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

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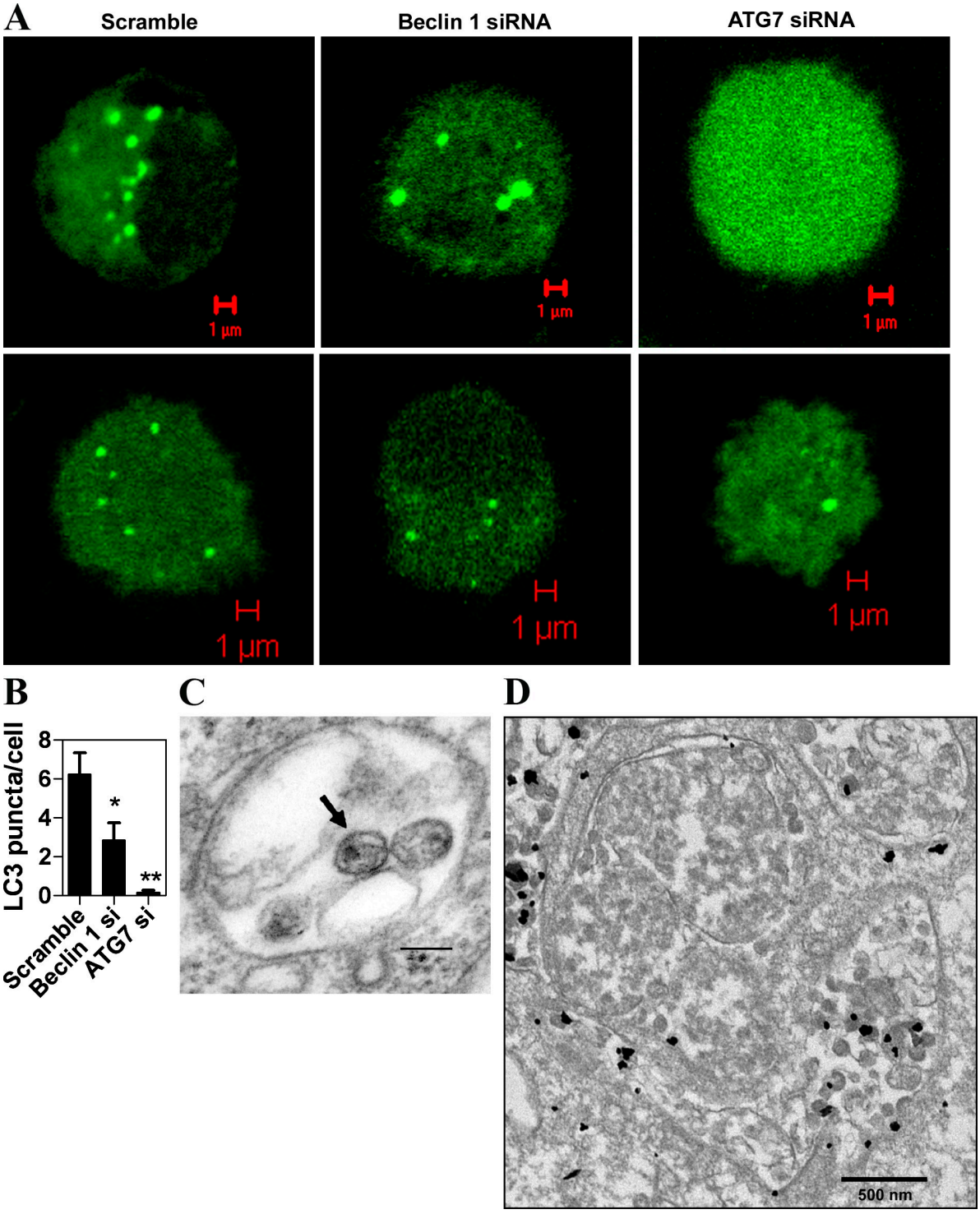

Figure S1. **Diminished autophagy in cells subjected to Atg7 and Beclin 1 knockdowns and transmission, and immunoelectron microscopy analysis of HIV profiles in macrophages.** (A and B) Atg7 and Beclin 1 knockdowns inhibit autophagy in U937 cells. U937 cells were cotransfected with GFP-LC3 and scrambled 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-γ). 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).

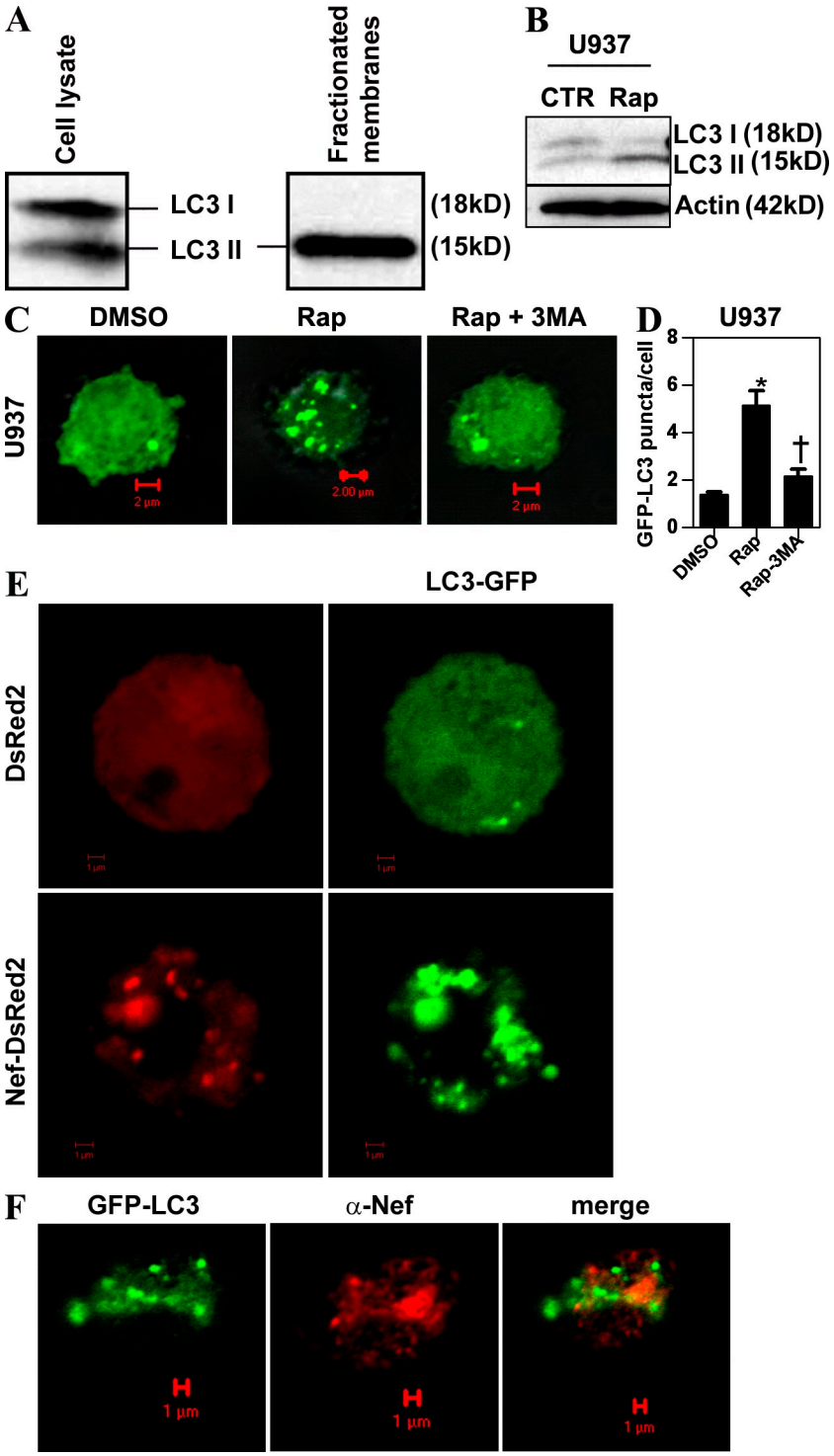

Figure S2. **Comparison of LC3 forms in whole cell lysate versus LC forms associated with membranes, control for autophagy induction in cells during acute rapamycin treatment, and effects of Nef on accumulation of LC3 puncta.** (A) Comparison of LC3 forms in whole cell lysate versus LC forms associated 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. (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 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 transected with Nef-Myc.

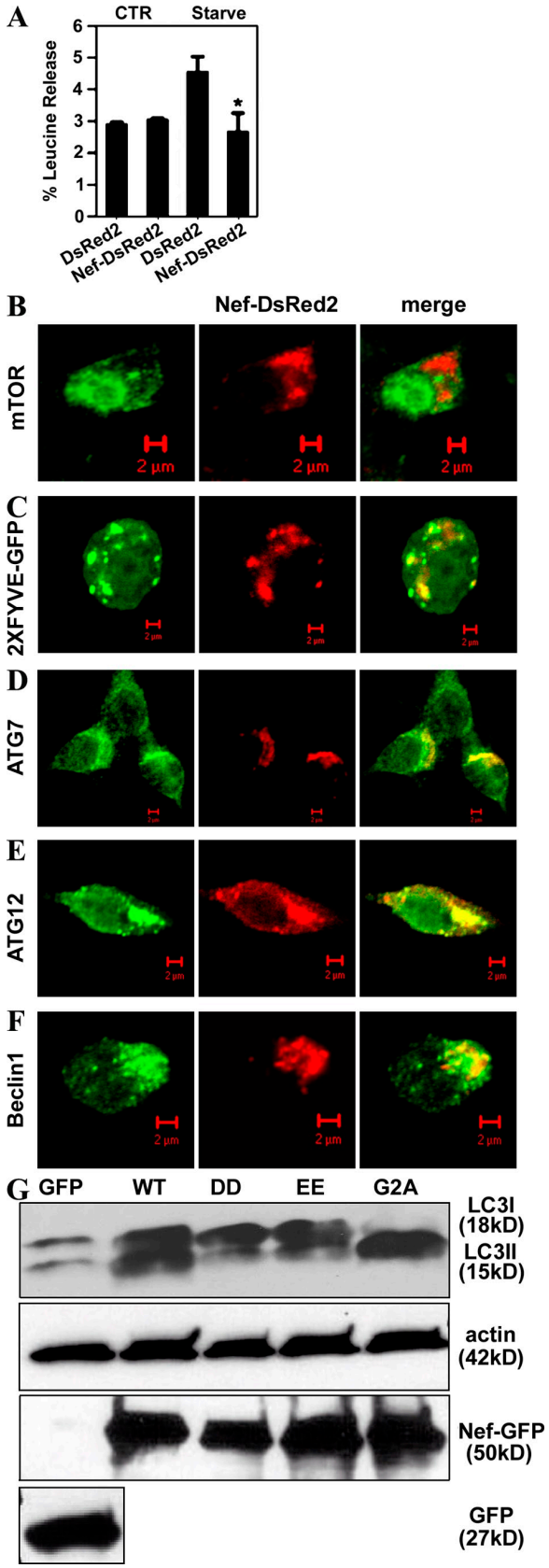

Figure S3. **Inhibition of autophagic proteolysis by Nef, intracellular localization of Nef, and the role of Nef motif ^{174}DD^{175} for Nef-dependent increase in LC3II levels.** (A) Nef inhibits autophagic proteolysis. RAW264.7 macrophages, used as the only established autophagic proteolysis system 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 [^{3}H]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

Table S1. **HIV molecular clones, viruses[a], viral preparations[b], and use[c]**

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

NA, not applicable.

[a]HIV clones and viruses (Barre-Sinoussi, F., J.C. Chermann, F. Rey, M.T. Nugeyre, S. Chamaret, J. Gruest, C. Dauguet, C. Axler-Blin, F. Vezinet-Brun, C. Rouzioux, et al. 1983. *Science.* 220:868–871; Nguyen, M.H., R.F. Schinazi, C. Shi, N.M. Goudgaon, P.M. McKenna, and J.W. Mellors. 1994. *Antimicrob. Agents Chemother.* 38:2409–2414). The HIV-1 NL4-3 molecular clones pMSMBA and its derivative pMSMBA-Vpu-null have been described previously (Callahan, M.A., M.A. Handley, Y.H. Lee, K.J. Talbot, J.W. Harper, and A.T. Panganiban. 1998. *J. Virol.* 72:5189–5197; McBride, M.S., and A.T. Panganiban. 1996. *J. Virol.* 70:2963–2973). GFP-HIV-Nef-null has been described previously (Valentin, A., W. Lu, M. Rosati, R. Schneider, J. Albert, A. Karlsson, and G.N. Pavlakis. 1998. *Proc. Natl. Acad. Sci. USA.* 95:8886–8891). DsRed2 and Nef-DsRed2 (from K. Collins, University of Michigan, Ann Arbor, MI) are derivatives of DsRed2 (Clontech Laboratories, Inc.) with reduced spontaneous aggregation, and have been described previously (Williams, M., J.F. Roeth, M.R. Kasper, R.I. Fleis, C.G. Przybycin, and K.L. Collins. 2002. *J. Virol.* 76:12173–12184). The intracellular localization of Nef-DsRed2 is similar to that shown for Nef-GFP (Cohen, G.B., V.S. Rangan, B.K. Chen, S. Smith, and D. Baltimore. 2000. *J. Biol. Chem.* 275:23097–23105) or immunostained Nef (Stumptner-Cuvelette, P., M. Jouve, J. Helft, M. Dugast, A.S. Glouzman, K. Jooss, G. Raposo, and P. Benaroch. 2003. *Mol. Biol. Cell.* 14:4857–4870); Nef-DsRed2 shows biological properties indistinguishable from untagged Nef when tested for effects on MHC class I (Williams et al., 2002). Other plasmids were p2xFYVE-EGFP from H. Stenmark (The Norwegian Radium Hospital, Oslo, Norway), pEGFP-LC3, and tandem RFG-GFP-LC3 from T. Yoshimori (Osaka University, Japan). Nef-GFP and associated mutants are derivatives of pcNEFsg25GFP. SF162 macrophage-tropic virus (Pelchen-Matthews, A., B. Kramer, and M. Marsh. 2003. *J. Cell Biol.* 162:443–455) 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 (Olivetta, E., and M. Federico. 2006. *Exp. Cell Res.* 312:890–900). 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.

were transfected with DsRed2 and Nef-DsRed2 plasmids, seeded at 7×10^4 cells/well, and then incubated for 24 h in DME with [^{3}H]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 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 (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 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 [Kim, J.E., and J. Chen. 2000. *Proc. Natl. Acad. Sci. USA.* 97:14340–14345; Bernardi, R., I. Guernah, D. Jin, S. Grisendi, A. Alimonti, J. Teruya-Feldstein, C. Cordon-Cardo, M.C. Simon, S. Rafii, and P.P. Pandolfi. 2006. *Nature.* 442:779–785], shows significant nuclear localization, augmented upon Tor inhibition under certain conditions [Bernardi et al., 2006]). (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 LC3II 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 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.