Supporting Information

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SI Methods

Cell Culture, Reagents, and Antibodies. HeLa cells were grown in HeLa media corresponding to DMEM (Invitrogen) containing 10% FCS (HyClone/Thermo Fisher Scientific), 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen). TRVb-1 cells were grown in Ham F-12 medium (Invitrogen), 5% FCS, penicillin/streptomycin, and 100 μg/mL G418 (Invitrogen). The following were obtained from Jackson ImmunoResearch: mouse mAb against vsv-g epitope (clone P5D4; Roche Diagnostics), mouse mAb against His epitope (Sigma-Aldrich), mouse mAb against myc epitope (clone 9E10; Roche Diagnostics), FITC and Texas red conjugated antibodies. Iron-loaded Texas red-conjugated human transferrin was from Molecular Probes/Fisher Scientific. Asolectin, unlabeled nucleotides, and egg dioleyl-phosphatidylcholine were from Sigma. Liver phosphatidylethanolamine and brain phosphatidylserine were from Avanti Polar Lipids. [^{35}S]GTPγS was from Perkin-Elmer.

GST Pull-Down Experiments. HeLa cells were mock-transfected or transfected with plasmids encoding myc-tagged endophilin B1, myc-tagged endophilin A2, and vsv-g–tagged exchange factor for Arf6 (EFA6) as indicated by using Jet Pei reagent. After 24 h, cells were lysed in 50 mM Tris, pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, 1% Triton-X100, 2 mM DTT, and a mixture of protease inhibitors (Roche Diagnostics), and centrifuged at 15,000 × *g* for 10 min at 4 °C. Supernatants were incubated with 0.25 mM GST constructs in the presence of 0.75% BSA and glutathione–Sepharose beads overnight at 4 °C.

For direct binding assay with purified recombinant proteins, 0.05–0.15 μM His fusion proteins containing various fragments of endophilin or EFA6 as indicated were incubated in 50 mM Tris, pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, 2 mM DTT, and a mixture of protease inhibitors with 0.25 μM GST constructs with or without 0.5 mM liposomes in the presence of 0.75% BSA and glutathione–Sepharose beads for 2 h 30 min at 4 °C.

Beads were washed, and bound proteins were eluted by using SDS sample buffer and separated on SDS/PAGE. The presence of EFA6 and endophilin in the eluate was detected by Western blotting by using the anti-tag antibodies.

Confocal Immunofluorescence Microscopy. HeLa or TRVb-1 cells plated on 11-mm round glass coverslips were transiently transfected with pcDNA3 or pEGFP-N1/C1 constructs by using the Jet-PEI transfection reagent (Polyplus Transfection) as described by the manufacturer. Unless otherwise stated, the cells were washed twice in PBS solution 24 h after transfection and then fixed in 3% paraformaldehyde and processed for immunofluorescence analysis as described previously (1). Confocal microscopy was carried out with a TCS-SP5 laser scanning confocal microscope (Leica Microsystems).

Preparation of Phospholipid Vesicles. Large unilamellar vesicles of asolectin were prepared as described elsewhere (2) and extruded through a 0.4- or 0.05-μM pore-size polycarbonate filter (Isopore; Millipore). Vesicles of defined composition (dioleyl-phosphatidylcholine/phosphatidylethanolamine/phosphatidylserine at 35/35/30 weight ratio) were prepared as previously described previously (3) at the concentration of 2 mg/mL.

[^{35}S]GTPγS Binding Assay. MyrArf6 (2 μM) was incubated at 30 °C with [^{35}S]GTPγS (20 μM; ∼2,000 cpm/pmol) in 50 mM Hepes (pH 7.5), 2 mM $MgCl_2$, 100 mM KCl, with asolectin (2 mM) with or without (as indicated in the Fig. 1 legend) His-tagged EFA6A (0.5 μM) and GST-endophilin (2 μM). At the indicated times, samples of 25 μL were removed and measured for radioactivity as described previously (4).

Flotation Experiments. Proteins (1.5–4 μM) and liposomes (1 mM) were incubated in HK buffer (50 mM Hepes pH 7.2; 120 mM K-acetate) with DTT (1 mM) and $MgCl_2$ (0.5 mM) at room temperature for 20 min in a total volume of 150 μL. The suspension was adjusted to 30% sucrose by adding and mixing 100 μL of a 75% wt/vol sucrose solution in HK buffer. The resulting high-sucrose suspension was overlaid with 200 μL HK containing 25% wt/vol sucrose and 50 μL HK containing no sucrose. The sample was centrifuged at 55,000 rpm (240,000 × *g*) in a swinging rotor (TLS 55; Beckman Coulter) for 1 h. The bottom (250 μL), middle (150 μL), and top (50 μL) fractions were manually collected from the bottom by using a Hamilton syringe, and analyzed by SDS-PAGE before and after staining with SYPRO Orange (Molecular Probes). The presence of proteins was detected by Western blot.

Dynamic Light Scattering. Measurements were made by using a Dynapro MSX instrument (Protein Solutions) equipped with a Peltier temperature controller. A 15-μL solution of Hepes 50 mM, pH 7.2, K-acetate 120 mM, and $MgCl_2$ 1 mM containing His-N-BAR (2 μM), with or without asolectin liposomes (0.1 mg/mL) and GST-Sec7 (5 μM) as indicated in Fig. 3, was placed in a quartz cuvette. After equilibration at 25 °C, 10 autocorrelation functions of the scattered light were determined at the optimal laser intensity, each for 10 s. From multiexponential fits of the autocorrelation functions, an intensity graph was produced, and the average size of particles represented (Dynamic V 5.0 software; Protein Solutions).

EM. Samples containing His-N-BAR (2μM) were incubated for 20 min at room temperature in HKM buffer (50 mM Hepes pH 7.5; 100 mM KCl; 2 mM $MgCl_2$) with or without asolectin liposomes (0.1 mg/mL) and GST-Sec7 (5 μM). Samples were deposited on glow discharge carbon-coated grids and negatively stained with 1% aqueous uranyl acetate. They were observed with a JEOL 1400 transmission electron microscope. Acquisitions were made with a Morada digital camera (Olympus SIS).

1. Franco M, et al. (1998) ARNO3, a Sec7-domain guanine nucleotide exchange factor for ADP ribosylation factor 1, is involved in the control of Golgi structure and function. *Proc Natl Acad Sci USA* 95(17):9926–9931.
2. Szoka F, Jr., Papahadjopoulos D (1978) Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc Natl Acad Sci USA* 75(9):4194–4198.
3. Macia E, Paris S, Chabre M (2000) Binding of the PH and polybasic C-terminal domains of ARNO to phosphoinositides and to acidic lipids. *Biochemistry* 39(19):5893–5901.
4. Franco M, Chardin P, Chabre M, Paris S (1995) Myristoylation of ADP-ribosylation factor 1 facilitates nucleotide exchange at physiological Mg2+ levels. *J Biol Chem* 270(3):1337–1341.

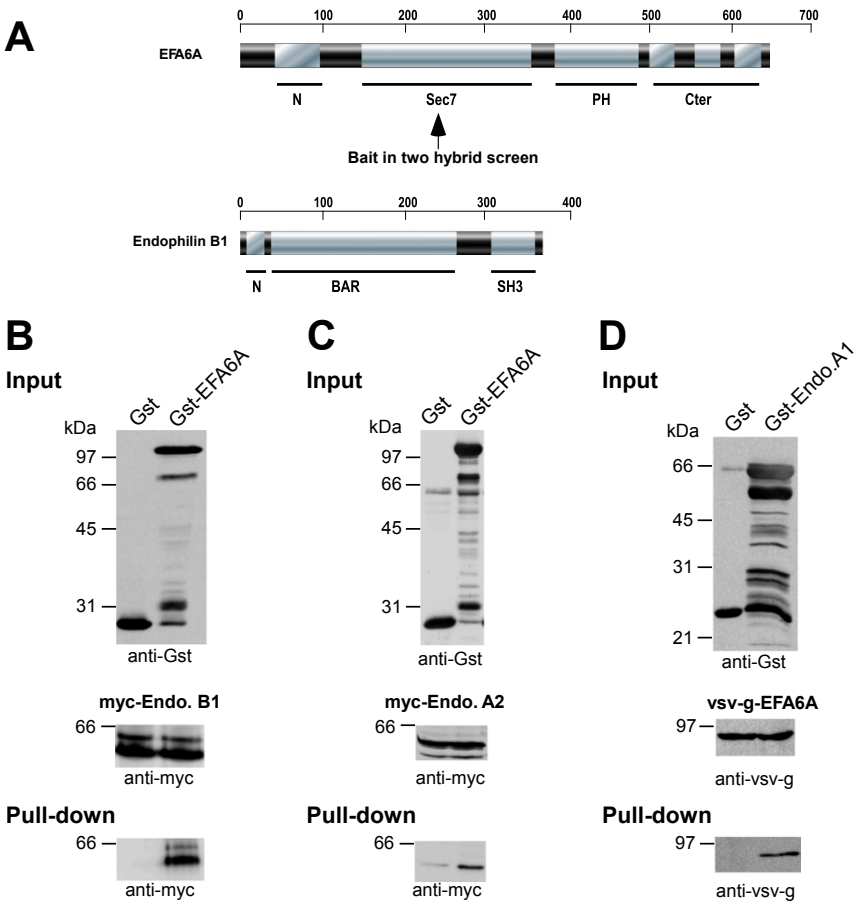

Fig. S1. Endophilin interacts with EFA6. (*A*) Schematic representation of the domain organization of EFA6A and endophilin B1. (*B* and *C*) GST pull-down of myc-tagged endophilin B1 (*B*) or A2 (*C*) expressed in HeLa cells by EFA6A fused to GST. (*D*) GST pull-down of vsv-g–tagged EFA6A expressed in BHK cells by endophilin A1 fused to GST. The results shown are representative of three independent experiments.

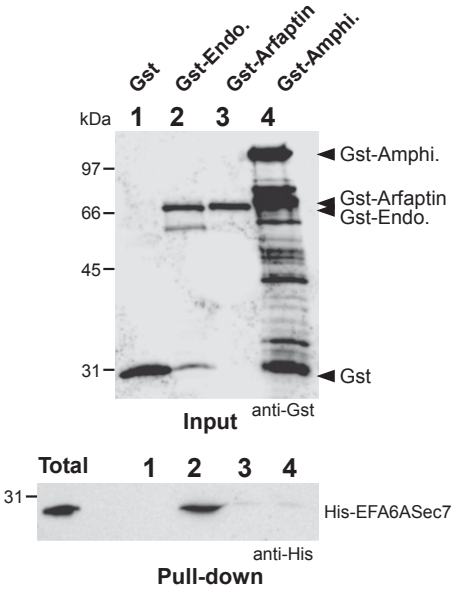

Fig. S2. EFA6A-Sec7 domain interacts specifically with GST-endophilin but not with GST-arfaptin 2, neither with GST-amphiphysin II. GST pull-down of purified His-tagged EFA6A-Sec7 domain using three BAR-domain containing proteins: endophilin A1, arfaptin 2, and amphiphysin II fused to GST. The results shown are representative of four independent experiments.