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# Supplemental Data

# GGA and Arf Proteins Modulate

# Retrovirus Assembly and Release

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## SUPPLEMENTAL EXPERIMENTAL PROCEDURES

**Reagents:** siRNAs against GGAs, Arfs 1-5 and Tsg101 have been described elsewhere and were obtained from Qiagen as custom-synthesized oligos (Ghosh et al., 2003; Hewitt et al., 2002; Puertollano and Bonifacino, 2004; Volpicelli-Daley et al., 2005). Arf6 siRNA was obtained as a validated siRNA from Qiagen. HIV immunoglobulin (HIV-Ig) was obtained from the NIH AIDS Research and Reference Reagent Program. Anti-EIAV horse serum was kindly provided by Dr. R. Montelaro (University of Pittsburgh). The following Abs or antisera were obtained from the indicated sources: goat anti-MLV Gag p30, ViroMed Biosafety Laboratories, Camden, NJ; anti-HIV-1 p17 and p24, Advanced Biotechnologioes Inc, Columbia, MD; anti-Myc (A-14, 9E10) and HA (HA-7, Y11), Sigma or Santa Cruz Biotechnologies; horseradish peroxidase (HRP)-conjugated anti-mouse Ig, Amersham Biosciences (Uppsala, Sweden); anti-GGA1, a kind gift from Dr. R. Kahn (Emory University, Atlanta); anti-GGA2, GGA3 and AP-3, BD Biosciences; mouse anti-Tsg101 (C-2); Santa Cruz Biotechnologies; mouse anti-GM130, Transduction Laboratories (Lexington, Ky.); and sheep anti-TGN46, Serotec (Oxford, United Kingdom). Molecular clones expressing full-length GGAs have been described (Dell'Angelica et al., 2000; Puertollano and Bonifacino, 2004). The aggresome marker GFP-250 was kindly provided by Dr. E. S. Sztul (University of Alabama at Birmingham,

Birmingham). Mammalian expression constructs for WT and dominant-active Arf proteins were kindly provided by Dr. K. Nakayama (Kyoto University, Kyoto, Japan) and J. Donaldson (NIH, Bethesda, MD). The construction of EIAV Gag expression plasmids (pPRE-Gag), (Patnaik et al., 2002) containing WT (YPDL) and PTAP late domains have been described (Li et al., 2002; Shehu-Xhilaga et al., 2004). The full-length Tsg101 expression vector (TSG-F), (Goila-Gaur et al., 2003) and the HIV-1 molecular clone pNL4-3 (Adachi et al., 1986) have been described previously. The Fyn10deltaMA Gag chimera, provided by Dr. A. Ono (University of Michigan, Ann Arbor, Michigan), was constructed by fusing the 10 N-terminal residues of Fyn directly to the CA domain of a MA-deleted pNL4-3 derivative (Chukkapalli et al., 2008). The WT MLV expression vector was obtained from the NIH AIDS Research and Reference Reagent Program. Intracellular $PI(4,5)P_2$ levels were monitored by using a GFP derivative fused to the N-terminal pleckstrin homology (PH) domain derived from phospholipase C δ1 (PH-GFP), a kind gift of Dr. T. Balla (NIH, Bethesda, MD) (Varnai and Balla, 1998). BFA was purchased from Calbiochem.

**Immunofluorescence and EM.** For fluorescence microscopy, cells were plated and transfected in 8-well chamber slides (Nunc). Transfected cells were fixed 24-48 h posttransfection for 20 min with freshly prepared formaldehyde buffer (3.7% formaldehyde in 100 mM sodium phosphate buffer) and permeabilized with 0.1% Triton X-100 in phosphate buffered saline (PBS). Following incubation with appropriate primary and secondary Abs, cover slips were mounted with Aqua Poly Mount (Polysciences Inc, Warrington, PA). Cells were examined and images acquired using a

Delta Vision RT deconvolution microscope. When dual staining with different Abs, the extent of colocalization between two markers was determined by using the softWoRx co-localization tool, with R = Pearson coefficient of correlation. For EM analysis, 24-48 h posttransfection, cells were washed once with PBS and fixed with buffer containing 2% glutaraldehyde and 0.1M sodium cacodylate. Transfected cell samples were prepared for EM as described (Freed et al., 1994).

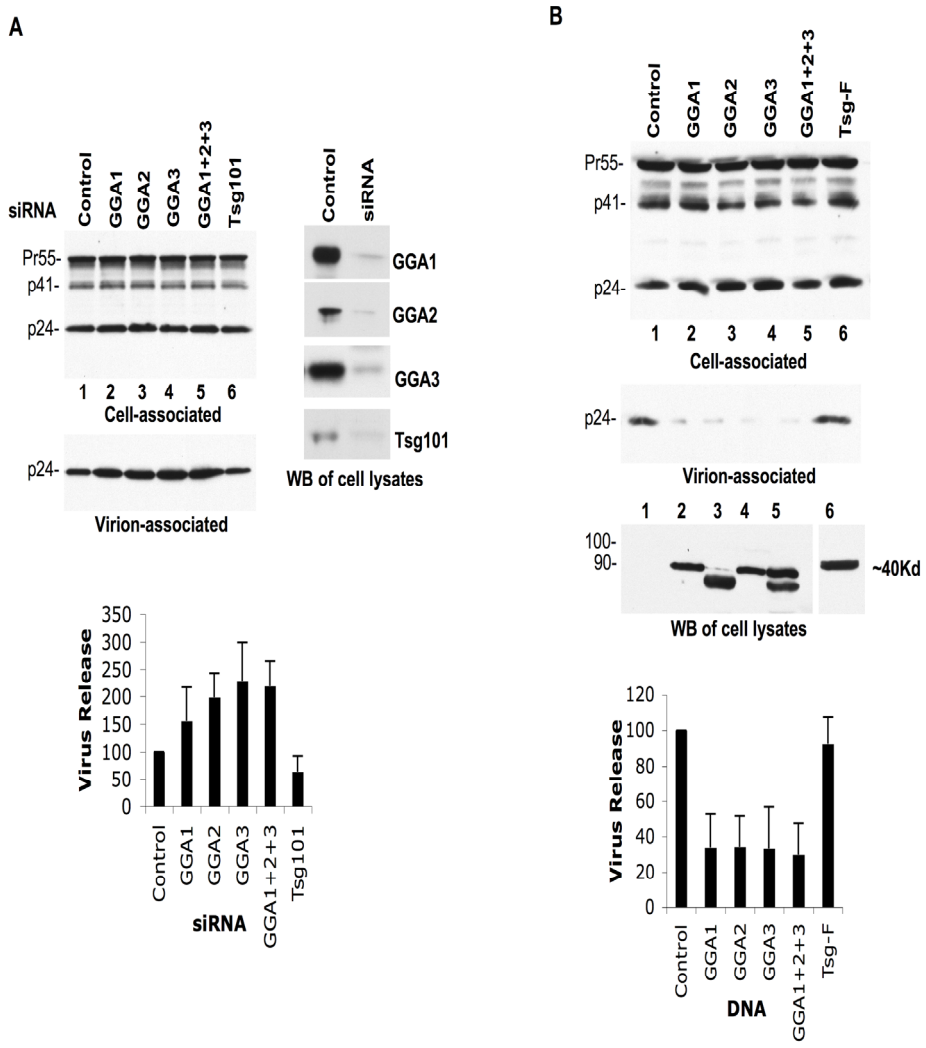


**Figure S1.** GGA depletion leads to an increase in HIV-1 release from MDMs. **(A)** Cells were transfected with pNL4-3 along with the indicated siRNAs using an Amaxa electroporator. Cell and virus lysates were resolved by SDS-PAGE, blotted onto membranes and probed with HIV-Ig. Bands were quantified using the Alpha Innnotech digital imager. Data ± SD; n = 3. Efficiency of siRNA-mediated depletion of GGAs in MDMs, determined by immunoblot analysis using Abs to detect endogenous GGAs or Tsg101, is shown in the right panel. **(B)** GGA overexpression inhibits HIV-1 production in MDMs. Cells were transfected with pNL4-3 along with control vector or GGA or

Tsg101 (Tsg-F) expression plasmids using an Amaxa electroporator. Cell and viral lysates were prepared and analyzed as described above. Data ± SD; n = 3. Lower gel panel shows GGA (lanes 2-5) and Tsg101 (lane 6) expression detected by anti-Myc or anti-HA WB, respectively.

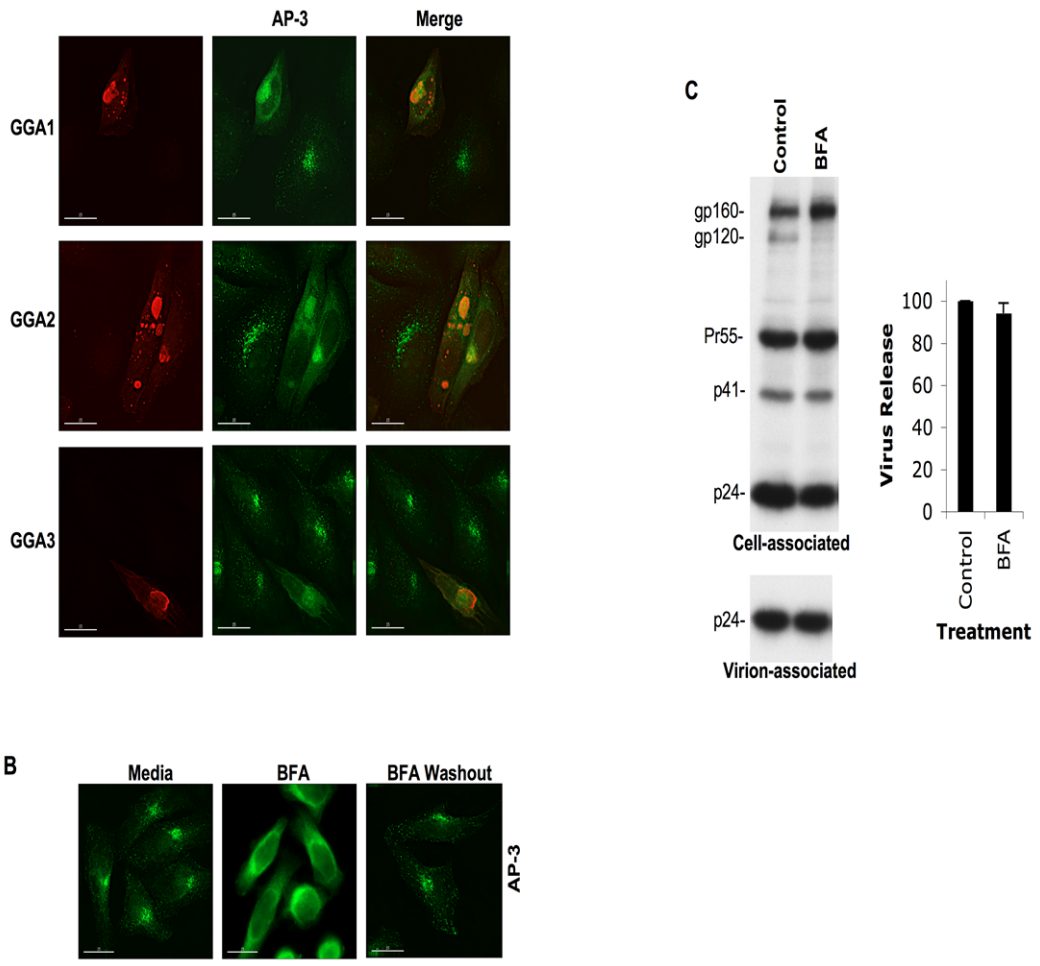


**Figure S2.** Effect of GGA overexpression and BFA on AP-3 localization. (**A**) HeLa cells transfected with Myc-tagged GGA expression constructs were immunostained with anti-Myc (red) and anti-AP-3 (green) Abs. (**B**) HeLa cells were treated with 20 µg/ml BFA for 1 h. Cells were either fixed immediately post-treatment or allowed to recover for 1 h (BFA washout) and were then immunostained for endogenous AP-3 (green). (**C**) Effect of BFA treatment on HIV-1 particle production. HeLa cells transfected with pNL4-3 were metabolically labeled with [$^{35}$S]Met/Cys in the presence or absence of 20 µg/ml BFA. Quantitation of virus release efficiency; ± SD , n=3.

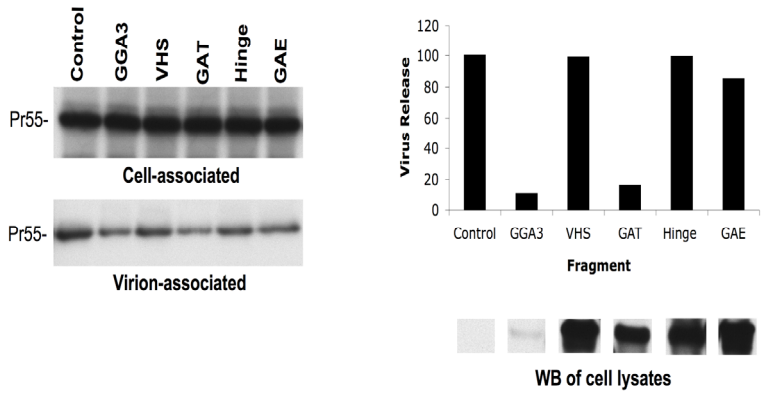


**Figure S3.** Overexpression of the GAT domain alone inhibits pNL4-3/PR$^-$ particle production. HeLa cells were transfected with the PR-defective molecular clone pNL4-3/PR$^-$ along with vectors expressing full-length GGA3 or the indicated GGA domains at a 5:1 DNA ratio. Virus release was determined by radioimmunoprecipitation assay with HIV-Ig (left panel) and quantitation was obtained by phosphorimager analysis (top right panel), normalized to individual GGA fragment expression levels (lower right panel) determined by anti-Myc Western blot (WB) followed by quantitation with an Alpha Innnotech digital imager. Pr55 indicates position of the HIV-1 Gag precursor Pr55$^{Gag}$. This experiment was performed twice, with similar results in both assays.

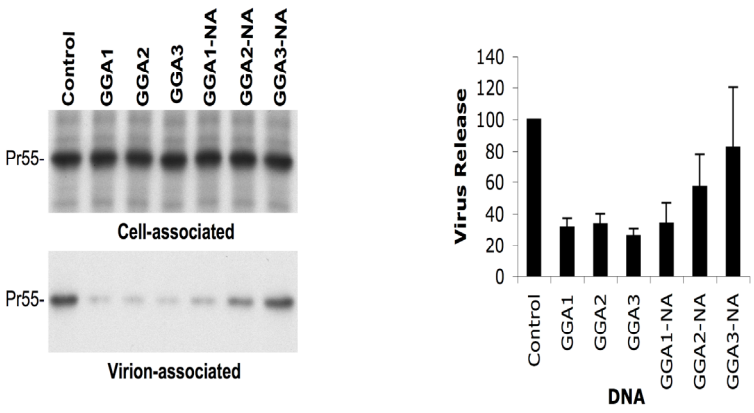


**Figure S4.** A GGA3 mutant defective in Arf binding does not inhibit EIAV particle production. HeLa cells were transfected with the EIAV Gag expression vector along with either WT GGA expression plasmids or their Arf-binding-defective (NA) counterparts. Virus release was determined by radioimmunoprecipitation assay, and quantitation was obtained by phosphorimager analysis. Position of the EIAV Gag precursor $Pr55^{Gag}$ (Pr55) is indicated.

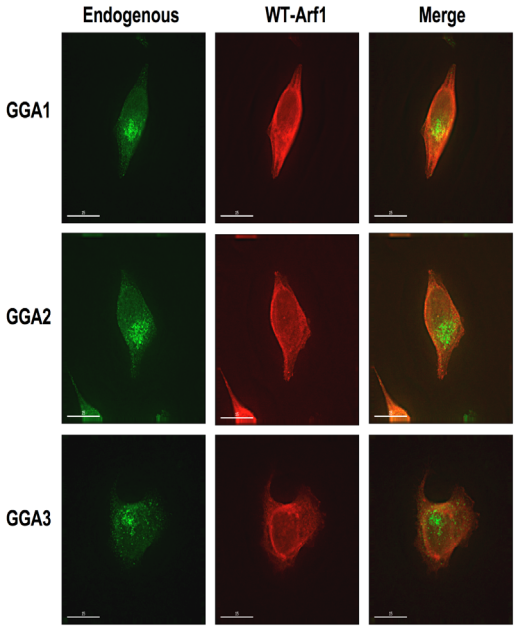


**Figure S5.** Endogenous GGAs do not sequester WT Arfs. HeLa cells were transfected with HA-tagged Arf1 expression vector. Cells were then immunostained with anti-HA (red) and Abs specific for the indicated endogenous GGAs (green).

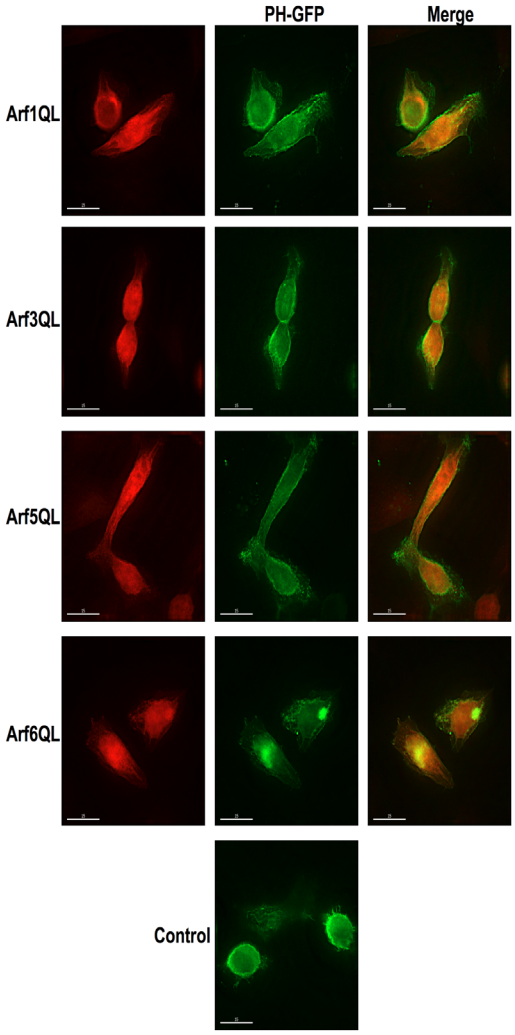


**Figure S6.** Disruption of class I or II Arfs does not induce a detectable shift in PI(4,5)$P_2$ localization. HeLa cells were transfected with PH-GFP, a probe for cellular PI(4,5)$P_2$, along with vectors expressing HA-tagged dominant-active isoforms of either Class I and II (Arf1-5) or class III (Arf6) Arfs. Cells were fixed, immunostained with anti-HA Ab, and analyzed by fluorescence microscopy. PH-GFP signal is in green, anti-HA signal in red. Bottom panel shows PH-GFP localization in cells not transfected with an Arf expression vector.

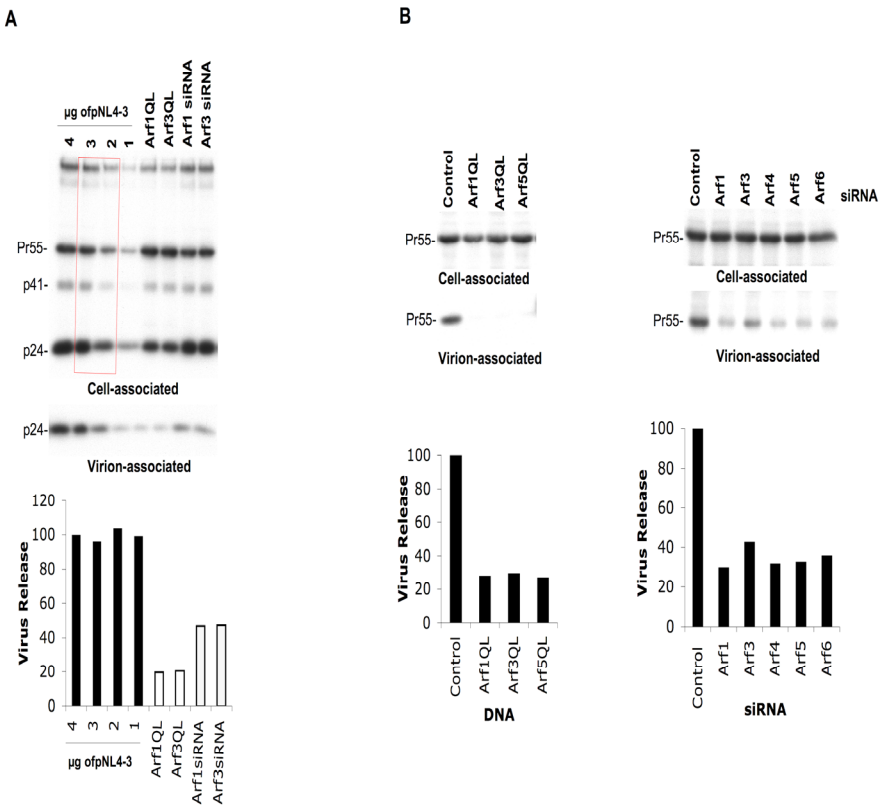


**Figure S7.** Inhibition of virus production by Arf disruption is not due to decreased Gag expression. **(A)** HeLa cells were transfected with decreasing amounts of pNL4-3 (lanes 1-4) or pNL4-3 along with the indicated dominant-active Arf expression vectors or Arf siRNAs (lanes 5-8). Virus release efficiency was calculated after radioimmunoprecipitation with HIV-Ig and phosphorimager analysis as described in the Figure 1 legend. Red inset depicts the Gag expression levels that correspond to those in the Arf1QL- and Arf3QL-transfected samples. In this experiment, Gag expression levels in the pNL4-3 titration spanned a 6-fold range whereas the dominant-active Arfs and Arf siRNAs had only a 2-fold effect on Gag expression levels. **(B)** HeLa cells were transfected with pNL4-3/PR$^-$ along with the indicated dominant-active Arfs or Arf siRNAs. Virus release efficiency was determined as described above.

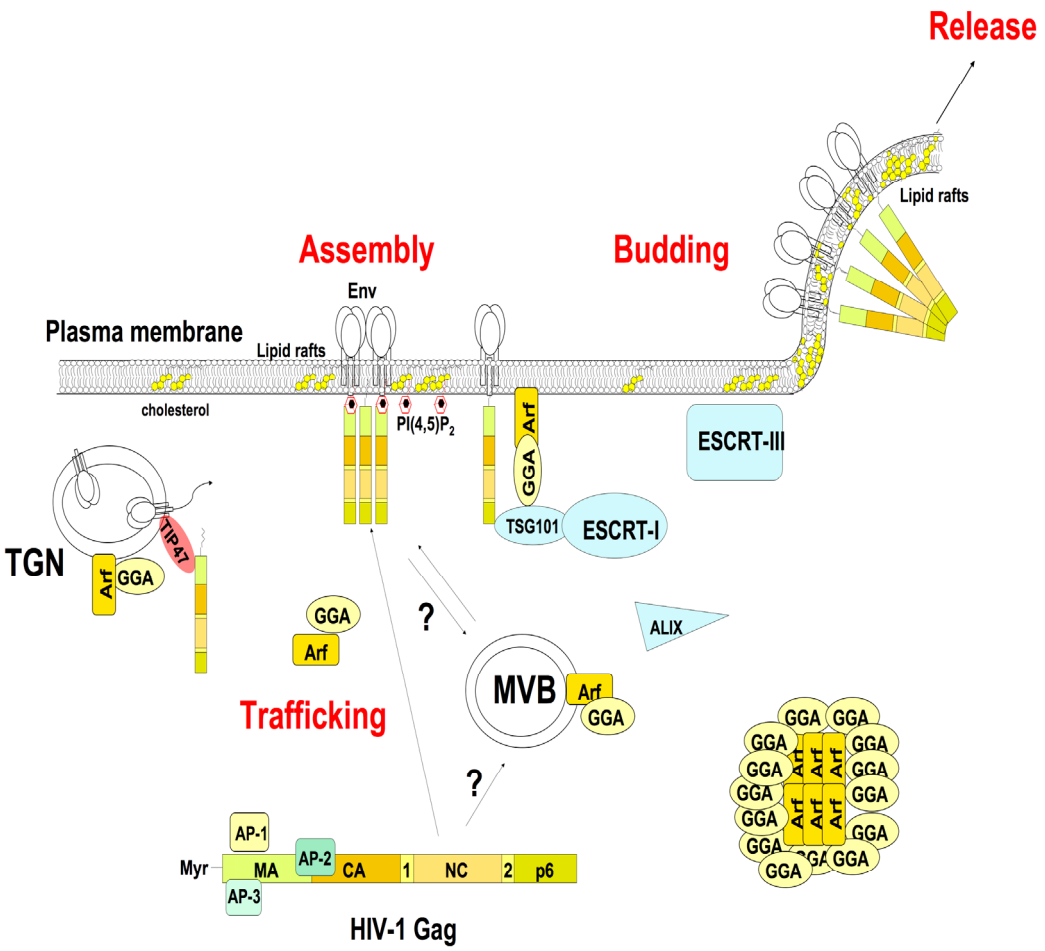


**Figure S8.** Schematic representation of host proteins involved in HIV-1 assembly and release. At the bottom of the figure is depicted $Pr55^{Gag}$, with major domains matrix (MA), capsid (CA), nucleocapsid (NC), and p6 and spacer peptides SP1 (1) and SP2 (2) indicated (Adamson and Freed, 2007). The MA domain has been reported to interact with clathrin adaptor protein complexes AP-1, AP-2, and AP-3 (Batonick et al., 2005; Camus et al., 2007; Dong et al., 2005). Interaction of Gag with membranes takes place in cholesterol-enriched lipid rafts (Ono and Freed, 2005) via the phospholipid $PI(4,5)P_2$ (Ono et al., 2004; Saad et al., 2006). Association between Gag and the Env glycoprotein complex has been reported to require TIP47 (Lopez-Verges et al., 2006). The GGA and

Arf proteins, demonstrated here to modulate virus budding and regulate Gag trafficking to the plasma membrane, respectively, are shown at various subcellular locations. Virus budding requires components of the ESCRT machinery of which ESCRT-I (whose Tsg101 subunit binds the p6 domain of Gag), ESCRT-III, and the ESCRT-associated factor Alix are shown (Fujii et al., 2007). At the lower right is illustrated the sequestration of endogenous Arf proteins by exogenously expressed GGAs. TGN, trans-Golgi network; MVB, multivesicular body. Modified from Future HIV Research, Joshi and Freed, 2007 (Joshi and Freed, 2007) with permission of Future Medicine Ltd., and from the cover of the Cold Spring Harbor 2006 Retroviruses abstract book, courtesy W. Sundquist and H.-Y. Chung, with permission.

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