Supplementary Figure and Movie Legends

Figure S1. Structural basis for GTP regulation of the Arf1:AP-1 complex. A hypothetical model of Arf1-GDP bound to the β1 subunit constructed by superimposing the Arf1-GDP crystal structure (1HUR) on the AP-1:Arf1-GTP complex, illustrating the steric collision that prevents Arf1-GDP from binding to AP-1. Associated with Fig. 2

Figure S2. Protein purification for in vitro studies. (A) SDS gel showing that each sample used for pull-down experiments in Fig. 3A, B was equally loaded and near homogeneously pure. (B) SDS gel showing that each sample used for pull-down experiments in Fig. 3C, D was equally loaded and near homogeneously pure. Associated with Fig. 3.

Figure S3. The AP-1 cores used for in vivo experiments are thermally stable. The melting curves of AP-1 core wild-type and mutants were measured by DSF. Normalized fluorescence is shown as a function of temperature. The calculated T_m is listed in the inset. Associated with Fig. 3.

Figure S4. The γ recruitment site is incompatible with the docked, open dimer. The membrane-docked open dimer is shown as in Fig. 6C, with the addition of the Arf1 molecules bound to the γ recruitment sites. The γ recruitment site-bound Arf1 molecules collide sterically with the membrane, as indicated by the black arrow. Associated with Fig. 6.

Movie S1. Animation of the conformational change between the crystal structures of the locked and Arf1-bound AP-1 core. The starting state for the animation was derived by docking the locked conformation of AP-1 (PDB 1W63) (Heldwein et al., 2004) into the open state crystal lattice. This docking leaves one of the key β1 contacts unsatisfied. In order to move towards completing the network of interactions with Arf1, the β1 trunk (together with the μ1 NTD) of the left-hand AP-1 core pivots, bringing the β1 Arf1-

binding site closer to the lower molecule of Arf1. The interaction is completed when the γσ1 subcomplex of the right-hand molecule pivots so as to bring the lower Arf1 molecule into direct contact with the left-hand AP-1 core. After each pivoting event, a steric collision is induced between the cargo-binding μ1 CTD and either the β1μ1 NTD subcomplex (first close-up) or the γσ1 (second close-up). To avoid these energetically disallowed collisions, the μ1 CTD is forced into the open conformation. Associated with Fig. 5.

Table S1. Statistics of data collection and crystallographic refinement.

	Carboxymethylated AP-1A core:Arf1-GTP
Construct	AP-1: σ1C (1-154), β1 (1-584), γ1 (1-595), μ1 (1-423) Arf1 (17-181)-Q71L
Data collection	
X-ray source	APS 22-ID
Wavelength (Å)	1.0000
Space group	$P6_4$
Cell dimensions	a=b=267.49 Å, c=191.41 Å, α=β=90°, γ=120°
Resolution (Å) (last shell)	50.00 – 7.00 (7.12 – 7.00)
Unique reflections	12533
R_{sym} [a] (%)	15.8 (69.5)
I/σ	13.1 (1.9)
Completeness	98.7 (94.4)
Redundancy	7.1 (5.4)
Refinement	
Resolution (Å) (last shell)	20.0 – 7.0 (7.24 – 7.0)
R-factor	0.21 (0.38)
Free R-factor	0.25 (0.45)
Bond lengths r.m.s.d.	0.0033 Å
Bond angles r.m.s.d.	1.0 degrees

Associated with Fig. 1

Table S2. Plasmids used in this study

GST-/His-tag AP1core (AP1 core-WT)	Human σ1C (1-154) in cassette1/pST44 6xHis-TEV site- human β1 (1-584) in cassette2 Mouse γ1 (1-595)-TEV site-GST tag in casette3 Mouse μ1 (1-423) in cassette4
AP1Acore-β1_I85D/V88D	β1_I85D/V88D mutation in the core
AP1Acore-γ1_L68D/L71E	γ1_L68D/L71E in the core
AP1Acore-γ1_L102E	γ1_L102E in the core
AP1Acore-γ1_L68D/L71E/L102E	γ1_L68D/L71E/L102E in the core
AP1Acore-β1_γ1 ΔArf1 mutant	β1_I85D/V88D & γ1_L68D/L71E/L102E in the core
Arf1-WT	6xHis-TEVsite- human Arf1 (17-181)_Q71L/phis2 (BamHI/XhoI)
Arf1-I49D	6xHis-TEVsite-Arf1 (17-181)_Q71L_I49D/phis2
Arf1-V53D	6xHis-TEVsite- Arf1 (17-181)_Q71L_V53D/phis2
Arf1-K73D	6xHis-TEVsite- Arf1 (17-181)_Q71L_K73D/phis2
Arf1-L77D/H80D	6xHis-TEVsite- Arf1 (17-181)_Q71L_L77D/H80D/phis2
Arf1- A136P/A137H	6xHis-TEVsite- Arf1 (17-181)_Q71L_ A136P/A137H/phis2
Arf1-W172D	6xHis-TEVsite- Arf1 (17-181)_Q71L_W172D/phis2
Arf1Δ16-Q71L	6xHis-human Arf1 (17-181)_Q71L/phis2 (NdeI/BamHI)
His-GB1-VAMP4-LL	6xHis-GB1 tag-human VAMP4 $_{20}$SERRNLLED$_{28}$/phis2 (NdeI/XhoI)
VAMP4-cys-GST	Human VAMP4 (1-51)-cys-TEV site-GST/phis2 (NdeI/BamHI)
β1/pEGFP-N1	Human β1 adaptin/pEGFP-N1 (HindIII/EcoRI)
β1 $^{\Delta Arf1}$/pEGFP-N1	Human β1 I85D/V88D/pEGFP-N1
γ1/pmCherry-N1	Mouse γ1 adaptin/pmCherry-N1 (XhoI/EcoRI)
γ1 $^{\Delta Arf1}$/pmCherry-N1	Mouse γ1 L68D/L71E/L102E/pmCherry-N1

Associated with the Extended Experimental Procedures.

Extended Experimental Procedures

Plasmid construction

Plasmids were constructed by the restriction cloning. DNAs coding for the four subunits of AP-1A core were as follows: human σ1C (1-154), human β1 adaptin (1-584) fused to an N-terminal His_6 tag, mouse γ1 adaptin (1-595) as a C-terminal GST fusion, and mouse μ1A adaptin (1-423). All four DNAs were subcloned into a single pST44 polycistronic vector (Tan et al. 2005). TEV cleavage sites were introduced between the affinity tags and the protein. A mutation I488F was present in the β1 subunit template DNA which is referred to as wild-type in this study. The β1-Phe488 background was used for all structural and *in vitro* experiments, but the Ile488 sequence was used for *in vivo* studies.

Human $Arf1^{\Delta 1\text{-}16\text{-}Q71L}$ was subcloned into the pHis2 vector (Sheffield et al., 1999) in order to express it as a fusion with an N-terminal His_6 tag and a TEV cleavage site. The Q71L mutant is referred to as Arf1 wild type in this study for *in vitro* experiments. For protein crystallization, one plasmid, $Arf1^{\Delta 1\text{-}16\text{-}Q71L}$ was constructed with a short His_6 tag as a N-terminal fusion. Both AP-1 and Arf1 mutants were generated using the Quick-change mutagenesis kit (Agilent). Plasmids designed for this study are listed in Table S2, all of which were verified by DNA sequencing.

For mammalian cell experiments, a Kozak consensus sequence was incorporated 5' to the ATG start codon of the appropriate gene subcloned into either pEGFP-N1 or pmCherry-N1 vector (Clontech). The cDNA of human β1 adaptin was inserted into the HindIII/EcoRI digest of pEGFP-N1; mouse μ1A adaptin cDNA with a 3' fusion to DNA coding for a 10-amino-acid linker was subcloned in the EcoRI/BamHI digest of pEGFP-N1; and mouse γ1 adaptin cDNA was subcloned into the XhoI/EcoRI digest of pmCherry-N1.

Protein expression and purification

The AP-1 core complex in pST44 vector was expressed in BL21 (DE3) star cell (Invitrogen), and induced with 0.3 mM IPTG at 20°C overnight. The cells were lysed in EmulsiFlex-C3 homogenizer (Avestin) in 1xPBS buffer, pH 7.4, 10% glycerol, 5mM β-mercaptoethanol (β-ME), 5mM EDTA, and a protease inhibitor cocktail (Sigma). The clarified supernatant was first purified on glutathione-Sepharose 4B resin (GE healthcare). After TEV cleavage at 4°C overnight, the sample was passed through 1ml of glutathione-Sepharose 4B and Ni-NTA column (Qiagen) to capture the GST and His-tag. Final protein was subjected to a HiLoad 16/60 Superdex 200 column (GE healthcare) chromatography in 20 mM Tris pH 7.4, 200 mM NaCl, 0.3 mM TCEP.

Cysteine S-carboxymethylation of AP-1 core was utilized for AP-1:Arf1 crystallization to improve the crystals and prevent protein aggregation, which was modified from the procedure of (Simpson, 2007). Briefly, the AP-1 core protein was first reduced with 20 mM DTT at room temperature for 3 hours, and then *S*-alkylated with freshly prepared iodoacetic acid for 30 min in the dark. After quenching by 1 M DTT, the sample was further purified on a Tricon 10/300GL column (GE healthcare) in the same buffer as above.

His-tagged Arf1 constructs were expressed in BL21 (DE3) star cells by induction at 20°C overnight. The cell pellet was lysed by sonication and purified on Ni-NTA column in 50 mM Tris pH 7.4, 300 mM NaCl, 20 mM imidazole, 5 mM $MgCl_2$, 3 mM β-ME, 10% glycerol and a protease inhibitor cocktail. The protein was eluted with 0.1 M imidazole, then loaded to a HiLoad 16/60 Superdex 75 column (GE healthcare) in sample buffer containing 5 mM $MgCl_2$. Proteins were quantified by the bicinchoninic acid assay (BCA; Pierce) using bovine serum albumin as standard.

Differential scanning fluorimetry (DSF)

The thermal stabilities of AP-1 wild-type and mutant cores were assayed by differential scanning fluorimetry (DSF) (Niesen et al., 2007). Protein unfolding as a function of temperature was monitored by the increase in the fluorescence of SYPRO orange (Invitrogen, Carlsbad, CA). The ABI 7900HT RT-PCR machine (Applied Biosystems, Carlsbad, CA) was used to increase the temperature of the samples in a gradient from 25

to 95 °C with 1 °C increments. Experiments were carried in 20 mM Tris pH 7.4, 200 mM NaCl, 0.3 mM TCEP in a reaction volume of 20 µl in 96-well microplates. Fluorescence intensity was measured every 1°C and normalized SYPRO orange fluorescence intensities were plotted as a function of temperature. The inflection point of the transition curve (T_m) was fitted using the Boltzmann equation in Origin (OriginLab, Northampton, MA). Each DSF experiment was repeated three times.

GST pull-down assays

15 µg of recombinant wild-type or mutant GST-AP-1 core was immobilized on 35 µl glutathione-Sepharose and incubated with wild-type or mutant His-tagged Arf1 proteins (5 µM) at 4°C overnight in 20 mM Tris pH 7.4, 200 mM NaCl, 0.3 mM TCEP, 5 mM $MgCl_2$ and 2 mM GTP. The beads were washed 4 times, mixed with 70 µl of 2x lithium dodecylsulfate (LDS)/ β-ME buffer and heated at 70°C for 5 min. 15 µl of each sample was subjected to SDS/PAGE, and immunoblotted with mouse monoclonal anti-polyHis antibody (R&D systems). Blots were developed using HRP-labeled anti-mouse secondary antibody and enhanced chemiluminescence (ECL, GE Healthcare). ImageJ (NIH) was used to quantify the intensity of bands (normalized to wild type).

For Arf1:AP-1 binding curves, the immobilized GST-AP-1 core was incubated overnight with 1 to 60 µM His-Arf1 at 4 ^{o}C. After washing three times, ¼ of the beads were subjected to SDS-PAGE and immounoblotting as described above. The amount of Arf1 bound was derived from the quantification of the band intensity and normalized to the Arf1 loading control (1.6 pmol). The Arf1 binding curve was plotted with relative Arf1 binding at the function of the Arf1 input concentration, and fitted using the Hill equation.

In order to test the effect of Arf1 in promoting recognition of dileucine cargo signals by AP-1, DNA coding for human VAMP4 (1-51) fused at its C-terminus to Cys, a TEV recognition site, and GST, in that order, was subcloned into pHis2 vector using NdeI and BamHI. The recombinant VAMP4 (1-51)-GST protein was expressed in BL21 (DE3) cells and purified using glutathione-Sepharose 4B. The eluate was further purified on a HiLoad 16/60 Superdex 75 column (GE healthcare) equilibrated in 20mM Tris pH

7.4, 200 mM NaCl, 0.1mM TCEP. 100 nM of recombinant VAMP4 (1-51)-GST protein was immobilized on 35 μl glutathione-Sepharose, then incubated overnight with tag-cleaved AP-1 core (0.5 μM) and His-tagged Arf1 at 4 ^{o}C. The concentration of His-Arf1 was varied from 0 to 20 μM. After three wash steps, the beads were boiled in 60 μl of 2x LDS/β-ME buffer for 3min. 5 μl of each sample was subjected to SDS-PAGE, and immunoblotted with rabbit polyclonal antibody to μ1 (clone RY/1, gift from L. Traub, University of Pittsburgh) or mouse anti-polyHis antibody to detect His-tagged Arf1.

Preparation of peptidoliposomes

To chemically conjugate the VAMP4 N-terminal tail sequence to lipid, 1% 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl)butyramide] (MPB-PE, Avanti Polar Lipids) was added into the lipid mixture consisting of 5% DOGS-NTA: 74% phosphatidylcholine (POPC): 20% (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine) POPE, or 5% DOGS-NTA: 69% POPC: 20% POPE: 5% phosphatidylinositol-4-phosphate **(**PI4P). 2 μmol of lipids were mixed and dried under nitrogen gas, and organic solvents were removed by drying under vacuum overnight. Dried lipids were resuspened in 1 ml of 0.4 M sucrose and 10mM Tris pH 7.4 at 4 °C for 1 hr with periodic vortexing. After freezing and thawing 5 times, the liposome solution was passed 11 times through a mini-extruder using a 100 nm membrane (Avanti Polar Lipids). The VAMP4-Cys peptide at two fold molar excess over MPB-PE lipid was incubated with liposomes at room temperature for 3 hours. The solution was then diluted with 2 ml of H_2O added to pellet and centrifuged at 60,000 rpm at 4 °C for 30 min (TLA 100 rotor, Beckman contour). The pellets were resuspended into 1ml of the buffer containing 20 mM Tris pH 7.4, 200 mM NaCl, 0.3 mM TCEP. 2mM β-ME was included in the buffer to quench the unreacted maleimide group at room temperature for 30 min. The lipids were then extruded 11 times using 100 nm filter membrane at a final lipid concentration of 2 mM. The labeled adduct was confirmed by mass spectrometry. Labeling efficiency was 30% as judged by the VAMP4 band intensity of pellet and supernatant fractions after ultracentrifugation in SDS/PAGE gel. All liposomes were used within 3 days.

Liposome sedimentation assays

Wild type or mutant AP-1 core (20 nM), His-tagged Arf1 (50 nM) and peptidoliposomes at 1 mM final lipid concentration were incubated in a 50-μl volume in 20mM Tris pH 7.4 200 mM NaCl, 0.3 mM TCEP, 1 mM $MgCl_2$, 2 mM GTP. After a 30 min incubation at room temperature, samples were centrifuged at 60, 000 rpm at 4 ^oC for 30 min, followed by one wash step. The final pellet was resuspended in 50 μl of 2x LDS buffer supplemented with β-ME. 10 μl of each supernatant (S) or pellet (P) fraction was subjected to SDS-PAGE for immunoblotting with rabbit polyclonal antibody to μ1. The intensity of bands on Western blots was quantified by ImageJ. The pellet and supernatant fraction for each sample was used to calculate the percentage of AP-1 binding to the liposome (100% x P/(P+S)). Results were obtained from three separate experiments.

Cell Culture and Transfection

HeLa (American Type Culture Collection, Manassas, VA) and MDCK cells expressing HA-tagged μ1A (gift of Xiaoli Guo, NICHD, NIH) were grown in Dulbecco's modified Eagle's medium, supplemented with 10% v/v fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM l-glutamine at 37°C and 5% CO_2. G418 was used to maintain the MDCK-μ1A-HA cell line. Cells were transiently transfected with plasmids encoding fluorescently-tagged μ1A-GFP, β1 or γ subunits using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), and analyzed 48h after transfection.

Immunofluorescence microscopy

HeLa cells were fixed with 4% v/v paraformaldehyde, 4% w/v sucrose in PBS for 20 min, permeabilized with 0.2% Triton X-100 for 15 min, blocked with 0.2% w/v gelatin, and stained with monoclonal antibody to γ-adaptin (BD Biosciences, Franklin Lakes, NJ) for 30 min at 37°C, followed by Alexa555-conjugated anti-mouse IgG (Invitrogen). DAPI (Invitrogen) was used to identify nuclei. Digital images were captured with a confocal microscope (LSM710, Zeiss, Oberkochen, Germany) using a 63x/1.4 numerical aperture oil-immersion objective.

Immunoprecipitation

MDCK-μ1A-HA cells were lysed with RIPA buffer (50mM Tris HCl pH 8, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors cocktail). Immunoprecipitation was performed for 4 h at 4°C using 1μg of rabbit anti-GFP antibody (Invitrogen) followed by Protein-A-Sepharose beads (GE Healthcare) for 4 h. After incubation, the bead mixture was washed three times in lysis buffer. Denatured samples were run on a SDS-PAGE and immunoblotted with HRP-coupled antibodies to HA- and GFP (MACS Miltenyi Biotec, Cambridge, MA). Co-precipitation was quantified from three separate experiments using ImageJ (NIH) (normalized to wild type).

References for Extended Experimental Procedures

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