The Conserved Bardet-Biedl Syndrome Proteins Assemble a Coat that Traffics Membrane Proteins to Cilia

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SUMMARY

The BBSome is a complex of Bardet-Biedl Syndrome (BBS) proteins that shares common structural elements with COPI, COPII, and clathrin coats. Here, we show that the BBSome constitutes a coat complex that sorts membrane proteins to primary cilia. The BBSome is the major effector of the Arf-like GTPase Arl6/BBS3, and the BBSome and GTP-bound Arl6 colocalize at ciliary punctae in an interdependent manner. Strikingly, Arl6GTP-mediated recruitment of the BBSome to synthetic liposomes produces distinct patches of polymerized coat apposed onto the lipid bilayer. Finally, the ciliary targeting signal of somatostatin receptor 3 needs to be directly recognized by the BBSome in order to mediate targeting of membrane proteins to cilia. Thus, we propose that trafficking of BBSome cargoes to cilia entails the coupling of BBSome coat polymerization to the recognition of sorting signals by the BBSome.

INTRODUCTION

Primary cilia are microtubule-based projections found on nearly every cell in the human body. Since primary cilia are required for phototransduction, olfaction, planar cell polarity, and Hedgehog signaling and since the receptor for each of these signaling pathways has been localized to the primary cilium (Fliegauf et al., 2007), the targeting of signaling receptors to cilia is thought to be crucial for signal sensing and transduction. Yet, our understanding of membrane protein targeting to cilia remains fragmented (Nachury et al., 2010).

The delivery of membrane proteins to cilia sequentially entails sorting and packaging into carrier vesicles, docking, and fusion of vesicles with the base of the cilium and intraflagellar transport (IFT) from cilia base to cilia tip (Rosenbaum and Witman, 2002). The step of docking and fusion requires the GTPase Rab8 and its guanine nucleotide exchange factor (GEF) Rabin8 (Moritz et al., 2001; Nachury et al., 2007). The sorting step most likely relies on the recognition of a ciliary targeting signal (CTS) by a sorting complex and CTSs have been identified in several ciliary membrane proteins (Tam et al., 2000; Berbari et al., 2008b; Follit et al., 2010). While the GTPases Arf4 and Rab8 have been shown to recognize the CTSs of rhodopsin and fibrocystin, respectively (Mazelova et al., 2009; Follit et al., 2010), it is expected that coat complexes resembling COPI, COPII, and clathrin carry out the sorting of membrane proteins to cilia. Canonical coat complexes are recruited to membranes by phosphoinositides (PIPs) and—in most cases—by a GTP-bound Arf family GTPase and the direct recognition of sorting signals by coat complexes ensures that coat polymerization packages transmembrane cargoes into a carrier vesicle (McMahon and Mills, 2004). Thus far, no coat complex has been identified for trafficking to cilia.

Recently, we discovered the BBSome, an octameric complex consisting of the seven highly conserved Bardet-Biedl syndrome (BBS) proteins BBS1, BBS2, BBS4, BBS5, BBS7, BBS8, and BBS9 and of the novel protein BBIP10 (Nachury et al., 2007; Loktev et al., 2008). Bardet-Biedl Syndrome is an autosomal recessive disorder characterized by retinal degeneration, polydactyly, kidney cysts, and obesity that can be caused by mutations in any of 14 known genes and whose etiology is associated with cilium dysfunction (Fliegauf et al., 2007). Since the BBSome binds Rabin8 and associates with the ciliary membrane and since BBS5 binds PIPs on protein-lipid overlays, we have proposed that the BBSome functions in vesicular trafficking to the cilium (Nachury et al., 2007). In support of this hypothesis, the G protein-coupled receptor (GPCR) Somatostatin receptor 3 (SSTR3) fails to reach the cilium of hippocampal neurons in bbs2 and bbs4 knockout mice (Berbari et al., 2008a). However, a role for the BBSome in vesicular transport remains controversial, with alternative roles in microtubule anchoring (Kim et al., 2004), intraflagellar transport (Ou et al., 2005; Lechtreck et al., 2009), and ubiquitination (Gerdes et al., 2007) having been proposed. Regardless of the model considered, the definite molecular activity of the BBSome remains unknown.

Interestingly, BBS3 encodes the small Arf-like GTPase Arl6, which is not part of the BBSome and whose function remains uncharacterized (Fan et al., 2004; Chiang et al., 2004). We now show...
that Arl6\(^{\text{GTP}}\) recruits the BBSome onto membranes to assemble an electron-dense coat and that the BBSome sorts SSTR3 to cilia by directly recognizing SSTR3's CTS. Thus, the BBSome constitutes a coat complex that sorts membrane proteins to cilia.

**RESULTS**

**The BBSome Is the Major Effector of Arl6 in Retinal Extracts**

We first sought to identify effectors of Arl6 by affinity chromatography. Mutations were introduced into Arl6 to preclude GTP hydrolysis (Q73L) and to limit aggregation of the GTP-bound form (ΔN16). We chose retinal extract as a starting material because of the tremendous rates of membrane protein trafficking to cilia in photoreceptors. Remarkably, eight protein bands were recovered specifically in the eluate of the Arl6\(^{\text{GTP}}\) column and were identified as the eight subunits of the BBSome (Figure 1A). Further, direct “in-solution” mass spectrometry analysis of the eluates failed to identify any Arl6 effector besides the BBSome subunits. TACT1, the only other protein specifically recovered in the Arl6\(^{\text{GTP}}\) eluate, binds directly to the BBSome and likely binds to Arl6\(^{\text{GTP}}\) indirectly (Figure 1B; T.S. and M.V.N., unpublished data). Furthermore, immunoblotting showed that over 75% of the BBSome was depleted by the Arl6\(^{\text{GTP}}\) column and recovered in the Arl6\(^{\text{GTP}}\) eluate, while no BBSome binding to Arl6\(^{\text{GDP}}\) and GST was detected (Figure 1C). Thus, the BBSome is the major Arl6 effector in retinal extracts. We further confirmed the BBSome-Arl6\(^{\text{GTP}}\) interaction by showing that BBS1 was the BBSome subunit most efficiently captured by Arl6\(^{\text{GTP}}\) and by mapping the interaction domain to the N terminus of BBS1 (Figure 1D). Since Arl6 is the only BBS gene besides the BBSome subunits to be universally conserved in ciliated organisms, these results tie all of the conserved BBS proteins into two connected biochemical units and suggest a conserved function for the BBSome/Arl6\(^{\text{GTP}}\) interaction.

**Fold Recognition Analyses Reveal Coat-like Structural Elements in the BBSome**

The finding that the BBSome is the major effector of an Arf-like GTPase suggested that the molecular activity of the BBSome may be related to that of coat complexes. We therefore set out to validate the BBSome coat hypothesis at the biochemical, structural, and functional levels. First, we explored the structural anatomy of the BBSome using sensitive structure-prediction algorithms. We extended previous findings (Kim et al., 2004) and discovered that BBS4 and BBS8 are almost entirely comprised of TPR repeats (Uliek et al., 2004) and are therefore predicted to fold into extended rod-shaped α solenoids.
structure and the modeled fold of the human BBS7 platform-like module. Intriguingly, β strand H and helix 1 are missing from BBS2/7/9, but in turn these gain an additional, conserved C-terminal β strand (labeled N) that is predicted to form an edge strand (gray) in the platform β sheet.

(D) Recurring membrane recruitment machinery and structural elements of the canonical coats and of the BBSome. The PIPs that participate in coat complexes gain an additional, conserved C-terminal structure and the modeled fold of the human BBS7 platform-like module. Intriguingly, β strand H and helix 1 are missing from BBS2/7/9, but in turn these gain an additional, conserved C-terminal β strand (labeled N) that is predicted to form an edge strand (gray) in the platform β sheet.

Figure 2. The BBSome and Canonical Coat Complexes Share a Related Structural Organization

(A) Schematics of the predicted domain organization of each BBSome subunit. BBS4 and BBS8 have 13 and 12.5 tetratricopeptide repeats (TPR), respectively. BBS1, BBS2, BBS7, and BBS9 each consist of a β propeller followed by an amphipathic helical linker and a γ-adaptin ear domain (GAE). In BBS2, BBS7, and BBS9, the GAE is followed by an α/β platform domain and α helix domain. In BBS1, a four-helix bundle is inserted between the second and third blades. BBS5 contains two pleckstrin homology (PH) domains and a three-helix bundle, while BBIP10 is predicted to fold into two α helices.

(B) Structure-based alignment of the proposed GAE modules of BBS1, BBS2, BBS7, and BBS9. The structures used to generate the model of the GAE domain of BBS1 were those of GGA1, AP2γ1, AP2ζ2, AP2α2, and γ-COP. Only the N- and C-terminal six β strands of the Ig-like folds (respectively labeled A–C and E–G) are shown, in a color gradient that matches the chain topology of the human BBS1 GAE domain model. While the primary sequence identity between solved or predicted structures remains quite low, hydrophobic positions (highlighted) are well conserved. See the Extended Experimental Procedures for details on fold recognitions and secondary structure predictions.

(C) The platform-like modules of BBS2, BBS7, and BBS9 are topological variants of the appendage domains. The C-terminal platform domains of γ-COP, β-COP, AP2ζ2, and AP2α2 were structurally aligned to the proposed platform-like modules of BBS2, BBS7, and BBS9 as above. β strands are shown as arrows (labeled 1–M) and helices as cylinders (labeled 1–3), color matched to the AP2α GAE domain of BBS1 were those of GGA1, AP2γ1, AP2ζ2, AP2α2, and γ-COP. Only the N- and C-terminal six β strands of the Ig-like folds (respectively labeled A–C and E–G) are shown, in a color gradient that matches the chain topology of the human BBS1 GAE domain model. While the primary sequence identity between solved or predicted structures remains quite low, hydrophobic positions (highlighted) are well conserved. See the Extended Experimental Procedures for details on fold recognitions and secondary structure predictions.

Aril6 Is Found in Punctae at the Ciliary Membrane Together with the BBSome

We next sought to identify the compartment(s) where the BBSome/Arl6 interaction takes place. We raised a polyclonal antibody against Aril6 and validated its specificity by immunoblotting lysates of RPE1-hTERT (RPE) cells transfected with Aril6 small interfering RNA (siRNA) (Figure S1A available online). RPE cells grow a primary cilium when switched into quiescence, and we previously showed that the BBSome subunits BBS4 and BBIP10 localize to cilia in RPE cells. We extended these findings by showing that endogenous BBS1 (Figure 3C), BBS2 (Figure 3A) and BBS5 (Figure 3B), all localized to cilia. Thus, we conclude that the BBSome is present in mammalian cilia as a complex as was recently shown in Chlamydomonas (Lechtreck et al., 2009). Remarkably, our anti-Arl6 antibody stained cilia (Figure 3A), and cilia staining was lost after siRNA-mediated depletion of Aril6 (Figure 4A, left panels). To accurately determine the distribution of Aril6 and the BBSome within cilia, a structure

(Figure 2A) . Meanwhile, BBS1, BBS2, BBS7, and BBS9 share a related β propeller fold (Chaudhuri et al., 2008) in their N termini (Figure 2A) and a domain distantly related to the immunoglobulin (Ig)-like β sandwich fold of the γ-adaptin ear (GAE) motif in their C termini (Figure 2B). In BBS2, BBS7, and BBS9, the GAE domain is further accompanied by an α/β platform domain (Figure 2C). In several clathrin adaptors and in COPI, the GAE motif—either by itself or fused to the α/β platform—constitutes the so-called appendage domain that recruits either regulators of coat assembly or factors that program the coated vesicle for subsequent targeting events (McMahon and Mills, 2004). In the BBSome, Rabin8 binds to the C terminus of BBS1, and it is conceivable that Rabin8 serves as a BBSome accessory factor. Since rigid α solenoids and β propellers form the architectural scaffolds of COPI and clathrin cages (Stagg et al., 2007), the abundance of β propellers, α solenoids, and appendage domains inside the BBSome suggests an ancient evolutionary relationship between the BBSome and canonical coat complexes (Figure 2D).
Figure 3. Arl6 Is Found in Punctae Together with the BBSome Inside Primary Cilia
(A) Arl6 localizes to the primary cilium in RPE cells. Serum-starved RPE cells were immunostained for Arl6 and acetylated α-tubulin (acTub). Scale bars represent 5 μm.
(B) Arl6 is found in punctae flanking the axoneme inside primary cilia. RPE cells immunostained as in (A) were imaged by structured illumination microscopy. The scale bar represents 5 μm. A 3D view of the same cilium is shown in Movie S1.
(C) Arl6 colocalizes with the BBSome inside cilia. Serum-starved RPE cells stably expressing GFP-tagged Arl6 were immunostained for BBS1 (top), BBIP10 (bottom), and acetylated α-tubulin (acTub). GFP-Arl6 was visualized in the green channel without antibody staining. Scale bars represent 5 μm. A line scan through these cilia is shown in Figures S1B and S1C. See also Figure S2.

whose 300 nm diameter cannot be resolved by conventional light microscopy, we resorted to structured illumination microscopy, a “super-resolution” technique that lowers the optical resolution to less than 50 nm (Schemmell et al., 2008). Arl6 staining appeared in a pattern of punctae flanking the microtubule axoneme that likely correspond to small membrane-associated patches (Figure 3B and Movie S1). Further, deconvolution microscopy could resolve a discrete pattern of Arl6 staining within cilia that precisely mirrored the distribution of the BBSome subunits BBS1 and BBIP10 (Figure 3C and Figures S1B and S1C). Thus, the interaction between Arl6 and the BBSome may take place within these intraciliary patches.

Arl6\textsuperscript{GTP} and the BBSome Are Dependent on One Another for Targeting to Cilia
Together with our biochemical data, the colocalization studies suggested that Arl6\textsuperscript{GTP} may recruit the BBSome to cilia. Here, we found that the BBSome subunits BBS1 and BBIP10 failed to localize to cilia when we depleted Arl6 by siRNA (Figures 4A–4C). While treatment with two different siRNAs targeting Arl6 dramatically decreased the abundance of Arl6 protein, the abundance of the BBSome subunit BBS4 remained unaffected (Figure 4B), and BBS4 still migrated as part of a 500 kDa complex on size-exclusion chromatography in the absence of Arl6 (Figure S3A). Thus, Arl6 is specifically required for BBSome localization to cilia but not for BBSome assembly. Next, we generated clonal RPE cell lines expressing moderate levels of Arl6 variants to determine whether GTP binding and hydrolysis by Arl6 were required for targeting the BBSome to cilia (Figure 4E and Figure S3B). While Arl6-GFP and Arl6[Q73L]-GFP were both found inside cilia, Arl6[T31R]-GFP, a variant deficient in GTP but not GDP binding (Kobayashi et al., 2009), was absent from primary cilia (Figure 4D). To assess the contribution of Arl6-GFP to BBSome targeting to cilia, we selectively depleted endogenous Arl6 using an siRNA targeting the 3′ untranslated region (UTR) of Arl6 messenger RNA (mRNA) (Figure 4E). While the localization pattern of the Arl6-GFP variants remained unaffected, only Arl6-GFP and Arl6[Q73L]-GFP supported BBSome targeting to cilia (Figures 4D and 4F). Furthermore, measurements of BBS1 immunoreactivity inside cilia showed that Arl6[Q73L]-GFP recruited a greater amount of BBSome to the cilium than Arl6-GFP (Figure S3C). We conclude that Arl6 and BBSome targeting to cilia both require Arl6 binding to GTP but not Arl6 GTPase activity. Conversely, depletion of the BBSome subunits BBS2, BBS4, and BBS5 resulted in a dramatic decrease of Arl6 staining within cilia (Figure S4A).

Interestingly, we noted that the fraction of cells with BBSome- or Arl6-positive cilia varied from 15% to 60% depending on the experiment (compare Figures 4C and 4F and Figure S4A). While the source of the variability remains unknown, we hypothesized that the levels of Arl6 and the BBSome in most cilia fall below the detection threshold of our traditional immunofluorescence protocol. We therefore developed a method that decreases background staining while preserving the signals in cilia (see the Extended Experimental Procedures and Figures S4C–S4G), and we now find that nearly every RPE cilia stains positive for Arl6 and BBS1 (Figure S4G). This increase in the proportion of Arl6- and BBS1-positive cilia is not simply an artifact of the new staining procedure, since Arl6 and BBS1 staining are still lost from cilia when Arl6 is depleted by siRNA. Together, these results demonstrate the interdependence of Arl6\textsuperscript{GTP} and BBSome targeting to cilia and suggest that Arl6\textsuperscript{GTP} and the BBSome synergize in binding to the membrane of the cilium.

Arl6\textsuperscript{GTP} Is Necessary and Sufficient to Efficiently Recruit the BBSome to Liposomes
We first tested whether Arl6 behaves like Arf1 and Sar1, i.e., binds to membranes upon GTP binding by exposing an amphipathic helix that inserts itself in the lipid shell and terminates in a basic collar that interacts with phospholipid headgroups (Gillingham and Munro, 2007). Helical representation of the N terminus of Arl6 demonstrates the amphipathic nature of the N terminal helix of Arl6 and its termination with several positively charged residues (Figures 5A and 5B). Since Arl6 is predicted not to be myristoylated (Gillingham and Munro, 2007), we expressed Arl6 in bacteria and purified it to homogeneity (Figure S5A). As mammalian cilia...
cannot be isolated in sufficient quantities to generate a pure lipid fraction, we conducted sedimentation assays with liposomes made from brain lipids (Folch fraction I). Here, we found that recombinant Arl6 efficiently bound to liposomes in the presence of the slowly hydrolyzable analog GMP-PNP but not in the presence of GDP or when the N-terminal amphipathic helix was removed (Figure 5C). We therefore conclude that Arl6 conforms to the Arf1/Sar1 paradigm and interacts with membranes through its N-terminal amphipathic helix when GTP bound.

To determine the minimal requirements for BBSome binding to liposomes, we next needed a highly purified BBSome fraction. Such a fraction was obtained by fractionating retinal extract over the Arl6-GTP column (Figure 1A) followed by cation exchange chromatography (Figure 5D). The purified BBSome was nearly free of contaminants as assessed by silver staining and behaved as a monodisperse complex devoid of aggregates by rate zonal sedimentation (Figure S5B). We then performed sedimentation assays with purified BBSome, Arl6, guanine nucleotides, and liposomes made from brain lipids and found that efficient binding of the BBSome to liposomes required Arl6 and GMP-PNP (Figure 5E). We have thus reconstituted the recruitment of the BBSome to membranes from purified components in vitro and no protein factor other than GTP-bound Arl6 is required for this binding.

**Phospholipid Requirement for BBSome Binding to Liposomes**

Given the robust interaction between Arl6-GTP and the BBSome on one hand and between Arl6-GTP and liposomes on the other, it was conceivable that the BBSome recruitment to liposomes...
Figure 5. Arl6GTP Recruits the BBSome to Liposomes Made from Pure Lipids
(A) Helical-wheel representation of the N-terminal 13 amino acids of Arl6. Hydrophobic, nonpolar residues are on one side of the helix, while charged or polar residues are on the opposite side. Nonpolar residues are yellow, polar residues are purple, acidic residues are red, basic residues are blue, and glycine is gray. The diameter of each circle is proportional to the bulk of each residue.
(B) Sequence alignment of the N terminus from selected Arf/Arl family members. Hydrophobic amino acids are highlighted in dark gray, and basic residues are light green.
(C) Arl6GTP binds to liposomes through its N terminus. Liposomes (20 μM) made from brain lipids were incubated with 2 μM Arl6 or Arl6ΔN in the presence of 100 μM GMP-PNP or GDP in a 100 μM reaction at 30°C for 1 hr. The reactions were centrifuged at 385,000 × g for 30 min at 24°C, and equal portions of the resulting supernatants (S) and pellets (P) were resolved by SDS-PAGE and stained with Coomassie. As control for protein precipitation during the course of the experiment, Arl6 or Arl6ΔN were incubated without lipid and processed as above.
(D) Purification of retinal BBSome. Eluates from the Arl6GTP affinity column were loaded onto a cation exchange column (MonoS), and the BBSome (red dots mark subunits) was eluted with a salt gradient. Fractions were analyzed by SDS-PAGE and silver staining.
(E) The BBSome binds to liposomes in an Arl6- and GTP-dependent manner. Various combinations of Arl6 (0.5 μM), GMP-PNP, or GDP (100 μM) were incubated with 4 μg brain lipid liposomes in a 50 μl reaction at 30°C for 30 min. Reactions were diluted to 100 μl, supplemented with BBSome (50 nM final), and returned to 30°C for a further 15 min. Liposomes and bound proteins were sedimented at 140,000 × g for 30 min at 24°C, and pellets were resolved by SDS-PAGE and stained with silver. Red dots denote BBSome subunits (except for BBIP10 which was not resolved), while the blue dot denotes Arl6.
(F) Phosphoinositide specificity of BBSome binding to liposomes. Liposomes (167 μM final lipid concentration) containing 3 mol% of various PIPs were incubated with Arl6 (0.25 μM), BBSome (50 nM), and GMP-PNP (100 μM) in a 60 μl reaction before flotation on iodixanol gradients. Liposome-bound proteins were analyzed by SDS-PAGE and silver staining. Although Arl6 binding was similar for all eight liposomes, BBSome binding was maximal when liposomes contained PI(3,4)P₂. Quantitation of Arl6 and BBSome binding is shown in Figure S5C.

The composition of PC/PE/PS/PA/PI liposomes is as follows: 53% DOPC, 22 mol% DOPE, 1 mol% Texas-Red DHPE, 8 mol% DOPS, 5 mol% DOPA, and 11 mol% DPPI. PIPs were substituted for 3 mol% PI in PIP liposomes.

(G) The BBSome requires acidic phospholipids to efficiently bind to liposomes. Liposomes (167 μM final lipid concentration) were incubated with Arl6, BBSome (50 nM), and GMP-PNP or GDP (100 μM) in a 60 μl reaction before flotation on iodixanol gradients. In order to achieve similar recoveries of Arl6 with different liposomes, 1.25 μM Arl6 were used for PC/PE liposomes, while 0.25 μM Arl6 were used for PI(3,4)P₂ liposomes. Liposome compositions are as follows. PC/PE: 88 mol% DOPC, 11 mol% DOPE, 1 mol% Texas-Red DHPE. PC/PE/PS/PA/PI: see (F).

See also Figure S5.

was strictly indirect and did not involve any contact between the BBSome and lipid headgroups. However, COPI and COPII coats and clathrin adaptors have all been shown to directly contact acidic phospholipids or specific PIPs (Matsuoka et al., 1998; Spang et al., 1998; Bremser et al., 1999; McMahon and Mills, 2004), and those contacts are probably important in the sculpting of buds and vesicles by the polymerizing coat. To determine whether specific lipids are required for the binding of the BBSome to membranes, we made liposomes from synthetic phospholipids. Given that the lipid composition of mammalian cilia is not known, we started with a base mixture (dubbed “major mix”) that allows for the efficient capture of COPI, COPII, and exomer coat complexes and that functions in COPI and COPII budding reactions (Matsuoka et al., 1998; Spang et al., 1998; Wang et al., 2006). The phospholipid part of the major mix contains 76 mol% of neutral phospholipids (53 mol% phosphatidylcholine [PC] and 23 mol% phosphatidylethanolamine [PE]) and 24 mol% of acidic phospholipids (8 mol% phosphatidylserine [PS], 5 mol% phosphatidic acid [PA], and 11 mol% phosphatidylinositol [PI]). The cholesterol/phospholipid molar ratio is 23:77, and all acyl chains are oleoyl (18:1) to keep fluidity high through the 4°C–30°C range of temperatures. To preclude any background signal
stemming from protein precipitation during the course of the incubation with liposomes, we isolated the protein complexes bound to liposomes by buoyant density flotation on miniature iodixanol step gradients.

Initial experiments showed moderate binding of the BBSome to major mix liposomes in the presence of Arl6\(^{\text{GMP-PNP}}\) (Figure 5F, lane 1). Since we have previously shown that the BBSome subunit BBSS binds to PIPs on protein lipid overlays, we replaced a portion of the PI in the major mix with one of seven individual PIPs. While the specificity of BBSome binding for a specific PIP was somewhat variable (Figure S5C), multiphosphorylated PIPs [in particular PI(3,4)P\(_2\)] enhanced BBSome binding to liposomes by as much as 3-fold. This enhanced BBSome binding did not result from a general stickiness of PIP liposomes as BBSome binding to PI(3,4)P\(_2\) liposomes was strongly Arl6 and GMP-PNP dependent (Figure 5G, lanes 3 and 4, and Figure S5D). We note that recombinant BBSS exhibits a different PIP specificity on lipid blot overlays (Nachury et al., 2007) than the BBSome does on liposomes and conclude that BBSS and individual lipids taken out of their physiological environments may not faithfully recapitulate the specificity of the BBSome complex for lipids in hydrated bilayers.

The preference for a lipid bearing a strong negative charge by the BBSome suggested that other acidic phospholipids in the major mix might participate in BBSome recruitment to membranes. We therefore tested Arl6- and GMP-PNP-dependent binding of the BBSome to liposomes made from neutral lipids. Since PC/PE liposomes were less effective than PI(3,4)P\(_2\) liposomes at recruiting Arl6\(^{\text{GMP-PNP}}\), the molarity of Arl6 was adjusted to recover similar amounts of Arl6\(^{\text{GMP-PNP}}\) regardless of the liposome composition. Despite the 5-fold increase in Arl6 molarity, the binding of Arl6 to liposomes was still strongly dependent upon the addition of GMP-PNP (Figure 5G, lanes 1 and 2). While the amount of Arl6\(^{\text{GMP-PNP}}\) recovered remained relatively unchanged, BBSome binding was drastically reduced in the absence of charges on the bilayer surface (Figure 5G, lanes 1 and 3). Thus, Arl6\(^{\text{GMP-PNP}}\) binds membranes through neutral and acidic phospholipids and Arl6\(^{\text{GMP-PNP}}\) and acidic lipids (in particular multiphosphorylated PIPs) synergize to recruit the BBSome to membranes.

**Ultrastructure of Liposomes Incubated with BBSome and Arl6\(^{\text{GTP}}\)**

We next investigated the morphological consequences of BBSome binding to liposomes. Incubation of COPI, COPII, clathrin, and exomer coat components with liposomes leads to the formation of coated profiles and, in the case of COPI and COPII, the budding of 50 nm diameter coated vesicles (Matsuoka et al., 1998; Spang et al., 1998; Bremser et al., 1999; Wang et al., 2006). Liposomes consisted of uni- and multilamellar structures with smooth bilayer surfaces when visualized by thin-section electron microscopy. After incubation with BBSome, Arl6, and GMP-PNP, close to 10% of all liposomes showed coated surfaces (Figure 6A). The coated profiles appeared as continuous and well-delineated patches clearly separated from noncoated surfaces (Figure 6A and Figure S6), and repeat units could be distinguished at high magnification (Figure 6B), suggesting formation of an ordered polymer. Similar to the exomer coat, no membrane deformation was seen with the BBSome coat proteins, and coated profiles retained the normal liposome curvature. When GMP-PNP was replaced with GDP (Figures 6C and 6D) or when BBSome was omitted (Figures 6E and 6F), no coated profiles were visible. Thus, upon recruitment to membranes by Arl6, the BBSome appears to polymerize into an electron-dense coat associated with the liposome surface.

**SSTR3\(^{131}\) Is an Arl6- and BBSome-Dependent CTS**

The polymerization of the BBSome into a membrane coat strongly implied that the BBSome sorts specific membrane
proteins (i.e., cargoes) inside the cell. A strong candidate for BBSome cargo is SSTR3, which is lost from cilia in bbs2−/− and bbs4−/− hippocampal neurons (Berbari et al., 2008a). We extended these results by showing that the number of SSTR3-positive cilia decreases dramatically when Arl6 is depleted from cultured hippocampal neurons by lentivirus-mediated short hairpin RNA (shRNA) (Figures 7A and 7B).

The hypothesis that SSTR3 is a bona fide BBSome cargo predicts that the BBSome directly recognizes the CTS of SSTR3, which was previously mapped to the third intracellular loop (l3) (Berbari et al., 2008b). We tested this prediction by expressing SSTR3l3 fused to GST in bacteria and conducting a GST capture assay with purified retinal BBSome. While GST-SSTR3l3 efficiently captured the purified BBSome, GST alone or GST fused to the third intracellular loop of the closely related GPCR SSTR5 failed to recover detectable amounts of BBSome (Figure 7C). Further, each SSTR5 subunit expressed in HEK cell bound to SSTR3l3 but not to SSTR3l3 (Figure 7A), indicating that SSTR3l3 is not recognized by a single BBSome subunit but rather by the homo-BBSome, which assembles around BBSome subunits expressed in HEK cells (See et al., 2010).

Sequence analysis of multiple GPCRs targeted to cilia has suggested that the CTS of ciliary GPCRs centers around the motifs of this motif to phenylalanine within SSTR3 (Figure 7D). Surprisingly, SSTR3AQA-FP bound to the BBSome more efficiently than its wild-type counterpart (Figure 7D). We therefore targeted alternative amino acids within the API/S[A]CO motifs of SSTR3 (Figure 7E and Figure 7B) and found that the cysteine at the fourth position was the only amino acid required for BBSome binding (Figure 7D). To then assess the CTS activity of the various SSTR3 variants, we spliced them into the cytoplasmic tail of the plasma membrane protein CD8α and transiently expressed the chimeras in IMCD3 cells. While CD8α and CD8α-SSTR3l3 failed to efficiently target to cilia, CD8α-SSTR3l3 was transported to cilia in more than 90% of transfected cells. Most importantly, the targeting of CD8α-SSTR3l3/A6-A1 to cilia was severely impaired compared to CD8α-SSTR3l3 (Figure 7F), while targeting of CD8α-SSTR3l3/A6-A1 to cilia remained unaffected (Figure 7B). Thus, the molecular recognition of SSTR3l3 by the BBSome is essential for the full CTS activity of SSTR3l3.

Finally, we wished to pinpoint the compartment where CD8α-SSTR3l3 accumulates in the absence of BBSome or Arl6 function. To this end, we generated a stable cell line expressing the chimeras in IMCD3 cells. While CD8α-SSTR3l3 was targeted to cilia in >95% of cells treated with a control siRNA, depletion of Arl6 or BBS4 led to a pronounced decrease in ciliary targeting of CD8α-SSTR3l3 (Figure 7G and Figure S7C). Importantly, the total levels of CD8α-SSTR3l3 remained unchanged by depletion of Arl6 or BBS4 (Figure 7H, “Total”), thus supporting the interpretation that the BBSome coat sorts the synthetic cargo to cilia rather than stabilizes it. Interestingly, examination of CD8α-SSTR3l3 localization in cells depleted of Arl6 or BBS4 revealed significant plasma membrane staining not observed in cells treated with control siRNA (Figure 7F). To rigorously test whether CD8α-SSTR3l3 accumulated at the plasma membrane in the absence of BBSome or Arl6, we conducted surface biotinylation followed by capture on avidin beads. Remarkably, we found that the levels of surface-exposed CD8α-SSTR3l3 remained unchanged in the absence of Arl6 or BBS4 (Figure 7H, “Surface,” and Figures S7D and S7E). Thus, we conclude that a prototypical BBSome cargo normally localized in the ciliary membrane accumulates at the plasma membrane in the absence of BBSome coat function.

Together, these results establish SSTR3l3 as an Arl6- and BBSome-dependent CTS and suggest that the BBSome carries out the trafficking of SSTR3l3-containing cargoes from the plasma membrane to the ciliary membrane.

**DISCUSSION**

**The BBSome as a Planar Coat for Lateral Transport between Plasma and Ciliary Membranes**

Since the BBSome forms a coat and reads the sorting signals of its cargoes to direct them to the cilium, the decision to sort membrane proteins such as SSTR3 toward the cilium is almost certainly made on the compartment where the BBSome coat assembles. Since a synthetic BBSome cargo is detected at the plasma membrane in the absence of BBSome function, a plausible model would have BBSome cargoes diffuse laterally in the plasma membrane until their sorting signals become recognized by the BBSome. The ensuing assembly of a planar BBSome coat would cluster these cargoes into a patch that can be dragged through the periciliary diffusion barrier separating plasma and ciliary membranes (Nachury et al., 2010).

Once inside the cilium, the patch of BBSome coat is predicted to become transported by the IFT machinery. In nematodes, Chlamydomonas, and human cells, the BBSome has been shown to undergo intraflagellar motility at the same rates as known IFT polypeptides, and it has been proposed that the BBSome functions as an adaptor between the IFT complexes and IFT cargos (Blacque et al., 2004; Nachury et al., 2007; Lechtreck et al., 2009). The observation that the BBSome assembles a coat in the absence of IFT polypeptides suggests that polymerization of a BBSome layer could drive polymerization of an IFT layer. These BBSome/IFT patches would possess one layer of BBSome coat connected to the membrane-bound cargoes and one layer of IFT-A and -B complexes bound to the IFT motors (Ou et al., 2005).

While Arl6 and the BBSome are generally not required for ciliogenesis (see the Extended Discussion), IFT function is universally required for cilium formation. A possible explanation resides in the fact that BBSome only transports a specific set of transmembrane proteins to cilia while the IFT complexes are likely required for all transport processes inside cilia.

**The BBSome as a Canonical Coat**

Unlike COPI and COPII coat formation, BBSome coat assembly in vitro did not sculpt membranes into buds and 50 nm vesicles. While the BBSome may strictly resemble the clathrin plaques...
Generation of a point mutant in SSTR3 was stained with Ponceau S to show equivalent amounts of fusion moiety cleaved off from GST. Four and a half input equivalents of each eluate were loaded. and the material cleaved off from GST was resolved on SDS-PAGE and probed with antibodies against BBS4, BBS8, and BBS9. The bottom part of the membrane bars represent 5

In the region around the cilium. Images were acquired with a 63

per experiment were counted and the percentages of CD8

directed against the extracellular domain of CD8

were transfected into IMCD3 cells, and cells were serum starved to induce ciliation. Surface exposed CD8α chimeras were visualized by incubating cells with the OKT8 antibody directed against the extracellular domain of CD8α before fixation. Cilia were visualized by Glu-Tubulin staining. Insets show an enlargement of the CD8α channel in the region around the cilium. Images were acquired with a 63 x 1.4 NA objective and planes containing cilia are shown. At least 85 ciliated and transfected cells per experiment were counted and the percentages of CD8α chimeras targeting to cilia were plotted. Error bars represents the SD between microscopic fields. Scale bars represent 5 μm.

Figure 7. Direct Recognition of SSTR3

(A) Arl6 is required for SSTR3 trafficking to cilia. Hippocampal neuron cultures were infected with shArl6-lentivirus or control lentivirus at DIV2 (2 days in vitro) and immunostained for adenylate cyclase III (ACIII), a marker of neuronal cilia, and SSTR3 at DIV8. Stacks of ten Z sections were acquired at 0.5 μm interval with a 63 x 1.4 NA objective and planes containing cilia are shown. At least 85 ciliated and transfected cells per experiment were counted and the percentages of CD8α-SSTR3

and CD8α-SSTR5

were plotted. Error bars represents the SD between microscopic fields. Scale bars represent 5 μm.

(B) Top: at least 156 cilia were counted for each condition in the experiment shown in (A). Error bars represent the SD between microscope fields. Bottom: protein extracts from hippocampal neuron cultures prepared as in (A) were immunoblotted for Arl6 and actin (loading control). At least 75% of Arl6 protein was depleted upon infection with shArl6-lentivirus compared to control virus. Nonetheless, the large amounts of Myc-BBS2 recovered with SSTR3

are highlighted, the conserved AX[A/S]XQ motifs are shown in bold and the mutated amino acids are red. The complete set of sequences is shown in Figure S7B.

(F) The CTS activity of SSTR3 depends upon intact BBSome binding. CD8α, CD8α-SSTR3

and CD8α-SSTR5

were transiently transfected into IMCD3 cells, and cells were serum starved to induce ciliation. Surface exposed CD8α chimeras were visualized by incubating cells with the OKT8 antibody directed against the extracellular domain of CD8α before fixation. Cilia were visualized by Glu-Tubulin staining. Insets show an enlargement of the CD8α channel in the region around the cilium. Images were acquired with a 63 x 1.4 NA objective and planes containing cilia are shown. At least 85 ciliated and transfected cells per experiment were counted and the percentages of CD8α chimeras targeting to cilia were plotted. Error bars represents the SD between microscopic fields. Scale bars represent 5 μm.
that cluster cargoes at the plasma membrane without deforming membranes (Saffarian et al., 2009), technical issues may have prevented vesicle budding upon BBSome coat formation in our in vitro system. First, there may be specific soluble factors that assist the BBSome coat in deforming membranes. In the example of clathrin, the large GTPase dynamin and local actin polymerization are required for the invagination and scission of clathrin-coated pits and patches (Koenig and Ikeda, 1996; Saffarian et al., 2009). Second, the presentation of sorting signals on the surface of the lipid bilayer and their capture by the BBSome may be necessary for budding coupled to BBSome coat assembly, as is the case for COPI when using liposomes mimicking Golgi membranes (Bremser et al., 1999). Finally, the concentrations of BBSome used in the present study (50 nM) may not be sufficiently high to permit the assembly of a BBSome coat competent to deform membranes. COPI-mediated budding was conducted using concentrations approaching 1.5 μM of coatamer (Bremser et al., 1999).

If the BBSome assembles a canonical coat that sculpts membranes into buds and vesicles, where would this budding reaction take place within the cell? Since the steady-state localization of all known coat complexes is the organelle where they sort cargo and deform membranes, one would predict that the BBSome buds endocytic vesicles off of the ciliary membrane. As the passage of vesicles between the ciliary lumen and the cytoplasm is topologically unfeasible (Nachury et al., 2010), it appears more plausible that the BBSome buds vesicles off of the base of the cilium to remove membrane proteins from cilia, as suggested by Lechtreck et al. (2009). However, to account for BBSome-mediated sorting of SSTR3i3 to cilia, one needs to invoke the budding of vesicles by the BBSome from a donor compartment that is distinct from cilia. Since we have never observed any BBSome or Arl6 staining on endomembrane compartments or at the plasma membrane in a number of different cell lines using well-validated antibodies, we would need to postulate that, unlike all known coat complexes, the steady-state localization of the BBSome does not correspond to the donor compartment for BBSome-mediated budding. Although unlikely, it is formally possible that the population of BBSome and Arl6 we observe within cilia corresponds to a slowly recycling pool that becomes injected into cilia after the fusion of BBSome-coated vesicles with the base of the cilium.

Restricting BBSome Coat Assembly within the Cell

Regardless of the model considered, only one protein, the small GTPase Arl6, is necessary and sufficient for recruiting the BBSome to membranes. No other soluble protein and no membrane proteins are required for BBSome binding to liposomes. Most strikingly, the biochemical output of the BBSome binding to membranes is the assembly of a thin electron-dense coat on the bilayer surface whose morphology is clearly distinct from COPI, COPII, clathrin, and exomer coats. While the process we have recapitulated in the test tube informs the minimal requirements for BBSome coat formation, BBSome coat assembly in vivo must occur with a high degree of spatial and temporal specificity. First, the loading of GTP onto Arl6 is likely to be rate-limiting for BBSome coat assembly in vivo, and an Arl6-GFP may locally activate Arl6 to enable BBSome coat assembly in vivo. Second, PCM-1, a major BBSome-associated protein, does not copurify with the BBSome on Arl6GTP chromatography. PCM-1 may therefore prevent BBSome binding to Arl6GTP, and removal of PCM-1 from the BBSome may be a prerequisite for BBSome coat assembly. Third, the requirement for acidic lipids—possibly specific PIPs—to efficiently recruit the BBSome to membranes in vitro suggests that local lipid composition may dictate where the BBSome coat assembles. While no PIPs have been detected in cilia thus far, the 5-phosphatase INPP5E that converts PI(3,4,5)P3 to PI(3,4)P2 is found in cilia, and loss of INPP5E leads to several ciliopathies closely related to BBS (Jacoby et al., 2009; Bielas et al., 2009). Furthermore, loss of the PI(3,4)P2-binding protein tubby leads to obesity and retinal degeneration in mice (Santagata et al., 2001). Since tubby genetically interacts with bbs-1 in worms (Mak et al., 2006), it is tempting to speculate that tubby and the BBSome may function on the same membrane compartment.

The BBSome coat model suggests that the variety of symptoms found in BBS patients is likely to result from the failure to transport signaling receptors to the cilium. While the relevance of SSTR3 to the etiology of BBS is currently unknown (Einstein et al., 2010), the observation that the leptin receptor interacts with BBS1 (Seo et al., 2008) provides a tantalizing hypothesis for the molecular basis of obesity in BBS, namely that leptin signaling may take place within primary cilia in a BBSome-dependent manner. The discovery of signaling receptors transported by the BBSome promises to uncover the signaling defects that underlie BBS and to provide mechanistic insights into the interplay between ciliary trafficking and signaling pathways.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents
Arl6 antibodies were raised in rabbits and purified according to standard protocols. All other antibodies are described in the Extended Experimental Procedures. All chemicals were purchased from Sigma unless otherwise indicated. 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS), and 1,2-dioleoyl-sn-glycero-3-phosphate (DOPA) were synthesized at Yale University or were gifts from M. Kobilka (Stanford University).

(G) Ciliary targeting of CD8x-SSTR3i3 is BBSome dependent. IMCD3-[CD8x-SSTR3i3] cells were treated with control siRNA or siRNA against Arl6 or BBS4 and serum starved to induce ciliation. Surface exposed CD8x-SSTR3i3 was visualized with the OKT8 antibody and cilia were stained with Glu-Tubulin as in (F). Insets show an enlargement of the CD8x channel in the region around the cilium. Images were acquired with a 100x/1.4 NA objective and planes containing cilia are shown. At least 110 cilia per treatment were counted and the percentages of CD8x-SSTR3i3-positive cilia were plotted. Error bars represent the SD between microscopic fields. Scale bars represent 5 μm.

(H) A missorted BBSome cargo accumulates at the plasma membrane. Surface biotinylation was performed on IMCD3-[CD8x-SSTR3i3] cells treated as in (G). Equal portions of cell lysates were either immunoprecipitated with the OKT8 antibody (Total) or captured with Neutravidin (Surface). The strips were excised from the same membrane. Arl6 and BBS4 were efficiently depleted by siRNA treatment as shown in Figure S7C. Note that total or surface exposed CD8x-SSTR3i3 protein levels is not changed in siArl6- or siBBS4-treated cells. See also Figure S7.
purchased from Avanti polar lipids. PI and PIPs were from Echelon or AG Scientific. Texas Red 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (DHPE) was from Invitrogen. Chemicals for electron microscopy were from EM Sciences.

**Immunofluorescence and Microscopy**

Cells were fixed in PBS containing 4% paraformaldehyde for 5 min at 37°C, followed by extraction with cold methanol at -20°C for 5 min and processing for immunofluorescence as described (Nachury et al., 2007). Nuclei were stained with Hoechst 33258. Unless otherwise indicated in the figure legend, stacks of 24 Z sections were acquired at 0.25 μm interval with a 100×/1.45 NA objective and deconvolved by constrained iterative, and the section containing the cillum was selected for each figure panel. Enhanced immunofluorescence and CD8 staining are detailed in the Extended Experimental Procedures.

**Affinity Chromatography and BBSome Purification**

Affinity chromatography onto immobilized GST-Arl6 was performed as in Christoforidis and Zerial (2000) with modifications. Bovine retinas (50 g) were thawed in 150 ml NS500 (25 mM Tris [pH 8.0], 500 mM KCl, 5 mM MgCl₂, 1 mM DTT) supplemented with 250 mM sucrose and protease inhibitors (1 mM AEBSF, 10 μg/ml each of Leupeptin, Pepstatin A, Bestatin), homogenized by buncing and centrifuged for 2 h at 184,000 × g at 4°C in a Ti70 rotor. The retinal extract was loaded onto 1 ml GSTrap HP columns (GE) previously saturated with GST fusion proteins, and columns were washed with 20 ml NS500 containing 50 μM nucleotide and eluted at 22°C with 4 column volumes of EB (25 mM Tris [pH 8.0], 2.5 M NaCl, 10 mM EDTA, 5 mM MgCl₂, 1 mM DTT, protease inhibitors). Eluates were run on SDS-PAGE or concentrated by methanol/chloroform precipitation for nanoscale micropipette reverse-phase liquid chromatography with electrospray ionization tandem mass spectrometry (LC-MS/MS) as previously described (Haas et al., 2006). For BBSome purification, the eluate of the Arl6 column was then dialyzed against four successive buffers of decreasing ionic strength for 45 min each (final buffer: 25 mM HEPES [pH 7.0], 100 mM NaCl, 5 mM MgCl₂, 1 mM DTT) and cleared by ultracentrifugation. The dialyzed Arl6 eluate was then fractionated on a Mono S PC 1.6/5 column (GE) equilibrated in buffer H5 (25 mM HEPES [pH 7.0], 50 mM NaCl) and developed with a gradient of 50 mM to 500 mM NaCl spanning nine column volumes. GST pulldowns are described in the Extended Experimental Procedures.

**Preparation of Liposomes, Binding Assays, and Ultrastructural Analysis**

Liposomes were prepared according to Matsuoka et al. (1998) with minor modifications as detailed in the Extended Experimental Procedures. Liposomes were extruded through polycarbonate filters (100 nm pore size for brain lipids and 400 nm pore size for synthetic lipids) and kept at 4°C. All liposomes made from synthetic lipids contained 23 mol% of cholesterol and 77 mol% phospholipids, and Texas Red DHPE was included to normalize lipid concentrations. Liposome flotation assays were conducted as detailed in the Extended Experimental Procedures. Ultrastructural analysis of protein/liposome mixtures by thin-section electron microscopy was performed as described Matsuoka et al. (1998).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Extended Discussion, Extended Experimental Procedures, seven figures, and one movie and can be found with this article online at doi:10.1016/j.cell.2010.05.015.

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Supplemental Information

EXTENDED DISCUSSION

Arl6 and the BBSome Are Generally Not Required for Ciliogenesis

We previously published that knockdown of most BBSome subunits does not affect cilium assembly in RPE cells (Figure 3 of (Loktev et al., 2008)). The exceptions were BBS1, BBSS and BBIP10, whose knockdown resulted in fewer cilia. Since then, we have shortened the serum starvation period of our ciliation protocol from 48h to 24h and we now find that knockdown of BBSS preserves cilium assembly (Figure S2) while knockdown of BBS1 still prevents cilium assembly (not shown). Mouse knockout for arl6, bbs2, bbs4 and bbs7 are all viable and assemble cilia normally in most tissues (Mykytyn et al., 2004; Nishimura et al., 2004; Zhang Q, Nachury MV, Loktev AV, Jackson PK, Stone EM and Sheffield VC. 2007. American Society of Human Genetics Annual Meeting. Abstr. 1270; Vogel T, Nishimura DY, Searby CC, Swiderski RE, Mullins RF, Pretorius PR, Seo S, Zhang Q, Bugge K, Thedens DR, Cassell MD, Wemmje JA, Sheffield VC. 2009. American Society of Human Genetics Annual Meeting. Abstr. 2394). In BBS patients, nonsense mutations presumed to yield null alleles have been found at the homozygous state in Arl6/BBS3 and in every BBSome subunit except for BBS1 where a missense mutation (mostly BBS1[M390R]) is always found on at least one allele (Mykytyn et al., 2002). In addition, no mutations in BBIP10 have so far been found in human patients (Loktev et al., 2008). Thus, BBS1—and possibly BBIP10—are likely to be the sole BBSome subunits essential for cilium assembly and consequently for vertebrate development. Given that null mutants of BBS1 in Chlamydomonas and in nematode assemble structurally normal cilia and flagella (Ou et al., 2007; Lechtreck et al., 2009), the essential role of BBS1 in cilium assembly appears restricted to vertebrates. In summary, the ciliogenic functions of BBS1 and BBIP10 are likely the sole exceptions to Arl6 and BBSome functions not being required for ciliogenesis.

EXTENDED EXPERIMENTAL PROCEDURES

Plasmids

Human Arl6 (NM_032146) was used for all subcloning purposes. 19-mer shRNAs targeting mouse Arl6 were designed using pSicoO-ligomaker 1.5 and subcloned into lentiviral vector pSicoR-GFP (human Arl6 (NM_032146) was used for all subcloning purposes. 19-mer shRNAs targeting mouse Arl6 were designed using pSicoO-ligomaker 1.5 and subcloned into lentiviral vector pSicoR-GFP (Ventura et al., 2004). The third intracellular loops (i3) of mouse SSTR3 (aa 231-266) and of mouse SSTR5 (aa 223-247) were cloned into pGEX6P by gene synthesis. CD8α-SSTR3/5i3 chimeras were generated by insertion of SSTR3/5i3 into the C terminus of human CD8α at position 213 and subcloned into a pEF5α.FRT derivative.

Cell Culture, Transfection and Virus Production

To generate RPE clones stably expressing Arl6 C-terminally fused to GFP, retroviruses were produced using derivatives of pBabe-Puro. RPE cells were infected and single cell clones were selected for moderate expression levels. RPE and RPE-[Arl6GFP] cells were transfected with 20 nM siRNA duplexes using Lipofectamine RNAiMAX (Invitrogen) following the manufacturer’s instructions. RPE cells were shifted from 10% serum to 0.2% serum 48-52 hr after transfection to induce ciliation and fixed 10-12 hr post starvation. Hippo-campi from P1 or P2 mice were dissected and cultured as described (Malgaroli and Tsien, 1992). Lentivirus were produced by cotransfecting HEK293 cells with transfer vector pSicoR-GFP, packaging vector pCMVdeltaR8.74 and envelope vector pMD2.6 (Dull et al., 1998). Neuron cultures were infected with lentiviruses after 2 days in vitro (DIV), and fixed and processed for immunofluorescence at 7-8 DIV.

CD8α and its derivatives were transiently transfected into IMCD3 cells using lipofectamine 2000 (Invitrogen). IMCD3 cells stably expressing CD8α-SSTR3i3 were generated by cotransfecting IMCD3 Flp-in cells (Invitrogen) with pEF5.FRT.CD8α-SSTR3i3 and pOG44. For BBSome depletion, IMCD3-[CD8α-SSTR3i3] cells were treated with 20 nM siRNA duplexes using Lipofectamine RNAiMAX (Invitrogen). To ensure efficient depletion, 20,000 cells per well of a 24-well plate were used. Cells were shifted from 10% serum to 0.2% serum 48-52 hr after transfection to induce ciliation and fixed 10-12 hr post starvation.

siRNA sequences targeting Arl6 and mouse BBS4 are available upon request. All siRNA controls were siGenome non-targeting #2 (Dharmacon) and siRNAs targeting human BBSome subunits are described elsewhere (Nachury et al., 2007).

Enhanced Immunofluorescence

Cells were prefixed in PBS containing 0.4% paraformaldehyde for 5 min at 37˚C, and extracted with 0.5% TX-100 in PHEM (50 mM PIPES, 50 mM HEPES, 10 mM EGTA and 10 mM MgCl2, pH 6.9) for 2 min at 37°C. After briefly washing with PBS, cells were processed following the conventional immunofluorescence protocol described in Experimental Procedures.

CD8 Immunofluorescence

Cells were washed three times with cold PBS and treated with 1 μg/ml OKT8 antibody in PHEM for 10 min on ice. After briefly washing with PBS, cells were fixed in PBS containing 4% paraformaldehyde for 5 min at 37˚C, followed by extraction with cold methanol at −20˚C for 5 min and processing for immunofluorescence as described in Experimental Procedures.

Surface Biotinylation

Surface-exposed amino groups were biotinylated using the membrane-impermeable EZ-Link Sulfo-NHS-SS-biotin (Pierce) for 30 min on ice at 0.6 mg/ml in PBS (1.2 mg per 1 well of a 6 well plate). After incubation, the reaction was stopped by washing cells three
times with 50 mM glycine in PBS. Cells were washed three times with PBS and scraped in lysis buffer (1% Triton X-100, 40 mM HEPES pH 7.4, 150 mM NaCl, 5 mM EDTA and 10 μg/ml each of Leupeptin, Pepstatin A, Bestatin, Aprotinin, AEBSF and E-64) and kept for 40 min at 4°C. After centrifugation at 16,000 x g_{max} for 10 min in a microcentrifuge, equal portions of the supernatant were subjected to immunoprecipitation with the OKT8 antibody or affinity capture with Neutravidin-Sepharose (Pierce). For immunoprecipitation, 1 μg OKT8 antibody was added to 100 μg lysate and incubated 90 min on ice followed incubation with 20 μl protein G-Sepharose for 1 hr at 4°C. Beads were washed five times with lysis buffer, and bound material was eluted with 2 X SDS sample buffer. For affinity capture, lysates were incubated with Neutravidin-Sepharose for 150 min at 4°C. After washing five times, the bound material was eluted with 2 X SDS sample buffer.

### Size Exclusion Chromatography

RPE cells treated with control siRNA or Arl6 siRNA were serum starved and harvested. All subsequent manipulations were done at 4°C. Cell pellets were resuspended in LAP300 (50 mM HEPES pH 7.4, 300 mM KCl, 1 mM EGTA, 1 mM MgCl₂, 10 μg/ml each of Leupeptin, Pepstatin A, Bestatin, Aprotinin, AEBSF and E-64) and lysed by adding NP-40 to 0.3% final concentration. After centrifugation at 16,000 x g_{max} for 10 min in a microcentrifuge, the supernatants were diluted to 200 mM KCl and spun at 36,600 x g_{max} for 1 hr in a TLA100 rotor. The clarified lysates were fractionated on a Superose 6 column (GE healthcare) equilibrated with LAP200N (50 mM HEPES pH 7.4, 200 mM KCl, 1 mM EGTA, 1 mM MgCl₂, 0.05% NP-40).

### Sucrose Gradient Fractionation of BBSome

10%–40% sucrose gradients were prepared by overlaying 500 μl each of 40%, 30%, 20% and 10% w/w sucrose, respectively, in LAP150N (50 mM HEPES, pH 7.4, 150 mM KCl, 1 mM EGTA, 1 mM MgCl₂, 0.05% NP-40) and made continuous by incubation at room temperature for 2 hr. Purified BBSome (25 μl) was layered on top of the gradient and centrifuged at 166,000 x g_{ave} for 8 hr at 4°C. 150 μl fractions were taken from the top.

### Antibody Sources and Dilutions

Antibodies against BBS1, BBS4 and BBIP10 were previously described (Nachury et al., 2007; Loktev et al., 2008). Commercial antibodies were against acetylated α-tubulin (mAb 6-11B-1, Sigma), ACII (sc-588, Santa Cruz), SSTR3 (for IF: sc-11617, Santa Cruz; for immunoblotting CD8α-SSTR3/C, Alomone), c-Myc (A-14, Santa Cruz), actin (Sigma), BBS2 (Proteintech), BBS5 (Proteintech), Glu-tubulin (AB3201, Millipore) and CD8α (OKT8, eBioscience). Antibodies against the following proteins were diluted as indicated for immunofluorescence: Arl6 (0.2 μg/ml), BBS1 (1 μg/ml), BBIP10 (1 μg/ml), Acetylated tubulin (1: 10,000), BBS2 (1: 50) and BB5 (1: 50), ACII (1 μg/ml), Goat anti-SSTR3 (1 μg/ml) and Glu-tubulin (1 μg/ml).

### Protein expression, Purification and GST Capture Assays

GST-Arl6ΔN16 and GST-Arl6 variants were expressed from derivatives of pGEX6P in Rosetta2(DE3)-pLysS cells following (Shimoni and Schekman, 2002) with minor modifications. Cells were resuspended in TBS+ (50 mM Tris pH 7.4, 150 mM NaCl, 50 μM GDP, 5 mM MgCl₂, 1 mM DTT) and lysed by sonication. The clarified lysate was loaded onto Glutathione Sepharose 4B (GE Healthcare) and Arl6 proteins were eluted by cleavage with HRV3C protease. Eluates were concentrated by ammonium sulfate precipitation and Arl6 was further purified on Superdex 75 equilibrated in CB (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM MgCl₂) for 2 hr (50 mM HEPES pH 7.4, 200 mM KCl, 1 mM EGTA, 1 mM MgCl₂, 0.05% NP-40).

GST-S3R3/C5fusions were expressed from derivatives of pGEX6P in Rosetta2(DE3)-pLysS cells. Cells were resuspended in 4XT (800 mM NaCl, 200 mM Tris pH 8.0, 1 mM DTT) and lysed by sonication.

**GST pull-down assays were conducted by saturating 10 μl of Glutathione Sepharose beads with GST-Arl6ΔN16[Q73L] or GST-S3R3/C5. HEK cells expressing individual Myc-tagged BBSome subunits were lysed in NSD250 (25 mM Tris pH 8.0, 250 mM KCl, 5 mM MgCl₂, 0.5% NP-40, 1 mM DTT), mixed with Arl6 or S3R3/C5 beads and rotated for 1 hr at 4°C. For binding to Arl6, lysates were supplemented with 100 μM GTP. Binding of S3R3/C5 to purified BBSome was assessed by mixing beads with a 10 mM solution of pure BSosome made in IB buffer (20 mM HEPES pH 7.0, 5 mM MgCl₂, 1 mM EDTA, 2% glycerol, 300 mM KOAc, 1 mM DTT, 0.2% Triton X-100). After extensive washing, proteins bound to Glutathione Sepharose were eluted in SDS sample buffer (Figure 1) or the moiety fused to GST was cleaved off by addition of 10 μg GST-HRV3C protease in 50 μl IB and incubation at 4°C for 2 hr (Figure 7 and Figure S7).

### Preparation of Liposomes and Binding Assays

All lipid mixtures were assembled in chloroform and dried under an argon stream followed by in vacuo desiccation for > 2 hr to remove all traces of organic solvents. The dried brain lipid film was then hydrated with HKS buffer (20 mM HEPES pH 7.0, 150 mM KOAc, 250 mM Sorbitol) at a final concentration of 1 mg/ml lipids while the synthetic lipid films were hydrated in HK buffer (20mM HEPES, pH 7.0, 160mM KOAc) at a final concentration of 1 mM lipids. The resulting liposome suspensions were extruded through polycarbonate filters with 100 nm or 400 nm pore size (Avanti polar lipids) and spun at 16,000 x g_{max} for 5 min to remove aggregates. Resulting liposomes were kept at 4°C, and used within a week. 1 mol% Texas Red DHPE was included in all synthetic lipid mixtures in order to normalize lipid concentrations in all liposome preparations and to normalize the rate of liposome recovery in the flotation assays. All liposomes from synthetic lipid contain 77 mol% phospholipids and 23 mol% of cholesterol.
To test Arl6 binding to liposomes, 2 μM Arl6 or Arl6ΔN were incubated with 20 μg brain lipid liposomes in HKS buffer containing 3.5 mM MgCl\(_2\) and 100 μM guanine nucleotides at 30°C for 60 min in a 100 μl reaction. Liposomes and bound proteins were recovered by centrifugation at 100,000 rpm for 30 min in a TLA100 rotor at 24°C, and equal portions of supernatant and pellet were loaded on NuPAGE gels. For BBSome binding to brain lipid liposomes, 0.5 μM Arl6 (final concentration in 100 μl reaction) was first incubated with 4 μl liposomes for 30 min at 30°C in 50 μl HKS buffer with 3.5 mM MgCl\(_2\) and 100 μM GMP-PNP or GDP. 4.3 pmol BBSome in 50 μl HKS buffer were then added to the liposome/Arl6 mixture and the reaction were returned to 30°C for 15 min. Reactions were spun at 60,000 rpm for 30 min in a TLA100 rotor at 24°C and the pellets were resuspended in HKS buffer and loaded on NuPAGE gels.

Binding to synthetic lipid liposomes was assayed by combining 167 μM liposome, 0.25 μM Arl6, 50 nM fresh BBSome, 0.5 mM MgCl\(_2\) and 100 μM GMP-PNP or GDP in a 60 μl reaction buffered with T40/K (40 mM Tris pH 8.6, 160 mM KOAc). After incubation at 30°C for 30 min, the reaction was mixed with 60 μl of 55.6% iodixanol in T20KM (20 mM Tris.HCl pH8.6, 160 mM KOAc, 0.5mM MgCl\(_2\)). 110 μl of this mixture were transferred to a TLA100 tube, and layered with 130 μl of 20% iodixanol in T20KM buffer. The resulting step gradient was centrifuged at 100,000 rpm for 15 min at 24°C. 30 μl were harvested from the top of the gradient and lipidosome recovery was measured using the 532 nm laser line on the Typhoon Trio (GE healthcare). Normalization was calculated so that equivalent amounts of lipids were loaded in each wells of the protein gel.

**Ultrastructural Analysis of Liposome-Protein Mixtures**

Liposome binding reactions were fixed after the last 30°C incubation by addition of 2% glutaraldehyde and fixation took place on ice for 10 min. 1% OsO\(_4\) was then added and samples were kept on ice for a further 30 min. Fixed samples were sedimented by centrifugation at 50,000 rpm for 30 min in a TLA100.3 rotor. Pellets were postfixed with 1% OsO\(_4\) on ice for 30 min in cacodylate buffer pH 7.4 and stained with 1% tannic acid for 8 min at room temperature and 1% uranyl acetate overnight at 4°C. The fixed samples were dehydrated with a series of ethanol and embedded with Epon. Thin sections were obtained and further contrasted with uranyl acetate and lead citrate. Images were acquired using an Orius cooled CCD camera mounted onto a Joel TEM 1230.

**Structure-Based Alignments and Modeling of Tertiary Structures**

The Ig-like folds of the proposed GAE modules of BBS1, BBS2, BBS7 and BBS9 were structurally aligned using PROMALS 3D (http://prodata.swmed.edu/promals3d/; (Pei et al., 2008)), and further matched by HHPred (http://toolkit.tuebingen.mpg.de/hhpred; (Söding, 2005)) to the corresponding segments of GGA1 (pdb 1NA8), AP1γ domain 1 (pdb 1GYV), AP2δ domain 2 (pdb 1E42), AP2ζ domain 1 (pdb 1KYF) and γCOP domain 1 (pdb 1RX4). GAE X-ray structures were further superposed by SSM (http://www.ebi.ac.uk/msd-srv/ssm/; (Krissinel and Henrick, 2004)) and visualized with PyMOL (http://www.pymol.org). The human BBS1 GAE domain model was constructed with MODELER (http://salilab.org/modeller/; (Eswar et al., 2006)). The modeled fold of the human BBS7 platform-like module was built using I-TASSER (http://zhang.bioinformatics.ku.edu/I-TASSER/; (Zhang, 2007)).

**Supplemental References**


Figure S1. Related to Figure 3
(A) Validation of the Arl6 antibody by Western blot. Protein extracts (10 μg per lane) from RPE cells treated with control siRNA or siRNA against Arl6 coding sequence (siArl6ORF) or Arl6 3′UTR (siArl6UTR) were immunoblotted for Arl6 or actin (loading control). Arl6 protein is specifically depleted by the Arl6 siRNAs. The asterisk denotes a 70kDa cross-reacting band whose level is unaffected by Arl6 siRNAs.
(B) Line profiles of BBS1 and Arl6 fluorescence intensities inside the cilium shown in Figure 3C (top panel).
(C) Line profiles of BBIP10 and Arl6 fluorescence intensities inside the cilium shown in Figure 3C (bottom panel). A line was drawn along the cilium and the line profile of fluorescence intensity was generated in Slidebook 5.0.
Figure S2. BBS2 and BBS5 Localize to Primary Cilia, Related to Figure 3C
RPE-hTERT cells treated with control siRNA or siRNAs against BBS2 (A) or BBS5 (B) were serum-starved and immunostained for BBS2 (A) or BBS5 (B) and co-stained for acetylated α-tubulin (acTub). Stacks of images were acquired at 0.25 μm interval with a 100x/1.45NA objective and the section containing the cilia was selected for each figure panel. Scale bars, 5 μm.
Figure S3. Related to Figure 4
(A) Arl6 is not required for BBSome assembly. RPE cells treated with control siRNA or siRNA against Arl6 were serum starved to induce ciliation. Cell lysates were fractionated by size-exclusion chromatography on Superose 6 and fractions were immunoblotted for BBS4. The relative protein concentration is shown as OD_{280}. The excluded volume (Ve) and the elution volumes of size markers are indicated.

(B) Full Western blot for Figure 4E. RPE, RPE-[Arl6-GFP], RPE-[Arl6(Q73L)-GFP], RPE-[Arl6(T31R)-GFP] clonal cell lines treated with control siRNA or siRNA against Arl6 3'UTR were serum starved and immunoblotted with anti-Arl6 antibody. Arl6-GFP, endogenous Arl6 and a nonspecific band of 70 kDa (double asterisk) that serves as a loading control are shown. The asterisk denotes a weak non-specific band that runs slightly below Arl6-GFP. When treated with siRNA against Arl6 3'UTR, endogenous Arl6 is depleted and Arl6-GFP proteins are slightly upregulated.

(C) Arl6[Q73L]-GFP recruits BBS1 more effectively to cilia compared to Arl6-GFP. RPE-hTERT cells stably expressing Arl6-GFP or Arl6[Q73L]-GFP were treated with siArl6UTR to specifically deplete endogenous Arl6. After serum starvation, cells were immunostained for BBS1 and acetylated α-tubulin as in Figure 4D. Fluorescence intensities of cilia-localized BBS1 were plotted in a histogram.
Figure S4. Related to Figure 4

(A) RPE cells treated with control siRNA or siRNA against BBS2, BBS4 and BBS5 were serum starved to induce ciliation. Cells were immunostained for Arl6 and acetylated α-tubulin (acTub). Insets show enlargements of the Arl6 channel around the cilia. Scale bars, 5 μm.

(B) RPE cells were prepared and stained as in (A). At least 150 cilia per siRNA were counted and the percentages of Arl6-positive cilia were plotted. Error bars represents field-to-field standard deviations.

(C to F) RPE cells treated with control siRNA were serum starved to induce ciliation before immunostaining for Arl6 (C and D) or BBS1 (E and F). Cells were subjected to either conventional immunofluorescence (IF) (C and E) or enhanced IF (D and F). Cilia were visualized with acetylated α-tubulin (acTub). Scale bars, 5 μm.

Note that the enhanced IF procedure significantly reduces background staining.

(G) RPE cells were prepared and stained as in (C–F). At least 150 cilia per experiment were counted and percentages of Arl6- or BBS1-positive cilia were plotted. RPE cells treated with siRNA against Arl6 and processed for enhanced IF were also quantified. Error bars represents field-to-field standard deviations.
Figure S5. Related to Figure 5

(A) Purification of recombinant Arl6 to near homogeneity. Arl6ΔN16 was cleaved off from GST-Arl6ΔN16 with HRV3C protease, concentrated by ammonium sulfate precipitation and fractionated on Superdex 75 size exclusion chromatography. Proteins were resolved by SDS-PAGE and stained with Coomassie. Similar results were obtained with full-length Arl6.

(B) The purified retinal BBSome is monodisperse. MonoS-purified BBSome was fractionated on a linear 10%–40% sucrose gradient by velocity sedimentation. The BBSome purified from retina sediments at 14S, similar to the BBSome from RPE cells (Nachury et al., 2007), and is nearly free of aggregates in the pellet fraction. Proteins of known sedimentation coefficient were run simultaneously on an identical gradient.

(C) Quantitation of BBSome binding to various PI/PIP-containing liposomes. Three independent experiments were conducted as in Figure 5F using PIPs from two different sources. The intensities of BBS9, BBS2, BBS1 and Arl6 were measured by densitometry and the (I_{BBS9} + I_{BBS2} + I_{BBS1})/I_{Arl6} ratios (“relative BBSome recovery”) were normalized to the value for PI liposomes. Error bars denote standard deviation between independent experiments.

(D) Sar1 fails to recruit the BBSome to liposomes. Liposomes (167 μM final lipid concentration) were incubated with Arl6 (0.25 μM) or Sar1 (1 μM), BBSome (50 nM) and GMP-PNP or GDP (100 μM) in a 60 μl reaction before flotation on iodixanol gradients. Liposome-bound proteins were analyzed by SDS-PAGE and silver staining.
Figure S6. Related to Figure 6
Gallery of BBSome-coated profiles on liposomes processed as in Figure 6A. Scale bars: 50 nm.
Figure S7. Related to Figure 7

(A) BBSome subunits bind specifically to the ciliary targeting signal of SSTR3. Extracts of HEK293 cells transfected with Myc-BBSome subunits were applied to beads decorated with GST-SSTR3<sup>i3</sup> or GST-SSTR5<sup>i3</sup> for capture assays. Total cell extracts (Input, top panel) and captured materials (bottom panels) were immuno-blotted for Myc. All three panels are extracted from the same exposure scan. 25 input equivalents of each eluate were loaded.

(B) Summary of the SSTR3/5<sup>i3</sup> variants and their behavior in BBSome binding and ciliary targeting activity. The conserved (AX[A/S]XQ) motifs are shown in bold and the mutated amino acids are red.

(C) Cell lysates (5 μg /lane) corresponding to Figures 7G and H were immunoblotted with anti-Arl6 and anti-BBS4 antibodies. Note that siArl6 and siBBS4 efficiently depleted Arl6 and BBS4, respectively. The asterisk denotes a cross-reacting band.

(D and E) Selectivity of the surface labeling protocol. IMCD3 cells (lane 1) and IMCD3-CD8<sub>α</sub>-SSTR3<sup>i3</sup> (lane 2) cells were subjected to surface biotinylation as in Figure 7H and the input of the avidin capture (input) or the proteins captured on avidin (surface) were immunoblotted for the plasma membrane markers E-cadherin (D) and Na<sup>+</sup>/K<sup>+</sup>-ATPase (E). Both anti E-cadherin and anti Na<sup>+</sup>/K<sup>+</sup>-ATPase antibodies recognize non-specific bands in the input (denoted as asterisks) that were not recovered on avidin beads, suggesting that only surface exposed proteins were selectively biotinylated.

(F) Stacks of 16 z-sections that were acquired at 0.5 μm interval with a 100x/1.4NA objective as in Figure 7G were projected using sum over z-axis. The dynamic range in the CD8<sub>α</sub>-SSTR3<sup>i3</sup> channel was adjusted identically in all micrographs to reveal plasma membrane staining in cells treated with siArl6 and siBBS4. Scale bars, 5 μm.