Supporting Information

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SI Text

Antibodies. Polyclonal anti-Hsp90 (H114) was from Santa Cruz Biotechnology, monoclonal anti-α-Tubulin (DM1a) was from Sigma, hybridoma anti-p24 Gag (183-H12–5C) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. Monoclonal anti-GFP (7.1/13.1) was from Roche. Polyclonal anti-Cep55 (A01) was from Abnova. Polyclonal anti-Alix was a kind gift from W. Sundquist (University of Utah, Salt Lake City). Alexa- and HRP-conjugated secondary antibodies were from Molecular Probes and Cell Signaling Technology, respectively. For infrared imaging, Alexa-conjugated secondary antibodies were from Licor, and membranes were imaged and quantified by using an Odyssey imaging system (Licor).

Plasmids. pCR3.1 mCh-Cep55 has been described (1) and was additionally cloned into pCAGGS/GST. siRNA-resistant versions of Alix ($Alix^R$) were created through the introduction of silent mutations K96K, D97D, A98A, F99F, D100D, K101K. Mutagenized versions of $Alix^R$ were created through standard PCR-based methodologies. Specifically, we mutated both F199D and I212D in Alix's BroI domain to prevent interaction with the ESCRT-III subunits Chmp4a, Chmp4b, and Chmp4c (2, 3). We also mutated F676D to prevent Alix-binding viral Gag L domains (2, 4), disrupted the Tsg101-binding PSAP motif (5) through mutation of P717A, S718A, and P720A, mutated P744V and R745D to stop binding to CD2AP and Cin85 (3), and abolished binding to the family of endophilins by introducing R757G, P758A, and P759A (2). All other mutations and deletions described were created by standard PCR methodologies and cloned EcoRI-NotI into necessary vectors. $Alix^R$ δPRR and 1–863 were additionally amplified with C-terminal SalI-NotI sequences. Minimal leucine zipper sequences from GCN4 were amplified as described (7) and cloned SalI/NotI into these vectors. Oligonucleotides encoding Alix residues 794–813 were synthesised with SalI and NotI flanking sequences and cloned SalI/NotI into $Alix^R$ δPRR to create AlixR δPRR + Peptide. All other constructs have been described (6) and, where necessary, were subcloned as required. Chmp4 nomenclature follows that of Katoh *et al.* (8).

Transfections. HeLa cells were transfected with Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. The 293T cells were transfected by using linear 25-kDa polyethylenimine (PEI; Polysciences) by combining 1 μg of plasmid DNA with 4 μg of PEI in 50 μl of serum free DMEM for 15 min before addition to cells.

Generation of Stable Cell Lines. The retroviral packing vector pCMS28-mCh has been described (1), and siRNA-resistant versions of Alix were cloned into this vector. The 293T cells were transfected with 100 ng of pHIT-VSVG, 700 ng of MLV-GagPol, and 200 ng of pCMS28-mCh constructs for 48 h. The 293T supernatants were filtered (0.2 μm) and used to transduce HeLa cells. Selection with puromycin (200 ng/ml) was applied 24 h later. Clonal HeLa pMSCVneoYFP-Cep55 cell lines have been described (1).

Imaging. HeLa cells were seeded onto glass coverslips 24 h beforehand and were fixed with 3% paraformaldehyde for 15 min. Cells were permeabilized with PBS 0.1% Tx100 for 5 min and treated sequentially with PBS 100 mM glycine for 30 min, PBS 1% BSA for 30 min and then stained with primary antibodies in PBS 1% BSA for 2 h. $Alexa^{488}$-, $Alexa^{594}$-, or $Alexa^{633}$-conjugated secondary antibodies were applied in PBS for 1 h. Nuclei were visualized by using Hoechst 33258, and coverslips were mounted in Mowiol. Slides were imaged by using a magnification ×63, 1.4-N.A. oil-immersion objective connected to a Leica AOBS SP2 confocal microscope. The AOTF was used to collect relevant narrow emission-λ windows for each flurophore. Greyscale images were pseudocolored and combined in Adobe Photoshop to create the merged images.

Yeast Two-Hybrid Assays. Yeast Y190 cells were cotransformed with plasmids encoding the indicated proteins fused to the VP16 activation domain (pHB18) or the Gal4 DNA-binding domain (pGBKT7). Cotransformants were selected on SD-Leu-Trp agar for 3 days at 30°C and harvested, and *LacZ* activity was measured by using a liquid β-galactosidase assay employing chlorophenol-red-β-D-galactopyranoside (Roche) as a substrate.

Coprecipitation Assays. 293T cells were transfected with plasmids encoding the indicated pCAGGS/GST fusion proteins and pCR3.1-YFP fusion proteins for 48 h. pCR3.1 Myc-Vps28 was included to stabilize YFP-Tsg101 expression if required. Cells were lysed in lysis buffer [50 mM Tris·HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 5% glycerol, 1% Triton X-100, and a protease inhibitor mixture (complete mini EDTA-free; Roche Applied Science)]. Clarified lysates were incubated with glutathione-Sepharose beads (Amersham Biosciences) for 3 h at 4°C and washed three times with wash buffer [50 mM Tris·HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 5% glycerol, 0.1% Triton X-100). Bead-bound proteins were resolved by SDS/PAGE and examined by Western blot analysis or Coomassie staining as described.

1. Carlton JG, Martin-Serrano J (2007) Parallels between cytokinesis and retroviral budding: A role for the ESCRT machinery. *Science* 316:1908–1912.
2. Fisher RD, *et al.* (2007) Structural and biochemical studies of ALIX/AIP1 and its role in retrovirus budding. *Cell* 128:841–852.
3. Usami Y, Popov S, Göttlinger HG (2007) Potent rescue of human immunodeficiency virus type 1 late domain mutants by ALIX/AIP1 depends on its CHMP4 binding site. *J Virol* 81:6614–6622.
4. Lee S, Joshi A, Nagashima K, Freed EO, Hurley JH (2007) Structural basis for viral late-domain binding to Alix. *Nat Struct Mol Biol* 14:194–199.
5. von Schwedler UK, *et al.* (2003) The protein network of HIV budding. *Cell* 114:701–13.
6. Martin-Serrano J, Yarovoy A, Perez-Caballero D, Bieniasz PD (2003) Divergent retroviral late-budding domains recruit vacuolar protein sorting factors by using alternative adaptor proteins. *Proc Natl Acad Sci USA* 100:12414–12419.
7. Accola MA, Strack B, Göttlinger HG (2000) Efficient particle production by minimal Gag constructs which retain the carboxy-terminal domain of human immunodeficiency virus type 1 capsid-p2 and a late assembly domain. *J Virol* 74:5395–5402.
8. Katoh K, *et al.* (2003) The ALG-2-interacting protein Alix associates with CHMP4b, a human homologue of yeast Snf7 that is involved in multivesicular body sorting. *J Biol Chem* 278:39104–39113.

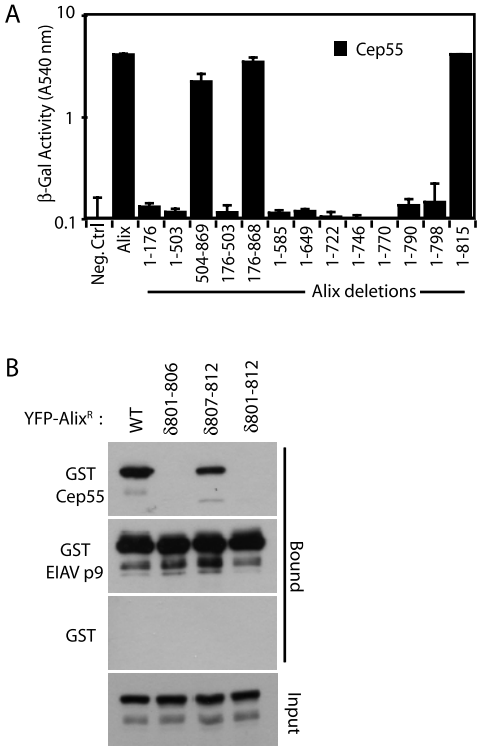

Fig. S1. Mapping the interaction of Cep55 with Alix. (*A*) Cep55 was fused to the VP16 activation domain and tested for interaction with the indicated Alix constructs fused to Gal4 DNA-binding domain by yeast two-hybrid assay ($n = 3 \pm$SD). (*B*) 293T cells were transfected with plasmids encoding the indicated GST and YFP fusion proteins. Cell lysates and glutathione-bound fractions were analyzed with α-GFP antisera.

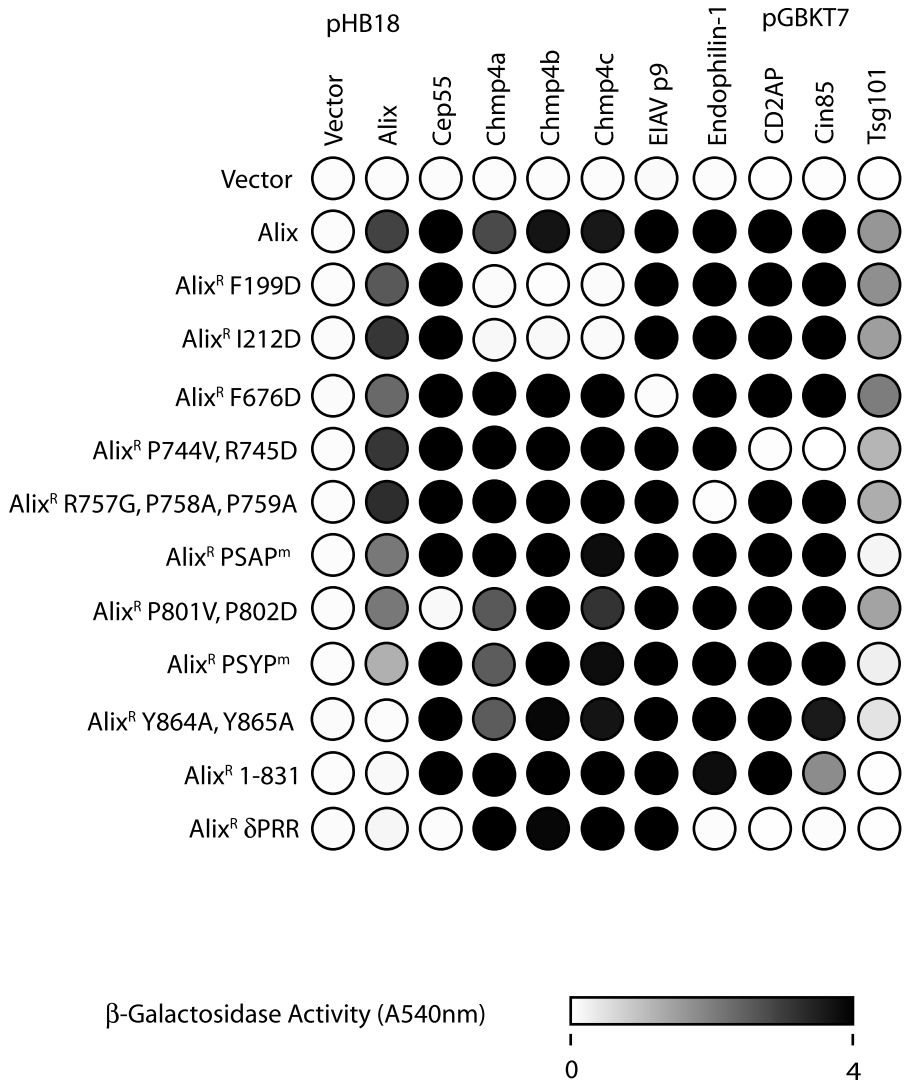

Fig. S2. Generation of a panel of Alix mutants. Alix and mutant forms of Alix^R were tested for interaction with the indicated Alix-binding proteins by yeast two-hybrid assay (mean of $n = 3$). Alix, Cep55, Chmp4a, Chmp4b, Chmp4c, EIAVp9, and Endophilin-1 were fused to the VP16 activation domain. Alix, CD2AP, Cin85, and Tsg101 were fused to the Gal4 DNA-binding domain. Both sets of constructs were tested for interaction with Alix or mutant versions of Alix^R fused to the Gal4 DNA-binding domain or the VP16 activation domain as required.

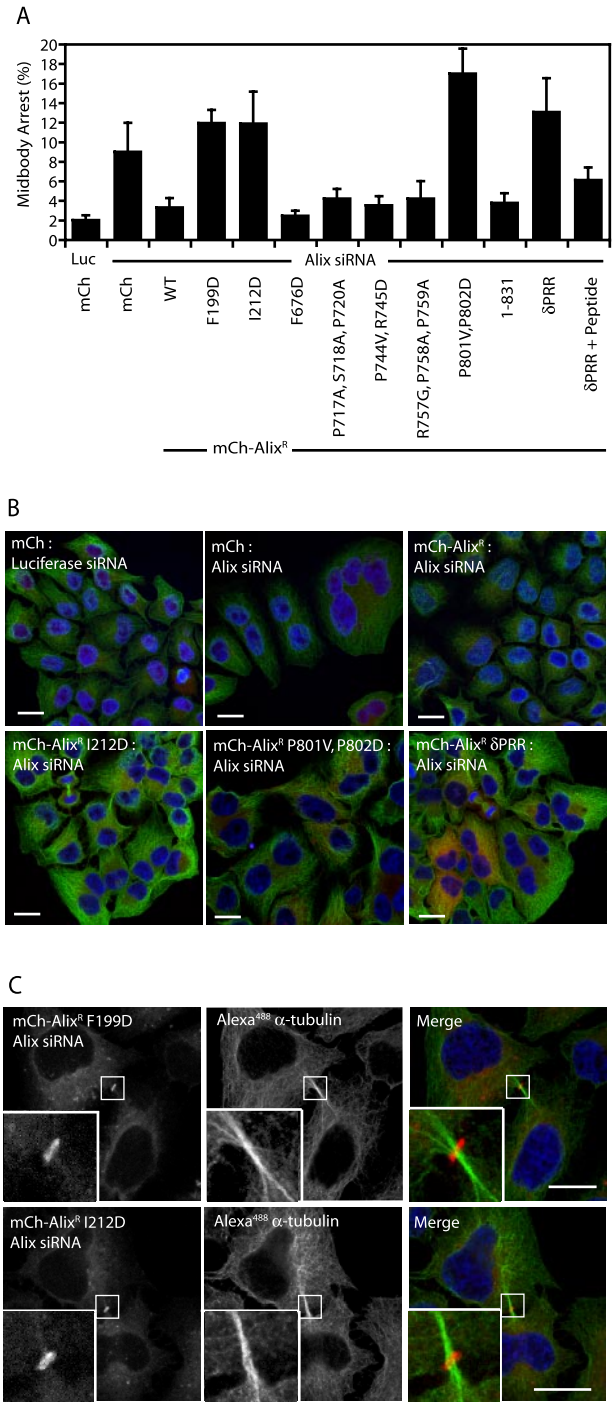

Fig. S3. Requirements of Alix interaction partners in cytokinesis. (*A*) Fixed cells from Fig. 2 were scored for midbody-arrest ($n > 4$ ±SD). (*B*) Representative micrographs of multinucleated cells from Fig. 2*A*., (Scale bars: 10 μm.) (*C*) Representative micrographs of mCh-Alix^R-F199D and I212D recruitment to the midbody in Alix-siRNA-treated cells. (Scale bars: 10 μm.)

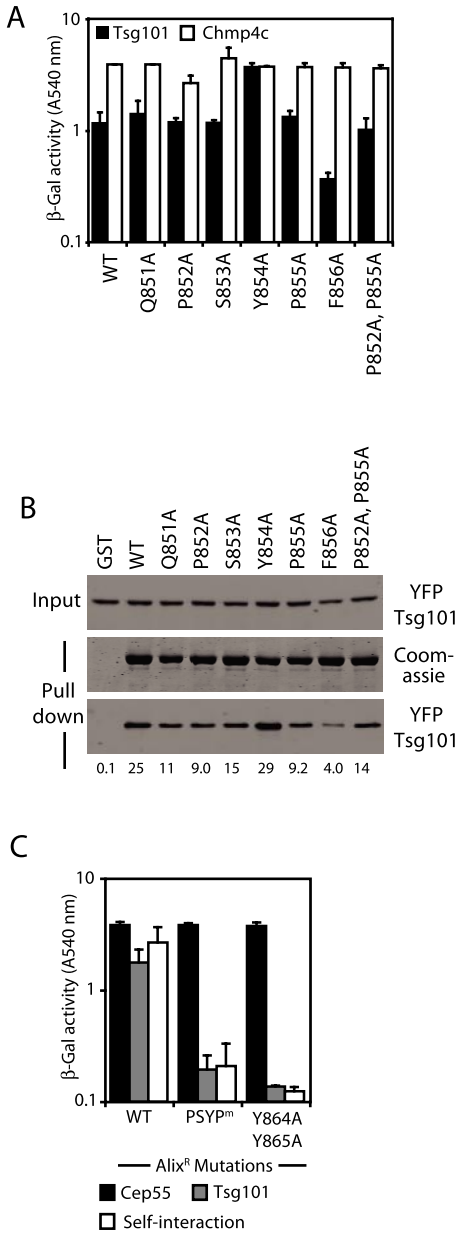

Fig. S4. The PSYP sequence does not bind directly to Tsg101 but is required for multimerization. (*A*) The indicated Alix^R mutants were fused to the VP16 activation domain and tested for interaction with Tsg101 or Chmp4c fused to the Gal4 DNA-binding domain by yeast two-hybrid assay ($n = 3 \pm$SD). (*B*) 293T cells were transfected with the pCR3.1 myc-Vps28, pCR3.1 YFP-Tsg101, and the indicated pCAGGST-Alix^R constructs. Resolved cells lysates and bead-bound fractions were analyzed by Western-blotting with α-GFP antisera. Bead-bound fractions were also analyzed by Coomassie staining to visualize GST-Alix^R expression, and relative binding was quantified by infrared imaging. (*C*) The indicated Alix^R mutations were fused to the VP16 activation domain and tested for autointeraction or interaction with Tsg101 or Cep55 fused to the Gal4 DNA-binding domain by yeast two-hybrid assay ($n = 3 \pm$SD).

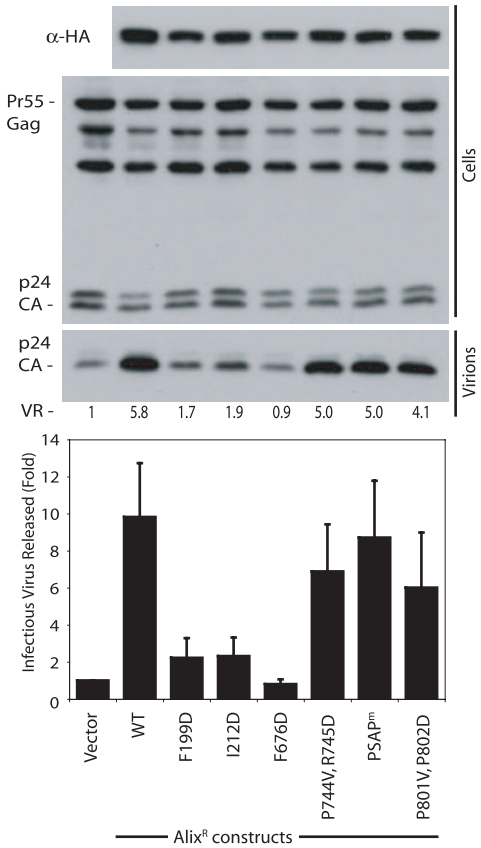

Fig. S5. Mutation of the Cep55-binding site on Alix does not prevent it from supporting HIV-1 release. 293T cells were cotransfected with the indicated Alix expression constructs and HIV-1 p6 (P7L, P10L) for 24 h. Resolved cell lysates and released virions were examined by Western blot analysis with α-Gag antisera. Virion release (VR) was quantified by using an infrared imaging system. β-Galactosidase assay was performed on HeLa-TZM-bl cells infected with 293T supernatant ($n = 3 \pm$SD).

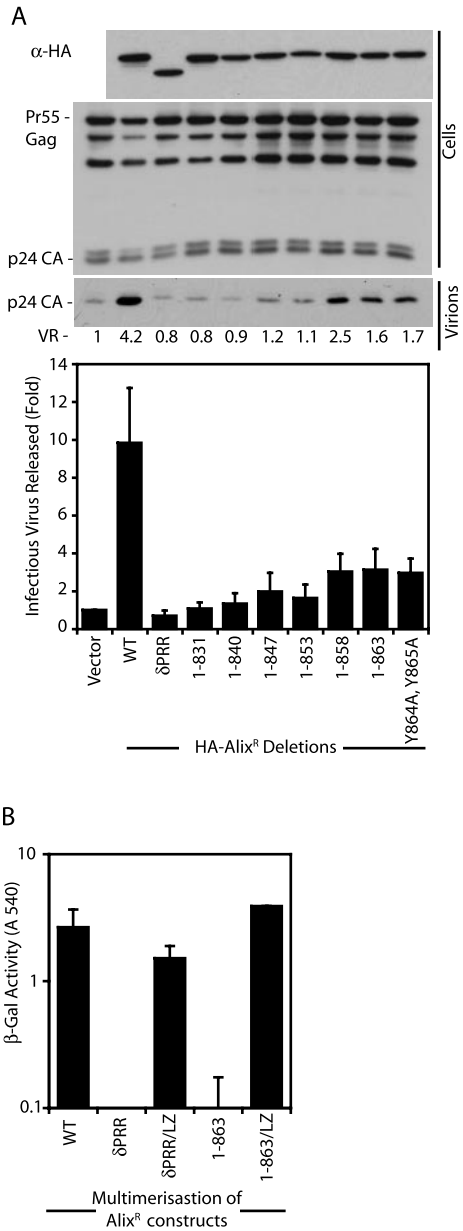

Fig. S6. Deletion analysis of Alix-dependent HIV-1 release. (*A*) 293T cells were cotransfected with plasmids encoding the indicated HA-tagged Alix^R constructs and an HIV-1 pNL/HXB p6(P7L, P10L) plasmid. Resolved cell lysates and released virions were examined by Western blott analysis with α-Gag antisera. Cell lysates were examined by Western blot analysis with α-HA antisera. Virion release (VR) was quantified using an infrared imaging system. β-Galactosidase assay was performed on HeLa-TZM-bl cells infected with 293T supernatant ($n = 3 \pm$SD). (*B*) The indicated Alix constructs were fused to both the VP16 activation domain and Gal4 DNA-binding domain and tested for self-interaction by yeast two-hybrid assay ($n = 3 \pm$SD).

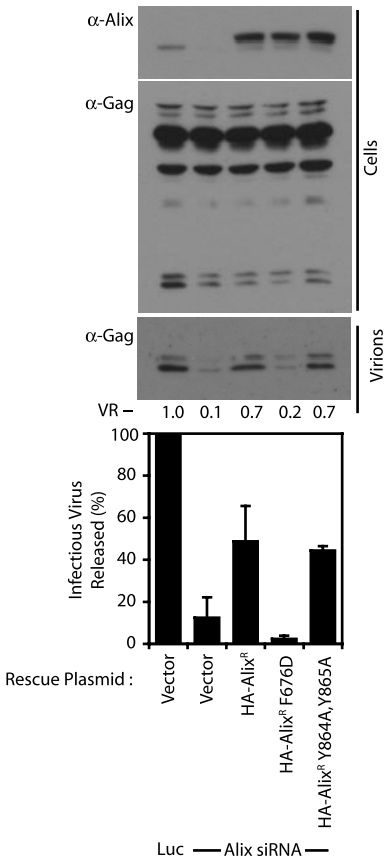

Fig. S7. Alix multimerization is not required for EIAVp9-dependent viral budding. 293T cells were treated with Luciferase or Alix-targeting siRNA and cotransfected with plasmids encoding pNL/HXB δp6, a truncated HIV-1 pNL/HXB Gag protein fused to the EIAVp9 late domain, and the indicated HA-Alix^R constructs. Resolved cell lysates and released virions were examined by Western blot analysis with α-Gag antisera. Quantification of virion release (VR) via densitometry (NIH Image) is given. Cell lysates were examined by Western blotting analysis with α-Alix antisera. β-Galactosidase assay was performed on HeLa-TZM-bl cells infected with 293T supernatant ($n = 3$ ±SD).