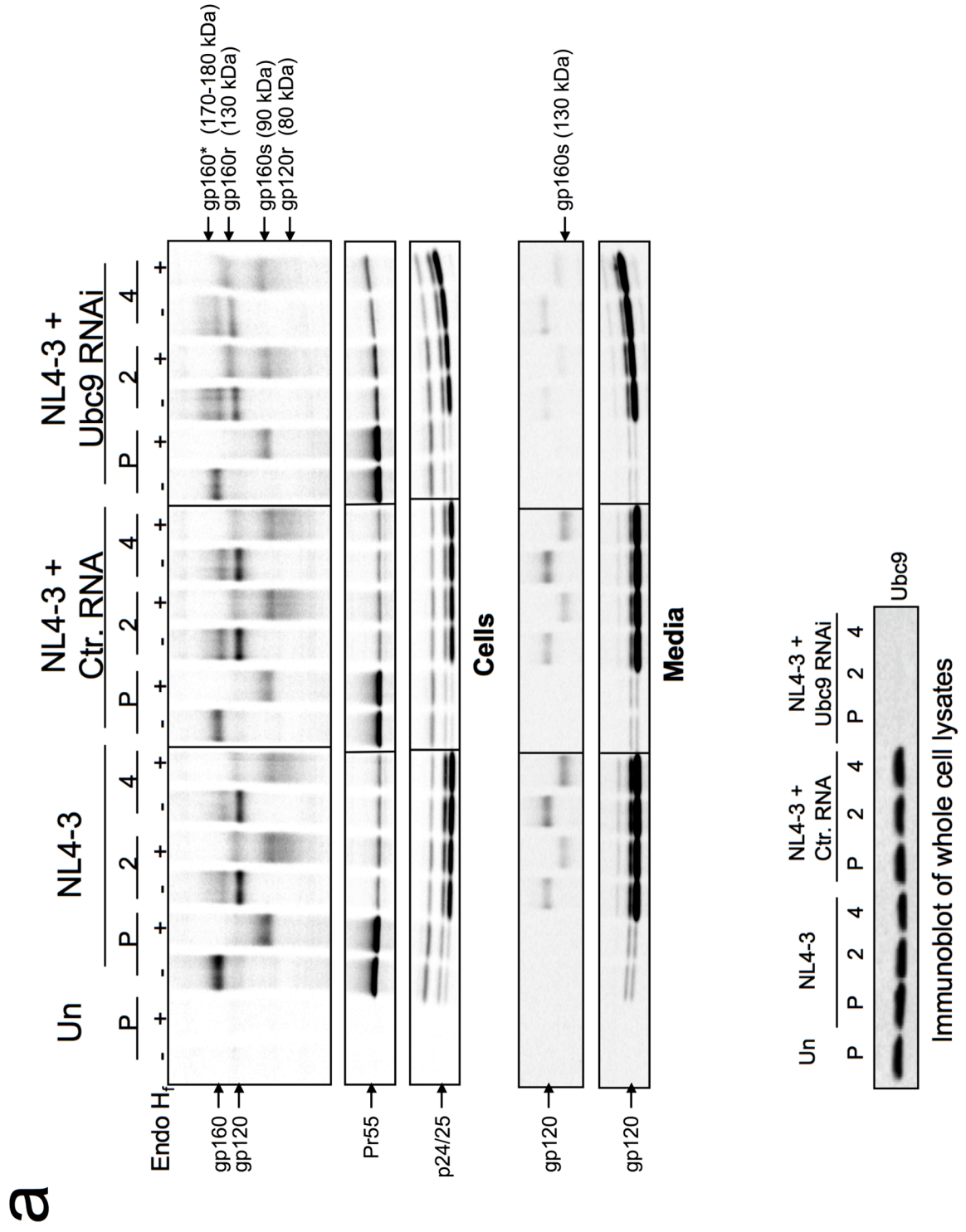

a
Un
NL4-3
NL4-3 + Ctr. RNA
NL4-3 + Ubc9 RNAi
P
2
4
Endo Hf
gp160
gp120
gp160* (170-180 kDa)
gp160r (130 kDa)
gp160s (90 kDa)
gp120r (80 kDa)
Pr55
p24/25
Cells
gp160s (130 kDa)
gp120
gp120
Media
Ubc9
Immunoblot of whole cell lysates

Figure 3 (continued)

b

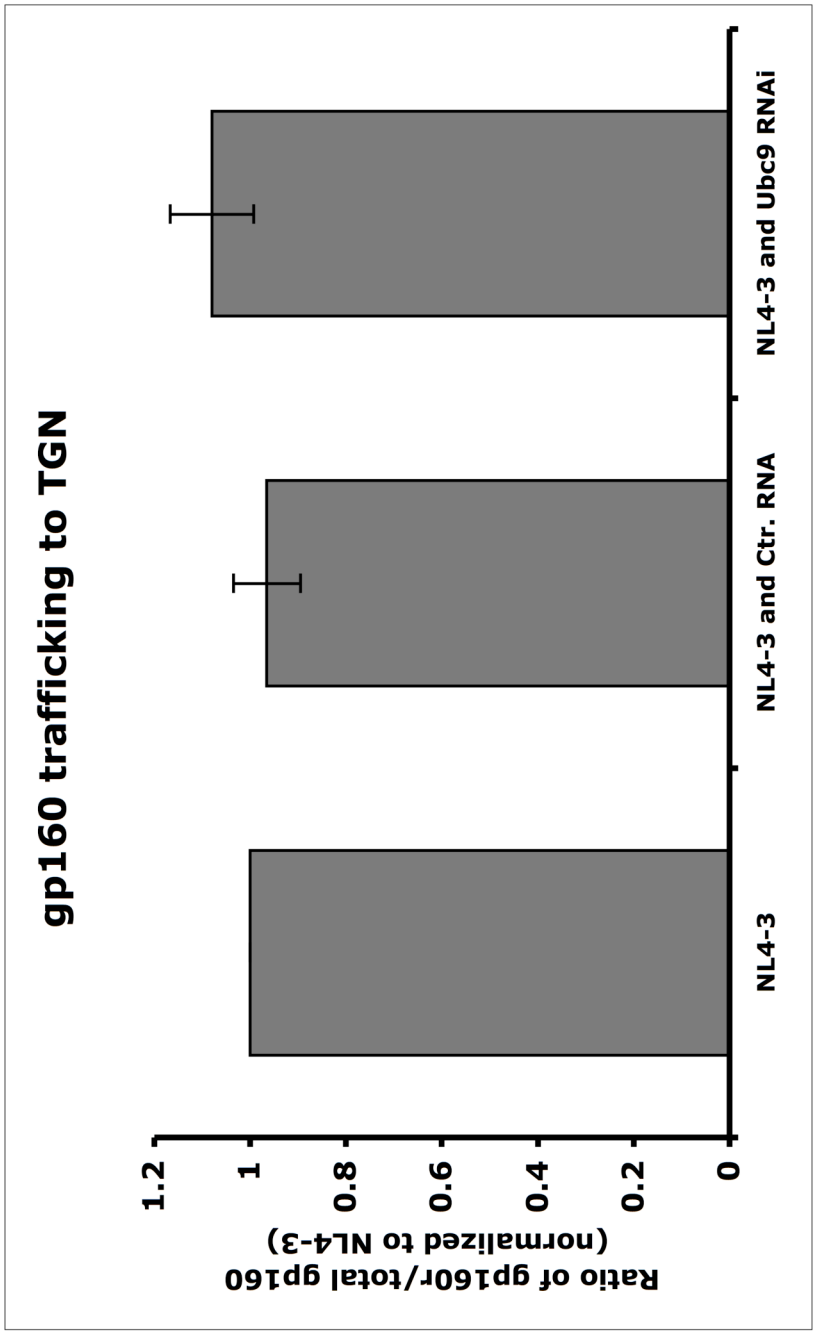


Figure 3: HIV-1 gp160 traffics to the Golgi network in Ubc9 knockdown cells. (a) Env trafficking through the secretory pathway. 293T cells were transfected with pNL4-3 alone, or in combination with either Ctr. RNA or Ubc9 siRNA (Ubc9 RNAi), or left untransfected (Un). Cells were pulse (P) labeled with [^{35}S] methionine/cysteine for 30 minutes and chased for 2 and 4 hours. Cell and media associated viral proteins were solublized and immunoprecipitated with pooled AIDS patient sera, split equally, and incubated for 3.5 hours at 37° C in the presence, or absence of Endo H_f. Samples were separated by SDS PAGE and visualized by phosphorimaging using The Discovery Series Quantity One software. A representative, over-exposed gel is shown so that partially Endo H_f resistant Env can be more easily visualized. Viral proteins and their positions in the gel are labeled on the left. The identity of Endo H_f, untreated viral proteins and their positions in the gel are labeled on the right. Deglycosylated Endo H_f sensitive forms of gp160 residing in the ER are labeled as gp160s. Partially deglycosylated, Endo H_f resistant forms of gp160 and gp120 that have undergone glycan modification in the TGN are labeled as gp160r and gp120r. gp160r in Endo H_f untreated samples is labeled as gp160*. (b) The gp160 trafficking to TGN. The ratio of gp160r/total gp160 during the 2-hour chase period, normalized to NL4-3.

Figure 4

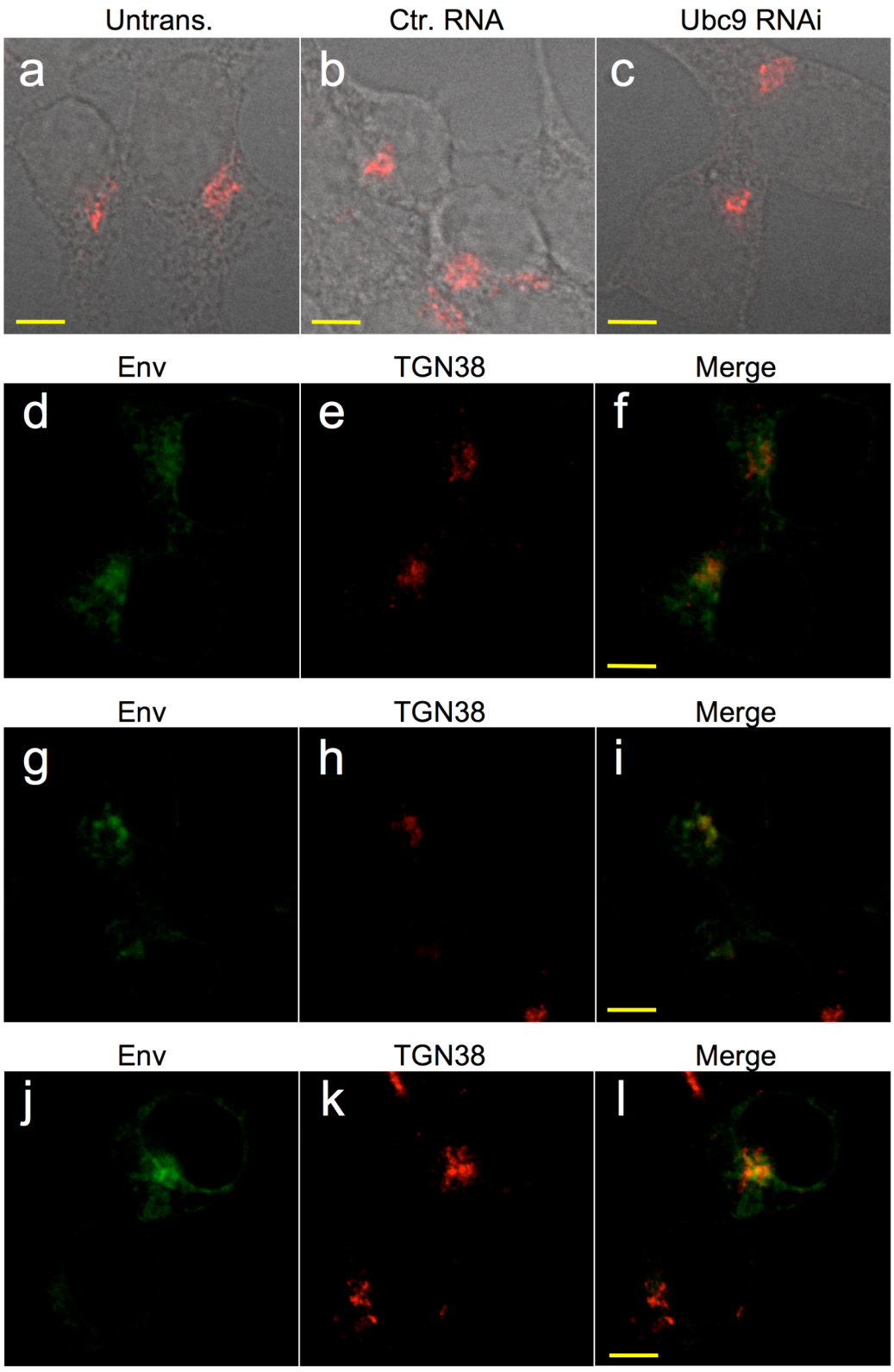

Untrans.
Ctr. RNA
Ubc9 RNAi
a
b
c
Env
TGN38
Merge
d
e
f
Env
TGN38
Merge
g
h
i
Env
TGN38
Merge
j
k
l

Figure 4: HIV-1 Envelope proteins traffic to the TGN in Ubc9 knockdown cells. (a) Untransfected 293T cells, transfected with (b) Ctr. RNA, or transfected with (c) Ubc9 siRNA (Ubc9 RNAi). (d-f) 293T cells transfected with pNL4-3. (g-i) 293T cells transfected with pNL4-3 and Ctr. RNA. (j-l) 293T cells transfected with pNL4-3 and Ubc9 siRNA. Cells were fixed, permeabilized, and prepared for immunofluorescence assays using anti-TGN38 PAb and anti-gp120 MAb. Cy2-conjugated donkey anti-mouse and Cy5-conjugated donkey anti-rabbit antibodies were used as secondary antibodies to visualize subcellular localization by confocal microscopy. Medial, 0.3-μm representative optical z-sections are shown. Scale bars, 10-μm.

Figure 5

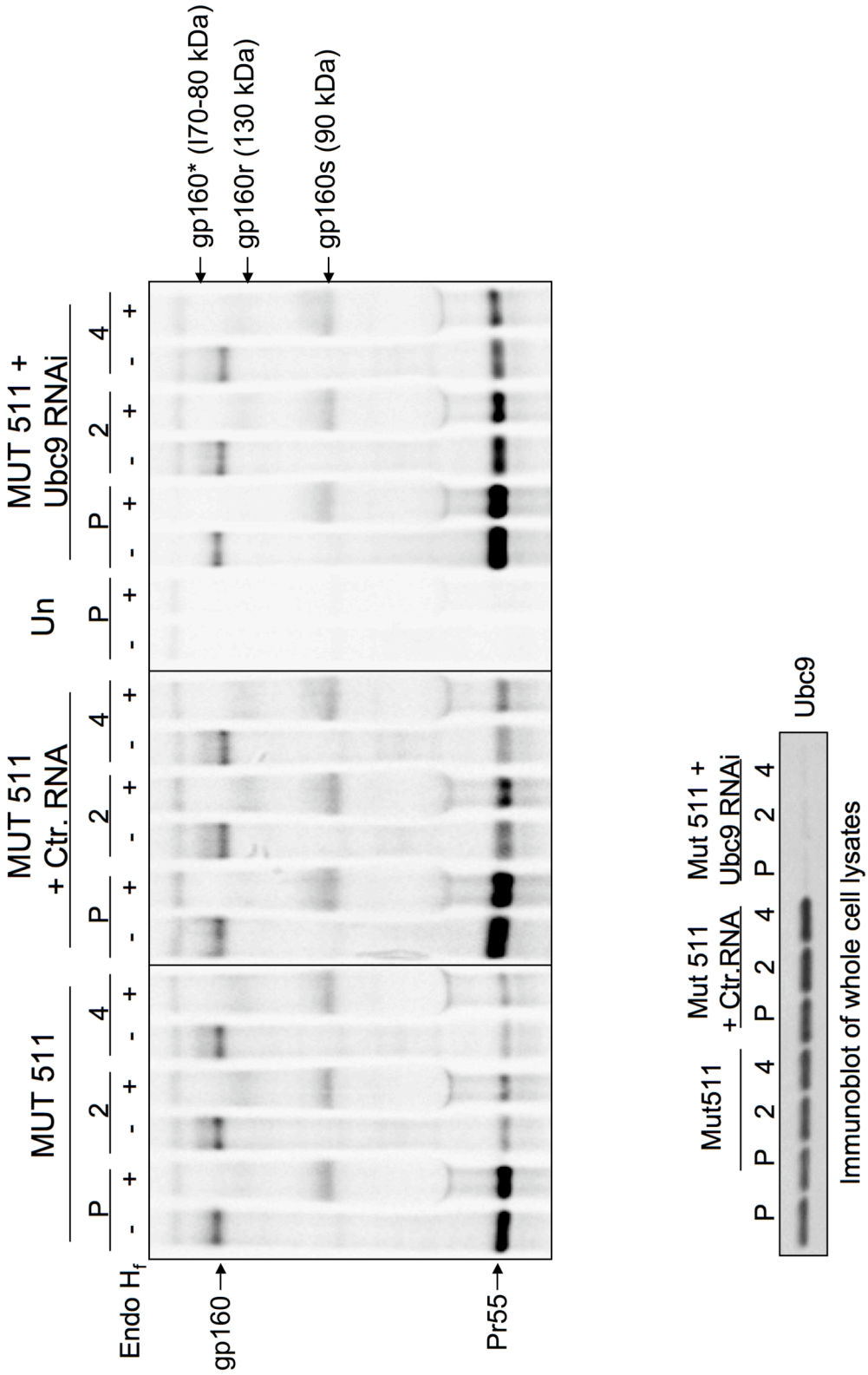

gp160* (l70-80 kDa)
gp160r (130 kDa)
gp160s (90 kDa)
MUT 511
MUT 511 + Ctr. RNA
Un
MUT 511 + Ubc9 RNAi
P 2 4
Endo Hf
gp160
Pr55
Mut511
Mut 511 + Ctr.RNA
Mut 511 + Ubc9 RNAi
Ubc9
Immunoblot of whole cell lysates

Figure 5: Stability of gp160 is unchanged in Ubc9 knockdown cells. (a) 293T cells were transfected with pNL4-3 MUT 511 alone, or in combination with Ctr. RNA or Ubc9 siRNA (Ubc9 RNAi), or left untransfected. Cells were pulse (P) labeled with [^{35}S] methionine/cysteine for 1 hour and then chased for 2 and 4 hours. Cell associated viral proteins were solublized and immunoprecipitated with pooled AIDS patient sera, split equally, and incubated for 3.5 hours at 37° C in the presence, or absence of Endoglycosidase H_f (Endo H_f). Samples were separated by SDS PAGE and visualized by phosphorimaging using The Discovery Series Quantity One software. A representative, over-exposed gel is shown so that partially Endo H_f resistant Env can be more easily visualized. The identity of Endo H_f, untreated viral proteins and their positions in the gel are labeled on the right. Deglycosylated Endo H_f sensitive forms of gp160 residing in the ER are labeled as gp160s. Partially deglycosylated, Endo H_f resistant forms of gp160 that have undergone glycan modification in the TGN are labeled as gp160r. These forms of gp160 denoted as gp160* in Endo H_f untreated samples.

Figure 6

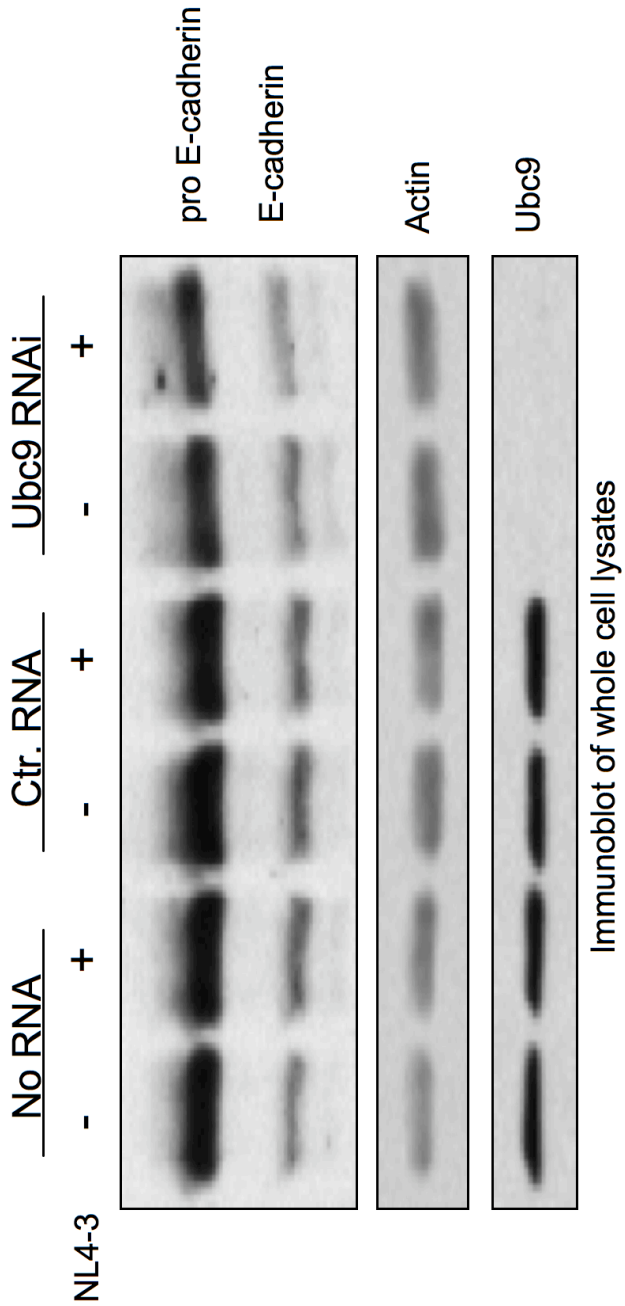

pro E-cadherin
E-cadherin
Actin
Ubc9
No RNA
Ctr. RNA
Ubc9 RNAi
NL4-3
- + - + - +
Immunoblot of whole cell lysates

Figure 6: Furin activity is not dependent upon Ubc9 expression. 293T cells were transfected with pNL4-3 alone, or in combination with either Ctr. RNA or Ubc9 siRNA (Ubc9 RNAi), or left untransfected. Cells were lysed 24 hrs post transfection with pNL4-3, separated by SDS-PAGE and transferred to nitrocellulose. Membranes were immunoblotted with antibodies against Ubc9, Actin, and E-Cadherin. Immature, uncleaved E-Cadherin is designated as pro E-Cadherin.

Figure 7

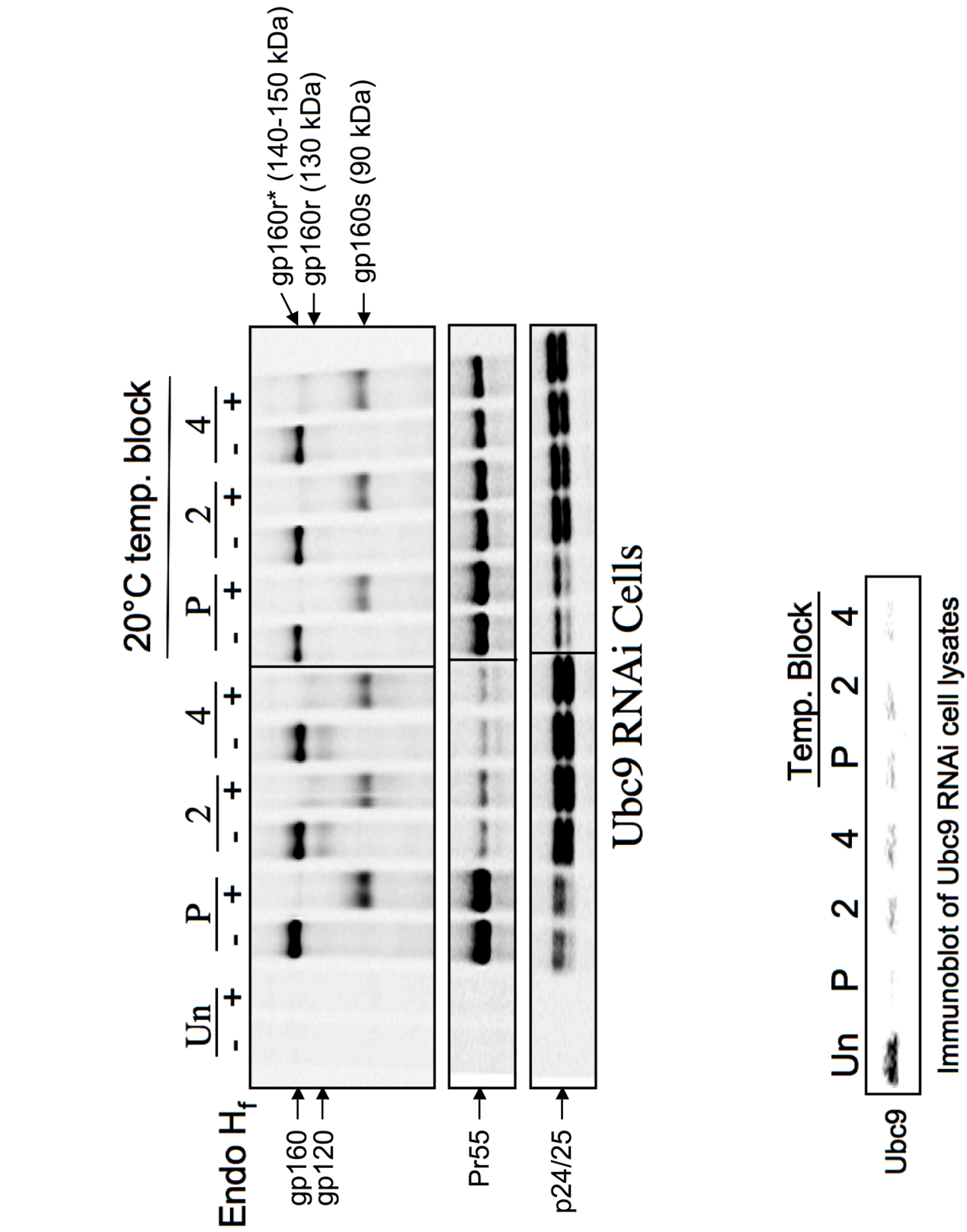

20°C temp. block
Un
P
2
4
gp160r* (140-150 kDa)
gp160r (130 kDa)
gp160s (90 kDa)
Endo Hf
gp160
gp120
Pr55
p24/25
Ubc9 RNAi Cells
Temp. Block
Un P 2 4
Ubc9
Immunoblot of Ubc9 RNAi cell lysates

Figure 7. HIV-1 Env processing and stability in Ubc9 knockdown cells during a TGN trafficking temperature block at 20°C. 293T cells were transfected with Ubc9 siRNA and pNL4-3 as in previous experiments. Cells were pulse (P) labeled with [^{35}S] methionine/cysteine for 1 hour, shifted to 20°C, and chased for 2 and 4 hours. Cell associated viral proteins were solublized and immunoprecipitated with pooled AIDS patient sera, split equally, and incubated for 3.5 hours at 37° C in the presence, or absence of Endoglycosidase H $_f$ (Endo H $_f$). Samples were separated by SDS PAGE and visualized by phosphorimaging using The Discovery Series Quantity One software. A representative, over-exposed gel is shown so that partially Endo H_f resistant Env can be more easily visualized. The identity of Endo H_f, untreated viral proteins and their positions in the gel are labeled on the right. Deglycosylated Endo H_f sensitive forms of gp160 residing in the ER are labeled as gp160s. Partially deglycosylated, Endo H_f resistant forms of gp160 that have undergone glycan modification in the TGN are labeled as gp160r and gp160r*.

Figure 8

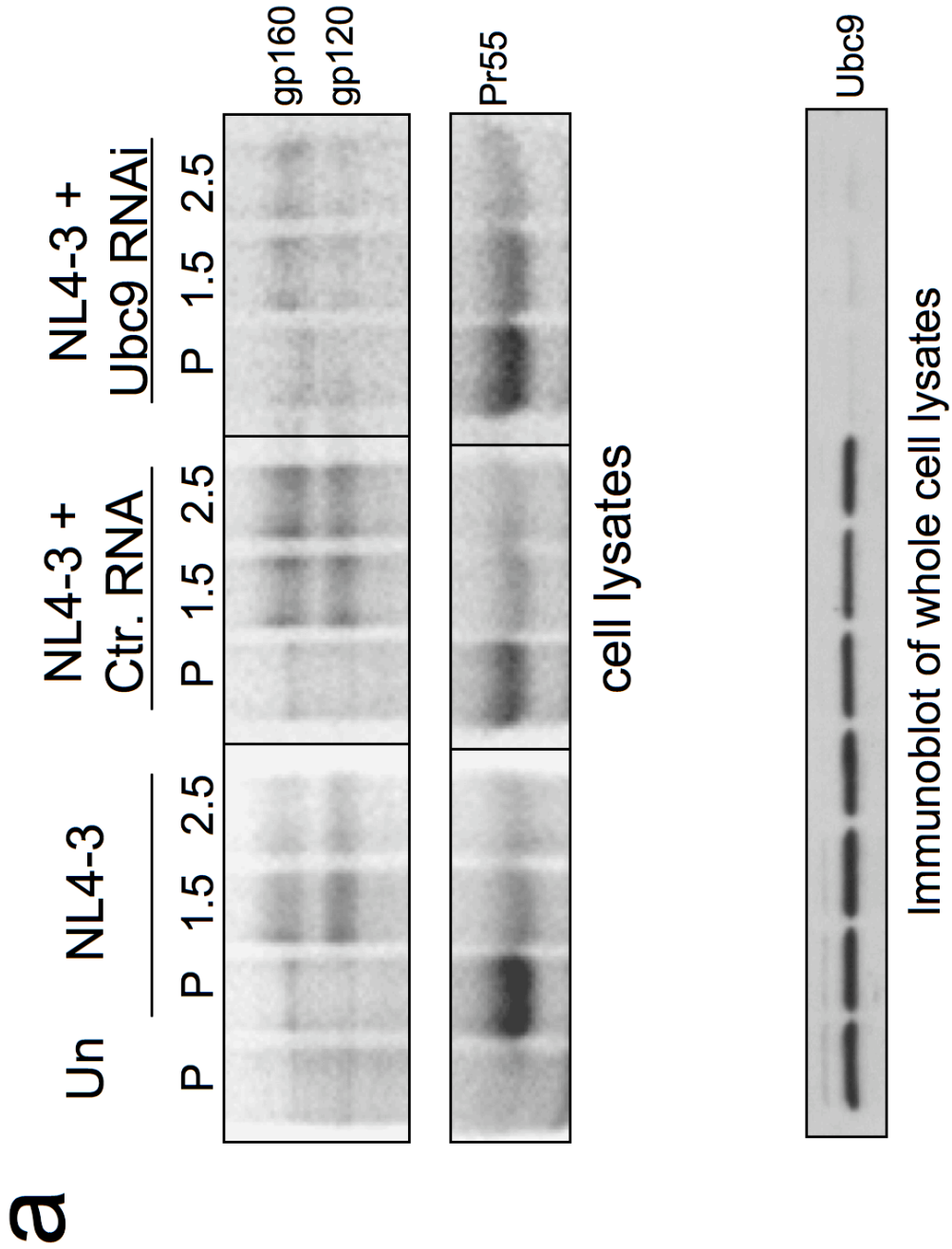

a
Un
NL4-3
P
P 1.5 2.5
NL4-3 +
Ctr. RNA
P 1.5 2.5
NL4-3 +
Ubc9 RNAi
P 1.5 2.5
gp160
gp120
Pr55
cell lysates
Ubc9
Immunoblot of whole cell lysates

Figure 8 (continued)

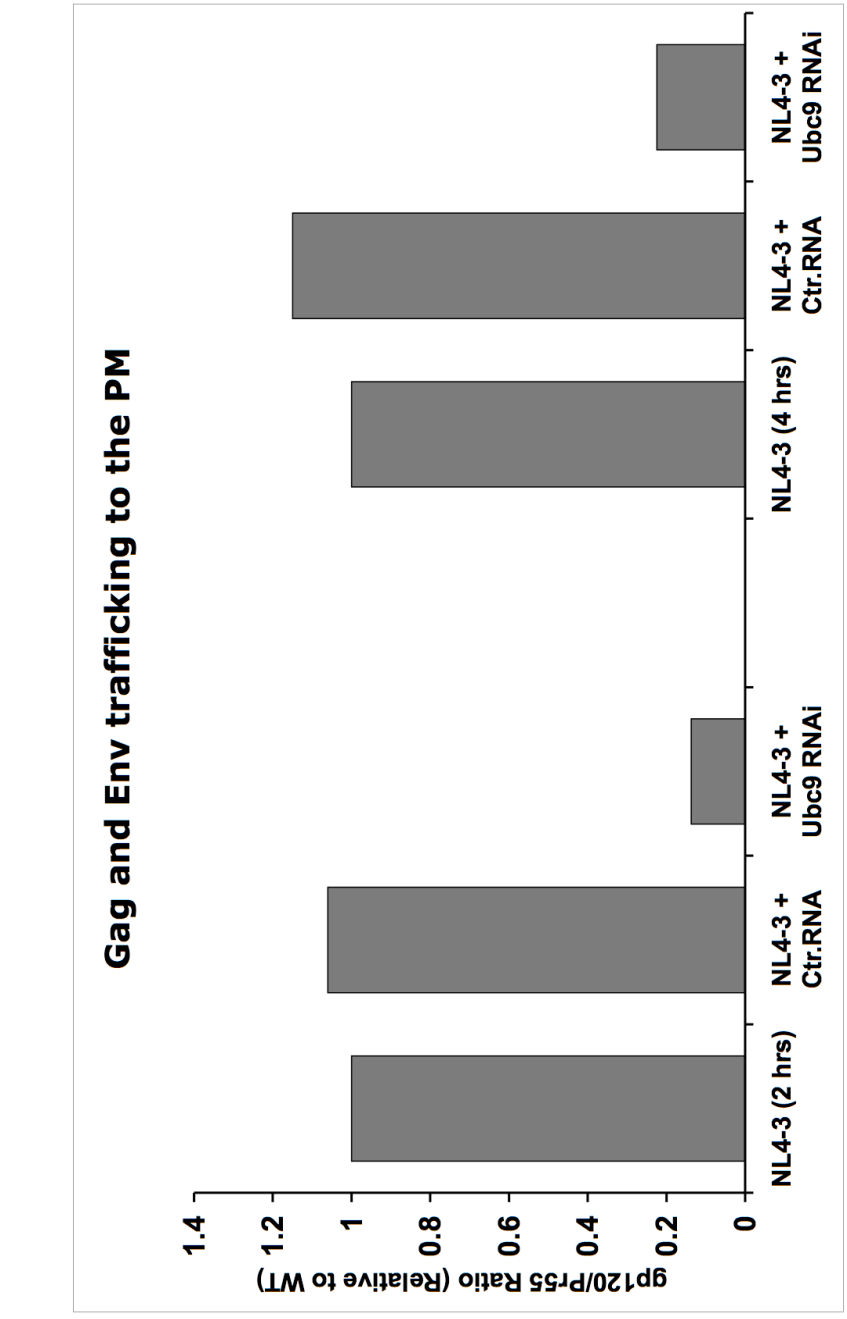


Figure 8: HIV-1 proteins exhibit altered trafficking to the plasma membrane in Ubc9 knockdown cells. (a) 293T cells were transfected with pNL4-3 alone, or in combination with either Ctr. RNA or Ubc9 siRNA, or left untransfected (Un). Cells were pulse (P) labeled with [^{35}S] methionine/cysteine for 30 minutes and chased for 1.5 and 2.5 hours. At indicated times, cells were quickly cooled on ice. Medium was removed from the culture dishes and cells were collected in ice-cold sucrose buffer (16% by weight), ruptured with 100 strokes in a dounce homogenizer, and followed with Benzonase treatment (50 units) for 0.5 hours. Samples were centrifuged at 4°C, for 20 hours at 34,500 RPMs in a ML-130 rotor. Supernatants were carefully collected leaving behind the pellet containing cellular organelles and adjusted to 1X lysis buffer. Viral proteins were immunoprecipitated with patient serum, separated by SDS PAGE, and visualized by phosphorimaging using The Discovery Series Quantity One software. (b) Quantitation of viral proteins associated with the plasma membrane. Pr55/gp120 ratios of the PM fraction during the 2 hour and 4 hour chase times.

Figure 9

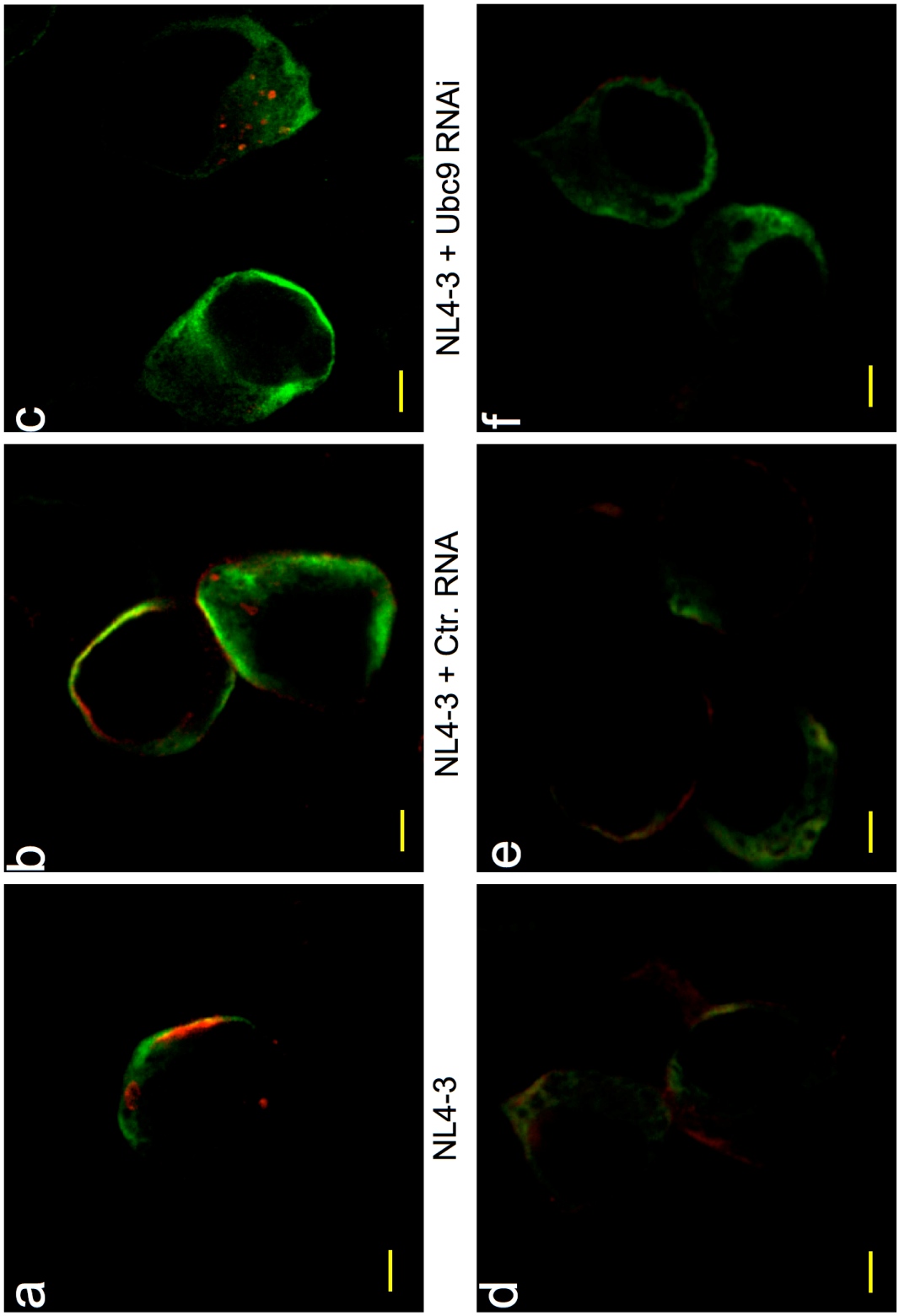


Figure 9: Gag and Env exhibit altered surface staining in Ubc9 knockdown cells. 293T cells were transfected with pNL4-3 alone (a and d), or in combination with either Ctr. RNA (b and e) or Ubc9 siRNA (c and f). Cells were fixed with 4% paraformaldehyde and processed for immunofluorescence using a combination of anti-p17 PAb and anti-gp41 MAb (a-c), or anti-p17 PAb and anti-gp120 MAb (d-f). Cy-2 conjugated donkey anti-rabbit (green) and Cy-5 donkey anti-mouse were used as secondary antibodies. Surface colocalization was visualized by confocal microscopy. Representative 0.3-μm medial sections are shown. Yellow scale bar, 10-μm.

Figure 10

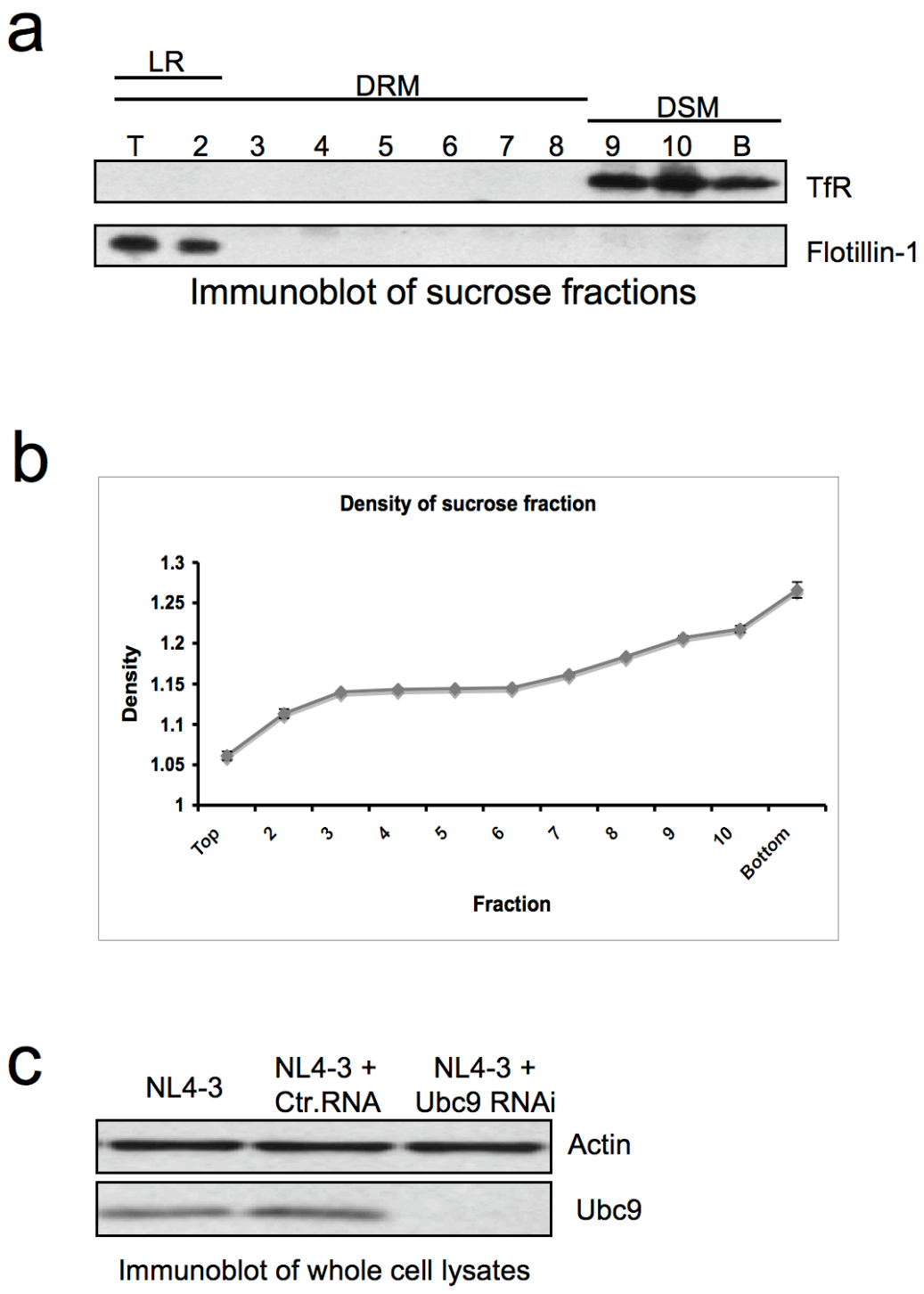

a
LR
DRM
DSM
T 2 3 4 5 6 7 8 9 10 B
TfR
Flotillin-1
Immunoblot of sucrose fractions
b
Density of sucrose fraction
Density
1.3
1.25
1.2
1.15
1.1
1.05
1
Top 2 3 4 5 6 7 8 9 10 Bottom
Fraction
c
NL4-3
NL4-3 + Ctr.RNA
NL4-3 + Ubc9 RNAi
Actin
Ubc9
Immunoblot of whole cell lysates

Figure 10 (continued)

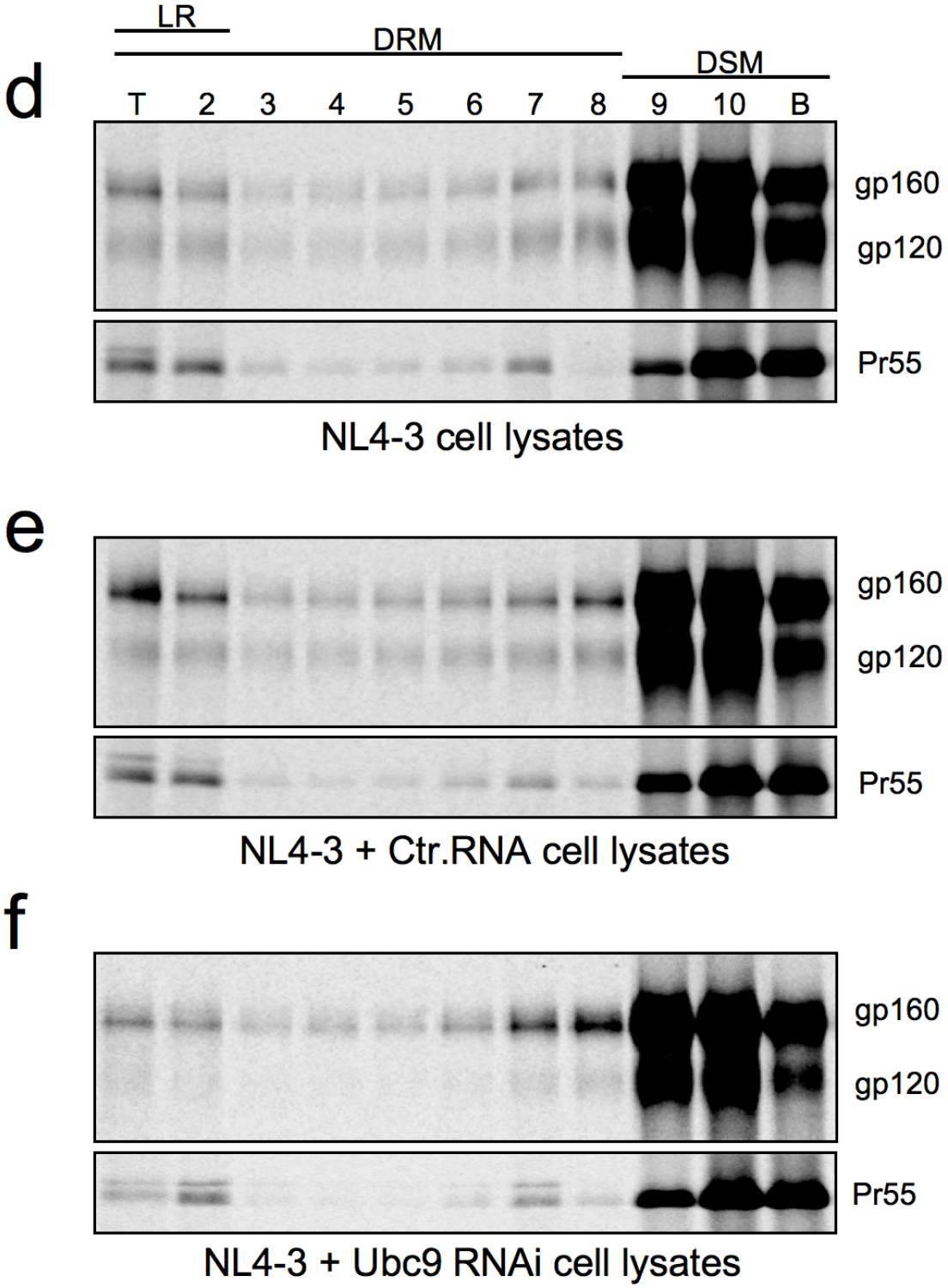
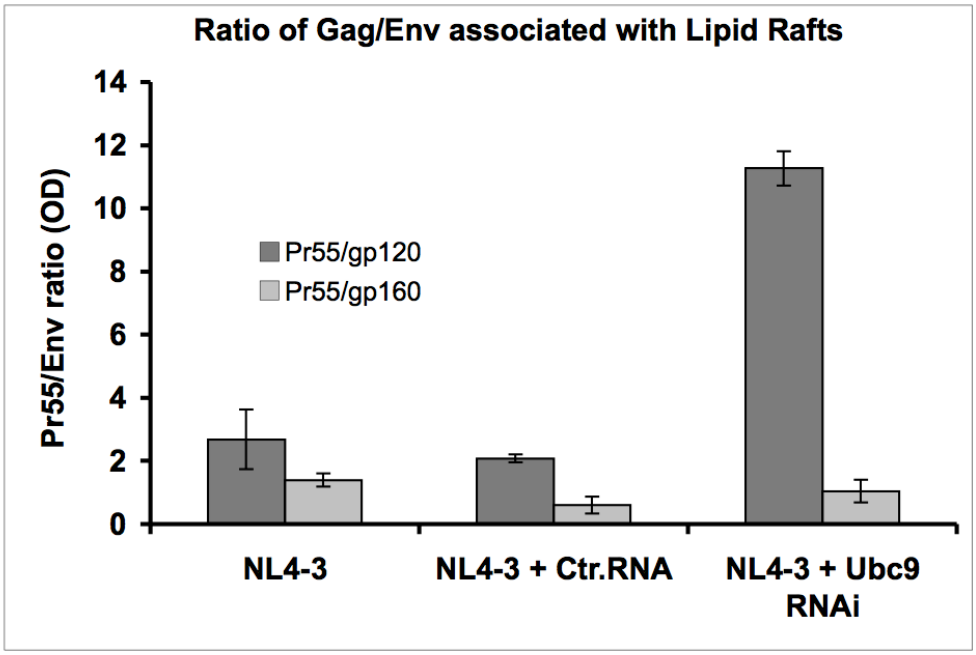


Figure 10 (continued)

g



h

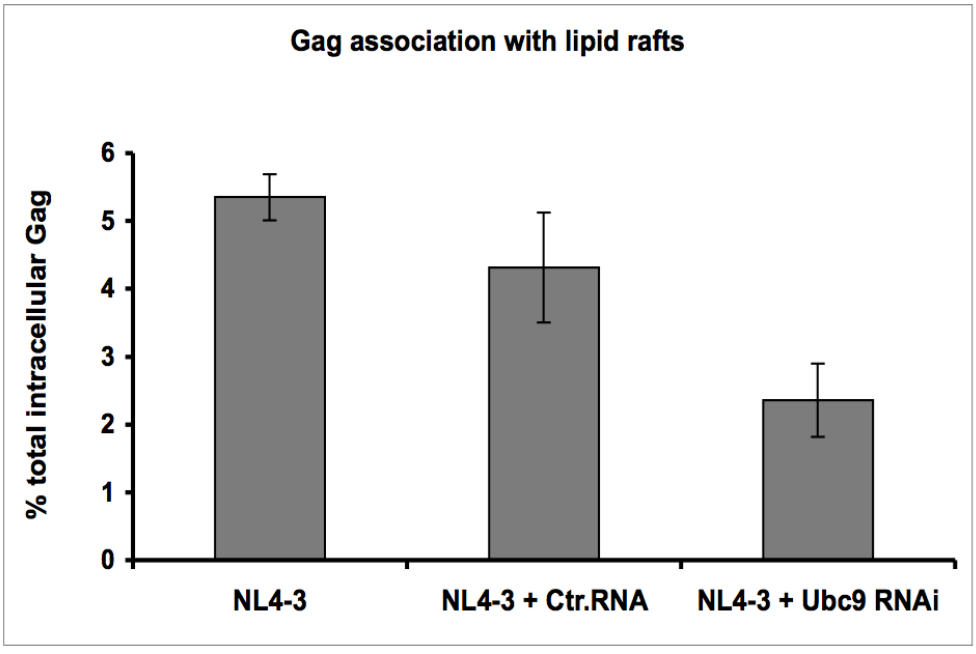


Figure 10: HIV-1 Gag and Env exhibit altered association with lipid rafts in Ubc9 knockdown cells. (a) Lipid raft isolation. Representative immunoblot of sucrose fractions for lipid raft markers Flotillin-1 and non-raft marker transferrin receptor 1 (TfR). Fractions containing lipid raft markers (LR), detergent soluble membranes (DSM; raft excluded), and detergent resistant membranes (DRM; non DSM). (b) Average density of all sucrose fractions prior to being adjusted to 1X lysis buffer. (c) immunoblot of transfected cell lysates. 293T cells were transfected with pNL4-3 alone (d), or in combination with either Ctr. RNA (e) or Ubc9 siRNA (f). Cells were labeled with [^{35}S] methionine/cysteine for 4 hours. Cells were placed on ice and transferred to a 4°C cold room where they were lysed with ice cold TNE buffer for 30 minutes on ice followed by homogenization with a 25G needle. Lysates were clarified in a cooled micro centrifuge and then adjusted to 60% sucrose and overlaid with a discontinuous sucrose gradient and centrifuged at 100,000 X g at 4C for at least 18 hrs. Gradients were fractioned into 11 equal samples using a Biocomp piston fractionator and adjusted to 1x lysis buffer. Viral proteins were immunoprecipitated with patient serum, separated by SDS PAGE, and visualized by phosphorimaging using The Discovery Series Quantity One software. Lipid raft floatation experiments were carried out in triplicate. Representative, over-exposed gels are shown so that viral proteins associated with lipids rafts can be more easily visualized. (g) Ratio of Gag and Env proteins associated with lipid rafts. (h) Percent of total cellular Gag associated with lipid rafts.

Figure 11

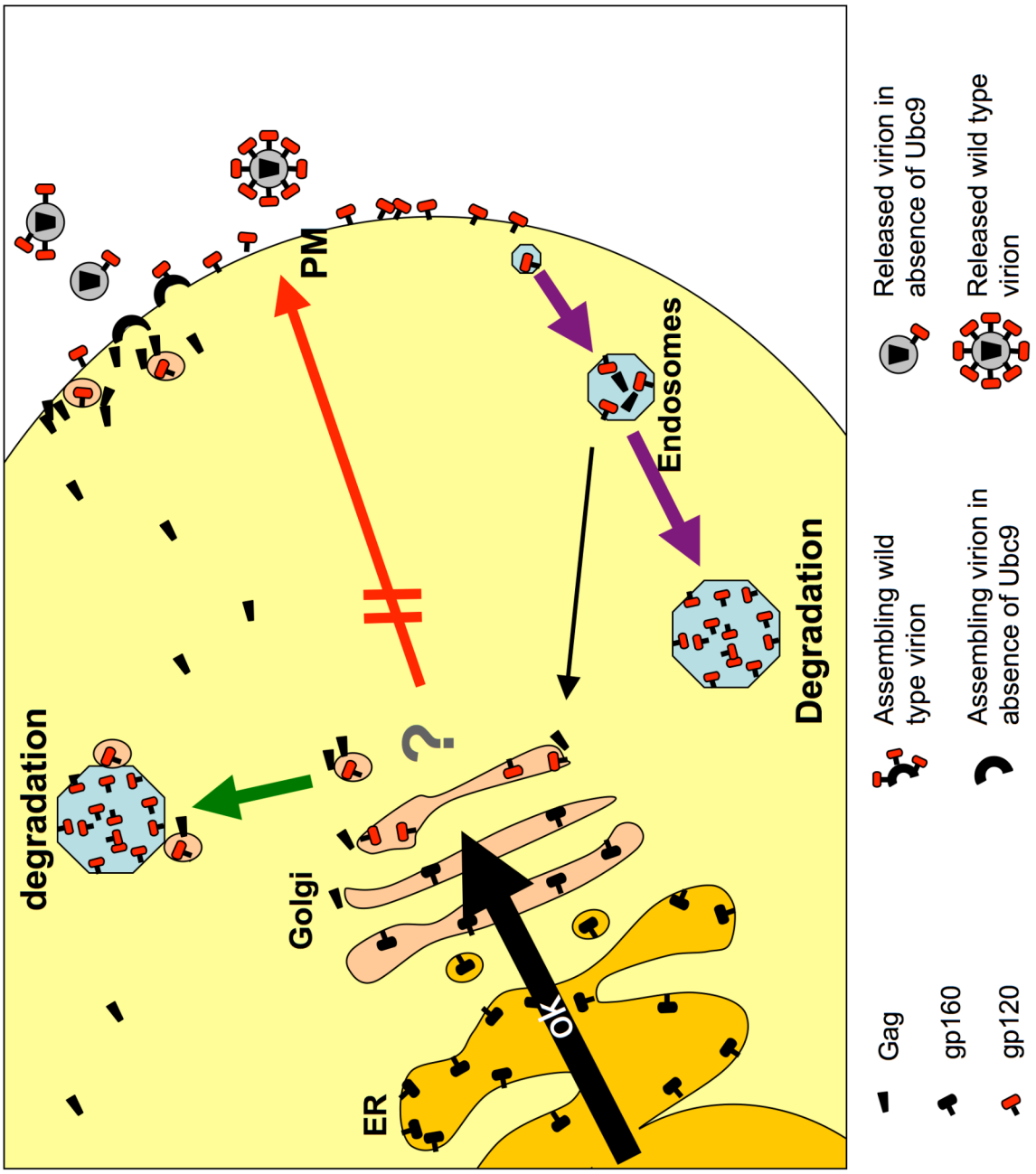

PM
Endosomes
Degradation
degradation
Golgi
ER
OK
?
Gag
gp160
gp120
Assembling wild type virion
Assembling virion in absence of Ubc9
Released virion in absence of Ubc9
Released wild type virion

Figure 11. Models for mature Env degradation in Ubc9 knockdown cells. In Ubc9 knockdown cells HIV-1 Env trafficking from the ER to the TGN is normal (black arrow). After cleavage into gp120/gp41 and transport out of the TGN, Env may be directly targeted for degradation instead of the assembly site at the PM. Mistrafficking of Env for degradation could be due to altered interactions with Gag, as Gag itself is mistrafficked to the assembly site in Ubc9 knockdown cells (green arrow). Another potential mechanism could be due to Gag being mistrafficked to the assembly site in Ubc9 knockdown cells, Gag may not stabilize Env at the PM and mature Env may be quickly endocytosed. In Ubc9 knockdown cells, a portion of endocytosed Env may then be targeted for degradation instead of recycling back to the TGN (violet arrows). Alternatively, the trafficking or function of yet unknown cellular factor(s) that are involved in Env stability and packaging into assembling virions may have been disrupted by altering Gag/Ubc9 interactions. (gray question mark).

Chapter IV

Mature HIV-1 Env is degraded through the lysosomal pathway in Ubc9 knockdown cells.

Unpublished data

Abstract

We previously have shown that Gag and Ubc9 are important for mature intracellular Env stability. We found that in Ubc9 knockdown cells, Env underwent increased degradation at an intracellular site prior to insertion into the plasma membrane, but only after Env was trafficked out of the trans-Golgi network and cleaved. We have continued to examine the effect that Ubc9 knockdown has on intracellular Env stability to further understand the underlying mechanism. Env stability in Ubc9 knockdown cells was examined and compared to control cells in the presence or absence of specific inhibitors of known cellular pathways that lead to degradation. We found that the proteasome inhibitor MG132, or the clathrin-dependent endocytosis inhibitor chlorpromazine (CPZ), did not restore intracellular gp120 levels to those of control cells. Intracellular gp120 levels were restored to that of control cells with the treatment of Ubc9 knockdown cells with a combination of lysosome inhibitors; however, inhibition of gp120 degradation did not restore Env packaging into released virions. Since lysosomal degradation is linked with autophagy, we examined autophagy in Ubc9 knockdown cells and found that autophagy was increased approximately 3-fold when compared to control cells. Commonly used autophagy inhibitors were not able to inhibit autophagy in Ubc9 knockdown cells and it is not clear whether intracellular mature Env is degraded in the lysosome through an autophagy dependent or independent mechanism.

Introduction

The HIV-1 assembly pathway is a complex web of viral and cellular interactions that makes use of a wide range of cellular pathways to traffic the essential viral components and correctly assemble then into immature virions on the plasma membrane of HIV-1 producer cells. The assembly process culminates in the release and maturation of nascent infectious HIV-1 particles capable of infecting new target cells. During virus assembly, various viral proteins have been shown to evade or use the host degradative pathways to their advantage in order to complete virion assembly and release of infectious virions. One such pathway is the ubiquitin-dependent pathway.

The ubiquitin dependent proteasome pathway has been shown to be an important pathway utilized by the HIV-1 in multiple ways. The proteasome pathway is the predominant ubiquitin-dependent degradation pathway of short-lived proteins in eukaryotic cells. The proteasome functions in both the cytoplasm and nucleus. The 26S proteasome is a large multi-subunit complex that degrades proteins that are damaged, misfolded, or unneeded by the cell. The covalent attachment of polyubiquitin chains to a target protein is the molecular signal for degradation by the proteasome. Ubiquitin is a small globular protein and its covalent addition to target proteins is regulated by the E2-Ubiquitin conjugating enzymes and the E3-Ubiquitin ligases. The E2 and E3 bind with the target protein, E3 then covalently transfers the ubiquitin from the E2 to the target protein and the transfer continues until a chain of multiple ubiquitins is formed, the ubiquitinated protein is then targeted to the proteasome for degradation. Ubiquitination and degradation occur constitutively at a basal level in a highly regulated manner. However, this process can be upregulated rapidly in a response to various stimuli, such as

during the cell cycle, cell stress and damage, and virus infection and replication (reviewed in 207, 302, 368).

It has been shown that blocking the ubiquitin dependent proteasome pathway with inhibitors led to defects in Gag maturation and release at the late stage of budding (331). Gag is known to be ubiquitinated, and the ubiquitin ligase POSH has been shown to play an important role during assembly (8). However, the exact mechanism of how ubiquitin regulates the late stages of assembly is not yet fully understood, although it has been shown to involve the endosomal sorting pathway (reviewed in 240). In addition, there are HIV-1 accessory proteins such Vif and Vpu that have been shown to utilize the proteasome pathway. Vif has been shown to utilize the proteasome pathway to degrade apoplipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G (APOBEC3G) to limit its packaging into the released virions (346). APOBEC3G is a host restriction factor that blocks HIV-1 replication at multiple steps during early events (reviewed in 127). Vpu has been demonstrated to utilize the proteasome to degrade CD4 in the ER to prevent CD4 expression on the cell, and to prevent premature interactions of CD4 with HIV-1 Env as it passes through the secretory pathway (239, 330).

In addition to the proteasome pathway, the endocytic/endosomal pathway is also an essential cellular sorting and degradation pathway. It is involved in the transport of vesicles and their contents through the cytoplasm, and has been implicated to play essential roles during HIV-1 assembly. The endocytic/endosomal pathway includes a set of functionally distinct membrane vesicles that are involved in the sorting and trafficking of various non-cytoplasmic proteins. The vesicles involved in the endosomal pathway can be broadly classified as early endosomes, recycling endosomes, multivesicular

bodies, and late endosomes. These vesicles are classified by shape, intracellular location, and the presence of specific host factors/markers. Proteins in the endocytic pathway can be targeted to recycling endosomes and trafficked back to the TGN or plasma membrane, to their intracellular sites of function, to be sequestered in storage vesicles, or they can be trafficked to late endosome and/or lysosomes for degradation (reviewed in 69, 70, 177, 361, 390). Proteins can enter the pathway either through endocytosis from the cell surface or they can be trafficked directly into the endocytic pathway from the trans-Golgi network (TGN). Numerous cellular factors are involved with the pathway, and function by regulating the assembly of vesicles, selecting their contents/cargo, trafficking of the vesicle through the cell, and vesicle-vesicle fusion.

The HIV-1 Gag protein has been shown to localize to various endosomal compartments and utilize the endosomal pathway during assembly (93, 172, 253). Virion assembly is dependent upon a large number of cellular factors involved in the endosomal pathway. For example, Rab9 and Rab11a are proteins involved with protein sorting from the late endosome to TGN, and then to plasma membrane (260). The heterotetrameric adaptor protein complexes AP-1, AP-2, and AP-3 that are involved with protein sorting between organelles, have been found to bind the matrix domain within Gag, and are involved with Gag transport to the assembly site and budding from the cell surface (19, 51, 93). In addition, TSG101, AIP1, and various proteins involved with endosomal sorting complex required for transport (ESCRT complex) have been shown to be essential in virus budding from the cell surface (122, 347, reviewed in 27, 66, 340). The kinesin family of motor proteins, involved with vesicular movement across microtubules have also been shown to play an important part in Gag trafficking and assembly (15, 41,

242). The endocytic pathway has been also shown to interact with the cytoplasmic tail of Env, which is involved with trafficking Env through the cell. AP-1, AP-2, AP-3, and Tip47 were found to interact with the cytoplasmic tail of Env to regulate trafficking to the plasma membrane, endocytosis, and recycling back to the TGN (21, 29, 271). The Nef accessory protein also interacts with and alters the protein trafficking and sorting pathways resulting in the down regulation of CD4 and MHC-I through degradation in the lysosome (reviewed in 312). Vpu has been shown to antagonize tetherin, another host restriction factor involved with blocking virion release at a very late state of budding. Vpu binds tetherin and targets it for degradation in the lysosome, however the mechanism is not fully elucidated and the proteasomal degradation has also been implicated to be involved (94, 158, 237).

The third pathway involved in protein degradation is autophagy, which has also been shown to play a role in HIV-1 assembly. Autophagy is normally induced in response to cellular stress or activation of the cell signaling pathways. Unregulated autophagy activation is believed to eventually lead to apoptosis. Autophagy is a degradation/recycling pathway that degrades long-lived proteins, damaged organelles, and cytoplasm. Old or damaged organelles and the surrounding cytoplasm are engulfed in a double, or multi-membrane vesicle known as the autophagosome, which fuses with lysosome to form the autophagolysosome. The contents of the autophagolysosome are degraded and recycled for anabolic cellular metabolism. The formation of an autophagic vacuole requires a sequential cascade of various protein-protein interactions and protein-lipid interactions leading to the formation of the double membraned autophagic vacuole. The upregulation of autophagy is commonly initiated by cellular stress through inhibition

of mTOR kinase activity, which activates the phosphatidylinositol 3-kinase (PI3K) and Atg1/ULK complex at the phagophore assembly site followed by phagophore elongation. Phagophore elongation involves a series of steps where Atg12 is conjugated to Atg5, which then forms a complex with Atg6 at the edges of the elongating phagophore. During this time, LC3-I is lipidated by Atg4, forming LC3-II to associate with the elongating phagophore. LC3-II levels correlate well with the induction and formation of autophagosomes and it is commonly used as a marker for autophagy (178). The phagophore elongates and curves around to form an enclosed vesicle forming the double membraned autophagosome, which fuses with the lysosome for degradation of the internal contents of the autophagosome. (autophagy reviewed in 95, 230, 231, 252, 289). Autophagy is highly regulated and is constitutively active at a basal level in cells.

HIV-1 Gag and Nef have been shown to interact with LC3 and Beclin-1 respectively, which are important components in the autophagy pathway. It was demonstrated that when autophagy was blocked through the use of inhibitors, or siRNA against autophagy structural components, there was decreased virion assembly and infectivity. In addition, Nef was shown to interact with Beclin-1 and block the fusion of the autophagosomes with the lysosome to inhibit autophagic degradation. It was hypothesized that the formation of autophagic vacuoles were important for increased levels of assembly and release of infectious HIV-1 virions (201). However, the exact role of autophagy in affecting HIV-1 infection is still not clear, as Zhou and Spector have demonstrated that HIV-1 infection leads to decreased autophagy in target cells (406).

We have previously shown that in Ubc9 knockdown cells, intracellular mature Env levels (gp120/gp41) were decreased in a Gag dependent mechanism. We found that

the decreased intracellular levels of mature Env was not due to altered gp160 trafficking or stability in Ubc9 knockdown cells, but due to selective degradation of the mature Env after it trafficked out of the TGN. We also found that both Gag and mature Env showed altered trafficking and association with the plasma membrane and lipid rafts, which are domains found to be important for virion assembly (reviewed in 273, 370). This led us to hypothesize that Env is mistargeted for degradation, through an unidentified pathway, potentially due to altered Gag and Env interactions within the cell, either at the assembly site on the plasma membrane or at an intracellular site where Gag and Env co-trafficked. We hypothesized that the altered Gag and Env interactions could be due to an altered intracellular Gag trafficking as a result of interrupted Gag/Ubc9 interaction in the knockdown cells.

In this study we have examined the functional role of Ubc9 during HIV-1 assembly. Using a wide variety of degradation inhibitors, we systematically examined which degradative pathway(s) are responsible for mature Env degradation in Ubc9 knockdown cells using pulse chase experiments. We have shown that MG132 (26S proteasome inhibitor), chlorpromazine (CPZ) (endocytosis inhibitor), 3-MA and wortmannin (autophagy inhibitors) were unable to restore intracellular Env levels. However, a combination of E63d, Pepstatin A, and Leupeptin (lysosome) were able to restore intracellular gp120 to wild type levels, indicating that the lysosome is the predominant pathway that degrades mature Env in Ubc9 knockdown cells. We also observed that LC3-II levels were increased approximately 3-fold in Ubc9 knockdown cells compared to control cells, suggesting that Ubc9 may also play a role in autophagy.

However, whether increased autophagy played an active role in Env degradation and lysosomal degradation of Env needs to be further elucidated.

Materials and Methods

Cell culture and transfections. 293T cells were cultured and transfected as previously described (159).

Immunoblotting. Cells were lysed directly in lysis buffer as previously described (159). Total protein content from whole cell lysates, or post-immunoprecipitation cellular lysates were normalized by BCA assays (Thermo) and equal amounts of total protein were solublized in 2x protein sample buffer (PSB) unless other wise described, separated by SDS PAGE, and transferred to nitrocellulose (GE Water & Process Technologies). Immunoblotting was performed as previously described (159), or with a LI-COR Odyssey infrared imaging system using the appropriate IRDye conjugated secondary antibodies for detection.

Antibodies. Anti-Ubc9 (N-15) goat polyclonal antibodies (PAb), anti-actin goat PAb, HIV-1 anti-gp41 (10E9) MAb, horseradish peroxidase (HRP)-conjugated chicken anti-goat PAb were purchased from Santa Cruz Biotechnology, Inc. Anti-LC3B PAb were purchased from Abcam. IRDye 800CW conjugated donkey anti-goat PAb were purchased from LI-COR. Pooled AIDS patient sera were obtained from a patient cohort.

Metabolic Labeling, immunoprecipitation, and Endoglycosidase treatments. Metabolic labeling experiments were carried out as previously reported but with different metabolic labeling times with [^{35}S] methionine/cysteine (>1,000 Ci/nMol; NEN) (159). Briefly, transfected and untransfected 293T cells were pulse labeled for either 30 minutes or 1 hour with 300μCi and 600 μCi respectively. For pulse chase experiments, labeling

medium were removed and chased for 2 and 4 hours in complete culture medium. The culture medium was removed, clarified by centrifugation (13,000 RPM for 2 min), and adjusted to 1x lysis buffer (320). Cells were lysed with 1X lysis buffer containing Halt protease inhibitors cocktail (Pierce) and clarified by centrifugation. Viral proteins were immunoprecipitated from the culture supernatant and cell lysates, using AIDS patient sera and ultralink A/G beads (Thermo Scientific). The beads were washed 4X with lysis buffer. Following the final wash, the viral proteins were eluted from the beads by boiling for 5 minutes in 0.04% SDS and 200mM 2-mercapitoethanol (319), split into two equal volumes, and treated with Endo H_f (NEB) per manufacturers suggestions for 3.5 hours, or left untreated. All samples were adjusted to 1X PSB, analyzed by SDS-PAGE, and the proteins were visualized by phosphor imaging. Alternatively, viral proteins were solublized directly from the A/G beads using 2X PSB, boiled, separated and examined as above.

Inhibitor treatments. Transfected and untransfected cells were treated with various protease inhibitors: proteasome inhibitor MG132 (10 μM), endocytosis inhibitor Chlorpromazine (CPZ) (10μg/ml), autophagy inhibitors 3-MA (10mM) or wortmannin (50 nM), and lysosome inhibitors E63d (10 μg/ml), Pepstatin A (10 μg/ml), Leupeptin (5 μg/ml), or NH_4Cl (20 mM). All inhibitors were purchased from Sigma. Cells were pretreated with the appropriate inhibitors for various times prior to metabolic labeling and maintained throughout the pulse-chase experiment. Pulse-chase samples were processed and analyzed as above.

Results

Intracellular gp120 in Ubc9 knockdown cells was not degraded through the proteasome pathway. We have previously shown that in Ubc9 knockdown cells there was a defect in HIV-1 assembly that led to the production of virions with lower specific infectivity. We had determined that the defect in infectivity was due to an increase in intracellular degradation of mature Env (gp120/gp41). To further understand how Env stability is affected in Ubc9 knockdown cells, we examined whether any of the predominant degradation pathways are involved. For these experiments we examined which inhibitors impeded the degradation of mature Env in an effort to determine which degradative pathway was involved with Env degradation in Ubc9 knockdown cells.

To examine the potential participation of the proteasomal degradation pathway, MG132 (10μM) was used to inhibit the ubiquitin dependent 26S proteasome, and Env stability was assayed by pulse-chase experiments. We began by examining the proteasome as it has been previously demonstrated that the proteasomal pathway plays a crucial role during virion assembly and release (331). Schubert et al. found that when the proteasomal degradation pathways were disrupted in HIV-1 producing cells, the amount of virions released into the media was decreased by approximately 3-fold and the released virions were up to 50-fold less infectious. Interestingly, no significant changes in Env maturation or stability was observed in the presence of the inhibitors, even though it has been reported by Bultmann et al. that a small fraction of Env is ubiquitinated and is degraded through the proteasome pathway (47). In cells transfected with Ubc9 siRNA and NL4-3, in the presence of MG132, Gag displayed an assembly phenotype similar to what was described by Schubert et al. when cells were treated with proteasome inhibitors

(Fig. 1). Less Env and Gag proteins were expressed. Gag processing was slightly slower than in the untreated cells, less cell associated p24/25, and virion release was decreased by approximately 3.5-fold in the presence of MG132 (data not shown). However, no alteration in the Endo H resistant forms of gp160 (gp160r) or gp120 (gp120r) was observed, indicating that the viral glycoproteins trafficked out of the endoplasmic reticulum (ER) and to the TGN where the high mannose sugars were modified into complex forms. Env expression levels did not appear to be altered in the presence of MG132, suggesting that the proteasome may not be the major degradation pathway involved in affecting the stability of intracellular gp120 in the absence of Ubc9.

Endocytosis inhibitor chlorpromazine did not restore intracellular Env levels in Ubc9 knockdown cells. We previously hypothesized that Env was degraded before reaching the plasma membrane, however we could not exclude the possibility that the increase in Env degradation could be through an upregulation of the endocytosis pathway. To examine the possibility that an increase in endocytosis was the cause of decreased Env levels, we examined Env trafficking and stability in the presence of chlorpromazine (CPZ). The CPZ was shown to inhibit clathrin-dependent endocytosis by disrupting AP-2 and clathrin at the plasma membrane (reviewed in 157). Transfected 293T cells were treated with CPZ (10μg/ml) for 2 hours prior to and during the pulse chase experiment. Interestingly, less intracellular p24/25 was detected in CPZ treated cells (Fig. 2) without significant changes in intracellular Pr55 or p24 levels in the culture supernatants when compared to untreated cells. We observed a decrease in intracellular p24/25 levels regardless of whether cells were transfected with control RNA or Ubc9 siRNA (data not shown). This suggests that a portion of the intracellular p24/25 levels

were degraded when endocytosis was disrupted by treatment with CPZ. We did not observe an increase in virion release as reported by Molle et al., when they used monensin or a dominant-negative dynamin to block the endocytic pathway (253). However, the use of different cell lines and methods of inhibition are possible reasons for the discrepancies. Unexpectedly, in CPZ treated Ubc9 knockdown cells, very little gp160 cleavage was observed in cells transfected with either Ubc9 siRNA (Fig. 2) or control RNA (data not shown) during the duration of the chase. In addition, no Endo H_f resistant forms of gp160 or gp120 could be detected throughout the chase after treatment with CPZ, which suggested that gp160 did not traffic out of the ER to the TGN. We did however, detect a band that migrated slightly faster in the gel than gp120 (Fig. 2) during the chase, and we hypothesize that this band could be the partially deglycosylated form of gp160 undergoing degradation, possibly through endoplasmic reticulum associated degradation (ERAD), since gp160 is being trapped for a longer period of time in the ER. It is unknown at this time how CPZ may affect gp160 transport out of the ER.

Intracellular gp120 was degraded through the lysosomal pathway in the absence of Ubc9. The demonstration that proteasome and endocytosis inhibitors were unable to restore intracellular Env levels, prompted us to examine the potential role of the lysosomal degradation pathway. The lysosome pathway has been previously implicated as having a role in HIV-1 Env degradation and virion assembly. A combination of specific lysosomal protease inhibitors, E64d, Pepstatin A, and Leupeptin (10, 245, 307) were used to determine if lysosomal degradation was involved in Env degradation in Ubc9 knock down cells. Lysosomal inhibitors were added to the culture medium 2 hours prior to the pulse chase experiment followed by metabolic labeling as in previous

experiments. Since newly synthesized viral aspartyl proteases may affect the inhibition of lysosomal aspartyl proteases by Pepstatin A, concentrations of Pepstatin A were increased 5X during the pulse chase (334). Interestingly, in the presence of these lysosome inhibitors, the intracellular gp120 levels were restored to levels similar to the control cells, (92-98%) at the 4-hour chase time in cells transfected with Ubc9 siRNA (Fig. 3a upper panels and 3b). However even with the restoration of in intracellular gp120 to normal levels in Ubc9 knockdown cells, this did not completely restore the packaging of gp120 into released virions to the control levels. This suggests that even though the mature Env degradation was blocked, the fraction of Env that was targeted for degradation in Ubc9 knockdown cells was not capable of being packaged into the assembling virions. Inhibition of the lysosome also did not appear to have any affect on Gag function as similar levels of p24 were released into the media (Fig. 3a middle panels).

To confirm the role of the lysosome in mature Env degradation in Ubc9 knockdown cells, we used another lysosome inhibitor NH_4Cl, which is a classical lysosomotropic endosomal-lysosomal acidification inhibitor that is functionally different from the effects of E64d, Pepstatin A, and Leupeptin. NH_4Cl is a weak base that functions by blocking lysosomal pH-dependent degradation by raising the pH within the secretory and endocytosis-lysosomal system. Transfected cells were incubated for 2 hours prior to the pulse chase experiments and maintained in NH_4Cl continuously throughout the experiment. Using a low dose of NH_4Cl, we found that in the absence of Ubc9, gp160 cleavage was completely blocked (Fig. 3c). Over exposure of the gels did not indicate any partially Endo H_f resistant forms, suggesting that gp160 did not traffic to

the TGN. However it has been shown previously that treatment with NH_4Cl can cause the redistribution of glycosyltransferases from the TGN to endosomal-like vesicles (14), which might change the glycosylation patterns, alter glycosylation trafficking, and cleavage of HIV-1 Env (79, 105, 386), or its susceptibility to Endo H_f. We did however detect a band that migrated slightly faster in the gel than normal gp120, which was likely to be the deglycosylated forms of gp160 undergoing degradation, possibly though ERAD.

Autophagy pathways were upregulated in Ubc9 knockdown cells. Our data using lysosomal inhibitors strongly suggests that gp120 is degraded via the lysosomal pathway in Ubc9 knockdown cells. Since the lysosomal degradation is linked to the autophagy pathway (253, 385), it is possible that the autophagy pathway is affected by Ubc9 knockdown to lead to degradation of gp120. To determine the affect of Ubc9 knockdown on autophagy, the conversion of LC3-I to LC3-II was measured in Ubc9 siRNA and control RNA transfected 293T cells (Fig. 4a). We found that cells transfected with Ubc9 siRNA alone or in combination with NL4-3, exhibited a 3-fold increase in the amount of LC3-II (Fig. 4b), suggesting that Ubc9 either directly or indirectly regulated autophagy through a PI3K independent manner. It is unlikely that the increase in autophagy in Ubc9 knockdown cells is non-specific, as cell transfected with control RNA did not show a similar increase in LC3-II under identical conditions (Fig. 4 and b). Increased LC3-II levels in Ubc9 knockdown cells could be due to either an increase autophagy, or a defect in the degradation and accumulation of autophagosomes. Autophagosomes that can complete autophagy are degraded through the fusion with lysosomes to form autophagolysosomes. Comparing LC3-II levels in the presence or absence of lysosome inhibitors can therefore determine if increased LC3-II levels was due to increased

autophagy, or defects in autophagolysosome formation and degradation (185). We found that LC3-II levels were stabilized in the presence of a combination of lysosome inhibitors, which indicated that the increased LC3-II levels in Ubc9 knockdown cells were due to increased autophagy and not because of defects in autophagolysosome formation and the accumulation of autophagosomes (Fig. 4c).

To further understand if Env degradation in Ubc9 knockdown cells was indeed due to an induction of autophagy, we treated transfected 293T cells with autophagy inhibitor 3-MA (PI3K inhibitor) for 2 hours prior to, and during the pulse-chase experiments. Cells treated with 3-MA did not show a decrease in p24 released into the media, similar to what was observed with HIV-1 infected macrophage treated with 3-MA (data not shown) (201). However, in Ubc9 siRNA transfected cells, the intracellular Env was not restored to the levels of control RNA transfected cells in the presence of 3-MA (Fig. 5a and 5b). When LC3-II levels were assayed to examine the efficiency of 3-MA inhibition of autophagy, it was found that LC3-II levels were still elevated in Ubc9 knockdown cells, and treatment with 3-MA did not inhibit autophagy based on LC3-II conversion levels (Fig. 6). Therefore, it is likely that the inability of 3-MA to inhibit Env degradation may be due to its inability to inhibit autophagy with the concentration used in the experiments.

Similar results were obtained with another PI3K inhibitor for autophagy, wortmannin. When wortmannin was used at concentrations previously published to efficiently block autophagy in complete medium (50nM) (394), it was able to block autophagy activation under amino acid and serum starvation conditions in control cells (Strv.) (Fig. 7), but did not block Env degradation (data not shown), or autophagy when

Ubc9 expression was knocked down. Interestingly, we also observed a gradual increase in LC3-II levels in wortmannin treated cells after 5 hours of treatment, which may indicate that wortmannin was losing its effect with time, or it may have induced autophagy after a prolonged incubation with the cells.

Discussion

In this study we have continued to examine the role of Ubc9 during HIV-1 assembly and the mechanism in which mature Env is selected for degradation in a Gag dependent manner in Ubc9 knockdown cells. There are four possible mechanisms by which Env degradation may have occurred upon Ubc9 knockdown: the first is degradation via the proteasome pathway; the second is the recycling of Env from the plasma membrane into the endosome and degradation in the lysosome; the third is the direct Env trafficking from the TGN to the lysosome for degradation; and fourth is the induction and degradation through the autophagy pathway. Based on our findings, we suggest that the lysosomal pathway is mostly likely involved in increased Env degradation in Ubc9 knockdown cells. Inhibition of the proteasome and endocytic pathways in Ubc9 knockdown cells failed to restore intracellular Env levels to those found in control cells. However, inhibition of the lysosome stabilized intracellular Env levels, yet it did not increase the amount of Env packaged into released virions. This suggests that once targeted for degradation, Env can no longer be packaged into the assembling virions. This is most likely due to the sequestering of Env in vesicles that cannot be trafficked to the assembly site. However, we did not observe an increase in virion release as described by Molle et al. when they blocked lysosomal degradation with

chloroquine. This could be due to the use of different cell lines and methods of lysosomal inhibition (253).

Interestingly, we found that autophagy had increased by approximately 3-fold in Ubc9 knockdown cells. The finding that autophagy had increased over steady state levels in Ubc9 knockdown cells supports the possibility that mature Env is degraded in Ubc9 knockdown cells. The observed degradation of Env was specific because viral gp160 and Pr55, cellular Actin, p53, and mature E-cadherin were unaffected (159), suggesting that additional factors may have been involved in the specific degradation of Env if the autophagy and lysosomal pathway are involved. However, the autophagy inhibitors tested were unable to inhibit autophagy or restore intracellular Env to wild type levels in Ubc9 knockdown cells, therefore we were not able to determine if increased Env degradation in the lysosome was due to increased autophagy, or if degradation occurred in spite of an increase in autophagy.

Our data, if further substantiated would suggest that Ubc9 and/or SUMOylation down regulates or inhibits the activation of autophagy in wild type cells under normal growth conditions. The possible mechanism involved is unknown at this time, but it is possible that in Ubc9 knockdown cells, autophagy regulation can be affected by either negatively affecting cellular factors that normally repress autophagy, or Ubc9 knockdown deregulated pro-autophagy factors that led to the activation of the autophagy pathway. The simplest interpretation of our data would be that in the Ubc9 knockdown cells, autophagy inhibition is deregulated at, or downstream of Vps34 activation, as inhibition of Vps34 PI3K activity by wortmannin or 3-MA did not appear to block autophagy, even though similar levels of inhibitors were found to inhibit the PI3K activity (28, 394). The

condition was also sufficient to inhibit autophagy in control cells where autophagy was induced by serum and amino acids starvations. This data also does not support the notion that an altered mTOR activity in Ubc9 knockdown cells could have led to the increase in autophagy, because mTOR acts upstream of Vps34 in the activation of autophagy. Other autophagy triggers such as reactive oxygen species (17, 33, 38, 92, 228, 328, 365), increased free Ca^{2+} (89, 151), altered cell signaling through the MEK/ERK pathways (198, 364, 371), activation of phosphorylation pathways (PKA, PKB, PKC, and AMPK) (12, 45, 77, 165, 209, 214, 249, 344), or toll-like receptor signaling (196, 197, 278, 305, 359), could all be potentially altered or involved in Ubc9 knockdown cells. Interestingly, all these pathways intersect with the SUMOylation pathways at some point, either directly or indirectly. Based on what is known about potential autophagy regulation pathways and their intersections with the SUMOylation pathway, some of these potential autophagy regulation pathways may or may not be involved in the upregulation of autophagy in Ubc9 knockdown cells. Given the far-reaching role of Ubc9 in cellular metabolism and replication, it is likely that one or a combination of a number of potential mechanisms may contribute to the increased autophagy in Ubc9 knockdown cells. To our knowledge, there are no other reports demonstrating that Ubc9 or SUMOylation having an effect on on the proteins that are involved with autophagic vesicle elongation or fusion with the lysosome (Atg3, Atg4, Atg5, Atg7, Atg9, Atg12, Atg16, Atg18, or LC3). Further experiments will be needed to decipher the mechanisms involved in increased autophagy in Ubc9 knockdown cells.

Although autophagy is commonly recognized as a catabolic pathway, the autophagy pathway has been shown to be an important pathway involved in the

replication of a number of viruses, including HIV-1 (96, 152, 201, 243, 246, 267, 299, 311, 326, 338, 350, 379, 391, 398, 404). Autophagy has been implicated as a pathway that enhances HIV-1 assembly in macrophages. It was demonstrated that inhibition of autophagy resulted in a 2 -to 3-fold decrease in virus released and a 3-fold decrease in infectious virions. Nef was found to be important for this phenotype by blocking the formation of autophagolysosomes. In our study we did not observe an obvious increase in autophagy in the presence of HIV-1 NL4-3 as observed by others in macrophage, nor did we detect any changes in the levels of virus released from the cell (159). Our data would suggest that autophagy is detrimental for HIV-1 assembly in Ubc9 knockdown cells, which is in contrast to what Kyei et al. have demonstrated in macrophage (201). The difference may be due to the use of different cell types and methods used to analyze the affects of autophagy on the assembly of HIV-1 virions.

Based on our findings we can propose several pathways leading to the lysosome being the site of Env degradation and the potential involvement of autophagy in Ubc9 knockdown cells (Fig. 8). Degradation of Env in the lysosome could potentially occur through three mechanisms. In Ubc9 knockdown cells Env could be directly targeted to the lysosome after trafficking out of the TGN, but before reaching the plasma membrane. Alternatively, Env could traffic to the plasma membrane, where it can be quickly endocytosed. Once Env enters the endocytic pathway, it could be trafficked through the late endosome for degradation in the lysosome, instead of recycling to the TGN. Lastly, Env could be degraded in the lysosome through the induction of the autophagy pathway, as the contents of the autophagolysosome are degraded and recycled in the lysosome. It is possible that Env could enter the autophagy pathway either directly after transport from

the TGN by selective autophagosome engulfment of Env containing secretory vesicles through crinophagy, or autophagosomes could engulf the Env containing endocytic vesicles and to degrade Env in the lysosome. Crinophagy is the degradative process where secretory vesicles are redirected to lysosomes instead of the plasma membrane (91). Crinophagy is not well understood in 293T cells, and its potential role in HIV assembly is unknown.

Our data supports the previously proposed model where the interruption of Ubc9-Gag interactions altered Gag trafficking to the assembly site, thus affecting Gag-Env interactions to enhanced Env degradation at an intracellular site that we have now identified as the lysosome (Fig. 8). The direct involvement of autophagy is a likely possibility, even though our data cannot confirm that an induction of autophagy directly leads to Env degradation, in spite of a 3-fold increase in autophagy in Ubc9 knockdown cells. Our data still can not completely rule out other alterative mechanisms involved, such as a mechanisms were Env is not stabilized at the assembly site on the plasma membrane by interactions with Gag, and is quickly endocytosed and degraded in the lysosome. Further experiments are underway to examine if Gag dependent Env degradation in the lysosome is due to an increase in autophagic flux in Ubc9 knockdown cells, or if Env degradation is occurring in an autophagy independent lysosomal mechanism.

Acknowledgments

We thank members of the Wood lab for thoughtful discussions. This study was supported in part by PHS grants P30 RR031151 and T32 AI060547 to CW. C.R.B is a NIH Ruth L. Kirschstein Fellow.

Figures and Figure Legends

Figure 1

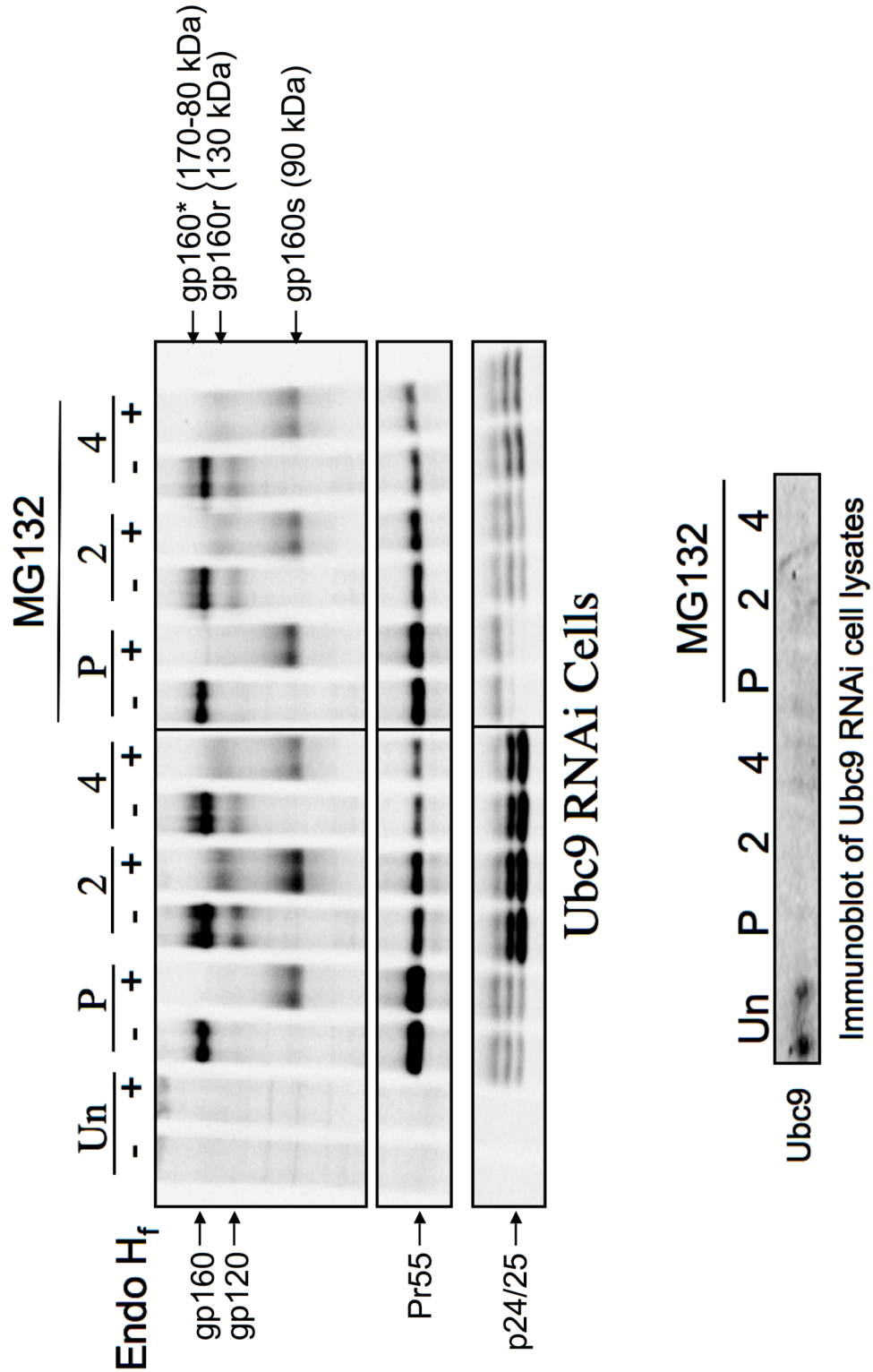


Figure 1: HIV Env stability and Gag assembly in Ubc9 knockdown cells in the presence of proteasome inhibitor MG132. 293T cells were transfected with Ubc9 siRNA and pNL4-3 as in previous experiments. MG132 (10uM) was added to the culture media for 1 hour prior to pulse chase experiments and was maintained throughout the experiment. Cells were pulse (P) labeled with [^{35}S] methionine/cysteine for 1 hour and then chased for 2 and 4 hours. Cell and media associated viral proteins were solublized and immunoprecipitated with pooled AIDS patient sera, split equally, and incubated for 3.5 hours at 37° C in the presence, or absence of Endoglycosidase H $_f$ (Endo H $_f$). Samples were separated by SDS PAGE and visualized by phosphorimaging using The Discovery Series Quantity One software. A representative, over-exposed gel is shown so that partially Endo H_f resistant Env can be more easily visualized. Viral proteins and their positions in the gel are labeled on the left. The identity of Endo H_f, untreated viral proteins and their positions in the gel are labeled on the right. Deglycosylated Endo H_f sensitive forms gp160 residing in the ER are labeled as gp160s. Partially deglycosylated, Endo H_f resistant forms of gp160 that have had their glycans modified in the TGN are labeled as gp160r. Forms of gp160 that have undergone glycan modification in the TGN but have not been cleaved are denoted as gp160* in Endo H_f untreated samples.

Figure 2

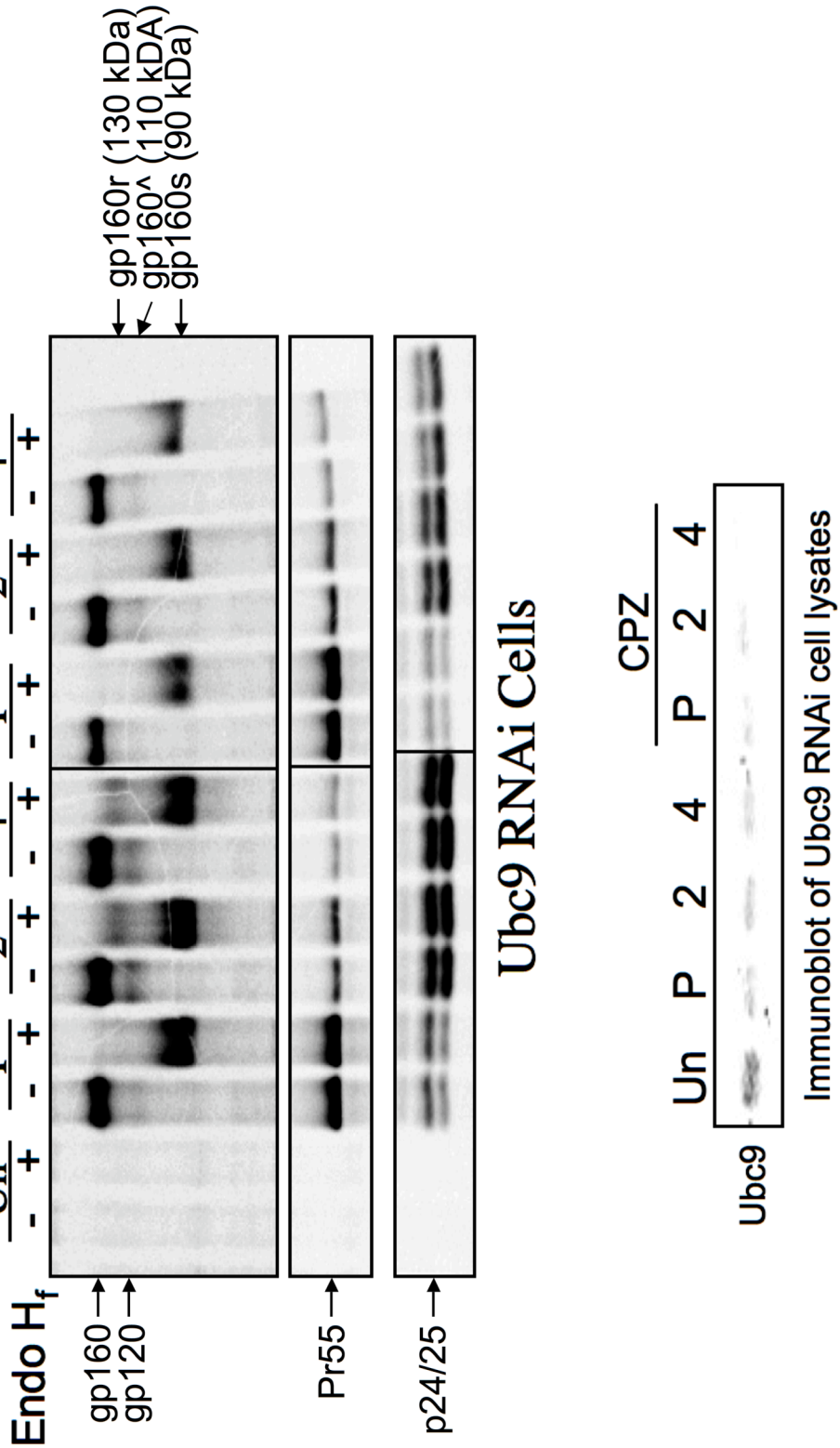

CPZ
Un
P
2
4
- + - + - + - +
gp160r (130 kDa)
gp160^ (110 kDA)
gp160s (90 kDa)
Endo Hf
gp160
gp120
Pr55
p24/25
Ubc9 RNAi Cells
CPZ
Un P 2 4
Ubc9
Immunoblot of Ubc9 RNAi cell lysates

Figure 2: HIV Env processing and stability in Ubc9 knockdown cells in the presence of endocytosis inhibitor chlorpromazine. 293T cells were transfected with Ubc9 siRNA and pNL4-3 as in previous experiments. Chlorpromazine (CPZ, 10ug/ml) was added to the culture media for 2 hour prior to pulse chase experiments and was maintained throughout the experiment. Cells were pulse (P) labeled with [^{35}S] methionine/cysteine for 1 hour and then chased for 2 and 4 hours. Cell associated viral proteins were solublized and immunoprecipitated with pooled AIDS patient sera, split equally, and incubated for 3.5 hours at 37° C in the presence, or absence of Endo H $_{f}$. Samples were separated by SDS PAGE and visualized by phosphorimaging using The Discovery Series Quantity One software. A representative, over-exposed gel is shown so that partially Endo H_f resistant Env can be more easily visualized. Viral proteins and their positions in the gel are labeled on the left. The identity of Endo H_f, untreated viral proteins and their positions in the gel are labeled on the right. Deglycosylated Endo H_f sensitive forms gp160 residing in the ER are labeled as gp160s. Partially deglycosylated, Endo H_f resistant forms of gp160 that have had their glycans modified in the TGN are labeled as gp160r. Forms of gp160 that have potentially undergone partial deglycosylation are denoted as gp160^ in Endo H_f untreated samples.

Figure 3

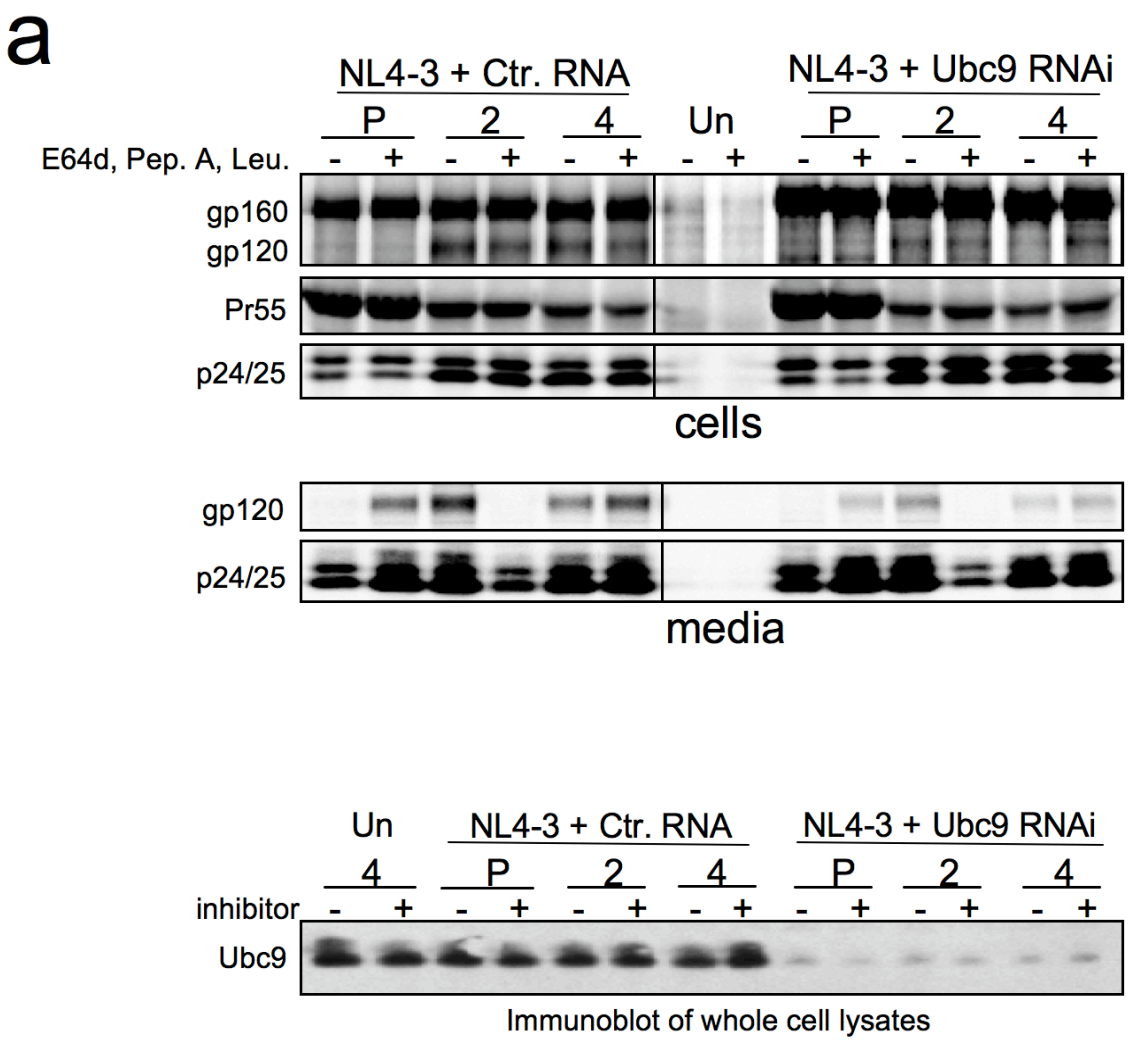

a
NL4-3 + Ctr. RNA
NL4-3 + Ubc9 RNAi
P
2
4
Un
P
2
4
E64d, Pep. A, Leu.
- + - + - + - + - + - + - +
gp160
gp120
Pr55
p24/25
cells
gp120
p24/25
media
Un
NL4-3 + Ctr. RNA
NL4-3 + Ubc9 RNAi
4
P
2
4
P
2
4
inhibitor
- + - + - + - + - + - + - +
Ubc9
Immunoblot of whole cell lysates

Figure 3 (cont.)

b

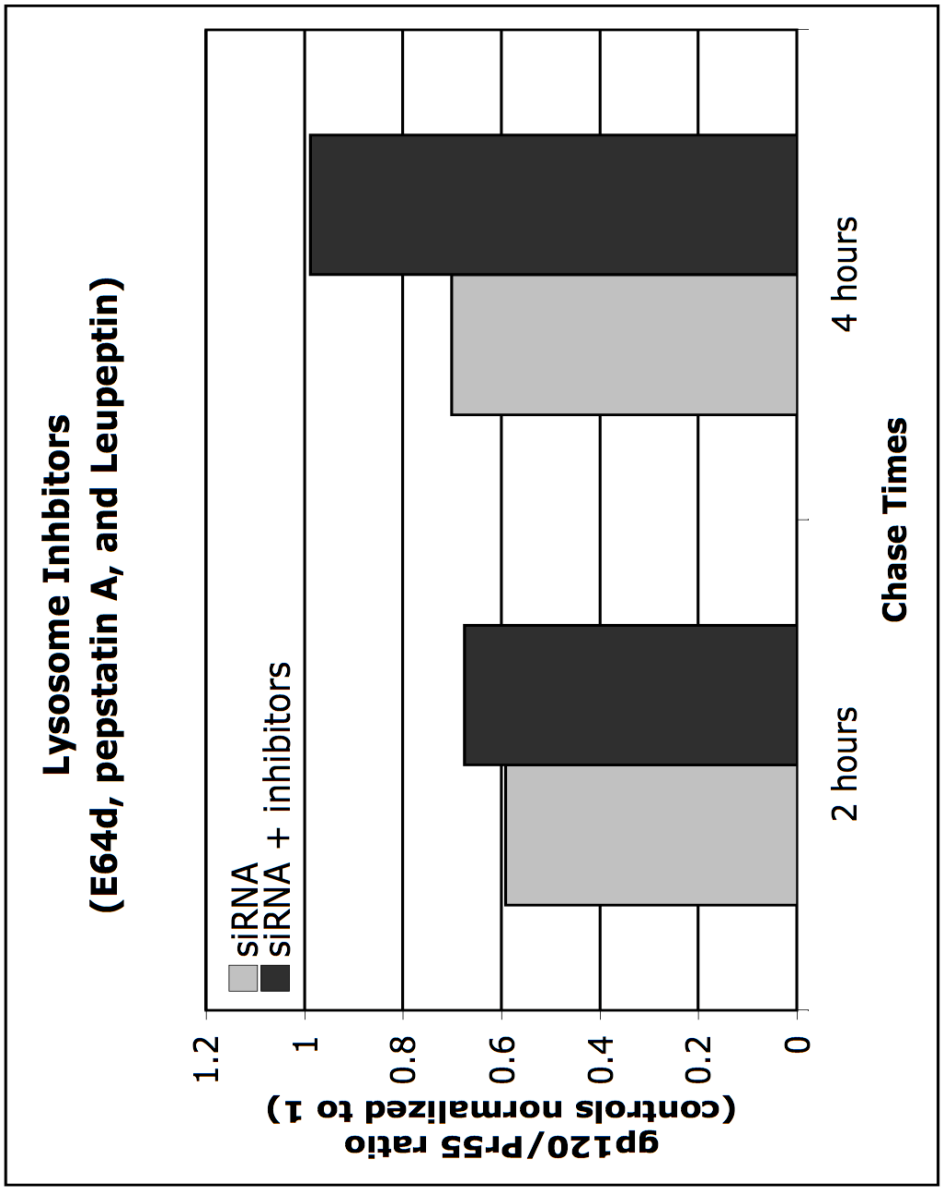


Figure 3 (cont.)

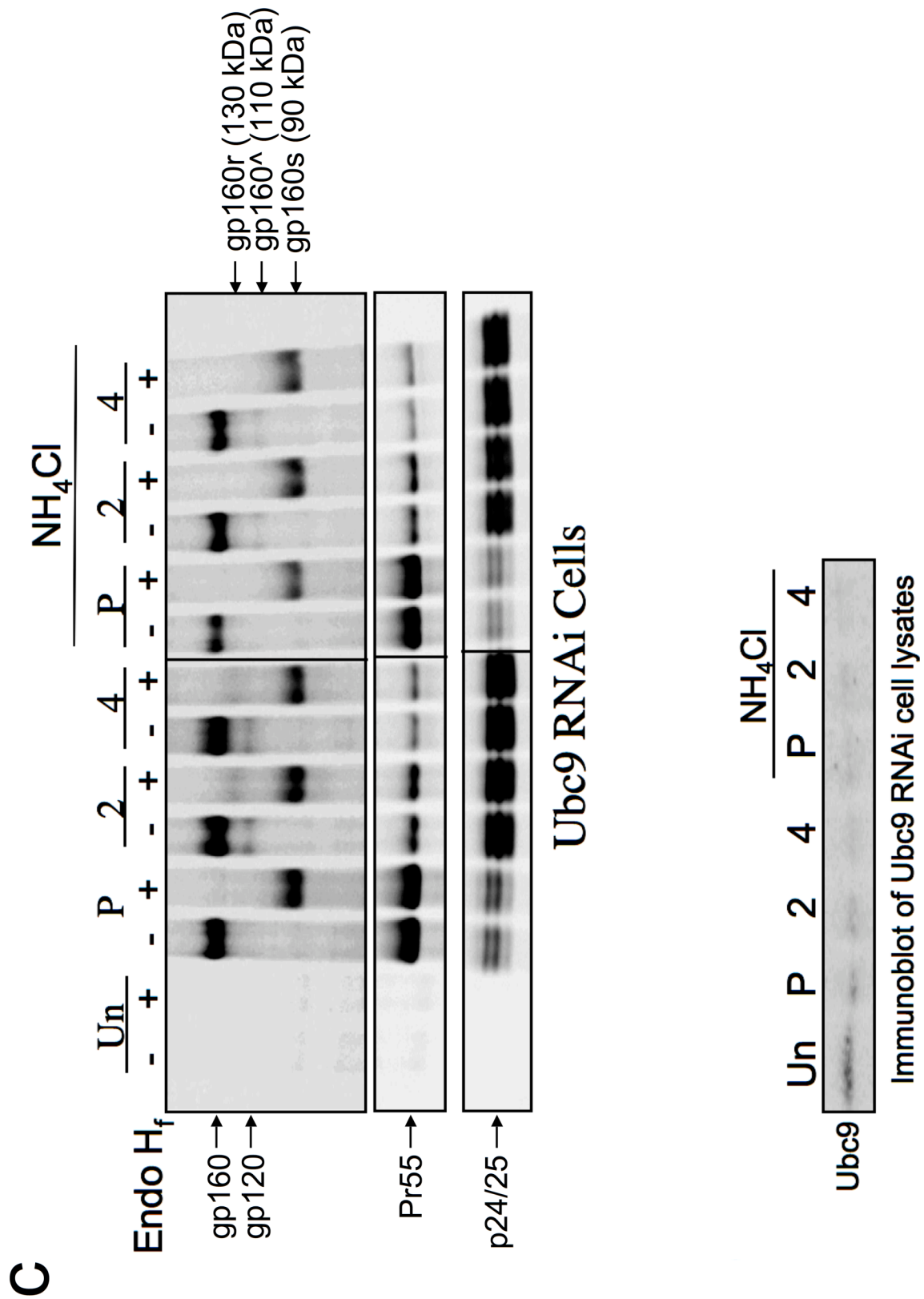
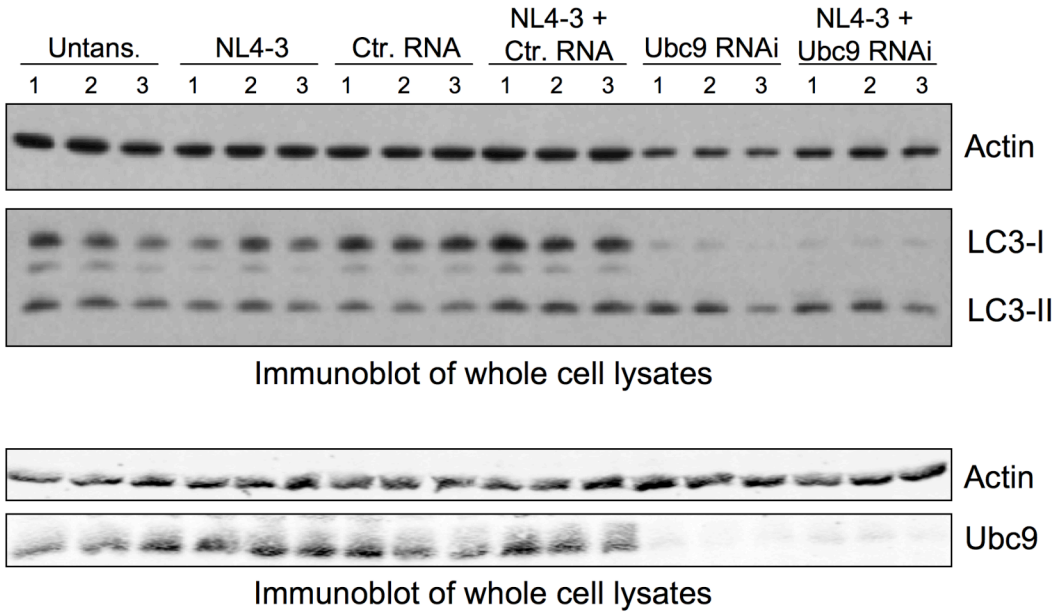


Figure 3: HIV-1 gp120 is degraded through the lysosomal pathway in Ubc9 knockdown cells. 293T cells were transfected with Ubc9 siRNA and NL4-3 as in previous experiments. (a) A combination of E64d (10μM), Pepstatin A (10μM), and Leupeptin (5μM), was added to the culture media 2 hours, or 45 minutes prior to pulse chase experiments. The amount of Pepstatin A was increased to 50 μM during the pulse chase experiment, while the amounts of E64d and Leupeptin were maintained at 10 μM and 5 μM respectively. Cells were pulse (P) labeled with [^{35}S] methionine/cysteine for 1 hour and then chased for 2 and 4 hours. Cell and media associated viral proteins were solublized and immunoprecipitated with pooled AIDS patient sera. Samples were separated by SDS PAGE and visualized by phosphorimaging using The Discovery Series Quantity One software. (b) Quantitation of intracellular gp120/Pr55 ratios of Ubc9 siRNA knockdown cells compared to control cells. The gp120/Pr55 ratios of controls cells were normalized to 1. (c) HIV Env processing and stability in Ubc9 knockdown cells in the presence of Lysosome acidification inhibitor NH_4Cl. Pulse chase experiments were carried out as before after a 45 minute pretreatment with NH_4Cl. A representative, over-exposed gel is shown so that partially Endo H_f resistant Env can be more easily visualized. Viral proteins and their positions in the gel are labeled on the left. The identity of Endo H_f, untreated viral proteins and their positions in the gel are labeled on the right. Deglycosylated Endo H_f sensitive forms gp160 residing in the ER are labeled as gp160s. Partially deglycosylated, Endo H_f resistant forms of gp160 that have had their glycans modified in the TGN are labeled as gp160r. Forms of gp160 that have potentially undergone partial deglycosylation are denoted as gp160^ in Endo H_f untreated samples.

Figure 4

a


Untans.
NL4-3
Ctr. RNA
NL4-3 + Ctr. RNA
Ubc9 RNAi
NL4-3 + Ubc9 RNAi
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3
Actin
LC3-I
LC3-II
Immunoblot of whole cell lysates
Actin
Ubc9
Immunoblot of whole cell lysates

b

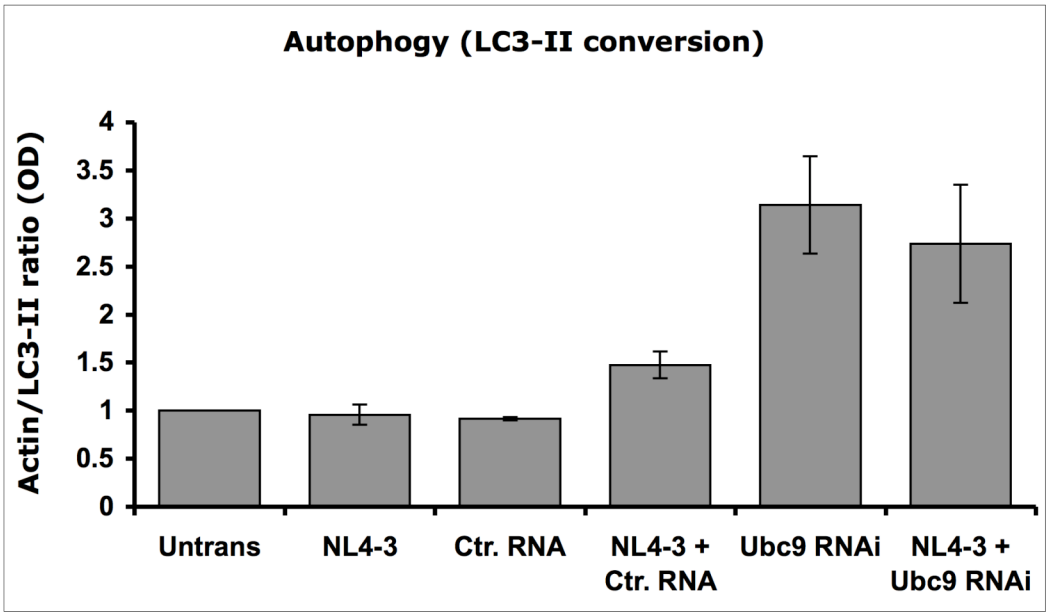

Autophogy (LC3-II conversion)
Actin/LC3-II ratio (OD)
4
3.5
3
2.5
2
1.5
1
0.5
0
Untrans
NL4-3
Ctr. RNA
NL4-3 + Ctr. RNA
Ubc9 RNAi
NL4-3 + Ubc9 RNAi

Figure 4 (continued)

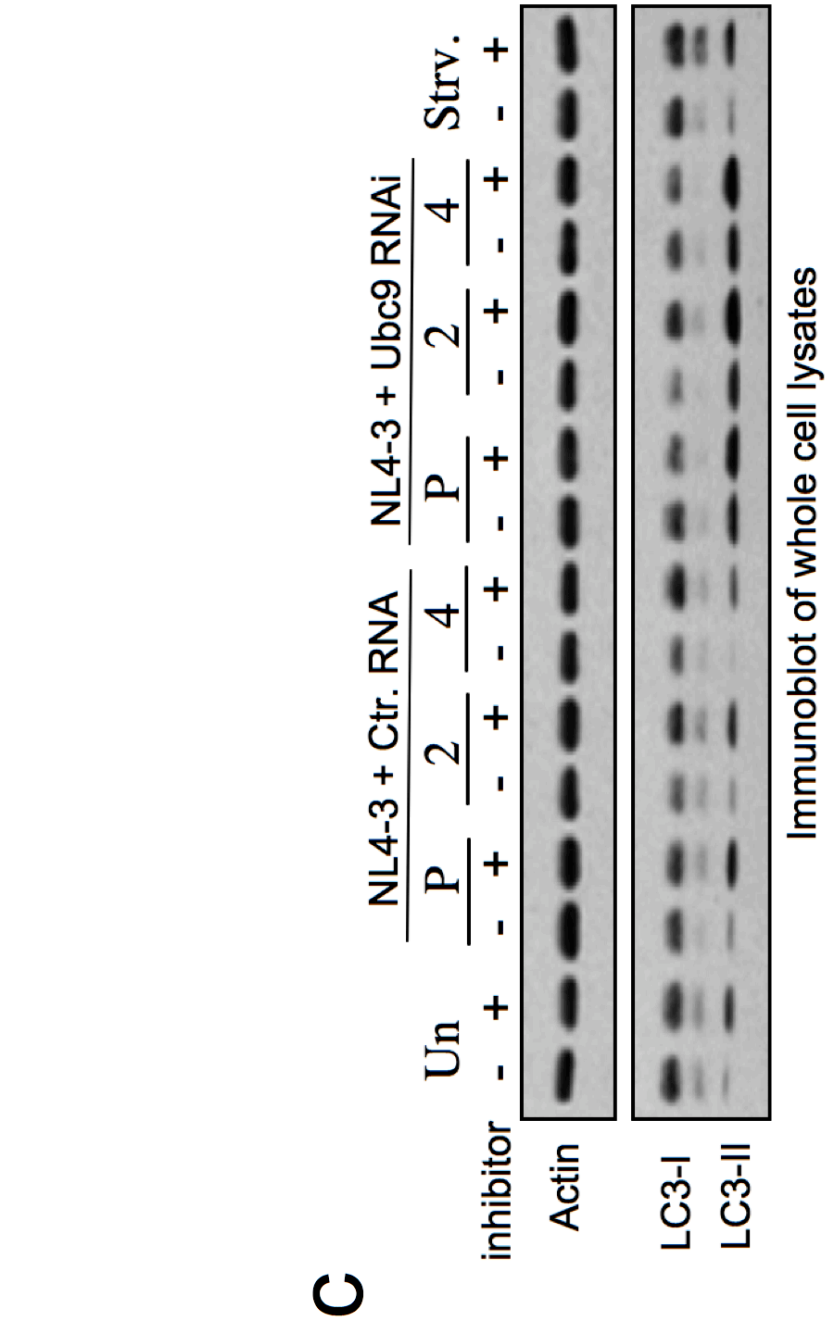


Figure 4: Ubc9 is involved in the regulation of autophagy. (a) Immunoblot for Actin, LC3, and Ubc9 levels in Ubc9 knocked downed cells. 239t cells were transfected with Ctr. RNA, or siRNA in combination with pNL4-3, RNA alone, pNL4-3 alone, or left untransfected. 24 hours after transfection with pNL4-3, cells were lysed in lysis buffer, and protein concentration in the lysates was assayed by BCA assays. Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for Actin, Ubc9, and LC3 expression. Unequal loading of whole cell lysates for LC3 immunoblotting, approximately 1/3 less Ubc9 RNAi transfected cells lysates compared to control cells (upper panels). Equal loading of whole cell lysates for Ubc9 immunoblotting (lower panels). (b) Ratio of Actin/LC3-II levels normalized to untransfected cells. (c) Autophagosomes complete autophagy by fusing with lysosomes to form autophagolysosomes. Immunoblot of cellular actin and LC3-II levels in cell lysates from pulse-chase experiments using a combination of lysosome inhibitors E64d, Pepstatin A, and Leupeptin.

Figure 5

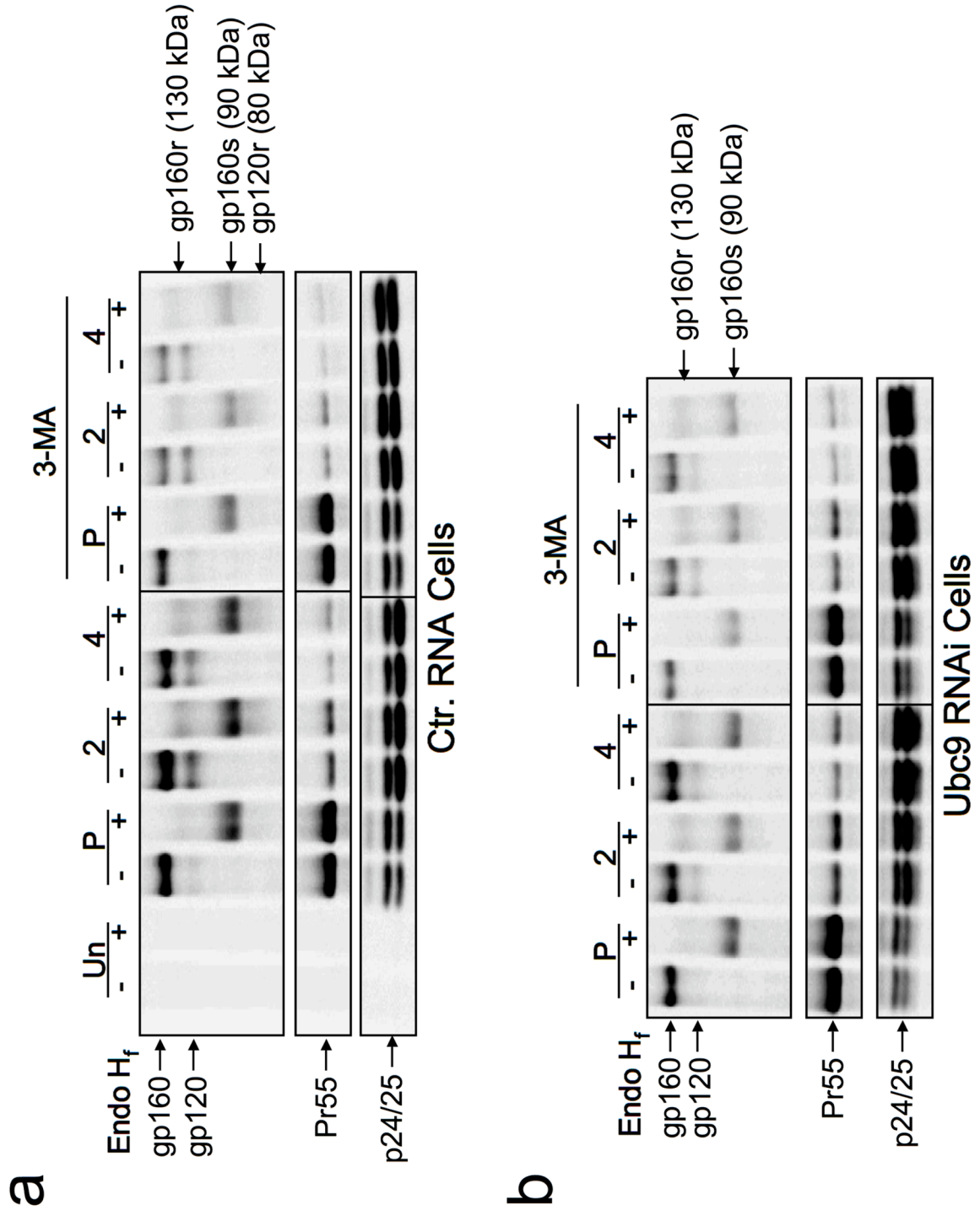

a
Endo H$_f$
gp160
gp120
Un
P
2
4
3-MA
gp160r (130 kDa)
gp160s (90 kDa)
gp120r (80 kDa)
Pr55
p24/25
Ctr. RNA Cells
b
Endo H$_f$
gp160
gp120
P
2
4
3-MA
gp160r (130 kDa)
gp160s (90 kDa)
Pr55
p24/25
Ubc9 RNAi Cells

Figure 5: HIV Env processing and stability in Ubc9 knockdown cells in the presence of autophagy inhibitor 3MA (PI-3K inhibitor). 293T cells were transfected with pNL4-3 in combination with Ctr. RNA (a) or Ubc9 siRNA (b) as in previous experiments. 3MA (10mM) was added to the culture media for 2 hours prior to pulse chase experiments and was maintained throughout the experiment. Cells were pulse (P) labeled with [^{35}S] methionine/cysteine for 1 hour and then chased for 2 and 4 hours. Cell associated viral proteins were solublized and immunoprecipitated with pooled AIDS patient sera, split equally, and incubated for 3.5 hours at 37° C in the presence, or absence of Endo H $_{f}$. Samples were separated by SDS PAGE and visualized by phosphorimaging using The Discovery Series Quantity One software. A representative, over-exposed gel is shown so that partially Endo H_f resistant Env can be more easily visualized. Viral proteins and their positions in the gel are labeled on the left. The identity of Endo H_f, untreated viral proteins and their positions in the gel are labeled on the right. Deglycosylated Endo H_f sensitive forms gp160 residing in the ER are labeled as gp160s. Partially deglycosylated, Endo H_f resistant forms of gp160 and gp120 that have had their glycans modified in the TGN are labeled as gp160r and gp120r.

Figure 6

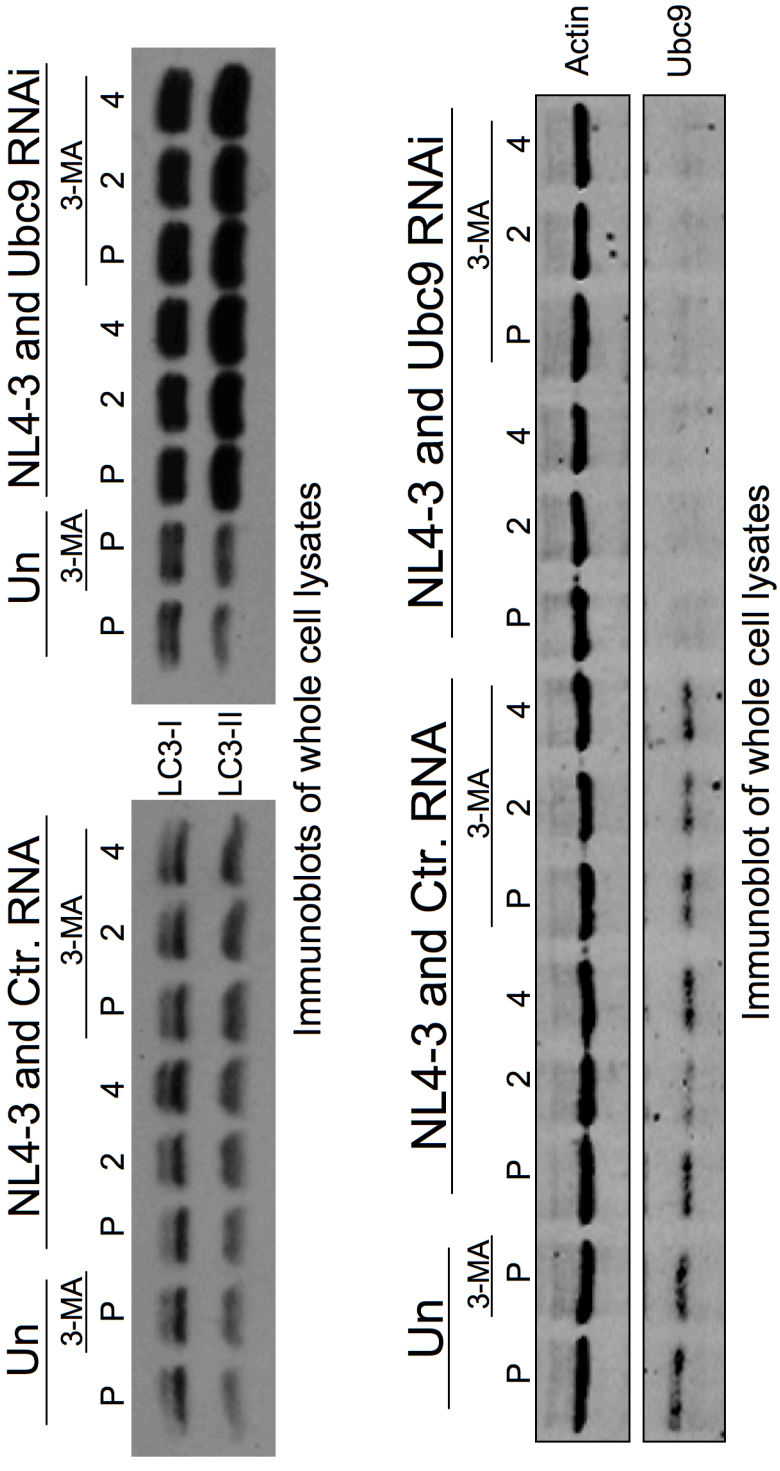

Un
NL4-3 and Ctr. RNA
NL4-3 and Ubc9 RNAi
3-MA
P P 2 4 P 2 4
LC3-I
LC3-II
Immunoblots of whole cell lysates
Actin
Ubc9
Immunoblot of whole cell lysates

Figure 6: Efficiency of autophagy inhibitor 3-MA (PI-3K inhibitor) to inhibit autophagy in Ubc9 knockdown cells. Immunoblot of LC3, actin, and Ubc9 in cell lysates from pulse-chase experiments using autophagy inhibitor 3-MA (10mM).

Figure 7

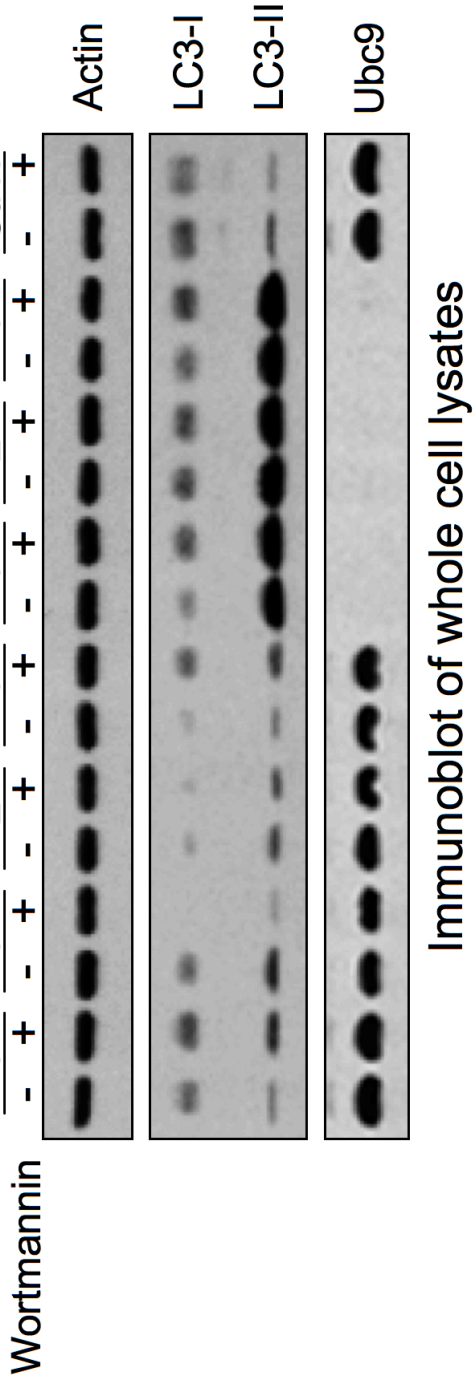

Un
NL4-3 + Ctr. RNA
NL4-3 + Ubc9 RNAi
4
P
2
4
P
2
4
Strv.
Wortmannin
Actin
LC3-I
LC3-II
Ubc9
Immunoblot of whole cell lysates

Figure 7: Efficiency of autophagy inhibitor Wortmannin (PI-3K inhibitor) to inhibit autophagy in Ubc9 knockdown cells. 293T cells were transfected with Ubc9 siRNA and pNL4-3 as in previous experiments. Wortmannin (50nM) was added to the culture media for 2. (b) Immunoblot of Actin, LC3 and Ubc9 expression in whole cell lysates. Cells were cultured in PBS for 2.5 to activate autophagy through FBS and amino acid starvation (Strv.).

Figure 8

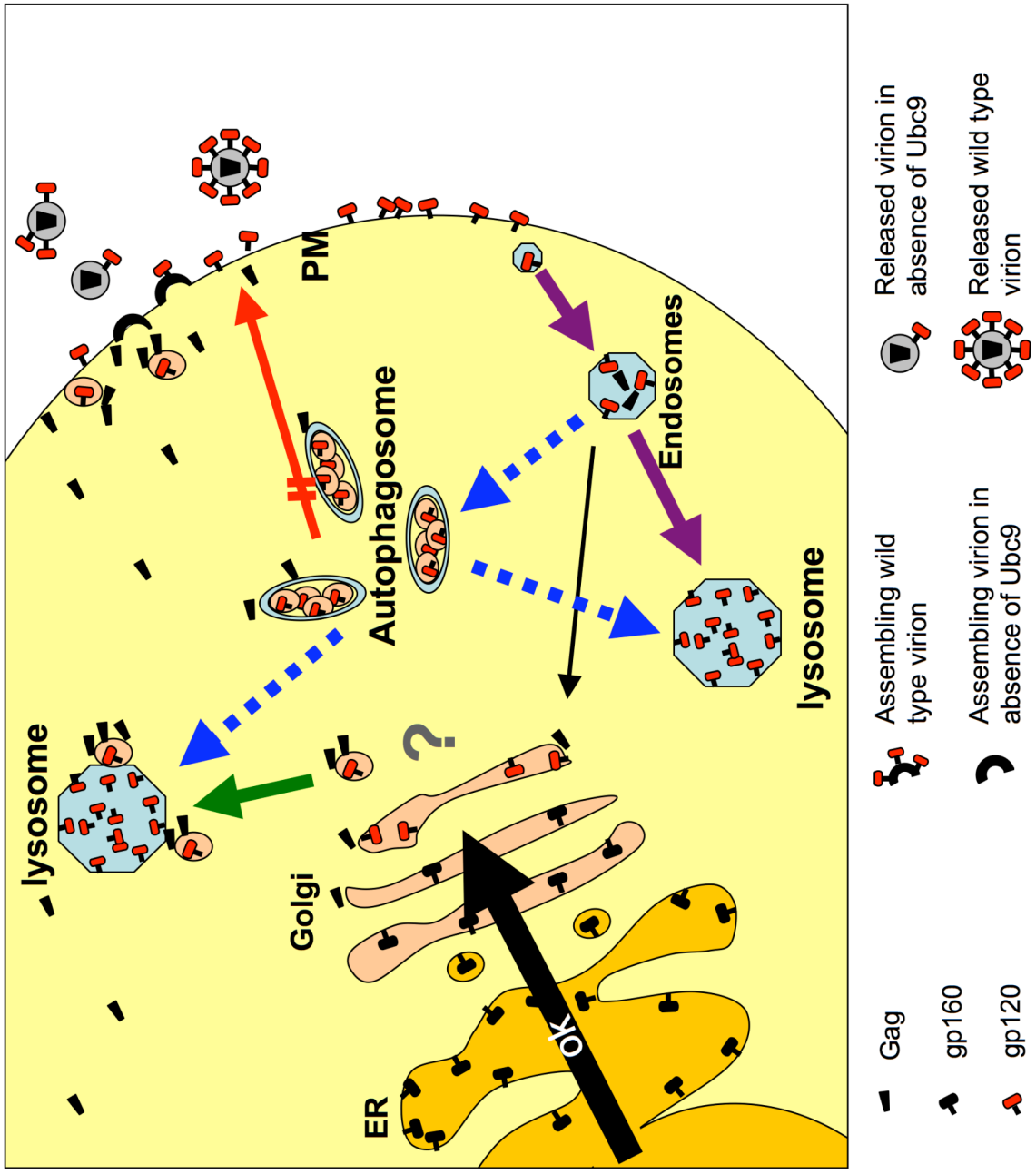

PM
Endosomes
Autophagosome
lysosome
lysosome
Golgi
ER
?
ok
Gag
gp160
gp120
Assembling wild type virion
Assembling virion in absence of Ubc9
Released virion in absence of Ubc9
Released wild type virion

Figure 8. Revised models for mature Env degradation by the lysosome in Ubc9 knockdown cells. In Ubc9 knockdown cells HIV-1 Env trafficking from the ER to the TGN is normal (black arrow). After cleavage into gp120/gp41 and transport out of the TGN, Env may be directly targeted for degradation in the lysosome instead of the assembly site of the plasma membrane. This mistrafficking of Env for degradation could be due to altered interactions with Gag, as Gag itself is mistrafficked to the assembly site on the plasma membrane in Ubc9 knockdown cells (green arrow). Another possibility is that because Gag is mistrafficked to the assembly site in Ubc9 knockdown cells, and may not stabilize Env at the plasma membrane and may be quickly endocytosed. In Ubc9 knockdown cells, a portion of endocytosed Env may then be targeted for degradation in the lysosome instead of recycling back to the TGN (violet arrows). Alternatively, the trafficking or function of yet unknown cellular factor(s) that are involved in Env stability and packaging into assembling virions may have been disrupted by altering Gag/Ubc9 interactions (gray question mark). With the observation that autophagic flux is increased in Ubc9 knockdown cells, autophagy could be an additional mechanism that results in degradation of mature Env in the lysosome (blue arrows).

Chapter V

Conclusions and future directions

Unpublished data

Summary of results

The role of Ubc9 in late events of HIV-1 infection.

In the previous chapters, we have identified Ubc9 as a HIV-1 Gag interacting partner and have found that it is an important host factor involved in the assembly of fully infectious HIV-1 virions. We have examined the role of Ubc9 during HIV-1 assembly, specifically showing how Ubc9 functions with Gag to stabilize mature intracellular Env and package it into assembling virions. Using various biochemical and microscopic imaging techniques, we showed that in Ubc9 knockdown cells, gp160 stability, glycosylation, and oligomerization in the endoplasmic reticulum (ER) was unaffected and that gp160 trafficked out of the ER to the trans-Golgi network (TGN) normally. However, after Env underwent cleavage maturation by Furin, and trafficked out of the TGN, gp120/gp41, the lack of Ubc9 caused Env to be specifically degraded in the lysosome. Env degradation likely occurred before Env was trafficked to the plasma membrane. Interestingly, during the course of these experiments, we serendipitously found that Ubc9 knockdown cells undergo increased autophagic flux. However, we were unable to block autophagy using common autophagy inhibitors (3-MA and wortmannin) did not block autphagy, thus we could not conclude if the increased autophagy in Ubc9 knockdown cells played a direct role in mature intracellular Env degradation in the lysosome, or if Env degradation occurred in spite of the increased autophagy.

We have proposed a mechanism that when interactions between Gag and Ubc9 are disrupted in knockdown cells, the Ubc9-dependent Gag function was uncoupled, resulting in the mistargeting of Env to the lysosome. This model is based on our observations that Env biosynthesis was normal until trafficked out of the TGN, and that

Gag and Env were observed to have altered association with the plasma membrane and lipid rafts. There are multiple reports in the literature demonstrating that Gag and Env affect the co-trafficking and stability of each other at the assembly site (99, 143, 171, 203, 210, 221, 222, 374). However, our data cannot rule out a mechanism where Env and Gag are both targeted to the plasma membrane, but because of the interruption of Gag and Ubc9 interactions in knockdown cells, Gag does not stabilize Env at the assembly site and it is quickly endocytosed, trafficked through the late endosome (LE), and subsequently degraded in the lysosome.

Future experiments to study the role of Ubc9 in HIV-1 late infection events.

In order to refine these models, I would first perform experiments to determine if endocytosis and recycling of Env is involved in the decreased intracellular levels of mature Env in Ubc9 knockdown cells. I would examine the surface expression and stability of Env in Ubc9 knockdown cells using Env mutants that have been demonstrated to inhibit endocytosis from the plasma membrane. Mutation of the tyrosine at position 712 in the dominant endocytosis signal in the cytoplasmic tail (CT) of gp41 (Y/C, tyrosine to cysteine) has been previously shown to decrease the endocytosis of Env by up to 2-fold (382). If Gag affects Env stability and trafficking to the lysosome at an intracellular site before co-trafficking to the plasma membrane, we would expect that in Ubc9 knockdown cells, intracellular Env (Y/C) stability and expression at the cell surface would still be disrupted when compared to control cells. However, if Env degradation in Ubc9 knockdown cells is dependent upon increased endocytosis, due to defects in the ability of Gag to stabilize Env on the surface of the cells, we would expect the levels of

Env (Y/C) on the plasma membrane to be higher relative to wild type Env in Ubc9 knockdown cells. We might also hypothesize that these mutations would increase Env stability, since less Env would undergo endocytosis, leading to less being trafficked through the late endosome and degraded in the lysosome. Results from these experiments could also be further confirmed by examining surface expression and stability of WT Env in Ubc9 knockdown cells in the presence of endocytosis inhibitors other than chlorpromazine (CPZ), which we found to have affected Env biosynthesis in the endoplasmic reticulum leading to defects in transport to the TGN. Inhibitors such as Chloroquine, Methyl-b-cyclodextrin, bafilomycin A1, or dynosore would be some alternatives to test.

I would also perform experiments that could confirm our hypothesis that interactions between the cytoplasmic tail (CT) of Env and the Matrix domain of Gag are important for the degradation of Env in Ubc9 knockdown cells. These experiments could be carried out in Ubc9 knockdown cells using Env CT deletion mutants (CT-del-63 or CT-del-144-2) (117, 258). We would hypothesize that if sequences within the CT of HIV-1 Env are important for stability in a Gag and Ubc9 dependent manner, deleting the CT should make the mutant Env insensitive to degradation in Ubc9 knockdown cells, and a similar amount of mutant Env should be packaged into virions compared to virions produced in control cells. We would also hypothesize that the CT mutants would restore specific infectivity of virions produced in Ubc9 knockdown cells compared to virions produced in control cells. These results could strengthen our published data using VSV-G pseudotyped virions, where we demonstrated that neither the packaging of VSV-G, a

heterologous viral glycoprotein, into assembling virions nor the infectivity of the pseudotyped virions was affected by Ubc9 knockdown (159).

As mentioned earlier, we observed that Ubc9 knockdown cells were undergoing increased autophagy and that neither of the autophagy inhibitors tested, 3-MA nor wortmannin, were able to inhibit autophagy in Ubc9 knockdown cells. These results cannot determine if autophagy plays a direct role in the degradation of mature intracellular Env in Ubc9 knockdown cells. 50nM of wortmannin might not be efficient in blocking a 3-fold increase in autophagy and we may have used sub-optimal concentrations. However, 50nM of wortmannin was sufficient to block serum and amino acid starvation induced autophagy in control cells. Thus experiments should be carried out to determine optimal wortmannin concentrations to use to inhibit autophagy in Ubc9 knockdown cells. We have planned to examine if increasing the concentration of autophagy inhibitors might block autophagy in Ubc9 knockdown cell and if Env levels could be restored to normal levels. If autophagy activation in Ubc9 knockdown cells is dependent upon PI3K activity, we would expect that higher concentrations of the inhibitors would lead to a decrease in LC3-II levels as compared to untreated Ubc9 knockdown cells. Once the concentration that blocks autophagy is optimized, these experiments should be able to clarify whether autophagy is involved in Env degradation in Ubc9 knockdown cells. These experiments would also determine if the initiation of autophagy is activated through PI3K dependent, or independent pathways.

Alternatively, if increased inhibitor concentrations are found not to inhibit autophagy, we would perform experiments to determine if increased autophagy might be playing a role in the lysosomal degradation of Env in Ubc9 knockdown cells. We are in

the process of utilizing confocal microscopy to examine potential co-localization between Env and autophagic vacuoles to determine if there is an increase in their co-localization in Ubc9 knockdown cells. For these experiments we are using 293 cell lines, which stably express LC3-GFP. Cells undergoing autophagy should show increased punctate staining correlating with LC3-GFP lipidation and increased formation of autophagic vacuoles. The number of GFP positive puncta has been found to be an indicator of the levels of cellular autophagy (185). If we observe an increase in co-localization or close association between Env and LC3-GFP puncta in Ubc9 knockdown cells, it would support the hypothesis that HIV-1 Env is degraded through the upregulated autophagy pathways. However, if Env is found not to co-localize with punctate LC3-GFP, it would suggest that autophagy is not involved with Env degradation and Env may be degraded in the lysosome in an autophagy independent mechanism. We will also carry out these experiments in the presence of lysosome inhibitors, since Env turnover might be too dynamic to capture by microscopy. We could also examine the potential co-localization of Gag and punctate LC3-GFP. We have demonstrated that Gag trafficking was altered in Ubc9 knockdown cells, and it is not known whether altered Gag trafficking has an effect on Gag-LC3-GFP colocalization. If the hypothesis that Env is degraded through increased autophagy in Ubc9 knockdown cells, we would also expect an increase in Gag association/co-localization with LC3-GFP as Env degradation is Gag dependent.

Furthermore, if we find an increase in Env and Gag co-localization with punctate LC3-GFP in Ubc9 knockdown cells, we would use thin-section electron microscopy (EM) to further substantiate the confocal co-localization data. Using EM with immunogold labeling techniques to identify Env and Gag localization to the autophagic

vacuoles would support the hypothesis that autophagy is involved in Env degradation in Ubc9 knockdown cells.

Another possible limitation of our data is that they were obtained using 293T cells because these are easily manipulatable with high transfection efficiencies and can express high levels of exogenous proteins, as well as very efficient Ubc9 knockdown. However, the natural target cells for HIV-1 are primarily T-cells and macrophages, and using more relevant cell types to confirm our findings in 293T cells is important. Nevertheless, there have been many discoveries about the retroviral replication cycles through the use of common laboratory cell lines that were later confirmed in more relevant HIV-1 target cells. Some examples are the Late domains and the role of TSG101 in budding, Env packaging signals, role of restriction factor Tetherin, function of various host factors involved with replication, and general characteristics of Env and Gag function (3, 41, 83, 98, 122, 128, 258, 341).

The role of Ubc9 in early events during HIV-1 infection.

The early events of the HIV-1 replication cycle begin by the attachment of the virus to the target cell through interactions between the host CD4 and viral Env. After attachment and engagement with the host co-receptor, the viral Env undergoes conformational changes, facilitating the fusion of the viral membrane with the host plasma membrane. The fusion of the membranes releases the viral core into the cytoplasm, where the viral RNA is uncoated and is reverse transcribed into viral DNA (vDNA). The viral DNA in complex with viral proteins is transported into the nucleus. In the nucleus, the vDNA is integrated into the host chromosome by the viral integrase, and the viral genes are expressed using the host transcriptional and translational

machinery (early events reviewed in 71). During these early events, the viral proteins utilize specific host proteins to their advantage and avoid others that are detrimental to complete the infection of new target cells (reviewed in 349, 262, 202).

The role of Ubc9 in HIV-1 target cells has not been examined, however SUMOylation had been implicated to be involved in the early infection events of replication (137, 400). These studies were done with infectious retroviral clones that contained lysine mutations that where shown to block SUMOylation of p30 in MoMuLV, p6-GFP in HIV-1, or HIV-1 Integrase. We have begun experiments to examine if Ubc9 is also an important host factor during HIV-1 early infectious events. Our experiments are potentially more far reaching because we will be determining if Ubc9 expression is needed in target cells during infection and not just the role of lysine mutations and potential SUMOylation of viral proteins during early events.

For these experiments, we have examined the ability of HIV-1 to complete the early steps of the replication cycle and express new proteins from the integrated provirus in Ubc9 knockdown cells. Ubc9 knockdown cells were infected with VSV-g pseudotyped NL4-3-green fluorescent protein (GFP) and assayed for the expression of GFP in the infected cell by flow cytometry. NL4-3-GFP is an infectious proviral clone that had GFP cloned in-frame 5' of the Nef open reading frame. VSV-G pseudotyped virions were used because 293T cells do not express the HIV-1 receptor and co-receptor, and thus cannot support HIV-1 Env dependent entry. Pseudotyping NL4-3-GFP with VSV-G is a common technique used by the HIV-1 field to increase virion infectivity and expand cell tropism for early infectious event studies (18).

To examine the role of Ubc9 in HIV-1 early events, 293T cells transfected as in previous knockdown experiments, or left untransfected and were infected with equal amounts of VSV-G pseudotyped NL4-3-GFP at either 24 or 48 hours after the second Ubc9 siRNA transfection. We allowed the virus to infect the cells for approximately 30 hours before the cells were fixed in 4% paraformaldehyde for 20 minutes at room temperature, washed 3-times with phosphate buffered saline (PBS), and analyzed for GFP expression by flow cytometry. The percentage of cells that expressed GFP after being infected 24 hours after the second Ubc9 siRNA transfection were decreased approximately 3-fold in Ubc9 knockdown cells compared to control cells (Fig. 1a). The percentage of GFP positive cells decreased by approximately 6-fold compared to control cells when cells were infected 48 hours after the second siRNA transfection (Fig. 1b). During the course of the experiment, Ubc9 was efficiently knocked down compared to control cells (Fig. 1c). These results suggest that Ubc9 is important for HIV-1 early infectious events leading to the integration of vDNA and the LTR based expression of GFP.

Interestingly, LC3-II levels increased over time, which correlated with a decrease in infection and GFP expression (Fig. 1c). These results suggest that increased autophagy may play a role in the decreased completion of early infectious events in Ubc9 knockdown cells, however further experiments are needed to confirm the observation, and determine if autophagy is the cause of decreased infectivity. However, our preliminary finding do not support those of other groups, demonstrating that the autophagy components Atg7, Atg8, Atg12, and Atg16L2 were required for HIV-1 infection (41).

Other than autophagy, there are other possible mechanisms that might explain the observed decrease in infection by pseudotyped HIV-1 in Ubc9 knockdown cells. First, the defect could be directly related to Ubc9 expression levels, and Ubc9 knockdown will result in an interruption of its interactions with viral components during early infectious events. Second, the defect could be due to changes in SUMOylation of viral or host proteins, and these changes may have altered their functions (SUMOylation reviewed in 124, 405). Third, defects could be due to the alteration of the mRNA of the host or viral mRNA transcriptional levels, including miRNA, which might have affected the expression of host factors utilized by the virus to enter the host cell and complete early events. Lastly, Ubc9 knockdown could increase the expression of host restriction factors.

Future experiments to investigate the role of Ubc9 in early events of viral infection.

As stated above, there could be a number of mechanisms involved with the decrease in HIV-1 infectivity in Ubc9 cells. Recently, Zamborlini et al. described lysine mutations in HIV-1 Integrase that affected SUMOylation by SUMO-2 and led to defects in viral integration (401). Our data is in agreement with the report by Zamborlini et al. Based on their results, we would expect a decrease in Integrase SUMOylation in Ubc9 knockdown cells, which could lead to the defects in integration and transduction of GFP in Ubc9 knockdown cells. However, our knockdown system might also affect HIV-1 infection at various early events if Ubc9 plays a role in these events in addition to integration, such as nuclear import of the pre-integration complex (PIC). Zamborlini et al. reported that there was no change in nuclear import of the PIC, which suggests that the SUMOylation of integrase is not involved with nuclear import, however Ubc9 could play

a role in nuclear import independent of the SUMOylation of integrase. Ubc9 is known to also localize to the nuclear pore (402), an alteration in nuclear pore composition and function in Ubc9 knockdown could be another mechanism to explain the decrease in infectivity because of defects in nuclear import of the pre-integration complex (PIC). However, nuclear pore function did not appear to be affected as viral and host transcripts were expressed and translated at normal levels. The potential role of Ubc9 in nuclear import could be tested by assaying for the completion of the various steps of reverse transcription in infected Ubc9 knockdown cells. If Ubc9 plays a role in nuclear import of the PIC, we would expect to detect lower levels of 2-LTR circles, which were used as a marker for nuclear localization of the PIC. If 2-LTR levels are found to be unchanged, this would suggest that the defect is occurring during integration of the provirus, and would be in agreement with the results of Zamborlini et al. However, if lower levels of 2-LTRs are detected, we would need to examine the completion of the earlier steps in reverse transcription, since defects earlier in reverse transcription would also lead to lower 2-LTR levels. Further work will need to be carried out to fully understand how Ubc9 is involved in the early events of the HIV-1 replication cycle.

Potential role of Ubc9 in autophagy regulation and potential mechanisms involved.

During the course of the autophagy inhibitor experiments we found that autophagy was increased in Ubc9 knockdown cells by approximately 3-fold based on LC3-II levels. It was also interesting to find that the commonly used inhibitors 3-MA and wortmannin, which inhibit autophagy by inhibiting PI3K, did not block autophagy under these conditions. It is possible that the concentrations of the inhibitors used were

not optimized and thus further experiments will be need to examine if higher amounts can lead to the inhibition of autophagy. Alternatively, Ubc9 knockdown can activate, or deregulate autophagy in a PI3K independent manner. Interestingly, an abstract by Rao et al. suggested that Vps34, a PI3K involved in autophagy activation, is SUMOylated with SUMO-1 by E3 SUMO ligase KAP1, and that SUMOylation is important for autophagy initiation. It is possible that the SUMOylation function of Ubc9 is involved in the regulation of autophagy.

To our knowledge, there are no published reports implicating that Ubc9 or SUMOylation can directly affect the cellular proteins, which are involved with autophagic vesicle elongation or fusion with the lysosome such as Atg3, Atg4, Atg5, Atg7, Atg9, Atg12, Atg16, Atg18, or LC3. The mechanism behind increased autophagy in Ubc9 knockdown cells is not known, and many of the proteins that have been demonstrated to play a role in regulating autophagy are also part of the Ubc9/SUMOylatio pathway. Given the far reaching role of Ubc9 in cellular metabolism and replication, it is likely that more than one of the potential mechanisms contributes to increased autophagy in Ubc9 knockdown cells, and a combination of one or all of them could be contributing to the induction of autophagy in Ubc9 knockdown cells.

Future experiments to examine the role of SUMOylation in the induction of autophagy by Ubc9 knockdown.

We will further examine the role of SUMOylation in the activation of autophagy in Ubc9 knockdown cells through various methods. To examine if the enzymatic E2 SUMO conjugase activity of Ubc9 is involved in autophagy regulation, the trans-dominant negative Ubc9 mutant (C93A) will be used to study its effect on autophagy.

C93A is a point mutation in the enzymatic active site that blocks Ubc9 function as an E2 SUMO conjugase. If SUMOylation is a key factor in regulating autophagy, autophagy would be reduced in the presence of the C93A mutant. Autophagy activation could also be examined when SUMOylation is knocked down using siRNA specific for SUMO-1, SUMO-2, SUMO-3, and SUMO-4 individually and in combination with each other. If SUMO knockdown by siRNA leads to increased autophagy, this would support the hypothesis that SUMOylation of host factors regulate autophagy activation and would also be in agreement with our Ubc9 knockdown data. However, detection of endogenous SUMOylation is already difficult, and the detection of knocked down SUMOylation could be more difficult. The SUMO siRNA experiments might also be difficult to interpret, because Ubc9 itself is SUMOylated and SUMOylation causes changes in binding characteristic with target proteins (144, 186). Alternatively, gain of function experiments can be carried out, where autophagy activation would be monitored in cells that have undergone endogenous Ubc9 knockdown, and then restore Ubc9 expression with either exogenous Ubc9 or C93A that is resistant to siRNA knockdown. We would hypothesize that if SUMOylation were important in the upregulation of autophagy in Ubc9 knockdown cells, we would expect that autophagy will be induced upon expression of the SUMOylation minus Ubc9 mutant in knockdown cells. However, if SUMOylation is not important for the activation of autophagy in Ubc9 knockdown cells, we would expect that the expression of resistant C93A would block the activation of autophagy in Ubc9 knockdown cells similar to endogenous Ubc9. Another experiment that could be carried out is to overexpress the various SUMO isoforms, either individually or in combination to examine autophagy activation. If SUMOylation is important for

autophagy regulation, we would hypothesize that with excess SUMOylation, the excessive SUMOylation of repressors may inhibit the activation of autophagy upon exogenous stimulation, since intracellular SUMO levels are the limiting factor in protein SUMOylation (87).

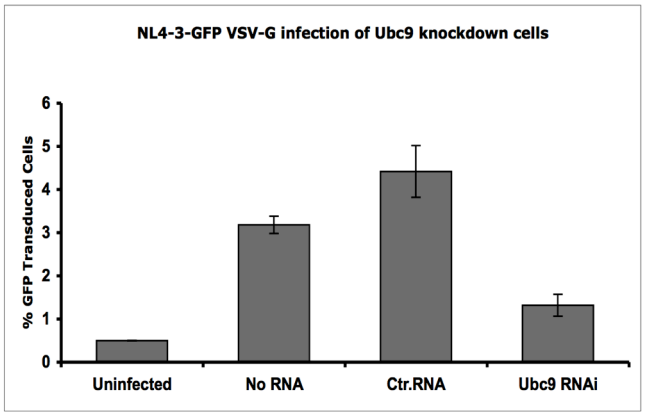
If SUMOylation is found to be an important factor for autophagy regulation, it will still be very difficult to identify which cellular protein(s) may be involved with the activation of autophagy in Ubc9 knockdown cells. Examining the activation state of known regulators of autophagy, such as some of the potential pathways detailed above, will first be carried out. These will include examining the levels of ROS, activation of the MEK/ERK signal pathway, activation of the PKA, PKB and PKC pathways, activation state of c-Jun signal pathway, and Ca2+ release from the ER and mitochondria in Ubc9 knockdown cells. If specific pathways are found to be activated in Ubc9 knockdown cells, further analysis of the pathways by data mining to identify potential interaction/complex partners based on previously published results may reveal proteins that are already known to interact with Ubc9 and/or are known targets of SUMOylation. The known components of the pathway(s) could also be analyzed for potential SUMOylation consensus motifs in an effort to narrow down which components may be involved in the increased autophagy.

Based on the outcomes of these proposed experiments, new experiments could then be designed to study the role of Ubc9 and the newly identified component, and its affect on autophagy regulation.

Figure and Figure Legend

Figure 1

a



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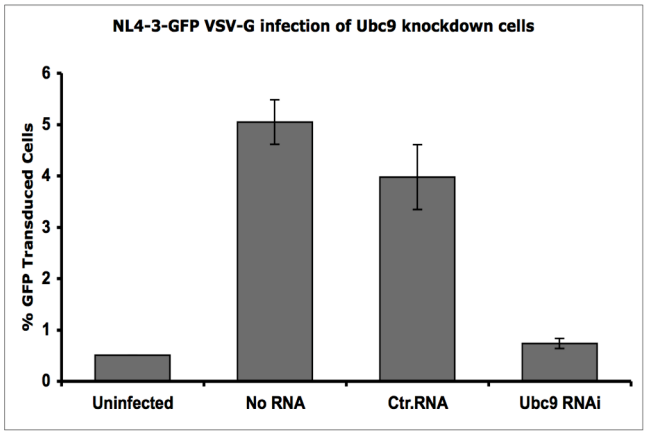

c

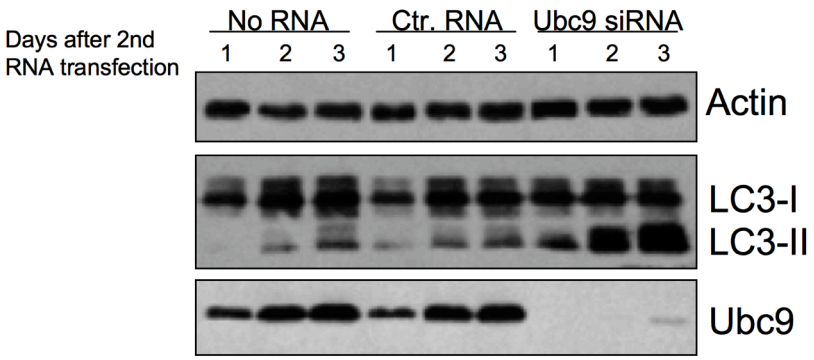

Figure 1. Ubc9 expression is important during the early infectious events in HIV-1 target cells. 24 (a) and 48 (b) hours post second RNA transfections, 293T cells transfected with Ctr. RNA, Ubc9 siRNA, or left untransfected were infected with VSV-G pseudotyped NL4-3-GFP virions. Cells were infected for approximately 30 hours, fixed with 4% paraformaldehyde in PBS for 20 minutes, washed multiple times with PBS, and analyzed for GFP expression by flow cytometry. (c) Ubc9, and LC3-II expression in 293T whole cell lysates at 1, 2, and 3 days post second RNA transfections.

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