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The interplay between RPGR, PDE δ and Arl2/3 regulate the ciliary targeting of farnesylated cargo

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Defects in primary cilia result in human diseases known as ciliopathies. The retinitis pigmentosa GTPase regulator (RPGR), mutated in the most severe form of the eye disease, is located at the transition zone of the ciliary organelle. The RPGR-interacting partner PDE δ is involved in trafficking of farnesylated ciliary cargo, but the significance of this interaction is unknown. The crystal structure of the propeller domain of RPGR shows the location of patient mutations and how they perturb the structure. The RPGR·PDE δ complex structure shows PDE δ on a highly conserved surface patch of RPGR. Biochemical experiments and structural considerations show that RPGR can bind with high affinity to cargo-loaded PDE δ and exposes the Arl2/Arl3-binding site on PDE δ . On the basis of these results, we propose a model where RPGR is acting as a scaffold protein recruiting cargo-loaded PDE δ and Arl3 to release lipidated cargo into cilia.

Keywords: ciliary trafficking; retinitis pigmentosa; RPGR; Arl2/Arl3; PDE δ

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INTRODUCTION

The cilium is a microtubule-based hair-like sensory organelle found on almost all human cells. Defects in the structure or function of cilia result in several human diseases collectively defined as ciliopathies. Ciliopathies include polycystic kidney disease, nephronophthisis, Bardet–Biedl syndrome (BBS) and various retinal degeneration syndromes [1]. Retinitis pigmentosa is a genetically heterogeneous eye disease causing the progressive degeneration of photoreceptor cells, in which the outer segments

are specialized forms of cilia in the retina. The disorder is characterized by night blindness, progressive loss of the peripheral visual field leading to complete blindness [2]. Some retinitis pigmentosa patients manifest more ciliopathy phenotypes as abnormal sperm development, respiratory tract infections and hearing defects [3–5].

The retinitis pigmentosa GTPase regulator (RPGR) gene is involved in the most severe form of the disease, X-linked retinitis pigmentosa [6]. In photoreceptors, RPGR localizes to the connecting cilium (CC) of rod and cones [7]. RPGR was also found in primary cilia of other cell types and the transition zone of motile cilia [2,7]. Knockout of RPGR in mice showed degeneration of rod and cone photoreceptor cells [8]. In addition, lack of RPGR is associated with opsin mislocalization pointing to an involvement of RPGR in regulation of ciliary trafficking [9].

The RPGR gene encodes several isoforms sharing an amino-terminal domain homologous to the RanGEF regulator of chromosome condensation 1 (RCC1), referred to as RCC1-like domain (RLD), and was thus predicted to also have GEF activity. One of the two main isoforms is the widely expressed RPGR^{1–19} consisting of 19 exons encoding a protein of 815 amino acids where exons 2–11 comprise the RLD [10]. A carboxy-terminal CAAX box, specific for geranylgeranylation, was shown to be essential for the localization and function of the protein [11]. By alternative splicing, the RPGR^{ORF15} isoform is generated, which has a highly repetitive acidic terminal exon, referred to as ORF15. While this exon is identified as a mutational hotspot in RPGR patients [12], most of the more severe patient mutations are localized in the RLD underscoring the physiological importance of this domain [13].

RPGR interacts via its RCC-like domain with a variety of cilia-related proteins, such as nephrocystin6 (NPHP6/CEP290), RPGRIP1 and nucleophosmin, and microtubule-associated proteins, such as IFT88, dynein, kinesin II and with PDE δ [10].

The phosphodiesterase 6 delta subunit is a ubiquitous and highly conserved prenyl-binding protein that was originally identified by co-purification with the prenylated catalytic subunits of PDE6 and hence its name [14]. It has been shown that PDE δ is a solubilizing and shuttling factor for several prenylated membrane-associated proteins, like Ras, RheB, the catalytic subunits of

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phosphodiesterases and rhodopsin kinase (GRK1), and recently the inositol polyphosphate 5-phosphatase E [15,16]. The small G proteins Arl2 and Arl3 allosterically regulate the release of farnesylated cargo from PDE δ in a GTP-dependent manner [17]. Nevertheless, the interplay of RPGR, PDE δ , Arl2/Arl3 and farnesylated cargo is not known.

To gain insights into the structure and function of RPGR and the interplay between its interacting partners, we determined the X-ray crystal structures of RPGR alone and in complex with PDE δ . Structural and biochemical analysis show that RPGR binds to the open conformation of PDE δ , which in contrast to the Arl2/3-bound closed conformation allows farnesylated cargo binding. Furthermore, the RPGR-binding site on PDE δ is not overlapping with the Arl2/Arl3 allosteric binding site suggesting the formation of a transient, fast dissociating quaternary complex. On the basis of these data, we propose a model where RPGR is recruiting PDE δ -bound farnesylated cargo at the transition zone (or CC) where the ciliary Arl3·GTP is releasing the cargo and hence targeting the farnesylated cargo into cilia.

RESULTS

Overall structure of RPGR

Owing to the difficulty to obtain the full-length 815 residue main RPGR isoform, we designed a shorter RPGR construct consisting of amino acids 1–392 comprising the RLD (referred to as RPGR from now on). Almost all RPGR patient mutations^{1–19} as well as the presently described protein interaction sites are located within the RLD. This construct was crystallized and the crystal structure was solved at a resolution of 1.7 Å by molecular replacement using the RCC1 structure (PDB code 1A12) as a search model.

As expected, RPGR shows an RCC1-like seven bladed propeller similar to the homologous RCC1 protein (Fig 1A). Each blade is formed by one repeat unit and consists of four antiparallel β -strands where the inner strands surround a central tunnel filled with water molecules. The structure shows the characteristic 2 + 2 arrangement of the first/last β -sheet with two strands from the N-terminal and two from the C-terminal half repeat, similar to RCC1 and unlike the WD40 propeller fold that has a 3 + 1 arrangement. The four molecules found in the asymmetric unit superimpose well (r.m.s.d. of 0.32, 0.37 and 0.29 Å). Compared with RCC1, the connecting loops show different conformations, and the positions of the β -sheets slightly differ (r.m.s.d. of 1.8 Å) (supplementary Fig S1A online). RPGR lacks the N-terminal extension, which is required in RCC1 for DNA binding. In addition, the RCC1 β -hairpin extension, referred to as β -wedge required for its GEF activity, is not found in RPGR; however, an unstructured loop between blades B3 and B4 is found instead (supplementary Fig S1B online). Surface representation of RCC1 shows a doughnut-like structure. In contrast to RCC1, the tunnel shaft of the RLD propeller in RPGR is closed on one side, where Tyr199, Tyr200, Arg96 and Asp344 are involved in the tunnel closure.

A ring of six highly conserved cysteine residues and another ring of six histidines in close proximity surround the RPGR tunnel entrance (Fig 1B). Two cysteine residues (C250R/Y and C302R/Y) and a histidine (H98Q) are mutated in patients with retinitis pigmentosa (RP). The cysteine and histidine residues most likely have a structural role stabilizing the protein fold. Indeed, purification of the proteins containing such patient mutations

showed the protein to be partly insoluble and degraded suggesting that mutations of the Cys/His ring destabilize the structure (supplementary Fig S2 online). Sequence alignment of RPGR from different vertebrates shows that the propeller surface is highly conserved, with two surface patches with the highest conservation located at the top of the molecule as viewed from the Cys/His ring (Fig 1C, patch1) and at one side of the molecule (Fig 1D, patch2).

Patient mutations and PDE δ interaction

Mapping the patient mutations to the RPGR structure shows that they are distributed over the entire molecule and are not restricted to particular regions. Many of the mutations are located in the β -strands, most likely contributing to the integrity of the propeller structure, as shown above for the cysteine mutations. In Fig 1C,D, we map all the surface mutations that are likely to be involved in the binding of RPGR-interacting proteins. Surprisingly, only some of these residues (R127G, F130C and P235S) are highly conserved among vertebrates (Fig 1C,D). One particularly interesting interactor is PDE δ as it is involved in ciliary trafficking of farnesylated cargo proteins and is regulated by the small G proteins Arl2 and Arl3, the latter of which is a *bona fide* ciliary protein [15,18]. We thus wanted to investigate the effect of patient mutations on RPGR binding to PDE δ . The affinity of RPGR to a fluorescently labelled Cy5-PDE δ determined by a fluorescence-based polarization experiment is 500 nM (supplementary Fig S3 online). This dissociation constant is in line with that reported before using a glutathione S-transferase (GST)-tagged PDE δ [19].

Unlike the Cys mutants mentioned above, the surface patient mutants could be purified. To analyse the effect of mutations on the interaction with PDE δ , we performed GST pull-down assays. RPGR mutants were pulled down by GST-PDE δ similarly to wild-type RPGR (supplementary Figs S3,S4 online). Densitometric analysis (data not shown) of the bands showed no significant differences (<3%) between wild-type and mutant proteins. In addition, we determined and compared the binding affinities using surface plasmon resonance. As expected, we did not observe significant changes in affinities compared with wild-type RPGR (data not shown). From this, we conclude that the patient mutations located at the surface of RPGR are not involved in the RPGR–PDE δ interaction.

Structure of RPGR in complex with PDE δ

To further investigate the RPGR–PDE δ interaction, we set out to solve the crystal structure of the RPGR–PDE δ complex. Good diffracting crystals were obtained with a construct of RPGR comprising amino acids 8–368, by mixing equimolar amounts of RPGR^{8–368} and PDE δ . The crystals belonged to space group C2 and diffracted to 1.9 Å. Using the RPGR structure as a search model for molecular replacement, we could identify one molecule of RPGR in the asymmetric unit. For the PDE δ molecule, a solution could only be found by using the PDE δ ‘open’ conformation model from the PDE δ ·cargo complex (PDB code 3T5G), and not the closed conformation from the PDE δ ·Arl2 complex (PDB code 1KSH). This and an ambiguous electron density in the open farnesyl-binding pocket (Fig 2A,B) imply a conformation-specific binding of PDE δ to RPGR (see below) [17,20].

PDE δ binds to one of the two most highly sequence-conserved patches (patch1) of RPGR, with a buried surface area of 1632 Å²

Fig 1 | Overall structure of RPGR. (A) Structure of the RLD domain of RPGR shown in ribbon representation. Blades are numbered (B1–B7), as indicated, as are the N and C termini. (B) Close-up view of the RPGR tunnel opening, showing the conserved cysteine and histidine ring in ball and stick representation and their residue number. Mutations of the Cys/His ring residues found in RP patients are indicated by asterisks. (C–D) Surface presentation of RPGR where conserved residues >90% are shown in red, residues with 60% are shown in white and residues with <20% are in blue (as demonstrated by the colour scale below). Two patches with the highest sequence conservation are at the top of the RPGR molecule, patch 1 (C) and at the side of the protein, patch 2 (D), as indicated. Patient mutations located on the surface are boxed. The relative orientations of the two views are indicated schematically above by the ribbons. RLD, RCC1-like domain; RP, retinitis pigmentosa; RPGR, retinitis pigmentosa GTPase regulator.

(Fig 2C,D). This side is topologically similar to where Ran binds to RCC1. Residues involved in complex formation are summarized in Fig 2E. Except for the W64-I30 and K51 (aliphatic side chain)–Y200 interactions, the interface is mostly formed by polar interactions. As expected from our pull-down-binding studies, patient mutations of RPGR are not located in but rather away from the interface (c supplementary Fig S3B online).

To verify the interface of the crystallized RPGR · PDE δ complex, one residue each from PDE δ and RPGR located in the interface, Arg48 and Glu216, respectively, were mutated by charge reversal. Indeed, both the PDE δ (R48E) and the RPGR(E216R) mutations

completely abolished interaction of both proteins as analysed by GST pull-down experiments (Fig 2F).

RPGR interaction with cargo-loaded PDE δ

PDE δ solubilizes and shuttles farnesylated cargo between membranes and is involved in their ciliary targeting. It is not known, however, how cargo bound to PDE δ is targeted to cilia. RPGR localization in the transition zone/CC makes it a potential candidate for recruiting cargo-loaded PDE δ . We thus wanted to study the connection between RPGR, PDE δ and cargo, and if RPGR would be able to recruit cargo-bound PDE δ . As PDE δ

Fig 2 | Structure of RPGR·PDE δ complex. (A,B) Slice through the electrostatic surface presentation of PDE δ in either the complex with farnesylated cargo (A, PDB code 3T5G) or with Arl2•GppNHp (B, PDB code 1KSH) to show the hydrophobic pocket in the open (A) and closed (B) conformations with the farnesyl moiety modelled into the closed pocket. (C) Ribbon representation of RPGR in cyan in complex with PDE δ in grey. (D) Ribbon representation of PDE δ (grey) and RPGR as surface representation similar to the position in Fig 1D (slightly rotated towards the viewer). PDE δ binds to conserved patch1 (encircled) of RPGR. (E) Schematic diagram showing the residues involved in the interface between RPGR (cyan) and PDE δ (grey) where solid lines represent nonpolar interactions and dotted lines show polar interactions. (F) The interface residues Arg48 of PDE and Glu216 of RPGR are shown as ball and stick presentation. GST pull-down experiments using wt and mutant PDE(R48E) (upper) and wt and mutant RPGR(E216R) (lower) are shown on the right side. GST, glutathione *S*-transferase; RPGR, retinitis pigmentosa GTPase regulator.

is in the open conformation in both structures, we can superimpose RPGR·PDE δ on the structure of the PDE δ ·F-RheB (for farnesylated RheB) cargo complex using PDE δ as a reference molecule. We could obtain a ternary complex model without any steric clashes (Fig 3A). We then wanted to test if the ternary complex can indeed be formed in solution. To do so, we performed GST pull-down experiments using GST-PDE δ , RPGR and F-RheB. Indeed, the pull-down experiment indicated the presence of a ternary 1:1:1 complex (Fig 3B). In addition, fluorescence-based polarization experiments using Cy5-labelled PDE δ showed a stepwise increase in the polarization signal after stepwise addition of RPGR and F-RheB (Fig 3C), confirming the existence of a ternary complex. Finally, a fluorescence polarization-based stopped-flow experiment using fluorescein-labelled F-RheB to study the kinetics of the PDE δ interaction with RheB in the presence and absence of RPGR showed no effect of RPGR for neither the dissociation nor association rate constants of the F-RheB/PDE δ interaction (supplementary Fig S5 online), which is in line with the observation that RPGR binds PDE δ in the 'open' but not the 'closed' conformation.

The interplay between RPGR, PDE δ and Arl2 or Arl3

We recently reported that Arl2 and Arl3 regulate the binding of farnesylated cargo to PDE δ [17]. We thus wanted to know if and how Arl2/Arl3 affects the RPGR·PDE δ interaction. By superimposing the crystal structure of RPGR·PDE δ on that of PDE δ ·Arl2•GppNHp, we can show that Arl2 binding does not sterically overlap with the RPGR-binding site and that a ternary complex of RPGR·PDE δ ·Arl2•GppNHp should in principle be structurally feasible (Fig 3D). On the other hand, the conformation of PDE δ in complex with RPGR is in the 'open' conformation, contrary to the closed conformation reported for PDE δ ·Arl2•GppNHp. This suggests that Arl2/3 binding to PDE δ might affect the RPGR–PDE δ interaction. To investigate this, we performed pull-down experiments using GST-PDE δ as bait and Arl2/3 and RPGR as prey. Increasing amounts of either Arl2 or Arl3 in the GppNHp-bound conformation are seen displacing RPGR from the complex with GST-PDE δ as shown in supplementary Fig S6A,B online. This seems to suggest that the two conformations of PDE δ are mutually exclusive for either Arl2/Arl3 or RPGR binding. However, as the binding sites of Arl2/3 and RPGR are

Fig 3 | The interplay between RPGR, PDE δ and Arl2/3. (A) Superimposition of the PDE δ ·RheB (farnesylated F-RheB) on the RPGR·PDE δ complex with RheB in yellow, RPGR in cyan and PDE δ in grey (using PDE δ as a reference molecule). (B) GST pull-down experiments using GST-PDE δ , RPGR and/or F-RheB as indicated. (C) Fluorescence polarization experiments using 0.25 μ M Cy5-labelled PDE δ where 10 μ M of RPGR and F-RheB, respectively, were added at the indicated time points. (D) Superimposition of Arl2·GppNHp on the RPGR·PDE δ complex with Arl2·GppNHp in green, RPGR in cyan and PDE δ in grey (using PDE δ as a reference molecule). (E–G) Gel filtration and SDS-PAGE analysis of mixtures containing equal amounts of RPGR (E–G), wild-type (E,F) or double mutant (F94A/I98A) (G) PDE δ and Arl3·GppNHp (E) or Arl2·GppNHp (F,G). Elution profiles for the controls are shown in supplementary Fig S7 online. PAGE analysis of the numbered elution fractions are shown below the elution profiles. GST, glutathione *S*-transferase; RPGR, retinitis pigmentosa GTPase regulator.

Fig 4 | Cargo release from the RPGR · PDE δ · F-RheB complex by Arl2/Arl3. **(A)** Superimposition of complexes of PDE δ with Arl2•GppNHp (green), F-RheB (yellow) and RPGR · in cyan using PDE δ (grey) as a reference molecule. **(B)** Fluorescence polarization experiments where 0.25 μ M fluorescein-labelled F-RheB peptide were titrated by stepwise addition of PDE δ and RPGR, at the indicated time points, to final concentrations of 0.25, 0.5 and 10 μ M for PDE δ and 1.5, 11.5 and 21.5 μ M for RPGR. Following addition of 5 μ M Arl3•GppNHp, a decrease of the polarization signal can be observed. RPGR, retinitis pigmentosa GTPase regulator.

non-overlapping, in the structural model (Fig 3D), it is more likely that Arl2/3 allosterically displaces RPGR via the formation of an intermediate ternary complex. To investigate this, we used size exclusion chromatography. Equal amounts of RPGR, PDE δ and Arl3•GppNHp were incubated and applied to a gel filtration column. The elution profile and PAGE analysis showed the appearance of three peaks (Fig 3E). The first peak contained high amounts of the ternary RPGR · PDE δ · Arl3 complex followed by the binary PDE δ · Arl3 complex and free Arl3•GppNHp (Fig 3E), as confirmed by control elution profiles for the individual proteins and the binary complexes (supplementary Fig S7 online). In contrast to Arl3•GppNHp, a ternary complex could not be identified by using Arl2•GppNHp. Here, the three peaks represent the binary RPGR · PDE δ complex, the PDE δ · Arl2 complex and free Arl2•GppNHp (Fig 3F). This is most likely owing to the fast dissociation of RPGR · PDE δ · Arl2 complex.

The pull-down experiments (supplementary Fig S6A,B online) had shown that Arl2 also displaces PDE δ from RPGR, which suggest that Arl2 is also acting allosterically and that the difference between Arl2 and Arl3 is a difference in quantity rather than quality. Previously, we have shown that Arl2 and Arl3 are unable to allosterically release farnesylated cargo from a PDE δ (F94A/I98A) double mutant but rather form a stable ternary PDE δ · cargo · Arl2/3 complex [17]. This indicates that Arl2•GTP (and Arl3•GTP) binding does not change the conformation of PDE δ from an open to a closed conformation, and that correspondingly such a mutant should be able to stably bind to both Arl2•GTP and RPGR. Using this mutant, a stable ternary complex of RPGR, PDE δ and Arl2•GppNHp could indeed be separated by gel filtration (Fig 3F). This confirms the presence of two distinct binding sites for Arl2 and RPGR on PDE δ and the formation of an intermediate, fast dissociating transient ternary complex. While a ternary complex could not be identified by

pull-down experiments using His-Arl3/2 as bait with RPGR and wt PDE δ as prey, such a complex was formed using the PDE δ (F94A/I98A) double mutant in the presence of Arl3•GppNHp, supporting the allosteric control of Arl3 (supplementary Fig S6C online). A minor band corresponding to RPGR being pulled down using His-Arl3 on nickel beads in the absence of PDE δ could be seen (supplementary Fig S6C online), but it was also seen in the absence of Arl3. Thus, we consider this to be nonspecific as we also did not observe direct binding between Arl3 and RPGR in solution in fluorescence polarization using fluorescently labelled mGppNHp-Arl3 and RPGR (data not shown) nor in size exclusion chromatography experiments (supplementary Fig S7 online). From all of the above, we conclude that although Arl2/3 and RPGR bind to PDE δ using two different binding sites and can in principle form a ternary complex, the binding sites are allosterically coupled. Both, Arl2•GppNHp and Arl3•GppNHp decrease the affinity of RPGR to PDE δ , with Arl3 apparently forming a more stable ternary complex.

RPGR as a scaffold for Arl3-mediated cargo release

Finally, we wanted to investigate the regulatory role of Arl2/Arl3 on the RPGR · PDE δ · cargo ternary complex. By superimposing the structures of the three PDE δ complexes with Arl2•GppNHp, F-RheB and RPGR on the PDE δ template, we obtain a model of a quaternary complex of RPGR · PDE δ · cargo · Arl2/Arl3•GppNHp in which the four proteins, sterically, do not significantly interfere with each other, apart from the highly mobile N terminus of Arl2 (Fig 4A). This suggest that RPGR could function as a scaffold for the PDE δ -mediated, Arl3-controlled transport of farnesylated cargo into cilia. Using fluorescence polarization and a fluorescein-labelled F-RheB peptide, we could clearly demonstrate the formation of a ternary complex between the peptide, PDE δ and RPGR, indicated by the increase in polarization after addition

of the proteins (Fig 4B). On addition of Arl3•GppNHp, the signal decreased indicating dissociation of the complex and release of F-RheB peptide. Although not relevant for ciliary transport, the same effect was also observed for Arl2 (data not shown).

DISCUSSION

RPGR mutations result in the most severe form of retinitis pigmentosa (X-linked RP), with 80% of the patient mutations located in this gene. In addition to retinal degeneration, a number of patients showed respiratory tract infections, hearing defects and abnormal sperm development as well. A recent study demonstrated that gene therapy using RPGR^{ORF15} was successful to rescue retinal degeneration in dogs [21]. This underscores the role of RPGR in photoreceptors and perhaps other cilia. While a large number of interacting proteins have been identified, the function of RPGR is still elusive.

As an approach to delineate the effect of patient mutations on the structure and possible function of RPGR, we have solved the three-dimensional structure of its propeller domain. We can show that a number of patient mutations are located in the structural core and are expected to destabilize the protein. While the surface shows two highly sequence-conserved patches, none of the mutations described so far are located in these patches, suggesting that they do not perturb a crucial interaction with a partner protein. We show that PDE δ is bound to one of the highest conserved surface patches where we cannot locate any of the patient mutations. This might indicate that the loss of this interaction is not tolerated [22].

The crystal structure of the RPGR·PDE δ complex showed binding of RPGR to the PDE δ 'open' conformation, and biochemical experiments confirmed the formation of a stable ternary complex of RPGR·PDE δ ·F-RheB. As RPGR is located in the transition zone of cilia in general and the CC of photoreceptor cells in particular, this suggests a role for the RPGR RCC1 domain, as a docking platform for the delivery of farnesylated cargo, such as the catalytic subunits of PDE6 or the γ -subunit of transducin. We have shown earlier that Arl2/3 allosterically induce a conformational change of PDE δ into the closed binding conformation. As both cargo and RPGR favour the open conformation, this would also suggest how cargo and the binding of PDE δ to RPGR are simultaneously weakened by Arl2/3 in the GTP-bound conformation. Our structures and structural models show that the PDE δ allosteric site for Arl2 and Arl3 is exposed and indeed our biochemical studies revealed release of farnesylated cargo bound to the RPGR·PDE δ complex by Arl2/Arl3, and at the same time the weakening of the interaction between RPGR and PDE δ . Although the binding sites of RPGR and Arl2/3 are on distinct faces of PDE δ , they are allosterically coupled such that they can inhibit the binding of each other via an intermediate ternary complex from which one of the partners is released.

Arl2 and Arl3 seem to be very similar in terms of biochemical and structural properties, and interact with the same set of effectors, such as BART, PDE δ and Unc119/HRG4. The identification of a more stable ternary complex between Arl3•GppNHp, wt PDE δ and RPGR, as compared with Arl2•GppNHp, is a further indication of differences in biochemical properties and biological function that were previously inferred from overexpression of GTPase-negative mutants and RNAi-knockdown studies [23]. Previously, we and others have shown that the release of

myristoylated ciliary cargo, such as GNAT1 and Cystin, from its complex with UNC119/HRG4 is only mediated by Arl3, although both Arl2 and Arl3 bind to UNC119 with similar affinities [24,25]. As Arl3 is a ciliary protein, whereas Arl2 is not, it suggests that Arl3 has a very specific function in ciliary trafficking. Our studies reported here suggest that farnesylated ciliary cargo transported by PDE δ is specifically captured in the transition zone/CC by the scaffolding protein RPGR and released by Arl3GTP. For validating such a pathway, further biochemical, structural and cell biological analysis of these and other RPGR-containing interactions are required.

METHODS

Constructs and plasmids. C-terminal His-tagged human RPGR (amino acids 1–392), and full-length murine Arl2 and Arl3 were cloned in pET21d and pET20a vectors, respectively; the GST fusion of human PDE δ was cloned in pGEX4T5-TEV as described [19,26]. For RPGR, a second construct comprising amino acids 8–368 with a C-terminal His-tag was cloned into pET21d.

Protein purification. Affinity chromatography followed by size exclusion chromatography was performed (for details see supplementary information online). Cy5-labelled fully modified farnesylated RheB protein and fluorescein-labelled farnesylated carboxymethylated RheB peptide was obtained as described before [17].

Structure. All crystals were obtained by the hanging drop vapour diffusion method, and microseeding was performed for optimization (for details for crystallization and structure determination please see supplementary information online). For data collection and refinement statistics, see supplementary Table S1 online.

In vitro pull-down assays. GST fusion protein (GST-PDE δ or mutants) were used for the pull-down assays following standard procedures (for details see supplementary information online).

Fluorescence measurements. Fluorescence polarization measurements were carried out at 20 °C in a buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl₂ and 2 mM β -ME. Data were recorded using a Fluoromax-4 spectrophotometer (Jobin Yvon, Munich, Germany). For kinetics measurements, the dissociation or association of fluorescein-labelled RheB peptide·PDE δ in the presence and absence of RPGR was followed by recording the polarization fluorescence in a stopped-flow apparatus (Applied Biophysics). The data were fitted to a single exponential function using Grafit 5.0.

Accession codes: The PDB access code for the RPGR structure is 4JHN and 4JHP for the RPGR-PDE complex.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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Author contributions: D.W. crystallized and solved the crystal structures, with I.V. and S.I., and performed the biochemical experiments, I.V. solved the crystal structures with D.W. and S.I., K.G. and M.M. contributed in the biochemical experiments, Y.-X.C. synthesized and purified lipid-modified proteins and peptides, A.W. and S.I. designed the experiments, supervised the project and wrote the paper with D.W.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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