

Structural basis for Arf6–MKLP1 complex formation on the Flemming body responsible for cytokinesis

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A small GTPase, Arf6, is involved in cytokinesis by localizing to the Flemming body (the midbody). However, it remains unknown how Arf6 contributes to cytokinesis. Here, we demonstrate that Arf6 directly interacts with mitotic kinesin-like protein 1 (MKLP1), a Flemming body-localizing protein essential for cytokinesis. The crystal structure of the Arf6–MKLP1 complex reveals that MKLP1 forms a homodimer flanked by two Arf6 molecules, forming a 2:2 heterotetramer containing an extended β -sheet composed of 22 β -strands that spans the entire heterotetramer, suitable for interaction with a concave membrane surface at the cleavage furrow. We show that, during cytokinesis, Arf6 is first accumulated around the cleavage furrow and, prior to abscission, recruited onto the Flemming body via interaction with MKLP1. We also show by structure-based mutagenesis and siRNA-mediated knockdowns that the complex formation is required for completion of cytokinesis. A model based on these results suggests that the Arf6–MKLP1 complex plays a crucial role in cytokinesis by connecting the microtubule bundle and membranes at the cleavage plane.

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Introduction

Cytokinesis is the final stage of cell division, during which cells exhibit drastic morphological changes and remodelling of the cytoskeleton (Balasubramanian *et al*, 2004; Glotzer, 2005). In anaphase/telophase, an actomyosin contractile ring constricts the plasma membrane in the equatorial region of a dividing cell to form a cleavage furrow, while an overlapping region of antiparallel microtubules from the central spindle gradually forms a dense structure, the Flemming body, in the middle of the intercellular bridge (note that, although this dense structure is often referred to as the midbody, we hereafter refer to it as the Flemming body to avoid confusion between the terms ‘midbody’, the entire central spindle and midbody microtubules). Finally, the cell undergoes abscission on either side of the Flemming body to generate two daughter cells. The drastic shape changes and membrane fission event during cytokinesis entail local delivery and/or removal of membranes and specific proteins (Prekeris and Gould, 2008; Steigemann and Gerlich, 2009). For example, proteins involved in membrane tethering, fusion and fission, such as exocyst components, SNARE proteins and ESCRT subunits, accumulate around the intercellular bridge and the Flemming body (Prekeris and Gould, 2008; Neto *et al*, 2011). Furthermore, coordination of changes in the organization of membranes and cytoskeleton suggests that interactions occur between components of the membrane trafficking and cytoskeleton remodelling machineries.

Members of the Arf (ADP-ribosylation factor) family of small GTPases regulate various aspects of membrane traffic. Mammals have six Arf isoforms, which are divided into three classes based on sequence similarity (Kahn *et al*, 2006): class I, Arf1–Arf3 (humans lack Arf2); class II, Arf4 and Arf5; and class III, Arf6. Arf6 is divergent from Arf1–Arf5, and localizes to the plasma membrane and the endocytic system, where it regulates endosome recycling and remodelling of actin and membranes (D’Souza-Schorey and Chavrier, 2006). In addition to these roles in interphase cells, Arf6 has been suggested to be required for the final phase of cytokinesis in mammalian cells (Schweitzer and D’Souza-Schorey, 2002, 2005). Furthermore, a *Drosophila arf6* mutant exhibits male sterility due to a cytokinesis defect during spermatocyte meiosis, although somatic cell mitosis appears to be normal (Dyer *et al*, 2007).

During cytokinesis, Arf6 transiently localizes to the Flemming body; however, the molecular mechanism under-

lying its targeting remains controversial. The roles of FIP3 and FIP4 (Rab11 family-interacting protein 3 and 4), which are dual effectors of Rab11 and Arf6 and associate with recycling endosomes in interphase cells, are the subject of debate. One previous study suggested that Arf6 is recruited to the Flemming body independently of Rab11- and FIP3-containing endosomes (Fielding *et al*, 2005), whereas other studies have proposed that Arf6 requires an interaction with Rab11- and FIP3-positive endosomes to be targeted to the cleavage furrow (Schonteich *et al*, 2007; Montagnac *et al*, 2009).

In the course of our previous study on the roles of Rab11 and Arf6 in the localization of FIP3 during cytokinesis, we obtained evidence indicating that Arf6 is recruited onto the Flemming body independently of Rab11 and FIP3 (Takahashi *et al*, 2011). In this study, we extend these observations to show that Arf6 targeting to the Flemming body depends on its direct binding to MKLP1 (mitotic kinesin-like protein 1; also known as KIF23), which together with MgcRacGAP/Cyk4 constitutes the centralspindlin complex at the Flemming body (Balasubramanian *et al*, 2004; Glotzer, 2005). We have determined the crystal structure of a complex between the MKLP1 C-terminal domain and Arf6, revealing a unique extended β -sheet of 22 strands that spans the entire 2:2 Arf6–MKLP1 complex. This interaction of MKLP1 with Arf6 is unique among Arf–effector interactions. Structure-based mutagenesis and siRNA-mediated knockdowns allowed us to critically test models of Arf6 recruitment to the Flemming body. Our findings demonstrate that formation of the Arf6–MKLP1 complex is crucial for faithful completion of cytokinesis.

Results

Specific association of GTP-bound Arf6 to the Flemming body

As a first step towards characterizing the specific localization of Arf6 to the Flemming body, we examined the localization of Arf proteins during cytokinesis in HeLa cells (Figure 1A and B). C-terminally mCherry-tagged Arf6 exhibited a distinct localization to the Flemming body in a late phase of cytokinesis, whereas no such Flemming body localization of other Arf proteins examined (Arf1, Arf3 and Arf5) was evident. At high levels of expression of Arf1–mCherry, however, a weak signal could be seen at the Flemming body. We next asked whether the activation status of Arf6 was critical for localization at the Flemming body. In order to address this issue, we took advantage of the dominant activating and inactivating mutants, respectively, Arf6(Q67L) and Arf6(T27N) (Figure 1C and D). Both the wild-type (WT) (top row) and activated Arf6 mutant (middle row) localized to the Flemming body. In contrast, Arf6(T27N), which is a nucleotide-free form (Macia *et al*, 2004), exhibited little or no staining at the Flemming body (bottom row).

We next examined spatiotemporal changes in the localization of Arf6 during mitosis, using three-dimensional time-lapse imaging to follow Arf6–EGFP expressed in HeLa cells (Figure 1E and Supplementary Movie S1). In metaphase, when the cells were nearly spherical, Arf6–EGFP was uniformly distributed throughout the cytoplasm (Figure 1E, top panels (0:00), the dividing cell marked with a yellow asterisk). At the onset of cytokinesis, Arf6–EGFP transiently

accumulated at the ingressing cleavage furrow (0:35, cyan arrow). Three-dimensional image analysis of fixed cells suggested that Arf6–EGFP is localized in the cortical region of the cleavage plane but not on the central spindle (Supplementary Movie S2). Arf6–EGFP then became increasingly concentrated at the Flemming body as cytokinesis proceeded (Figure 1E, green arrows). Upon abscission, Arf6–EGFP was incorporated into one of the daughter cells as a Flemming body remnant (3:40, red arrows in the cell with a magenta asterisk; 6:00, in the cell with a yellow asterisk). Intriguingly, the Flemming body remnant subsequently crawled up along the cortical surface of the daughter cell (4:30 and 6:00, red arrows in the cells with magenta asterisks) (see Discussion).

Phenotype of Arf6-knockout cells

Two different laboratories have published studies using Arf6 siRNAs, but their experiments yielded contradictory results: one study showed that Arf6-knockdown cells exhibited a two-fold increase in the percentage of binucleate cells (Schweitzer and D'Souza-Schorey, 2005), whereas the other study showed that a similar siRNA treatment did not change the number of binucleate cells (Yu *et al*, 2007). To circumvent problems inherent to the siRNA approach (i.e., incomplete protein depletion and off-target effects), we exploited mouse embryonic fibroblasts (MEFs) established from an Arf6-knockout (KO) mouse (Suzuki *et al*, 2006) to evaluate the role of Arf6 in cytokinesis. As shown in Figure 1F, multinucleate cells (cells with \geq nuclei) were observed in a fraction of Arf6-KO MEFs. Counting the numbers of both binucleate and multinucleate cells revealed that, as compared with WT MEFs, Arf6-KO MEFs frequently exhibited a multinucleate phenotype indicative of failed cytokinesis (Figure 1G); note that, the percentage of multinucleate cells might be underestimated because cytokinesis failure potentially reduces cell viability. Taken together, these data indicate that Arf6 contributes to cytokinesis.

Characterization of the Arf6–MKLP1 interaction

Approximately a dozen years ago, Boman *et al* (1999) reported that Arf isoforms interacted with MKLP1 in the yeast two-hybrid system. MKLP1 constitutes the centralspindlin complex together with MgcRacGAP/Cyk4 and plays an essential role in cytokinesis by localizing to the Flemming body (Matulienė and Kuriyama, 2002; Mishima *et al*, 2002; Glotzer, 2005). However, the physiological relevance of this interaction has never been clearly established. Given the Flemming body localization of Arf6, its direct binding to MKLP1, and the role of MKLP1 in the centralspindlin complex, we set out to reevaluate the functional implications of the Arf6–MKLP1 interaction.

In humans, there are two splice variants of MKLP1 (Figure 2A); a longer form of 960 amino acids, and a shorter form of 856 amino acids that lacks residues 690–793 of the longer one. We used the shorter form in the following experiments because our preliminary experiments indicated that it is predominant in HeLa cells; the region missing in the shorter form is dispensable for the MKLP1 interaction with Arf6 (see below); and each form localizes to the Flemming body. Hereafter, residue numbering corresponds to the sequence of the shorter form.

We found that a region near the C-terminus of MKLP1 was necessary and sufficient for binding to Arf6; residues 690–807 of MKLP1 are required for binding to Arf6(Q67L), as the 118-

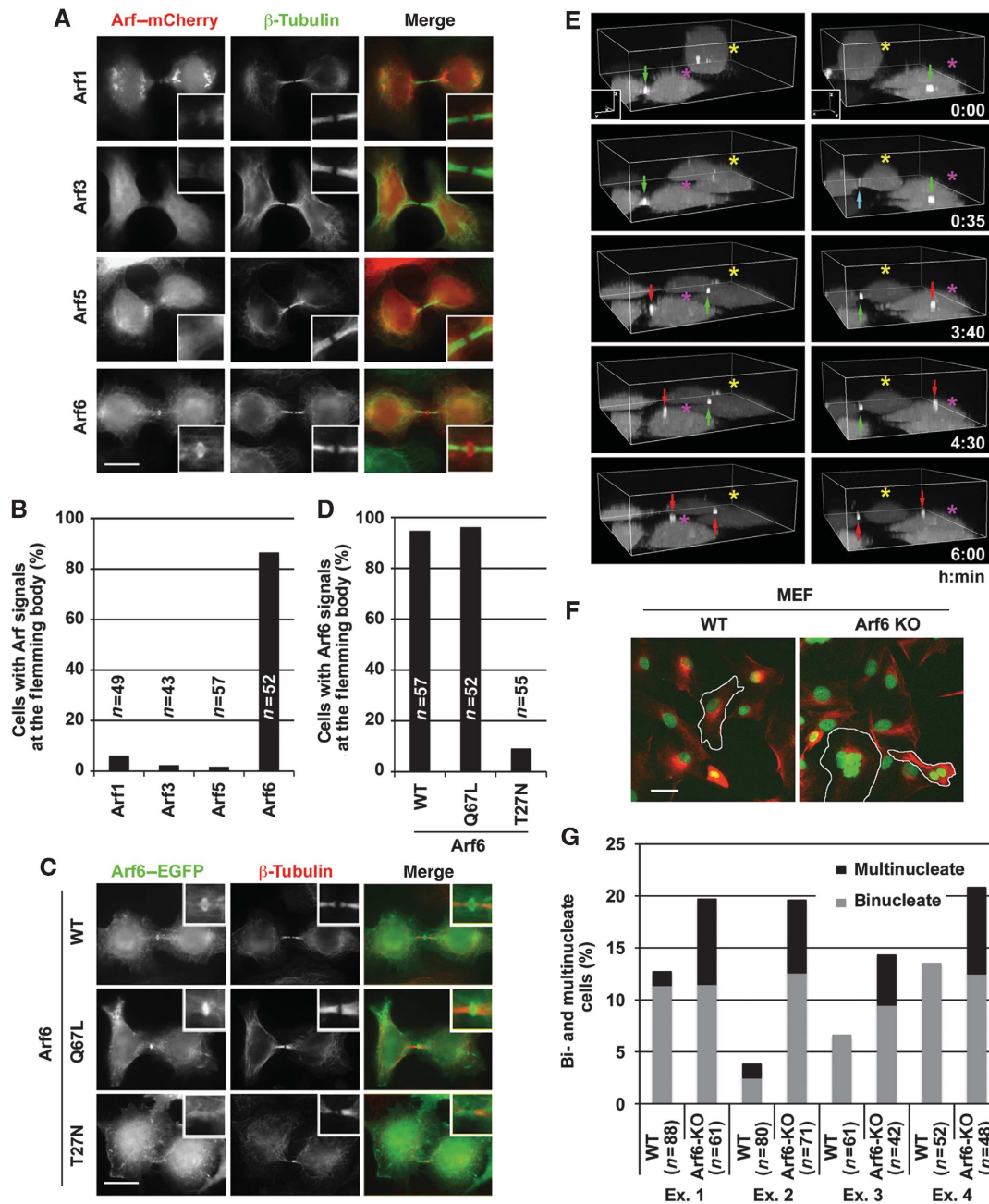


Figure 1 Localization of Arf6 to the Flemming body and phenotype of Arf6-KO cells. (A) HeLa cells transfected with an expression vector for C-terminally mCherry-tagged Arf1, Arf3, Arf5 or Arf6 were stained with anti- β -tubulin antibody. (B) The cells in late cytokinesis phase in the experiment shown in (A) were classified as those with and without Arf-mCherry signals at the Flemming body. Percentages of cells with Arf signals at the Flemming body are expressed as bar graphs. (C) HeLa cells transfected with an expression vector for C-terminally EGFP-tagged Arf6(WT), Arf6(Q67L) or Arf6(T27N) were immunostained for β -tubulin. (D) The cells in late cytokinesis phase in the experiment shown in (C) were classified as those with and without Arf6-EGFP signals at the Flemming body. Percentages of cells with Arf6-EGFP signals are expressed as bar graphs. (E) Images from a three-dimensional time-lapse series of HeLa cells expressing Arf6-EGFP. HeLa cells transfected with an expression vector for Arf6-EGFP were subjected to three-dimensional time-lapse recording. Representative images from Supplementary Movie S1 are shown. Left and right images, rotated by 90° with respect to the z axis, show the same two cells (cells identified by yellow and magenta asterisks). Cyan, green and red arrows indicate Arf6-EGFP signals on the cleavage furrow, Flemming body and its remnant, respectively. (F) Multinucleate phenotype of Arf6-KO MEFs. WT or Arf6-KO MEFs were stained with anti- β -tubulin to reveal microtubules (red) and SYTOX Green to reveal nuclei. (G) Increase in the population of multinucleate cells by Arf6 KO. The cells in the experiment shown in (F) were classified as mono-, bi- and multinucleate (≥ 3 nuclei), and counted. Percentages of bi- and multinucleate cells in four independent examinations are expressed as bar graphs. In (A, C, F); bars, 10 μ m.

residue region fused to GST was sufficient to specifically pull down Arf6(Q67L)-HA from solution. C-terminal truncation of this domain by only 18 residues (MKLP1(690-789)) abolished the ability to pull down Arf6(Q67L)-HA, suggest-

ing a particularly important role for the C-terminal peptide region (Figure 2B). This result was unexpected, because Boman *et al* (1999) reported that the region of MKLP1 C-terminal to residue 720 was dispensable for the

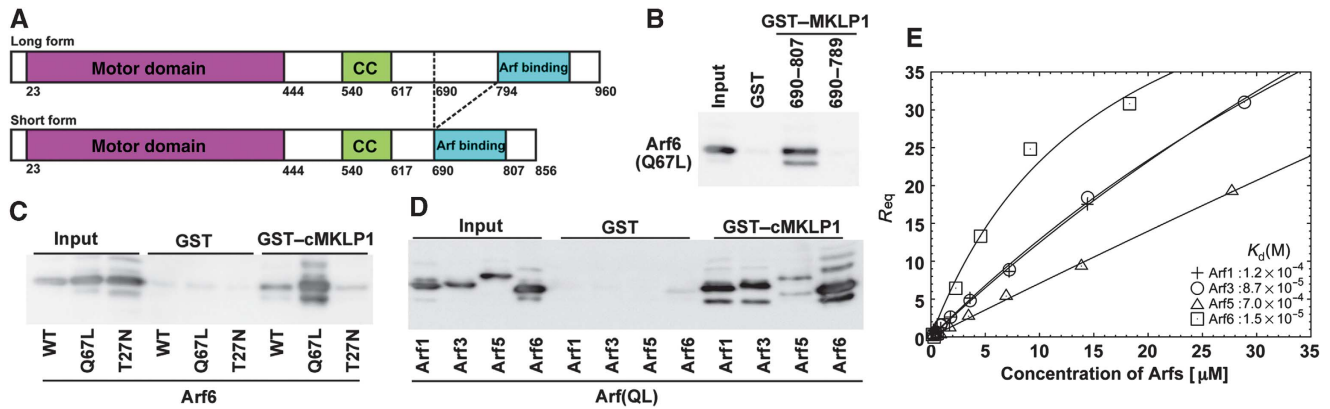


Figure 2 Interaction of MKLP1 with Arf6 *in vitro*. (A) Domain organization and comparison of the structures of the long and short forms of MKLP1. (B) A MKLP1 region encompassing residues 690–807 is responsible for interaction with Arf6. Lysates of HeLa cells expressing C-terminally HA-tagged Arf6(Q67L) were pulled down with GST, GST–MKLP1(690–807) (cMKLP1) or GST–MKLP1(690–789) and subjected to immunoblotting with anti-HA antibody. (C) GTP-bound Arf6 interacts with MKLP1. Lysates of HeLa cells expressing C-terminally HA-tagged Arf6(WT), Arf6(Q67L) or Arf6(T27N) were pulled down with GST or GST–cMKLP1 and subjected to immunoblotting with anti-HA antibody. (D) MKLP1 interacts with all Arf isoforms examined. Lysates of HeLa cells expressing C-terminally HA-tagged Arf1(Q71L), Arf3(Q71L), Arf5(Q71L) or Arf6(Q67L) were pulled down with GST or GST–cMKLP1 and subjected to immunoblotting with anti-HA antibody. (E) SPR analysis of the GST–cMKLP1 interaction with Arf1, Arf3, Arf5 or Arf6. Steady-state resonance (R_{eq}) levels were plotted against each Arf concentration. K_d values were calculated from the fitted curves.

interaction with Arf proteins in yeast two-hybrid assays (discussed below). The Arf-binding domain of MKLP1 (residues 690–807; hereafter referred to as cMKLP1) was sufficient to pull down WT Arf6 or Arf6(Q67L), but not Arf6(T27N) (Figure 2C). This domain also interacted with all Arf isoforms examined (Arf1, Arf3, Arf5 and Arf6) when they were expressed in HeLa cells as constitutively activated proteins (Figure 2D). In addition, surface plasmon resonance (SPR) measurements revealed that cMKLP1 showed the highest affinity to Arf6 (with a K_d value of 15 μ M) among the Arf isoforms examined (Figure 2E); the K_d values of Arf1, Arf3 and Arf5 were 120, 87 and 700 μ M, respectively.

Characterization of Arf6 and MKLP1 on the Flemming body

Next, we compared the localization of endogenous Arf6 and MKLP1 by immunofluorescence analysis. In the majority of cells during cytokinesis, Arf6 and MKLP1 were colocalized at the Flemming body (Figure 3A, lower row). However, some cells exhibited MKLP1, but not Arf6, on the Flemming body (upper row). After closer examination of this variability in results, we noted a strong correlation between the presence of a relatively thick central spindle, suggestive of an earlier phase of cytokinesis, and the absence of Arf6 colocalization with MKLP1 at the Flemming body (Figure 3A, upper row, and Figure 3B). In contrast, cells with a relatively thin central spindle, indicating a later phase of cytokinesis, exhibited colocalization of MKLP1 and Arf6 (Figure 3A, lower row, and Figure 3B). These observations reveal a clear temporal distinction between the times at which MKLP1 and Arf6 are recruited to the Flemming body, and suggest that Arf6 may be recruited to the Flemming body through its binding to MKLP1.

To begin to test this model, we next performed time-lapse analysis of cells co-expressing Arf6–EGFP and mRFP–MKLP1. As shown in Figure 3C and Supplementary Movie S3, mRFP–MKLP1 appeared first on the Flemming body (e.g., see 0:10). After that time point, Arf6–EGFP started accumulating around the ingressing cleavage furrow (0:15

and 0:20). As cytokinesis proceeded, Arf6–EGFP became colocalized with mRFP–MKLP1 on the Flemming body (0:30–1:10). After abscission, Arf6–EGFP and mRFP–MKLP1 were incorporated *en bloc* into one of the daughter cells as a Flemming body remnant (2:00 and 3:00). The time-lapse data indicate that the localization of MKLP1 to the Flemming body is independent of Arf6. To confirm this, we examined the localization of MKLP1 in Arf6–KO MEFs (Figure 3D). MKLP1 was found on the Flemming body in Arf6–KO MEFs (lower row), as in WT MEFs (upper row), confirming Arf6-independent localization of MKLP1 to the Flemming body.

Overall structure of the Arf6–MKLP1 complex

To elucidate the molecular basis for the GTP-dependent interaction of Arf6 with MKLP1, we determined the crystal structure at 3.0 Å resolution of an N-terminal truncation of Arf6(Q67L) (residues 13–175; referred to as Arf6 in the context of X-ray crystallography and small-angle X-ray scattering (SAXS) measurements) in complex with cMKLP1. The crystals of the Arf6–cMKLP1 complex belonged to the space group $P2_1$, and its overall structure is shown in Figure 4A. The asymmetric unit contains four Arf6–cMKLP1 complexes, which are similar to one another: $C\alpha$ -atom root-mean-square deviations (RMSD) of four Arf6(Arg15–Asn172)–cMKLP1(Arg714–Pro799) complexes were between 0.21 and 0.42 Å. The cMKLP1 structure is composed of five strands (β 1:Trp715–His718, β 2:Ala737–Val740, β 3:Lys753–Leu761, β 4:Ile767–Lys778, β 5:Gln784–Lys795), one short helix (α 1:Glu745–Lys750), and a long loop (Pro720–Val734) including the short β A region (Val729–His733) (Supplementary Figure S1A), which is assigned to constitute a small β -sheet in the cMKLP1 homodimer (Figure 4A and B). In the crystal, two cMKLP1 molecules form a homodimeric core held together by two layers of antiparallel β -strand interactions, the major interaction between two β 5-strands and the minor one between two β A-strands of cMKLP1 (Figure 4B). The two Arf6 molecules flank the cMKLP1 homodimer on either side, and do not make direct contact with each other. The overall heterotetramer is a

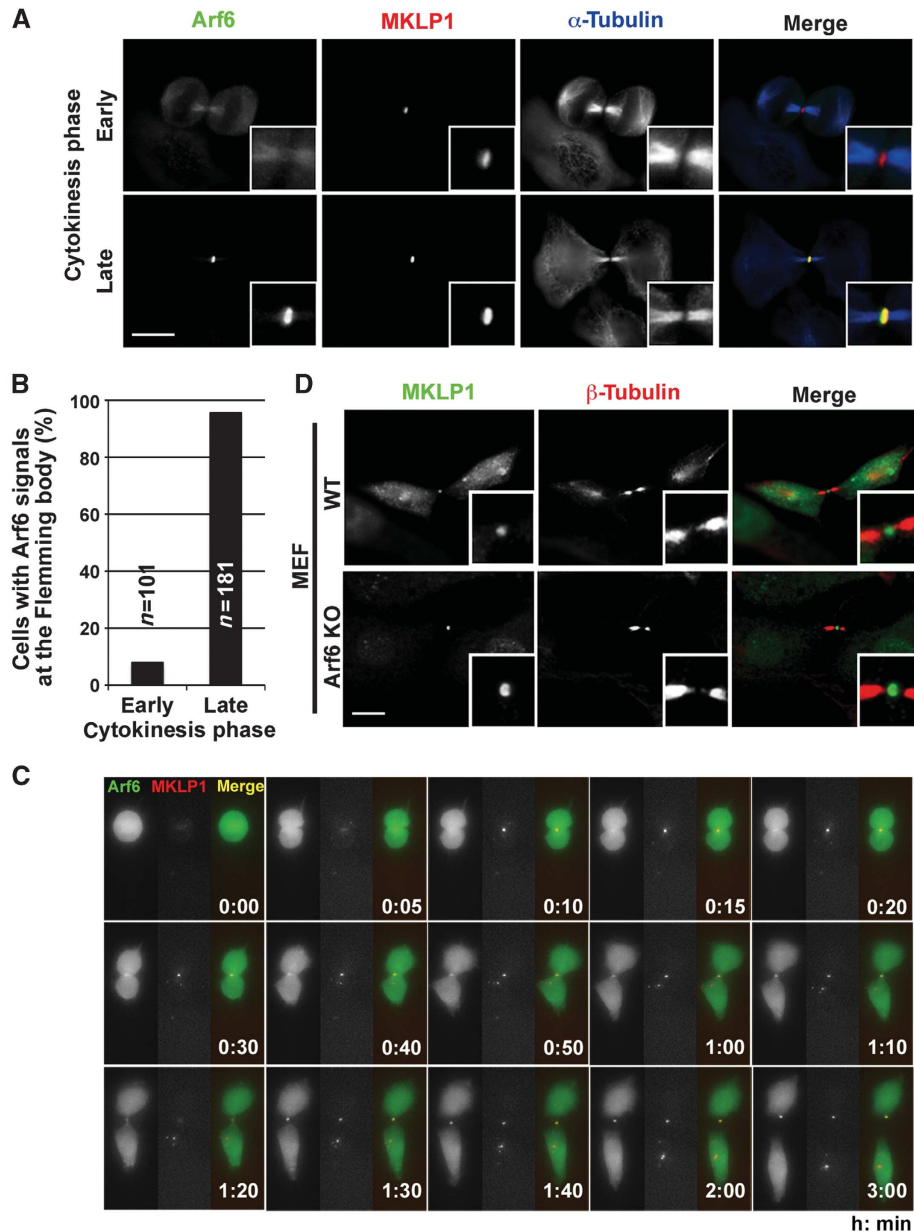


Figure 3 Colocalization of Arf6 and MKLP1 on the Flemming body and Arf6-independent localization of MKLP1. **(A)** Localization of endogenous Arf6 and MKLP1 during cytokinesis. HeLa cells were processed for triple immunostaining for Arf6, MKLP1 and α -tubulin. Note that Arf6 was found on the Flemming body in cells in later phase of cytokinesis (lower row), but not in early phase (upper row), as judged by the width of the central spindle. **(B)** The cells in early and late cytokinesis phases in the examination shown in **(A)** were classified as those with and without Arf6 signals at the Flemming body. Percentages of cells with Arf6–EGFP signals are expressed as bar graphs. **(C)** A time-lapse analysis of HeLa cells expressing Arf6–EGFP and mRFP–MKLP1. An image sequence from Supplementary Movie S3 is shown. HeLa cells transfected with expression vectors for Arf6–EGFP and mRFP–MKLP1 were subjected to time-lapse recording. **(D)** Normal Flemming body localization of MKLP1 in Arf6-KO cells. WT or Arf6-KO MEFs were doubly immunostained for MKLP1 and β -tubulin. In **(A, D)**, bars, 10 μ m.

rectangular parallelepiped with approximate dimensions of 51 Å \times 54 Å \times 96 Å (Figure 4A). One striking feature of the 2:2 Arf6–cMKLP1 heterotetrameric complex is an extended β -sheet of 22 β -strands that spans the entire complex (Figure 4B, a shadowed region). The buried surface areas between Arf6 and cMKLP1, and between cMKLP1 and cMKLP1, are respectively 2241 Å² (surface complementarity (Sc) values: 0.69) and 2080 Å² (Sc values: 0.78) (Supplementary Table S1).

SAXS measurements were carried out in order to detect the formation of the Arf6–cMKLP1 heterotetramer in solution (Figure 4C). Guinier analysis revealed that the molecular

size of the Arf6–cMKLP1 complex changes as a function of its concentration: at higher concentrations, size increased gradually and eventually reached that of the heterotetramer. We also confirmed formation of the cMKLP1 dimer in solution by chemical cross-linking experiments (Supplementary Figure S2), indicating that the heterotetramer in the crystal reflects a form that occurs naturally in solution, rather than a crystallographic artifact. Statistics regarding the structure determination and its refinement are summarized in Supplementary Table S2. Multiple alignment, secondary structure and binding sites of the solved Arf6 and cMKLP1 structures are summarized in Supplementary Figure S1. Unless otherwise noted, we here-

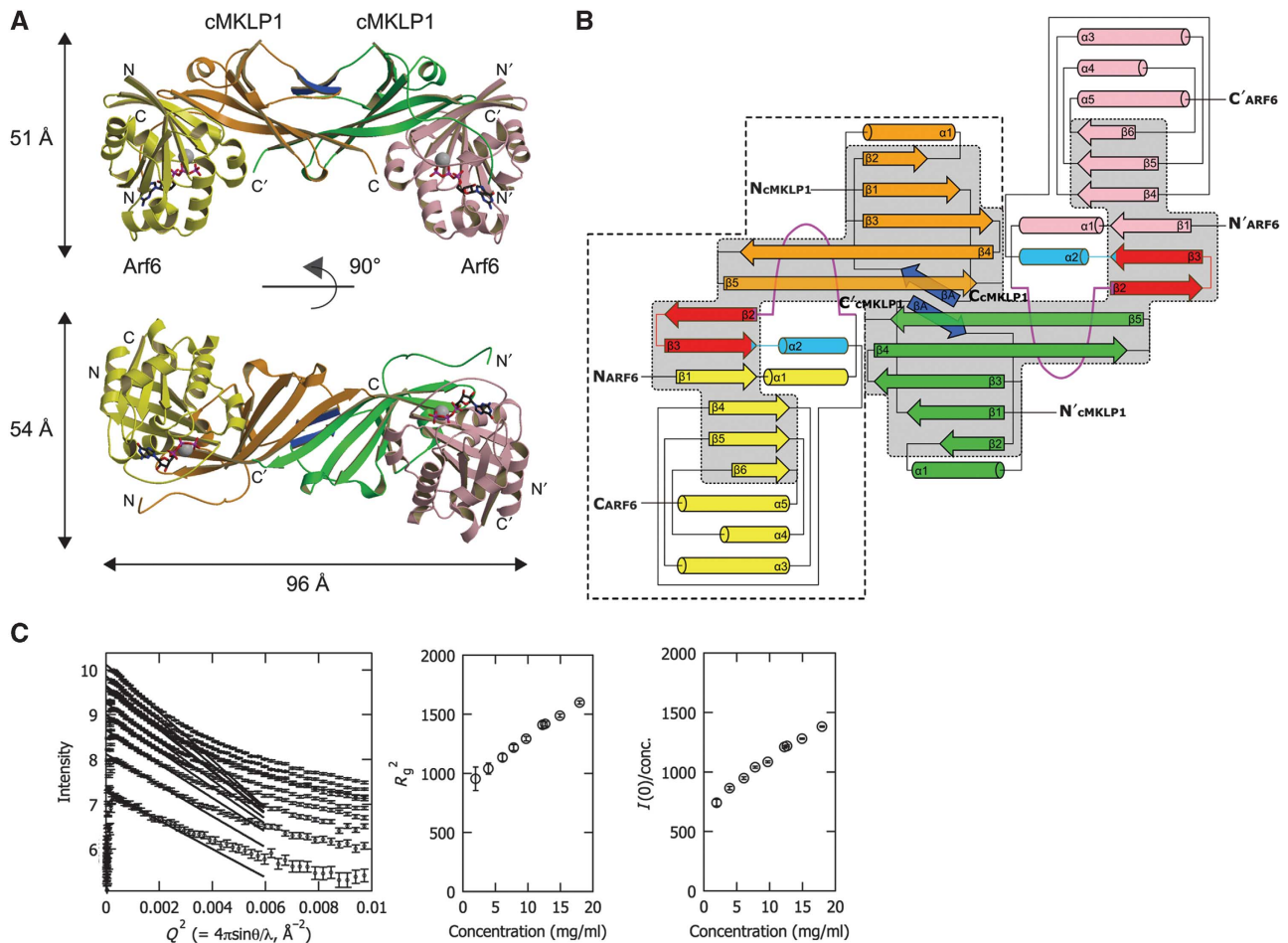


Figure 4 Overall structure and SAXS experiments of the Arf6-cMKLP1 complex. **(A)** Cartoon representation of the heterotetrameric Arf6-cMKLP1 complex is shown in two orthogonal views (side and bottom views). The two Arf6 molecules are coloured in yellow and pink, and the two cMKLP1 molecules are coloured in orange and green. The βA region is coloured in royal blue. In each Arf6 molecule, a GTP molecule and Mg^{2+} are rendered using ball and stick representations. **(B)** Schematic representation of the heterotetrameric Arf6-cMKLP1 complex structure. Arf6 and cMKLP1 proteins are coloured in the same way as in **(A)**. In Arf6, Sw1, interswitch and Sw2 regions were coloured in purple, red and cyan, respectively. One Arf6-cMKLP1 complex is indicated by a dashed line enclosure. The region shaded in grey shows the inter-protein β -sheet extending the entire Arf6-cMKLP1 complex. The βA region coloured in royal blue is represented as making a small β -sheet in the heterotetrameric Arf6-cMKLP1 complex structure. **(C)** Results of SAXS measurements of the Arf6-cMKLP1 complex. Guinier analysis shows that the values of R_g and $I(0)$ increase as a function of the protein concentration. At the lowest concentration, the apparent molecular weight of the complex was estimated to be 26 kDa, slightly lower than the value (33 kDa) calculated from the amino-acid sequence, still suggesting a 1:1 complex. At higher protein concentrations, the apparent molecular weight reaches a value approximately twice that of the heterodimer, indicating a 2:2 heterotetramer in solution. (Left) Guinier plots of Arf6-cMKLP1 complex. (Middle) Concentration dependence of R_g^2 . R_g^2 for the Arf6-cMKLP1 complex was plotted against protein concentration. (Right) Concentration dependence of $I(0)/\text{conc}$ for the Arf6-cMKLP1 complex.

after describe the structural details using chain A (Arf6) and chain B (cMKLP1) in the crystal.

Interactions between Arf6 and cMKLP1

The interface between Arf6 and cMKLP1 in the complex (Figure 5A-C) effectively explains the GTP-dependence of the interaction between the two proteins (Figure 2C) (Boman *et al*, 1999). The GDP-GTP cycle of Arf6 results in significant conformational changes in the switch 1 (Sw1) and switch 2 (Sw2) regions, as well as an additional interswitch toggle that causes a two-residue register shift (Pasqualato *et al*, 2001). In the GDP-bound state, the Sw1 region retracts, resulting in formation of a new β -strand ($\beta 2'$) (Figure 5C) (Ménétrey *et al*, 2000; Pasqualato *et al*, 2001, 2002). In the complex between GTP-bound Arf6 and MKLP1, the $\beta 2'$ -strand overlaps with the

$\beta 5$ -strand of MKLP1 (Figure 5C), thus interfering with the $\beta 2^{\text{Arf6}}-\beta 5^{\text{MKLP1}}$ inter-strand interaction.

Although the hydrophobic residues of Arf6 involved in the Arf6-cMKLP1 interaction are similar to those playing analogous roles in the Arf6-CTA1 and Arf6-JIP4-LZII complexes (Supplementary Figure S1) (O'Neal *et al*, 2005; Isabet *et al*, 2009), a search of the Dali server (Holm and Rosenström, 2010) revealed no structures similar to that of cMKLP1. The unique structure of cMKLP1 allows hydrogen bond interactions between residues in the hydrophobic pocket and Phe47^{Arf6}, Trp62^{Arf6} and Tyr77^{Arf6} (Figure 5D); these residues are conserved in the Arf and Rab GTPase families and are collectively called a 'triad patch' (Merithew *et al*, 2001; Kawasaki *et al*, 2005; Ménétrey *et al*, 2007; Chavrier and Ménétrey, 2010). First, $\beta 2^{\text{Arf6}}$ and $\beta 5^{\text{cMKLP1}}$ make an antiparallel inter-protein β -sheet interaction (Figure 5D and

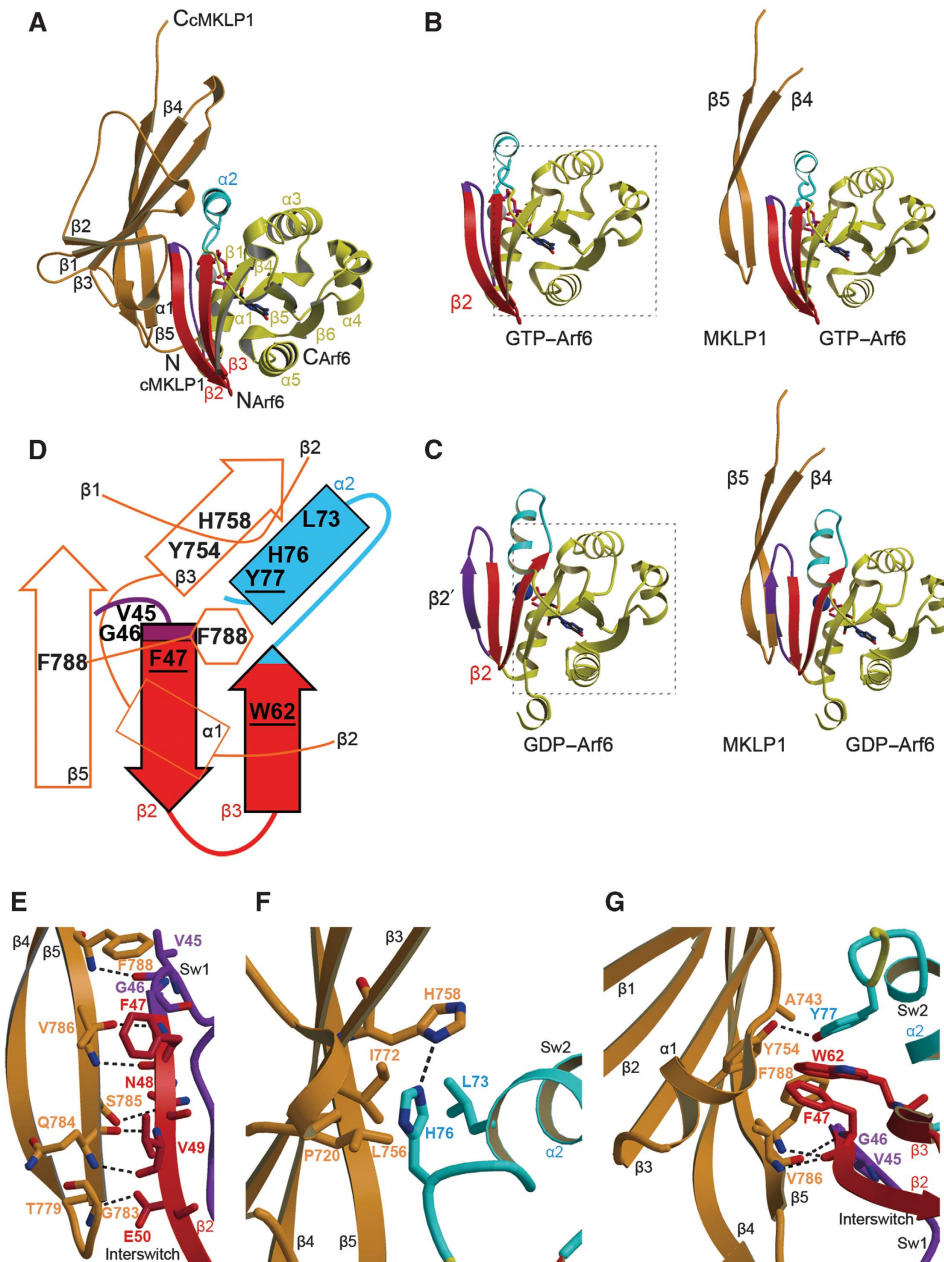


Figure 5 Interaction between Arf6 and cMKLP1 in the complex. (A) Overall structure of a heterodimeric portion of the Arf6-cMKLP1 complex. In this and following panels, cMKLP1 is shown in orange, and Arf6 is shown in yellow with Sw1 (purple), interswitch (red) and Sw2 (cyan). A GTP molecule and Mg^{2+} are represented by ball-and-stick models. (B) Structure of GTP-bound Arf6 alone, or along with β 4- and β 5-strands of cMKLP1. The outer portion of the switch region of Arf6 is enclosed by a dashed square. (C) Structure of GDP-bound Arf6 alone or along with β 4- and β 5-strands of cMKLP1. GDP-Arf6 (PDB 1E0S) is superimposed on GTP-Arf6 in the Arf6-cMKLP1 complex. The conformational changes in the Sw1 region create an additional β -strand (β 2') in the GDP-Arf6 structure. The β 2'-strand of GDP-Arf6 overlaps with the β 5-strand of cMKLP1, making it impossible for GDP-Arf6 to bind cMKLP1. (D) Schematic representation of the interface between Arf6 and cMKLP1. Boxes, arrows and lines represent helices, β -strands and loops, respectively. Phe788^{cMKLP1}, located between Sw1 and Sw2, is labelled in black letters in the hexagon, and two other important residues (Tyr754^{cMKLP1} and His758^{cMKLP1}) on β 3^{cMKLP1} are labelled in black letters. Residues of the effector-binding patch on Arf6 are also labelled in black letters and underlined. (E) Interaction between β 2^{Arf6} and β 5^{cMKLP1}. Secondary structure elements are drawn as cartoons and residues involved in the binding as sticks. Hydrogen bonds are represented as black dashed lines. (F) Interaction between His76^{Arf6} and His758^{cMKLP1}. (G) Interaction between Tyr77^{Arf6} and Tyr754^{cMKLP1} in the hydrophobic triad patch region.

E). Val45^{Arf6}, Phe47^{Arf6} and Val49^{Arf6} make main-chain hydrogen bonds with Phe788^{cMKLP1}, Val786^{cMKLP1} and Gln784^{cMKLP1}, respectively. There are also two side-chain hydrogen bonds (Asn48^{Arf6}-Ser785^{cMKLP1} and Glu50^{Arf6}-Thr779^{cMKLP1}) on this interface. Second, His758^{cMKLP1} makes a hydrogen bond to His76^{Arf6} in the GTP-sensitive

Sw2 (Figure 5F). In this region, the hydrophobic interface includes Leu73^{Arf6} and His76^{Arf6} along with Pro720^{cMKLP1}, Leu756^{cMKLP1}, His758^{cMKLP1} and Ile772^{cMKLP1}. His758^{cMKLP1} interacts with both Leu73^{Arf6} and His76^{Arf6}. A third interface is present at the triad patch formed by Phe47^{Arf6}, Trp62^{Arf6} and Tyr77^{Arf6}. Ala743^{cMKLP1}, Tyr754^{cMKLP1} and

Val786^{cMKLP1} are involved in the interaction with the triad patch, with Phe47^{Arf6} and Tyr77^{Arf6} forming three hydrogen bonds with Val786^{cMKLP1} and Tyr754^{cMKLP1}, respectively (Figure 5G). Trp62^{Arf6} does not engage in hydrogen bond interactions with the residues of effector proteins such as cMKLP1 and CTA1, with the exception of JIP4–LZII. Phe788^{cMKLP1} is located between the Sw1 and Sw2 regions, and its main chain forms a hydrogen bond with that of Val45^{Arf6} (Figure 5D, E and G). The position of the Phe788^{cMKLP1} residue is conserved not only in various Arf effectors but also in Rab effectors (Kawasaki *et al*, 2005).

Arf6 – MKLP1 interaction is required for Arf6 localization to the Flemming body and for cytokinesis

Based on the crystal structure, we introduced mutations into Arf6 and MKLP1 that were designed to perturb the Arf6 – MKLP1 interaction. We focused on the hydrophilic interactions between His76^{Arf6} and His758^{MKLP1} and between Tyr77^{Arf6} and Tyr754^{MKLP1}, because our previous structural study of Arf1 in complex with the GGA1–GAT domain revealed that the corresponding His and Tyr residues of Arf1 are critical for this interaction (Shiba *et al*, 2003). When assayed by pull down with GST–GGA1, GST–JIP4 or GST–FIP4 (Figure 6A), both H76A and Y77A mutations in Arf6(Q67L) abolished the interaction with the GST-fusion proteins. The Y77A mutation also abolished Arf6 binding to MKLP1, when assayed by pull down with GST–cMKLP1 (Figure 6A). However, Arf6(H76A) retained binding to cMKLP1 in the same assay (Figure 6A). When the Y754A or H758A mutation was introduced into cMKLP1, only the former mutation substantially reduced the binding to Arf6 (Figure 6B). These results highlight the conclusion that the interaction between Tyr77^{Arf6} and Tyr754^{MKLP1} is critical to formation of the complex, while that between His76^{Arf6} and His758^{MKLP1} is dispensable (see Discussion).

We then used the Arf6 and MKLP1 mutants to test whether the Arf6 – MKLP1 interaction is crucial for targeting of Arf6 to the Flemming body (Figure 6C and D). When expressed in HeLa cells, Arf6–EGFP (Figure 6C, top row) and the MKLP1 binding-competent Arf6(H76A)–EGFP (middle row) both localized to the Flemming body, like endogenous Arf6. In contrast, the MKLP1 binding-defective Arf6(Y77A) mutant was unable to localize to the Flemming body (bottom row).

We also exploited MKLP1 mutants to determine whether MKLP1 is responsible for recruiting Arf6 to the Flemming body, and whether the loss of Arf6 recruitment is associated with any defects in cytokinesis. We first knocked down endogenous MKLP1 in HeLa cells using siRNAs, and examined the localization of Arf6. A number of control cells exhibited immunostaining for both MKLP1 and Arf6 on the Flemming body or its remnant (Figure 6E, upper panels; green for MKLP1, red for Arf6). Because MKLP1 is essential for Flemming body formation and completion of cytokinesis (Zhu *et al*, 2005), knockdown of MKLP1 abolished not only the MKLP1 signals but also the Arf6 signals on the Flemming body and its remnants, and increased the population of multinucleate cells (Figure 6E, lower panels, and Figure 6H). We determined whether exogenous expression of MKLP1 mutants restored the Flemming body localization of endogenous Arf6 in the context of MKLP1 knockdown (Figure 6F and G). Like WT MKLP1 (Figure 6F, top row), the Arf6 binding-competent MKLP1(H758A) mutant (bottom

row) localized to the Flemming body where it was able to recruit Arf6. In contrast, the Arf6 binding-defective mutant, MKLP1(Y754A) (middle row), could not restore the Flemming body localization of Arf6, even though this mutant itself localized to the Flemming body and was able to recruit MgcRacGAP/Cyk4 to the Flemming body like WT MKLP1 (Supplementary Figure S3). In the rescue experiment, we counted the numbers of bi- and multinucleate cells. Co-transfection of the WT MKLP1 or MKLP1(H758A) vector together with the MKLP1 siRNAs significantly decreased the percentage of multinucleate cells (Figure 6H). In contrast, co-transfection of the MKLP1(Y754A) vector led to a marginal, if any, rescue of the multinucleate phenotype. These observations demonstrate that Arf6 must bind to MKLP1 in order to be recruited to the Flemming body. Furthermore, loss of Arf6 recruitment diminishes the ability to faithfully complete cytokinesis and is correlated with an increase in polyploidy.

Discussion

In this study, we have shown that Arf6 is recruited onto the Flemming body by interacting with MKLP1 prior to abscission during cytokinesis. We have also revealed the molecular basis for the Arf6–MKLP1 interaction, and demonstrated that Arf6 and MKLP1 form a 2:2 heterotetramer containing a unique β -sheet composed of 22 strands that spans the entire heterotetramer. Furthermore, using mutants of Arf6 and MKLP1 designed on the basis of the crystal structure, we have confirmed that the Arf6 – MKLP1 interaction is crucial for proper cytokinesis.

Targeting of Arf6 to the MKLP1-positive Flemming body and its implication in cytokinesis

We have shown that Arf6 targeting to the Flemming body requires its interaction with MKLP1. Arf6 appears first to be concentrated around the ingressing cleavage furrow (Figure 1E, cyan arrow, and Supplementary Movies S1 and S2), and is subsequently recruited onto the Flemming body, where MKLP1 has been previously localized (Figure 3C; Supplementary Movie S3). Together with experiments using Arf6-KO MEFs (Figure 3D) and MKLP1 siRNAs (Figure 6E and F), these observations support a model in which Arf6 targeting to the Flemming body depends on MKLP1.

MKLP1 is required for formation of the Flemming body and completion of cytokinesis (Matulienė and Kuriyama, 2002; Zhu *et al*, 2005). In HeLa cells depleted of MKLP1, not only MKLP1 signals but also Arf6 signals on the Flemming body and its remnants disappear (Figure 6E); the population of multinucleate cells is increased as compared with control siRNA-treated cells (Figure 6H). Both the Arf6 delocalization and multinucleate phenotype are significantly rescued by exogenous expression of MKLP1(WT) or MKLP1(H758A), which retains the Arf6 binding ability. By contrast, expression of the Arf6 binding-defective mutant MKLP1(Y754A) apparently does not restore localization of Arf6 to the Flemming body and marginally rescues the multinucleate phenotype (Figure 6F–H).

The transient association of Arf6 with the cleavage furrow prior to its attachment to the Flemming body (Figures 1E and 3C; Supplementary Movies S1–S3) suggests that Arf6 is activated in a spatially specific manner when it is near the cleavage furrow. In this context, it is noteworthy that

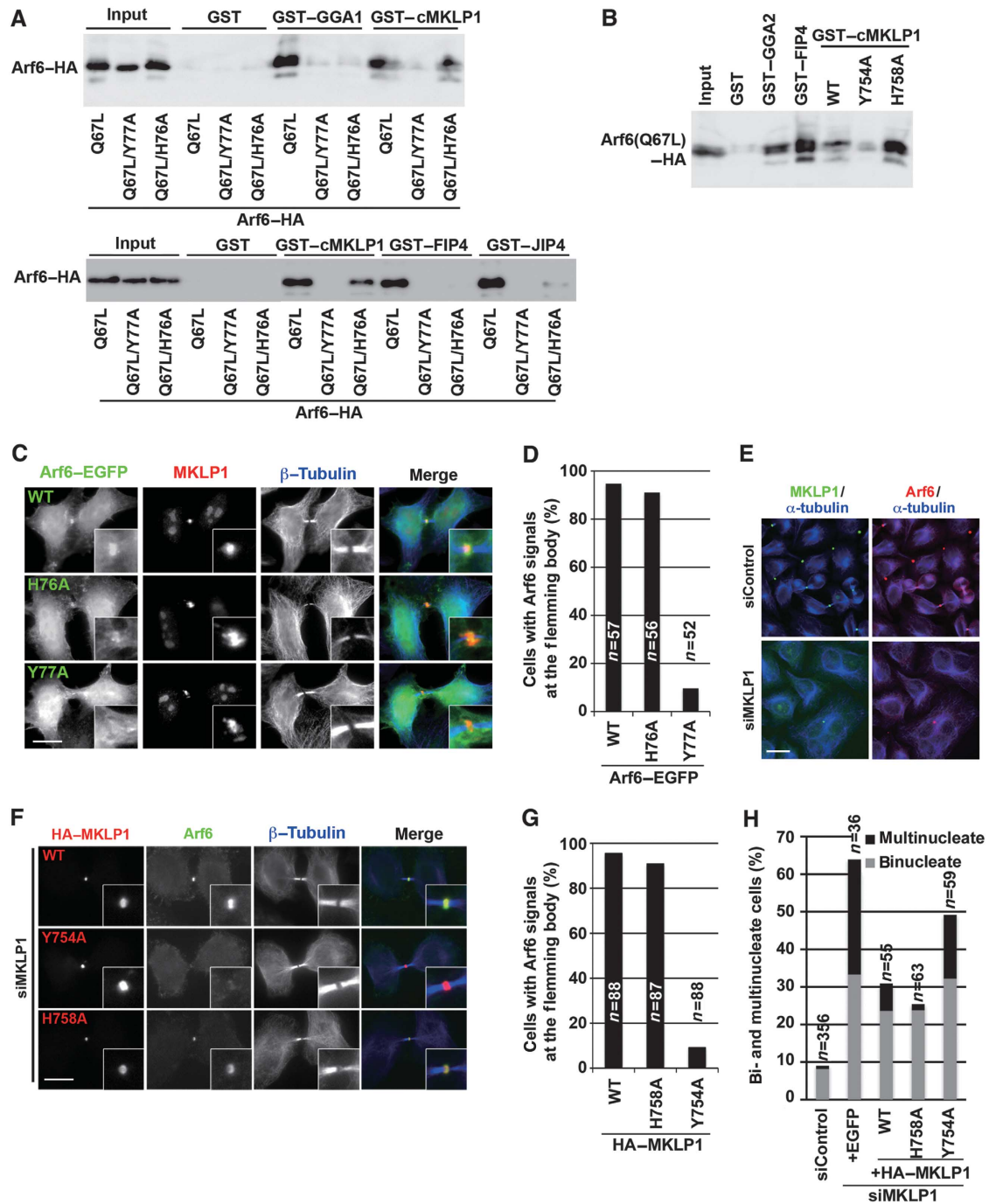


Figure 6 Mutational analysis of the Arf6-MKLP1 interaction and Arf6 localization to the Flemming body. **(A)** Tyr77 of Arf6 is important for Arf6's interaction with MKLP1. Lysates of HeLa cells expressing C-terminally HA-tagged Arf6(Q67L), Arf6(Q67L/Y77A) or Arf6(Q67L/H76A) were pulled down with GST, GST-cMKLP1, GST-GGA1 (GAT), GST-FIP4(484-613) or GST-JIP4(LZII) and subjected to immunoblotting with anti-HA antibody. **(B)** Tyr754 of MKLP1 is important for MKLP1's interaction with Arf6. Lysates of HeLa cells expressing Arf6(Q67L)-HA were pulled down with GST, GST-GGA2(GAT), GST-FIP4(484-613), or WT, Y754A mutant, or H758A mutant of GST-cMKLP1, and subjected to immunoblotting with anti-HA antibody. **(C)** HeLa cells transfected with an expression vector for Arf6(WT)-EGFP, Arf6(H76A)-EGFP or Arf6(Y77A)-EGFP were doubly stained with anti-MKLP1 and anti- β -tubulin. In this experiment, we used the Arf6(H76A) and Arf6(Y77A) mutants in the context of Q67L. **(D)** The cells in late cytokinesis phase in the experiment shown in **(C)** were classified as those with and without Arf6-EGFP signals at the Flemming body. Percentages of cells with Arf6-EGFP signals are expressed as bar graphs. **(E)** MKLP1-dependent localization of Arf6 to the Flemming body. HeLa cells treated with a pool of siRNAs for LacZ (siControl) or MKLP1 were triply immunostained for MKLP1 (green), Arf6 (red) and α -tubulin (blue). Note that a number of the control cells exhibit both the MKLP1 and Arf6 signals on the Flemming body or its remnant. **(F)** HeLa cells were transfected with an expression vector for HA-MKLP1(WT), HA-MKLP1(Y754A) or HA-MKLP1(H758A), together with siRNAs for MKLP1. After 48 h, the cells were fixed and triply immunostained for HA, Arf6 and β -tubulin. **(G)** The cells in late cytokinesis phase in the experiment shown in **(F)** were classified as those with and without Arf6 signals at the Flemming body. Percentages of cells with Arf6-EGFP signals are expressed as bar graphs. **(H)** The control siRNA-treated cells and MKLP1 siRNA-treated cells with exogenous expression of EGFP or an HA-MKLP1 construct in the experiment shown in **(F)** were classified as mono-, bi- and multinucleate (≥ 3 nuclei), and counted. Percentages of bi- and multinucleate cells are expressed as bar graphs. In **(C, E, F)**, bars, 10 μ m.

PtdIns4P 5-kinase and its product, PtdIns(4,5)P₂, accumulate near the cleavage furrow during cytokinesis (Emoto *et al*, 2005; Field *et al*, 2005). Previously, we showed that Arf6 can directly activate PtdIns4P 5-kinase (Honda *et al*, 1999). Because several Arf guanine-nucleotide exchange factors, including EFA6 (exchange factor for Arf6), have PH domains that bind PtdIns(4,5)P₂ (Klarlund *et al*, 2000; Derrien *et al*, 2002), Arf6 can be locally activated by these exchange factors. Thus, it is possible that a small increase in activated Arf6 leads to an amplification in the signal through a localized increase in the phosphoinositide.

How does Arf6 regulate cytokinesis? Arf6 may mediate, by vesicular transport, local delivery of membranes and specific proteins such as the exocyst complex, which allows targeting of vesicles to the plasma membrane domains where ingression and abscission occur (Prekeris and Gould, 2008; Steigemann and Gerlich, 2009). Arf6's role in cytokinesis may be primarily a matter of fine-tuning, since Arf6-KO MEFs retain the capacity for division, although the population of multinucleate cells is elevated.

Comparison of interfaces between GTP-bound Arf6 and effector proteins

The overall structure of the Arf6-cMKLP1 complex, including the β -sheet extending throughout the complex, is unique among Arf-effector complexes. However, comparison between the Arf6-cMKLP1 complex and two Arf6-effector complexes, Arf6-CTA1 and Arf6-JIP4-LZII, whose structures have already been solved (O'Neal *et al*, 2005; Isabet *et al*, 2009), reveals some intriguing similarities (Figure 7A). First, the overall structures of Arf6 in complex with these effectors, as well as the monomeric Arf6-GTP γ S structure (PDB 2J5X), are quite similar to one another, with an RMSD of 0.42 Å over all the C α atoms. Thus, MKLP1 binding, or binding of effectors in general, does not significantly affect the conformation of the activated GTPase. Second, the same interface of Arf6, involving the Sw1, Sw2 and interswitch regions, is employed not only in its complex with cMKLP1 but also in its complexes with CTA1 and JIP4-LZII (Figures 5A and 7A), with the surface areas varying from 1658 Å² (Arf6-JIP4-LZII) to 2241 Å² (Arf6-cMKLP1) (Supplementary Table S1). The corresponding surface regions of Arf1 and Arl (Arf-like) GTPases are also engaged in formation of complexes with their effectors (Figure 7B) (Hanzal-Bayer *et al*, 2002; Panic *et al*, 2003; Shiba *et al*, 2003; Kawasaki *et al*, 2005; Zhang *et al*, 2009). The interfaces between Arf/Arl molecules and their effectors include invariant hydrophobic residues that constitute the hydrophobic pocket, as well as the triad patch involved in effector binding (Kawasaki *et al*, 2005; Ménétrey *et al*, 2007; Chavrier and Ménétrey, 2010). In the Arf6-cMKLP1, Arf6-CTA1 and Arf6-JIP4-LZII complexes, these hydrophobic residues of Arf6 are largely conserved (Figure 5D; Supplementary Figure S1B).

Despite the extensive conservation of Arf6 residues involved in its interaction with effector proteins, the effector structures are quite different from one another (Figures 5A and 7A). The CTA1 interface includes an activation loop between an α -helix and a β -sheet (Kawasaki *et al*, 2005; O'Neal *et al*, 2005), and JIP4-LZII forms an all-helical homodimer that constitutes the interface for Arf6 (Isabet *et al*, 2009). The cMKLP1 interface is mainly composed of five β -strands (Figures 4B and 5A). The two hydrogen bonds

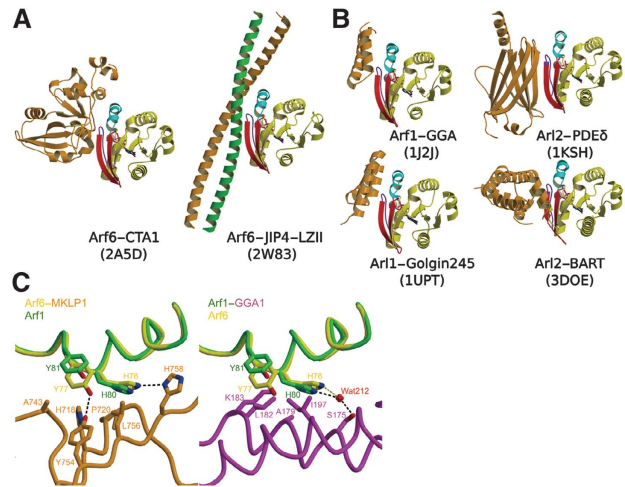


Figure 7 Structures of complexes of Arf and Arl proteins with their effectors. (A) Interfaces of Arf6 for CTA1 and JIP4-LZII. The orientation of the Arf6 molecule is the same as that in the Arf6-cMKLP1 complex (Figure 5A), and colouring of Arf6 regions and representation of a GTP molecule and Mg²⁺ are the same as in Figure 5A. CTA1 is coloured in orange (left), and helices of the JIP4-LZII dimer are in orange and green (right). (B) Structures of Arf/Arl complexes with their effector proteins as indicated. The orientation of the Arf/Arl molecule is the same as in the Arf6-cMKLP1 complex (Figure 5A); colouring of Arf/Arl regions and representation of GTP and Mg²⁺ are the same as in Figure 5A. The effector proteins are coloured in orange. (C) The interfaces between Arf6/Arf1 and cMKLP1 (left) and between Arf1/Arf6 and the GGA1-GAT domain (right) are shown. Arf6, Arf1, cMKLP1 and GGA1 are coloured in yellow, green, orange and magenta, respectively. In the left panel, the Arf1 molecule in the Arf1-GGA1 complex (PDB 1J2J) is superimposed on Arf6 of the Arf6-cMKLP1 complex, and black dashed lines indicate hydrogen bonds between Arf6 and cMKLP1. In the right panel, a red ball represents a water molecule (Wat212), black dashed lines indicate hydrogen bonds between GGA1 and Arf1 via Wat212, and a yellow dashed line indicates a hydrogen bond between Arf6 and Wat212.

between Phe47^{Arf6} within β 2 and Val786^{cMKLP1} within β 5 make major contributions to the β -strand interaction (Figure 5E and G). In contrast, Phe47^{Arf6} forms a water-mediated hydrogen bond with Gly118^{CTA1} and a hydrogen bond with Lys423^{JIP4} in only one (chains A/C) of the two complexes in the crystal (O'Neal *et al*, 2005; Isabet *et al*, 2009).

We have shown that the interaction between Tyr77^{Arf6} and Tyr754^{cMKLP1} is more critical for the formation and function of the Arf6-cMKLP1 complex than that between His76^{Arf6} and His758^{cMKLP1} (Figure 6). Tyr77^{Arf6} is assigned as one of the key residues in the invariant hydrophobic triad (Figure 5D) (Kawasaki *et al*, 2005; Ménétrey *et al*, 2007; Chavrier and Ménétrey, 2010) and participates in direct hydrogen bond formation in the Arf6-cMKLP1 complex (the distance between OH atom of Tyr77^{Arf6} and OH atom of Tyr754^{cMKLP1} is 2.8 Å) (Figure 7C, left), whereas it makes a water-mediated hydrogen bond with Pro92^{CTA1} or Asp419^{JIP4}. His76^{Arf6} that has been identified as a residue of the hydrophobic pocket (Kawasaki *et al*, 2005; Ménétrey *et al*, 2007; Chavrier and Ménétrey, 2010) contributes less significantly to the cMKLP1 interaction than does Tyr77^{Arf6}. However, His76^{Arf6} is indeed important for interactions with other effectors; it is one of the residues that form an effector-

binding patch in the Arf6-CTA1 complex (O'Neal *et al*, 2005), and its carbonyl main chain forms an additional hydrogen bond to Lys41^{7JIP4} to further stabilize the Arf6-JIP4-LZII complex (Isabet *et al*, 2009). His76^{Arf6} (His80^{Arf1}) is critical for the Arf interaction with the GGA1-GAT domain (Figure 7C, right). Superimposing the Arf6 structure on the Arf1-GGA1 complex (PDB 1J2J) (Shiba *et al*, 2003) reveals that His76^{Arf6} forms a water-mediated hydrogen bond with Ser175^{GGA1}, whereas Tyr77^{Arf6} can interact with GGA1 only through van der Waals contacts (Figure 7C, right). Thus, when His76^{Arf6} is mutated, the van der Waals contacts of Tyr77^{Arf6} are not sufficient to maintain the Arf6-GGA1 complex. By contrast, in the Arf6-cMKLP1 complex, His76^{Arf6} forms a hydrogen bond with His758^{cMKLP1}, whereas Tyr77^{Arf6} interacts with cMKLP1 through a hydrogen bond with Tyr754^{cMKLP1} and through van der Waals contacts. It is therefore likely that the Tyr77^{Arf6}-Tyr754^{cMKLP1} interaction is able to maintain the Arf6-cMKLP1 complex in the absence of the His76^{Arf6}-His785^{cMKLP1} interaction (Figure 6A and B).

We then compared the Arf6-cMKLP1 structure with that of the Arl2-PDE δ complex (Hanzal-Bayer *et al*, 2002). Although β -sheet interactions contribute to formation of both complexes, a parallel β -sheet is formed in the Arl2-PDE δ complex (β 2^{Arl2}- β 7^{PDE δ}) (Figure 7B), while an antiparallel one in Arf6-cMKLP1 (β 2^{Arf6}- β 5^{cMKLP1}) (Figure 5). Furthermore, the residue that corresponds to His76^{Arf6} is conserved in all Arfs, but not in Arl proteins, including Arl2 (Supplementary Figure S1B). In the crystal structures, Asn79^{Arl2} (corresponding to the His76^{Arf6} position) has hydrophobic interactions with Phe96^{PDE δ} and Ile98^{PDE δ} , and Tyr80^{Arl2} (corresponding to Tyr77^{Arf6}) makes a hydrogen bond with the main chain of Phe96^{PDE δ} . Overall, the molecular basis for the complex formation of Arf6-cMKLP1 is different from that of Arl2-PDE δ .

Model for function of the Arf6-MKLP1 complex

We propose that two molecules of activated Arf6 bind to each homodimer of MKLP1 at the Flemming body, resulting in a higher fidelity of cytokinesis. In the crystal, the Arf6-MKLP1 complex consists of a heterotetramer with two Arf6 and two MKLP1 molecules (Figure 4A). Our SAXS (Figure 4C) and cross-linking data (Supplementary Figure S2) support the conclusion that the heterotetramer is present in solution and is not a crystallographic artifact. We speculate that the heterotetrameric Arf6-MKLP1 complex is anchored to membranes, most likely the plasma membrane, through myristoylated N-terminal helices of the two Arf6 molecules (Figure 8A). Note that each of the Arf6 molecules in the heterotetramer are positioned such that their N-terminal helices and covalently attached myristates, each of which are involved in membrane binding, are optimally positioned, pointed away from the rest of the MKLP1 molecule and towards a membrane anchoring site for the activated GTPase. This model suggests that on the Flemming body, Arf6 and the C-terminal domain of MKLP1 are positioned close to the membrane, while the N-terminal motor domain associates with the microtubule bundle (Figure 8A). The simultaneous association of the Arf6-MKLP1 complex with the microtubule bundle and the plasma membrane is indirectly supported by the observation that, after abscission, the Flemming body remnant migrates up along (probably beneath) the cortical membrane of a daughter cell (Figure 1E,

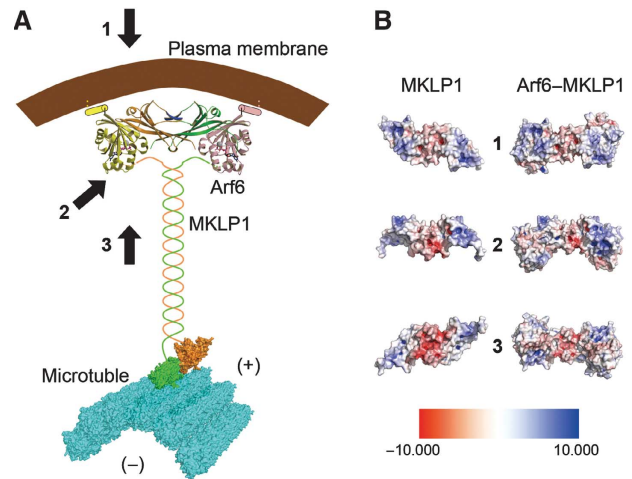


Figure 8 Model for membrane and microtubule interactions of the Arf6-MKLP1 complex and its electrostatic potential. **(A)** Model for simultaneous interactions of the heterotetrameric Arf6-MKLP1 complex with membrane and microtubules. During cytokinesis, the complex is likely to be anchored to the plasma membrane, and the MKLP1 motor domain is associated with microtubules of the Flemming body. MKLP1 molecules are coloured in green, orange and royal blue. Arf6 molecules are coloured in yellow and pink with schematically represented N-terminal helices, and their myristates are represented as dotted lines. The numbered arrows indicate viewing directions in **(B)**. **(B)** The electrostatic potential representations of MKLP1 and the Arf6-cMKLP1 complex, coloured according to the calculated surface potential, from -10 kT (red) to 10 kT (blue).

red arrows, and Supplementary Movie S1). The electrostatic surface potential map indicates that Arf6 binding to cMKLP1 enlarges its positively charged surface area relative to cMKLP1 alone (Figure 8B, top panels), making more favourable the association between the positively charged protein surface and negatively charged membrane surface. In this context, it is noteworthy that, as described above, PtdIns(4,5) P_2 accumulates around the cleavage furrow during cytokinesis (Emoto *et al*, 2005; Field *et al*, 2005). It will be therefore important to address whether membrane association of the Arf6-MKLP1 complex is under the regulation of changes in the local lipid composition.

There is another possible function of Arf6 at the Flemming body, although not mutually exclusive with the above model. We previously showed that Arf6-depleted cells accumulate FIP3-positive vesicles around the central spindle in early cytokinesis phase, but then failed to proceed with cytokinesis (Takahashi *et al*, 2011). On the other hand, Montagnac *et al* (2009) have proposed that FIP3-positive endosomal vesicles are trafficked towards the plus ends of the central spindle in a kinesin-1/JIP4-dependent manner. Together with the results in the present study, it is therefore possible that, through interacting with FIP3/4 or JIP3/4, Arf6 at the Flemming body serves as an acceptor for incoming endosomal vesicles, the accumulation of which is required for subsequent abscission (Prekeris and Gould, 2008; Steigemann and Gerlich, 2009; Schiel *et al*, 2011). We previously showed that, after abscission, FIP3 as well as Arf6 is incorporated into one of the daughter cells as a Flemming body remnant (Takahashi *et al*, 2011), supporting an interaction of FIP3 with Arf6 on the Flemming body. However, because an Arf6 molecule is unlikely to bind simultaneously to MKLP1 and FIP3/4 or JIP3/4, this model requires local switching of the Arf6 partners.

Materials and methods

Cell culture, DNA transfection and RNA interference

MEFs were established from WT and Arf6-KO mice (Suzuki *et al*, 2006). Knockdown of MKLP1 was performed in a previously described manner (Ishizaki *et al*, 2008; Man *et al*, 2011). Briefly, a 3'-untranslated region of human MKLP1 cDNA was amplified using a primer set (5'-GCCATGAAGTACAGTCCCAG-3' and 5'-AGT GCTTTTGATTTAATCTTTTGG-3') and used for preparation of a pool of siRNAs with BLOCK-iT RNAi TOPO Transcription and Dicer RNAi kits (Invitrogen). HeLa cells grown on coverslips in a 3.5-cm dish were transfected with siRNAs (0.25 µg/dish) using Lipofectamine 2000 (Invitrogen) and incubated for 4 h. The medium was then changed, and 48 h after transfection the cells were processed for immunofluorescence and immunoblot analyses. For recovery experiments, a pCAG vector for HA-MKLP1 (0.4 µg/dish) was transfected into cells along with the siRNAs.

Pull down assay

Escherichia coli BL21(DE3) cells transformed with the GST-fusion protein vector were treated with 0.1 mM IPTG for 4 h at 25°C to induce protein expression, lysed and used to purify the recombinant protein with glutathione–Sephacryl 4B beads (GE Healthcare). Lysates of HeLa cells expressing HA-tagged Arf were prepared as described previously (Takatsu *et al*, 2002; Shiba *et al*, 2006; Man *et al*, 2011) and incubated at 4°C for 2 h with the GST-fusion protein coupled to glutathione–Sephacryl 4B beads. The beads were washed four times with Hepes-based buffer, and subjected to SDS–PAGE and immunoblotting.

Immunofluorescence microscopy and time-lapse imaging

For immunofluorescence microscopy, cells were fixed, permeabilized and immunostained as described previously (Shin *et al*, 2004; Ishizaki *et al*, 2008; Takahashi *et al*, 2011). For time-lapse imaging, cells were placed on a microscope stage that had been pre-warmed at 37°C in a 5% CO₂ atmosphere. The cells were observed using EM-CCDs (Hamamatsu Photonics) on an Axiovert 200M microscope (Carl Zeiss). Images were acquired sequentially every 5 min and analysed using IP-Lab™ ver. 4.0.8 (SOLUTION Systems). Three-dimensional time-lapse imaging was performed using an A1RMP confocal microscope (Nikon).

Crystallization and data collection

Crystallization conditions were initially searched using a crystallization robot (Hiraki *et al*, 2006). The final crystallization conditions for the native and Se–Met cMKLP1–Arf6 complexes were 12% w/v PEG 4000, 0.1 M sodium cacodylate, pH 6.5, 50 mM (NH₄)₂SO₄, 5 mM MgCl₂, 0.25–1.0% v/v ethyl acetate at 20°C. Crystals were then transferred to cryosolutions containing 20% glycerol or ethylene glycol. Data were collected at 100 K using beamline BL-5A or AR-NW12A at the Photon Factory, KEK (Tsukuba, Japan). All diffraction data were processed using HKL2000 (Otwinowski and Minor, 1997). Data collection and processing statistics are summarized in Supplementary Table S2.

Phasing, model building and refinement

The MR-SAD method was applied to solving the Se–Met data of the Arf6–cMKLP1 complex (Schuermann and Tanner, 2003). An Arf6 structure (PDB 2J5X) was used as a molecular replacement (MR) template. The MR analysis was performed using Phaser (CCP4, 1994). The initial model was built using Buccaneer (CCP4, 1994) after density modification by DM or Parrot (CCP4, 1994). MR was

applied to the native data using the Arf6–cMKLP1 complex structure generated from the Se–Met data, using Phaser. NCS restraints were applied during refinement before the final refinement. The final model was built with Coot (Emsley *et al*, 2010) and refined using Phenix (Adams *et al*, 2010) and REFMAC5 (CCP4, 1994) with TLS (Painter and Merritt, 2006) (Supplementary Figure S5) at 3.0 Å resolution. There are four Arf6 (chains A, C, E and G)–cMKLP1 (chains B, D, F and H) complexes in the asymmetric unit. Finally, the refined structure of the Arf6–cMKLP1 complex was validated by MolProbity (Chen *et al*, 2010). The refinement statistics are summarized in Supplementary Table S2. Figures (4, 5, 7 and 8) were drawn using MOLSCRIPT (Kraulis, 1991), Raster3D (Merritt and Bacon, 1997), Pymol (Schrodinger, 2010) and CCP4MG (Potterton *et al*, 2004).

SAXS experiments

SAXS data were collected at BL-10C at the Photon Factory, KEK using bovine serum albumin (BSA) as a calibration standard. Arf6–cMKLP1 and BSA were dissolved in Buffer A. Protein concentrations varied within a range of 2.0–21.3 mg/ml. The sample cell was 50 µl in volume and had a 1-mm path-length. The X-ray wavelength was 1.488 Å, and the scattered X-rays were recorded by PSPC with an exposure of 600 s at 900 mm from the sample position. Guinier analysis was carried out by the standard procedure (Guinier and Fournet, 1955). Data processing was performed using the IGOR Pro data analysis program (Wavemetrics). X-ray scattering intensities in the small-angle region are given as $I(Q) = I(0) \exp(-R_g^2 Q^2/3)$, where Q and $I(0)$ are momentum transfer and the intensity at zero scattering angle, respectively. Q is defined by $Q = 4\pi \sin\theta/\lambda$, where 2θ and λ are the scattering angle and the wavelength of the X-rays, respectively. The radius of gyration (R_g) was obtained from the slope of the Guinier plot, which is a plot of $\ln[I(Q)]$ against Q^2 .

Accession code

Protein Data Bank: Coordinates for the Arf6–MKLP1 complex have been deposited with accession code 3VHX.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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