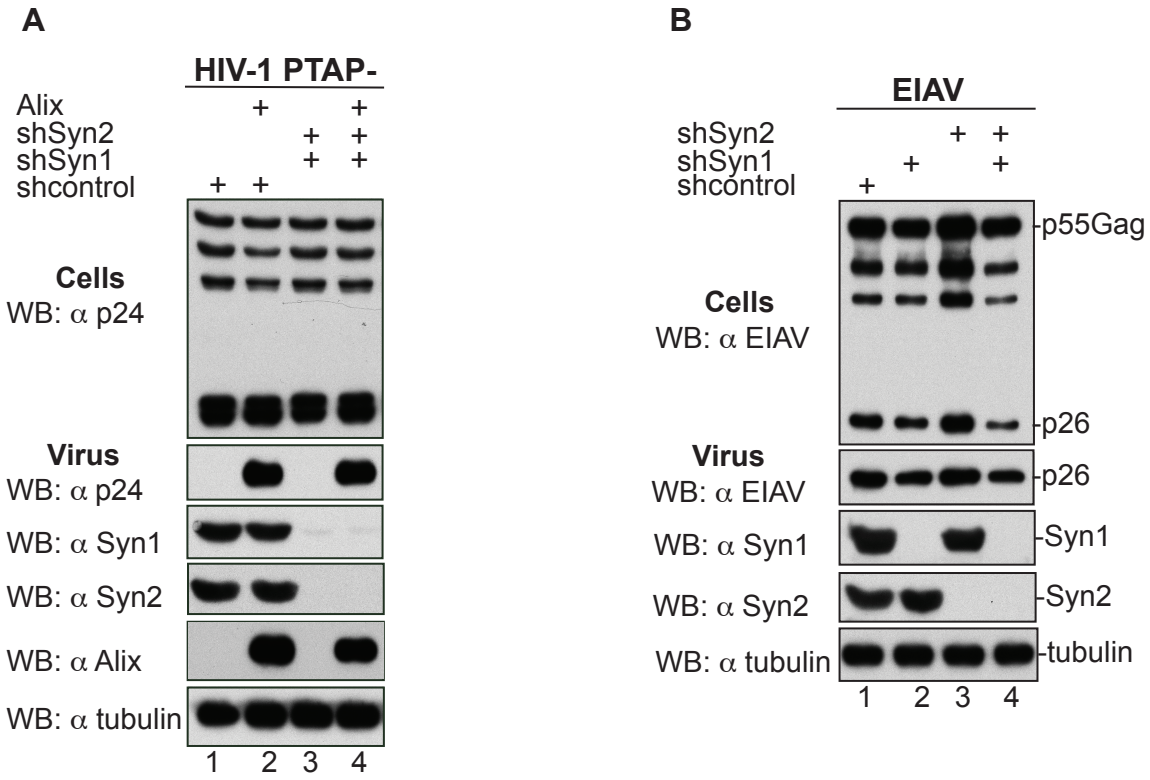
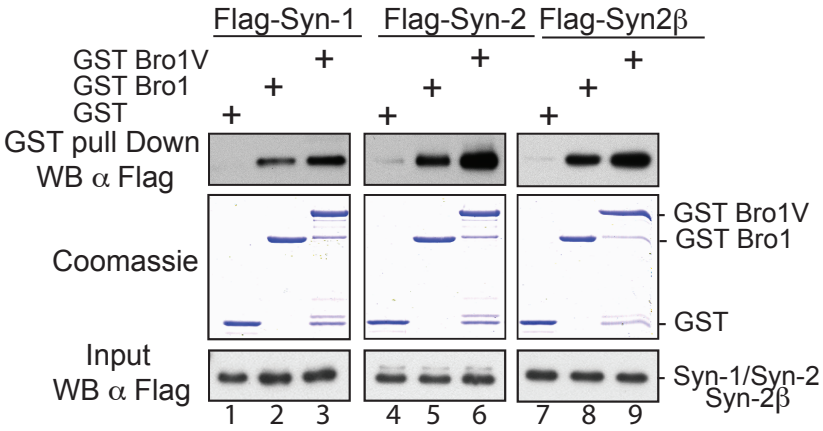
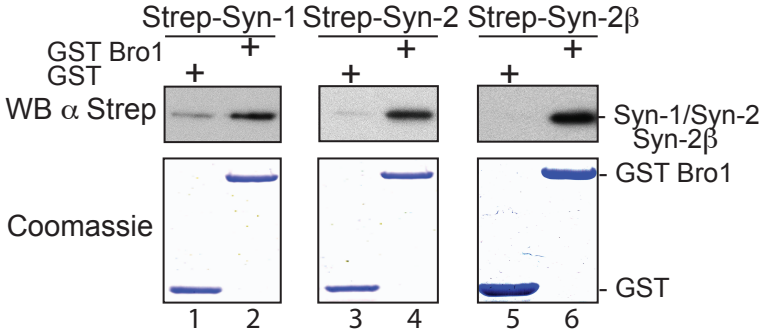


Figure S1 Sette et al. (related to Figure 1)

A



B



C

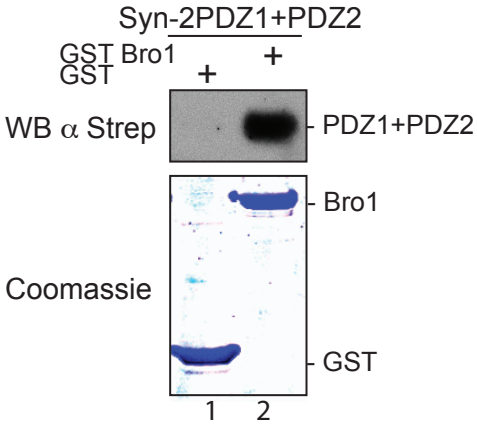


Figure S2 Sette et al. (related to Figure 1)

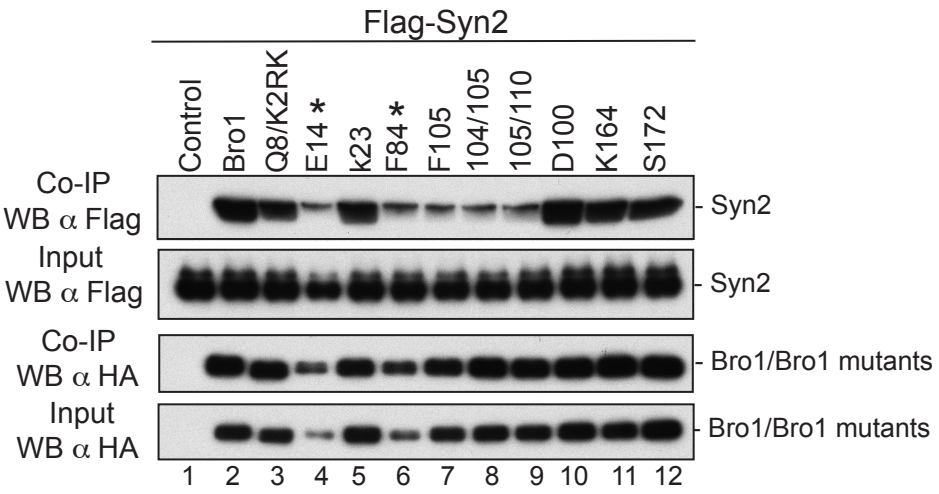


Figure S3 Sette et al. (related to Figure 2)

Figure legends of supplemental figures S1, S2 and S3:

Figure S1, related to Figure 1: shRNA silencing of endogenous syntenin isoforms had no effect on virus budding. **(A)** 293T cells were treated with control shRNA (lanes 1 and 2) or with shRNA targeting Syn-1 and Syn 2 (lanes 3 and 4). After 6 days of treatment with puromycin to select cells expressing syntenin shRNAs, cells were transfected with HIV-1 PTAP- alone (lanes 1 and 3) or in combination with WT HA-ALIX--to stimulate virus release (lanes 2 and 4). **(B)** Cellular syntenin proteins are dispensable for EIAV release. 293T cells were treated with control shRNA (lane 1), with shRNA against Syn-1 or Syn-2 individually or together (lanes 2, 3 and 4, respectively). After 6 days puromycin treatment, cells were transfected with EIAV proviral DNA and harvested 24-48h post-transfection. Protein content of cells and virus was analyzed as previously described and proteins were detected by western blot using the indicated antibodies.

Figure S2, related to Figure 1: (A) Syn-1, Syn-2 and Syn-2β retain binding to ALIX Bro1 and Bro1-V domains. GST (lanes 1, 4 and 7), GST-Bro1 (lanes 2, 5 and 8) and GST-Bro1-V fusion proteins (lanes 3, 6 and 9) were captured on Glutathione conjugated beads and then incubated with lysates from 293T cells expressing Flag-Syn-1 (lanes 1-3), Flag-Syn-2 (lanes 4-6) or Flag-Syn-2β (lanes 6-9). Captured proteins and cell lysates were analyzed by western blot using the anti-Flag antibody. GST fusion proteins were visualized by Coomassie blue staining. **(B)** Purified syntenin proteins interact with ALIX Bro1. GST (lanes 1, 3 and 5) and GST-Bro1 fusion proteins (lanes 2, 4 and 6) captured on beads were incubated with purified strep-tagged version of syntenin proteins. The captured complexes were analyzed by western blot using an anti-strep antibody. **(C)** Purified syntenin PDZ domains bind ALIX Bro1. GST (lane 1) or GST-Bro1 fusion protein (lane 2) were captured on Glutathione conjugated beads and then incubated with strep-tagged version of recombinant Syn-2 PDZ domains purified from *E.coli* (lane 2). Captured proteins were analyzed by western blot using anti-strep antibodies. GST fusion proteins were visualized by Coomassie blue staining.

Figure S3, related to Figure 2: The Phe105 loop plays key role(s) in ALIX Bro1-Syntenin interactions. 293T cells were transfected with Flag-Syn 2 alone (lane 1), in combination with wild type HA-Bro1 (lane 2) or the indicated Bro1 mutants (lanes 3 to 12—see material and methods for description). Cells were lysed in RIPA buffer and clear lysates were incubated with anti-HA antibody-conjugated beads. Both input and immunoprecipitated complexes were analyzed by SDS-PAGE and western blot using the anti-Flag or anti-HA antibodies. Mutants marked with the (*) symbol exhibited low expression levels in cells and were therefore excluded from further analysis (lanes 4 and 6).

Supplemental Experimental Procedures:

Proviral and expression vectors: Syntenin-1 and Syntenin-2 expression genes were amplified by PCR from cDNAs (Open Biosystems). Syntenin-2β cDNA, which lacks the first 255 bp of syntenin-2, syntenin-1 and syntenin-2 PDZ domains (amino acids 114 to 271 and 103 to 267, respectively) were amplified from full-length cDNA and all sequences were subcloned into p3XFLAG-myc-CMV-26 vector (Sigma) between *NotI/BglII* sites to obtain the N-terminally 3XFLAG tagged protein expression plasmids. A similar strategy was followed to clone syn-2 PDZ1 (108-187) or PDZ2 (192-267). PDZ1 and PDZ2 domains of syntenin-1 and syntenin-2 and the full-length version of both proteins were also cloned in a modified pET30a(+) vector (EMD) between the SalI/NotI sites. The expressed proteins carried a N-terminal GB1 soluble tag, a Strep tag, a 6 × Histidine tag followed by a cleavage site for the tobacco etch virus (TEV) protease. The HA-tagged version of ALIX full length, ALIX-105 mutant, the Bro1 (1–367) domain and the Bro1-105 mutant as well as all other Bro1 mutants shown in figure S3 were described previously in (Sette et al., 2012; Sette et al., 2011a). To generate GST expression vectors, the ALIX Bro1 and ALIX Bro1-V (1- 702) domains were subcloned in pGEX-5X-2 (GE) between (Sette et al., 2011a) *EcoRI*/*NotI* sites. We used the wild-type (WT) $EIAV_{UK}$, the L-Domain singly defective HIV-1 mutant $PTAP^{-}$, or the doubly defective PY- (where both the PTAP and the LYPXnL were mutated; proviral constructs were previously described in (Huang et al., 1995). When immunogold labeling was used, viral particles were produced using the HIV-1 proviral vector pBruΔenv, or the GagΔp2-NCp1-p6 and GagΔp1-p6 mutants previously described in (Reed et al., 2012). RKI and RKII mutants were generated following a strategy previously described in Dussupt et al., 2011; overlapping PCR was used to substitute a select number of R or K to A residues in either NC proximal (RKI) or distal (RKII) zinc finger as depicted in figure 3B.

Immunoprecipitation assays: Immunoprecipitation assays were conducted as previously described (Sette et al., 2011b). Briefly, 293T cells (2×10^6 cells/T25) were seeded into T25 flasks and transfected the following day using Lipofectamine 2000. Forty-eight hours post-transfection, the cells were lysed in Lysis buffer (1% (v/v) Igepal, 0.1% (w/v) SDS, 0.5% (w/v) Sodium deoxycholate and Complete protease inhibitor cocktail [Roche, Indianapolis, IN]). Immunoprecipitation complexes and cell lysates (input fractions) were analyzed by SDS-PAGE and western blot using anti-HA, anti-Flag M2 (Sigma), anti-syntenin-1 (S-31, Santa Cruz Biotechnology) and anti-syntenin-2 (Proteintech) antibodies.

Protein expression and purification: Syntenin-1, syntenin-2 and syntenin-2β were purified as previously described (Sette et al., 2011b). The expression vectors were transformed into *E. coli*

BL21 (DE3) cells and protein expression was induced with 0.5 mM isopropyl-β-d-thiogalactoside (IPTG) at OD600 = 0.6–0.8 and grew overnight at 18°C. Cells were lysed in a buffer containing 50 mM Tris-HCl pH8.0, 1% sucrose, 10% Glycerol, 0.1 mM DTT, 500 mM NaCl, 10 mM imidazole and protease inhibitor cocktail and lysed by sonication. Cleared cell lysates were purified by Ni-NTA Agarose beads and the proteins were eluted with 250 mM of Imidazole.

GST pull down assays: GST-Bro1 and Bro1-V fusion proteins were expressed in BL21(DE3) pLysS *E. coli* (Stratagene) and their interaction with Flag-syntenin-1, Flag-syntenin-2 and Flag-syntenin-2β were examined in GST-pull down assays following the protocol previously described (Sette et al., 2012). Bacterial pellets were resuspended in Bacteria Lysis Buffer (BLB) [50 mM Tris-HCl pH8.0, 1% (w/v) sucrose, 10% Glycerol, 0.1 mM DTT, 300 mM (w/v) NaCl and protease inhibitor cocktail]. GST-NC, GST-NC-p6 or mutant counterparts were prepared and pull down assays were performed as previously described (Sette et al., 2012). When cells were lysed mechanically, a tissue homogenizer (pestle B) was used after cells were swollen in hypotonic buffer (20 mM Tris HCL pH 7.4, 25 mM NaCl, 1 mM MgCl2) on ice for 1h. When cells were lysed in presence of detergent, 0.5% of Igepal was used. Nuclease treatment was performed following the protocol previously described (Sette et al., 2012) with RNAse A (EMD) (100 μg/ml) or Benzonase (100units) (Novagen) following the manufacturer recommendations at 37C for 1h. Captured protein complexes and cell lysates (input fractions) were analyzed by SDS-PAGE and western blot using the indicated antibodies.

Virus release analysis: 293T cells were maintained and transfected as previously described (Sette et al., 2011b). Twenty-four hours after transfection, cells and culture media were harvested and their protein content was analyzed by SDS-PAGE and Western blot using the indicated antibodies. HIV-1 proteins were detected using an anti-HIV-1 p24 monoclonal antibody (clone 183-H12-5C) or NEA-9306 (PerkinElmer). EIAV proteins were detected using a horse anti-EIAV serum.

RNAi knockdown: 293T cells (2.5×10^6 cells/T25) were transfected with 400 pmol or with 100 pmol of Stealth siRNA duplexes against cellular syntenin-1 and cellular syntenin -2, respectively (Sigma and Invitrogen life technologies). After 36 h, cells were cotransfected with the same amount of siRNA duplexes and 500 ng of $EIAV_{UK}$ or 1 mg of HIV-1 PTAP- proviral DNA. Cells and virus were harvested and processed as described above. When shRNA technique was used to deplete gene expression, lentiviruses were produced by transfecting 293T cells with a mixture of p8.91, pMD.G and pGIPZ lentiviral vector (Open Biosystems) containing the shRNA sequence. Viruses were harvested 48h post-transfection, filtered and titrated using the GFP reporter. For establishment of stable lines, 293T cells were transduced at M.O.I. ranging from 10 to 20 and

selected in 2µg/ml puromycin for 6 days. All knockdowns were assessed by western blot analysis of cell extracts by probing for endogenous proteins.

Bro1 and NC binding to LUVs: 1-palmitoyl-2-oleoyl phosphatidylethanolamine (POPE), 1 -palmitoyl-2-oleoylphosphatidylserine (POPS),1-palmitoyl-2-oleoylphosphatidylcholine (POPC), cholest-5-en-3ß-ol (cholesterol) and 1,2-dihexanoyl-*sn*-glycero-3-phospho-(1'-myo-inositol-3',5'-bisphosphate) were purchased from Avanti Polar Lipids (Alabaster, AL). Large unilamellar vesicles (100 nm) composed of POPE: POPS: POPC (1:1:1) or POPE: POPS: POPC: Cholesterol (28:34:28:10) were prepared as previously described (Philip and Scarlata, 2006). For membranes containing 5% PI(4,5)P2, PI(4,5)P2 solubilized in chloroform was placed in a round bottom flask and dried to a thin film using a stream of nitrogen. After, freshly extruded LUVs were added and the flask was subjected to extensive vortex or low power sonication. The GST-tagged proteins were labeled with 7-diethylamino-3- (4′-maleimidylphenyl)-4-methylcoumarin (CPM, Invitrogen) to yield proteins with an ~1:1 probe:protein ratio as described in (Philip and Scarlata, 2006). RNAse was then added to the labeled protein to a final concentration of 0.1 µg/µl. Binding measurements were performed on an ISS spectrofluorometer (Urbana, IL) using a 3 mm cuvette. Binding affinity was determined by the increase in fluorescence intensity of a 130 nM CPM-labeled purified protein solution, as freshly extruded LUVs were incrementally added. The sample spectra were corrected by subtracting identical spectra in control cuvettes that substituted the protein domains for the free GST tag similarly labeled with CPM. The area under the emission peaks of the corrected spectra was calculated using ISS Vinci software. These values were then plotted as a function of concentration of added lipid, and the resulting curves were fit using Sigma Plot (Jandel, Inc.).

Immunogold labeling and transmission electron microscopy: Methods for virus infection followed by double immunolabeling have been described previously in detail (Reed et al., 2012). For immunogold labeling, grids were double labeled with a murine mAb directed against HIV-1 Gag p24 (AIDS Reagents hybridoma 183-H12-5C) and an antiserum directed against ALIX. Grids were blocked with 5% BSA in PBS, and labeled with ALIX antiserum diluted 1:1 with antibody buffer (phosphate buffered saline containing 0.1% BSA-C), followed by labeling with anti-Gag (0.125 mg/ml in phosphate buffered saline containing 0.1% BSA-C). Antibody to ALIX was detected using a-rabbit F(ab)2', conjugated to 15nm gold particles (EMS); antibody to Gag was detected with a-mouse F(ab)2', conjugated to 6nm gold particles (EMS). Sections were then fixed and post-stained as described previously (Reed et al., 2012). Stained sections were examined using a JEOL-1400 transmission electron microscope. Images were acquired using a Gatan UltraScan 1000XP (2K x 2K) camera and Gatan Digital Micrograph software. Two

independent immunolabeling experiments were performed, each containing 2 sections from each group. Counting of ~ 300 particle assembly events at the membrane in 30 distinct fields has been performed as previously described (Reed et al., 2012) to quantify Gag-ALIX colocalization at the membrane. TEM of 293T cells expressing HIV-1 or the indicated mutant was performed as previously described in Dussupt et al., 2011.

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