Supporting Information

Beck *et al.* 10.1073/pnas.0805182105

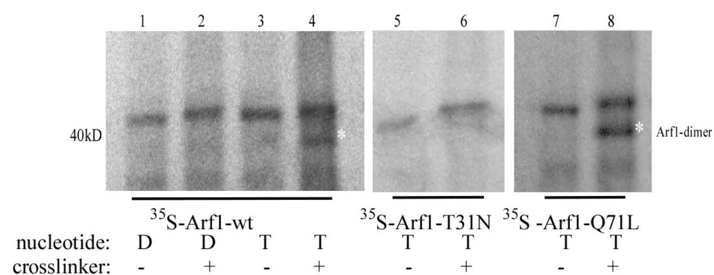

Fig. S1. ^{35}S-labeled Arf1 dimerizes on Golgi membranes in a nucleotide-dependent manner. [35S]-methionine-labeled Arf1-wt, Arf1-T31N, and Arf1-Q71L were produced and purified in an *in vitro* transcription and translation system (1, 2). Golgi-enriched membranes from HeLa cells were incubated for 15 min at 37°C with *in vitro* translated 35S-labeled full-length myristoylated Arf1-wt (lanes 1–4) and GDPβS (D) or GTPγS (T) as indicated. To further analyze nucleotide specificity, the GDP-locked mutant Arf-T31N (lanes 5 and 6) and the GTP-locked mutant Arf-Q71L (lanes 7 and 8) were investigated (3). Membranes were collected by centrifugation, resuspended in PBS, and incubated in the presence or absence of the homobifunctional thiol-reactive cross-linker BMH for 1h at room temperature (RT). Thereafter the samples were analyzed by SDS/PAGE and autoradiography. In lane 4, a cross-linker- and GTPγS-dependent band is visible below the band of actin, present in all lanes. This band is the expected size for a dimer of Arf1 (asterisks). No such band is visible in lanes when mutant T31N was used (lanes 5 and 6). The dominant positive mutant Q71L gave rise to a cross-link product at the expected size (lanes 7 and 8). A window of the gels is shown representing a molecular mass range at ≈40 kDa.

Arf1-dimer

2
GNIFANLFKG LFGKKEMRIL MVGLDAAGKT TILYKLKLGE IVTTIPTIGF
NVETVEYKNI SFTVWDVGGQ DKIRPLWRHY FQNTQGLIFV VDSNDRERVN
EAREELMRML AEDELRDAVL LVFANKQDLP NAMNAAEITD KLGLHSLRHR
159 181
NWYIQATCAT SGDGLYEGLD WLSNQLRNQK

Arf1-monomer

2
GNIFANLFKG LFGKKEMRIL MVGLDAAGKT TILYKLKLGE IVTTIPTIGF
NVETVEYKNI SFTVWDVGGQ DKIRPLWRHY FQNTQGLIFV VDSNDRERVN
EAREELMRML AEDELRDAVL LVFANKQDLP NAMNAAEITD KLGLHSLRHR
159 181
NWYIQATCAT SGDGLYEGLD WLSNQLRNQK

Fig. S2. The Arf1 dimer interface is shielded from proteolytical digestion. The experiment described in Fig. 2 was scaled up so that bands could be detected by Coomassie-staining. The bands corresponding to cross-linked dimeric Arf1 and to monomeric Arf1 were excised, digested with trypsin, and analyzed by MALDI-TOF mass spectrometry according to standard procedures. The detected peptides are indicated in red letters within the sequence of full-length Arf1. Two peptides missing in the dimeric Arf1 sample are highlighted with boxes.

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Fig. S3. Nucleotide exchange activity on Golgi membranes of Arf1-wt and Arf1-Y35A. Arf1 proteins were incubated with Golgi-enriched membranes, radiolabeled GTP, and the Arf-GEF ARNO. Protein-bound and free nucleotide was separated by gel filtration, and the fractions collected were analyzed in a scintillation counter. Twenty micrograms of Golgi-enriched membranes from rat liver were extruded through 200-nm pore-size polycarbonate filter membranes (Avestin) and incubated with 0.2 μM Arf1, 20 μM GTP, 0.2 μM [8–3H]-GTP (0.1 μCi), and 1 nM ARNO with assay buffer [25 mM Hepes-KOH (pH 7.4), 150 mM KCl] in a final volume of 50 μl. After incubation for 5 min at 25°C, the reactions were subjected to gel filtration on Sephadex G-50. The GTP exchange activity of Arf1-Y35A is ≈70% of Arf1-wt.

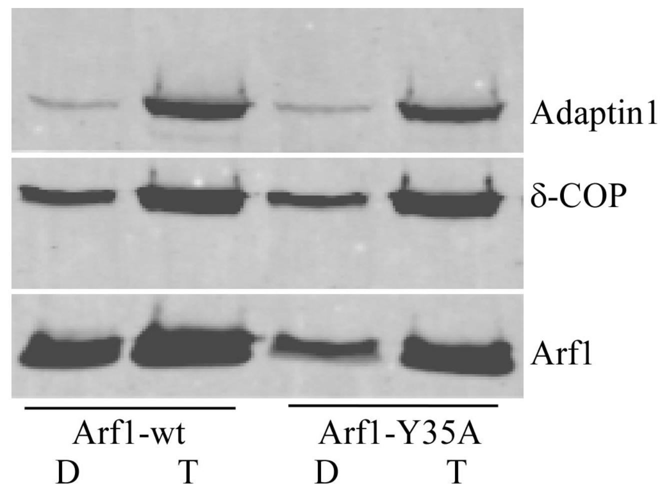


Fig. S4. Recruitment to Golgi membranes of coatomer and adaptin-1 by Arf1-wt and Arf1-Y35A. Golgi-enriched membranes were incubated with Arf1-wt, Arf1-Y35A, GDPβS (D), or GTPγS (T), and Arf1-depleted cytosol. Golgi-bound material was analyzed by Western blotting with antibodies against Arf1, adaptin-1, and the coatomer subunit δ-COP. Binding of Arf1 and coatomer to HeLa and rat-liver Golgi membranes was performed as described in ref. 1. After a 3-min incubation at 37°C of 10 μg of HeLa Golgi membranes with 0.6 μg of either Arf1-wt or Arf1-Y35A in the presence of 50 μM nucleotide (GDPβS or GTPγS) in assay buffer [25 mM Hepes-KOH (pH 7.2), 2.5 mM magnesium acetate, 20 mM KCl, ovalbumin (1 mg/ml), 1 mM DTT, and 200 mM sucrose], 2 μg of rabbit-liver coatomer was added to an end volume of 50 μl. After an additional 15-min incubation, Golgi membranes were pelleted by loading the sample onto a 300-μl cushion of 15% sucrose (wt/vol), followed by a 30-min centrifugation at 16,000 × *g* (4°C). Pellets were resuspended in SDS sample buffer and, after separation by SDS/PAGE, analyzed by Western blotting.

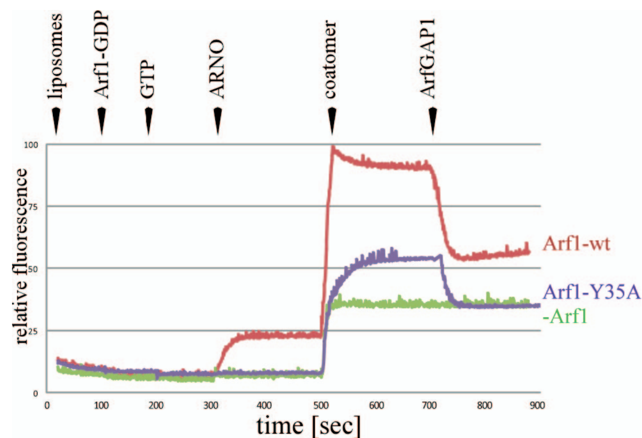


Fig. S5. Monitoring Arf1-mediated coat recruitment and release in real time by static light scattering according to ref. 4. Light scattering of Golgi-like liposomes containing 1 mol% PI(4,5)P_2 and 2 mol% p23 lipopeptide was monitored in 25 mM Hepes-KOH (pH 7.4), 2.5 mM potassium acetate by using a Jasco FP-6500 spectral photometer over time. 1 μM Arf1-wt or 2 μM Arf1-Y35A was added as indicated (after 100 sec), followed by 100 μM GTP (after 200 sec). Nucleotide exchange was started by the addition of 0.1 μM ARNO (after 300 sec), and after a further 200 seconds, coatomer was added at a concentration of 160 nM. The signal observed after the addition of coatomer is composed of the light scattering of the soluble coatomer plus the light scattering of the recruited coatomer, as shown by the response in the absence of Arf1. Interestingly, whereas a signal is seen for Arf1-wt after addition of ARNO, nucleotide exchange of Arf1-Y35A seems to be stimulated by coatomer. From these experiments we conclude that on liposomes, the activity of Arf1-Y35A to recruit coatomer is $\approx$20% of that of Arf1-wt. To further challenge the nucleotide specificity of the binding observed, recombinant ArfGAP1 (0.2 μM) was added after 750 sec. Both, the signals with Arf1-wt and Arf1-Y35A were reversed with similar kinetics and efficiency, indicating that in both cases the proteins were bound via Arf1-GTP and released by GTP hydrolysis.

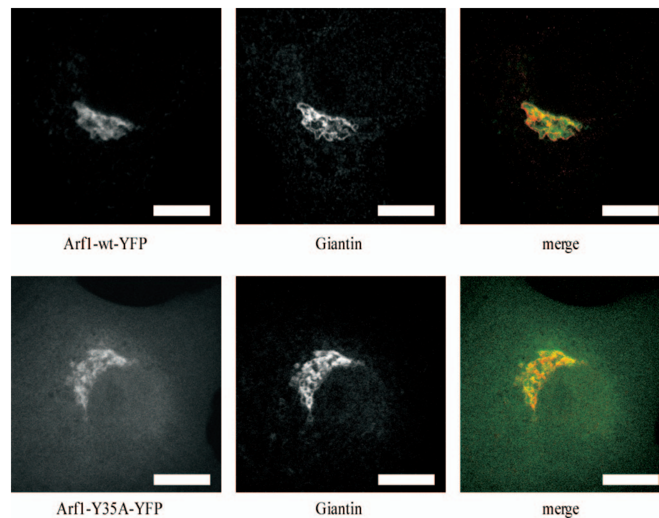


Fig. S6. Arf1-wt and mutant localize to the Golgi. Immunofluorescence of Vero cells transfected with Arf1-wt YFP (*Upper*) and Arf1-Y35A YFP (*Lower*). The localization was analyzed by immunofluorescence with an antibody directed against Giantin. Vero cells (ATCC CCL-81) were routinely cultured in DMEM supplemented with 10% FCS, 100 units of penicillin/ml, 100 μg of streptomycin/μl, 2 mM L-glutamine, at 5% CO_2 at 37°C. For electroporation, cells of one confluent 10-cm dish were trypsinized, washed three times with PBS, and transferred into 300 μl of internal medium [10 mM K_2HPO_4, 100 mM potassium glutamate, 2 mM EGTA, 5 mM $MgCl_2$, 0.15 mM $CaCl_2$, 25 mM Hepes-KOH (pH 7.4), and 0.2 mM ATP]. Fifteen micrograms of the respective plasmids were added to this suspension, and one pulse of 600 V and 50 μF was applied. Electroporated cells were kept at RT for 10 min, plated on coverslips in full DMEM, and subsequently incubated at 37°C for 24 h. The ARF1-wt-YFP plasmid was received as a kind gift by Rainer Duden (Royal Holloway University, London) (5). The ARF1-Y35A-YFP plasmid was generated by site directed mutagenesis. Cells were washed with PBS, fixed in 3% paraformaldehyde for 20 min, and subsequently permeabilized in 0.5% Triton X-100 for 5 min. After blocking with 5% BSA in PBS for 15 min, the samples were incubated with anti-Giantin antibody diluted 1:500 in PBS containing 5% BSA for 30 min. After washing twice with PBS for 10 min, the samples were incubated with Alexa Fluor 546-labeled anti-rabbit secondary antibody for another 30 min, washed twice with PBS for 10 min, and subsequently mounted in Mowiol 4–88 (Calbiochem). All steps were carried out at RT. Images were taken with an inverted laser scanning confocal microscope (LSM 510, Carl Zeiss) with a ×63 objective lens, ×4 zoom, and a pinhole size equivalent to one Airy disk diameter.

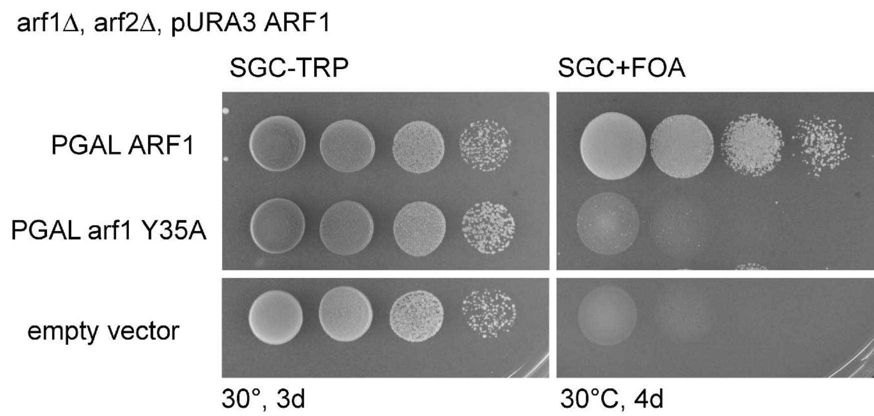
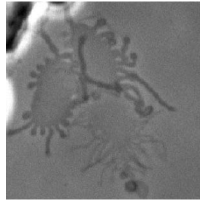


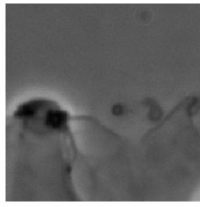
Fig. S7. GAL1 overexpression of Arf1-Y35A does not rescue the loss of Arf1-wt. To test whether overexpression of the arf1 Y35 mutant can rescue arf1 function, we transformed the *arf1Δarf2Δ* (pURA3 ARF1) strain with plasmids that encode ARF1-wt and Y35A allele, under control of the GAL1 promotor. The transformants were then plated on synthetic media complete, supplied with 2% galactose and 0.1% 5-FOA (5-fluoroorotic acid) to remove pURA3 ARF1 plasmid. Although ARF1-wt did restore growth under overexpression conditions, the Y35A mutant could not.



Movie S1. Arf1-mediated tubulation of synthetic lipid sheets. Lipids containing p23 lipopeptide were spotted on a glass surface and hydrated with buffer containing either GTP or GTP and the exchange factor ARNO (50 nM). After addition of myristoylated Arf1-GDP (1 μM), the lipid surface was observed by light microscopy. Shown are real-time recordings in the presence of Arf1-wt with GTP (*Movie S1*), Arf1-wt with GDP (*Movie S2*), and Arf1-Y35A with GTP (*Movie S3*).

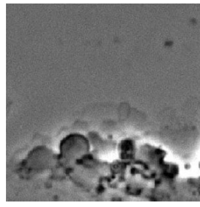
1. Zhao L, *et al.* (1997) Direct and GTP-dependent interaction of ADP ribosylation factor 1 with coatomer subunit beta. *Proc Natl Acad Sci USA* 94:4418–4423.
2. Zhao L, Helms JB, Brunner J, Wieland FT (1999) GTP-dependent binding of ADP-ribosylation factor to coatomer in close proximity to the binding site for dilysine retrieval motifs and p23. *J Biol Chem* 274:14198–14203.
3. Sohn K, *et al.* (1996) A major transmembrane protein of Golgi-derived COPI-coated vesicles involved in coatomer binding. *J Cell Biol* 135:1239–1248.
4. Bigay J, Antonny B (2005) Real-time assays for the assembly-disassembly cycle of COP coats on liposomes of defined size. *Meth Enzymol* 404:95–107.
5. Majoul I, Straub M, Hell SW, Duden R, Soling HD (2001) KDEL-cargo regulates interactions between proteins involved in COPI vesicle traffic: Measurements in living cells using FRET. *Dev Cell* 1:139–153.

Movie S1 (AVI)



Movie S2. Arf1-wt with GDP.

Movie S2 (AVI)



Movie S3. Arf1-Y35A with GTP.

Movie S3 (AVI)