Ciliary targeting motif VxPx directs assembly of a trafficking module through Arf4

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Dysfunctions of primary cilia and cilia-derived sensory organelles underlie a multitude of human disorders, including retinal degeneration, yet membrane targeting to the cilium remains poorly understood. Here, we show that the newly identified ciliary targeting VxPx motif present in rhodopsin binds the small GTPase Arf4 and regulates its association with the trans-Golgi network (TGN), which is the site of assembly and function of a ciliary targeting complex. This complex is comprised of two small GTPases, Arf4 and Rab11, the Rab11/Arf effector FIP3, and the Arf GTPase-activating protein ASAP1. ASAP1 mediates GTP hydrolysis on Arf4 and functions as an Arf4 effector that regulates budding of post-TGN carriers, along with FIP3 and Rab11. The Arf4 mutant I46D, impaired in ASAP1-mediated GTP hydrolysis, causes aberrant rhodopsin trafficking and cytoskeletal and morphological defects resulting in retinal degeneration in transgenic animals. As the VxPx motif is present in other ciliary membrane proteins, the Arf4-based targeting complex is most likely a part of conserved machinery involved in the selection and packaging of the cargo destined for delivery to the cilium.

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Introduction

Primary cilia are specialized organelles in vertebrate cells, which house arrays of signal-transduction modules that sense a wide variety of signals. Despite ciliary involvement in a

broad range of human diseases (ciliopathies), including retinal degeneration, polycystic kidney disease, Bardet-Biedl syndrome and neural tube defects (Rosenbaum and Witman, 2002; Singla and Reiter, 2006; Christensen *et al*, 2007; Fliegauf *et al*, 2007), the molecular basis for selection and packaging of sensory receptors and membrane cargo targeted to the primary cilia and cilia-derived sensory organelles remains elusive. Nonetheless, the directed delivery of ciliary cargo most likely involves vesicular transport regulated by the small GTPases of the Rab and Arf families, which have a central function in coordinating intracellular membrane trafficking (Gillingham and Munro, 2007; Leroux, 2007).

Upon GTP binding, Rabs recruit a multitude of effectors that organize membrane domains involved in the tethering of membranes to other membranes and to cytoskeletal elements, thus conferring directionality to membrane traffic (Zerial and McBride, 2001; Grosshans *et al*, 2006; Markgraf *et al*, 2007). Rab8 (Wandinger-Ness and Deretic, 2008) has recently emerged as a central regulator of the biogenesis of primary cilia (Nachury *et al*, 2007; Yoshimura *et al*, 2007; Omori *et al*, 2008). In retinal rod photoreceptors, Rab8 regulates the biogenesis of cilia-derived sensory organelles, the rod outer segments (ROSs), by regulating trafficking of post-TGN (trans-Golgi network) rhodopsin transport carriers (RTCs) (Deretic *et al*, 1995; Moritz *et al*, 2001). RTCs travel vectorially through the cell body, the rod inner segment (RIS) (Figure 1A), to the base of the cilium and fuse with the specialized domain separating the ciliary membrane from the surrounding RIS plasma membrane (Deretic and Papermaster, 1991), thereby regulating the replenishment of light-sensitive ROS membranes, the major component of which is rhodopsin.

The continuous renewal of ROS membranes, which are removed through daily shedding and phagocytosis by retinal pigment epithelial cells, necessitates the hypertrophy of the Rab8/RTC-mediated pathway and renders photoreceptors vulnerable to mutations that affect membrane trafficking (Deretic, 2006). Among the mutations with the most severe phenotypes are those that alter the rhodopsin C-terminal VxPx targeting motif (Deretic *et al*, 1998), which causes rapid retinal degeneration and blindness in a group of diseases known as autosomal dominant retinitis pigmentosa (adRP) (Berson *et al*, 2002). Within the targeting motif, V345 and P347 are the primary sites of C-terminal adRP mutations involving single amino-acid substitutions (<http://www.retina-international.com/sci-news/rhomut.htm>).

Abundant evidence points to the role of the VxPx motif in rhodopsin sorting into RTCs at the TGN, and delivery to the cilium and the ROS (Deretic *et al*, 1998; Green *et al*, 2000; Tam *et al*, 2000; Shi *et al*, 2004). Similar to rhodopsin, polycystin-2 (PC2), a protein affected in autosomal dominant polycystic kidney disease, and the cyclic nucleotide-gated (CNG) channel CNGB1b subunit in olfactory neurons, also possess a functional ciliary targeting VxPx motif (Geng *et al*, 2006; Jenkins *et al*, 2006).

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Figure 1 Rhodopsin VxPx motif engages Arf4 at the TGN to regulate RTC budding. **(A)** Diagram of the photoreceptor cell. RTCs travel from the Golgi/TGN (arrow) to the base of the cilium (C). BB, basal body; M, mitochondria; N, nucleus; Sy, synapse; AJ, adherens junction. **(B)** adRP mutations in the rhodopsin VxPx targeting motif are indicated with asterisks. **(C)** Frog retinas were pulse-labelled for 60 min and retinal PNS was incubated for 30 min with 50 μM peptides, as indicated in the panel, prior to a 2 h cell-free chase. Radiolabelled membrane proteins from two retinas were fractionated into Golgi/TGN and RTCs and analysed by SDS-PAGE and autoradiography ($[^{35}\text{S}]\text{Rh}$). **(D)** Immunoblots of a duplicate gel were probed successively with anti-Arf3 and anti-Arf4, which specifically recognizes an ~20-kDa protein (left panel) and quantified (lower right panels). **(E)** Golgi/TGN and RTCs were separated on sucrose density gradients, and distribution of rhodopsin ($[^{35}\text{S}]\text{Rh}$) was visualized as in (C). Duplicate gels were blotted and probed with antibodies, as indicated in the panel (partial loss of the Rab11 band in RTC fractions is due to a cut to allow Arf3 detection on the same immunoblot). The differential distribution of calnexin, galactosyltransferase (Gal T) and sialyltransferase (Sial T), markers for the ER, trans-Golgi and TGN, respectively, in membranes from identically run sucrose gradients, as reported (Deretic and Papermaster, 1991, 1993; Deretic *et al*, 2004). **(F)** Quantification of autoradiograms and immunoblots from (E).

The VxPx targeting motif of rhodopsin binds directly and specifically to Arf4, suggesting a distinct role for this Arf in

the generation of RTCs (Deretic *et al*, 2005). The Arf family members generally regulate membrane trafficking, lipid metabolism, organelle morphology and cytoskeleton dynamics (Nie *et al*, 2003; Volpicelli-Daley *et al*, 2005; D'Souza-Schorey and Chavrier, 2006; Kahn *et al*, 2006); however, the function of Arfs 4 and 5 remains poorly understood. The class II Golgi Arfs, Arf4 and Arf5, bind Arfophilins/FIPs, the effectors of the small GTPase Rab11 (Shin *et al*, 1999; Meyers and Prekeris, 2002). Arfophilin 1/FIP3 coordinates Arf- and Rab11-regulated trafficking pathways (Hickson *et al*, 2003; Fielding *et al*, 2005; Eathiraj *et al*, 2006). Rab11 has been localized to the photoreceptor TGN and the RTCs (Deretic *et al*, 1996), and has been implicated in the regulation of plasma membrane trafficking through the recycling endosomes (Prekeris *et al*, 2000; Pasqualato *et al*, 2004) or through the TGN (Urbe *et al*, 1993; Chen *et al*, 1998; de Graaf *et al*, 2004).

Cargo recruitment through Arf4 most likely depends on GTP hydrolysis mediated by Arf GTPase-activating proteins (GAPs) (Inoue and Randazzo, 2007). Arf GAPs are essential for coupling the proof-reading of cargo incorporation to carrier budding, and are often incorporated into protein coats (Lee *et al*, 2004). ASAPs are Arf GAPs containing ankyrin repeats, pleckstrin-homology (PH), proline-rich and SH3 domains that belong to a family of multifunctional scaffold proteins regulating membrane trafficking and actin remodelling (Randazzo and Hirsch, 2004; Nie and Randazzo, 2006). The BAR (Bin/amphiphysin/Rvs) domain (Peter *et al*, 2004), at the N terminus of ASAP1, mediates homodimerization and, in concert with the GAP domain, functions as a coincidence detector sensing the membrane phospholipid composition and the presence of GTP-Arf and responding by increasing membrane curvature (Nie *et al*, 2006). Thus, ASAP1 functions both as an Arf GAP and an Arf effector that may regulate carrier budding (Luo *et al*, 2007).

In the present study, we identify components of the molecular machinery that assembles at the TGN upon binding of Arf4 to the conserved rhodopsin C-terminal VxPx motif and examine the possibility that this machinery regulates ciliary targeting, a specialized form of the TGN-to-plasma membrane trafficking. We report that Arf4, ASAP1, FIP3 and Rab11 interact to form a complex that regulates budding of post-TGN carriers targeted to the primary cilia.

Results

The association of Arf4 with TGN membranes is dependent on its access to the VxPx targeting motif of rhodopsin

To gain further insight into interactions of the rhodopsin VxPx targeting motif (Figure 1B) with Arf4 at the TGN, we employed our established cell-free assay, which reconstitutes RTC budding in the retinal post-nuclear supernatant (PNS), enriched in biosynthetic membranes that mediate rhodopsin trafficking, in an ATP-, GTP-, cytosol-, Rab- and Arf4-dependent manner (Deretic *et al*, 1996, 1998, 2005). Consistent with our published reports, the peptide corresponding to the α helix 3 unique to Arf4, and the rhodopsin C-terminal peptide inhibited RTC budding *in vitro*, whereas the peptide bearing the adRP mutation P347S in the VxPx motif had no effect (Figure 1C). Remarkably, both inhibitory peptides nearly completely displaced Arf4 from the Golgi/TGN membranes (Figure 1D), whereas the P347S peptide had no effect

on Arf4 membrane association. The peptide effect was specific for Arf4, as Arf3 was unaffected (Figure 1D). Thus, association of Arf4 with TGN membranes is dependent on its access to the VxPx sorting motif of rhodopsin.

Arf3 and Arf4 are differentially distributed among membrane fractions separated on sucrose density gradients (Figure 1E and F). Arf4 co-fractionated with Rab11 and the sialyltransferase-enriched TGN membranes, but was absent from RTCs, which contained Rab11, as reported (Deretic *et al*, 1996). The absence of Arf4 from RTCs suggests that it most likely hydrolyses GTP and dissociates from the membrane during RTC budding from the TGN.

ASAP1 functions as a GAP for Arf4 and regulates RTC budding

To identify the candidate GAP for Arf4, we examined the retinal expression of Arf GAPs ARAP1 and 2, and ASAP1 and 2. We determined that ASAP1 was abundant in photoreceptor Golgi/TGN and cytosolic fractions, and was also present on RTCs (Figure 2A). ASAPs display phosphatidylinositol 4,5-bisphosphate (PIP₂)-dependent activity on Arf1 and Arf5 (Brown *et al*, 1998), but the activity of ASAP1 towards Arf4 has not been tested. To test it, we employed ASAP1 BAR-PZA, a recombinant protein containing the BAR, PH, GAP and ankyrin repeat domain (Figure 2B), which stimulates membrane tubulation and regulates trafficking in a BAR- and GAP-dependent manner (Nie *et al*, 2006). We determined that ASAP1 BAR-PZA has a comparable GAP activity towards Arf4, Arf1 and Arf5 (Figure 2C), but not Arf6, as reported (Brown *et al*, 1998). Therefore, ASAP1 is a good candidate for an Arf4 GAP.

To examine the function of ASAP1 in rhodopsin trafficking, we tested the ability of ASAP1 BAR-PZA to stimulate RTC budding in our established cell-free assay. The full cytosol present in the PNS supports maximum RTC budding *in vitro*, equivalent to the maximum RTC budding *in vivo* in the same time period (Deretic *et al*, 1996). To test the activity of ASAP1 BAR-PZA, we reduced the amount of cytosol present in the assay. We separated membranes from the cytosol, followed

by the add-back of the limited (25%) or the full cytosol complement. Under limited cytosol conditions, ASAP1 BAR-PZA stimulated RTC budding by ~50%, almost to the level observed with full cytosol (Figure 2D). EM analysis of the budded carriers (Figure 2E–G) showed that RTCs formed with ASAP1 BAR-PZA were identical to the ones formed in full cytosol, and that these structures were undetectable when the cytosol was omitted from the budding assay. Moreover, similar to the control post-TGN carriers, RTCs budded in the presence of ASAP1 BAR-PZA were devoid of the Golgi marker GM130 (Figure 2H), indicating that the observed increase in budding was not due to nonspecific Golgi fragmentation. We next prepared ASAP1-immunodepleted cytosol (Figure 2I), and assayed its activity in RTC budding (Figure 2J). RTC budding was diminished (<50%), but was

Figure 2 ASAP1 is a GAP for Arf4 and regulates RTC budding from the TGN. (A) Membrane proteins from one retina were separated by SDS-PAGE and immunoblotted with anti-ASAP1, which recognized an ~120-kDa protein. Upon fractionation (lower panel), ASAP1 was detected in the G/TGN and the cytosol, with a small portion associated with RTCs. (B) Schematic representation of the ASAP1 BAR-PZA construct. (C) ASAP1 BAR-PZA was incubated with recombinant Arfs 1, 4, 5 or 6, and the hydrolysis of [α^{32} P]GTP bound to Arf was measured. The data are presented as the means $\pm$ s.d. of three separate experiments. (D) Radiolabelled retinal PNS was separated into membrane and cytosolic fractions, which were reconstituted as indicated in the panel. ASAP1-BAR-PZA (1 μ M) was added to the assay in limited (25%) cytosol during the cell-free chase, and incorporation of rhodopsin into RTCs was determined ([35 S]Rh). The quantification data are presented as the means $\pm$ s.e. of three separate experiments (* P = 0.01). (E–G) Negatively stained *in vitro* budded RTCs (~250 nm) examined by EM. (H) Following *in vitro* budding, Golgi and RTCs were immunoblotted with anti-GM130. (I) Cytosol was immunodepleted with anti-ASAP1 and protein A beads, reducing ASAP1 to ~10% of control. (J) After *in vitro* budding in ASAP1-immunodepleted cytosol, membrane proteins from two retinas were separated by SDS-PAGE and autoradiographed ([35 S]rhodopsin, left panels). Duplicate gels were immunoblotted with ASAP1 (right panels).

not completely inhibited due to the remaining cytosolic and TGN-associated ASAP1 (Figure 2I and J). Because of high retinal tissue requirements, the effect on RTC budding was established using a single concentration of ASAP1-immuno-depleted cytosol. Importantly, ASAP1 was detected on budded RTCs in quantities proportional to the amount of radiolabelled rhodopsin (Figure 2J). Taken together, these data indicate that ASAP1 regulates budding and is incorporated into budded RTCs.

ASAP1 associates with Golgi/TGN-derived buds and with microfilaments

As ASAP1 regulates both membrane trafficking and actin remodelling, we examined its association with photoreceptor biosynthetic membranes and with the actin cytoskeleton by confocal microscopy (Figure 3; Supplementary Figure S1). Photoreceptor actin filaments, which are anchored at the adherens junctions (AJs), encircle the RIS (Figure 3A) and extend to the calycal processes (CPs), the villous structures

that evaginate from the RIS and surround the ROS. ASAP1 was detected in the structures resembling actin filaments (arrowheads, Figure 3B; Supplementary Figure S1A), in the cytoplasm, as well as in punctate structures within the RIS area occupied by the Golgi (G, arrow, Figure 3B; Supplementary Figure S1A). ASAP1 localized along phalloidin-labelled microfilaments (arrowheads, Figure 3C; Supplementary Figure S1B and C), from the AJs to the CPs. We also examined ASAP1 association with the Golgi/TGN in further detail. ASAP1 localized to the punctate structures tightly juxtaposed to the trans-Golgi labelled by Rab6 (arrows, Supplementary Figure S1D, E and H), and to RTCs (arrowhead, Supplementary Figure S1D). In retinas treated with latrunculin or nocodazole to disrupt photoreceptor cytoskeleton, ASAP1-positive puncta remained associated with the Golgi (arrows, Supplementary Figure S1F and G). However, upon treatment with brefeldin A (BFA), which we showed leads to Golgi disruption and the inhibition of rhodopsin trafficking (Deretic and Papermaster, 1991), ASAP1 was distributed diffusely in the cytosol (Supplementary Figure S1K–M). Conversely, treatment with propranolol (Deretic *et al*, 2004) to enlarge the TGN and RTCs (Supplementary Figure S1O) resulted in uniform distribution of ASAP1 in these enlarged membrane structures (arrows, Supplementary Figure S1P and Q). Taken together, the Arf-dependent localization at the photoreceptor TGN and its presence on RTCs are consistent with a role for ASAP1 as a regulator of Arf4 in RTC budding.

TGN-derived nascent RTCs contain ASAP1, Rab11 and FIP3

By confocal microscopy, ASAP1 and rhodopsin colocalized at the Golgi/TGN in photoreceptor cells (Figure 3D, arrows). Surprisingly, Rab11 was similarly detected in Golgi-associated puncta, as well as in RTCs (Figure 3E). This prompted us to examine whether ASAP1 and Rab11 reside in the same TGN-associated bud-like structures. We first performed double labelling of Rab6 and Rab11 (Supplementary Figure S2A), which revealed Rab6 in the *trans*-Golgi cisternae and Rab11 in punctate structures at the TGN in control retinas (arrows, Supplementary Figure S2B–G) and in propranolol-treated retinas (Supplementary Figure S2H and I). Next, we examined the colocalization of Rab11 and ASAP1 by triple labelling with Rab6 (Figure 3F–H). Buds containing both Rab11 and

ASAP1 localized to the tips of the Rab6-positive *trans*-Golgi cisternae (arrows, Figure 3G and H; Supplementary Figure S3). Propranolol treatment produced greatly enlarged buds and RTCs (Figure 3K), in which Rab11 and ASAP1 completely overlapped (arrows, Figure 3L–N).

The localization of Rab11 at the sites of Arf4 function led us to probe these structures for the presence of FIP3, a member of the family of Arf- and Rab11-interacting proteins (Hales *et al*, 2001; Junutula *et al*, 2004). By confocal microscopy, FIP3 was present on the ASAP1/Rab11-positive buds (Figure 3I and J). By immunoblotting, FIP3 was detected on purified RTCs (Figure 3O). On sucrose density gradients, Rab11, ASAP1 and FIP3 co-fractionated with the Golgi/TGN-enriched fractions and with RTCs (Figure 3P and Q), which segregated into two major fractions. Although higher buoyant density RTCs contained all three proteins, ASAP1 and FIP3 were nearly absent from the lower density RTCs (Figure 3P and Q). Because the fusion regulator Rab8 predominantly associates with the low-density membrane carriers (Deretic *et al*, 1995), we surmise that the higher density RTCs correspond to the nascent post-TGN carriers, which mature, possibly by losing protein coats, and give rise to the low-density Rab8-positive periciliary RTCs.

Arf4, ASAP1, Rab11 and FIP3 form a complex

Colocalization and co-fractionation of ASAP1, Rab11, FIP3 and Arf4 at the TGN prompted us to ask whether they form a complex at the RTC budding sites. In co-immunoprecipitation experiments we determined that anti-Arf4 immunoprecipitated Rab11 and ASAP1 from retinal PNS (Figure 4A). Moreover, Rab11 and ASAP1 were mutually co-immunoprecipitated, and anti-FIP3 (Wilson *et al*, 2005) precipitated ASAP1, along with Rab11, a known FIP3-binding protein (Figure 4A). A control Golgi-associated protein G20 was not co-immunoprecipitated (Figure 4A). These data suggest that Arf4, ASAP1, Rab11 and FIP3 not only colocalize at the TGN but also interact to form at least one complex.

To determine whether Arf4 and Rab11 associate through their interacting protein FIP3, we tested the ability of Arf4 to bind FIP3 directly. FIP3 was shown to have significantly higher affinity for Rab11 and Arf6 than for Arf5, a class II Arf (Fielding *et al*, 2005). Our pull-down assay confirmed strong binding of recombinant FIP3 to GST–Arf6, and showed a direct, albeit weak binding of FIP3 to GST–Arf4. Retinal

Figure 3 ASAP1 is associated with both the Golgi/TGN and actin cytoskeleton, and colocalizes with Rab11 and FIP3 on nascent RTCs at the TGN. (A) A diagram representing the microfilaments (red) and calycal processes (CPs) that evaginate from the RIS and surround the base of the ROS (modified from Deretic *et al*, 1995). (B) A confocal optical section (0.7 μ m) of frog retina. ASAP1 (green) is detected in the RIS cytoplasm, in punctate structures around the Golgi (G, arrow), and in axially aligned structures (arrowheads), resembling microfilaments. N, nucleus (TO-PRO-3, blue). (C) ASAP1 (green) and phalloidin (red), colocalize (yellow, arrowheads) along microfilaments, including the CPs (arrow). Microfilaments are anchored at the adherens junctions (AJs). (D) Rhodopsin C-terminal mAb 11D5 (red) labels the ROS and the Golgi (G), where ASAP1-positive puncta (yellow, arrows) line up with regular periodicity. ASAP1 is also detected in CPs. (E) Rab11-positive puncta (yellow, arrows) aligned with rhodopsin-laden Golgi. Rab11 is also present on RTCs (arrowheads). (F) ASAP1 (blue) and Rab11 (red) colocalize in the bud-like profiles at the tips of the *trans*-Golgi (Rab6, green) (boxed area magnified in G). (G) Magnified *trans*-Golgi area from (F), with ASAP1- and Rab11-positive buds (arrows). (H) Rab11 (red) and ASAP1 (blue) are shown separately. (I) FIP3 (green, rabbit Ab; Wilson *et al*, 2005), overlaps with Rab11 (red) and ASAP1 (blue) in the punctate structures within the Golgi region (boxed area magnified in J). (J) FIP3, Rab11 and ASAP1 are shown in separate channels to better visualize their colocalization (arrows). (K) An EM image showing a greatly enlarged nascent bud at the TGN (arrowhead) and enlarged RTCs (arrows) in propranolol-treated retina (modified from Deretic *et al*, 2004). (L) Rab11 (red) and ASAP1 (green) colocalize (arrows, yellow) in propranolol-enlarged RTCs and buds lining Rab6-positive *trans*-Golgi (blue). (M, N) Rab11 and ASAP1 shown individually. Insets demonstrate identical distribution of Rab11 and ASAP1. (O) Goat anti-FIP3 detects an ~80-kDa protein on isolated RTCs. (P) Following *in vitro* budding and subcellular fractionation, the distribution of rhodopsin was determined by autoradiography (35 S)Rh). Duplicate gels were immunoblotted as indicated in the panel. (Q) Quantification of autoradiograms and immunoblots. Bar, 4 μ m in (B, C), 3 μ m in (D–F, I), 2 μ m in (L–N), 1 μ m in (H, J), 0.7 μ m in (G, K) and insets in (L–N).

Figure 4 Arf4, Rab11, FIP3 and ASAP1 form a complex and regulate RTC budding. (A) Specific antibodies were incubated with retinal PNS and proteins bound to protein-A beads were separated by SDS-PAGE and analysed by immunoblotting, as indicated. Left panels, pAb IPs; right panels, mAb IPs. (B) Arf4 and Arf6 were expressed as GST fusion proteins and incubated with 6His-FIP3. Proteins bound to glutathione beads were separated by SDS-PAGE and analysed by immunoblotting with anti-FIP3. (C) FIP3-F1 C-terminal fragment (aa 441–756) (1 μM) was added to the assay in limited (25%) cytosol during the cell-free chase, and rhodopsin incorporation into RTCs was determined (35 S]rhodopsin). Autoradiograms and immunoblots of duplicate gels probed for Rab11 and ASAP1 were quantified (right panel). (D) Cytosol was immunodepleted with anti-Rab11, reducing it to nearly undetectable levels. (E) Following *in vitro* budding in Rab11-immunodepleted cytosol, membrane proteins from two retinas were separated by SDS-PAGE and autoradiographed (35 S]rhodopsin, left panels). Rab11 immunoblots of duplicate gels (right panels).

cytosol greatly enhanced FIP3 binding to GST-Arf4 (Figure 4B), but did not affect its binding to GST-Arf6 (not shown). ASAP1- or Rab11-immunodepleted cytosol did not facilitate the binding of GST-Arf4 to FIP3 (Figure 4B). These data suggest that Rab11 and ASAP1 stabilize the Arf4-FIP3 interaction and that these proteins associate to form a single complex, most likely at the TGN.

A FIP3 fragment containing Arf and Rab11 interaction domains, along with Rab11, stimulates RTC budding

To further define the roles of the individual components of the complex, we tested the effect on RTC budding of the FIP3-F1 C-terminal fragment, encompassing the Arf- and Rab11-

binding sites (Schonteich *et al*, 2007). In low cytosol, the addition of the FIP3-F1 fragment nearly doubled *in vitro* RTC budding measured by the radiolabelled rhodopsin content (Figure 4C). The budded RTCs displayed a proportional increase in membrane-associated Rab11 and ASAP1 (Figure 4C). As Rab11 is predominantly membrane-associated in photoreceptors (Supplementary Figure S2J and K), a greater fraction of total Rab11 was bound to RTCs than the fraction of total ASAP1, which exists in equilibrium between the membranes and the cytosol (see Figure 2A). Budded carriers were devoid of GM130 (not shown), suggesting that sorting was not compromised in the presence of the FIP3-F1 fragment.

Surprisingly, we found that the cytosolic pool of Rab11 increases when RTC budding is inhibited (Supplementary Figure S2J and K). We thus tested the capacity of Rab11-immunodepleted cytosol (Figure 4D) to support RTC budding. In a representative experiment, RTC budding was decreased by ~50% (Figure 4E), suggesting that Rab11 is also a component of the budding machinery. The residual RTC budding in Rab11-depleted cytosol was most likely due to the remaining TGN pool of Rab11 (Figure 4E). Membrane-associated Rab11 cannot be removed by cytosol immunodepletion, but it is readily removed by the Rab GDP dissociation inhibitor (Chen *et al*, 1998), which we showed completely blocks RTC budding (Deretic *et al*, 1996). Taken together, our data support the notion that Arf4, ASAP1, FIP3 and Rab11 form a functional complex that regulates RTC budding.

An Arf4 mutant that selectively affects ASAP1-mediated GTP hydrolysis interferes with ciliary targeting and causes rhodopsin mislocalization and retinal degeneration in transgenic animals

Mutational analysis of Arf1 identified the I46D mutation in the switch 1 domain, which selectively reduced ASAP1-induced GTP hydrolysis by ~10 000-fold, but had a modest effect on catalysis by other GAPs (Luo *et al*, 2005). We introduced the I46D mutation into Arf4, which rendered ASAP1-induced GTP hydrolysis undetectable, while minimally affecting the activity of AGAP1 (Figure 5A).

We generated transgenic *Xenopus laevis* expressing WT or I46D mutant Arf4-GFP fusion proteins in their rod photoreceptors using an established protocol (Tam *et al*, 2006). Arf4-GFP had no discernable effect on the transgenic retinas (Figure 5B), whereas [I46D]Arf4-GFP caused photoreceptor cell loss and robust retinal degeneration (Figure 5C), indicating its dominant-negative effect on endogenous Arf4. Arf4-GFP (Figure 5D) and [I46D]Arf4-GFP (Figure 5E) had similar subcellular localization. Expression of [I46D]Arf4-GFP did not cause detectable de-localization of ASAP1, Rab11 or FIP3 (not shown). However, the RIS was often constricted in photoreceptors expressing [I46D]Arf4-GFP (Figure 5E, arrows), suggesting that it negatively impacted the actin cytoskeleton, which controls the photoreceptor cell shape (see Figure 3A and C). Arf4-GFP localized predominantly at the Golgi (G, Figure 5F). In Arf4-GFP-expressing cells, actin bundles, revealed by phalloidin staining, extended the length of the RIS (large bracket, Figure 5F) from the AJs that form the outer limiting membrane (OLM, dotted line) to the CPs. Conversely, in photoreceptors expressing [I46D]Arf4-GFP, cytoskeleton perturbation was evident and the OLM (dotted lines, Figure 5G and H) was displaced towards the ROS. RIS

Figure 5 Expression of [I46D]Arf4 deficient in ASAP1-mediated GTP hydrolysis disrupts rhodopsin trafficking and causes retinal degeneration in transgenic *X. laevis*. (A) Arf4 or [I46D]Arf4 was incubated with ASAP1 BAR-PZA, or with AGAP1, and the hydrolysis of [α^{32} P]GTP bound to Arf was measured. The data are presented as the means $\pm$ s.d. of three separate experiments. (B) Transgenic expression of Arf4-GFP (green) in retinal photoreceptors. WGA (red) detects glycosylated membrane proteins. Nuclei are stained with DAPI (blue). (C) Expression of [I46D]Arf4-GFP (green) causes photoreceptor loss and retinal degeneration. (D) Arf4-GFP colocalizes with WGA-labelled membranes in the RIS. ONL, outer nuclear layer. (E) RIS is constricted (arrows) in [I46D]Arf4 mutants. (F) Arf4-GFP is present in the Golgi (G). Microfilaments (phalloidin, red), span the RIS (large bracket) from the outer limiting membrane (OLM, dotted line) to the CPs. N, nucleus. (G, H) In [I46D]Arf4 retinas, microfilaments (phalloidin, red) do not span the RIS (indicated by large brackets). The OLM (dotted line) is displaced and RIS is constricted (arrow in H). (I) Rhodopsin (red) colocalizes with Arf4-GFP in the Golgi (G, arrows). N, nucleus (TO-PRO-3, blue). (J) [I46D]Arf4-GFP and rhodopsin (red) colocalize (arrow) at the site of RIS constriction. (K) Rhodopsin-rich membranes (red, arrow) are elaborating from the [I46D]Arf4 Golgi (G), towards the nucleus (N). (L–N) Arf4-GFP (green, L) and rhodopsin (red, M) extensively colocalize in the Golgi (G, arrows, yellow in N). Inset in N: surface density of Golgi membranes (S_v^G) containing Arf4, rhodopsin or both, determined within the RIS area occupied by the Golgi, indicated by a large bracket in (N). The data from a representative transgenesis experiment are presented as the means $\pm$ s.e., $n = 11$. (O–Q) [I46D]Arf4 (green, O) and rhodopsin (red, P) partition into distinct membranes. Arrows in (O–Q) indicate atypical rhodopsin-rich membranes juxtaposed to the Golgi/TGN-containing Arf4. Inset in Q: surface density of Golgi membranes (S_v^G) in photoreceptors expressing [I46D]Arf4-GFP. The data are presented as in the inset in (N), $n = 11$. Bar, 500 μ m (B, C), 20 μ m (D, E), 5 μ m (F–H, J), 4 μ m in (I, K) and 3 μ m in (L–Q).

was constricted at the OLM (Figure 5H, arrow) and actin filaments were fragmented, no longer spanning the full length of the RIS (large brackets, Figure 5G and H).

Expression of [I46D]Arf4-GFP caused dysfunctional rhodopsin trafficking, but the mutant phenotype was significantly different from the previously observed morphological changes in transgenic *X. laevis* models (Tam *et al*, 2000, 2006; Moritz *et al*, 2001). Arf4-GFP was detected in the cytosol and colocalized with rhodopsin in the Golgi (Figure 5I, arrows), as reported for Arf4 (Deretic *et al*, 2005), whereas [I46D]Arf4-GFP caused delivery of rhodopsin to the plasma membrane around displaced AJs (Figure 5J, arrow). [I46D]Arf4-GFP had a detrimental effect on RTC budding,

as substantially enlarged budding profiles containing rhodopsin were detected emanating from the Golgi towards the nucleus and the opposite pole of the photoreceptor (Figure 5K, arrow). In contrast to Arf4-GFP, which extensively colocalized with rhodopsin in the Golgi (arrow, Figure 5L–N), [I46D]Arf4 localized in the Golgi that appeared disorganized (Figure 5O). Rhodopsin accumulated in separate enlarged tubulo-vesicular structures (arrows, Figure 5P and Q), which were devoid of Arf4 (arrow, Figure 5O). To quantify the effect of the I46D mutant on Arf4-rhodopsin colocalization, we determined the surface density (Deretic *et al*, 2004) of Golgi membranes (S_v^G) containing Arf4, rhodopsin or both. S_v^G was defined as a measure of surface

occupied by the Golgi in a volume of cell cytoplasm outlined with brackets in Figure 5N and Q. Here, 90% of Arf4-GFP colocalized with rhodopsin in the Golgi ($S_V^G = 0.33 \pm 0.03/\mu\text{m}$, $n = 11$; Figure 5N, inset). In [I46D]Arf4-GFP retinas, their colocalization was significantly reduced ($S_V^G = 0.19 \pm 0.01/\mu\text{m}$, $n = 11$, $P = 8.7 \times 10^{-4}$; Figure 5Q, inset). The Golgi region of [I46D]Arf4-GFP photoreceptors was filled with atypical membrane structures, which were not seen in cells expressing Arf4-GFP, and either contained Arf4 ($S_V^G = 0.31 \pm 0.01/\mu\text{m}$ in I46D, versus 0.01 ± 0.01 in WT, $P = 2.5 \times 10^{-14}$), or rhodopsin ($S_V^G = 0.21 \pm 0.04/\mu\text{m}$ in I46D, versus 0.02 ± 0.01 in WT, $P = 3.4 \times 10^{-5}$). Taken together, our data indicate that [I46D]Arf4 impairs rhodopsin post-TGN trafficking and that other Arf GAPs that are capable of GTP hydrolysis on [I46D]Arf4 cannot substitute for the effector function of ASAP1 in RTC budding at the Golgi/TGN.

Discussion

In this study, we show that Arf4, which binds to the VxPx motif present in membrane proteins targeted to primary cilia, including rhodopsin, functions within a novel trafficking module. We demonstrate that the Arf GAP ASAP1 serves both as an Arf4 GAP and its effector and, along with Rab11 and an Arf/Rab11-binding effector FIP3, generates a protein complex. The functional site of this complex is at the TGN, where it regulates the packaging of membrane cargo into post-TGN carriers targeted to the sensory cilia.

Little is known about the function of Arf4, except in photoreceptors, where it was shown to regulate rhodopsin trafficking (Deretic *et al*, 2005). We have now determined that ASAP1, an Arf GAP that couples Arf-GTP binding with membrane deformation (Nie *et al*, 2006), mediates GTP hydrolysis on Arf4 and functions as an Arf4 effector, which regulates budding from the TGN *in vitro* and *in vivo*. A trigger for budding of post-TGN RTCs appears to be the formation of the functional complex comprised of Rab11, FIP3, Arf4 and ASAP1 at the TGN. Remarkably, we find that ASAP1 and Rab11 stabilize direct interaction of Arf4 and FIP3. Consistent with our data, a recent study shows that binding of Rab6 promotes direct association of Arl1 with the GRIP domain of the Golgi tether GCC185 (Burguete *et al*, 2008), suggesting that cooperation between the Arf and Rab families may be realized through similar molecular interactions at multiple stages of trafficking.

FIP3 can simultaneously bind Rab11 and an Arf, linking Rab11- and Arf-positive microdomains and triggering Arf-mediated carrier budding (Shiba *et al*, 2006). A recent study shows that FIP3 forms a ternary complex with Rab11 and ASAP1 (Inoue *et al*, 2008). The BAR domain of ASAP1 binds to FIP3 N-terminally to the Arf-binding site. FIP3 binding stimulates Arf GAP activity of ASAP1 towards Arf1, but not Arf6 (Inoue *et al*, 2008). In this context, our data suggest that ASAP1 and FIP3 may cooperate to provide temporally and spatially restricted hydrolysis of GTP bound to Arf4 at the TGN. As ASAP1 and FIP3 function as homodimers, they may oligomerize to form a protein coat that regulates TGN-to-plasma membrane trafficking, or a functional module that serves as a coincidence detector for membrane microdomains and cargo when engaged by the site-specific activation of Arf4. Rhodopsin provides the spatial control for this module by targeting Arf4 to the carrier

budding sites at the TGN, where the membrane-docked Arf4 is most likely activated by the BFA-inhibited GEFs. The rhodopsin-binding site on Arf4 is at its unique α helix 3 (Deretic *et al*, 2005), suggesting that there is no functional redundancy with the closest homologue Arf5. Interestingly, the α helix 3 of Arf1 binds the ER/Golgi fusion regulator membrin and targets Arf1 to the Golgi (Honda *et al*, 2005). Thus, our finding that the TGN association of Arf4 is dependent on its access to the rhodopsin VxPx sorting motif is consistent with the role of this motif in the targeting of Arf4 to the TGN.

It is possible that Arf4 could simultaneously interact with rhodopsin, FIP3 and ASAP1, although our current experiments do not demonstrate this conclusively. A most likely binding site for FIP3 is at the C terminus of Arf4, as was demonstrated for Arf6 (Schonteich *et al*, 2007). This site is distinct from α helix 3, which binds rhodopsin, or the switch 1 domain, which is the ASAP1-binding site (Luo *et al*, 2005). The expression of mutant ASAP1 that prolongs Arf activation was shown to be accompanied by membrane recruitment of Rab11 (Nie *et al*, 2006), which is targeted to the Golgi/TGN by an Arf effector phosphatidylinositol 4-kinase β (PI4K β) (de Graaf *et al*, 2004). We have observed a linkage between the cytosolic pool of Rab11 and RTC budding, suggesting that *de novo* recruitment of Rab11 into the regulatory complex may be coupled to its recruitment to the TGN, possibly through Arf-dependent activation of PI4K β .

Our data suggest that at the TGN Rab11 may regulate the timing of Arf4 GTP hydrolysis by ASAP1 through FIP3, thereby providing a pacemaker for cargo concentration and sorting into ciliary-targeted carriers. Persistence of Rab11 on RTCs supports its involvement in post-budding events as well. These could include interactions with the conserved exocyst complex (Novick *et al*, 2006), which tethers the Rab11/FIP3-positive membranes (Fielding *et al*, 2005). Exocyst complex localizes to the cilium in polarized epithelial cells (Rogers *et al*, 2004) and in photoreceptors (Mazelova *et al*, *J Cell Sci.*, in revision). Its interaction with Rab11 has a function in the tethering of membranes carrying rhodopsin (Zhang *et al*, 2004; Beronja *et al*, 2005; Satoh *et al*, 2005). Rab11 may cooperate with a major player in ciliogenesis, Rab8, possibly through common effectors (Roland *et al*, 2007). Alternatively, a handover from Rab11 to Rab8, or Rab conversion (Rink *et al*, 2005), may occur at the base of the cilium to couple successive stages of traffic along the ciliary pathway.

Emerging evidence points to multiple pathways for targeting membrane proteins to the cilium. The conserved FR motif present in GPCRs regulates ciliary targeting of receptors from the Frizzled/Smoothed family (Corbit *et al*, 2005). Although rhodopsin has both the FR and VxPx motifs, only the latter has been shown to be functional in ciliary targeting. The VxPx sorting motif is also present in PC2 (Geng *et al*, 2006) and in CNG channel CNGB1b subunit (Jenkins *et al*, 2006), and it regulates their targeting to the primary cilia in polarized epithelial cells and olfactory neurons, respectively. We found that the N-terminal domain of PC2, which contains the VxPx targeting motif can bind Arf4 *in vitro* (our unpublished data). As the assembly of the Arf4/ASAP1/FIP3/Rab11 complex at the TGN is dependent on recognition of the VxPx motif, the factors that form the complex with Arf4 might be widely employed in trafficking to the cilium. Delineating the

hierarchy and molecular interactions of ciliary targeting motifs with the regulatory complexes remains a future challenge.

In summary, we have identified the minimal components of the Arf4-based molecular complex, which regulates ciliary targeting through recognition of the conserved VxPx motif and couples cilium-directed cargo sorting to carrier budding at the TGN. This targeting module is most likely conserved in polarized cells containing primary cilia.

Materials and methods

Retinal subcellular fractionation and RTC budding in photoreceptor-enriched PNS

Frogs (*Rana berlandieri*) were used for the preparation of PNS enriched in photoreceptor biosynthetic membranes (Deretic and Papermaster, 1991). The standard assay for RTC cell-free budding (Deretic *et al*, 1998, 2005) is described in Supplementary data.

Arf GAP activity assay

Arfs 1, 4, 5 and 6, [1-724]ASAP1 (ASAP1 BAR-PZA) and AGAP1 were expressed and purified from bacteria as described (Nie *et al*, 2006). Hydrolysis of [α^{32} P]GTP bound to Arfs was measured and GAP activity was determined as described in Supplementary data.

Generation of transgenic *X. laevis* expressing Arf4-GFP and [I46D]Arf4-GFP

Transgenic *X. laevis* were generated as described earlier (Tam *et al*, 2006) and analysed 14 days post-fertilization, as described in

Supplementary data. One eye was used for the analysis of total rhodopsin content (Tam *et al*, 2006), whereas the other eye was fixed for analysis by confocal microscopy.

Confocal microscopy

Confocal microscopy on frog retinas and quantitative analysis of confocal images were performed as described earlier (Deretic *et al*, 2004), and in Supplementary data. Digital images were cropped, and brightness and contrast were adjusted on entire panels using Adobe Photoshop CS (Adobe Systems Inc.).

All other Materials and methods are described in Supplementary data.

Supplementary data

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

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