Supplemental Materials

Experimental Procedures

Cells

HeLa and COS-7 cells were cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin/glutamine (Life Technologies, Inc). Mouse embryonic fibroblasts (MEF-1, LRP-1-expressing and MEF-2, LRP-1-dificient) were obtained from the ATCC and cultured in DMEM with 10% FBS and penicillin/streptomycin/glutamine. LR73 parental cells and stable clones expressing GST-GULP and GST-GULP PTB or antisense RNA of GULP were described previously [1].

Antibodies

Antibody to human GULP was generated by immunizing rabbits with purified GST PTB domain of GULP. Polyclonal antibody against Arf6 has been described previously [2]. Goat anti-ACAP1, goat anti-ERK2 and antibodies against GFP, HA, Myc, His and GST-tagged proteins were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). M2 anti-Flag antibody was from Sigma-Aldrich. Secondary antibodies conjugated to horseradish peroxidase were purchased from Amersham Biosciences. All immunoblots were developed using enhanced chemiluminescence (Pierce).

Plasmids and Transfections

Wild type ACAP1 and GAP-deficient mutant (ACAP1-GD) were obtained from Paul Randazzo (NCI). The cDNAs encoding ACAP1 individual regions/domains, i.e., BAR (amino acids 1-264), PH (amino acids 264-381), GAP (amino acids 381-532), ankyrin repeats (amino acids 532-741, ANK) and GAP+ANK (amino acids 381-741) were amplified by PCR and subcloned into pFLAG-CMV2 (Sigma-Aldrich). ACAP2 and AGAP1 were also generous gifts from Paul Randazzo at NCI. Plasmids encoding Git1 and Git2 were obtained from Rick Horwitz (University of Virginia), and EFA6 was from Julie Donaldson (NHLBI, NIH). pEGFPN1 constructs encoding wild-type, Q67N and T27N Arf6 were obtained from David Castle

(University of Virginia). pEGFPN1 construct encoding T44N Arf6 was obtained by QuikChange site-directed mutagenesis kit from Stratagene. HeLa cells or COS-7 cells were transfected using Lipofectamine 2000 (Invitrogen) according to manufacturer's recommendations and cultured for 24-48h before analysis.

Protein expression and purification

GST and GST fusions of GULP, GULP-PTB domain, GULP-LZ+C or $\Delta N^{1\text{-}24}$ and RAP (receptor associated protein) were expressed in BL21 cells and purified using glutathione Sepharose 4B (Amersham Biosciences). Myristoylated wild-type Arf6 was expressed and purified as previously described [3, 4], with the addition of a Sephacryl S-200 gel filtration column as a final step. To generate bacterial construct expressing Arf6 mutant (T27N and Q67L), the coding region of each individual Arf6 mutant from pEGFPN1 construct mentioned above were subcloned into pET15b without a His-tag. Non-myristoylated Arf6 mutant proteins were produced in BL21 cells and purified as previously described [3]. The coding region of ACAP1 amino acids 264-741 was subcloned into pET32EK/LIC and fused to His-tag. The construct was then transformed into ArcticExpression (DE3) (Stratagene) competent cells and soluble ACAP1 proteins were produced according to manufacturer's instructions. His-ACAP1 was purified using His•Bind resin from Novagen.

In vitro protein interactions

Bacterially produced and purified Arf6 mutant proteins and/or His-ACAP1 were incubated with GST fusions on glutathione Sepharose beads in a buffer containing 10 mM Tris-HCl, pH 7.9, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, 0.5% NP-40 and 10% glycerol at 4°C for 2 hours. The beads were washed three times with the same buffer. The bound Arf6 was immunoblotted with rabbit anti-Arf6 polyclonal antibody. His-ACAP1 was immunoblotted with rabbit anti-His antibody. GST fusions on the membranes were visualized by Ponceau S staining.

Sequential trimeric complex formation was assessed by first incubating Arf6 mutant proteins with GST-GULP beads in the above buffer for 2 hours at 4°C. The beads were washed

three times before the addition of His-ACAP1 and then incubated in the same buffer for another 2 hours at 4°C. The bound Arf6 and His-ACAP1 were immunoblotted as mentioned above. The GST-GULP on the membrane was visualized by Ponceau S staining.

siRNA mediated knockdown of GULP expression

To generate siRNA against GULP, nucleotides 125 to 145 (from the starting codon) of GULP was targeted to generate 64-base complementary oligonucleotides with ends staggered to form BamH I and Hind III restriction sites when annealed for subsequent subcloning into the p*Silencer*2.1-U6 puro plasmid (Ambion). Mouse embryonic fibroblasts (MEF-1) were transfected with the above plasmid and selected with 6 μg/ml of puromycin for stable GULP knockdown clones. The control clone was transfected with a control plasmid (pApuro) and selected under the same conditions.

Immunoprecipitations and GST precipitations

Stable GULP knockdown MEF-1 clones and the control clone were lysed on the tissue culture plates with the Arf6 lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate, 2 mM $MgCl_2$, 1 mM DTT, 10% glycerol with 5 mM sodium fluoride, 1 mM sodium orthovanadate and 1 μM each aprotinin, leupeptin, pepstatin and AEBSF). The lysates were cleared by centrifugation at 10,000 g for 10 minutes at 4°C. The lysates were incubated with anti-GULP antibody for 1 hour at 4°C and precipitated with protein A/G-agarose conjugates (Santa Cruz Biotechnology) at 4°C overnight. Precipitates were washed three times with Arf6 wash buffer (PBS containing 0.5% Triton X-100, 1 mM DTT, 5 mM $MgCl_2$, 1 μM each aprotinin, leupeptin, pepstatin and AEBSF). Co-precipitated endogenous ACAP1 was detected by goat anti-ACAP1 immunoblotting. GULP knockdown was confirmed by rabbit anti-GULP immunoblotting. The interaction assays between bacterially produced GULP and the $Arf6^{T27N}$ or $Arf6^{Q67L}$ mutants were performed in the Arf6 lysis buffer. Lysates from HeLa cells were incubated with GST beads for 4 hours at 4°C. The beads were then washed three times with the Arf6 wash buffer. Precipitated endogenous ACAP1 was detected using goat

anti-ACAP1 antibody. Precipitated overexpressed Arf6, Rac1, Cdc42, RhoG, ARNO, EFA6B, ACAP2, Git1, Git2 or ACAP1 mutant proteins were immunoblotted with the respective epitope-tagged antibodies. To detect interaction between GULP and endogenous Arf6, MEF-2 cell lysates were incubated with GST fusion beads in a buffer containing 25 mM HEPES, pH 7.4, 100 mM NaCl, 2 mM $MgCl_2$, 10 mM EDTA, 1 mM DTT, 0.7 mg/ml azolectin vesicles (Sigma-Aldrich) and protease inhibitors at 4°C for 2 hours. The beads were washed in the same buffer. The endogenous Arf6 was detected by anti-Arf6 immunoblotting. All the GST fusions on the membranes were visualized by Ponceau S staining. For trimeric complex formation in cells, COS-7 cells were transfected with Flag-tagged ACAP1 and HA-tagged GULP and Arf6 mutant plasmids. 48 hours after transfection, the cells were lysed in Arf6 lysis buffer in the presence of Mg++ (see above) and anti-Flag immunoprecipitations were performed. The co-precipitated Arf6 and GULP was detected by anti-HA immunoblotting.

Migration Assays

Cell migration across membranes was performed using tissue culture-treated 6.5-mm Transwell chambers with 8.0 μm pore membranes (Costar). For LR73 cells, the underside of each membrane was coated with 20 μg/ml Fibronectin for 2 hours at 37°C. The membranes were then blocked with 1% BSA in α-MEM for 1 hour at 37°C. LR73 cells were dissociated, washed in serum free medium, and transferred to the top chambers of each Transwell unit at a density of 10^6 cells/ml in 100 μl α-MEM. The bottom chambers contained α-MEM and 10% FBS. Migration was allowed to proceed for 6 hours at 37°C. Non-migrating cells were removed from the top surfaces of the Transwell membranes using cotton swabs. The membranes were then fixed in methanol for 5 minutes and stained with 0.1% Crystal Violet for 15 minutes. The membranes were rinsed with water and the dye was eluted with 10% Acetic Acid. OD_{570nm} was measured using a microplate reader.

For HeLa cells, both sides of each membrane were coated with 20% FBS in DMEM through incubation for 2 hours at 37°C. Under these conditions, the Transwells become coated

primarily with vitronectin, which serves as the major attachment and spreading factor. The membranes were then blocked with 1% BSA in DMEM for 1 hour at 37°C. HeLa cells starved in DMEM with 0.1% BSA overnight were dissociated, washed in serum free medium, and transferred to the top chambers of each Transwell unit at a density of 10^6 cells/ml in 100 μl DMEM. The bottom chambers contained DMEM and 20% FBS. Migration was allowed to proceed for 4 hours at 37°C. Non-migrating cells were removed from the top surfaces of the Transwell membranes using cotton swabs. The membranes were then fixed in 4% paraformaldehyde for 15 minutes and counted by fluorescence microscopy.

For the wound-healing assay, 90% confluent HeLa cells cultured on coverslips were transfected with GULP together with either ACAP1 or ACAP1-GD plasmids using Lipofectamine 2000. The cells were cultured for 36 hours before starvation overnight in DMEM with 0.1% BSA. After denuding the cultures, the cells were washed and cultured in 20% FBS containing DMEM for 12 hours at 37°C. Pictures were taken immediately after denudation and at the end of the experiments. The wound gap between the edges in each picture was measured and the migration/wound closure was calculated as the percentage of the wound filled.

GGA pull down assay

GTP-bound state of the endogenous Arf6 in LR73 cells stably overexpressing GULP or the GULP knockdown MEF-1 and LR73 cells was assayed using a GST fusion to the VHS and ARF binding domains of GGA3 (Golgi-localized, γ ear-containing ARF-binding proteins) as described previously[5]. To detect effect of coexpressing GULP and ACAP1 in regulating Arf6-GTP level in LR73 cells, stable clones expressing GULP were transfected with ACAP1 or the control plasmid using Lipofectamine 2000 and cultured for 24 h before analysis. The lysates were incubated with GST-GGA and the bound Arf6 was assessed by immunoblotting.

Guanine nucleotide exchange assays

Guanine nucleotide exchange assays were performed as described previously [3]. Myristoylated wild type Arf6 (1 μM) was incubated with $[^{35}S]GTP\gamma S$ (5 μM) in the presence or

absence of GST-GULP or GST-GULP-PTB (10 μM each) in a buffer containing 25 mM HEPES, 100 mM NaCl, 1 mM EDTA, 2 mM $MgCl_2$ and 1 mM DTT. The assay was done at 37°C in the presence of large unilamellar vesicles (liposomes) with a lipid composition of 40% PC, 25% PE, 15% PS, 9.5% PI, 10% cholesterol and 0.5% PIP2 (all from Sigma-Aldrich) and the total lipid concentration was 500 μM. At various times, aliquots were removed and the amount of $[^{35}S]$GTPγS bound to Arf6 was determined by a nitrocellulose filter-binding assay.

References

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