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

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Introduction

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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Abbreviations used in this paper: 3MA, 3-methyl adenine; AIDS, acquired immune deficiency syndrome; ANOVA, analysis of variance; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HIV, human immunodeficiency virus; MDM, monocyte-derived macrophages; MVB, multivesicular body; PI3K, phosphatidylinositol 3-kinase; Tor, target of rapamycin; VLP, virus-like particle; VSV-G, vesicular stomatitis virus G.

(Levine and Klionsky, 2004; Levine and Kroemer, 2008). 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, thus promoting 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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conjunction 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 lipiddated form (LC3-II) covalently modified by phosphatidylethanolamine. The lipiddated 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 fusion with multivesicular body (MVB) compartments (Gruenberg and Stenmark, 2004) and other lysosomal organelles (Shintani and Klionsky, 2004). Most cells undergo baseline autophagy to remove protein aggregates and spuriously damaged mitochondria or other organelles, or to adjust the cellular biomass (Levine and Kroemer, 2008). 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 (Levine and Kroemer, 2008; and has emerged as an important player in inflammatory and infectious diseases (Levine and Deretic, 2007; Deretic and Levine, 2009).

Autophagy is now well recognized as an innate and adaptive immunity mechanism (Levine and Deretic, 2007; Schmid and Munz, 2007). Pharmacologically, physiologically, or immunologically induced autophagy can act as a powerful antimicrobial defense (Gutierrez et al., 2004; Nakagawa et al., 2004; Ogawa et al., 2005; Birmingham et al., 2006, 2008; Singh et al., 2006; Levine and Deretic, 2007; Yano et al., 2008; Deretic and Levine, 2009). Autophagy is under the control of immune receptors and cytokine signaling (Levine and Deretic, 2007; Schmid and Munz, 2007), and is stimulated upon microbial recognition by innate immunity pattern recognition receptors (Lee et al., 2007; Sanjuan et al., 2007; Xu et al., 2007; Delgado et al., 2008) or activation with Th1 cytokines (Harris et al., 2007). However, certain pathogens can harness this process to assist their own propagation (Jackson et al., 2005; Orvedahl et al., 2007; Birmingham et al., 2008; Deretic and Levine, 2009). Interestingly, a recent large scale siRNA screen of host cell factors required for human immunodeficiency virus (HIV) type 1 (HIV-1) replication has identified several Atg factors among >250 HIV dependency host genes (Brass et al., 2008). 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 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 (Daniel et al., 1992; Deacon et al., 1995; Kirchhoff et al., 1995). The methods by which the Nef protein acts as a pathogenic factor in vivo are not fully understood, but involve several mechanisms (Geleziunas et al., 2001; Swingler et al., 2003; Peterlin, 2006; Roeth and Collins, 2006). Recent findings suggest that the inability of lentivirus Nef to suppress CD4+ T cell activation correlates with viral pathogenesis (Schindler et al., 2006; Schindler et al., 2008). Our findings presented here uncover an additional, previously unappreciated Nef action in control of autophagy. Nef functions in preventing destruction of HIV components in autolysosomes, thus shielding 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 (Schmid et al., 2007). 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 threefold fewer infectious virions compared with the untreated control (Fig. 1 A). 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 optimal HIV yields was also assessed by determining extracellular release of the HIV capsid protein Gag p24 (Ono and Freed, 2004). The p24 released from live virus–infected primary macrophages was reduced in cells subjected to inhibition by 3MA (Fig. 1 B). 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 Beclin 1 (Atg6) in primary macrophages (Fig. 1, C and D) and in macrophages differentiated from monocyctic U937 cells (Fig. 1, E and F). Knockdown of Beclin 1 diminished p24 yields in both primary monocyte- and U937-derived macrophages (Fig. 1, D and F). A knockdown of another essential autophagy factor, Atg7, resulted in a similar effect on p24 yields (Fig. 1 F); Atg7 and Beclin 1 knockdowns affected autophagy in U937 cells, as determined by GFP-LC3 puncta/cell counts (Fig. S1). 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 NL4-3 HIV 1, cellular p24 levels were reduced compared with controls (Fig. 1 G). 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 (Gendelman et al., 1988; R appo et al., 2002; Pelchen-Matthews et al., 2003; Morita and Sundquist, 2004; Pelchen-Matthews et al., 2003; Morita and Sundquist,
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 macrophages to subcellular fractionation by isopycnic centrifugation in sucrose gradients. Fig. 2D shows that membranes 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 and Atg7 in cells infected with SF162 HIV-1 for 7 d, then p24 yields were quantified. (D) Western blots showing siRNA knockdown of Beclin 1 and Atg7 in 48 h after transfection in U937 cells. (E) 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 ≥ 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 performed for Gag processing analysis. *, P < 0.05, paired t-test.

Figure 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 and Atg7 48 h after transfection in U937 cells. (D) 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 ≥ 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 performed for Gag processing analysis. *, P < 0.05, paired t-test.

2004; Deneka et al., 2007; Jouvenet et al., 2006; Deneka et al., 2007; Welsch et al., 2007), which facilitates colocalization studies. The Gag-p17–specific antibody showed colocalization of the budded virus with autophagy marker LC3 (Atg8; Fig. 2A). By ultrastructural analysis, HIV virions were observed in these compartments (Fig. 2B), which, based on presence of clathrin-coated pits (Fig. 2B, asterisk; and Fig. S1C), were consistent with the previously reported plasma membrane connections (Deneka et al., 2007). These morphologically identified compartments also labeled for LC3 (Fig. 2C) and p24 (Fig. S1D). A dual labeling procedure was not practicable, as LC3-enhanced immunogold labeling resulted in globular, oval, and acicular shapes, and precluded clear distinction.
Figure 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. S1 C. Immunelectron 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. S1 D 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 organelar markers. The box with the broken line indicates peak band intensity fractions for LC3-II, Gag, and Gag-derived polypeptides, and CD9. (E) HIV Gag coimmunoprecipitates with LC3. U937 cells were infected with HIV and lysates immunoprecipitated for LC3. Immunoblotting with p24 and LC3 antibodies 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).
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. S2 A). The LC3-II–positive membranes enriched for Gag p55, Gag processing intermediate p41, 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 D; Deneka et al., 2007).

We next tested whether HIV Gag interacted with autophagy proteins in communoprecipitation experiments. Fig. 2 E shows that LC3 is found in protein complexes with the HIV Gag. These findings reinforce the subcellular fractionation experiments (Fig. 2 D), are in keeping with morphological analyses (Fig. 2, A–C), and demonstrate that HIV components and virions interact with the autophagic pathway with the functional consequence of augmenting Gag processing (Fig. 1 G) and HIV yields (Fig. 1, A–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 (Delgado et al., 2008). Infection of primary human peripheral blood MDM with HIV-1 strain SF162 virus increased LC3-II levels at 10 h after infection (Fig. 3, A and B), which coincided with the expected (Prasad and Kalpana, 2009) 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. 3, C and D). The increase in p24 was not caused by a nonspecific leakage of cytoplasmic contents, as the cytosolic enzyme lactate dehydrogenase levels in the medium were not altered by rapamycin treatment (Fig. 3 E). Similar results were obtained when two monocytic cell lines, THP-1 and U937, differentiated into macrophages were tested (Fig. 3 F). 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. 3 G). The autophagy machinery in these experiments was intact, as indicated by rapamycin both with response to rapamycin by LC3-I–to–LC3-II conversion and LC3 puncta formation (Fig. S2, B–D), and responsiveness of the effects to inhibition with the conventional autophagy inhibitor 3MA (Fig. S2, C and D). The enhancement effect of rapamycin on p24 release was counteracted by knocking down the key regulator of autophagy Atg7 (Fig. 3 H). 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 (Heredia et al., 2003; Roy et al., 2002), 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. 3 I) and H9 T cells infected with the CXCR4 coreceptor using (X4, T cell tropic) virus HIV LAI (Fig. 3 J).

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

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 (Gutierrez et al., 2004; Nakagawa et al., 2004; Ogawa et al., 2005; Birmingham et al., 2006, 2008; Singh et al., 2006; Levine and Deretic, 2007; Yano et al., 2008). 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. 4 A, 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. 4 A, inset). As a consequence, the ratios remained the same (Fig. 4 A, main graph), although the absolute levels of p24 (both cellular and released) were diminished (Fig. 4 A, inset). In contrast to nef deletion, HIV deleted for vpu still responded to rapamycin stimulation with increased p24 levels (Fig. 4 C). Furthermore, ΔNef virus, although showing reduced relative release of the viral p24 (Fig. 4 C) and cellular p24 levels (Fig. 4 D), showed no further change in yields, release, or cellular p24 when autophagy was inhibited by 3MA (Fig. 4 E, left two panels) or suppressed by Beclin 1 knockdown (Fig. 4 E, 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 based on monitoring biochemical and morphological changes that the autophagy protein LC3 undergoes (Kabeya et al., 2000). During the stages when autophagic isolation membranes (phagophores) begin to form and nascent autophagosomes elongate until they are 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 stages, 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 (Kabeya et al., 2000; Mizushima and Yoshimori, 2007). 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. 4 F), and LC3-II/loading control ratios (Mizushima and Yoshimori, 2007) using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the loading reference (Fig. 4 G). 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; Kabeya et al., 2000). 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 (Roeth et al., 2004). Transfection of U937 cells with Nef-DsRed2 resulted in an increased abundance of GFP-LC3 puncta versus the control DsRed2-transfected cells (Fig. 4, H and I; and Fig. S2, E). 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)
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 (Kimura et al., 2007). 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 stages 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 (Roberts and Deretic, 2008). Transfection with Nef-DsRed did not induce autophagic proteolysis (Fig. S3 A). Instead, Nef-DsRed inhibited autophagic protein degradation induced by starvation, a gold standard for assessment of autophagy function (Fig. S3 A). 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 (Kimura et al., 2007). 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; Kimura et al., 2007). 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. 5, A–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. 5 D). All red’green’ puncta (representing 31% of the total mRFP-GFP-LC3 puncta) were Lamp2 positive (Fig. 5 D). Expression of Nef-GFP resulted in an increase of LC3-II (Fig. 5 E). This was not or only slightly enhanced in the presence of bafilomycin A1 (Fig. 5 E, graph), an inhibitor of autophagosomal/autolysosomal acidification used to differentiate between effects on autophagy induction versus maturation (Mizushima and Yoshimori, 2007), 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. 5 F). The decrease in p24 was abrogated with bafilomycin A1, which blocks autophagic degradation (Fig. 5 F). Similar results were observed with cellular p24 levels (Fig. 5 F). 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. 5 S3 B), so it is unlikely that it affects Tor directly. Nef showed a partial colocalization with 2xFYVE-GFP (Fig. S3 C), a probe binding to membranes containing phosphatidylinositol 3-phosphate (PI3P), the enzymatic product of type III PI3K hVPS34 that binds 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 (Kihara et al., 2001; Furuya et al., 2005; Pattingre et al., 2005; Zeng et al., 2006). Nef showed colocalization with autophagy factors Atg7 and Atg12 (Fig. S3, D and E), and colocalized with the autophagic protein Beclin 1, which is the central regulator of autophagy at multiple stages (Liang et al., 1999; Pattingre et al., 2005). 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. 6 B, top left). GFP was absent from the control samples when Beclin 1 was immunoprecipitated from cells transfected with GFP alone (Fig. 6 B, 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. 6 B, bottom left) but not in extracts from cells transfected with GFP alone (Fig. 6 B, 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. 6 C). In all immunoprecipitation experiments, IgG control showed negative results for the specific proteins analyzed (Fig. 6). The blots shown with the IgG control were developed until a 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 bands were still detected. Importantly, HIV Nef also coimmunoprecipitated with Beclin 1 in extracts from cells infected with HIV virus (Fig. 6 D), demonstrating that Nef–Beclin 1 complexes form during viral infection. Thus, Beclin 1 and Nef colocalize (Fig. 6 A) and are present in a shared protein complex (Fig. 6, B–D), associating directly or indirectly via an intermediate partner. Furthermore, Nef affected hVPS34 distribution (Fig. 6, E and F), as a consequence of its association with Beclin 1, resulting in an increased presence of hVPS34 on membranes.

**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 (Figs. 7 A and S3 G). In an identical coimmunoprecipitation approach as in Fig. 6, the previously characterized Nef mutant construct (Olivetta and Federico, 2006) with changes in the diacidic motif (174DD175 → 174AA175), responsible for interactions with the V1 domain of vacuolar H+ ATPase and required for CD4 down-regulation (Roeth and Collins, 2006), lost the capacity to coimmunoprecipitate Beclin 1 (Fig. 7 A). In contrast, the mutation 154EE155 → 154QQ155, in another region of Nef, i.e., the diacidic motif required for β-COP interactions (Piguet et al., 1999; Roeth and Collins, 2006), did not significantly diminish the capacity of Nef to coimmunoprecipitate with Beclin 1 (Fig. 7 A). Another mutation 2G → 2A, abrogating the ability of Nef to be N-terminally myristoylated, a posttranslational modification assisting Nef in membrane localization and required for many Nef functions (Roeth and Collins, 2006), did not affect the capacity of Nef to coimmunoprecipitate with Beclin 1 (Fig. 7 A). Myristoylation of Nef is often considered a sine qua non posttranslational modification required for nearly all previously known functions of Nef (Roeth and Collins, 2006), with the exception of Hck activation by Nef (Briggs et al., 2001), and thus it may appear surprising that this did not nullify Nef’s action in our assays. However, it has been shown (Bentham et al., 2006) that membrane association of Nef2A 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.

The same set of Nef mutants was tested for their capacity to increase the LC3-II form (Fig. 7, B and C). The 174DD175 → 174AA175 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. S3 G). 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.
Figure 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 images). (B and C) Quantification of (red+green+) R⁺G⁺ puncta per cell, respectively. (D) Analysis of Lamp2 association with RGP-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. 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 ≥ 0.05 (ANOVA).
its function in autophagy (Orvedahl et al., 2007). However, the action of Nef is preferentially related to the maturation stages of 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 (Itakura et al., 2008), which appears to be autophagy initiation specific, and VPS38 (UVRAG), which acts at maturation and possibly initiation stages (Liang et al., 2008). 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 (Orvedahl et al., 2007), 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 (Brass et al., 2008).

It has been previously reported (Roy et al., 2002; Heredia et al., 2003) 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 affects its function in autophagy (Orvedahl et al., 2007). However, the action of Nef is preferentially related to the maturation stages of 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 (Itakura et al., 2008), which appears to be autophagy initiation specific, and VPS38 (UVRAG), which acts at maturation and possibly initiation stages (Liang et al., 2008). 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 (Orvedahl et al., 2007), 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 (Brass et al., 2008).
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 (Delgado et al., 2008). In terms of the mechanism of the antiautophagic degradation action of Nef, we found that the diacidic 174DD175 motif, responsible for interactions with the V1 domain of vacuolar H+ ATPase and needed for CD4 down-regulation (Roeth and Collins, 2006), 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 (Jouve et al., 2007). 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. 6 E) 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 (Peterlin, 2006; Roeth and Collins, 2006; Schindler et al., 2006, 2008), a less understood effect is the Nef-induced accumulation of MV-like organelles (Stumptner-Cuvelette et al., 2003; Sandrin and Cosset, 2006) and emergence of large vacuoles (Sanfridson et al., 2003; Sandrin and Sundquist, 2004; Jouvenet et al., 2006; Deneka et al., 2007; Jouve et al., 2007; Welsch et al., 2007). 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 (Brass et al., 2008). 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 autophagy indicate that this process plays a role in promoting 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 (Scott et al., 1996; Xie and Klionsky, 2007).

Nef also inhibits apoptosis and cell death in macrophages (Olivotta and Federico, 2006). 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 (Espert et al., 2006). This effect was recently narrowed down to gp41 (Denizot et al., 2008). 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 (Olivetta and Federico, 2006) and protecting virions from degradation may lead to higher HIV yields that are important for progression to AIDS (Daniel et al., 1992; Kirchhoff et al., 1995). 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 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 DMEM 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 reagents 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-LC3 puncta, LC3I→LC3II 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, DioRed2, NeDioRed2 (NeDoRed2) was provided by K. Collins, University of Michigan, Ann Arbor, MI and other constructs for 24 h. The total number of puncta (≥1 µm) per cell was counted.

HIV extracellular yield

Methods to monitor HIV p24 yields are described in the legend to Table S2. 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 the manufacturer’s instructions. Relative viral release was calculated as the ratio of extracellular-to-intracellular reverse transcription activity (Peden et al., 1991; Fan and Peden, 1992; Conti et al., 1998; Prasad and Kalpana, 2009).

Transfections and infections

Cells were transfected using the nucleoporation protocol (Amaxa) as described previously (Chua and Deretic, 2004), with 10 µg of DNA or 1.5 µg of siRNA, as required. Atg7 and Beclin 1 knockout knockdown protein was achieved using siGENOME SMART pool (Thermo Fisher Scientific). All effects of siRNA, as required. Atg7 and Beclin 1 knockdown protein was achieved using siGENOME SMART pool (Thermo Fisher Scientific). All effects of siRNA, as required. Atg7 and Beclin 1 knockdown protein was achieved using siGENOME SMART pool (Thermo Fisher Scientific). All effects of siRNA, as required. Atg7 and Beclin 1 knockdown protein was achieved using siGENOME SMART pool (Thermo Fisher Scientific). All effects of siRNA, as required. Atg7 and Beclin 1 knockdown protein was achieved using siGENOME SMART pool (Thermo Fisher Scientific). All effects of siRNA, as required. Atg7 and Beclin 1 knockdown protein was achieved using siGENOME SMART pool (Thermo Fisher Scientific). All effects of siRNA, as required. Atg7 and Beclin 1 knockdown protein was achieved using siGENOME SMART pool (Thermo Fisher Scientific). All effects of siRNA, as required. Atg7 and Beclin 1 knockdown protein was achieved using siGENOME SMART pool (Thermo Fisher Scientific). All effects of siRNA, as required. Atg7 and Beclin 1 knockdown protein was achieved using siGENOME SMART pool (Thermo Fisher Scientific). All effects of siRNA, as required. Atg7 and Beclin 1 knockdown protein was achieved using siGENOME SMART pool (Thermo Fisher Scientific). All effects of siRNA, as required. Atg7 and Beclin 1 knockdown protein was achieved using siGENOME SMART pool (Thermo Fisher Scientific). All effects of siRNA, as required. Atg7 and Beclin 1 knockdown protein was achieved using siGENOME SMART pool (Thermo Fisher Scientific). All effects of siRNA, as required. Atg7 and Beclin 1 knockdown protein was achieved using siGENOME SMART pool (Thermo Fisher Scientific). All effects of siRNA, as required. Atg7 and Beclin 1 knockdown protein was achieved using siGENOME SMART pool (Thermo Fisher Scientific). All effects of siRNA, as required. Atg7 and Beclin 1 knockdown protein was achieved using siGENOME SMART pool (Thermo Fisher Scientific). All effects of siRNA, as required. Atg7 and Beclin 1 knockdown protein was achieved using siGENOME SMART pool (Thermo Fisher Scientific). All effects of siRNA, as required.
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.

Online supplemental material
Fig. S1 shows that Atg7 and Beclin 1 knockdowns inhibit autophagy in U937 cells, as well as transmission and immunoelectron microscopy HIV profiles in macrophages. Fig. S2 shows a comparison of LC3 forms in whole cell lysate versus LC3 forms associated with membranes, that HIV profiles in macrophages. Fig. S2 shows a comparison of LC3 forms in U937 cells, as well as transmission and immunoelectron micrograph. Fig. S1 shows that Atg7 and Beclin 1 knockdowns inhibit autophagy in U937 cells, as well as transmission and immunoelectron micrograph. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200903070/DC1.

We thank N. Mizushima for Atg12 antibody. K. Collins for Nef clones, T. Howard and the electron microscopy facility for assistance, and M. Petelin for discussion. This work was supported by National Institute of Allergy and Infectious Diseases grants AI069345, AI52148, AI42999 to V. Deretic, AI06849 to L. Wu, and by amfAR grant 10160.44.52 and a Bill and Melinda Gates Foundation Grand Challenges Explorations grant 0652068 to V. Deretic. C. Dinkins was supported by National Institutes of Health Biology of Infectious Diseases and Inflammation training grant T32AI007538.

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References
Supplemental material

Kyei et al., http://www.jcb.org/cgi/content/full/jcb.200903070/DC1
Figure S1. **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 IFN-α). 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 B 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 membranous profile. This is in keeping with findings of HIV in membranous domains contiguous with the plasma membrane (Jouvenet, N., S.J. Neil, C. Bess, M.C. Johnson, C.A. Virgen, S.M. Simon, and P.D. Bieniasz. 2006. PloS Biol. 4:e435; Deneka, M., A. Pelchen-Matthews, R. Byland, E. Ruiz-Mateos, and M. Marsh. 2007. J. Cell Biol. 177:329–341; Welsch, S., O.T. Keppler, A. Habermann, I. Allespach, J. Krijnse-Locker, and H.G. Krausslich. 2007. PloS Pathog. 3:e36). (D) Immunoelectron micrographs of HIV-containing profiles labeled with p24 (enhanced immunogold).
Figure S2. 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 D. Note that the whole cell lysate displays both the unlipidated form LC3I and the lipidated form LC3II. Membrane fractions, identical to preparations shown in Fig. 2 D, display only LC3II. (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/m 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. (E) 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 cells were transfected with GFP-LC3 along with Nef-DsRed2 or DsRed2. The data in A are similar to those in Fig. 4 A, 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.
Figure S3. Inhibition of autophagic proteolysis by Nef, intracellular localization of Nef, and the role of Nef motif DD for Nef-dependent increase in LC3II levels. (A) Nef inhibits autophagic proteolysis. RAW264.7 macrophages, used as the only established autophagic proteolysis system in macrophages (Roberts, E.A., and V. Deretic. 2008. Methods Mol. Biol. 445:111–117), 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 [3H]leucine measured and expressed as the percentage of radiolabeled leucine released; n = 3. Data indicate means; error bars indicate ±SEM. *, P < 0.05. (Materials and methods) RAW264.7 macrophages...
were transfected with DsRed2 and Nef-DsRed2 plasmids, seeded at 7 × 10^4 cells/well, and then incubated for 24 h in DME with [3H]leucine as described previously (Roberts and Deretic, 2008). Cells were washed and incubated in unlabeled DME to allow degradation of short-lived proteins as described previously (Roberts and Deretic, 2008). Cells were then washed three times with 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 (Mizushima, N., A. Yamamoto, M. Hatano, Y. Kobayashi, Y. Kabeya, K. Suzuki, T. Tokuhisa, Y. Ohsumi, and T. Yoshimori. 2001. J. Cell Biol. 152:657–668.). Baseline degradation of stable proteins in non-liver 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 NefDsRed2 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 [Kim, J.E., and J. Chen. 2000. J. Biol. Chem. 275:23097–23105]). (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 17DD175 is but G3 motif is not required for a Nef-dependent increase in LC3II levels. 293T cells were transfected with the wild-type HIV-1 Nef fusion with GFP or the mutants GΦA/NefGΦ and VΦA/NefVΦ for 48 h, and LC3II 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. 7 B. 

Table S1. HIV molecular clones, viruses, viral preparations, and use

<table>
<thead>
<tr>
<th>Construct</th>
<th>Reference or source</th>
<th>Comment</th>
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<tbody>
<tr>
<td>pMSMBMA</td>
<td>Callahan et al., 1998</td>
<td>Same as NL4-3env</td>
</tr>
<tr>
<td>pMSMAB-vpu-null</td>
<td>McBride and Panganiban, 1996</td>
<td>Same as NL4-3envΔvpu</td>
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<td>HIVANef</td>
<td>Valentin et al., 1998</td>
<td>NL4-3ΔenvANef–OFF replaces Nef in this clone</td>
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<tr>
<td>HIV-1ΔOD8</td>
<td>E. Freed, National Cancer Institute</td>
<td>NA</td>
</tr>
<tr>
<td>SF162</td>
<td>Pelchen-Matthews et al., 2003</td>
<td>Full-length HIV virus</td>
</tr>
<tr>
<td>HIV 1ΔL</td>
<td>Barre-Sinoussi et al., 1983; Nguyen et al., 1994</td>
<td>CXCR4 co-receptor specific (T cell-tropic)</td>
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</table>

NA, not applicable.


6Virus generation and isolation. Replication-competent HIV-1ΔOD8 was generated by transfections of HEK293T cells with the proviral construct pNLAD8. Preparations of pNLAD8 and HIV−Nef−null virus (pseudotyped with the envelope glycoprotein of the VSVG) were obtained as cleared supernatants of 293T cells 48 h after calcium phosphate cotransfection; viral infections were carried out as described previously [Olivetta, E., and M. Federico. 2006. J. Virol. 80:4946–4954]. Replication-competent HIV−Nef−null virus (pseudotyped with the envelope glycoprotein of the VSVG) was obtained as cleared supernatants of 293T cells 48 h after calcium phosphate cotransfection; viral infections were carried out as described previously [Olivetta, E., and M. Federico. 2006. J. Virol. 80:4946–4954]. Replication-competent HIV−Nef−null virus (pseudotyped with the envelope glycoprotein of the VSVG) was obtained as cleared supernatants of 293T cells 48 h after calcium phosphate cotransfection; viral infections were carried out as described previously [Olivetta, E., and M. Federico. 2006. J. Virol. 80:4946–4954]. Replication-competent HIV−Nef−null virus (pseudotyped with the envelope glycoprotein of the VSVG) was obtained as cleared supernatants of 293T cells 48 h after calcium phosphate cotransfection; viral infections were carried out as described previously [Olivetta, E., and M. Federico. 2006. J. Virol. 80:4946–4954]. Replication-competent HIV−Nef−null virus (pseudotyped with the envelope glycoprotein of the VSVG) was obtained as cleared supernatants of 293T cells 48 h after calcium phosphate cotransfection; viral infections were carried out as described previously [Olivetta, E., and M. Federico. 2006. J. Virol. 80:4946–4954]. Replication-competent HIV−Nef−null virus (pseudotyped with the envelope glycoprotein of the VSVG) was obtained as cleared supernatants of 293T cells 48 h after calcium phosphate cotransfection; viral infections were carried out as described previously [Olivetta, E., and M. Federico. 2006. J. Virol. 80:4946–4954]. Replication-competent HIV−Nef−null virus (pseudotyped with the envelope glycoprotein of the VSVG) was obtained as cleared supernatants of 293T cells 48 h after calcium phosphate cotransfection; viral infections were carried out as described previously [Olivetta, E., and M. Federico. 2006. J. Virol. 80:4946–4954]. Replication-competent HIV−Nef−null virus (pseudotyped with the envelope glycoprotein of the VSVG) was obtained as cleared supernatants of 293T cells 48 h after calcium phosphate cotransfection; viral infections were carried out as described previously [Olivetta, E., and M. Federico. 2006. J. Virol. 80:4946–4954].