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Approved by the Dissertation Committee:

, Chairperson

07/08/11

ROLES OF AUTOPHAGY IN HIV-1 INFECTION

by

CHRISTINA ARCHER DINKINS

B.S., Biological Sciences, University of New Orleans, 2005

DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy
Biomedical Sciences

The University of New Mexico
Albuquerque, New Mexico

July 2011

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DEDICATION

Dedicated to my mother, Sharon Dunn, for teaching me the value of education, for always believing in me, and for her many sacrifices on my behalf.

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ACKNOWLEDGMENTS

I wish to thank my principal investigator, Dr. Vojo Deretic, and my committee members, Drs. Bryce Chackerian, David Peabody, Antonito Panganiban, Chien-An (Andy) Hu, and Rebecca Hartley for their confidence and support.

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ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) chronically infects approximately 30 million people worldwide. HIV-1 has become one of the most difficult viral infections to treat due to its high mutability and emerging drug resistance. Critical to our development of more effective therapeutic treatments, we must better understand the HIV life cycle in order to reveal better targets for drug development. Thus, the purpose of this work was to investigate the role of autophagy, a cellular homeostatic mechanism capable of eliminating intracellular pathogens, in HIV-1 infection of two relevant hosts, the human and the Old World non-permissive primate, the rhesus macaque. The ability of autophagy to eliminate HIV-1 in these two species was compared with the objective of identifying why rhesus macaques efficiently eliminate HIV-1 and humans do not. Here we report that human cells have lost autophagic protection against HIV, and identify a role for autophagy in supporting HIV-1 biogenesis and egress from human macrophages. We further identify the rhesus macaque restriction factor, rhesus TRIM5α, as a major player in targeting HIV-1 for degradation through p62-dependent recognition and capture by the autophagy machinery.

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CHAPTER 1

INTRODUCTION

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Autophagy acts as a defense against intracellular pathogens

Autophagy is a multi-purpose homeostatic process whereby cells gather intracellular components in a double membrane compartment and target the contents for degradation through convergence with the lysosomal pathway. First called "autophagy" by Cristian de Duve in 1963, the double membrane compartment was identified as a lysosome related structure containing mitochondria, endoplasmic reticulum, and dense structures indicative of cytosolic proteins and ribosomes. De Duve went on to show that glucagon treatment of liver cells induced the formation of autophagic vacuoles which could fuse with lysosomes [1]. Despite this early discovery of autophagy, intensive research on this important cellular pathway was not part of the research landscape until the identification of autophagy genes in yeast about 15 years ago [2-4]. Since that time, research on the mechanisms underlying autophagy have increased exponentially, yet important aspects of the autophagy pathway still remain unclear. For example, despite efforts to determine the origin of the membrane forming the autophagosome, the basic unit of the autophagy pathway consisting of a double membrane vacuole, it is still unresolved.

Three types of autophagy have been described. The first and primary type, macroautophagy, hereafter referred to simply as autophagy, has been researched primarily in mammalian cells. It is characterized by the large-scale engulfing of items in a double membrane vacuole. The second type, primarily studied in yeast, is microautophagy. Microautophagy is a process of budding directly into the lysosome. This type of autophagy allows only for the degradation of small amounts of cytoplasm. The third type of autophagy, called chaperone mediated autophagy (CMA), has also been

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researched mainly in yeast. In CMA, proteins are directly translocated into the lysosome by heat shock cognate protein 71kDa (hsc70).

Autophagy can be separated into three stages: initiation, elongation, and maturation. In initiation (Fig. 1.1), autophagy induction is regulated by two, seemingly independent, signaling pathways. The first is centered around Beclin 1, a component of the class III PI3K complex also containing human vacuolar protein sorting 34 (hVPS34, the class III PI 3-kinase) and its regulatory subunit p150, and the second is centered around mammalian target of rapamycin (mTOR), which acts as a suppressor of autophagy initiation. Beclin 1 is believed to promote colocalization of the components necessary for autophagosome formation [5] and its association with hVPS34 can be positively regulated by autophagy protein 14 (Atg14) and negatively regulated by Bcl-2 [6-8] as well as other factors, while mTOR inhibits Unc-51-like kinase 1 (Ulk1) activation through phosphorylation thereby preventing Ulk1-mediated induction of autophagy [9].

Once either signaling pathway is activated, nascent membrane will begin to form in a crescent shape known as a phagophore. It is commonly believed that the phagophore can initiate directly adjacent to autophagic targets [10]. The curvature of the phagophore is assisted by proteins encoded by autophagy (Atg) genes, specifically the Atg12-5/16L complex, covering the convex side of the initiating membrane. Atg5 and 12 are conjugated by the action of two other autophagy factors, Atg7 and Atg10, then associate hydrophobically with Atg16L [11]. Both the convex and concave sides of the phagophore are coated externally with a protein called LC3. LC3 exists in two forms, an

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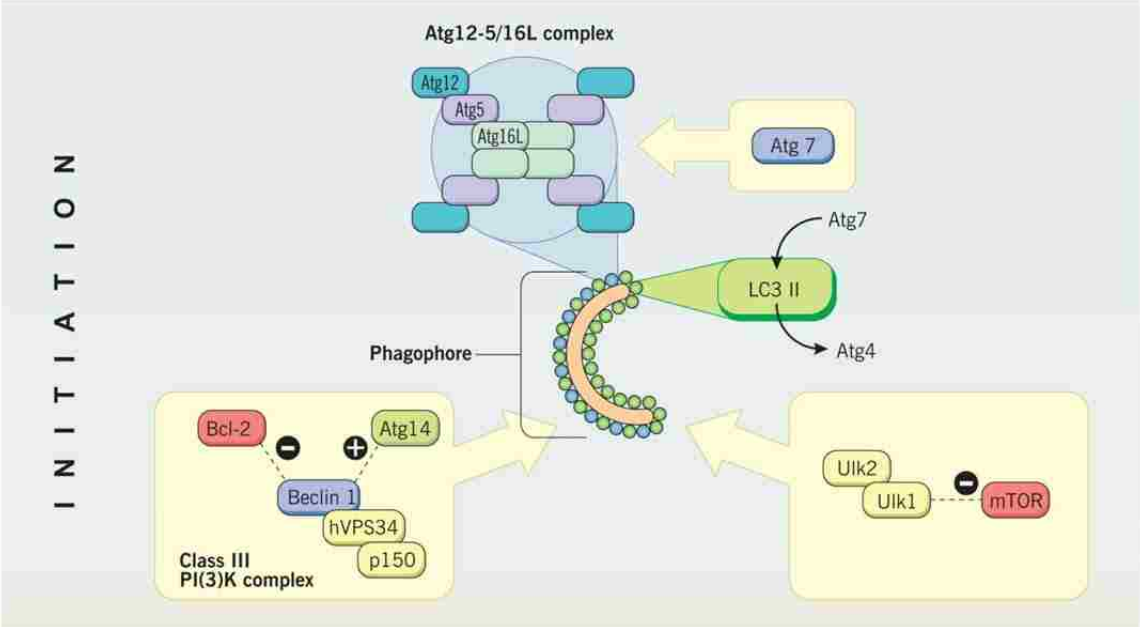

Figure 1.1. Initiation. Phagophore formation is centered around two proteins, Beclin 1 and mTOR. Beclin 1 forms a complex with hVPS34 and its regulatory subunit p150. Initiation of autophagy by Beclin 1 can be positively regulated by Atg14 and negatively regulated by Bcl-2. mTOR negatively regulates Ulk1, preventing its association with Ulk2 and suppressing autophagy initiation. The outer surface of the phagophore is covered by Atg12-5/16L protein complexes, which facilitate curvature of the phagophore. Atg7 assists in formation of the Atg12-5/16L complex. Around the entire phagophore is LC3 II, which is recycled on and off the membrane by the activity of Atg7 and Atg4, respectively.

unlipidated, cytosolic form called LC3 I, and a phosphatidylethanolamine lipidated, membrane bound form called LC3 II. These two forms of LC3 can be used to indicate the state of autophagy. An increased rate of conversion from the LC3 I form to the LC3 II form indicates that there is an enhancement or induction of autophagy from basal levels. In the presence of maturation inhibitors, LC3 II will not be degraded and its accumulation can be a positive indicator of treatment success.

In elongation (Fig. 1.2), the phagophore continues to grow around its cargo ending with fusion and closure of the double membrane structure into what is now called the autophagosome. Trapped within the autophagosome are LC3 II and the cargo, along with any autophagy adaptors or tags (for example, polyubiquitin, discussed later in this chapter

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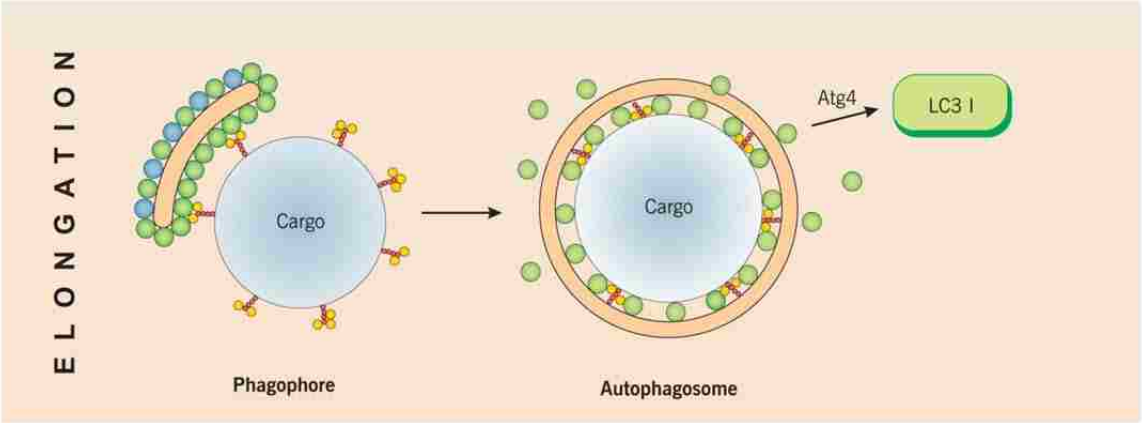

Figure 1.2. Elongation. The phagophore grows around the target (Cargo) for autophagic capture. In this example, the cargo is tagged with polyubiquitin chains (red, sequential circles) which are recognized by an autophagy adaptor (yellow circles), and in turn, associate with LC3 II on the phagophore membrane, thus resulting in recognition. After fusion and closure of the phagophore membrane, the structure is now called an autophagosome, where LC3 II, cargo, polyubiquitin, and autophagy adpators can be trapped inside. LC3 II on the outside of the autophagosome can be removed by the activity of Atg4 and recycled back into the cytosol as LC3 I.

and in Chapter 5) that may have been associated with the cargo during recognition and capture. The recognition and capture of targets for autophagic degradation, which occur during initiation and elongation, is mediated by a variety of autophagy cargo adaptor proteins. The first adaptor protein to be recognized was p62, also known as sequestosome 1 [12] (for a review and explanation of the domains of p62, see Fig. 1.3 and G1). Before the discovery of p62's role in autophagy, it was originally found in complex with aggregates in several diseases such as Paget's disease of bone, Parkinson's disease, Huntington's disease, and hepatocellular carcinoma. Subsequent to the discovery of p62, other autophagy adaptor proteins have been identified, including Nix, NBR1 [13], and ALFY [14, 15].

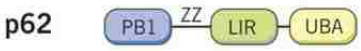

Figure 1.3. p62 and domains. P62, also called sequestosome 1, is an autophagic adaptor protein involved in recognition of cytoplasmic targets, typically aggregated proteins, for autophagy. Specifically recognizes the polyubiquitin tag of proteins through its UBA domain and associates with LC3 through it LIR domain. PB1 domain allows dimerization with itself and other autophagy adaptors. The ZZ type zinc finger is involved in other signaling functions of p62.

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The last stage of autophagy is maturation. During maturation (Fig. 1.4), lysosomes fuse with the autophagosome, where the inner membrane, cargo, and LC3 II proteins that were trapped inside are degraded by acidification and protease activity. The resulting basic cellular components are believed to be recycled back into other cellular pathways as needed for protein production, organelle formation, and other homeostatic processes. Recently, Beclin 1 has been shown to be involved in regulating this stage of autophagy as well. Here, Beclin 1 association with hVPS34 can be positively regulated by UVRAG and negatively regulated by Rubicon [6, 7].

Consequent to the ability of autophagy to regulate intracellular composition, the autophagy mechanism has been exploited in the management of a broad spectrum of diseases and physiological states including cancer, aging, neurodegeneration, inflammation and immunity. Autophagy's role in immunity includes both adaptive and innate immunity. In adaptive immunity, autophagy has been found to affect Th1 versus Th2 differentiation of the T cell response. It has also been shown to assist in antigen loading of MHC class II molecules for presentation. In innate immunity, autophagy has been shown to be involved in direct elimination of pathogens, and to function as both an effector and a regulator of the innate immunity machinery through interactions with toll-like receptors (TLR) and antigen presentation through MHC class I [16, 17].

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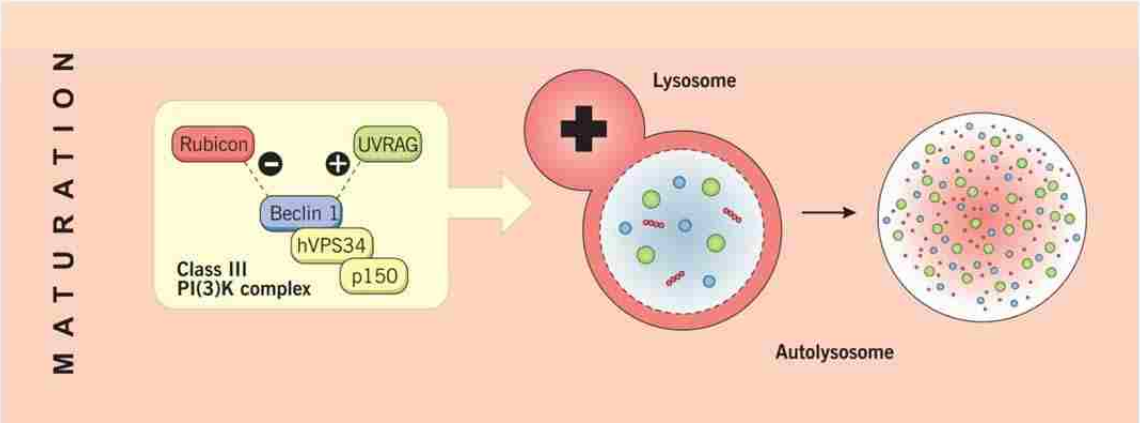

Figure 1.4. Maturation. The autophagosome formed during elongation will fuse with lysosomes, now called an autolysosome, resulting in degradation of the inner membrane and contents trapped inside. This process of maturation can be controlled by the Beclin 1, hVPS34, p150 complex through positive and negative regulation of this complex by UVRAG and Rubicon.

Studies on autophagy's role in elimination of pathogens have shown that autophagy can act as a cell-autonomous defense against intracellular pathogens, whereby invading microbes or their toxic products can be targeted by autophagy and degraded through fusion with lysosomes. Specifically, studies involving the knock down of essential autophagy genes have been shown to aggravate infections by vesicular stomatitis virus, tobacco mosaic virus, *Staphylococcus aureus*, *Listeria monocytogenes*, group A *Streptococcus*, *Bacillus anthracis*, *Helicobacter pylori*, *Vibrio cholerae*, and others [5].

However, in the case of pathogenic species, certain microbes are able to avert the degradative stages of autophagy. These successful pathogens have evolved anti-autophagic factors to protect against autophagic clearance. One of the first pathogens of this kind to be studied was herpes simplex virus type 1 (HSV-1) [18]. Orvedahl et al. showed that the HSV-1 neurovirulence protein ICP34.5 binds to and inhibits Beclin 1, thereby preventing proper clearance of the herpes virus in brain tissue and leading to lethal encephalitis in a mouse model. Yet other studies have shown that certain

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pathogens can use autophagy to assist in replication. For example, Jackson et al. showed that induction of autophagy during poliovirus infection actually increases production, as well as release of the virus [19]. They went on to hypothesize that autophagic membrane was being hijacked by the poliovirus as a source of membrane for envelope formation and as a site of assembly for biogenesis. These reports on autophagy involvement in pathogen life cycles led us to hypothesize that human immunodeficiency virus type 1 (HIV-1) intersects the autophagy pathway.

Elimination of HIV by autophagy should be escaped by the virus as a requirement to developing AIDS

Human immunodeficiency virus type 1 (HIV-1) is the most common cause of acquired immunodeficiency syndrome (AIDS) in humans (Fig. 1.5, showing structure and components of HIV). HIV-1 is believed to have evolved from simian immunodeficiency virus (SIV) variants that recombined in chimpanzees, while HIV-2 is believed to have evolved more directly from sooty mangabey monkeys [20]. Two important elements in the pathogenicity of the pandemic strain of HIV-1 (group M) that are generally impaired in the non-pandemic strains of HIV, including HIV-2, are the ability of the virus to (i)

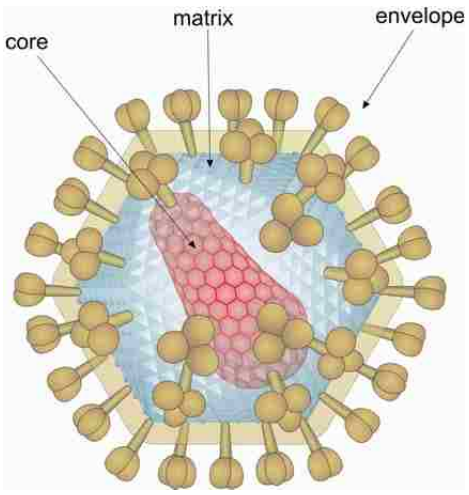

Figure 1.5. HIV structure and components. The HIV virion is an enveloped, double-stranded RNA virus. The envelope of HIV contains glycoprotein (gp) 120 heads and gp41 stalks. Inside the envelope is the matrix of the virus, composed of p17 (derived from HIV Gag) structural protein. Inside the matrix of the virus is the core, composed of p24 capsid protein (also derived from Gag) forming a Fullerene core shape.

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down-regulate antagonizers to the budding of HIV-1, CD4 and tetherin, despite the requirement for CD4 as the primary receptor for virion fusion and entry, and (ii) an evolved loss of the viral protein Nef to dampen immune activation through down-regulation of surface CD3 [21]. The enhanced budding allows the virus to promote host-wide infection more efficiently in the face of the greater immune activation. Paradoxically, the inability of Nef to down-regulate CD3 in pathogenic strains of HIV coincides with the expression of the viral protein Vpu, which is also responsible for the down-regulation of surface tetherin. Viruses that encode *Vpu* almost always encode a *Nef* that is deficient for down-regulation of CD3 [21].

Much of the pathogenicity of HIV-1 has been attributed to its regulator protein Nef. Perhaps the best demonstration of the pathogenicity enhancing effects of Nef have been in studies showing that *Nef*-deficient virus *in vivo* results in an attenuated strain of HIV, marked by reduced yields of the virus in the infected individual's blood plasma [22]. The most well-known of these studies has been the Sydney Blood Bank Cohort, where 8 long-term non-progressors of HIV, all containing mutations in their *Nef* alleles, were followed for more than 20 years. For more than 17 of these years these individuals had nearly undetectable plasma viral loads reflecting the impaired production of HIV deleted for *Nef* [22]. Studies on this cohort and others have hypothesized that the decreased viral load seen in these patients is due to a defect in viral replication as assisted by the Nef protein. Several follow-up studies sought to investigate the exact role of Nef in viral pathogenesis, with the majority of these indicating that Nef's effects on maintaining viral load are cumulative and primarily based on two effects: first, in Nef enhancing virion release and

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second, in Nef enhancing infectivity of those particles released. These activities would result in an enhanced host-wide infection through increased cellular transmission.

The most in-depth studies on the effect of Nef in promoting viral release to date have shown that Nef specifically mediates the following activities: (i) down-regulation of CD4, thereby preventing the CD4-mediated blockade of viral assembly and budding at the plasma membrane (further discussed below), (ii) the bridging of the HIV structural protein precursor GagPol (cleaved into Gag, which is further processed into structural proteins, and polymerase) to the ESCRT machinery, through AIPI/ALIX, to assist in HIV egress [23], and most recently, (iii) implications for Nef in enhancing the incorporation of fusogenic cholesterols into viral particles which could assist in budding, as well as subsequent fusion of the virion [24, 25]. In addition to enhancing release, and perhaps even fusion events, the gain in infectivity by Nef has also been shown to include altered trafficking of cellular components necessary for viral replication, altered trafficking of cellular components necessary for survival of infected cells, and trafficking and assembly of viral components necessary for viral replication [26].

One of the earliest studies on how CD4 impairs release of viral particles showed that CD4 can directly interact with and bind envelope proteins at the plasma membrane and prevent proper budding and egress of virions from the infected cell [27]. From this study, it was demonstrated that down-regulation of surface CD4 from the plasma membrane during infection required the presence of Nef. It had also been shown about this time that Nef could also interact with the endocytic machinery through interaction with adaptor

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protein (AP) complexes [28]. Thus, it was hypothesized that Nef bridged CD4 with the endocytic machinery via interaction with AP complexes, thereby mediating its endocytosis from the plasma membrane. After endocytosis of CD4, Nef then mediates its targeting to lysosomes through interaction with β-COP [29]. Because many of the factors involved in endocytosis are now known to also be involved in autophagy, it is therefore tempting to suggest that vesicular re-trafficking by Nef may represent a conserved mechanism by which Nef manipulates autophagy. Indeed, it has been recently shown that adaptor protein 2 (AP-2), the same AP now known to be required for Nef-mediated CD4 down-regulation [30], is involved in the endocytic delivery of plasma membrane to autophagy for autophagosome biogenesis [31].

The possible role of autophagy in HIV-1 pathogenesis was first investigated by Codogno and Biard-Piechaczyk. In their article published in 2006, the two authors showed that an interaction between the HIV envelope and $CD4^+$ T cell co-receptor CXCR4 induced autophagy-mediated cell death [32]. The authors suggested that this may reveal how uninfected bystander $CD4^+$ T cells are killed during the development of AIDS. Subsequent to this publication, a study published in 2008 identified the involvement of over 250 host genes as essential factors for HIV replication in a siRNA genomic screen [33]. The authors, Brass et al., called these genes HIV dependency factors (HDFs). Of the over 250 HDFs Brass identified were four autophagy genes: Atg 7, Atg 8, Atg 12, and Atg16L2. Because the cells this study used in the genomic screen were not primary targets of HIV infection, it is possible that more or different autophagy genes are

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involved in those cell types that are primary targets for HIV infection, $CD4^+$ T cells and macrophages.

Species-specific variation in the development of a productive infection exists among primates

Despite the ability of HIV-1 to antagonize human cells in a variety of ways, some species of non-human primates that become infected with HIV-1 never progress to AIDS, while other species are never infected after exposure to the virus [34]. Thus, it has been hypothesized that some non-permissive species of monkeys, primarily Old World monkeys, have evolved ways to restrict HIV-1 infection by encoding specific cellular restriction factors that block productive infection [35-38]. This hypothetical restriction factor to the lentivirus among non-permissive species of monkeys was first called *l*enti*v*irus susceptibility factor 1 (Lv1) [39].

The rhesus macaque TRIM5α (RhTRIM5α) protein was the first restriction factor definitively identified in a monkey to potently restrict HIV-1 infection at a post-entry, pre-integration stage in the viral life cycle [40]. Subsequent to the discovery of RhTRIM5α was the discovery of a variety of other species variants of TRIM5α among the Old World monkeys, such as the owl monkey TRIMCyp protein (a fusion of the RBCC motif of TRIM5 to cyclophilin A) [41, 42]. Various other restriction factors, such as Fv1 in mice, as well as APOBEC3G, Bst-2 (Tetherin), a variety of cellular micro RNAs in humans, and zinc-finger antiviral protein (ZAP) in rats, have been shown to inhibit retrovirus infections at various other stages in the virus life cycle [43].

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RhTRIM5α as a linking factor for HIV-1 to autophagic degradation

The TRIM (tripartite motif) family of proteins is a large class of zinc-finger proteins, several of which have been implicated in innate immunity [43-45]. Common to all TRIM proteins is an RBCC motif containing the RING, B-box 2, and coiled-coil domains (for a review and explanation of the RhTRIM5α domains, see Fig. 1.6 and G2). RhTRIM5α, along with several other virus restricting TRIM proteins, also contains a B30.2(SPRY) domain in addition to the RBCC motif. Studies have shown that the SPRY domain of RhTRIM5α is critically important for HIV restriction because it allows for direct interaction of RhTRIM5α with HIV-1 capsid (CA) protein p24 [46, 47], forming a complex around the HIV core particle in what is commonly referred to as the RhTRIM5α-HIV capsid complex (Fig. 1.7). This interaction has been shown to be essential for significant degradation of the incoming viral core to occur after fusion with the cell membrane and release into the cytoplasm. However, the exact mechanism leading to the subsequent degradation of the viral particle is still unresolved.

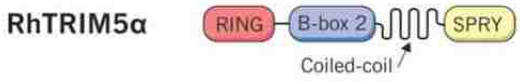

Figure 1.6. RhTRIM5α and domains. RhTRIM5α is a potent early entry restriction factor to HIV-1 expressed in rhesus macaque tissues. The RING domain of RhTRIM5α has E3 ubiquitin ligase activity. The B-box 2 domain is required for HIV restriction. The coiled-coil domain is the site of RhTRIM5α oligomerization. The SPRY domain is the site of specific

Figure 1.7. RhTRIM5α-HIV capsid complex. Illustration shows HIV's Fullerene core (made of self-associating p24 proteins) surrounded by a complex of RhTRIM5α oligomers.

The RING domain of this family has been of particular interest in recent years in that it was found to have E3 ubiquitin ligase activity, which in conjunction with an E2 ubiquitin

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ligase allows the protein to attach ubiquitins to lysine residues within proteins. Several TRIMs are known to self-ubiquitinate, including the newly discovered TRIM5α from rhesus macaques [43, 48-50]. However, the function of this self-ubiquitination to RhTRIM5α restriction of HIV, if any, is still unclear. One of these studies demonstrated that RhTRIM5α is rapidly turned over in the cell as a result of self-ubiquitination in the absence of infection [48]. This study went on to hypothesize that self-ubiquitination targeted RhTRIM5α to the proteasome for degradation. Several subsequent studies suggested that self-ubiquitination of RhTRIM5α could be a target for proteasomal degradation of the entire RhTRIM5α-HIV capsid complex [51-53]. Unfortunately, other studies were unable to resolve the fact that although proteasome inhibitors were able to alleviate some of the restrictive capabilities of RhTRIM5α, they were not able to alleviate all restriction, and, at the same time, were also equally able to alleviate the minimally restrictive capabilities of human TRIM5α (HuTRIM5α) [54-56]. Furthermore, the Diaz-Griffero study also showed that altering the rate of poly-ubiquitination of RhTRIM5α does not affect the rate of degradation of the HIV CA [48]. Thus, based on these collective works, we hypothesize that the proteasome is not the primary target for CA degradation, but rather, the autophagosome (referring to the basic physical unit of the autophagy pathway). We propose that the specific role of RhTRIM5α is to selectively target HIV-1 viral particles to the autophagosome for the purpose of degradation and clearance through autophagy. Consistent with our proposal, Chatterji et al suggested that RhTRIM5α action on the incoming viral particle is entirely proteasome independent and hypothesized that “proteases other than proteasomal proteases, possibly lysosomal proteases, are responsible for CA degradation in the vesicular compartment” [56].

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Interestingly, another TRIM has already been implicated in autophagy. TRIM63 (MuRF1), which regulates skeletal muscle protein, has been shown to do so in an mTOR-dependent manner [57]. Thus, it is possible that RhTRIM5α might also require mTOR to degrade HIV, thereby revealing a mechanism for autophagic degradation of HIV. Finally, another possible mechanism for RhTRIM5α to target HIV to autophagy was suggested by data showing that TRIM5α associates with hsc70. Hsc70 is the chaperone protein used in chaperone-mediated autophagy [58]. While this study did not state any effect of the association of TRIM5α with hsc70, it is possible that this interaction may mediate yet another alternative method for autophagic capture of incoming HIV core.

TRIM5α may regulate autophagy as part of the innate immune response

Many TRIM proteins have been shown to regulate innate immunity. Among these are: Pyrin (TRIM20), involved in suppressing inflammation by interacting with the inflammasome complex; Ro52 (TRIM21), identified as a unique Fc receptor for IgG that also interacts with IRF8 and is involved in Sjögren's syndrome and systemic lupus erythematosus, likely as a consequence of its Fc receptor activity; TRIM8, known to inhibit SOCS1-mediated down-regulation of IFNγ signaling; TRIM25, which promotes the antiviral response by enhancing RIG-I-mediated signaling (which itself can associate with autophagy proteins [59]); TRIM27, which down-regulates toll-like receptor (TLR) signaling by interfering with $IKK_{\alpha/\beta/\gamma}$ activity; and last, TRIM56, which has been shown to regulate double-stranded DNA-mediated type I interferon induction [44, 60, 61]. Many of the regulatory functions of TRIM proteins are mediated by the activity of their RING domain, especially in the case of TRIM25, which specifically mediates K63

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polyubiquitination of RIG-I to enhance its signaling [62, 63]. It may be that most, if not all, of these regulatory effects by TRIM proteins might be mediated, in part, through autophagy.

An interesting aspect of autophagic target recognition is that many proteins that end up in the autophagosome have K63 type polyubiquitin tags. In fact, some agree that K63 polyubiquitin itself is a tag specifically for autophagosome capture and clearance, much like the K48 type polyubiquitin tag has been strongly associated with proteasome-mediated degradation. The "K63 type" refers to single ubiquitin proteins connected to each other through a lysine residue at position 63, while the "K48 type" refers to ubiquitins connected through a lysine at position 48. Depending on the type of lysine linkage, the tertiary structure of the polyubiquitin varies and is believed to have effects on the tertiary structure of the protein it tags.

Specifically, studies on the association between K63 type polyubiquitin and autophagy have shown the following: K63 polyubiquitination of Beclin 1 (mediated by TRAF6, another RING domain containing protein, involved in the NFκB pathway and previously known to associate with p62 [64]) can induce autophagy [65], Parkin (yet another RING domain containing protein, where mutations can lead to Parkinson's disease) induces p62-dependent autophagy of mitochondria (mitophagy) through K63 polyubiquitination of itself and a mitochondrial target(s) [66], and most importantly that autophagic cargo adaptor p62 itself shows preference for K63 type polyubiquitinated proteins [64, 66-68].

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Thus, several lines of evidence indicate a role for the RING domain of TRIM proteins in mediating induction of autophagy: (i) many proteins that end up associated with autophagy contain K63 type polyubiquitin tags [65-71], (ii) TRIM proteins (TRIM25, 38, and 21) appear to be capable of mediating K63 type polyubiquitination [62, 63, 72, 73], (iii) E3 ubiquitin ligases, such as Parkin and TRAF6, can mediate targeting of proteins to autophagy by K63 polyubiquitination, and (iv) p62, which seems to prefer K63 polyubiquitinated proteins, is already known to associate with another TRIM protein, TRIM55, during regulation of cardiac myofibril assembly and turnover [74], and likely also TRIM1 and/or TRIM18 during autophagy of the midbody ring in cytokinesis [75].

Finally, since the Chatterji study showed that proteasome inhibitors were able to increase the presence of HIV capsid protein (p24) equally in cells expressing either RhTRIM5α or HuTRIM5α [56], this may indicate that studies with proteasome inhibition have revealed a second, non-specific mechanism of viral clearance that has previously gone unrecognized, but requires TRIM5α-induced autophagy. This second mechanism of viral clearance would be due to a non-specific capture of virus particles by an autophagy that is in an elevated, non-basal state. This may explain why other studies have reported a slight, inexplicable, restrictive capability of HuTRIM5α despite the lack of direct interaction with the viral capsid [40, 45, 76]. Induction of autophagy through TRIM5α might be accomplished either: directly, with plasmid transfection, leading to the over-expression of the TRIM5α proteins, or indirectly, through the treatment of cells with IFN_{β}. The use of type I interferons to stimulate an increase in endogenous TRIM5α expression has been previously reported [77-79]. These results might reveal a new

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therapeutic target for the prevention of HIV infections, which is that pharmacological induction of autophagy, at the time of infection, may entirely abrogate the acquisition of the virus and thus prevent infection with HIV.

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Hypothesis

Based on the published literature described here, I hypothesize that HIV-1 intersects with the autophagy pathway in a species-specific manner, and that the outcome in development to AIDS should reflect an ability of HIV-1 to circumvent autophagy and lead to a productive infection. The following studies investigate this by (i) exploring the role of autophagy in a primary human cell target for HIV-1, the human macrophage, (ii) exploring the role of autophagy in HIV infection in the presence of a non-human primate restriction factor, RhTRIM5α, and (iii) exploring a newly revealed ability for this restriction factor to regulate autophagy.

Dissertation Summary

Chapter 2: Autophagy pathway intersects with HIV-1 biosynthesis and regulates viral yields in macrophages

In this chapter, we explore the interactions between autophagy and the HIV-1 life cycle in the human macrophage. We reveal a mechanism by which autophagic elimination of HIV-1 is impaired and uncover a surprising contribution of the autophagy pathway to HIV-1 biosynthesis. Furthermore, we identify the factor responsible for these opportunistic effects on autophagy as the HIV-1 protein Nef and show that the actions of Nef require its diacidic motif ^{174}DD^{175}.

Chapter 3: Autophagy-mediated restriction of HIV-1 by RhTRIM5α

In this chapter, we explore a requirement for autophagy in the degradation, and subsequent restriction, of HIV-1 by the rhesus macaque restriction factor, rhesus TRIM5α

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(RhTRIM5α). Here we show that degradation of the incoming HIV-1 viral capsid (CA) by RhTRIM5α depends on an interaction with the autophagic machinery through p62 and that degradation of the viral CA requires an intact autophagy, which can be inhibited with siRNA, bafilomycin A1, or e64d and pepstatin A treatment.

Chapter 4: TRIM5α is an innate immunity regulator inducing autophagy

In this chapter, we uncover a surprising ability of the TRIM5α of either species to regulate autophagy. Specifically, we show that both species variants are capable of inducing autophagy and explore a possible mechanism by which autophagy could be controlled by TRIM5α. We find that interaction with the autophagic machinery through p62 is not unique to RhTRIM5α, as human TRIM5α (HuTRIM5α) also interacts with p62. Finally, we propose a mechanism by which either TRIM5α can induce autophagy, and present the hypothesis that regulation of autophagy by TRIM5α represents a unique form of autophagy-mediated innate immunity regulation.

Chapter 5: Discussion and future implications

Here we summarize our findings from the above studies and discuss future implications of our research to the treatment of HIV-1 infection. We discuss new literature published since these studies began that support our findings and shed new light on our understanding of the mechanisms revealed by each study. Finally, we discuss the future directions to the research and results uncovered here.

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CHAPTER 2

AUTOPHAGY PATHWAY INTERSECTS WITH HIV-1 BIOSYNTHESIS AND REGULATES VIRAL YIELDS IN MACROPHAGES

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Authorship requirements

General guidelines

1) To make substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data.

2) To participate in drafting the manuscript or revising it critically for important intellectual content.

3) To approve the final version of the submitted manuscript.

My contributions to the following publication consisted of:

- Experiments shown in Fig. 2.1G, 2.3J, 2.4E, 2.5D, A3G
- Original writing
  - figure legends
  - materials and methods
- Revising and editing
- Literature review
  - previously known functions of Nef
  - technical reviews
- Final approval

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AUTOPHAGY PATHWAY INTERSECTS WITH HIV-1 BIOSYNTHESIS AND REGULATES VIRAL YIELDS IN MACROPHAGES

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Abstract

Autophagy is a cytoplasmic degradative pathway that can participate in biosynthetic processes, as in the yeast Cvt pathway, but is more commonly known for its functions in removing damaged or surplus organelles and macromolecular complexes. Here, we find that autophagy intersects with human immunodeficiency virus (HIV) biogenesis, mirroring the above dichotomy. Early, nondegradative stages of autophagy promoted HIV yields. HIV Gag-derived proteins colocalized and interacted with the autophagy factor LC3, and autophagy promoted productive Gag processing. Nevertheless, when autophagy progressed through maturation stages, HIV was degraded. This, however, does not occur, as the HIV protein Nef acts as an antiautophagic maturation factor through interactions with the autophagy regulatory factor Beclin 1, thus protecting HIV from degradation. The dual interaction of HIV with the autophagy pathway enhances viral yields by using the early stages while inhibiting the late stages of autophagy. The role of Nef in the latter process enhances yields of infectious HIV and may be of significance for progression to clinical AIDS.

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Introduction

Autophagy is a general homeostatic process in eukaryotic cells whereby protions of the cytoplasm, containing cytosol or organelles, are sequestered into double membrane-bound autophagic vacuoles for fusion with lysosomal organelles and subsequent degradation of the captured contents in the resulting autolysosomes [80, 81]. A functional core autophagy pathway and associated processes are important for cell survival under starvation or growth factor withdrawal conditions, programmed cell death, elimination of aggregated proteins, and removal of surplus or damaged organelles [82, 83]. Autophagy is regulated by signaling pathways centered around the Ser/Thr protein kinase Tor (target of rapamycin) and phosphatidylinositol 3-kinases (PI3Ks), both type I (inhibitory to autophagy) and type III (essential for execution of autophagy). The type III PI3K hVPS34 acts in a complex with Beclin 1 (yeast Atg6), a factor endowing hVPS34 with its role in autophagy [8]. A detailed picture on these and other autophagy proteins (Atg) in mammalian cells is emerging, with the core pathway resembling that in yeast [80-82].

Autophagosome biogenesis and wrapping around autophagic targets is facilitated by the two specialized protein conjugation systems: the Atg5-12/16 complex stimulates a second conjugation system, whereby LC3 (Atg8) undergoes conversion from its free C-terminus state (LC3-I) to its C-terminally lipidated form (LC3-II) covalently modified by phosphatidylethanolamine. The lipidated LC3-II localizes to the membrane of a growing phagophore. Once a phagophore closes, this results in the formation of a double membrane-delimited autophagosome that typically matures into an autolysosome through

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fusion with multivesicular body (MVB) compartments [84] and other lysosomal organelles [80]. Most cells undergo baseline autophagy to remove protein aggregates and spuriously damaged mitochondria or other organelles, or to adjust the cellular biomass [83]. With a broad range of targets, ranging from protein complexes to whole organelles, autophagy is a process affecting a multitude of health and disease states; has been implicated in neurodegeneration, cancer, and aging [83]; and has emerged as an important player in inflammatory and infectious diseases [5, 85].

Autophagy is now well recognized as an innate and adaptive immunity mechanism [85, 86]. Pharmacologically, physiologically, or immunologically induced autophagy can act as a powerful antimicrobial defense [5, 85, 87-93]. Autophagy is under the control of immune receptors and cytokine signaling [85, 86], and is stimulated upon microbial recognition by innate immunity pattern recognition receptors [94-97] or activation with Th1 cytokines [98]. However, certain pathogens can harness this process to assist their own propagation [5, 18, 19, 91]. Interestingly, a recent large scale siRNA screen of host cell factors required for human immunodeficiency (HIV) type 1 (HIV-1) replication has identified several Atg factors among >250 HIV dependency host genes [33]. Thus far, no in-depth functional links between Atg proteins or processes and HIV have been established.

Here, we tested mechanistically whether and how autophagy affects HIV yields during de novo virion generation. We found that the Atg proteins LC3 and Beclin 1 (Atg6) are found in complexes with the HIV proteins Gag and Nef, respectively. The latter

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interaction provides the basis for Nef function in control of autophagy. The Nef protein of HIV-1 and simian immunodeficiency virus (SIV) is required for efficient viral replication and acquired immune deficiency syndrome (AIDS) pathogenicity in HIV-1-infected humans or SIV-infected macaques [99-101]. The methods by which Nef protein acts as a pathogenic factor *in vivo* are not fully understood, but involve several mechanisms [102-105]. Recent findings suggest that the inability of lentivirus Nef to suppress $CD4^+$ T cell activation correlates with viral pathogenesis [106, 107]. Our findings presented here uncover an additional, previously unappreciated Nef action in control of autophagy. Nef functions in preventing destruction of HIV from autophagy in its role of a cell autonomous antimicrobial defense.

Results

Basal autophagy augments HIV yields in macrophages

Basal autophagy is operational in all cell types, particularly in mononuclear phagocytic and dendritic cells [108]. To test whether basal autophagy can affect HIV yields, we pharmacologically inhibited autophagy in primary human macrophages differentiated from peripheral blood monocytes and infected with the macrophage-tropic HIV strain SF162, and determined yields of the infectious virus released from the macrophages. The macrophages treated with 3-methyl adenine (3MA), a conventional inhibitor of autophagy, yielded three-fold fewer infectious virions compared with the untreated control (Fig. 2.1A). The magnitude of this effect could be increased with higher inhibitor concentrations, but we used mild pharmacological and other treatments in these and subsequent experiments to avoid nonspecific effects. The autophagy requirement for

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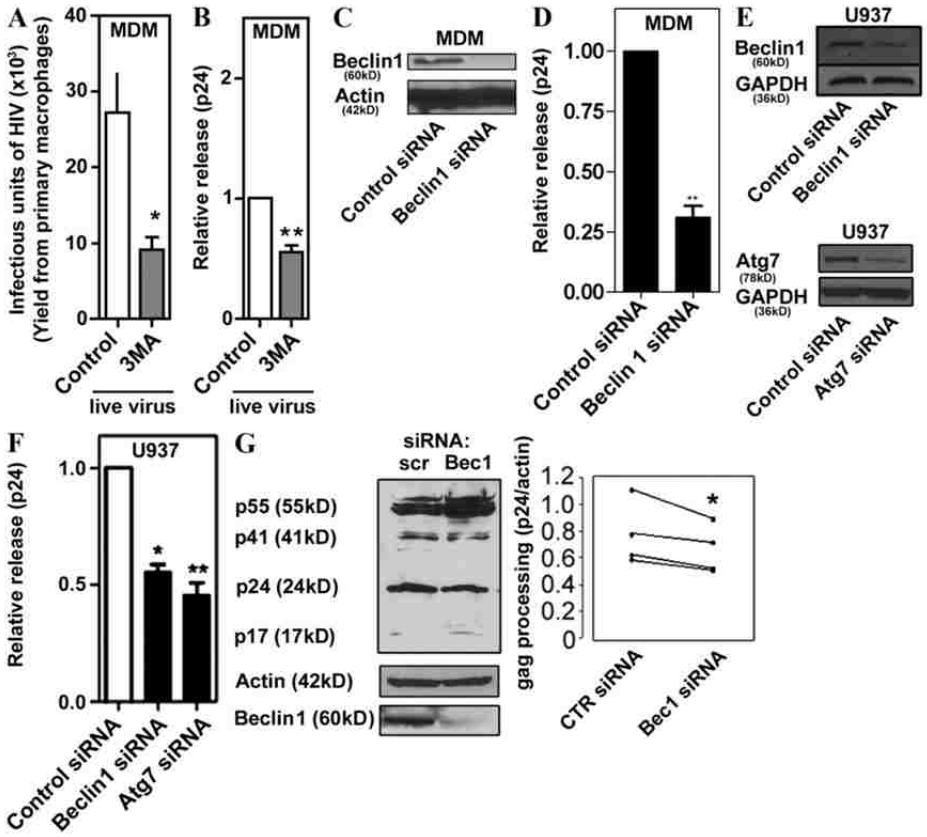

Figure 2.1. Autophagy is required for optimal HIV yields in macrophages. (A) Pharmacological blockage of autophagy inhibits release of infectious virions. Human peripheral blood MDM were infected with SF162 HIV-1 for 10 d, then washed and incubated with control media or 3MA for 4.5 h. Culture supernatants containing HIV particles were used for a MAGI infectivity assay as described in Materials and methods. (B) Relative viral release was calculated as a ratio of extracellular-to-intracellular Gag-derived core antigen capsid protein CA (p24) and normalized to the control. (C) Western blot showing siRNA knockdown of Beclin 1, 7 d after transfection in MDM. (D) Human MDM were transfected with siRNA to Beclin 1 and infected with SF162 HIV-1 for 7 d, then p24 yields were quantified. (E) Western blots showing siRNA knockdown of Beclin 1 and Atg7 48 h after transfection in U937 cells. (F) Knockdown of autophagy regulators Atg7 and Beclin 1 inhibits basal viral yields released from macrophages. U937 cells were cotransfected with Beclin 1 or Atg7 siRNA and pMSMBA (a clone of NL4-3). Data indicate means; error bars indicate ±SEM ($n \geq 3$). *, $P < 0.05$; **, $P < 0.01$; †, $P > 0.05$ (analysis of variance [ANOVA]). (G) U937 cells were knocked down for Beclin 1 expression and infected with VSV-G-pseudotyped HIV. Cell lysates were prepared for Gag processing analysis. *, $P < 0.05$, paired *t* test.

optimal HIV yields also assessed by determining extracellular release of the HIV capsid protein Gag p24 [109]. The p24 released from live virus-infected primary macrophages was reduced in cells subjected to inhibition by 3MA (Fig. 2.1B). Because pharmacological inhibitors such as 3MA may not affect only the autophagic pathway, we ascertained a role of the bona fide autophagy pathway by knocking down Beclin1 (Atg6)

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in primary macrophages (Fig. 2.1C and D) and in macrophages differentiated from monocytic U937 cells (Fig. 2.1E and F). Knockdown of Beclin 1 diminished p24 yields in both primary monocyte- and U937-derived macrophages (Fig. 2.1D and F). A knockdown of another essential autophagy factor, Atg7, resulted in a similar effect on p24 yields (Fig. 2.1F); Atg7 and Beclin 1 knockdowns affected autophagy in U937 cells, as determined by GFP-LC3 puncta/cell counts (Fig. A1). We next examined the intracellular Gag processing by monitoring Gag-derived p24 band intensity in immunoblots of cell extracts. When U937 cells knocked down for Beclin 1 were infected with vesicular stomatitis virus G (VSV-G)-pseudotyped NL4-2 HIV-1, cellular p24 levels were reduced compared with controls (Fig. 2.1G). Collectively, these results indicate that basal autophagy promotes optimal Gag processing and yields of HIV in macrophages.

HIV and HIV Gag-derived proteins colocalize with the autophagy marker LC3

To test whether and how the autophagy pathway intersects with HIV, we examined the relative distribution of HIV virions and Atg proteins. In macrophages, HIV virions are found in membranous domains [110-115] recently shown to be contiguous with plasma membrane [114, 116, 117], which facilitates colocalization studies. The Gag-p17-specific antibody showed colocalization of the budded virus with autophagy marker LC3 (Atg8; Fig. 2.2A). By ultrasctructural analysis, HIV virions were observed in these compartments (Fig. 2.2B), which, based on presence of clathrin-coated pits (Fig. 2.2B, asterisk; Fig. A1C), were consistent with the previously reported plasma membrane connections [114]. These morphologically identified compartments also labeled for LC3 (Fig. 2.2C) and p24 (Fig. A1D). A dual labeling procedure was not practicable, as LC3

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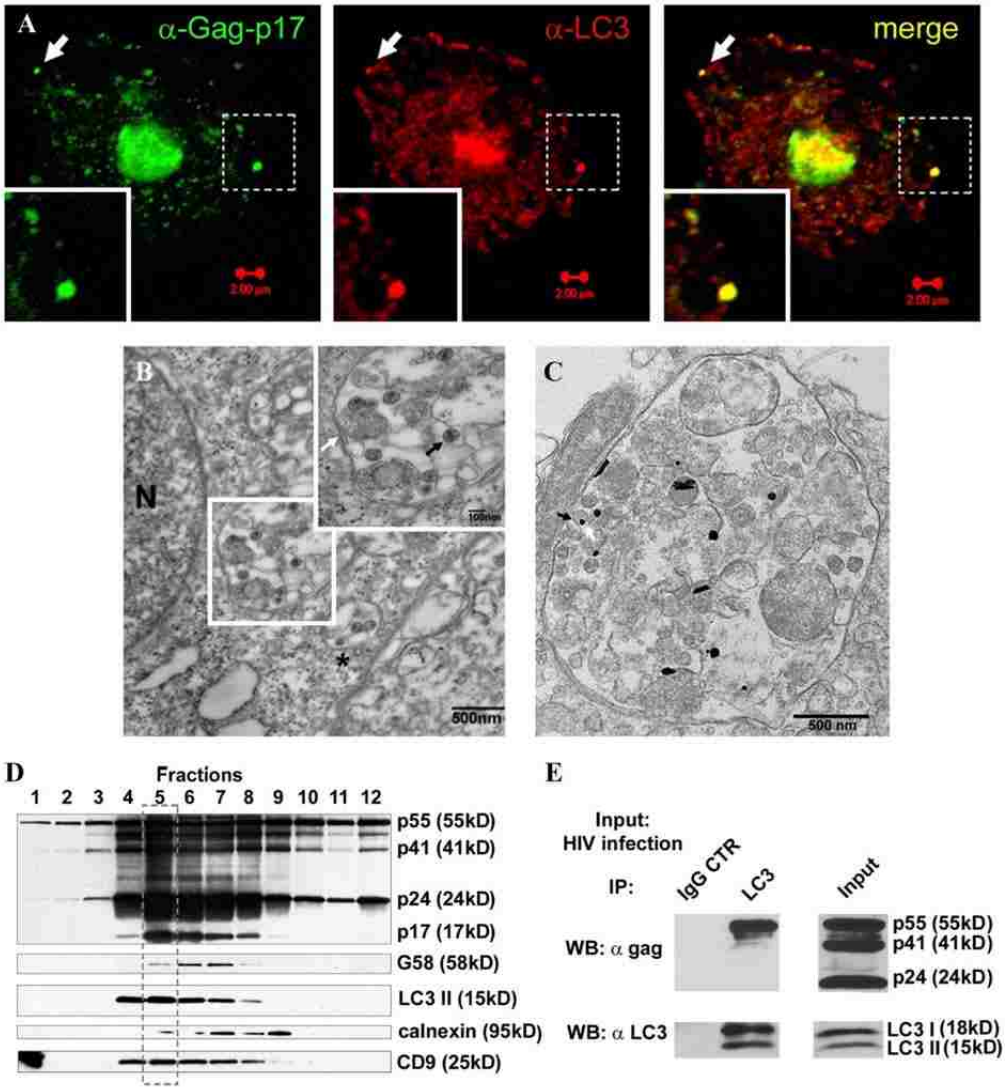

Figure 2.2. Autophagy protein LC3 colocalizes, copurifies, and coprecipitates with HIV Gag. (A) MDM were infected with VSV-G-pseudotyped HIV and immunostained for Gag-p17 and LC3. Arrows, a peripheral structure as an example of Gag-p17 and LC3 overlap. (B) Ultrastructural analysis of HIV virions in macrophages infected with HIV. U937 cells were infected with VSV-G-pseudotyped HIV. (inset) Enlarged region boxed in the electron micrograph. White arrow, membrane; black arrow, HIV virion; asterisk, HIV virions in a membranous compartment with a clathrin-coated pit consistent with plasma membrane origin. An enlarged image of this profile is shown in Fig. A1C. (C) HIV-containing compartments are positive for LC3. Immunoelectron microscopy showing gold particles (enhanced gold particles appear globular, oval, and acicular) of LC3 in HIV-containing compartments. Arrow: virion and LC3 gold particle. See Fig. A1D for p24 immunoelectron microscopy analysis. (D) HIV Gag precursor and Gag-derived proteins cofractionate with LC3 and the tetraspanin CD9. Subcellular organelle fractionation via isopycnic sucrose gradient separation was performed with lysates from HIV- infected cells (see Materials and methods). 12 fractions starting from the top were immunoblotted for the indicated proteins and organellar markers. The box with the broken line indicates peak band intensity fraction for LC3-II, Gag, and Gag-derived polypeptides, and CD9. (E) HIV Gag coimmunoprecipitates with LC3. U937 cells were infected with HIV and lysates immunoprecipitates for LC3. Immunoblotting with p24 and LC3 antibodies

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was performed on lysate and immunoprecipitate samples. The p24 antibody recognizes all three Gag proteins, as shown in the input. Note that only the precursor Gag-p55 comes down in immunoprecipitates with LC3 (n = 3).

enhanced immunogold labeling resulted in globular, oval, and acicular shapes, and precluded clear distinction.

Biochemical analysis of HIV Gag-derived proteins shows copurification and interactions with the autophagic protein LC3

To determine at the biochemical level whether HIV intersects with the autophagy pathway, we subjected HIV-infected marcophages to subcellular fractionation by isopycnic centrifugation in sucrose gradients. Fig. 2.2D shows that membranes containing HIV Gag polypeptides p24 and p17 cofractionated (note the coinciding peaks framed in Fig. 2.2D) with the autophagic protein LC3. These membranes were enriched for the lipidated for of LC3, LC3-II, normally generated during engagement in an autophagic conjugation cascade driving autophagy initiation and elongation [118], and the recently recognized plasma membrane-associated autophagic events in macrophages [95]. As expected, LC3-I, the soluble cytosolic form of LC3, was not found on these membranes, although it was detectable in whole cell lysates (Fig. A2A). The LC3-II-positive membranes enriched for Gag p55, Gag processing intermediate gp41, and Gag products p24 and p17 did not copurify with the ER marker calnexin, but did cofractionate with CD9, a tetraspanin previously reported to colocalize with HIV virions in monocyte-derived macrophages (MDM; Fig. 2.2D; [114]).

We next tested whether HIV Gag interacted with autophagy proteins in coimmunoprecipitation experiments. Fig. 2.2E shows that LC3 is found in protein

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complexes with the HIV Gag. These findings reinforce the subcellular fractionation experiments (Fig. 2.2D), are in keeping with morphological analyses (Fig. 2.2A-C), and demonstrate that HIV components and virions intersect with the autophagic pathway with the functional consequence of augmenting Gag processing (Fig. 2.1G) and HIV yields (Fig. 2.1A-F).

Pharmacological induction of autophagy enhances HIV yields

We next reasoned that although the basal autophagy is required for optimal HIV yields, physiologically, pharmacologically, and immunologically induced autophagy might affect HIV yields differently, i.e., by degrading HIV components en route to or at viral assembly sites. Significantly, induction of autophagy occurs during HIV infection of macrophages, as described previously [97]. Infection of primary human peripheral blood MDM with HIV-1 strain SF162 virus increased LC3-II levels at 10 d after infection (Fig. 2.3A and B), which coincided with the expected [119] peak HIV production in primary peripheral blood mononuclear cells.

To test the effects of induced autophagy, macrophages were treated with the conventional inducer of autophagy rapamycin, and extracellular HIV yields were determined. Primary macrophages treated with rapamycin yielded higher p24 levels than untreated control when infected with the live virus or transfected with a virus molecular clone (Fig. 2.3C and D). The increase in p24 was not caused by a nonspecific leakage of cytoplasmic contents, as the cytosolic enzyme lactate dehydrogenase levels found in the medium were not altered by rapamycin treatment (Fig. 2.3E). Similar results were obtained when two

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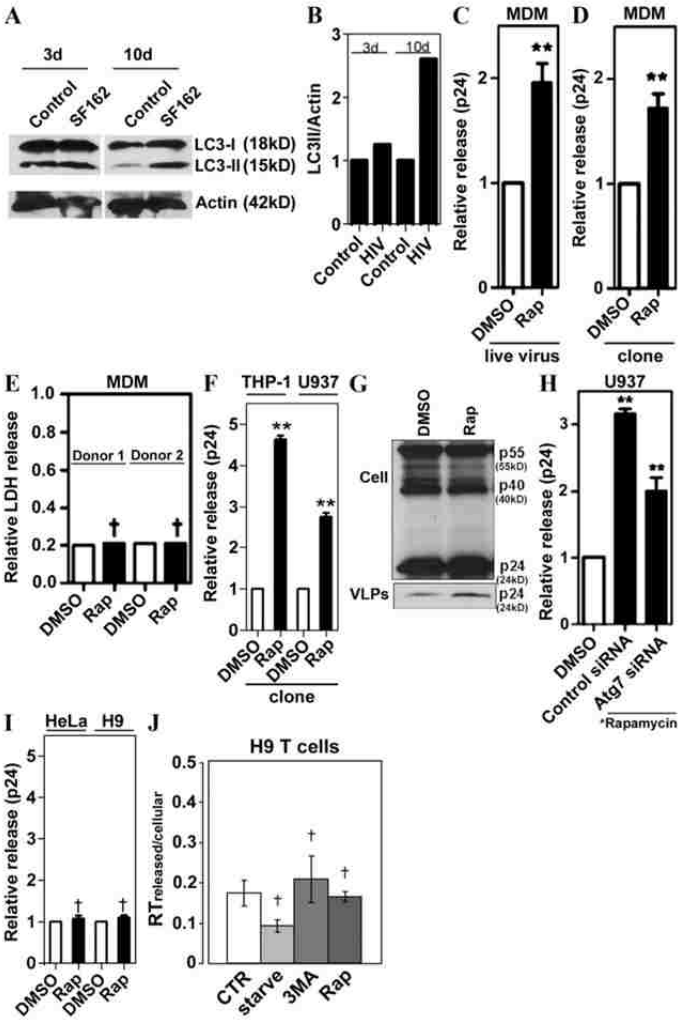

Figure 2.3. Induction of autophagy enhances HIV yields. (A) Human peripheral blood MDM were infected with SF162 HIV-1 for 3 or 10 d, and lysates were immunoblotted for LC3 (one of two equal experiments shown). (B) Quantification: LC3-II band intensity relative to actin. Representative data from one of two independent experiments. (C and D) Induction of autophagy promotes viral yields from MDM. (C) MDM were infected with SF162 HIV for 10 d, then washed and incubated with DMSO or 50 μg/ml rapamycin for 4 h. (D) MDM were transfected with a pMSMBA HIV clone for 48 h, then washed and incubated with DMSO or 50 μg/ml rapamycin for 4 h. (D) MDM were transfected with a pMSMBA HIV clone for 48 h, then washed and incubated with DMSO or 50 μg/ml rapamycin for 4 h. Core antigen capsid protein (p24) was measured as in Fig. 2.1. (E) Assessment of nonspecific cytosolic release. Extracellular lactate dehydrogenase (LDH) release assay was performed with a kit from Promega according to manufacturer's instructions using lysates and media from cells treated with DMSO or rapamycin. Relative LDH was calculated as the ratio between extracellular and total LDH, released from MDM from two different donors. (F) Induction of autophagy increases p24 yields in human monocytic cell lines. THP-1 and U937 cells differentiated into macrophages were transfected with pMSMBA for 48 h, then washed and incubated with DMSO or rapamycin for 4 h. (G) Induction of autophagy increases release of VLPs in human macrophages. U937 cells were infected with VSV-G-pseudotyped pMSMBA-derived virus for 48 h, then washed and treated for 5 h with rapamycin or DMSO alone. VLPs were isolated on a 20% sucrose cushion, and lysates and VLP were immunoblotted for Gag and p24, respectively. (H) U937 cells were co transfected with siRNA and pMSMBA for 48 h, then washed and incubated with DMSO alone or 50μg/ml rapamycin for 4 h. (I) HeLa and H9 cells were transfected with pMSMBA for 48 h, then

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washed and incubated with DMSO or rapamycin for 4 h, and relative viral release (p24) was determined. (J) H9 cells (a T cell line) were infected with T cell tropic HIV_{LAI} for 4 d and washed; relative viral release was found by determining the ratios of extracellular versus intracellular reverse transcription levels. Data indicate means; error bars indicate ±SEM ($n \geq 3$). *, $P < 0.05$; **, $P < 0.01$; †, $P > 0.05$ (ANOVA).

monocytic cell lines, THP-1 and U937, differentiated into macrophages were tested (Fig. 2.3F). Immunoblot analyses of cell-associated viral proteins and p24 in virus-like particles (VLP) in culture medium indicated that the increased p24 yield in macrophages treated with rapamycin was associated with VLP released from macrophages (Fig. 2.3G). The autophagy machinery in these experiments was intact, as indicated by response to rapamycin both with LC3-I-to-LC3-II conversion and LC3 puncta formation (Fig. A2B-D), and responsiveness of the effects to inhibition with the conventional autophagy inhibitor 3MA (Fig. A2C and D). The enhancement effect of rapamycin on p24 release was counteracted by knocking down the key regulator of autophagy Atg7 (Fig. 2.3H). Because Atg7 is a key autophagy factor, this indicates that the rapamycin effects on p24 are autophagy dependent. Thus, pharmacological induction of autophagy, in contrast to our predictions and previous findings of inhibitory effects on viral replication of rapamycin in low concentrations [120, 121], did not diminish but instead enhanced yields of the virus released from macrophages. The effects of autophagy induction appeared to be specific for macrophages, as we did not observe enhancement using rapamycin in HeLa or H9 T cell lines transfected with an HIV molecular clone (Fig. 2.3I) and H9 T cells infected with the CXCR4 coreceptor using (X4, T cell tropic) virus HIV_{LAI} (Fig. 2.3J).

HIV protein Nef is required for enhanced HIV yields in response to autophagy induction

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Given the observation that induced autophagy did not harm the virus, but further augmented its yields, we wondered whether the virus, in addition to using basal autophagy to increase its yields, also protected against induced autophagy, which can act as an antimicrobial cell-autonomous defense [82, 87-89, 91-93]. We investigated whether specific HIV-1 proteins affected autophagic machinery. A release of HIV deleted for Nef was not stimulated by rapamycin in cells transfected with pNL4-3ΔNef, as shown in Fig. 2.4A, where the data were normalized to represent fold change in relative p24 release. These data show that rapamycin has no additional effect on HIV yields when the virus lacks Nef. The absolute levels of both the released p24 and cell-associated p24 were proportionately reduced with HIVΔNef treated with rapamycin (Fig. 2.4A, inset). As a consequence, the ratios remained the same (Fig. 2.4A, main graph), although the absolute levels of p24 (both cellular and released) were diminished (Fig. 2.4A, inset). In contrast to Nef deletion, HIV deleted for Vpu still responded to rapamycin stimulation with increased p24 levels (Fig. 2.4C). Furthermore, ΔNef virus, although showing reduced relative release of the viral p24 (Fig. 2.4C) and cellular p24 levels (Fig. 2.4D), showed no further change in yields, release, or cellular p24 when autophagy was inhibited by 3MA (Fig. 2.4E, left two panels) or suppressed by Beclin 1 knockdown (Fig. 2.4E, right two panels), indicating that Nef is critical for the detectable effects of autophagy on HIV.

HIV causes Nef-dependent accumulation of early autophagic markers

We next examined HIV effects on the execution stages of autophagy. The gold standard for assessment of the early execution phases of autophagy (initiation and elongation) is

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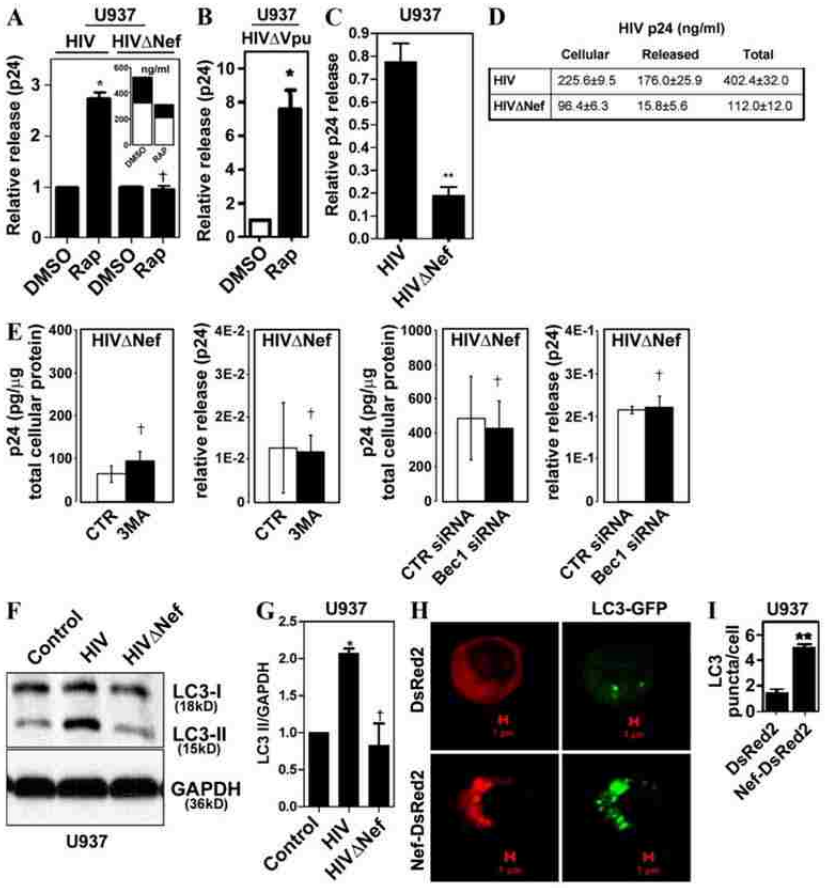

Figure 2.4. Nef is required for yield-enhancing effects of autophagy on HIV. (A) U937 cells were transfected with pGFP-NL43ΔNef (HIVΔNef) for 48 h and incubated with DMSO or 50 μg/ml rapamycin. (inset) Absolute values of p24 concentrations in cells (open bars) and released into the medium (shaded bars). Note that absolute levels of ΔNef virus are inhibitable by rapamycin but that the ratios of released versus cell-associated virus remain the same, as reflected in the main graph. (B) U937 cells were transfected with pMSMBA-Vpu-null (HIVΔVpu) and tested as in A for rapamycin effects. (C) U937 cells were infected with 100 ng/ml each of VSV-G-pseudotyped HIV or HIVΔNef for 48 h, and p24 yields were quantified. (D) Absolute levels of cell-associated, released, and total p24 from samples in B. (E) Absence of basal autophagy inhibition effects on HIVΔNef yields. For experiments with 3MA, U937 cells were infected with NL4-3ΔNef for 48 h, then washed and treated for 4 h. Cells in experiments with Beclin 1 knockdowns were first transfected with siRNA, infected 24 h later, and harvested 48 h after infection. CTR, control. (F and G) Nef increases LC3-II (lipidated form). U937 cells were infected with VSV-G-pseudotyped pMSMBA-derived virus (HIV) or HIVΔNef for 3 d and immunoblotted for LC3. (E) Immunoblot. (F) Quantification (ratio of LC3-II to GAPDH band intensities). (H) U937 macrophages were cotransfected with GFP-LC3 and either DsRed2 or Nef-DsRed2 for 24 h. GFP-LC3 puncta were quantified in three independent experiments. (I) Quantification of LC3 puncta (≥ 1μm) per cell. Data indicate means; error bars indicate ±SEM ($n \geq 3$). *, $P < 0.05$; **, $P < 0.01$; †, $P > 0.05$ (ANOVA).

based on monitoring biochemical and morphological changes that the autophagy protein LC3 undergoes [118]. During the stages when autophagic isolation membranes (phagophores) begin to form and nascent autophagosomes elongate until they are

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completed by closure, LC3 converts from the nonlipidated cytosolic species (LC3-I) to a predominantly membrane-associated form (LC3-II) covalently modified at the C terminus by phosphatidylethanolamine. During maturation stags, LC3-II is consumed as a portion of it gets degraded in the autolysosomes. The level of LC3 lipidation is monitored by immunoblotting, and detected as a conversion from the unmodified LC3-I band to the lipidated LC3-II form, which shows increased electrophoretic mobility [118, 122]. Complete HIV, but not HIV deleted for Nef, increased levels of lipidated LC3, as reflected in the increase of LC3-II band on Western blots (Fig. 2.4F), and LC3-II/loading control ratios [122] using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the loading reference (Fig. 2.4G). We next used another standard assay of autophagy, based on fluorescence microscopy detection of LC3 on autophagic membranes as punctate GFP-LC3 (LC3-II) vis-à-vis diffuse cytosolic GFP-LC3 (LC3-I; [118]). U937 cells were transfected with the previously well characterized expression clone of Nef-DsRed2, thoroughly documented in cell biological studies to fully correspond both in distribution and function to the untagged Nef [123]. Transfection of U937 cells with Nef-DsRed2 resulted in an increased abundance of GFP-LC3 puncta versus control DsRed2-transfected cells (Fig. 2.4H and I; and Fig. A2E). Thus, Nef was responsible for accumulation of the early autophagic markers, the lipidated LC3-II form and LC3 puncta.

Nef inhibits autophagic maturation

The observed increase in early autophagic markers associated with Nef action is consistent with: (a) induction of autophagy or (b) a blockage of the maturation stages of autophagy. We first examined whether Nef affected the maturation (degradative) stages

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of the autophagic pathway. This was performed by testing Nef effects on the marquee autophagic degradative function: proteolysis of long-lived, stable proteins that are normally turned over by autophagy. We tested whether Nef affected autophagic proteolysis using the published assay for stable protein autophagic proteolysis in macrophages, optimized and functional only in the mouse macrophage cell line RAW264.7 [124]. Transfection with Nef-DsRed2 did not induce autophagic proteolysis (Fig. A3A). Instead, Nef-DsRed2 inhibited autophagic protein degradation induced by starvation, a gold standard for assessment of autophagy function (Fig. A3A). Thus, Nef inhibits terminal, degradative stages of autophagy.

The role of Nef in inhibiting degradative stages of autophagy was further examined in human cells using the RFP-GFP-LC3 probe, a specialized tool for investigation of the autophagic flux, i.e., the maturation of autophagic organelles into degradative autolysosomal compartments [125]. Based on the sensitivity of GFP fluorescence to acidic pH and insensitivity of RFP fluorescence to low pH, it is possible to differentiate early, nonacidified autophagosomes (red^+green^+; yellow in merged images) from acidified, degradative autophagic organelles (red^+green^-; red in merged images; [125]). In cells infected with Nef^+ HIV, there was a pronounced accumulation of red^+green^+ (yellow) puncta, compared with uninfected cells or cells infected with ΔNef HIV (Fig. 2.5A-C). This is in keeping with the conclusion that Nef blocks maturation of early autophagic organelles into acidified, degradative autolysosomes. Of the Nef-dependent red^+green^+ puncta, 85% were negative for the lysosomal protein Lamp2 (Fig. 2.5D). All

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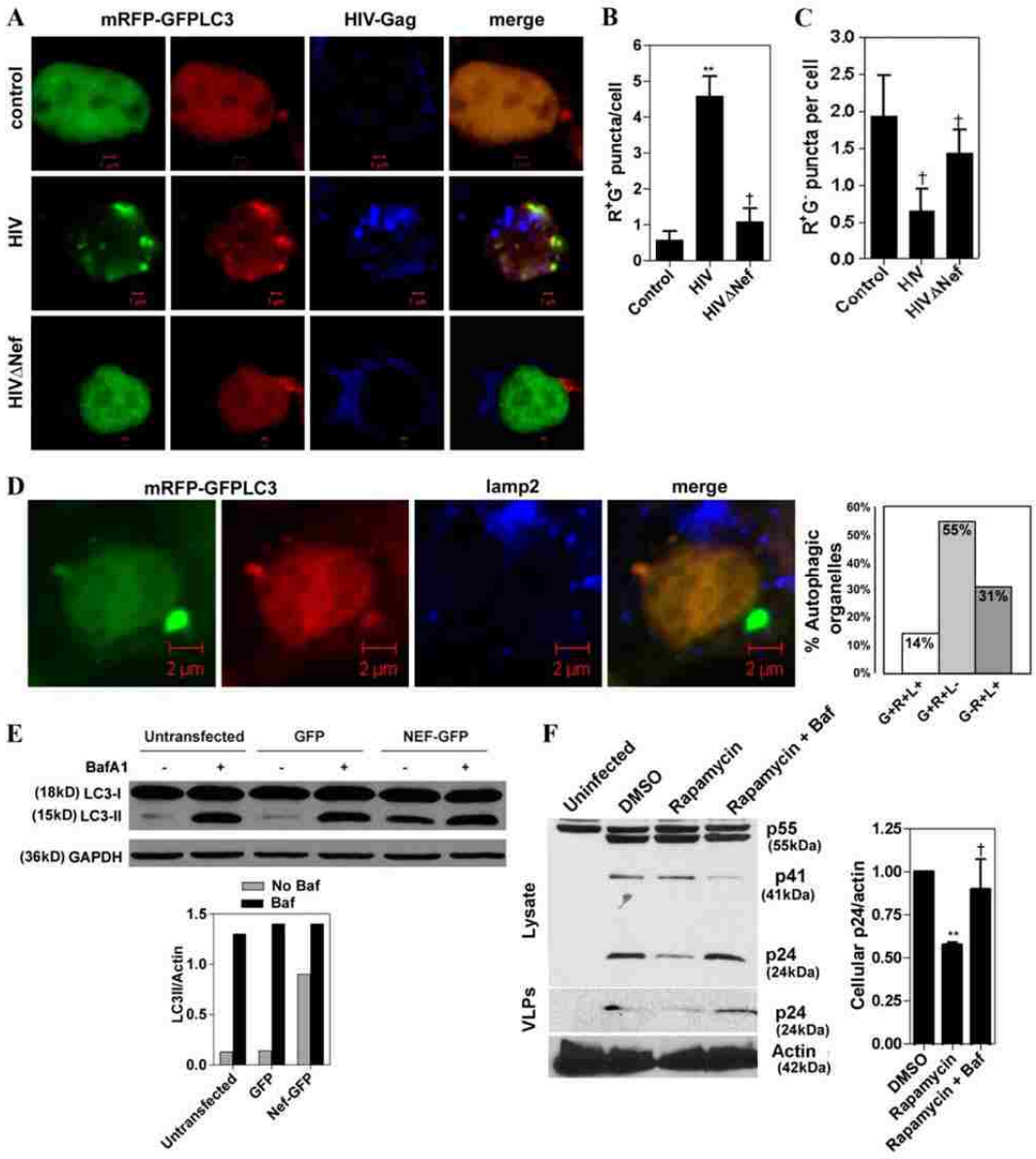

Figure 2.5. Nef inhibits autophagic maturation. (A) Nef blocks maturation of early autophagic organelles into autolysosomes. 293T cells were infected with VSV-G-pseudotyped HIV or HIVΔNef and transfected with mRFP-GFP-LC3 for 48 h, then immunostained for Gag and analyzed by confocal microscopy. Based on differential pH sensitivity of RFP and GFP, the mRFP-GFP-LC3 probe differentiates between early, nonacidified autophagosomes (red^+green^+; yellow in merged images) from acidified, degradative autolysosomes (red^+green^-; red in merged images0. (B and C) Quantification of (red^+green^+) R^+G^+ and R^+G^- puncta per cell, respectively. (D) Analysis of Lamp2 association with RFP-GFP-LC3 profiles in HIV-infected 293T cells (HIV infection of >90% determined by staining with antibody to Gag). L^+, percentage of Lamp2-positive profiles; L^-, percentage of Lamp2-negative profiles. Data are from 42 cells from three slides. (E) LC3-II levels in Nef-transfected cells in the presence or absence of bafilomycin A1. 293T cells were transfected with GFP alone or Nef-GFP for 48 h. Cells were then incubated with or without bafilomycin A1 (Baf A1 or Baf) for 4 h and immunoblotted for LC3 and GAPDH. (E, bottom) Quantification: LC3/GAPDH ratios, representative of one of two experiments. (F) Inhibition of autophagic flux/maturation protects Nef-null virus from degradation. U937 cells were infected with VSV-G-pseudotyped HIVΔNef for 72 h, then washed and treated with rapamycin or rapamycin plus bafilomycin A1 (100 nM) for 5 h.

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VLP and cell lysates were subjected to immunoblot analysis. (F, right) Quantification of cellular p24 (n = 3). Data indicate means; error bars indicate ±SEM. *, $P < 0.05$; **, $P < 0.01$; †, $P \geq 0.05$ (ANOVA).

red^{+}green^{-} puncta (representing 31% of the total mRFP-GFP-LC3 puncta) were Lamp2 positive (Fig. 2.5D). Expression of Nef-GFP resulted in an increase of LC3-II (Fig. 2.5E). This was not or only slightly enhanced in the presence of bafilomycin A1 (Fig. 2.5E, graph), an inhibitor of autophagosomal/autolysosomal acidification used to differentiate between effects on autophagy induction versus maturation [122], which suggests that the bulk of Nef effects on autophagy were based on blocking autophagic flux.

Nef blocks autophagic degradation of HIV

We next tested whether Nef blocks HIV-specific autophagic degradation by monitoring the yields of HIV p24. U937 cells were infected with VSV-G-pseudotyped Nef-null HIV and treated with rapamycin. This led to a marked decrease in intracellular p24 levels and in lower p24 levels in VLP preparations (Fig. 2.5F). The decrease in p24 was abrogated with bafilomycin A1, which blocks autophagic degradation (Fig. 2.5F). Similar results were observed with cellular p24 levels (Fig. 2.5F). These findings strongly indicate that Nef inhibits autophagic degradation of HIV biosynthetic intermediates or virions, and that this in turn enhances HIV yields.

HIV Nef colocalizes with autophagy regulators and is found in Beclin 1 protein complexes

We next investigated intracellular distribution of Nef in relationship to autophagy regulators. Nef did not colocalize with mTOR (Fig. A3B), so it is unlikely that it affects

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Tor directly. Nef showed a partial colocalization with 2xFYVE-GFP (Fig. A3C), a probe binding to membranes containing phosphatidylinositol 3-phosphate (PI3P), the enzymatic product of type III PI3K hVPS34 that plays a critical role in autophagy when complexed with Beclin 1 [8, 126-128]. Nef showed colocalization with autophagy factors Atg7 and Atg12 (Fig. A3D and E), and colocalized (Figs. 2.6A and A3F) with the autophagic protein Beclin 1, which is the central regulator of autophagy at multiple stages [8, 129]. Immunoprecipitation of Beclin 1 in extracts from cells transfected with Nef-GFP resulted in the presence of Nef-GFP in the precipitated protein complexes (Fig. 2.6B, top left). GFP was absent from the control samples when Beclin 1 was immunoprecipitated from cells transfected with GFP alone (Fig. 2.6B, top right). A converse experiment using immunoprecipitation of GFP revealed the presence of Beclin 1 in immune complexes in cells transfected with Nef-GFP (Fig. 2.6B, bottom left) but not in extracts from cells transfected with GFP alone (Fig. 2.6B, bottom right). In a different configuration, using cells transfected with C-terminally myc epitope-tagged Nef, Beclin 1 was found in immunoprecipitates generated with myc antibodies (Fig. 2.6C). In all immunoprecipitation experiments, IgG control showed negative results for the specific proteins analyzed (Fig. 2.6). The blots shown with the IgG control were developed until very faint band (representing background in any type of immunoprecipitation experiments) was revealed when possible; shorter development times left IgG controls completely blank, whereas the specifically coimmunoprecipitated band were still detected. Importantly, HIV Nef also coimmunoprecipitated with Beclin 1 in extracts from cells infected with HIV virus (Fig. 2.6D), demonstrating that Nef-Beclin 1 complexes form during viral infection. Thus, Beclin 1 and Nef colocalize (Fig. 2.6A) and

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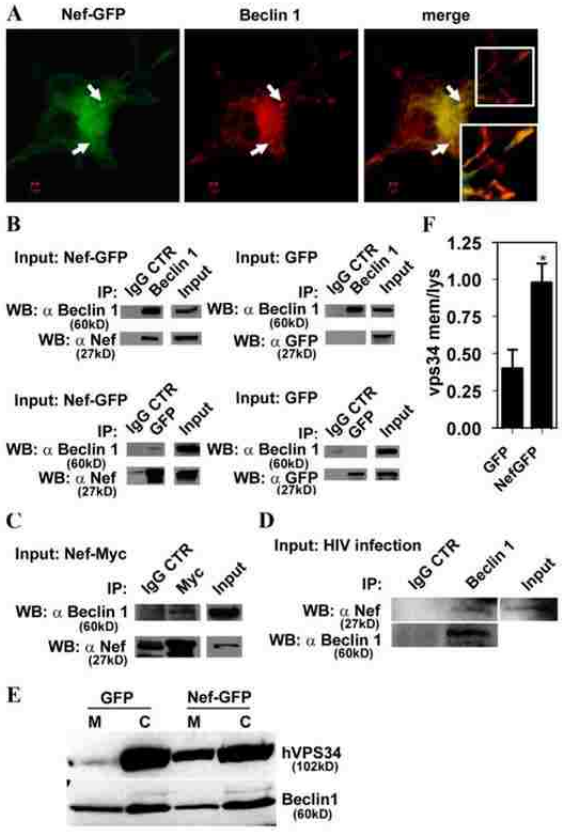

Figure 2.6. Nef is in protein complexes with autophagy regulator Beclin 1. (A) Macrophages were transfected with Nef-GFP and immunostained for Beclin 1. Arrows, perinuclear profiles exemplifying Nef and Beclin 1 colocalization. (inset) Peripheral colocalization. (B) 293T cells were transfected with Nef-GFP or GFP alone for 48 h. Lysates were immunoprecipitated either with Beclin 1 antibody and immunoblotted with Nef antibody (top) or with GFP and immunoblotted for Beclin 1 (bottom). Specific protein levels in cell lysates (input) and immunoprecipitations with IgG controls are shown. (C) Coimmunoprecipitation of Beclin 1 with Nef-Myc. 293T cells were transfected with Nef-Myc for 48 h. then cells were lysed and immunoprecipitation was performed using monoclonal Myc antibody or IgG control. The blost shown represent one of four independent experiments. (D) U937 cells were infected for 48 h with VSV-G-pseudotyped HIV. Cells were lysed and immunoprecipitated with Beclin 1-specific antibody or control IgG. Western blots of immunoprecipitated material were probed with Nef antibody. (E and F) 293T cells were cotransfected with Nef-GFP or GFP and Flag-hVPS34 for 48 h. Cells were fractionated into membranes (M) and cytosol (C) and immunoblotted with anti-Flag and Beclin 1 antibodies. (E) Immunoblots. (F) Quantification (ratios of membrane-associated hVPS34 to cytosolic hVPS34. Data indicated means; error bars indicate ±SEM. *, $P < 0.05$ ($n = 3$).

are present in a shared protein complex (Fig. 2.6B-D), associating directly or indirectly via an intermediate partner. Furthermore, Nef affected hVPS34 distribution (Fig. 2.6E and F), as a consequence of its association with Beclin 1, resulting in an increased presence of hVPS34 on membranes.

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Mutational analysis of HIV Nef-Beclin 1 interactions and Nef effects on autophagy

We next used a panel of Nef mutants to test whether any of the known motifs were necessary for Nef interactions with Beclin 1 and Nef effects on autophagy (Fig. 2.7A and A3G). In an identical coimmunoprecipitation approach as in Fig. 2.6, the previously characterized Nef mutant construct [130] with changes in the diacidic motif (^{174}DD^{175} → ^{174}AA^{175}), responsible for interactions with the V_1 domain of vacuolar H^+ ATPase and required for CD4 down-regulation [105], lost the capacity to coimmunoprecipitate Beclin 1 (Fig. 2.7A). In contrast, the mutation ^{154}EE^{155} → ^{154}QQ^{155}, in another region of Nef, i.e., the diacidic motif required for β-COP interactions [29, 105], did not significantly diminish the capacity of Nef to coimmunoprecipitate with Beclin 1 (Fig. 2.7A). Another mutation ^{2}G → ^{2}A, abrogating the ability of Nef to be N-terminally myristoylated, a posttranslational modification assisting Nef in membrane localization and required for many Nef functions [105], did not affect the capacity of Nef to coimmunoprecipitate with Beclin 1 (Fig. 2.7A). Myristoylation of Nef is often considered a *sine qua non* posttranslational modification required for nearly all previously known functions of Nef [105], with the exception of Hck activation by Nef [131], and thus it may appear surprising that this did not nullify Nef's action in our assays. However, it has been shown [132] that membrane association of NefG^2A is not fully abrogated despite the loss of myristoylation, but that instead it may be shifted from plasma membrane to endomembranes, which is compatible with the action of Nef within the autophagic pathway.

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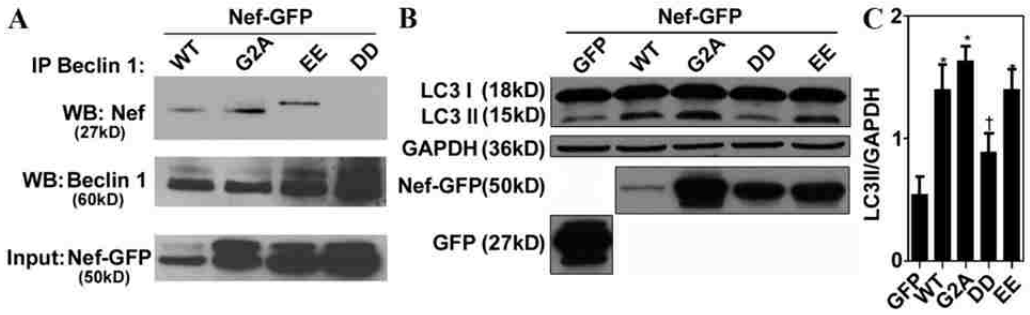

Figure 2.7. Nef ^{174}DD^{175} motif is required for interaction with Beclin 1 and inhibition of autophagic maturation. (A) 293T cells were transfected with the wild-type HIV-1 Nef fusion with GFP or the indicated mutants: G^{2}ANefGFP, ^{154}EE-GG^{155}NefGFP, and ^{174}DD-AA^{175}NefGFP for 48 h. Beclin 1 immunoprecipitates were analyzed by immunoblotting. (B and C) 293T cells were tranfected with the indicated constructs and immunoblotted for LC3. (B) Immunoblot. (C) Quantification (ratio of LC3-II to GAPDH band intensities). Data indicate means; error bars indicated ±SEM. *, $P < 0.01$; †, $P \geq 0.05$ (ANOVA; $n = 3$).

The same set of Nef mutants was tested for their capacity to increase the LC3-II form (Fig. 2.7B and C). The ^{174}DD^{175} → ^{174}AA^{175} mutant was again the only Nef variant tested that resulted in reduced increase in the autophagic marker LC3-II. Identical results were obtained when expression levels of Nef mutants were adjusted (Fig. A3G). Thus, based on interaction assays with Beclin 1, and functional analysis with LC3-I-to-LC3-II conversion, the diacidic motif at the positions 174 and 175 of Nef is critical for the ability of Nef to control autophagic flux.

Discussion

This study shows that autophagy factors interact with HIV components, that basal autophagy augments Gag processing and HIV yields in macrophages, and that HIV inhibits degradative stages of autophagy. Our data show that HIV proteins Gag and Nef interact with two different autophagy factors and that this controls the autophagic pathway in infected cells with the net result of enhancing HIV yields. The rationale for our study was based on a growing recognition of autophagy as an antimicrobial cell-

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autonomous defense mechanism endowing eukaryotic cells with the ability to eliminate invading microbes [5]. However, we found that autophagy, even when pharmacologically induced, could not decrease HIV yields in macrophages, and that instead it enhanced HIV production. HIV protein Nef binds to the protein complexes containing the mammalian autophagy protein Beclin 1. Nef inhibits the degradative stages of the autophagy pathway, thus protecting HIV or its biosynthetic intermediates from autophagic destruction. The net effect of Nef may at first appear akin to that of herpes simplex virus type 1 protein ICP34.5, which binds to Beclin 1 and inhibits its function in autophagy [18]. However, the action of Nef is preferentially related to the maturation stages of the autophagosomal pathway, as shown in our work. Recent studies have indicated that Beclin 1-hVPS34 complexes in mammalian cells include potential equivalents of yeast Atg14 [133], which appears to be autophagy initiation specific, and VPS38 (UVRAG), which acts at maturation and possibly initiation stages [129]. Thus, different parts of the autophagic pathway may be targeted by viral factors affecting Beclin 1: autophagy initiation or maturation as in the case of ICP34.5 [18], and Nef (this paper). Our findings also provide a functional and molecular basis for the results of a recent comprehensive siRNA screen in HeLa cells identifying among >250 host genes several Atg factors as playing a role in the HIV life cycle [33].

It has been previously reported [120, 121] that chronic treatment (for 3-7 d) of cells with low concentrations of rapamycin, which do not induce autophagy, may inhibit viral replication. The use of rapamycin in our study was limited to acute doses inducing autophagy, as we used rapamycin only as one of the agents to study how autophagy

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affects viral yields, irrespective of any long term effects that rapamycin may have on viral replication. Furthermore, induction of autophagy either with rapamycin or by starvation (unpublished data) both increased HIV yields, provided that the virus encoded Nef. Our work nevertheless indicates that, when unopposed by Nef, autophagy can act as a cell-autonomous anti-HIV defense. This is likely to be of importance, as autophagy is induced during HIV infection, as shown here and as recently noted in the context of TLR7 and TLR8 signaling [97]. In terms of the mechanism of the antiautophagic degradation action of Nef, we found that the diacidic ^{174}DD^{175} motif, responsible for interactions with the V1 domain of vacuolar H^+ ATPase and needed for CD4 down-regulation [105], is required for effects of Nef on autophagy. Hence, the simplest explanation would be that Nef influences H^+ ATPase assembly or activity, precluding autophagosomal acidification and maturation into autolysosomes. However, although HIV inhibits acidification of compartments with newly budded virions, the pH effect has been reported to be independent of Nef [115]. Thus, the protein complex containing Nef and Beclin 1 may act through a mechanism other than acidification. The effect of Nef on redistribution of hVPS34 (Fig. 2.6E) to membranes may be related to inhibition of autophagic maturation.

Within the portfolio of Nef effects, which includes down-regulation of MHC class I and CD4 cell surface expression, altered T cell activation, and augmented viral infectivity [104-107], a less understood effect is the Nef-induced accumulation of MVB-like organelles [134, 135] and emergence of large vacuoles [136]. This phenomenon can now

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be explained at least in part by the inhibition of MVB or amphisome consumption due to a Nef-dependent blockage of autophagic degradation.

In primary human macrophages, the virus transits through the intracellular compartments that intersect with autophagy factors such as LC3, as illustrated in Fig. 2.2. The intersection between HIV and the autophagic pathway is not limited to conditions when autophagy is induced to high levels. For example, the HIV precursor protein Gag is found in complexes with LC3 (Fig. 2.2E) even when macrophages are not pharmacologically stimulated for autophagy, which indicates engagement of the basal autophagy in biosynthesis, processing, or assembly of HIV intermediates. In other cell types, such as 293T cells used to dissect mechanistically molecular aspects of Nef action in the context of the autophagic pathway, the viral Gag did not show colocalization with the RFP-GFP-LC3 probe (Fig. 2.5A), reflecting the likely differences in intracellular trafficking of viral precursors in these cells versus macrophages [110-117]. Nevertheless, viral Nef did inhibit autophagic maturation even in 293T cells, indicating that this activity does not necessarily coincide with the location of the viral particles or Gag in relation to LC3.

This is further underscored by the effects of Atg7 and Beclin 1 knockdowns on total p24 yields in resting macrophages infected with HIV. This effect has been independently observed in HeLa cells upon knockdown of other Atg factors [33]. The enhancement by autophagy of HIV yields coincides with the association of Gag with LC3 uncovered in our work. Furthermore, our findings of enhanced Gag processing associated with

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autophagy indicate that this process plays a role in promoting certain steps in HIV biogenesis. Although autophagy is commonly viewed as a catabolic, degradative pathway primarily engaged in turning over macromolecules and removing toxic protein aggregates, or whole or parts of intracellular organelles and pathogens, it can also play a biosynthetic, anabolic role; this is clearly seen in the Cvt pathway in yeast, where Atg proteins are needed for completion of a functional vacuole [137, 138].

Nef also inhibits apoptosis and cell death in macrophages [130]. It has been shown that HIV Env induces death in bystander $CD4^+$ $CXCR4^+$ cells via a temporal succession of autophagy followed by apoptosis, and that completion of autophagy was a prerequisite for the execution of the subsequent apoptotic cell death [32]. This effect was recently narrowed down to gp41 [139]. Based on Nef's ability to inhibit terminal stages of autophagy, it follows that Nef may protect infected macrophages against cell death, in addition to guarding virions from autophagic elimination. Extending the life span of macrophages [130] and protecting virions from degradation may lead to higher HIV yields that are important for progression to AIDS [99, 101]. Pharmacological intervention to modulate autophagy in HIV-infected macrophages may help delay or prevent development of clinical AIDS.

Materials and methods

Cells

For macrophages, human monocytes were prepared from HIV-negative donors by density gradient centrifugation (400 *g* for 30 min) through a Ficoll-Hypaque gradient (GE

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Healthcare). Adherent monocytes were matured into macrophages for 7 d before infection. THP-1 and U937 cells were maintained in RPMI supplemented with glucose, glutamine, Hepes, and pyruvate. HeLa and 293T cells were maintained in DME supplemented with glutamine and FBS. MAGI cells were obtained from the National Institutes of Health AIDS reagent program.

Antibodies and chemicals

Atg7 and Beclin 1 antibodies were obtained from Santa Cruz Biotechnology, Inc.; monoclonal p24 antibody was obtained from Novus Biologicals; LC3 antibody was obtained from T. Ueno (Juntendo University School of Medicine, Tokyo, Japan) or from Sigma-Aldrich, and Atg12 antibody was obtained from N. Mizushima (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan); and the GAPDH antibody was obtained from Abcam. Gag monoclonal p17 and actin antibodies were obtained from Abcam. Secondary Alexa Fluor 488- and 568-conjugated antibodies were obtained from Invitrogen. Gag rabbit polyclonal antibody was obtained from the National Institutes of Health AIDS reagent program. Rapamycin, 3MA, bafilomycin, and lipopolysaccharide were obtained from Sigma-Aldrich.

Autophagy methods

Autophagy was triggered by treatment with 25-50 ng/µl rapamycin for 5 h in full nutrient medium. Alternatively, autophagy was induced by amino acid and serum starvation. Cells were washed three times with PBS and incubated in 1 ml Earle's balanced salts solution (starvation medium) at 37°C for 5 h. Autophagy was quantified by the GFP-

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LC3 puncta, LC3-I-to-LC3-II conversion, and proteolysis assays. Autophagy was inhibited with 10 mM 3MA. Where used, bafilomycin A1 was at a concentration of 100 nM. Cells were transfected or cotransfected with GFP-LC3, RFP-GFP-LC3, DsRed2, Nef-DsRed2 (Nef-DsRed2 was provided by K. Collins, University of Michigan, Ann Arbor, MI) and other constructs for 24 h. The total number of puncta (≥ 1μm) per cell was counted.

HIV extracellular yield

Methods to monitor HIV p24 yields are described in the legend to Table A2. For experiments with H9 T cells infected with live virus, the RT-PCR-based assay EnzChek (Invitrogen) was used to measure reverse transcription activity. Assays were performed according to manufacturer's instructions. Relative viral release was calculated as the ratio of extracellular-to-intracellular reverse transcription activity [119, 140-142].

Transfections and infections

Cells were transfected using the nucleoporation protocol (Amaxa) as described previously [143], with 10 μg of DNA or 1.5 μg of siRNA, as required. Atg7 and Beclin 1 knockdown protein was achieved using siGENOME SMART pool (Thermo Fisher Scientific). All effects of siRNA were compared with siCONTROL nontargeting siRNA pool (Thermo Fisher Scientific). For VSV-G pseudotyped HIV infections, U937 cells were differentiated overnight with 50 ng/ml of phorbol 12-myristate 13-acetate (Sigma-Aldrich), and viral infections were performed as described previously [130]. MDM were infected with 10^5 tissue culture infectious dose of SF162. Infections were allowed to go

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for 10 d with replacement of media every other day. Supernatants from these cells and cell lysates were frozen at -70°C until used for p24 ELISA or MAGI assays. The MAGI assay for HIV infectivity was performed as described previously [144]. HIV_{LAI} [145, 146] was expanded for 8 d in H9 T cells as previously described [140]. Supernatants from these cells were used to infect H9 T cells for experiments using titers as described by Prasad and Kalpana (2009) [119].

Fluorescence microscopy and image acquisition

Cells were fixed for 10 min with 1% paraformaldehyde, washed with PBS, and, when immunostained, permeabilized with 0.1% Triton X-100. Secondary antibody controls were routinely performed and showed no similarity to the pattern obtained when the primary antibody was included. Images were taken and processed on a confocal microscope system (META; Carl Zeiss, Inc.), equipped with 63x 1.4 NA oil differential interference contrast Plan-Apochromat objective, using Immersol (n = 1.518) at room temperature. Acquisition software was LSM 510, expert mode (Carl Zeiss, Inc.). Images were processed by Photoshop (Adobe) using proportional adjustments.

Electron and immunoelectron microscopy

Electron microscopy or viral budding in HIV-infected cells was performed as follows: control or U937 cells infected with VSV-G-pseudotyped pMSMBA were fixed with 3% formaldehyde (from paraformaldehyde) + 2% glutaraldehyde in 0.1 M cacodylate, pH 7.4. Cells were then washed and postfixed in 1% osmium tetroxide, 100 mM cacodylate buffer; dehydrated with increasing concentrations of ethanol; and gradually infiltrated

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with epon resin, embedded in straight resin, and examined using a transmission electron microscope (EM 900; Carl Zeiss, Inc.). Immunoelectron microscopy was performed using rabbit polyclonal LC3 antibody [147], applying the preembedding gold enhancement method as described previously [148]. U937 cells cultured on plastic coverslips (LF; Sumitomo Bakelite) were fixed with 4% paraformaldehyde (Nacalai Tesque) in 0.1 M sodium PBS, pH 7.4, for 30 min. After washing with the same buffer three times for 5 min, the fixed cells were permeabilized using 0.25% saponin in PBS. The cells were washed with PBS, blocked by incubating for 30 min in PBS containing 0.1% saponin, 10% BSA, 10% normal goat serum, and 0.1% cold water fish skin gelatin, then exposed overnight to 0.01 mg/ml of anti-LC3 rabbit polyclonal antibody or 0.01 mg/ml of nonimmunized rabbit IgG in the blocking solution. After washing with PBS containing 0.005% saponin, the cells were incubated with colloidal gold (1.4-nm diameter; Nanoprobes)-conjugated goat anti-rabbit IgG in the blocking solution for 2 h. The cells were then washed with PBS and fixed with 1% glutaraldehyde in PBS for 10 min. After washing with 50 mM glycine in PBS, 1% BSA in PBS, and finally with milliQ water (Millipore), gold labeling was intensified with a gold enhancement kit (GoldEnhance EM; Nanoprobes) for 3 min at room temperature according to the manufacturer's instructions. After washing with distilled water, the cells were postfixed in 1% OsO_4 containing 1.5% potassium ferrocyanide in PBS for 60 min at room temperature, and washed with distilled water. The cells were dehydrated in a series of graded ethanol solutions and embedded in epoxy resin. After the epoxy resin hardened, the plastic coverslip was removed from it. Ultrathin sections were cut horizontally to the

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cell layer and double stained with uranyl acetate and lead citrate. Samples were analyzed with an electron microscope (H7600; Hitachi).

Western blots and immunoprecipitations

Cells were washed in PBS and lysed with buffer containing 10 mM Tris HCl, pH 8.0, 150 mM NaCl, 0.5% deoxycholate, 2 mM EDTA, 2% NP-40, 1 mM PMSF, and protease inhibitor cocktail (Roche). 50 µg of protein was loaded and separated on a 12.5% SDS-polyacrylamide gel and transferred to nitrocellulose. The membrane was blocked overnight at 4°C in 5% milk in PBS/Tween 20 (0.1%) and probed with primary antibodies for 1 h at room temperature. After washing with PBS/Tween, the blot was probed with appropriate HRP-conjugating secondary antibody for 1 h at room temperature and stained with SuperSignal West Dura chemiluminescent substrate from Thermo Fisher Scientific. GAPDH was used as a loading control. For immunoprecipitations, transfected 293T cells were lysed with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% nonidet P-40, 0.25% sodium deoxycholate, and 1 mM EDTA, with protease and phosphatase inhibitors) for 1 h, followed by centrifugation to remove cell debris. Supernatants were precleared and incubated for 2 h with rabbit anti-Beclin 1 (Novus Biologicals), anti-LC3B, or rabbit anti-GFP (Abcam) at 4°C. The immune complexes were captured with protein G-agarose beads (EMD) overnight at 4°C. Immunoprecipitates were washed four times with PBS, eluted with Laemmli SDS-PAGE sample buffer for 5 min at 100°C, and subjected to immunoblot analysis with mouse anti-NEF (United States Biological), goat anti-Beclin 1 (Santa Cruz Biotechnology, Inc.), and mouse anti-GFP (Abcam). Note, in immunoprecipitation experiments with the ^{154}EE^{155}

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→ ^{154}QQ^{155} Nef mutant, this Nef variant consistently showed anomalous electrophoretic mobility.

Subcellular fractionation and cytosol preparation

U937 cells were infected with VSV-G-pseudotyped HIV for 2 d. Cells were lysed by passage through a tubing-interconnected two-syringe apparatus, and nuclei were cleared by low-speed centrifugation. The postnuclear supernatant was put on 20, 30, 40, 45, 50, 55, and 60% sucrose gradients, then centrifuged overnight at 100,000 *g*. 12 fractions collected from the top were pelleted at 100,000 *g* for 1 h and immunoblotted for the indicated organellar markers.

Appendix supplemental material

Fig. A1 shows that Atg7 and Beclin 1 knockdowns inhibit autophagy in U937 cells, as well as transmission and immunoelectron micrograph HIV profiles in macrophages. Fig. A2 shows a comparison of LC3 forms in whole cell lysate versus LC3 forms associated with membranes, that autophagy induction is operational in cells used to detect HIV yield-enhancing effects of acute rapamycin treatment, and that Nef causes accumulation of LC3 puncta. Fig. A3 shows that Nef inhibits autophagic proteolysis; intracellular localization of Nef relative to mTOR, 2xFYVE-GFP, and autophagy markers Atg7, Atg12, and Beclin 1; and that Nef motif ^{174}DD^{175} is but G^{2}A motif is not required for Nef-dependent increase in LC3II levels. Table S1 shows HIV molecular clones, viruses, and viral preparations and use. Table A2 shows ELISA (p24) and quantification of viral release. Online supplemental material is available at http://www.jcb.org/cgi/content/full/j

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cb.200903070/DC1.

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CHAPTER 3

AUTOPHAGY-MEDIATED RESTRICTION OF HIV-1 BY RHTRIM5α

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Abstract

With a broad spectrum of physiological and medical consequences, autophagy controls cytosolic and organellar quantity and quality through the capture of intracellular targets into autophagosomes for subsequent degradation in autolysosomes [5]. Among diverse immune functions, autophagy acts as a cell-autonomous process by capturing and eliminating intracellular microbes [5, 85]. Recently, autophagy has been identified as a process that can inhibit HIV-1 in infected cells at stages when it is unopposed by HIV-1 anti-autophagic factors [149, 150]. Although HIV-1 is the leading cause of acquired immunodeficiency syndrome (AIDS) in humans, it does not cause disease in certain species of monkeys [34], due in part to the existence of restriction factors. The recently identified rhesus macaque restriction factor, RhTRIM5α, has been shown to specifically bind HIV capsid and lead to degradation of the viral core by an unknown mechanism immediately after fusion and release of the core into the cytosol [40]. Here we show that RhTRIM5α acts through autophagy to restrict HIV-1, leading to its degradation soon after infection. This restriction requires the autophagy factors p62 [12], LC3 [151], Beclin 1 and Atg7, thereby revealing the preferred target for restriction of HIV-1 by RhTRIM5α. We furthermore uncover an inability of human TRIM5α to bridge HIV capsid to autophagy, of possible significance to the susceptibility of humans to HIV-1 infection.

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Results

In addition to roles in homeostasis and disease states, autophagy enables eukaryotes to defend against dangerous invaders, such as viruses, bacteria, and likely protozoa and fungi as well. Autophagy specifically recognizes and encloses microbes in a double membrane vacuole called an autophagosome, which then fuses with lysosomes to degrade and clear the threat from host cells [5]. During capture of targets slated for destruction, autophagy typically employs a variety of adaptor proteins, including p62 (also called sequestosome 1) [12]. P62 has recently been shown to associate with RhTRIM5α [152], but the possible contribution of p62-mediated autophagy to RhTRIM5α restriction of HIV-1 has not been considered. Already the contribution of autophagy to HIV pathogenesis has been shown in human cells [149, 150], but there remains the need for investigation into the role of autophagy in non-permissive non-human primates. Thus, delivery of incoming HIV-1 core particles to autolysosomes as a possible mechanism for RhTRIM5α-mediated restriction of HIV was explored in this study.

To investigate the possibility that restriction of HIV-1 by RhTRIM5α could be autophagy-mediated, a panel of inhibitors was tested for an ability to block degradation of the viral core (as measured by p24 capsid protein immunoblotting) during acute infection (Fig. 3.1). Monkey cells (FRhK4) endogenously expressing RhTRIM5α were infected with VSVG-pseudotyped HIV-1 in the presence of these inhibitors for 4 hours, prior to peak viral integration and *de novo* replication [153]. Both lysosomal inhibitor, bafilomycin A1 (an inhibitor of the vacuolar ATPase, labeled "Baf" in the figure), and

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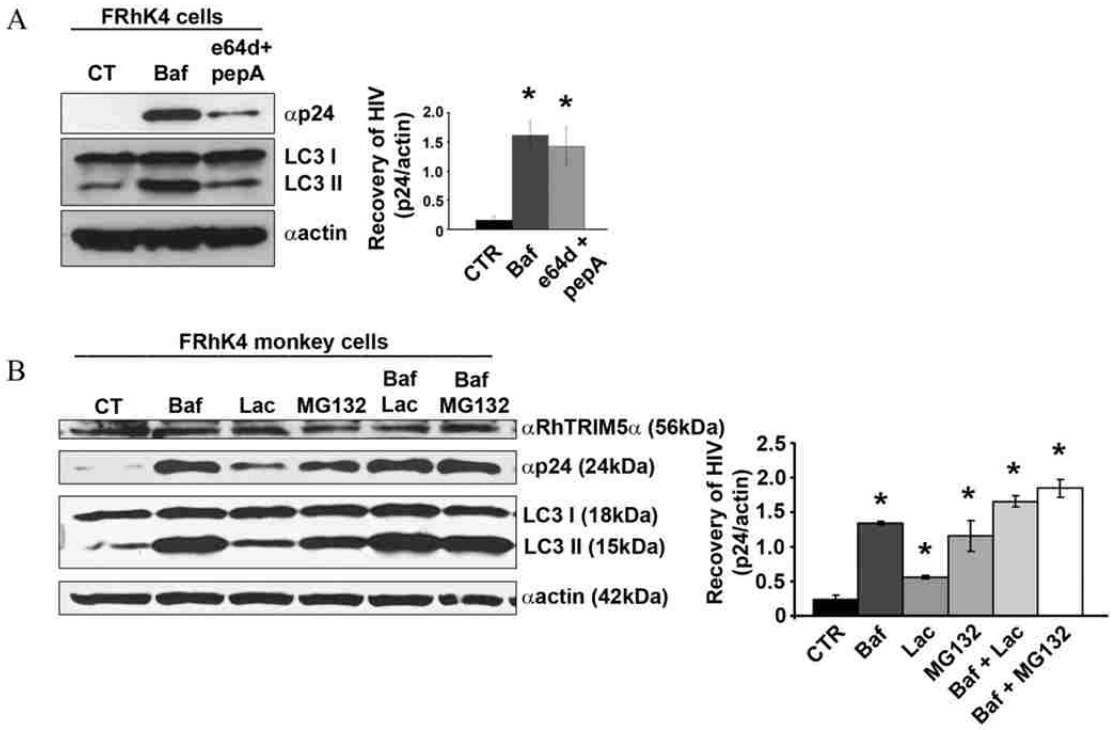

Figure 3.1. Lysosomal inhibitors block degradation of HIV in RhTRIM5α expressing cells. (A) FRhK4 monkey cells were infected with VSVG-pseudotyped HIV in the presence of vehicle control (CT or CTR), bafilomycin A1 (Baf) or e64d and pepstatin A (e64d + pepA). After 4 hours, the cells were collected and lysed. Whole cell lysates were Western blotted to detect the amount of remaining p24 after treatment. (B) FRhK4 cells were infected in the presence of either vehicle control (CT or CTR), bafilomycin A1 (Baf), lactacystin (Lac), MG132, or a combination of two and Western blotted as in A for the amount of p24 that was not degraded. Recovery of HIV for both graphs is expressed as the amount of p24 over the amount of actin (used as a loading control). Quantifications of p24 and actin for graphical analyses are explained in the materials and methods section. Both graphs represent at least three independent experiments. Asterisks indicate significance compared to control (p-value < 0.05, student's t-test). Error bars indicate ±SEM.

lysosomal protease inhibitors, e64d and pepstatin A (labeled "e64d+pepA"), protected HIV capsid (p24) from elimination in monkey cells (Fig. 3.1A). Recovery of p24 in the presence of lysosomal inhibitor bafilomycin was greater than the recovery of p24 in the presence of proteasome inhibitors, lactacystin (labeled "Lac") or MG132, indicating that the contribution of lysosomes to degradation of HIV is greater than that of the proteasome (Fig. 3.1B). The same protective effect on p24 by bafilomycin was also seen in HeLa cells stably expressing HA-tagged RhTRIM5α (HA-RhTRIM5α cells) (Christina

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Dinkins and George Kyei, unpublished). To definitively determine whether HIV core particles were being degraded by autophagy, as a route to lysosomal degradation, monkey cells were infected with HIV after knockdown of key autophagy initiators, Beclin 1 or Atg7, using siRNA. Beclin 1 is a master regulator of autophagic membrane formation, while Atg7 is responsible for regulating the conjugation of phosphatidylethanolamine (PE) to LC3, a protein. PE conjugation allows LC3, in this form called LC3 II, to anchor into and stabilize the newly forming autophagic membrane, a step essential for membrane growth. PE unconjugated LC3 is referred to as LC3 I, which is distributed free in the cytosol [10]. Without Beclin 1 or Atg7, p24 was, similar to bafilomycin treatment, not significantly degraded, indicating that RhTRIM5α-mediated restriction of HIV requires a functional autophagy pathway (John Arko-Mensah, unpublished).

In order to distinguish between an indirect effect on autophagy and a direct association with the autophagic machinery, we next asked if RhTRIM5α colocalized with two important autophagy factors, adaptor protein p62 and autophagy marker LC3 (Fig. 3.2). HA-RhTRIM5α cells were transiently transfected with either p62-GFP or LC3-GFP and immunostained for HA-RhTRIM5α via the HA tag. Normally, transiently transfected p62 or LC3 will appear as punctate structures in confocal microscopy images [154, 155]. P62 puncta indicate pre-autophagic and autophagic macromolecular complexes (in other words, structures which may or may not contain membrane, but are in transit to autophagy), whereas LC3 puncta specifically designate autophagic membrane as a function of LC3 II's ability to associate with membrane. In HA-RhTRIM5α cells, colocalization between RhTRIM5α and transiently transfected p62-GFP was nearly

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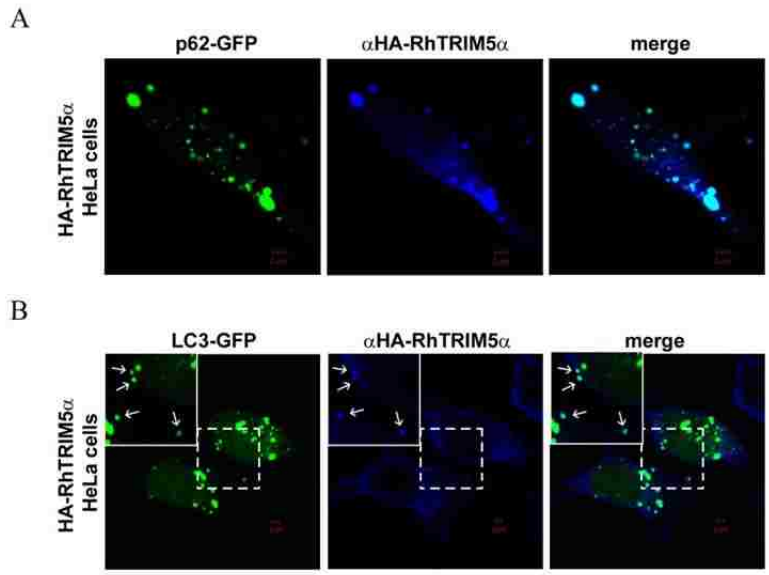

Figure 3.2. Autophagy factors colocalize with RhTRIM5α. (A) Confocal microscopy was used to examine the distribution of RhTRIM5α and autophagy adaptor protein p62. Stably-expressing HA-RhTRIM5α HeLa cells were immunostained for HA in the presence of p62-GFP, and fluorescently labeled with the appropriate secondary antibody as described in the Materials and Methods section. (B) Confocal microscopy was used to examine the distribution of RhTRIM5α and autophagy marker LC3. HA-RhTRIM5α cells were immunostained for HA and fluorescently labeled as in A and transfected with LC3-GFP. The inset at the top left of each image shows a magnification of the area shown in dashed outline. It is included to show a representative example of overlapping areas of RhTRIM5α and LC3 distributions indicating colocalizations, each region indicated by the white arrows.

absolute (Fig. 3.2A), indicating that RhTRIM5α and p62 occupy the same subcellular space. However, when compared to LC3-GFP, only certain populations of RhTRIM5α were seen to colocalize, perhaps indicating that RhTRIM5α and LC3 potentially occupy distinct, but overlapping areas within the cell under uninfected conditions (Fig. 3.2B, inset shows magnification of 4 puncta containing both LC3 and RhTRIM5α).

To determine whether colocalizations of RhTRIM5α with LC3 or p62 represent physical interactions in a protein complex, co-immunoprecipitation was performed on LC3-GFP or GFP-p62 transfected HA-RhTRIM5α cells (GFP transfection was used as a negative control) (Fig. 3.3). LC3-GFP protein was immunoprecipitated from cell lysates using a GFP specific antibody. The amount of RhTRIM5α in the precipitate was visualized after

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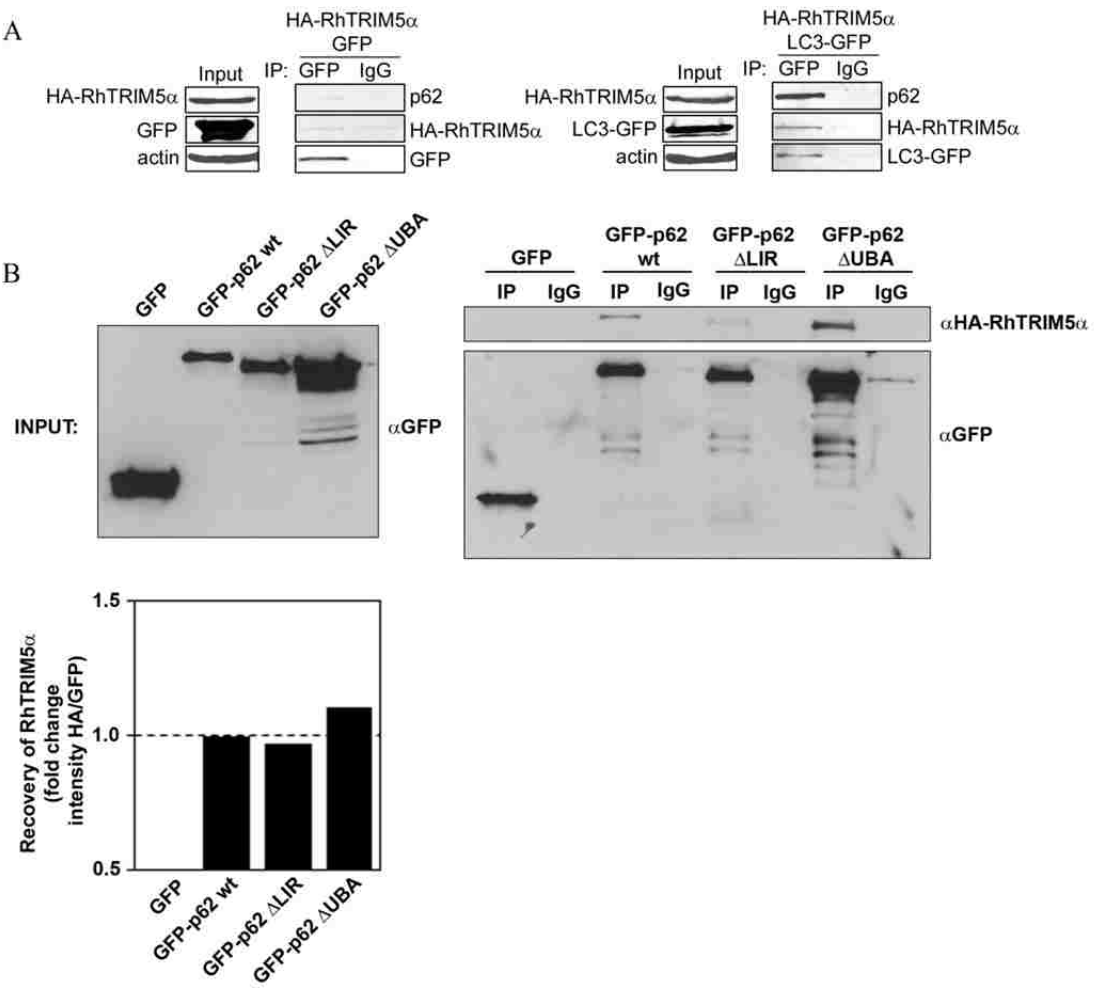

Figure 3.3. Autophagy factors co-precipitate with RhTRIM5α. (A) Complex formation between RhTRIM5α and LC3 was assessed by co-immunoprecipitation. HA-RhTRIM5α cells were transfected with either LC3-GFP or GFP. LC3-GFP or GFP was immunoprecipitated using a GFP specific antibody. The amount of HA-RhTRIM5α in the precipitate was visualized by Western blotting using anitbodies specific to the HA tag. (B) Complex formation between RhTRIM5α and p62 was assessed by co-immunoprecipitation. HA-RhTRIM5α cells were transfected with either GFP, p62-GFP wt (full length p62), p62-GFP ΔLIR (LIR domain deleted p62), or p62-GFP ΔUBA (UBA domain deleted p62). The various p62 proteins or GFP were immunoprecipitated using antibodies to the GFP tag and the amount of HA-RhTRIM5α in the precipitate was visualized by Western blotting. The graph represents the amount of HA-RhTRIM5α recovered for amount of GFP immunoprecipitated. For both immunoprecipitation experiments, GFP was used as a negative protein interaction control, while IgG from unimmunized animals was used as a negative antibody control.

Western blotting using an antibody specific to its HA tag. In precipitates containing purified LC3-GFP, HA-RhTRIM5α was seen to co-precipitate better with it than when compared to GFP transfected controls, supporting the idea that a population of RhTRIM5α associates with LC3 (Fig. 3.3A). When p62 was immunoprecipitated via its

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GFP tag, again RhTRIM5α (via it's HA tag) was seen to co-precipitate with it better than GFP control (compare amount of RhTRIM5α recovered from GFP versus GFP-p62 wt immunoprecipitates, Fig 3.3B, blot on right side, first lane and third lane). IgG antibody from unimmunized animals was used as a control to determine the amount of background recovery of each protein investigated in these experiments (Fig. 3.3A and B, lanes labeled "IgG").

P62, also known as sequestosome 1, bridges autophagic targets to the autophagosome through two direct interactions. First, p62 recognizes polyubiquitin tags on targets destined for autophagy through its ubiquitin binding area (UBA). Second, p62 directly interacts with LC3 through its LC3 interacting region (LIR) (for a review and explanation of the domains of p62, see Fig. G1). To investigate complex formation requirements between p62 and RhTRIM5α, we used p62 domain mutants at the same time we did the GFP-p62 wt immunoprecipitation experiments described above (Fig. 3.3B, last four lanes). The graph in Fig. 3.3B is an analysis of the above, right blot and shows the recovery of RhTRIM5α for the amount of p62 protein immunoprecipitated (expressed as fold change over GFP-p62 wt). The amount of RhTRIM5α recovered in immunoprecipitates from cells transfected with either GFP-p62 deleted for LC3 binding (GFP-p62 ΔLIR) or p62 deleted for ubiquitin binding (GFP-p62 ΔUBA) can be compared to the amount recovered in the presence of full length p62 (GFP-p62 wt) (Fig. 3.3B, see blot on right side, last six lanes and graphical analysis of the blot, lower left). RhTRIM5α associated with GFP-p62 wt efficiently (compared to GFP control), but lost some ability to associate with p62 when it was deficient for LC3 binding (GFP-p62

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ΔLIR) (Fig. 3.3B, see graph, GFP-p62 wt compared to GFP-p62 ΔLIR and blot on right side, comparing third lane to fifth lane) (supporting data by Manohar Pilli, unpublished). These results may indicate that complex formation of RhTRIM5α with p62 is supported by LC3, but in fact, when p62 is knocked down using siRNA, almost no RhTRIM5α can be seen in LC3 purified immunoprecipitate, indicating that RhTRIM5α association with LC3 instead requires p62 (Marisa Ponpuak, unpublished data). This is consistent with the microscopy data that shows RhTRIM5α preferentially localizes to regions containing p62, while only partially overlapping with regions containing LC3, suggesting that RhTRIM5α may form a complex with p62 first and then associate with LC3 as needed. Therefore, the requirement for the LIR domain of p62 may reveal a unique binding site for RhTRIM5α. Interestingly, it has been published that p62 binds to the RING domain of TRAF6 (involved in the NFκB pathway) through a span of amino acids that partially overlaps with the amino acids deleted in the LIR domain mutant used in this study [156]. Thus, it is possible that p62 also binds to the RING domain of RhTRIM5α through the same binding site. Finally, when the ubiquitin binding domain was deleted from p62 (GFP-p62 ΔUBA), RhTRIM5α, surprisingly, appeared to associate better with it (Fig. 3.3B, see graph, GFP-p62 wt compared to GFP-p62 ΔUBA and blot on right side, comparing third lane to seventh lane). This result may reflect some kind of competition for p62 between ubiquitinated proteins at the UBA domain and RhTRIM5α near the LIR domain.

When colocalization of RhTRIM5α with endogenous p62 and LC3 was investigated using confocal microscopy (Fig. 3.4), RhTRIM5α minimally colocalized with LC3 under

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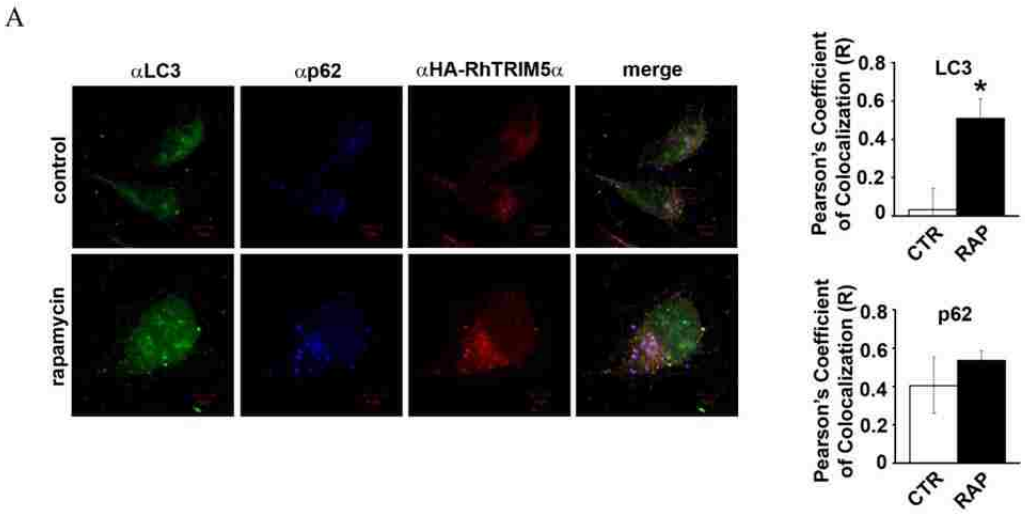

Figure 3.4. Endogenous autophagy factors colocalize with RhTRIM5α. (A) The degree of colocalization between RhTRIM5α and endogenous autophagy factors were evaluated in HA-RhTRIM5α cells using indirect immunofluorescence. HA-RhTRIM5α cells were immunostained for HA-RhTRIM5α, LC3 and p62 under control (CTR) and rapamycin (RAP) treated conditions. Graphs represent amount of colocalization between RhTRIM5α and LC3 or p62, expressed as Pearson's coefficient of colocalization, among 4 cells. All indirect immunofluorescence labeling procedures are explained in the Materials and Methods section.

basal autophagy, control conditions (Fig. 3.4A, see fluorescent images and graph on right, LC3 control (CTR)). However, RhTRIM5α colocalization with LC3 could be induced by stimulating autophagy with rapamycin treatment (Fig. 3.4A, images and graph on right, LC3 rapamycin (RAP)). Rapamycin is a pharmacological stimulator of autophagy and was used to induce autophagy to an elevated state where the rate of autophagic flux is maximized. These data suggest, with the co-immunoprecipitation data, that RhTRIM5α may associate with the autophagic machinery in response to stimuli inducing autophagy, perhaps indicating that infection with HIV could induce the same movement of RhTRIM5α to autophagosomes. When colocalization of RhTRIM5α with p62 was analyzed, it was found that RhTRIM5α colocalized with p62 without pharmacological induction of autophagy (Fig. 3.4B). The amount of colocalization among proteins was analyzed by measuring the Pearson's coefficient of colocalization, a

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measure of the amount of overlap of two fluoroscences per given object (puncta, ≥ 0.25 μm^2, 0 indicates no colocalization, while -1 and 1 indicate negative and positive colocalization, respectively). All fluorescent labeling in the study was done by indirect immunofluorescence as is described in the Materials and Methods section. Otherwise, fluorescence was visualized as a result of GFP fusion protein expression and not labeled (as in Fig. 3.2).

Next, confocal microscopy was used examine the distribution of HIV capsid (as measured by p24 immunolabeling) with p62 and LC3 in the presence of RhTRIM5α (Fig. 3.5). HA-RhTRIM5α cells or monkey cells were infected with HIV for 4 hours then fluorescently labeled for endogenous autophagy factors p62 and LC3. Colocalization of p62 and LC3 was compared to HIV p24 distribution, also fluorescently labeled. The Pearson's correlation coefficient (R) of colocalization between p24 and p62 in the monkey cells was 0.45, indicating positive colocalization, whereas the correlation of colocalization between p24 and LC3 was less at 0.21, although still positive (Fig. 3.5A). When compared to HA-RhTRIM5α cells, colocalization between p24 and p62 or LC3 increased (Fig. 3.5B, $R_{RhTRIM5\alpha/p62} = 0.49$, $R_{RhTRIM5\alpha/LC3} = 0.32$). These results indicate that association of HIV capsid with autophagy increases in a RhTRIM5α dose dependent manner, as stably expressing HA-RhTRIM5α in HeLa cells results in a greater expression level (about 1.6 fold increase) as compared to endogenous RhTRIM5α in monkey cells. To definitively determine whether RhTRIM5α-mediated restriction of HIV was p62 dependent, p62 was knocked down using siRNA during infection. In the absence of p62, HIV could not be significantly degraded (John Arko-Mensah, unpublished). Finally,

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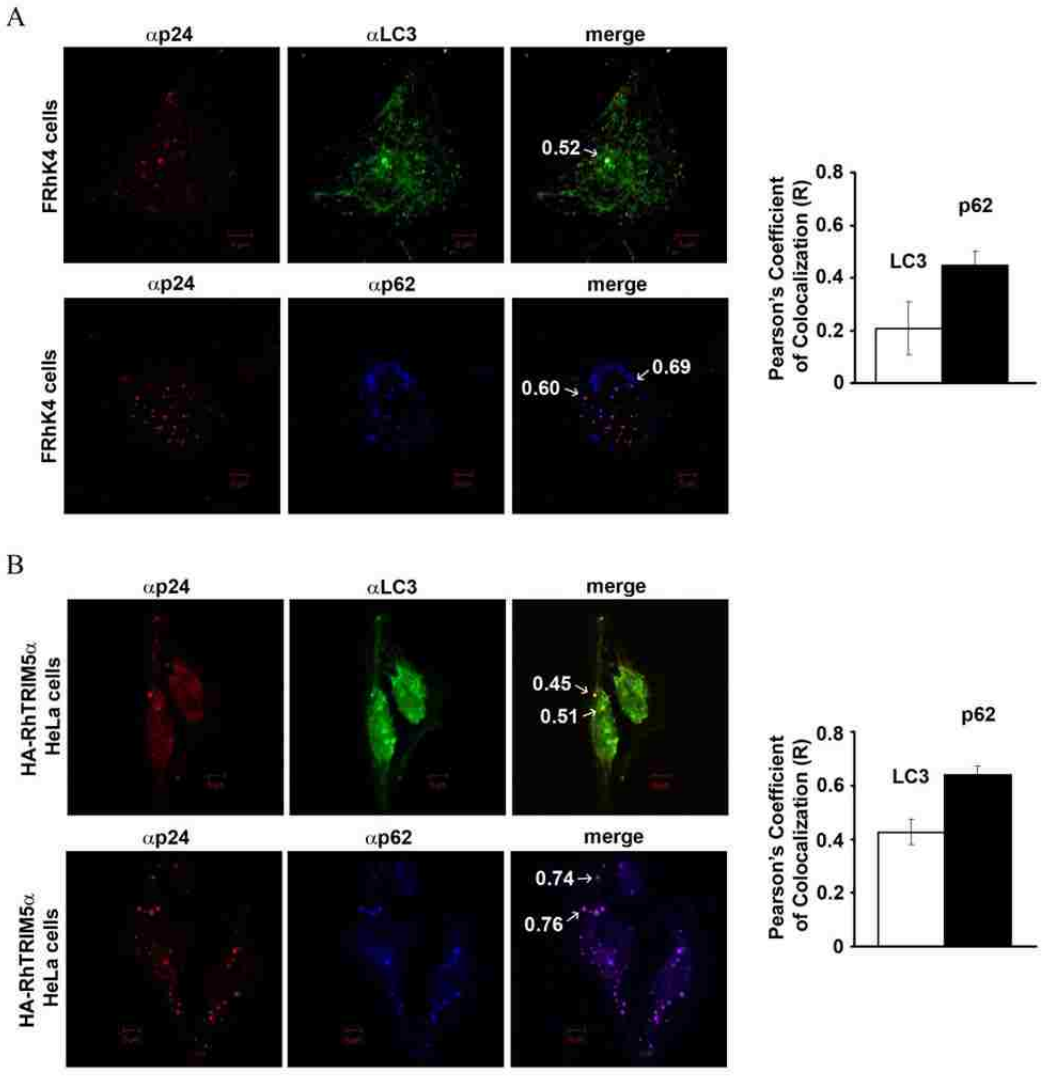

Figure 3.5. HIV colocalizes with autophagy in the presence of RhTRIM5α. (A) FRhK4 monkey cells were infected with HIV and immunostained for HIV p24, LC3 and p62. Colocalizations between HIVp24 and LC3 or p62 were quantified (graph on the right). (B) HA-RhTRIM5α HeLa cells were infected with HIV and immunostained for HIV p24, LC3 and p62. Colocalizations between HIV p24 and LC3 or p62 were quantified (graph on the right). Graphs represent amount of colocalization between p24 and LC3 or p62, expressed as Pearson's coefficient of colocalization, among 9-15 objects ($p24^+$ and $LC3^+$ or $p62^+$ puncta).

when HeLa cells not expressing RhTRIM5α were infected with HIV, no overlap in localization was seen between p24 and p62. In the absence of RhTRIM5α, HIV p24 distributed in a perinuclear fashion while p62 remained in the cytosol and, interestingly, did not form the puncta seen in previous experiments (Fig. 3.6).

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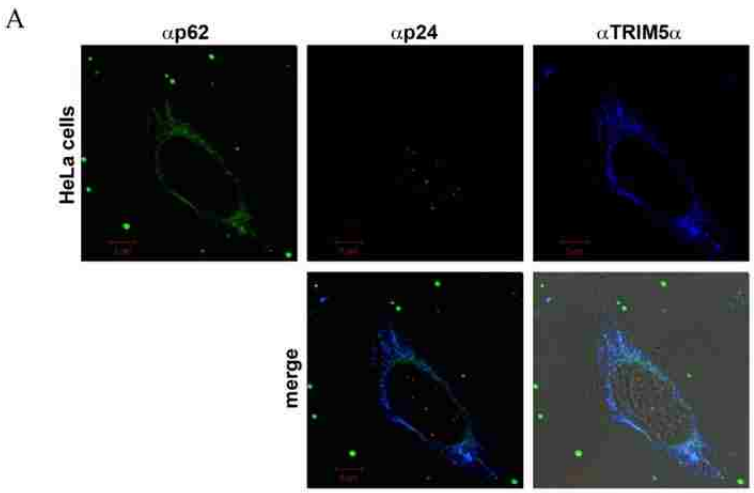

Figure 3.6. HIV does not colocalize with autophagy protein p62 in the absence of RhTRIM5α. (A) HeLa cells were infected with HIV and immunostained for HIV p24, p62, and HuTRIM5α. Colocalization between p24 and p62 or HuTRIM5α was not analyzed.

These results indicate that autophagy may be the primary target for degradation of incoming HIV core particles through capsid recognition by RhTRIM5α and subsequent engagement with the autophagic machinery. The specific recognition of HIV capsid that is unique to RhTRIM5α, while impaired with HuTRIM5α, may reveal a loss in the link between HIV and autophagy in human cells. The requirement of RhTRIM5α for HIV association with the autophagy adaptor p62 suggests that the failure of autophagy to recognize HIV capsid in the presence of HuTRIM5α may be a reflection of the inability of HuTRIM5α to significantly associate with HIV capsid. The consequences of recognition failure to autophagic capture are best demonstrated by the experiment showing that HIV cannot associate with the autophagy adaptor p62 in the absence of RhTRIM5α, while in the presence of HuTRIM5α. As the mechanism of restriction by RhTRIM5α is further elucidated, it may become a viable target for therapeutic intervention perhaps through pharmacological manipulation of autophagy or through promoting the recognition of HIV core by autophagy.

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Materials and Methods

Cells and virus

HeLa cells stably expressing HA-RhTRIM5α were kindly provided by Joseph Sodroski. These cells were maintained in complete DMEM media containing 1 μg/ml of puromycin as a positive selection agent [40]. FRhK4 cells were used as a rhesus macaque model of endogenous RhTRIM5α restriction [76], and were purchased from ATCC. They were maintained in complete DMEM. VSV-G pseudotyped HIV-1 was prepared from co-transfection of plasmids encoding NL4.3Δenv HIV-1 (NIH) and vesicular stomatitis virus G protein into HEK 293T cells. Supernatants from these cells were used to infect HA-RhTRIM5 HeLa, HeLa or FRhK4 cells for experiments using titers as previously described (Chapter 2).

Infection and drug treatments

All experiments with HIV infection were synchronized at 4°C for 1 hour. After synchronization, cells were washed 2-3 times with cold 1x PBS before experimental treatment in full media. Cells were then incubated in treatment conditions at 37°C for 4 hours. For the various drug treatments the following concentrations were used: 100 nM bafilomycin Al, 50 μg/ml rapamycin, 10 μg/ml e64d and 10 μg/ml pepstatin A, 10 μM lactacystin, and 10 μM MG132 as previously described [155]. Stocks of the various pharmacological agents were diluted to their final concentrations using DMSO. DMSO was used as a control for all drug treatment experiments.

Immunoblotting and immunoprecipitation

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Whole cell lysates were collected and lysed in a non-denaturing buffer containing protease inhibitors. Lysis was performed either by freeze fracturing overnight at -70°C for immunoblotting or by incubating the samples on ice for 30 minutes for immunoprecipitations. The lysis buffer was composed of 1% Triton X-100, 50 mM Tris-HCl (pH 7.4), 300 mM NaCl, and 5 mM EDTA. Protease inhibitors were added to the lysis buffer at the following final concentrations: 100 μM leupeptin, 1 μM pepstatin A, 10 μM e64 and 100 μM TLCK. Whole cell lysates were then prepared for immunoblotting with Laemmli buffer or diluted for immunoprecipitation. For immunoblotting, 50 μg of protein for each sample was separated on a 15% polyacrylamide gel and transferred to nitrocellulose. Immunoblotting was then performed as previously described (Chapter 2). The following primary antibodies were used for immunoblotting: mouse anti-p24 (Abcam), rabbit anti-LC3 (Sigma), mouse anti-actin (Abcam or Sigma), rabbit anti-TRIM5α (Imgenex), mouse or rabbit anti-HA (Abcam or Sigma, respectively), mouse anti-GFP (Abcam), and guinea pig anti-p62 (Progen). For immunoprecipitations, 0.5-1.5 mg of protein was diluted in ice cold PBS containing protease inhibitors in concentrations as described above. Samples were then pre-cleared on protein G agarose beads (Calbiochem) using non-specific rabbit IgG. Immunoprecipitation was then performed on Invitrogen Dynabeads® Protein G for IgG capture using rabbit anti-GFP (Abcam) for samples and non-specific rabbit IgG for controls. The procedure for immunoprecipitation was then performed as previously described (Chapter 2).

Fluorescence microscopy and image acquisition

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The immunofluorescence procedure was performed as previously described (Chapter 2). The following primary antibodies were used for immunostaining: mouse anti-HA (Covance), guinea pig or sheep anti-p62 (Progen or Abcam, respectively), rabbit anti-LC3 (MBL), rabbit anti-TRIM5α (Abcam), and mouse anti-p24 (Abcam). The following secondary antibodies (Invitrogen) were used for immunofluorescence: Alexa Fluor 488 for green, Alexa Fluor 568 for red, and Alexa Fluor 647 for blue. Images were taken and processed on a confocal microscope system (META; Carl Zeiss, Inc.), equipped with 63x 1.4 NA oil differential interference contrast Plan-Apochromat objective, using Immersol (n = 1.518) at room temperature. Acquisition software was LSM 510, expert mode (Carl Zeiss, Inc.). Images were processed by Adobe Photoshop using proportional adjustments.

Visual and statistical analyses

Western blot band intensity was measured using the Image J software provided by NCBI. Recovery of p24 was calculated as the band intensity of p24 divided by the band intensity of actin per sample. Actin was used as a loading control. Recovery of HA-RhTRIM5α was calculated as the band intensity of HA-RhTRIM5α divided by band intensity of GFP or p62-GFP (wt, ΔLIR or ΔUBA) over band intensity of p62-GFP wt. The colocalization of fluorescent objects (puncta, $\geq$ 0.25 μm^2) on microscopy images were measured by calculating the Pearson's correlation coefficient (R) between fluorescence intensities using Slidebook 5.0 (Intelligent Imaging Innovations, Inc.). "0" indicates no colocalization. Values above 0 indicate positive colocalization. Colocalizations were

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measured among cells or objects as described in the figure legends. Significance was determined by performing a student's t-test, p-value < 0.05.

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CHAPTER 4

TRIM5α IS AN INNATE IMMUNITY REGULATOR INDUCING AUTOPHAGY

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Abstract

Defense against intracellular pathogens is a key feature of higher order eukaryotes. Complex mechanisms have evolved to protect the organism from parasitic infection. These protective mechanisms can be categorized as part of the immune system, falling into two basic groups: innate and adaptive immunity. Within the innate and adaptive immune systems, autophagy, as a degradative process, contributes to defense against pathogens in a variety of ways [5]. One way in which autophagy contributes to anti-microbial defense is through the direct elimination of pathogens. Prerequisite to this capture and clearance by autophagy is believed to be the recognition of pathogens through a set of pattern recognition receptors (PRRs) [16]. After recognition of the pathogenic information, the innate immunity machinery may then activate the autophagy pathway. TRIM proteins are increasingly gaining attention as a unique class of innate immunity regulators [44], with a variety of TRIM's capable of recognizing specific pathogens [43]. However, induction of autophagy by TRIM proteins as a function of their roles in innate immunity has yet to be investigated. Here we report TRIM5α as a unique autophagy regulator capable of engaging the autophagic machinery. Furthermore, we find supporting evidence that induction of autophagy by TRIM5α occurs through the generation of K63 type polyubiquitin chains.

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Results

After infection with an intracellular microbe, two steps must occur for the upregulation of autophagy. First, pattern recognition receptors (PRRs) must recognize pathogen-associated molecular patterns (PAMPs) and activate a signaling cascade that induces the innate immune response. Second, the innate immunity machinery (through a new signaling cascade) must respond to these signals by upregulating the autophagy pathway. The TRIM family of proteins represent a unique class of innate immunity factors implicated in both stages of the antimicrobial response [44, 59-61].

To investigate the possibility that TRIM5α may be capable of connecting innate immunity to autophagy, its ability to affect autophagy independent of infection was tested by measuring the amount of LC3 II due to the overexpression of TRIM5α (Fig. 4.1). LC3 II is an autophagy factor required for membrane stabilization and growth during autophagosome formation, the basic physical component of autophagy. LC3 exists in the cell in two forms: the LC3 I form, which is distributed free in the cytosol, and the LC3 II form, converted from LC3 I, and phosphatidylethanolamine (PE) lipidated. Conversion of LC3 I to LC3 II, regulated by other autophagy factors, reflects the rate of autophagic flux. During states of elevated autophagic flux (i.e. induction), LC3 I is converted to LC3 II at a higher rate than at basal autophagy levels. In this experiment, HeLa cells were transfected with human TRIM5α-GFP (HuT5-GFP), rhesus TRIM5α-GFP (RhT5-GFP), or GFP as a control. Transfection with either TRIM5α resulted in an increase in LC3 II compared to GFP control (HuT5-GFP CT and RhT5-GFP CT compared to GFP CT, Fig. 4.1A, blot and graph), indicating that TRIM5α proteins do have an effect on

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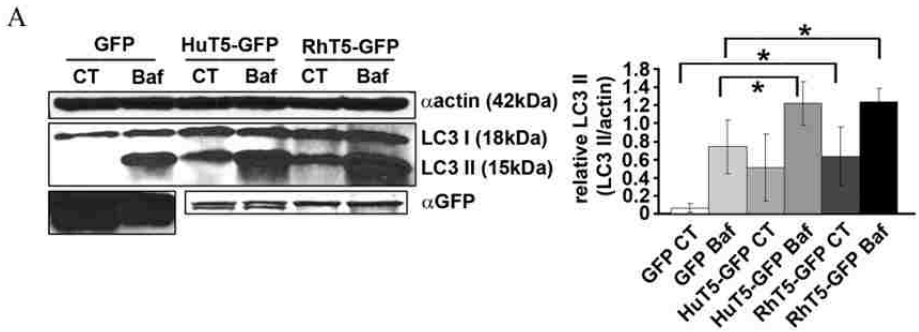

Figure 4.1. TRIM5α can induce autophagy. (A) HeLa cells were transfected with either GFP, human TRIM5α-GFP (HuT5-GFP), or rhesus TRIM5α-GFP (RhT5-GFP) in the presence or absence of bafilomycin A1. The levels of LC3 II that accumulated due to these treatments and transfections are graphically represented as the amount of LC3 II over the amount of actin (used as a loading control). The graph represents data from three independent experiments. Asterisks indicate significance among the data sets compared (indicated by brackets) (p-value <0.05, student's t-test). Error bars indicate ±SEM.

autophagy. Because the accumulation of LC3 II may be due to either an increased rate of conversion from LC3 I to LC3 II (caused by induction) or an impaired degradation of LC3 II (caused by a block in autophagic maturation), bafilomycin A1 was used to inhibit autophagic maturation, thereby controlling for possible effects on maturation and allowing distinction between the two. Treatment with bafilomycin A1 resulted in significantly higher levels of LC3 II in the presence of both TRIM5α compared to GFP alone, indicating that the accumulation of LC3 II in untreated conditions is due to an elevated rate of conversion from LC3 I to LC3 II (i.e. induction of autophagy) (HuT5-GFP Baf and RhT5-GFP Baf compared to GFP Baf, Fig. 4.1A, graph).

A common feature of all TRIM proteins is that they contain RING domains. Many of these RING domains have been shown to have E3 ubiquitin ligase activity, including human and rhesus TRIM5α [50]. Some of these TRIMs have been shown to be capable of generating K63 type polyubiquitin chains [62, 63, 72, 73]. This type of polyubiquitin chain describes ubiquitin proteins sequentially linked through lysine residues at amino

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acid position 63. Interestingly, some studies have indicated that autophagy adaptor protein p62 itself prefers this kind of lysine linkage through its UBA (ubiquitin binding area) domain and traffics proteins tagged with K63 type polyubiquitin chains to autophagy [64, 66-68]. Therefore, the possibility of an interaction between TRIM5α and p62 was investigated through a co-immunoprecipitation experiment (Fig. 4.2). HeLa cells stably expressing either human HA-TRIM5α (HA-HuTRIM5α) or rhesus HA-TRIM5α (HA-RhTRIM5α) were lysed and immunoprecipitated for TRIM5α protein using an antibody specific to the HA tag. From purified TRIM5α precipitates, endogenous p62 was found in complex with both variants of the TRIM5α protein upon immunoblotting (Fig. 4.2A). These results might suggest a role for TRIM5α in directly regulating autophagy through association with p62.

Next, to determine whether there was an association of TRIM5α with polyubiquitin and to evaluate the kind of polyubiquitin linkage, a final immunoprecipitation experiment was performed (Fig. 4.3). HeLa cells stably expressing HA-RhTRIM5α were lysed and immunoprecipitated for the TRIM5α protein as described above. When TRIM5α was purified, an abundance of K63 type polyubiquitin chains were found to associate with it. The blot was then stripped and probed for the presence of another type of polyubiquitin chain (K48, thought to be a signal for proteasomal degradation). K48 polyubiquitin chains were not detected and therefore cannot be confirmed to associate with TRIM5α.

K63 type polyubiquitination has been found to specifically promote protein inclusion formation [68, 71], which has been implicated in p62-mediated trafficking of targets to

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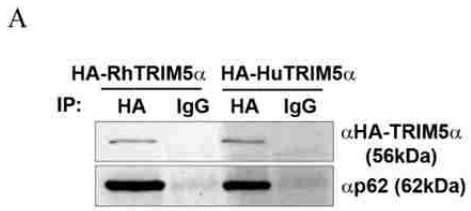

Figure 4.2. TRIM5α forms a complex with autophagy adaptor protein p62. (A) HA-TRIM5α was immunoprecipitated from HeLa cells stably expressing either human TRIM5α or rhesus TRIM5α and the amount of endogenous p62 in the precipitate was visualized by Western blotting.

autophagy [66, 67]. This type of polyubiquitin, found in association with RhTRIM5α, might indicate a mechanism whereby either TRIM5α variant could promote the formation of pre-autophagic aggresomal structures (as a function of their homologous RING domains) in response to some stimulus (such as HIV-1 infection in the case of the rhesus macaque variant), then attract p62 for targeted upregulation of autophagy. To fully understand the contribution of p62 and K63 type polyubiquitin to TRIM5α-mediated induction of autophagy, it will first be important to show whether or not these factors are definitively required for TRIM5α's effects. Part of this understanding will also require investigation into the nature of the association between TRIM5α and p62 and K63 polyubiquitin, especially direct versus indirect interactions. It is possible that the target for K63 polyubiquitination is not TRIM5α, as one could predict, but perhaps another factor involved in regulating autophagy. The identification of other factors involved in TRIM5α's effects on autophagy will help us to elucidate the mechanism of autophagic induction by TRIM5α and may reveal new targets for therapeutic intervention of HIV and possibly other diseases.

An important indicator of a possible role for TRIM5α in promoting pre-autophagic aggresomal formation is revealed by overexpression of either human or rhesus TRIM5α,

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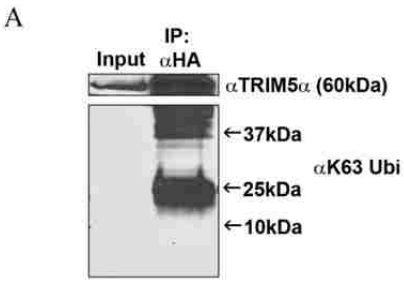

Figure 4.3. TRIM5α forms a complex with K63 type polyubiquitin. (A) HA-TRIM5α was immunoprecipitated from HeLa cells stably expressing rhesus TRIM5α and the amount of K63 type polyubiquitin in the precipitate was visualized by Western blotting.

where they are seen to distribute in the cell as punctate structures called cytoplasmic bodies [48]. These structures may reflect TRIM5α's propensity for aggresome-based autophagic induction, revealing another possible target for therapeutic intervention during HIV infection through the control of cytoplasmic body formation. Interestingly, expression of TRIM5α can be upregulated through IFN_β treatment. Indeed, future studies with IFN_β may prove an effective new treatment for infected individuals.

Materials and Methods

Cells

HeLa cells stably expressing either human or rhesus HA-TRIM5α were kindly provided by Joseph Sodroski. These cells were maintained as previously described (Chapter 3).

Plasmid constructs and drug treatment

Plasmids encoding human or rhesus TRIM5α-GFP were a kind gift from Joseph Sodroski. Transfections were performed as previously described (Chapter 2). For the bafilomycin A1 study, cells were treated with either 100 nM bafilomycin A1 or vehicle

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as a control for 4 hours then collected for Western blotting. Stocks of bafilomycin A1 were diluted in DMSO. DMSO was used as a control.

Immunoblotting and immunoprecipitation

Cells were collected and lysed as previously described (Chapter 3). Immunoblotting and immunoprecipitation protocols were performed as previously described (Chapter 2 & 3). For immunoprecipitations, rabbit anti-HA antibody was used to precipitate HA-TRIM5α. Non-specific rabbit IgG was used as an immunoprecipitation control. Because no cells were transfected or treated, no input control was performed. The following primary antibodies were used for immunoblotting: rabbit or mouse anti-GFP (Abcam), mouse anti-actin (Sigma or Abcam), rabbit anti-LC3 (Sigma), mouse or rabbit anti-HA (Abcam or Sigma, respectively), guinea pig anti-p62 (Progen), rabbit anti-TRIM5α (Imgenex), mouse anti-K63 type polyubiquitin (Enzo Life Sciences), and mouse anti-K48 type polyubiquitin (Enzo Life Sciences).

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CHAPTER 5

DISCUSSION AND FUTURE IMPLICATIONS

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Objective and hypothesis

The studies presented in this dissertation were designed and implemented to investigate the species-specific variation in the involvement of autophagy during HIV-1 infection. We hypothesized that HIV would escape autophagy in a host model for productive infection and, conversely, that HIV would not be able to escape autophagy in a host model where productive infection does not occur. We found that the HIV factor Nef inhibits autophagic maturation in the human macrophage, thus allowing the virus to escape degradation and elimination by autophagy. We also showed that the rhesus macaque restriction factor RhTRIM5α allows the rhesus macaque to utilize autophagy to eliminate HIV, an ability that is lost in the human cells studied. Finally, we explored a recently revealed role for both human and rhesus TRIM5α in regulating innate immunity through induction of autophagy and identify a previously an association of K63 type polyubiquitin in complex with TRIM5α (specifically, RhTRIM5α). Here we discuss the future implications of the research presented in this dissertation.

Chapter 2 Summary

In Chapter 2, we tested the specific hypothesis that autophagy could be manipulated to eliminate HIV in the human macrophage. Contrary to our hypothesis, we show that: (i) autophagy is essential in maintaining optimal viral yields from macrophages, (ii) autophagy promotes biogenesis of HIV by supporting the processing of HIV structural proteins, (iii) elimination of HIV by autophagic degradation is inhibited by HIV regulatory protein Nef, and (iv) Nef specifically inhibits acidification of autophagosomes through an as yet described mechanism requiring its ^{174}DD^{175} motif. These findings lead

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us to conclude that HIV is capable of circumventing elimination by autophagy in the human macrophage, one of two primary targets for HIV infection, thus allowing the virus to establish a productive infection in macrophages for significance to the development of AIDS.

HIV-1 factor Nef protects the virus from autophagic degradation in the human macrophage

To investigate the role of autophagy in human macrophages, we tested whether pharmacological induction of autophagy during a stable HIV infection reduces the replication rate. Unexpectedly, we found that treatment of cells with an autophagy inducer increased the amount of virus released into the culture media. We went on to show that this effect was due to the viral protein Nef; Nef was inhibiting the maturation stage of autophagy thereby preventing elimination of the virus by autophagic degradation.

Not only is the autophagy pathway altered by HIV during infection, but it also appears to be hijacked by HIV to assist in the processing of viral structural proteins required for proper virion assembly. We showed that when essential autophagy factor Beclin 1 is knocked down in these cells, processing of HIV Gag is decreased. How exactly Nef blocks the maturation stage of autophagy is not yet clear, but the experiments outlined in Chapter 2 suggest that Nef may either alter the preference of Beclin 1 for one complex versus another (initiation versus maturation or maturation inhibiting [marked by the presence of Rubicon] versus maturation promoting [marked by the presence of

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UVRAG]), redirect Beclin 1 from the maturation complex to lysosomes for degradation (discussed in the following paragraph), disrupt acidification of lysosomes directly by interacting with the V_1 subunit of the vacuolar ATPase, or act through altering the membrane preference of hVPS34 in some way that disrupts maturation. As far as the first scenario (altering Beclin 1 complex preference), Nef may, for example, prevent Beclin 1 from associating with UVRAG, thereby decreasing Beclin 1's bioavailability for association within the maturation complex, while stabilizing its association within the initiation complex. Alternatively, Nef may stabilize association of Beclin 1 with Rubicon, resulting in a decrease in the available pool of Beclin 1 that can associate with UVRAG. Both of these possible mechanisms would result in the down-regulation of autophagic maturation.

Interestingly, we found that Nef's ability to inhibit maturation of autophagy requires its ^{174}DD^{175} motif. This is the same motif known to interact with the recruitment factor AP-2, as well as the V_1 subunit of the vacuolar H^+ ATPase, during clathrin-mediated endocytosis of surface CD4 [28-30]. Nef's ability to interact with both, AP-2 and the vacuolar H^+ ATPase, allows Nef to mediate the endocytosis of CD4 from the plasma membrane. Once CD4 is endocytosed from the plasma membrane, Nef then interacts with β-COP through its ^{154}EE^{155} motif, which allows Nef to direct CD4 containing endosomes to lysosomes for degradation. Thus, one could imagine a mechanism whereby Nef down-regulates the Beclin 1 maturation complex (typically associated with membrane as well) in the same manner as CD4 down-regulation, thereby disrupting autophagic maturation. Similarly, Nef may also be capable of pulling in vast amounts of

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plasma membrane and directing it to autophagosomes to support biogenesis at different time points than those tested here.

Finally, studies on the origin of autophagic membrane have shown possible involvement of at least three sources including plasma membrane, endoplasmic reticulum, and mitochondria [31, 157-160]. The origin of the membrane may be of significance for pathogens requiring autophagic structures for biogenesis. Perhaps it may even be possible to identify the particular source of membrane by identifying certain organelle-specific markers assembled into or associated with viral or microbial pathogens. This may lead to greater understanding of the requirements for microbial biogenesis, revealing therapeutic targets for intervention.

Chapter 3 Summary

Here we tested the hypothesis that the rhesus macaque restriction factor TRIM5α could restrict HIV by targeting it to autophagy for degradation. We show that: (i) RhTRIM5α targets HIV to autophagy for elimination from the cell and (ii) restriction by RhTRIM5α requires a physical interaction with the autophagy machinery. These findings lead us to conclude that in the rhesus macaque, autophagy and HIV are still coupled, thus preventing HIV from establishing a productive infection and abrogating the ability of the virus to develop into AIDS.

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RhTRIM5α can direct HIV capsid to autophagy for degradation

In order to determine if autophagy is the primary target for viral clearance by RhTRIM5α, we used a combination of pharmacological inhibitors and genetic manipulation to test the ability of RhTRIM5α to restrict HIV while the autophagy pathway was impaired. We found that autophagy is required for restriction of HIV in the presence of RhTRIM5α, as inhibiting autophagy in either HA-RhTRIM5α stably expressing HeLa cells or FRhK4 monkey cells impaired the ability of HIV capsid protein p24 to be degraded. Furthermore, we identified autophagy proteins, LC3 and p62, associating with RhTRIM5α and HIV p24 in the presence of RhTRIM5α, while not in the absence of RhTRIM5α (only p62 was investigated here).

Interaction of RhTRIM5α with p62 might reveal something about the mechanism by which RhTRIM5α restricts HIV. P62, already found in complex with other TRIM proteins [75, 161] and recently with RhTRIM5α in another study [152], was first discovered in protein aggregates where autophagic clearance of these aggregates was impaired. Originally, p62 was reported to traffic proteins to the proteasome for degradation [64], but due to the intimate synergism between the proteasome and the autophagosome, it is often difficult to distinguish between these two processes. Studies where the effects of the proteasome have been controlled for demonstrate that the effect of p62 is to shuttle cargo into the autophagosome, not the proteasome [66, 68, 69, 71].

The specific action of p62 is revealed in studies of neurodegenerative diseases that show that p62 promotes aggregate formation of misfolded proteins [12, 66, 162]. Therefore, it

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is hypothesized by myself and others that p62 promotes aggresome formation through the formation of a scaffold enveloping misfolded proteins or other items, then directs the formation of nascent autophagic membrane around these structures [162, 163]. In the case of HIV-1 where RhTRIM5α is present, p62 would form around the RhTRIM5α-HIV capsid complex (consisting of HIV core covered by a network of RhTRIM5α oligomers, inner to the p62 shell) and direct autophagic membrane formation around these encased incoming core particles (Fig. 5.1). Assuming that this is the case, it will be important to explore ways of re-establishing the physical link between HIV core and p62 in human cells.

Even though human TRIM5α cannot specifically recognize HIV-1 capsid, studies have shown that it does have slight restrictive capability, as demonstrated when human TRIM5α is overexpressed [40]. One feature of overexpression of either TRIM5α is that these proteins distribute in the cells in punctate structures, called cytoplasmic bodies [48]. Literature that describes the activity of these structures shows that these dynamic bodies do not impair restriction of HIV by rhesus TRIM5α, but instead enhance its ability to restrict [48]. Therefore, cytoplasmic body formation by either TRIM5α likely represents a pre-autophagic structure with consequences for non-specific capture and clearance of HIV. It would be interesting to see if cytoplasmic body formation, which does not normally exist at endogenous expression levels in monkey cells, is induced in response to infection with HIV. Results from this experiment might suggest that artificially promoting cytoplasmic body formation of human TRIM5α *in vivo* is enough to result in physiologically relevant levels of HIV restriction.

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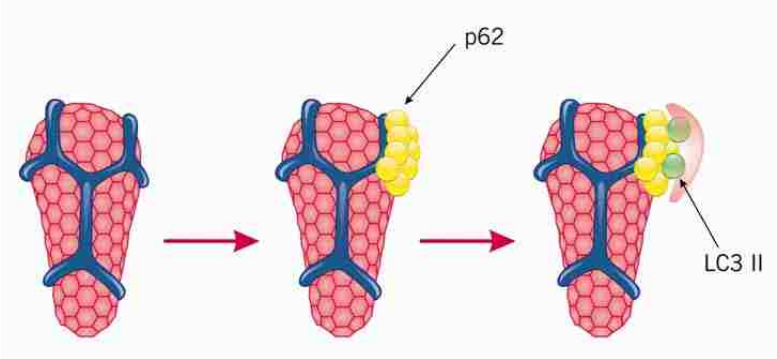

Figure 5.1. Model of RhTRIM5α-mediated autophagic recognition and capture of HIV core. The HIV core particle is recognized and coated by RhTRIM5α oligomers, which in turn are recognized by the autophagy adaptor p62 and LC3. Association with these autophagy factors allows the autophagic membrane to form around the HIV core, resulting in capture by the autophagy machinery and subsequent degradation.

Chapter 4 Summary

In the final study presented here, we hypothesized that TRIM5α could induce autophagy, thereby promoting the antiviral response during infection. We show that: (i) TRIM5αs can induce autophagy in the absence of infection and (ii) TRIM5αs interact with the autophagic machinery, including the autophagy adaptor p62 and an autophagy targeting/signaling tag, K63 polyubiquitin. These findings suggest a role for both species variants of TRIM5α in the innate arm of the immune system through an ability to induce autophagy.

TRIM5α may induce autophagy as part of the innate immune response

Previous literature has shown that RhTRIM5α is capable of mediating its own polyubiquitination through the E3 ubiquitin ligase activity of its RING domain [50], but the specific type has never been investigated. Additionally, studies have shown that both the RING and B-box 2 domains influence the rate of protein turnover and subcellular distribution [48, 164]. Specifically, it was shown that disruption of the RING domain

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results in an increase in the size of cytoplasmic bodies, with an overall decrease in diffuse cytoplasmic staining, while disruption of the B-box 2 domain results in an impaired ability of the RhTRIM5α protein to form cytoplasmic bodies (as seen by fluorescence microscopy) [48, 164]. Furthermore, disruption of either the RING or the B-box 2 domains results in significantly impaired restriction [40, 48]. In the context of autophagy, it may be that the B-box 2 domain controls the ability of TRIM5α to form pre-autophagic inclusion bodies (i.e. cytoplasmic bodies; pre-requisite to autophagic recognition), while the RING domain is required for subsequent recognition and clearance by autophagy.

Studies on the formation of pre-autophagic inclusion bodies show that the structures are enriched with p62 [12, 66, 162], while others have shown that they can also be enriched in K63 type polyubiquitin [165-167]. Kah Leong Lim explains the K63 phenotype best, "K63-linked polyubiquitin is the most consistent enhancer of inclusion formation. Moreover, K63 ubiquitin-modified inclusions are preferentially cleared by autophagy" [68]. One of the best autophagic adaptor proteins recognizing K63 type polyubiquitin is p62 [64], although NBR1 (another autophagy adaptor protein, similar to p62) has also been shown to have a strong preference for K63 type polyubiquitin as well [168]. Notably, studies on neurodegenerative diseases, particularly Huntington's disease, have shown that p62-enriched inclusion bodies induce the upregulation of autophagy [12, 169].

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Because the study outlined in Chapter 3 shows that RhTRIM5α associates with p62 and because it is already known that both human and rhesus TRIM5αs, when overexpressed, are capable of distributing in the cell as "cytoplasmic bodies", very closely resembling p62 pre-autophagic inclusion bodies, I considered the possibility that overexpressing either human or rhesus TRIM5α (thus, artificially promoting inclusion body formation, i.e. cytoplasmic bodies) would be able to stimulate autophagy. When this was tested, indeed this was the case. Next, knowing that TRIM5α is capable of ubiquitination, the possibility that it mediates induction of autophagy through K63 type polyubiquitination was tested. In a co-immunoprecipitation experiment, K63 type polyubiquitin was found to associate with purified RhTRIM5α in immunoprecipitates. Conversely, when the blot was stripped and re-probed for K48, no K48 was seen. These results suggest a preference for K63 type polyubiquitin by TRIM5α and may reveal the type of self-ubiquitination mediated by the TRIM5α RING domain.

Taken together, our results suggest a general model of RhTRIM5α restriction (Fig. 5.2): RhTRIM5α acts as a pattern recognition receptor for the HIV capsid lattice (the intricate molecular structure of the HIV core particle), allowing the indirect K63 polyubiquitination of HIV-1 capsid, thereby forcing viral core into pre-autophagic inclusion bodies capable of being recognized and captured by p62 for autophagy-mediated degradation and clearance from the cell. Furthermore, cytoplasmic body formation of either human or rhesus TRIM5α, either artificially induced or as a result of association with the HIV capsid, simultaneously acts as a signal inducing the upregulation of autophagy in a K63 type polyubiquitin-dependent manner.

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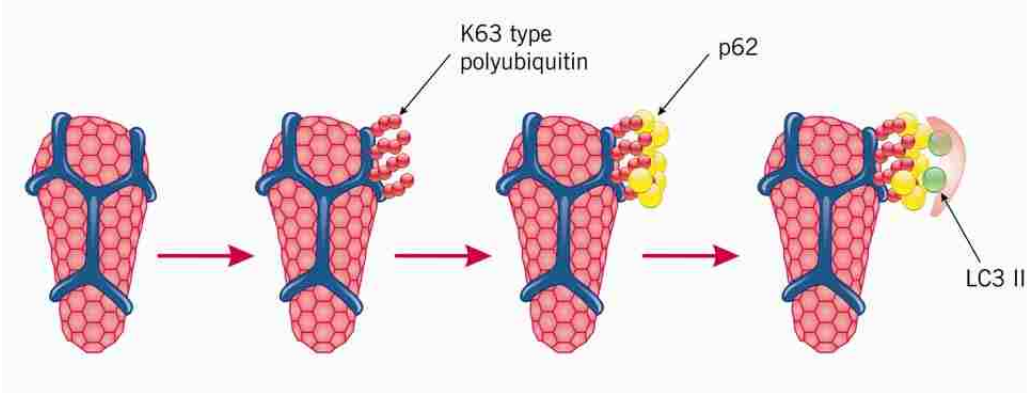

Figure 5.2. Model of RhTRIM5α-mediated K63 type polyubiquitin-dependent recognition and capture of HIV core by the autophagy machinery. The HIV core particle is recognized and coated by RhTRIM5α oligomers. RhTRIM5α then self-ubiquitinates with K63 type polyubiquitin, forcing RhTRIM5α-HIV capsid complexes into large inclusion bodies (not shown here) that are recognized by the ubiquitin binding domain (UBA) of p62 and promoted by its dimerization domain (PB1). P62 then (through its LC3 binding domain [LIR]) directs the formation of autophagic membrane around these inclusion bodies, trapping and degrading HIV core particles.

Support for the idea that TRIM5α might upregulate autophagy as a result of K63 type polyubiquitination comes from a recent paper showing that TRIM5α does indeed generate K63 type polyubiquitin chains, which then induce innate immune signaling (specifically, activator protein 1 (AP-1) and nuclear factor kappa B (NFκB) signaling) through transforming growth factor-beta-activating kinase 1 (TAK1) to upregulate the general antiviral response (and likely also autophagy [170]) [171].

Together, these studies suggest that promoting cytoplasmic body formation of endogenous human TRIM5α could result in autophagy induction as an innate immune response to viral infection. This could be tested pharmacologically through the treatment of cells with IFN_{β}. The use of type I interferons to stimulate an increase in endogenous TRIM5α expression has had been previously reported [77-79]. If the treatment of cells with IFN_{β} could overexpress HuTRIM5α enough to form cytoplasmic bodies, then autophagy should be induced and non-specific capture and clearance of HIV may occur.

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These results would be significant because they would suggest a prophylactic or therapeutic treatment for individuals immediately after exposure to HIV-1 through the HuTRIM5α-mediated induction of autophagy with IFN_{β}.

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APPENDIX A

Supplemental material

Kyei et al., http://www.jcb.org/cgi/content/full/jcb.200903070/DC1

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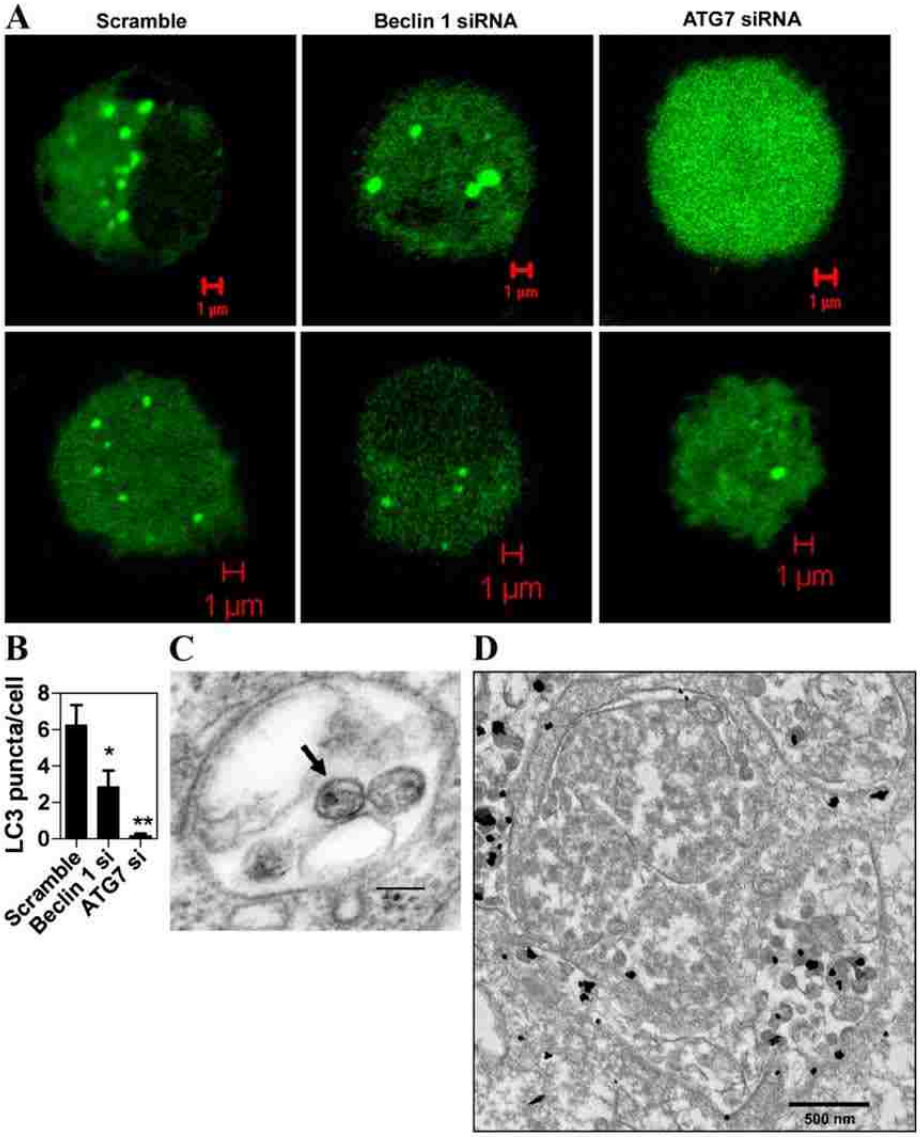

Figure A1. Diminished autophagy in cells subjected to Atg7 and Beclin 1 knockdowns and transmission, and immunoelectron microscopy analysis of HIV profiles in macrophages. (A and B) Atg7 and Beclin 1 knockdowns inhibit autophagy in U937 cells. U937 cells were cotransfected with GFP-LC3 and scrambled control, Beclin 1 siRNA, Atg7 siRNA, and LC3 puncta (A) quantified (B) in cells stimulated for autophagy. Similar data were obtained for two different autophagy induction stimuli (starvation and IFNγ). Data indicate means; error bars indicate ±SEM; **, $P < 0.01$; *, $P < 0.05$. (C and D) Transmission and immunoelectron micrograph HIV profiles in macrophages. (C) An enlarged area from Fig. 2.2B showing a membranous compartment with HIV virions (black arrow) and clathrin-coated pit budding from the profile (next to the scale bar) that is indicative of the plasma membranous nature of the plasma membranous profile. This is in keeping with findings of HIV in membranous domains contiguous with the plasma membrane [114, 116, 117]. (D) Immunoelectron micrographs of HIV-containing profiles labeled with p24 (enhanced immunogold).

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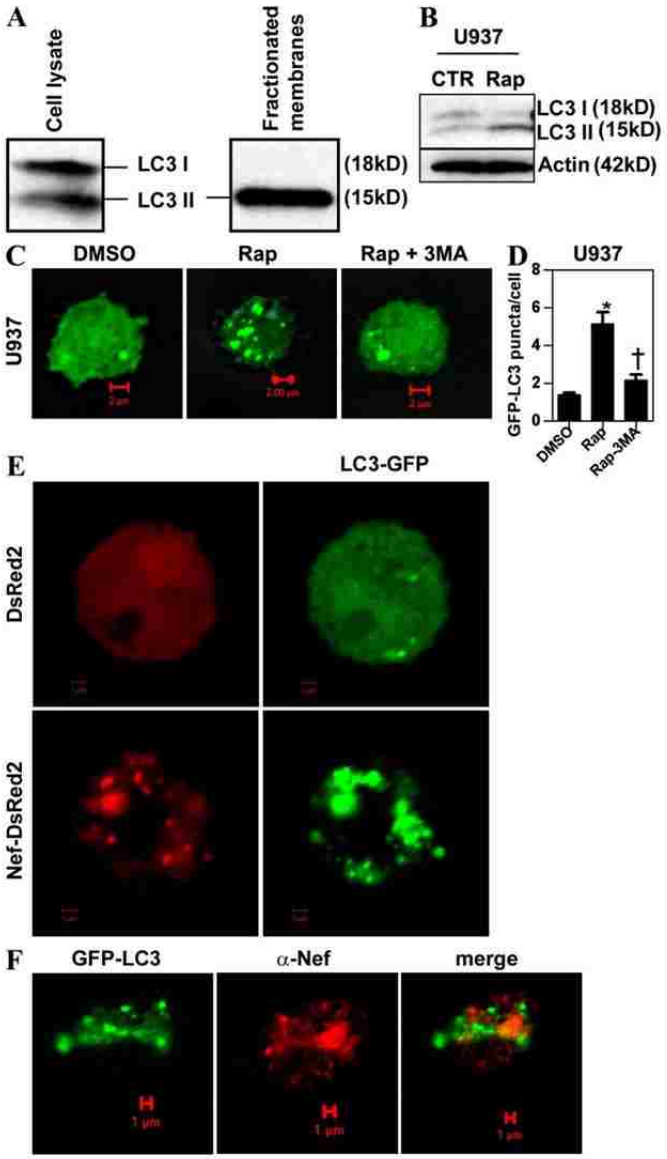

Figure A2. Comparison of LC3 forms in whole cell lysate versus LC forms associated with membranes, control for autophagy induction in cells during acute rapamycin treatment, and effects of Nef on accumulation of LC3 puncta. (A) Comparison of LC3 forms in whole cell lysate versus LC forms associated with membranes. Immunoblots with LC3 antibody of whole cell lysate and membrane fractions purified as described for the gradient shown in Fig. 2.2D. Not that the whole cell lysate displays both the unlipidated form LC3 I and the lipidated form LC3 II. Membrane fractions, identical to preparations shown in Fig. 2.2D, display only LC3 II. (B-D) Autophagy induction is operational in cells used to detect HIV yield-enhancing effects of acute rapamycin treatment. (B) U937 were treated with 50 µg/ml rapamycin for 2 h and immunoblotted for LC3 and actin. (C) U937 cells were transfected with GFP-LC3 and treated with rapamycin with or without 3MA for 2 h. (D) Quantification of GFP-LC3 puncta in B. Data indicate means; error bars indicate ±SEM. *, $P < 0.05$; †, $P > 0.05$; $n = 3$. (E and F) Nef causes accumulation of LC3 puncta. (E) U937 were transfected with GFP-LC3 along with Nef-DsRed2 or DsRed2. The data in A are similar to those in Fig. 2.4A, and are included to show additional cells, as dual versus single transfected cells were not found in the same field to allow comparisons by capturing a single field of view. (F) U937 cells were transfected with Nef-Myc and GFP-LC3, and immunostained with an antibody against Nef. The images in B are included to show additionally that GFP-LC3 puncta accumulation was independent of DsRed2, as they were detected in cells transfected with Nef-Myc.

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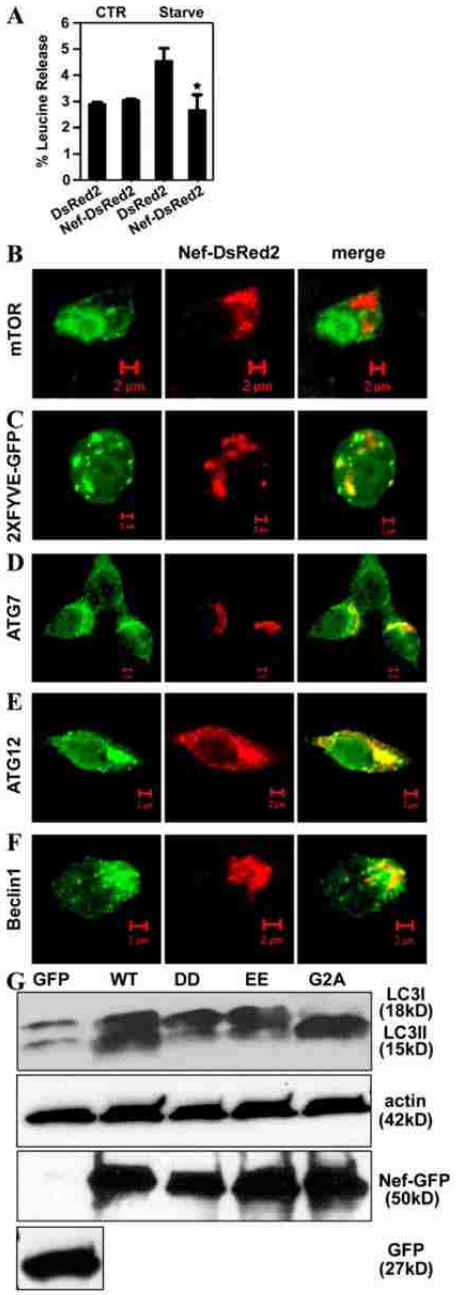

Figure A3. Inhibition of autophagic proteolysis by Nef, intracellular localization of Nef, and the role of Nef motif ^{174}DD^{175} for Nef-dependent increase in LC3 II levels. (A) Nef inhibits autophagic proteolysis. RAW264.7 macrophages, used as the only established autophagic proteolysis system in macrophages [124], were transfected with DsRed2 or Nef-DsRed2 for 48 h. Cells were induced for autophagy by starvation and autophagic proteolysis of stable polypeptides radiolabeled with [^{3}H] leucine measured and expressed as the percentage of radiolabeled leucine released; n = 3. Data indicate means; error bars indicated ± SEM. *, P < 0.05. (Material and methods) RAW264.7 macrophages were transfected with DsRed2 and Nef-DsRed2 plasmids, seeded at 7 x 10^4 cells/well, and then incubated for 24 h in DME with [^{3}H] leucine as described previously [124]. Cells were washed and incubated in unlabeled DME to allow degradation of short-lived proteins as described previously[124]. Cells were then washed three times for 10 min and incubated for 4 h in either complete DME medium or Earle's Balanced Salt Solution. TCA precipitation of the media fraction, cell lysis, and measurement of the percentage of leucine release was performed as described previously for autophagic proteolysis quantification [172]. Baseline

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degradation of stable proteins in nonliver cells normally converts 0.5% per 1 h (2% per 4 h) of the total radioactivity incorporated into macromolecules into TCA-soluble form; autophagy induction normally increases this rate by 50-200%. (B-F) Intracellular localization of Nef relative to mTOR, 2xFYVE-GFP, and autophagy markers Atg7, Atg12, and Beclin 1. (B and C) Macrophages were transfected with Nef-DsRed2 for 24 h and immunostained for mTOR or cotransfected with 2xFYVE-GFP, then confocal images were taken. (B) No colocalization is seen between Nef and mTOR (TOR, as previously described [173, 174], shows significant nuclear localization, augmented upon TOR inhibition under certain conditions [174]). (C) Partial but distinct colocalization is evident between Nef and 2xFYVE-GFP. 2xFYVE-GFP is a probe for phosphatidylinositol 3-phosphate, a phosphoinositide generated by hVPS34, the PI3K that complexes with Beclin 1 in regulation of autophagy. (D-F) Macrophages were transfected with Nef-DsRed2, and endogenous autophagy proteins Atg7, Atg12, and Beclin 1 (Atg6) were revealed by immunofluorescence. (G) Nef motif ^{174}DD^{175} is but G^{2}A motif is not required for a Nef-dependent increase in LC3 II levels. 293T cells were transfected with the wild-type HIV-1 Nef fusion with GFP or the mutants G^{2}ANefGFP, ^{154}EE-QQ^{155}NefGFP, and ^{174}DD-AA^{175}NefGFP for 48 h, and LC3 II levels were determined by immunoblotting. Note that in this experiment, Nef mutant and wild-type expression levels were adjusted to more equal levels than in Fig. 2.7B.

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Table A1. HIV molecular clones, viruses[a], viral preparations[b], and use[c]

Construct	Reference or source	Comment
pMSMBA	Callahan et al., 1998 [175]	Same as NL4-3Δenv
pMSMBA-Vpu-null	McBride and Panganiban, 1996 [176]	Same as NL4-3ΔenvΔvpu
HIVΔNef	Valentin et al., 1998 [177]	NL4-3ΔenvΔNef - GFP replaces Nef in this clone
HIV-1_{NLAD8}	E. Freed, National Cancer Institute	NA
SF162	Pelchen-Matthews et al., 2003 [112]	Full-length HIV virus
HIV_{LAI}	Barre-Sinoussi et al., 1983 [145]; Nguyen et al., 1994 [146]	CXCR4 co-receptor specific (T cell tropic)

NA, not applicable.

[a]HIV clones and viruses [145, 146]. The HIV-1 NL4-3 molecular clones pMSMBA and its derivative pMSMBA-Vpu-null have been described previously [175, 176]. GFP-HIV-Nef-null has been described previously [177]. DsRed2 and Nef-DsRed2 (from K. Collins, University of Michagan, Ann Arbor, MI) are derivatives of DsRed2 (Clontech Laboratories, Inc.) with reduced spontaneous aggregation, and have been described previously [178]. The intracellular localization of Nef-DsRed2 is similar to that shown for Nef-GFP [179] or immunostained Nef [134]; Nef-DsRed2 shows biological properties indistinguishable from untagged Nef when tested for effects on MHC class I [178]. Other plasmids were p2xFYVE-EGFP from H. Stenmark (The Norwegian Radium Hospital, Oslo, Norway), pEGFP-LC3, and tandem RFP-GFP-LC3from T. Yoshimori (OsakaUniversity, Japan). Nef-GFP and associated mutants are derivatives of pcNEFsg25GFP. SF162 macrophage-tropic virus [112] was obtained through the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH: SF162, No. 276.

[b]Virus generation and isolation. Replication-competent HIV-1_{NLAD8} was generated by transfections of HEK293T cells with the proviral construct pNLAD8. Preparations of pMSMBA and HIV-Nef-null virus (pseudotyped with the envelope glycoprotein of the VSV-G were obtained as cleared supernatants of 293T cells 48 h after calcium phosphate cotransfection; viral infections were carried out as described previously [130]. For isolation of VLPs, cell supernatants were harvested, spun down at 14,000 rpm for 30 s to remove any cells, and layered (500 µl) on to 400 µl of 20% sucrose in PBS, then centrifuged at 20,000 *g* for 90 min at 4°C.

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Table A2. HIV p24 yields as measured by ELISA

	MDM (3MA) (**Fig.2.1B**)		MDM (siRNA) (**Fig.2.1D**)		U937 (siRNA) (**Fig.2.1F**)			
	CTR ATG7	3MA	CTR	Beclin 1	Beclin CTR	Beclin 1	ATG7 CTR	
MEDIA(M)	145	96	519	87	1138	2940	369	264
LYSATE(L)	832	960	657	168	1162	4944	620	811
TOTAL	**977**	**1056**	**1176**	**255**	**2300**	**7884**	**989**	**1075**

Fig 2.3C and D

	MDM(CLONE)		MDM(SF162)		THP-1 -**Fig.2.3F**		U937-**Fig.2.3F**	
	DMSO	RAP	DMSO	RAP	DMSO	RAP	DMSO	RAP
MEDIA(M)	22.7	24.4	30.3	35.7	50.2	180	81	62
LYSATE(L)	85.3	60.3	381.7	231.5	490	340	175	55
TOTAL	**108**	**84.7**	**412**	**267.2**	**540.2**	**520**	**256**	**117**

	ATG7-rap-**Fig.2.3H**			HeLa and H9 cells-**Fig. 2.3I**			
				HeLa		H9	
	CTR	CTR-RAP	SI-RAP	CTR	Rap	CTR	Rap
MEDIA(M)	282	513	244	27	26	0.19	0.16
LYSATE(L)	1300	832	707	188	197	0.6	0.35
TOTAL	**1582**	**1345**	**951**	**215**	**223**	**0.79**	**0.51**

	U937 (Nef-Null) (**Fig.2.4A**)				U937 (Nef-Null) (**Fig.2.4D**)	
	HIV		HIVDNef		HIVDVpu	
	DMSO	RAP	DMSO	RAP	DMSO	RAP
MEDIA(M)	175	62	206	106	457	2940
LYSATE(L)	256	55	319	206	4568	3441
TOTAL	**431**	**117**	**525**	**312**	**5050**	**6381**

	U937 (Nef-Null) (**Fig. 2.4E**)				U937 (**Fig. 2.5F**)		
	DMSO	3MA	CTR	Beclin 1	DMSO	RAP	RAP + BAF
MEDIA(M)	6	3	42	51			
LYSATE(L)	337	336	197	232	283	158	241
TOTAL	**343**	**339**	**239**	**283**			

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GLOSSARY

adaptive immunity - A collection of cellular mechanisms evolved to combat microbial pathogens during sustained infection. Activation of adaptive immunity typically results in antibody production.

aggresome - A macromolecular cluster of proteins, not necessarily misfolded, distributing in microscopy images as large foci within the cell.

AIDS (acquired immunodeficiency syndrome) - Symptomatic disease associated with HIV-1 infection in humans characterized by an impaired immune system due to, among other things, depleted $CD4^+$ T cells levels.

autolysosome (autophagolysosome) - The mature double membrane vacuole of autophagy consisting of an autophagosome fused with a lysosome, where the contents are in a state of degradation.

autophagy (*n.*) - [autophagic (*adj.*)] A cellular homeostatic process by which cytoplasmic components, including aggregated proteins, portions of cytosol and organelles, whole organelles, and intracellular pathogens, are degraded in a double membrane vacuole through fusion with lysosomes. Consisting of the following three types: macroautophagy, microautophagy, and chaperone-mediated autophagy.

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autophagosome - The fundamental organelle of the autophagy pathway, a closed double membrane vacuole.

bafilomycin A1 - An inhibitor of autophagic maturation, specifically interfering with proper vacuolar ATPase formation, which results in improper acidification of autophagosomes.

Beclin 1 - A protein with undescribed activity that regulates autophagy initiation and maturation through association with various autophagy factors.

endocytosis - A process where extracellular materials are internalized in a single membrane vesicle. Trafficking involves a group of proteins commonly referred to as the ESCRT machinery. Acquisition of materials commonly requires utilization of clathrin.

HIV (human immunodeficiency virus) -

capsid, HIV - Structural portion of the HIV viral particle that encloses and protects the HIV genomic material, consisting of a complex of p24 structural protein. Exists in a Fullerene shaped structure.

core, HIV - The entire capsid structure, including cellular and other viral proteins believed to be incorporated with the capsid protein p24.

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p24 - HIV structural protein which forms the capsid of HIV.

HuTRIM5α (human TRIM5α) - Ortholog of rhesus TRIM5α without anti-HIV-1 activity. Can restrict N-tropic murine leukemia virus however. Contains the same domains as RhTRIM5α (see RhTRIM5α for an explanation of the domains).

hVPS34 - A class III PI3 kinase capable of generating PI3P, believed to be essential for autophagic membrane formation, typically found on complex with Beclin 1.

innate immunity - A collection of cellular response mechanisms evolved to combat microbial pathogens immediately after infection. Activation of innate immunity typically results in cytokine production.

K48 polyubiquitin - Individual ubiquitin proteins linked to each other through their lysine residue at position 48.

K63 polyubiquitin - Individual ubiquitin proteins linked to each other through their lysine residue at position 63.

LC3 - An autophagy marker commonly used in autophagy research. Exists in the cell in two forms: (i) the LC3 I (15kDa) form, which is unlipidated and free in the cytosol, and (ii) the LC3 II (18kDa) form, which is conjugated to phosphatidylethanolamine (PE) and

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is only found on autophagic membrane. Conversion from the LC3 I form to the LC3 II form indicates the rate of autophagic flux.

lysosome - An acidic vesicle also containing proteases used during endocytic and autophagic digestion.

mTOR (mammalian target of rapamycin) - Inhibits Ulk1 activity through phosphorylation.

multivesicular body - An endosome were vesicles bud internally from the membrane into the lumen of the endosome resulting in a multivesicular appearance. Thought to be important for sorting endosomal contents for recycling prior to fusion with and degradation by lysosomes.

Nef - HIV regulatory protein that potently enhances the pathogenicity of the virus. Previously shown to be involved in enhancing infectivity and egress of individual viral particles, as well as altering cellular pathways.

p150 - The catalytic subunit of hVPS34.

p62 (also called sequestosome 1) - Autophagic adaptor protein involved in recognition of cytoplasmic targets, typically aggregated proteins, for autophagy. Specifically

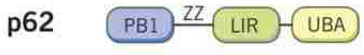

Figure G1. p62 domains.

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recognizes the polyubiquitin tag of proteins through its UBA domain and associates with LC3 through it LIR domain. PB1 domain allows dimerization with itself and other autophagy adaptors. The ZZ type zinc finger is involved in other signaling functions of p62.

phagophore - The immature double membrane structure of autophagy prior to fusion and formation of the autophagosome.

rapamycin - An inducer of autophagy, specifically inhibits mTOR suppression of autophagy initiation and results in an elevated rate of autophagic flux.

restriction factor - A host cellular protein capable of inhibiting a virus at a particular stage in the virus's life cycle.

RhTRIM5α (rhesus TRIM5α) - Potent early entry restriction factor to HIV-1 expressed in rhesus macaque tissues. The RING domain of RhTRIM5α has E3 ubiquitin ligase activity. The B-box 2 domain is required for HIV restriction. The coiled-coil domain is the site of RhTRIM5α oligomerization. The SPRY domain is the site of specific HIV capsid recognition.

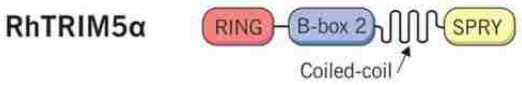

Figure G2. RhTRIM5α domains.

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VSV-G pseudotyped HIV - An engineered HIV virion consisting of an HIV core within a vesicular stomatitis virus (VSV) glycoprotein (G) envelope. Typically generated by co-transfection of plasmids encoding HIVΔenv and VSV-G protein in a producer cell.

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