Structural basis for membrane targeting of the BBSome by ARL6

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The BBSome is a coat-like ciliary trafficking complex composed of proteins mutated in Bardet-Biedl syndrome (BBS). A critical step in BBSome-mediated trafficking is recruitment of the BBSome to membranes by the GTP-bound Arl-like GTPase ARL6. We have determined crystal structures of Chlamydomonas reinhardtii ARL6–GDP, ARL6–GTP and the ARL6–GTP–BBS1 complex. The structures demonstrate how ARL6–GTP binds the BBS1 β-propeller at blades 1 and 7 and explain why GTP-bound but not GDP-bound ARL6 can recruit the BBSome to membranes. Single point mutations in the ARL6-GTP-BBS1 interface abolish the interaction of ARL6 with the BBSome and prevent the import of BBSome into cilia. Furthermore, we show that BBS1 with the M390R mutation, responsible for 30% of all reported BBS disease cases, fails to interact with ARL6–GTP, thus providing a molecular rationale for patient pathologies.

RESULTS

ARL6∆N–GTP binds BBS1N with ~0.5-µM affinity

To unravel the molecular basis for membrane recruitment of the BBSome and BBS disease phenotypes, we purified Homo sapiens (Hs) and Chlamydomonas (Cr) versions of ARL6 (full length or ∆N (residues 1–15 deleted)) in the GDP- or GTP-bound form; the N-terminal domain of BBS1 (HsBBS1N (residues 1–416) and CrBBS1N (residues 1–425)); and the ARL6∆N–GTP–BBS1N complex (Fig. 1a and Supplementary Fig. 1). Both Chlamydomonas and human ARL6∆N–GTP–BBS1N complexes were stable during size-exclusion chromatography (SEC) and eluted in peaks well separated from the peaks of excess ARL6–GTP (Fig. 1a and Supplementary Fig. 1a). To determine the affinity between the two proteins, we carried out isothermal titration calorimetry (ITC) measurements. The results demonstrated that CrBBS1N binds CrARL6∆N–GTP to form a stoichiometric complex with a KD of 0.35 µM (Fig. 1b). Consistently with this, HsBBS1N bound HsARL6∆N–GTP with a KD of 0.54 µM to form a stoichiometric complex (Supplementary Fig. 1a). Given that ARL6 and BBS1 proteins are well conserved across species (40–50%
conservation between *Chlamydomonas* and human proteins, we tested whether CrBBS1 interacts with *Hs*ARL6AN–GTP. Indeed, we found that *Hs*ARL6AN–GTP–CrBBS1N could be purified by SEC and that the *K*ₐ for this chimeric complex was 0.30 μM (Supplementary Fig. 1d). We conclude that ARL6–GTP forms a stable complex with the N-terminal domain of BBS1 and that the interaction is conserved between *Chlamydomonas* and human proteins.

**Structures of CrARL6AN–GTP and CrARL6AN–GTP–CrBBS1N**

We determined crystal structures of CrARL6AN–GTP and the CrARL6AN–GTP–CrBBS1N complex (Fig. 1c,d, Supplementary Fig. 2a,b and Table 1). We determined the CrARL6AN–GTP structure at 2.2-Å resolution by molecular replacement, using the available structure of HsARL6–GTP[32]. Crystals of the CrARL6AN–GTP–CrBBS1N complex reproducibly diffracted to resolutions of 3.1–3.5 Å, and we determined the structure by using experimental phasing on a mercury derivative (Supplementary Fig. 2f). The structures showed that CrARL6 adopts a classical small-GTPase fold and that CrBBS1N adopts the fold of a seven-bladed WD40-like β-propeller (Fig. 1c,d). A comparison of the CrARL6AN–GTP structure with previously published structures of human and *Trypanosoma brucei* ARL6–GTP[32,33] revealed largely identical structures with r.m.s. deviations of 0.7–0.9 Å (Supplementary Fig. 2a). The CrARL6AN–GTP–CrBBS1N structure reveals an elongated assembly with complex formation mediated...
Table 1 Data collection and refinement statistics

| Data collection and refinement statistics | ARL6αN–BBS1N | ARL6αN–GTP | ARL6αN–GDP CdCl₂ | ARL6αN–BBS1N EMP (Hg) | ARL6αN–BBS1N Pb(C₂H₃O₂)₂ |
|-----------------------------------------|--------------|------------|------------------|------------------------|--------------------------|

**Data collection**

| Space group | P₃₁,2₁ | P₃₂,2₁ | P₃₁,2₁ | P₃₁,2₁ | P₃₁,2₁ |
|-------------|--------|--------|--------|--------|--------|

**Cell dimensions**

| a, b, c (Å) | 124.8, 124.8, 441.8 | 119.4, 119.4, 147.3 | 65.7, 65.7, 185.6 | 123.6, 123.6, 443.6 | 123.7, 123.7, 439.0 |

α, β, γ (°) | 90.0, 90.0, 120.0 | 90.1, 90.0, 60.0 | 90.0, 90.0, 120.0 | 90.0, 90.0, 120.0 | 90.0, 90.0, 120.0 |

Wavelength | 1.0000 | 1.0332 | 1.0000 | 1.0075 | 0.9497 |

Resolution (Å) | 50–3.45 (3.55–3.45) | 50–2.20 (2.32–2.20) | 50–1.43 (1.51–1.43) | 50–3.13 (3.24–3.13) | 50–3.35 (3.47–3.35) |

| R(merge) | 0.12 (0.82) | 0.034 (0.55) | 0.11 (0.79) | 0.11 (0.81) | 0.11 (0.81) |

Completeness (%) | 99.1 (97.6) | 99.9 (99.1) | 96.2 (91.81) | 99.56 (96.76) | 99.56 (96.76) |

Redundancy | 6.5 (6.0) | 12.7 (12.2) | 12.6 (1.7) | 8.8 (8.5) | 13.2 (11.3) |

Refinement

| Resolution (Å) | 3.45 | 2.20 | 1.43 | 3.13 | 3.35 |

| No. reflections | 51,878 (6,201) | 29,942 (3,511) | 83,301 (11,179) | 70,112 (6,437) | 56,947 (5,395) |

| Rwork / Rfree | 0.222 / 0.277 | 0.183 / 0.217 | 0.157 / 0.185 | 0.213 / 0.249 | 0.221 / 0.268 |

| No. atoms | Protein: 14,270, Ligand/ion: 132, Water: 627 | Protein: 14,270, Ligand/ion: 66, Water: 127 | Protein: 14,270, Ligand/ion: 627, Water: 2,467 | Protein: 14,270, Ligand/ion: 67, Water: 14,399 | Protein: 14,270, Ligand/ion: 67, Water: 14,369 |

| B factors | Protein: 80.8, Ligand/ion: 79.9, Water: 1037 |

| r.m.s. deviations |

| Bond lengths (Å) | 0.011 | 0.007 | 0.007 | 0.11 | 0.012 |

| Bond angles (°) | 1.5 | 1.2 | 1.5 | 1.5 | 1.5 |

*One crystal was used for each measurement. Values in parentheses are for highest-resolution shell.*

by blades 1 and 7 of the CrBBSN1 β-propeller and helix α3, switch 2 and the loop preceding helix α1 from the GTP-binding region of CrARL6 (Fig. 1c,d). The finding that BBS1 binds at the switch regions of the GTP site of ARL6 is compatible with the BBSome being an effector for ARL6 (ref. 31). Comparison of ARL6αN–GTP and ARL6αN–GTP–BBS1N structures revealed that GTP-bound ARL6 is already in a conformation competent for BBS1 binding with no major structural changes occurring upon formation of the BBS1N complex (Supplementary Fig. 2b).

The structure of the CrARL6αN–GTP–CrBBS1N complex revealed a highly complementary but relatively small interaction surface with only 600 Å² of buried surface area (Supplementary Fig. 2e). The interaction interface is bipartite, with one hydrophilic patch closer to the GTP pocket and one hydrophobic patch farther away (Fig. 2a,b). The hydrophobic patch is formed by L100, V103 and V104 from helix α3 of CrARL6 contacting L41, I415 and M417 from blade 1 and T86 from blade 2 of CrBBS1N (Fig. 2a,b). The hydrophilic patch is made by D26 and N27 from the loop preceding helix α1, R77 from switch 2 and R101 and E108 from helix α3 of CrARl6 that interact with R399, E400 and R420 from blade 1 of the CrBBS1N β-propeller (Fig. 2a,b). The ARL6–BBS1N interface is highly conserved between different species (Fig. 2a and Supplementary Fig. 3). Of the eight residues from ARL6 directly involved in BBS1N binding, all are completely conserved between the *Chlamydomonas* and human proteins (Supplementary Fig. 3). Of the seven BBS1N residues that interact with ARL6, five are completely conserved between *Chlamydomonas* and human protein sequences, whereas one residue represents a conservative hydrophobic substitution (M417 in CrARL6) and T86 in CrBBS1N (Supplementary Fig. 3).
These data support the notion of an evolutionarily conserved ARL6–BBS1 complex.

**Mutation analysis of human ARL6ΔN–BBS1N**

We designed, on the basis of the structure of HsARL6–GTP–CrBBS1N described above, single point mutations of human ARL6 and BBS1N and tested them for their ability to form a protein complex. We introduced nonconservative substitutions in ARL6 and BBS1N and tested them for their ability to form a protein complex. Then we incubated HsARL6 variants with native BBSome from bovine retinal extract and probed for interaction in pulldown experiments (Fig. 2c). Whereas GST-tagged HsARL6Q73L without mutations in the interface efficiently captured the BBSome from retinal extracts, R77A, L100E or E108A single-point-mutant ARL6 failed to do so (Fig. 2c). (All mutants used in this study were tested for proper folding by SEC and for nucleotide binding by NMR (as shown for ARL6 mutants in Supplementary Fig. 4).) Residues of the interaction interface between ARL6 and BBS1N eluted from the CrARL6ΔN–GTP–CrBBS1N structure are thus required for the efficient binding of the mammalian BBSome to ARL6. To reciprocally probe for interaction, we showed that single ARL6 point mutations were sufficient to abolish complex formation, consistently with the results (Fig. 2d) demonstrated that a single point mutation in either the hydrophilic (R404A) or the hydrophobic (I399E) interface is sufficient to abolish complex formation, consistently with the relatively small buried surface area within the CrARL6ΔN–GTP–CrBBS1N complex.

**ARL6 E108A mutation prevents BBSome recruitment to cilia**

Previously published data have shown that active GTP-bound ARL6 is required to recruit the BBSome to membranes and to allow access of the BBSome complex to the ciliary compartment31. Because we showed that single ARL6 point mutations were sufficient to abolish the interaction with native BBSome from retinal extracts, we hypothesized that the ciliary entry of BBSomes might also be compromised. To test the functional implications of the ARL6–GTP–BBS1 interaction in a cellular system, we thus generated clonal RPE-hTERT cell lines that expressed either wild-type31 or E108A-mutant ARL6. After knocking down endogenous ARL6 by short interfering RNA (siRNA) targeting the 3′ untranslated region (UTR), we monitored the recruitment of the BBSome to cilia (Fig. 3). The knockdown of Arl6 reduced the percentage of BBSome-positive cilia from above 40% to below 5%, and this effect could be fully rescued by reintroduction of wild-type ARL6. In contrast, rescue experiments with the ARL6E108A interface mutant, which ablated BBSome interaction in pulldown experiments (Fig. 2c), failed to increase the number of BBSome-positive cilia (Fig. 3). These data confirmed the structural results and, given that the BBSome is the major effector of ARL6, suggested that the ARL6–GTP–BBS1 interaction is required to recruit the BBSome to cilia.

**Structure of CrARL6ΔN–GDP**

CrARL6 recombinantly expressed in *Escherichia coli* copurified with GTP bound at the GTPase site (Supplementary Fig. 2h). After treatment with EDTA, we replaced the GTP with GDP and determined the crystal structure of CrARL6ΔN–GDP at 1.4-Å resolution by experimental phasing with a cadmium derivative (Fig. 4 and Supplementary Fig. 2i). Small GTPases of the Arf family associate with membranes via an amphipathic N-terminal helix. The exchange from GDP- to GTP-bound Arf results in a shift of two amino acids in the interswitch region, which in turn pushes out and exposes the N-terminal amphipathic helix to allow membrane association34,35. To examine whether ARL6 is likely to use a similar mechanism for membrane attachment, we superposed the structures of CrARL6ΔN bound to either GDP or GTP with the equivalent structures of Arfl (refs. 34,35), revealing similar interswitch conformations (Supplementary Fig. 2c,d). The structural studies of CrARL6 presented here were carried out in the absence of the amphipathic N-terminal helix. Nevertheless, our analysis demonstrated a shift of two residues in the interswitch region of ARL6–GDP compared to ARL6–GDP (Fig. 4a and Supplementary Fig. 2c,d). Because this shift is a hallmark of Arf proteins, the structures thus support a canonical Arf mechanism of membrane association via the amphipathic N-terminal helix of ARL6.

Furthermore, in the structure of CrARL6ΔN–GDP, both switch 1 and switch 2 adopted substantially altered conformations in comparison to the CrARL6ΔN–GTP structure (Fig. 4a). The reason that GTP- but not GDP-bound CrARL6 can recruit the BBSome to membranes31 is probably a result of structural changes in switch 2. The conformational change in switch 2 upon hydrolysis of GTP to GDP results in the disruption of the ARL6R77–BBS1E400 salt bridge and would cause ARL6R77 to clash with BBS1N R420 (Fig. 4b). This

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**Figure 4** Nucleotide-dependent conformational changes in CrARL6. (a) Superposition of CrARL6ΔN in the GDP- and GTP-bound forms, revealing a canonical two-residue shift in the interswitch region. (b) Comparison of the switch regions of CrARL6ΔN–GTP in the context of the CrARL6ΔN–GTP–CrBBS1N complex with the CrARL6ΔN–GDP structure. The boxed region is shown in zoomed-in view at right.
observations explain why GTP-bound but not GDP-bound ARL6 have been shown to recruit the BBSome to membranes.\(^2^1\)\(^\text{–}\)\(^2^3\)\(^\text{–}\)\(^3^1\).

**CrBBS1N is not a GTPase-activating protein for CrARL6**

The crystal structures of CrARL6\(^{\text{ΔN}}\)–GTP and CrARL6\(^{\text{ΔN}}\)–GTP–CrBBS1N presented here both have GTP bound at the GTPase site of the CrARL6\(^{\text{ΔN}}\) subunit, consistent with the well-defined electron densities (Supplementary Fig. 2g,h). Because nucleotides were not added to the sample, we conclude that the crystal structures of CrARL6\(^{\text{ΔN}}\) and CrARL6\(^{\text{ΔN}}\)–BBS1N are in the GTP-bound state, as demonstrated by the CD spectra of wild type (WT) and E234K and M390R mutants of CrARL6\(^{\text{ΔN}}\) (Supplementary Fig. 2i). We confirmed this notion with GTPase assays (Supplementary Fig. 5). The crystal structures of CrARL6\(^{\text{ΔN}}\)–GTP–CrBBS1N structure, we found no residues from the CrBBS1N subunit inserted into the GTP-binding pocket of Arl6\(^{\text{ΔN}}\), a result in agreement with the slow hydrolysis rate by the CrARL6\(^{\text{ΔN}}\)–GTP–CrBBS1N complex. We conclude that CrBBS1N does not act as a GTPase-activating protein (GAP) for CrARL6\(^{\text{ΔN}}\), which is in contrast to COP11 coat recruitment by Sar1, in which the COP11 component Sec23 serves as a Sar1 GAP by introducing an arginine into the active site, thus promoting GTP hydrolysis.\(^3^5\) Membrane recruitment of BBSomes by ARL6–GTP may thus be long lived in the absence of an external GAP.

**BBS1\(^{\text{M390R}}\) mutant does not interact with ARL6–GTP**

Mutational analyses of patients with BBS have uncovered several point mutations in BBS proteins including variants that disrupt GTP binding by ARL6 (refs. 32,37,38). Interestingly, a single M390R point mutation in BBS1 represents ~80% of all BBS1 disease mutations and accounts for 18–32% of all BBS disease mutations.\(^3^9\)\(^4^0\).

- ARL6\(^{\text{ΔN}}\)–BBS1
- BBSomes
- K\(_d\) = 0.4–0.6 \(\mu\)M
- This work

| ARL6–BBS1 | BBSome | K\(_d\)  |
|-----------|--------|---------|
| Sar1–Sec23 | COP11  | ND      |
| Art1–γ-COP | COP1  | 1 \(\mu\)M |
| Art1–GAT | AP1  | 20 \(\mu\)M |

**Figure 5** HsBBS1 M390R disease mutant does not bind HsARL6–GTP. (a) Structural mapping of M390 and E234 residues mutated in BBS onto the model of the HsARL6\(^{\text{ΔN}}\)–GTP–HsBBS1N complex. M390 is located in blade 1 close to the ARL6–interaction interface, whereas E234 is located at the top of the β-propeller far away from the ARL6–interaction interface. (b) CD spectra of wild type (WT) and E234K and M390R mutants of the HsBBS1N protein. Secondary-structure content is tabulated at bottom. (c) GST pulldown of wild-type or mutant HsBBS1N with GST-tagged HsARL6\(^{\text{Q73L}}\)–GTP. (d) Tabulation of K\(_d\) from ITC titrations of HsARL6\(^{\text{Q73L}}\)–GTP to BBS1N, wild type, E234K and M390R. (s.d. from 3 independent experiments; ITC curves in Supplementary Fig. 1.)

**Figure 6** Structural comparison of membrane recruitment of coating complexes by Arf, Sar and Arf-like proteins. Recruitment of Arf, Sar and Arl GTPases (green) to lipid bilayers (top) requires the active GTP-bound state, which exposes an N-terminal amphipathic helix (not shown) and allows for effector binding. The complexes of Sar1–Sec23, Arf1–γ-COP, Arf1–AP1 (β1 subunit) and Arf1–GAT are shown in cartoon representation after structural superposition with the ARL6–BBS1 complex (with only GTPase domains superposed). Switch regions are labeled as in Figure 1. Affinities of the Arf–effector complexes are indicated below. ND, not determined.
We modeled the human ARL6ΔN–GTP–BBS1N structure on the basis of the CrARL6ΔN–GTP–CrBBS1N structure reported here and mapped reported BBS1 disease mutations onto the structure (Fig. 5a). This analysis revealed that HsBBS1 M390 is located at blade 1 of the β-propeller close to the GTP-binding site of ARL6 (Fig. 5a). The HsBBS1 M390 residue is not directly involved in the interaction interface with ARL6, but its position suggests that mutation to arginine might disrupt the structure of the β-propeller in the region around blade 1 of BBS1 and thus indirectly prevent complex formation with ARL6. To test this notion, we purified the HsBBS1NM390R mutant protein for interaction studies (Supplementary Fig. 1c). Although the HsBBS1NM390R purification resulted in a much lower yield than that for the wild-type protein, HsBBS1NM390R eluted in SEC as a broad peak well separated from the void volume in which aggregated proteins elute (Supplementary Fig. 1c). To assess the folding state of the HsBBS1NM390 protein in more detail, we carried out CD experiments. The CD spectrum of HsBBS1NM390 was that of an overall folded protein, but it indicated substantially lower β-strand content compared to that of wild-type HsBBS1, thus suggesting that the β-propeller of HsBBS1NM390 protein is partly unstructured (Fig. 5b).

The HsBBS1NM390R mutant protein failed to interact with GST-tagged HsARL6Q73L and HsARL6Q73LΔN in pulldown experiments (Fig. 5c and Supplementary Fig. 6). Additionally, ITC and SEC experiments did not detect any interaction between HsARL6Q73LΔN and HsBBS1NM390R; this suggested that the affinity is at least two orders of magnitudes lower than that for wild-type BBS1N (Fig. 5d and Supplementary Fig. 1c). The main defect of the BBS1 M390R mutation in patients with BBS thus appears to be misfolding of the β-propeller, which in turn disrupts the association with binding partners such as ARL6. For a positive control, we used the BBS1N224K mutant protein, representing an infrequent mutation in patients with BBS40, because E234 is located at the top of the β-propeller, far away from the ARL6-interaction site (Fig. 5a). BBS1N224K was efficiently pulled down by GST-tagged HsARL6Q73L and HsARL6Q73LΔN, and it bound untagged HsARL6Q73LΔN with a K_D of 0.35 μM in ITC experiments, which is similar to that of wild-type BBS1N (Fig. 5b,c and Supplementary Fig. 6). These results demonstrated that up to 30% of all BBS mutations in patients could result in failure of ARL6-mediated recruitment of BBSomes to membranes, which in turn would probably prevent the proper ciliary trafficking of several membrane proteins.

**DISCUSSION**

There are clear similarities in sequence and domain composition of subunits from membrane coat complexes (COPI, COPII and clathrin–AP1) and subunits from ciliary trafficking complexes (IFT and BBSome complexes), thus suggesting a common evolutionary origin31,41,42. Another commonality is the recruitment to membranes by Arf and Arf-like proteins, because COPI, AP-1 and GGA1 GAT are recruited by Arf1, and COPII is recruited by Sar1 (refs. 36,43–45). Whereas these complexes bind switch 1, switch 2 and the interswitch region (β-strands 2 and 3) of Arf1 and Sar1 mainly via α-helical structural elements, the recruitment of BBS1 by ARL6 is quite different (Fig. 6). The β-propeller of BBS1 binds switch 2, α-helix 3 and residues from the loop preceding helix α1 of ARL6, to position the β-propeller on the opposite side of ARL6 (Fig. 6). The recruitment mode of BBSomes by ARL6 is thus different from that found in other coating complexes, and it will be very interesting to see how the additional BBSome subunits related to COP and clathrin–AP1 subunits contribute to coat formation and recognition of membrane proteins for ciliary transport.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**ACCESSION CODES**

The authors declare no competing financial interests.

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**AUTHOR CONTRIBUTIONS**

A.M. carried out the protein biochemistry and structural biology under the supervision of A.R.N.; A.R.N. carried out the pulldown experiments of native BBSome with wild-type and mutant ARL6 and the cell biology experiments under the supervision of M.V.N.; A.M. and E.L. designed the experiments and wrote the paper with input from A.R.N. and M.V.N.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Protein purification and crystallization. Untagged or C-terminally histidine-tagged CrBBS1N (residues 1–425) and HsBBS1N (residues 1–417) were cloned into pFL vectors, and the proteins were recombinantly expressed in High Five insect cells (Invitrogen). Full-length or ΔN (lacking residues 1–15) CrArl6 and HsArl6 were cloned into pET vectors with cleavable N-terminal histidine tags and were recombinantly overexpressed in E. coli BL21 (DE3). Proteins were purified by Ni-NTA affinity chromatography after lysis of cells in buffer A (20 mM Tris- HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 10 mM imidazole and 5 mM MgCl₂). After elution with 10–500 mM imidazole, overnight dialysis and histidine-tag cleavage by TEV protease, the proteins were passed onto a Ni-NTA column, and the flow through was further purified by anion-exchange chromatography on a MonoQ column. As a last purification step, proteins were subjected to size-exclusion chromatography on a Superdex 75 column in buffer B containing 10 mM HEPES, pH 7.5, 150 mM NaCl, and 1 mM MgCl₂. CrArl6AN and CrBBS1N were cloned as a bicistronic construct for coexpression in High Five cells, and the CrArl6AN–GTP–CrBBS1N complex was purified as described above.

Crystallization experiments were done by sitting-drop vapor-diffusion methods at 18 °C. CrArl6AN that copurified with GTP was concentrated to 75 mg/ml, and crystals were obtained by mixing of the proteins with an equal volume of precipitant containing 30% (v/v) pentaerythritol ethoxylate, 50 mM Bis-Tris, pH 6.5, and 50 mM ammonium sulfate. To obtain GTP-bound CrArl6AN, purified CrArl6AN–GTP was incubated with buffer B containing 5 mM EDTA and no MgCl₂ for 3 h at 20 °C. This was followed by SEC in buffer B without MgCl₂. The eluted nucleotide-free protein was mixed with 1 mM GTP in buffer B, subjected again to SEC, concentrated to 15 mg/ml and crystallized by mixing with equal volumes of precipitant containing 12% PEG 3350, 0.1 M HEPES, pH 7.5, 5 mM CdCl₂, 5 mM MgCl₂, 5 mM NiCl₂ and 5 mM CoCl₂. The CrArl6–GTP–CrBBS1N complex was crystallized by mixing of the protein complex at 10 mg/ml with equal volumes of precipitant containing 29% PEG 400 and 0.1 M Tris, pH 8.0. Before flash cooling, crystals were cryoprotected by soaking in mother liquor supplemented with 25% glycerol (ARL6 crystals) or without the addition of glycerol (BBS1N crystals). The CrArl6–GTP–CrBBS1N crystals were subjected again to SEC, concentrated to 30 mg/ml and crystallized by mixing of the protein complex at 10 mg/ml with equal volumes of precipitant containing 29% PEG 400 and 0.1 M Tris, pH 8.0. Before flash cooling, crystals were cryoprotected by soaking in mother liquor supplemented with 25% glycerol (ARL6 crystals) or without the addition of glycerol (BBS1N crystals).

X-ray diffraction data collection and structure determination. X-ray diffraction data were acquired at the Swiss Light Source (SLS, Villigen, Switzerland) at beamlines PXII and PXIII. All data collections were performed at cryogenic temperatures (100 K) at wavelengths of 1.000 Å (CrArl6AN–CrBBS1N and CrArl6AN–GDP–CrBBS1N, 1.0322 Å (CrArl6AN–GTP), 1.0075 Å (CrArl6AN–GTP–CrBBS1N–Hg) or 0.9497 Å (CrArl6AN–GTP–CrBBS1N–Pb). The data were indexed with the XDS package46 before being scaled with Aimless in the CCP4 package47,48. The structure of CrArl6–GTP was determined at 2.2-Å resolution by molecular replacement (MR) with the human ARL6 structure (PDB 2H5T) as a search model in Phaser49 as implemented in the PHENIX package50. Two molecules of CrArl6 were found in the asymmetric unit, and the structure was completed by iterative cycles of model building in Coot51 and refinement in PHENIX. The CrArl6–GDP structure was determined at 1.4-Å resolution by single anomalous dispersion on cadmium-derivatized crystals. This was followed by autobuilding in PHENIX. For the CrArl6AN–GTP–CrBBS1N complex structure, crystals were soaked in mother liquor complemented with 1.3 mM of the mercury compound EMP and crystals were subjected again to SEC, concentrated to 30 mg/ml and crystallized by mixing of the protein complex at 10 mg/ml with equal volumes of precipitant containing 29% PEG 400 and 0.1 M Tris, pH 8.0. Before flash cooling, crystals were cryoprotected by soaking in mother liquor supplemented with 25% glycerol (ARL6 crystals) or without the addition of glycerol (BBS1N crystals).

The CrArl6–GTP–CrBBS1N structure was determined at 3.35-Å resolution and with a lead-derivatized crystal that diffracted to 3.35-Å resolution. The CrArl6–GTP–CrBBS1N lead-derivatized crystals gave only a weak anomalous signal that did not extend beyond 7-Å resolution because of low-occupancy lead sites, and this data set can thus be considered as near native. All crystal structures reported here have 90–97% of the residues in the most favored region of the Ramachandran plot with 0.1–0.6% in the disallowed regions (refinement statistics in Table 1). The human ARL6AN–GTP–BBS1N complex structure (Fig. 5a) was modeled with Modeller52 on the basis of the experimentally determined CrArl6AN–GTP–CrBBS1N crystal structure.

Isothermal titration calorimetry (ITC). ITC was done with an ITC200 instrument (MicroCal) at 25 °C with purified proteins in buffer B. Each experiment was independently carried out at least three times with different purification batches of protein. The titration protocol consisted of one initial injection of 0.2 µl followed by 39 injections of 1 µl of the ligand (350–450 µM) into the protein sample (35–45 µM) with intervals of 150 s to allow the titration peak to stabilize at the baseline. The data were fitted to titration curves with Origin v7.0 (MicroCal).

GST pulldown experiments. For GST pulldowns with recombinant proteins, GST-HsARL6Q73LΔN–GTP was mixed with wild-type or mutant forms of His6BBS1N and incubated with GSH beads for 1 h in a buffer containing 10 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM MgCl₂ and 1 mM GTP. After being washed three times with 40× the bead volume, proteins were eluted with 30 mM reduced glutathione-containing buffer. Analysis was done on an SDS-PAGE gel stained with Coomassie blue. Full-length GST-HsARL6Q73L–GTP was incubated with insect-cell extract with overexpressed wild-type or mutant His6BBS1N for 1 h at 4 °C with GSH beads. After being washed three times with 40× the bead volume of buffer, proteins were eluted with buffer containing 30 mM reduced glutathione. Visualization was done with SDS-PAGE and subsequent western blot analysis with an antibody that recognizes the hexahistidine tag of BBS1N (anti-His tag; Novagen 70796-3; 1:1000; validation provided on manufacturer’s website). For bovine retinal-extract pulldowns, HsARL6Q73LΔN constructs and bovine retinal extract were prepared as described previously51. Equal amounts of GST-HsArl6 in E. coli lysate were bound to GSH beads and incubated with retinal extract at 4 °C for 2 h. After being washed three times with 1× PBS, proteins were eluted in 6 M guanidine-HCl and analyzed by SDS-PAGE. Western blot analysis was done with anti-BBS4 as described in ref. 13. Full-size scans of western blots and SDS-PAGE gels are shown in Supplementary Data Set 1.

Stable cell lines and immunofluorescence. RPE clones and immunofluorescence experiments were done as described in ref. 2. In short, retroviruses were produced with pBabe-puro containing Arl6-GFP or Arl6-GST-HA as described in ref. 53 and used to infect RPE cells. Single cell clones were screened for low expression levels. RPE, RPE expressing Arl6-GFP, and RPE expressing Arl6-GST-HA were transfected with 20 nM siRNA duplex against the 3′ UTR of endogenous Arl6 for 24 h and then shifted from 10% to 0.2% serum media for 48 h. Cells were fixed by the enhanced immunofluorescence protocol described in ref. 31. Commercial antibodies against BBS5 (14569-1-AP, Proteintech) and polyglutamylated tubulin (GT335) were used. Anti-Arl6 antibody was used at 0.2 µg/ml; anti-BBS5 antibody was used with 1/50 dilution and validated in ref. 31. Antibody to polyglutamylated tubulin (GT335) was diluted to 1/10 and validated in ref. 54.

GTPase assay. The GTPass activity of CrArl6AN and the CrArl6AN–CrBBS1N complex were measured with the EnzCheck Phosphate kit (Invitrogen) by incubation of 50 µM of the proteins with 1 mM GTP at 20 °C. GTP hydrolysis was followed by monitoring the conversion of the released inorganic phosphate and 2-amino-6-mercaptop-7-methylpurine riboside into ribose 1-phosphate and 2-amino-6-mercaptop-7-methyl-purine by absorbance measurements at 360 nm. Measurements were recorded every minute over a 20-min time course. For the negative control, buffer without protein was added, and for the positive control, 100 µM of inorganic phosphate was added.

NMR spectroscopy. Prior to NMR data acquisition, protein samples were buffer exchanged into 20 mM NaPO₄, pH 6.5, 100 mM NaCl and 1 mM MgCl₂ and concentrated to 10–60 µM. GTP was titrated to each protein sample in a 1:2-fold molar excess. NMR data were acquired at 25 °C on a Bruker AV600 spectrometer equipped with a room-temperature triple-resonance probe. The spectra were processed with Topspin software (http://www bruker.com/products/mr/nmr/nmr-software/software/topspin/overview.html/).

Circular dichroism. CD spectroscopy was performed with a Jasco J-715 spectropolarimeter. Measurements were performed at 20 °C in a 0.1-cm quartz cuvette and were buffer corrected. Proteins were measured in buffer B at 1.5-µM concentration.
Data analysis and secondary-structure fractions were obtained with the CONTIN method of the CDPro analysis program (http://lamar.colostate.edu/~sreeram/CDPro/main.html). Although we observe no α-helices in BBS1N from the CrARL6–GTP–CrBBS1N structure, electron density is missing for the N-terminal 34 residues as well as for residues 150–212. Residues 150–212 are predicted to adopt the fold of four α-helices, thus probably accounting for the α-helical content of HsBBS1N. In case of the M390R mutant, the β-sheet content is significantly decreased, thus suggesting a mostly unfolded β-propeller. The increase in α-helical content may suggest that part of the unfolded β-propeller adopts an α-helical structure in the case of the HsBBSN M390R mutant.

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