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Supplemental Information

The Adaptor Protein-1 μ1B Subunit Expands the Repertoire of Basolateral Sorting Signal Recognition in Epithelial Cells

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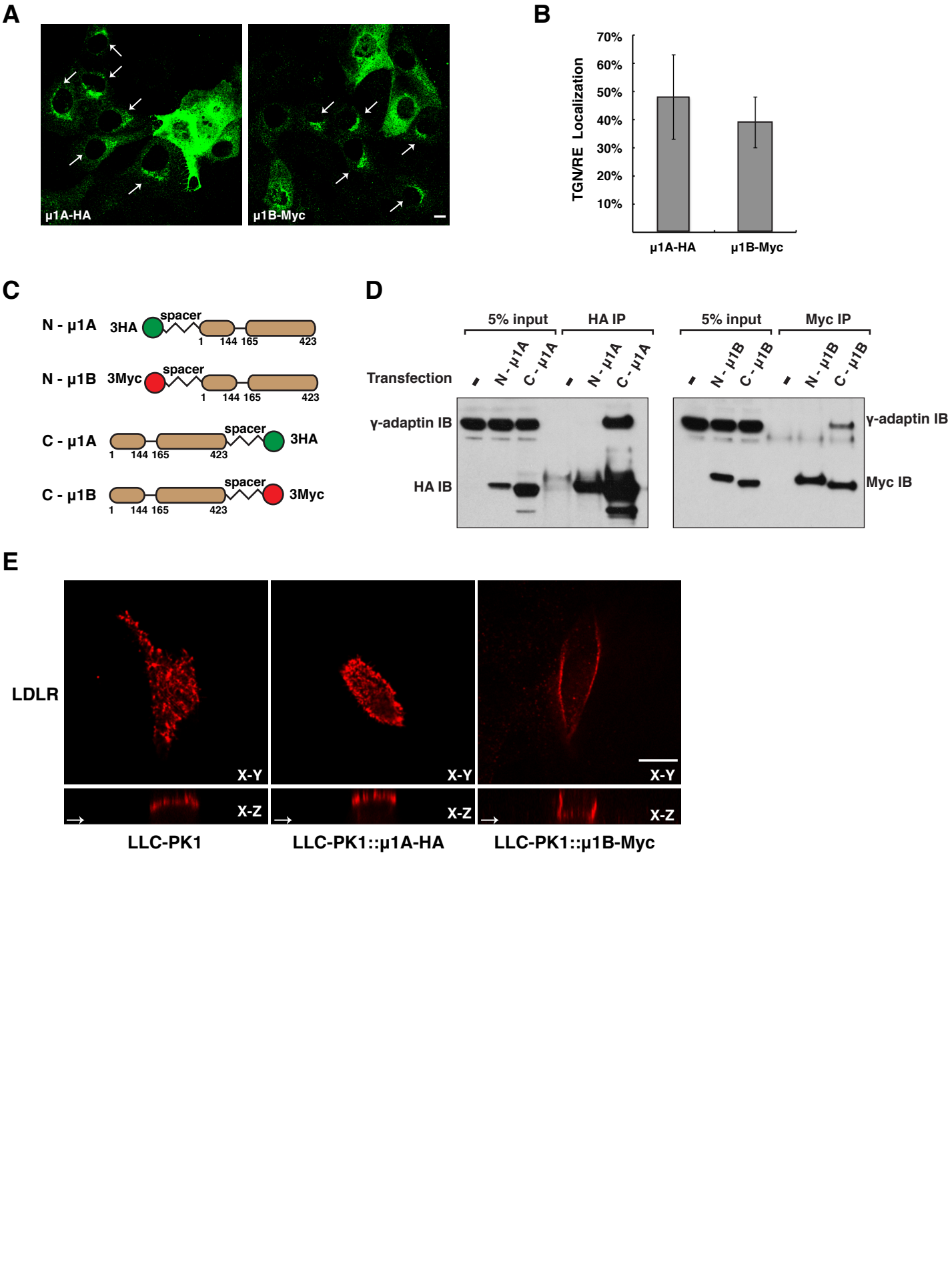
Inventory of supplemental materials

Figure S1


A
μ1A-HA
μ1B-Myc
B
TGN/RE Localization
70%
60%
50%
40%
30%
20%
10%
μ1A-HA
μ1B-Myc
C
N - μ1A
3HA
spacer
1
144 165
423
N - μ1B
3Myc
C - μ1A
3HA
C - μ1B
3Myc
D
5% input
HA IP
5% input
Myc IP
Transfection
-
N - μ1A
C - μ1A
N - μ1B
C - μ1B
γ-adaptin IB
HA IB
Myc IB
E
LDLR
X-Y
X-Z
LLC-PK1
LLC-PK1::μ1A-HA
LLC-PK1::μ1B-Myc

Supplemental Figure Legends

Figure S1, related to Figure 1. Expression of C-terminally tagged μ1A and μ1B in transfected epithelial cells. (A) MDCK cells were transiently transfected with C-terminally tagged μ1A-HA or μ1B-Myc. Twenty-four hours after transfection, cells were immunostained for the HA or Myc epitopes. Arrows indicate TGN/RE localization. Scale bar: 10 μm. (B) Quantification of cells with TGN/RE-localized μ1 constructs. Data are mean ± SD from three independent experiments (>50 cells analyzed per experiment). (C) Schematic representation of constructs. μ1A and μ1B were tagged at their N- or C-termini with three copies of the HA or Myc epitopes separated by a 10-amino-acid spacer (GSGSGGSGSG) as indicated in the figure. (D) MDCK cells transiently transfected with plasmids encoding N- or C-terminally tagged μ1A-HA or μ1B-Myc were lysed 24 h after transfection. Lysates were subsequently subjected to immunoprecipitation with antibodies to the HA or Myc epitopes followed by immunoblotting (IB) with antibodies to endogenous γ-adaptin or to the HA or Myc epitopes. (E) LLC-PK1 cells stably transfected with C-terminally tagged μ1A-HA or μ1B-Myc constructs were grown on Transwell filters for four days, transfected with a plasmid encoding HA-LDLR and cultured for three additional days. Cell surface staining with HA antibody was performed as described in Experimental Procedures. Specimens were analyzed by confocal microscopy and representative X-Y X-Z sections are shown. Arrows mark the position of the filters. Scale bar: 10 μm.

Figure S2

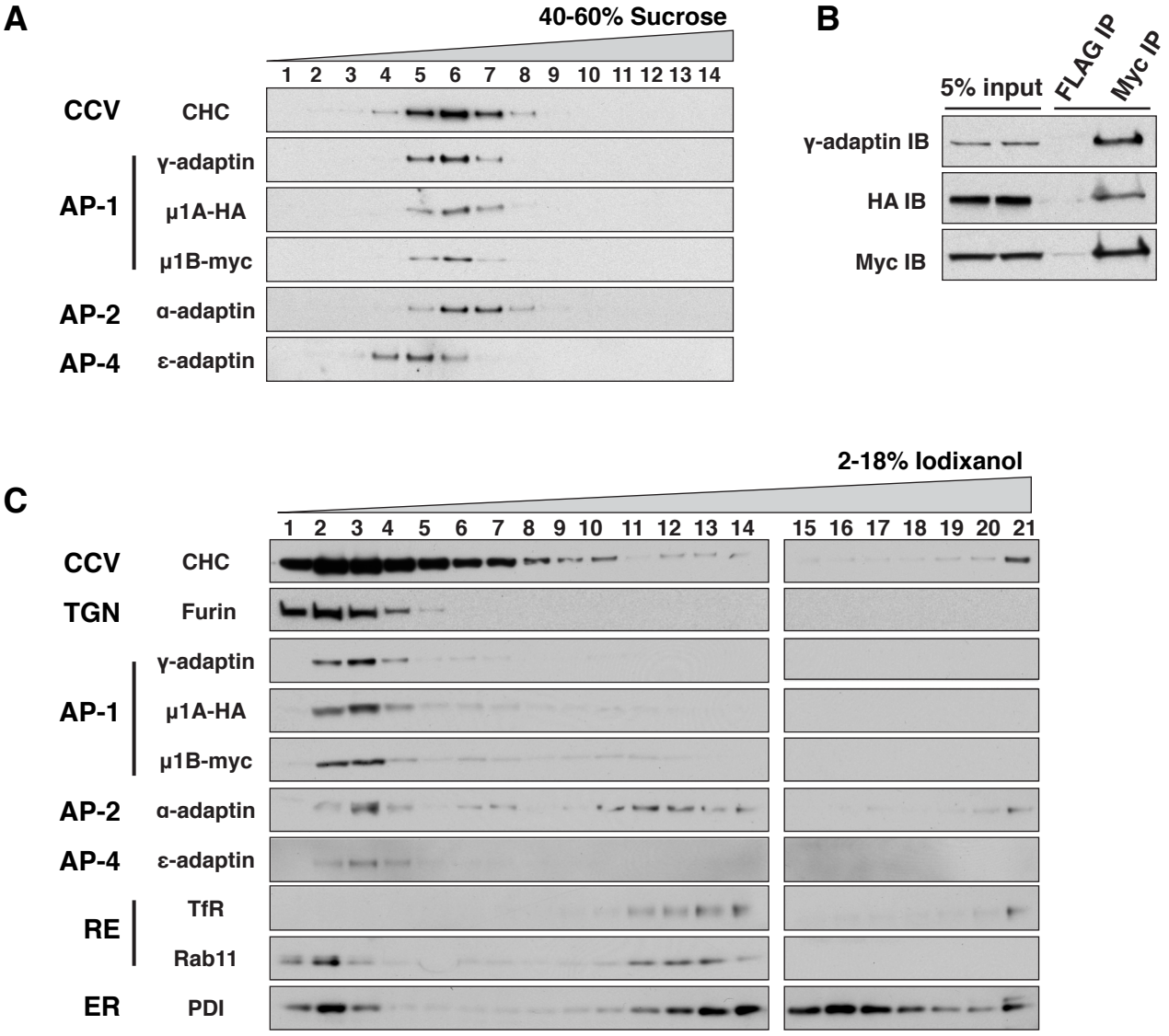

A
40-60% Sucrose
1 2 3 4 5 6 7 8 9 10 11 12 13 14
CCV
CHC
AP-1
γ-adaptin
μ1A-HA
μ1B-myc
AP-2
α-adaptin
AP-4
ε-adaptin
B
5% input
FLAG IP
Myc IP
γ-adaptin IB
HA IB
Myc IB
C
2-18% Iodixanol
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
CCV
CHC
TGN
Furin
AP-1
γ-adaptin
μ1A-HA
μ1B-myc
AP-2
α-adaptin
AP-4
ε-adaptin
RE
TfR
Rab11
ER
PDI

Fig. S2, related to Figure 2. Subcellular fractionation of μ1A- and μ1B-containing structures. (A) An enriched CCV fraction (Hirst et al., 2004) was prepared from MDCK cells stably co-expressing μ1A-HA and μ1B-Myc, and further resolved by centrifugation on 40-60% sucrose density gradients. Fractions were analyzed by SDS-PAGE and immunoblotting with antibodies to clathrin heavy chain (CHC), γ-adaptin, HA and Myc epitopes, AP-2-α and AP-4-ε. (B) An enriched CCV fraction from MDCK cells stably co-expressing μ1A-HA and μ1B-Myc was immunoprecipitated with anti-Myc or anti-FLAG (negative control) and subjected to SDS-PAGE and immunoblotting with the indicated antibodies. (C) Post-nuclear supernatants of MDCK cells stably co-expressing μ1A-HA and μ1B-Myc were layered on a 2-18% iodixanol gradient and centrifuged at 200,000 × g for 3 h. Fractions (0.5 ml) were collected from the top of the gradient and analyzed by SDS-PAGE and immunoblotting with antibodies to the indicated proteins. PDI; protein disulfide isomerase.

Figure S3

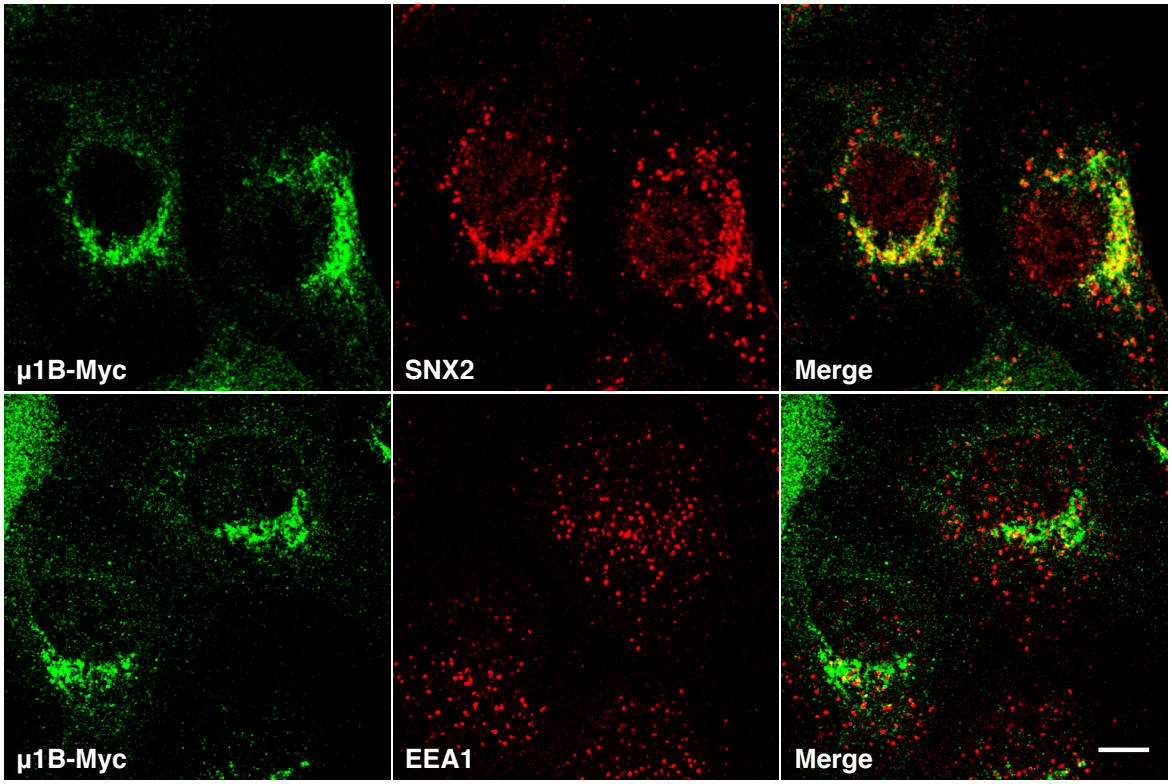


Figure S3, related to Figure 5. Analysis of μ1B co-localization with endosomal markers. MDCK cells stably expressing μ1B-Myc were immunostained with antibodies to the Myc epitope (left panels), SNX2 or EEA1 (middle panels). Merged images are shown in the right panels; yellow indicates colocalization. Scale bar: 10 μm.

Video 1, related to Figure 4. Co-localization of μ1A and μ1B in live non-polarized MDCK cells. MDCK cells stably co-expressing μ1A-GFP and μ1B-mCherry (mCh) were analyzed by TIRF microscopy within 200 nm of the plasma membrane. Images of μ1A-GFP (green) (150 ms exposure time) and μ1B-mCh (red) (200 ms exposure time) were continuously taken between the two channels for 35.6 s. The video is played at 2.8 frames per second.

Supplemental Experimental Procedures

Additional DNA constructs

AP-1A and AP-1B core complexes used in pull-down assays were generated by sequential cloning of subunits into a single pST44 polycistronic vector (Tan et al., 2005), as described (Ren et al., 2013). AP-1A-core included mouse γ1 (1-595)-GST, 6xHis-human β1 (1-584), full-length mouse μ1A and full-length human σ1C. AP-1B-core was generated by replacing mouse μ1A with full-length human μ1B. Sequences encoding N-terminal truncation mutants of active Arf forms containing an N-terminal His_6 tag and a TEV cleavage site were subcloned into the pHis2 vector (Sheffield et al., 1999). Human Arf1$^{\Delta 1\text{-}16\text{-}Q71L}$ PCR fragment was inserted into BamHI/XhoI sites, and fragments encoding human Arf4$^{\Delta 1\text{-}16\text{-}Q71L}$, Arf5 $^{\Delta 1\text{-}16\text{-}Q71L}$ and Arf6 $^{\Delta 1\text{-}14\text{-}Q67L}$ were subcloned into NcoI/XhoI sites. cDNAs encoding human LDLR cytosolic tail (residues 811-860) wild type and the corresponding Y845A/Y847A mutant were subcloned into a modified pHis2 vector containing a maltose-binding protein (MBP) coding sequence between the His tag and TEV cleavage site sequences. pcDNA3-HA-LDLR was a gift from Guojun Bu (Mayo Clinic, Jacksonville, FL). pCB6-LDLR-GFP was provided by Karl Matter (University College London, United Kingdom). pXS-Arf1 T31N and pXS-Arf6 T27N were gifts from Julie Donaldson (NHLBI, NIH, Bethesda, MD).

Immunoprecipitation and immunofluorescence

For immunoprecipitation, cells transiently or stably expressing μ1A-HA and/or μ1B-Myc were washed with PBS and lysed in ice-cold lysis buffer (0.5% Triton X-100, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA), supplemented with a protease inhibitor cocktail (Sigma-Aldrich). Cell lysates were spun for 15 min at 20,000*g*, and supernatants mixed with rabbit polyclonal antibody to the HA epitope (made in our laboratory) bound to protein A-Sepharose (Amersham Biosciences, Piscataway, NJ), or mouse monoclonal antibody to the Myc epitope (R950-25) (Invitrogen/Life Technologies) bound to protein G-Sepharose (Amersham Biosciences). Immunoprecipitates were eluted with NuPAGE SDS sample buffer (Invitrogen/Life Technologies) and heating at 85°C for 10 min, and analyzed by SDS-PAGE and immunoblotting. Details of immunoprecipitation and immunoblotting are as previously described (Rojas et al., 2008).

Non-polarized MDCK cells expressing different constructs were fixed in 4% paraformaldehyde, permeabilized with 0.1% saponin, and stained with mouse monoclonal antibodies to γ-adaptin (BD Biosciences, San Jose, CA), HA epitope (Covance, Berkeley, CA), TfR (Invitrogen/Life Technologies), and EEA1 (BD Biosciences), and rabbit polyclonal

antibodies to Myc epitope (made in our laboratory) and furin (Enzo Life Sciences, Farmingdale, NY), followed by appropriate fluorescently-labeled secondary antibodies (Invitrogen/Life Technologies). Fluorescence images were obtained using a confocal microscope (SP5, Leica, Wetzlar, Germany, or 710, Zeiss, Jena, Germany). Polarized MDCK cells expressing different constructs were fixed with methanol at -20ºC and stained as above. For cell surface staining of polarized LLC-PK1 cells, the Transwell cultures were fixed in 4% paraformaldehyde, and mouse monoclonal HA antibody was added to the apical and basolateral sides. Subsequently, filters were incubated with secondary antibody and analyzed using a Marianas spinning disc microscope. Digital images were acquired with an Evolve EM-CCD camera.

Subcellular fractionation

CCVs were isolated from MDCK cells stably expressing μ1A-HA and μ1B-Myc as described (Hirst et al., 2004). Subcellular fractionation on gradients of iodixanol (Axis shield, Oslo, Norway) was performed as described (Bakrac et al., 2010), with modifications following the manufacturer's recommendations to separate the lower density fractions (Golgi, PM and endosomes). Briefly, MDCK cells stably expressing μ1A-HA and μ1B-Myc were washed twice with pre-chilled PBS, and disrupted in 800 μl homogenization buffer (0.25 M sucrose, 1 mM EDTA, 10 mM HEPES-NaOH, pH 7.4, with a protease inhibitor mixture) by 20 passages through a 25-gauge needle on ice. Samples were centrifuged at 2000g for 10 min at 4°C. The resulting supernatants were layered on a 2-18% iodixanol gradient and centrifuged at 200,000g for 3 h at 4°C in a SW41Ti rotor (Beckman Coulter, Fullerton, CA). Fractions of 0.5 ml were collected for further analysis by SDS-PAGE and immunoblotting with antibodies to clathrin heavy chain (CHC) (BD Biosciences), furin (Enzo Life Sciences), γ-adaptin (BD Biosciences), HA epitope (Covance), Myc epitope (Cell Signaling Technology, Beverly, MA), α-adaptin (BD Biosciences), ε-adaptin (BD Biosciences), TfR (Invitrogen/Life Technologies), Rab11 (BD Biosciences) and protein disulfide isomerase (PDI) (Enzo Life Sciences).

Expression and purification of recombinant proteins

GST-His-tagged AP-1A or AP-1B core complexes were expressed from the pST44 vector in BL21 (DE3) Star *E. coli* (Invitrogen/Life Technologies) induced with 0.3 mM IPTG at 20°C overnight. Cells were lysed by sonication in 50 mM Tris-HCl pH 7.4, 300 mM NaCl, 20 mM imidazole, 10% glycerol, 3 mM β-mercaptoethanol (β-ME) and a protease inhibitor cocktail (Sigma-Aldrich) (lysis buffer). The clarified supernatants were first purified on Ni-NTA resin (Qiagen). The proteins were eluted with 0.1 M imidazole followed by purification on glutathione-Sepharose 4B resin (GE Healthcare, Piscataway, NJ) eluted with 20 mM glutathione. The concentrated

eluates were subsequently loaded onto a HiLoad 16/60 Superdex 200 column (GE Healthcare) and eluted with 20 mM Tris-HCl pH 7.4, 200 mM NaCl, 0.3 mM tris-(2-carboxyethyl)phosphine (TCEP) (sample buffer).

His-tagged Arf constructs were expressed in BL21 (DE3) Star cells by induction at 20°C overnight. Cell pellets were lysed by sonication in lysis buffer supplemented with 5 mM $MgCl_2$. The His-tagged proteins were initially purified on a Ni-NTA column eluted with 0.1 M imidazole, followed by chromatography in a HiLoad 16/60 Superdex 75 column (GE Healthcare) eluted with sample buffer containing 5 mM $MgCl_2$. Proteins were quantified by the bicinchoninic acid assay (Pierce) using bovine serum albumin as standard.

MBP and MBP fusion proteins were expressed in BL21 (DE3) Star cells induced at 18°C overnight. After sonication in lysis buffer, the clarified lysates were purified on Ni-NTA resin. The eluted samples were diluted 5-fold in 30 mM HEPES pH 7.4, 3 mM β-ME and purified in a HiTrap Q HP 5 ml column (GE healthcare) eluted with 70 ml of a 0 – 400 mM NaCl linear gradient in 30 mM HEPES pH 7.4, 3 mM β-ME. Eluates were analyzed by SDS-PAGE gel and electron spray mass spectrometry; fractions containing MBP proteins were concentrated and subsequently purified on a HiLoad 16/60 Superdex 75 column (GE Healthcare) eluted with sample buffer.

Pull-down assays

GST-His-tagged-AP-1A or -AP-1B core complexes (15 μg) were immobilized on 35 μl of glutathione-Sepharose and incubated with active (Q/L mutants) His-tagged Arf proteins (5 μM) at 4°C overnight in 200 μl of 20 mM Tris-HCl pH 7.4, 200 mM NaCl, 0.3 mM TCEP, 5 mM $MgCl_2$ and 2 mM GTP (Jena Bioscience GmbH, Jena, Germany). The beads were washed 4 times with the same buffer, mixed with 70 μl of 2x lithium dodecylsulfate (LDS, Invitrogen)/β-ME buffer and heated at 70°C for 5 min. Eluted samples were subjected to SDS-PAGE and immunoblotted with mouse monoclonal anti-poly His antibody (R&D systems, Minneapolis, MN).

MBP and MBP-LDLR tail WT and Y845A/Y847A (20 μg) were immobilized on 30 μl of amylose resin (New England Biolabs, Ipsiwich, MA) by incubation for 1.5 h at 4°C in 20 mM Tris-HCl pH 7.5, 200 mM NaCl, 0.3 mM TCEP supplemented with protease inhibitor cocktail (EDTA-free Complete, Roche Applied Science) (immobilization buffer). GST-His-tagged AP-1A and AP-1B core complexes were diluted to 1 μM in immobilization buffer and centrifuged for 15 min at 300,000g and 4°C. The supernatants were further diluted to 0.2 μM with immobilization buffer supplemented with 0.25% v/v Triton-X-100 and 0.15% w/v BSA (binding buffer) and precleared by incubation for 1 h at 4°C with amylose resin (0.5 ml of 0.2 μM AP-1 per 30 μl beads). Precleared AP-1 core supernatants were separated by centrifugation for 15 min at

21,000g and 4°C and subsequently incubated overnight at 4°C with 20 μg of MBP fusions immobilized onto amylose resin (0.5 ml of precleared 0.2 μM AP-1 per 20 μg of immobilized MPB's) in the presence or absence of 5-20 μM Arf1 Q71L, 1 mM GTP and 5 mM $MgCl_2$. Beads were washed three times with binding buffer containing 0.1% v/v Triton-X-100 and lacking BSA and protease inhibitors. Bound AP-1 core complexes were eluted by resuspension in 65 μl of 2X Laemmli buffer and incubation for 10 min at 90ºC. Eluted samples were separated from beads by centrifugation and subjected to SDS-PAGE and immunoblotting with rabbit anti-GST antiserum (Dell'Angelica *et al*., 1998).

Yeast two-hybrid assays

Complementary DNAs encoding cytosolic tail sequences from human LAMP1 (residues 406-417), human IL6R-α (residues 387-468), human IL6R-β (residues 642-918), human PVR (residues 368-417) and human LDLR (residues 832-860) were amplified by PCR and cloned into the Gal4-binding domain (BD) vector pGBKT7 (Clontech). The use of pGBKT7 provided greater sensitivity of detection because this vector yields higher expression levels (Tucker et al., 2009) and encodes an EED sequence within the Myc epitope that enhances interactions with Gal4-activation domain-μ1B fusions (Rafael Mattera, unpublished observations). Full-length mouse μ1A, mouse μ2, rat μ3A, rat μ3B, human μ4 as well as the C-terminal domain of human μ1B (residues 137-423) were cloned in the Gal4-activation domain (AD) vector pACT2 (Clontech). All amino-acid substitutions of the LDLR cytosolic tail were made by site-directed mutagenesis (QuikChange, Agilent, Santa Clara, CA) and verified by DNA sequencing. Y2H assays were performed as previously described (Mattera et al., 2003).

Supplemental references

Bakrac, B., Kladnik, A., Macek, P., McHaffie, G., Werner, A., Lakey, J. H., and Anderluh, G. (2010). A toxin-based probe reveals cytoplasmic exposure of Golgi sphingomyelin. J Biol Chem *285*, 22186-22195.

Dell'Angelica, E.C., Klumperman, J., Stoorvogel, W. and Bonifacino, J.S. (1998). Association of the AP-3 adaptor complex with clathrin. Science *280*, 431-434.

Hirst, J., Miller, S.E., Taylor, M.J. von Mollard, G.F. and Robinson, M.S. (2004). EpsinR is an adaptor for the SNARE protein Vti1b. Mol Biol Cell *15*, 5593-5602

Mattera, R., Arighi, C. N., Lodge, R., Zerial, M., and Bonifacino, J. S. (2003). Divalent interaction of the GGAs with the Rabaptin-5-Rabex-5 complex. EMBO J *22*, 78-88.

Ren, X., Farias, G. G., Canagarajah, B. J., Bonifacino, J. S., and Hurley, J. H. (2013). Structural Basis for Recruitment and Activation of the AP-1 Clathrin Adaptor Complex by Arf1. Cell *in press*,

Rojas, R., van Vlijmen, T., Mardones, G. A., Prabhu, Y., Rojas, A. L., Mohammed, S., Heck, A. J., Raposo, G., van der Sluijs, P., and Bonifacino, J. S. (2008). Regulation of retromer recruitment to endosomes by sequential action of Rab5 and Rab7. J Cell Biol *183*, 513-526.

Sheffield, P., Garrard, S., and Derewenda, Z. (1999). Overcoming expression and purification problems of RhoGDI using a family of "parallel" expression vectors. Protein Expr Purif *15*, 34-39.

Tan, S., Kern, R. C., and Selleck, W. (2005). The pST44 polycistronic expression system for producing protein complexes in Escherichia coli. Protein Expr Purif *40*, 385-395.

Tucker, C. L., Peteya, L. A., Pittman, A. M., and Zhong, J. (2009). A genetic test for yeast two-hybrid bait competency using RanBPM. Genetics *182*, 1377-1379.