Shuttling and sorting lipid modified cargo into the cilia

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Abstract

Primary cilia are hair-like microtubule-based organelles that can be found on almost all human cell types. Although the cilium is not separated from the cell by membranes, their content is different from that of the cell body and their membrane composition is distinct from that of the plasma membrane. Here we will introduce a molecular machinery that shuttles and sorts lipid modified proteins to the cilium, thus contributing in maintaining its distinct composition. The mechanism involves the binding of the GDI-like solubilising factors, UNC119a, UNC119b and PDE6D, to the lipid modified ciliary cargo and the specific release of the cargo in the cilia by the ciliary small G protein Arl3 in a GTP dependent manner.

Abbreviations Used

IFT – Intraflagellar transport, JBTS - Joubert Syndrome, PDE6D – Phosphodiesterase 6D, Homologue, INPP5E - Inositol Polyphosphate-5-phosphatase, Arl13b – ADP-Ribosylation factor-like 13b, GEF – Guanine nucleotide exchange factor, RP – Retinitis pigmentosis, RCC - Regulator of chromosome condensation, RPGR - Retinitis Pigmentosa GTPase Regulator, Arf - ADP-ribosylation factor, GSFs – GDI-like solubilising factor, GDI - GDP dissociation inhibitor, Uncoordinated – UNC, Rheb - RAS homologue enriched in brain, GAP – GTPase-Activating Protein, ELMOD - Engulfment and Motility Domain, TBCC - Tubulin Folding Cofactor C, GTP – Guanosine triphosphate, Leu – Leucine, Ser - Serine

Introduction

Primary cilia are microtubule-based sensory organelles that emerge from centrioles and can be found on almost all human cell types as a single hair-like protrusion. Defects in the structure or function of cilia result in a spectrum of diseases, including developmental abnormalities affecting multiple organs, collectively called ciliopathies [*1*]. Primary cilia are involved in the regulation of several signalling pathways including Hedgehog, Wnt and Notch pathways [*2*]. They communicate with the external environment by receiving external signals and stimuli, which are then transmitted into the cell via receptors and signalling proteins, which are concentrated in the cilia.

Unlike most organelles, cilia are not fully enclosed within membranes, and their membranes seem to be an extension of the plasma membrane, despite having a distinct composition. The distinction of ciliary composition from that of the non-ciliary plasma membrane and the cell body is achieved partly through a diffusion barrier at the base of cilia, whereby entry and exit of ciliary components are regulated [*3,4*]. Proteins destined to the cilium can be divided into four major groups; integral membrane proteins, small soluble proteins, large soluble proteins and membrane associated proteins. Here we will focus on membrane-associated proteins in particular lipid-modified proteins. Here, we will introduce and review a GTPase regulated machinery that is involved in shuttling and sorting of several myristoylated and prenylated ciliary proteins.

GDI-like solubilizing factors (GSFs)

GDI-like solubilising factors are a family of proteins which act to solubilise lipid-modified proteins and share homology with the Rho dissociation inhibitor (RhoGDI), a class of protein known to bind prenylated Rho proteins. PDE6D, UNC119a and UNC119b are proteins that share sequence homology, similar structural fold and interact with the two small G proteins Arl2 and Arl3. PDE6D, which is highly conserved in animals, is a small 17 kDa protein that was first co-purified with, and showed to solubilize, the rod photoreceptor-specific phosphodiesterase PDE6 [*5,6*]. PDE6D has immunoglobulin-like beta-sandwich fold similar to RhoGDI (Fig1). Based on this structural homology it was predicted that PDE6D functions as a GDI and hence bind and solubilise prenylated proteins [*7*]. Indeed throughout the years the majority of PDE6D interactors that were reported are prenylated and crystal structures of PDE6D in complex with farnesylated proteins or gerangeranylated peptide have been reported [*6,8*]. In these crystal structures the hydrophobic prenyl group is deeply buried in PDE6D with the five C-terminal amino acids of the prenylated cargo contacting PDE6D, explaining its solubilising effect (Fig1) [*9*]. Knocking out of PDE6D in mice resulted in mislocalisation of the prenylated proteins, PDE6 and GRK1, and impeded their delivery to photoreceptors outer segment [*6*]. Another ciliary prenylated protein reported to be targeted to cilia via PDE6D is the Inositol Polyphosphate-5-phosphatase (INPP5E) [*10*], confirming the role of PDE6D in targeting prenylated proteins to cilia. Indeed both PDE6D and INPP5E are disease genes in Joubert syndrome, a neurodevelopmental ciliopathy clinically defined as the presence of a “molar tooth sign” on axial MRI alongside one or more classic ciliopathy presentations [*11,12*]. Mutations in INPP5E have been reported in a number of JBTS patients [*11,13*] throughout the gene; widely in the phosphatase domain, but also in the CAAX domain, required for interaction with PDE6D, and the SH3 domain, required for interaction with Arl13b [*13-15*] suggesting the importance of GSF trafficking in healthy ciliogenesis.

The Retinitis pigmentosa GTPase regulator protein (RPGR), known to be mutated in majority of X-linked retinitis pigmentosa patients [*16*], is a prenylated PDE6D interactor; nevertheless it was reported to interact with PDE6D via its non prenylated regulator of chromosome condensation (RCC1)-like N terminal domain [*16,17*]. A crystal structure of the RCC1-like domain in complex with PDE6D has been reported; based on the structural and biochemical analysis the authors proposed RPGR to have a docking function at the ciliary base to recruit PDE6D in complex with prenylated cargo [*18*]. Contrary to these findings recently it has been reported that RPGR interacts with PDE6D through its prenyl group and the authors could not detect PDE6D interaction with the N terminal RCC1-like domain of RPGR by immunoprecipitation experiment [*19*]. One possibility is that PDE6D interaction with RPGR takes place through both interfaces and perhaps regulate the conformation of RPGR and hence its function. Nevertheless simultaneous interaction with both RCC1-like domain and the prenyl group of RPGR with PDE6D would exclude the proposed docking function of RPGR for PDE6D-cargo. Studies in cells in physiological context are instrumental to unravel the role of RPGR and its interplay with PDE6D and prenylated cargo. It's noteworthy that not all RPGR are prenylated, which makes it interesting to study the localisation of different RPGR isoforms in relation to prenylation and PDE6D.

In addition to its ciliary function, PDE6D is involved in trafficking of non-ciliary proteins as well. Knocking down PDE6D in pancreatic cancer cell lines resulted in mislocalisation of prenylated Ras from plasma membrane to endomembranes and the attenuation of its oncogenic signalling [*20*]. Capitalising on this observation, small molecules that inhibit the interaction of Ras and PDE6D are being developed to target cancer [*21*]. Since PDE6D is involved in different essential pathways as Ras and ciliary signalling, developing specific and safe drugs targeting one pathway over the other is quite challenging. Deeper understanding of the regulation of PDE6D and the cross talk between different pathways involving PDE6D is thus essential.

Two paralogues homologues of the C. elegans Uncoordinated (Unc)119 protein, UNC119a and UNC119b, show sequence homology to PDE6D and have the same immunoglobulin-like beta-sandwich fold. However, UNC119a and UNC119b selectively bind acylated cargo and have been shown to be involved in trafficking of acylated GNAT1 and myristoylated NPHP3 and cystin1 [*22,23*]. Mutation in UNC119a has been reported in a patient with rod-cone dystrophy, a late-onset degenerative retinal disease, underscoring its importance in the function of photoreceptors [*24*]. The crystal structure of UNC119a in complex with a lauroylated N-terminal Transducin-alpha peptide shows the acyl group to be deeply embedded in UNC119a [*23*]. The first six amino acids of the peptide form a 3_{10}-helix inside UNC119a where the amino acids are rather small with no bulky side chains, which might be the basis for cargo selectivity (Fig1). Superimposition of UNC119a and PDE6D bound to lipid modified proteins show the opening of the hydrophobic pocket to be on opposite sides (Fig1a&b).

UNC119a and UNC119b share a 60% sequence identity and differ mostly in the N terminal sequence. The difference in function between the two paralogous is not clear, nevertheless UNC119a is localised in the centrosome, and unc119b is localised in the cilia. The ciliary localisation of the myristoylated protein NPHP3, mutated in the ciliopathy renal nephronophthisis, is dependent on UNC119b and not UNC119a [*22*]. In addition to its ciliary function UNC119a is involved in trafficking of several Src kinases [*25*]. Based on the structural and functional similarities that PDE6D UNC119a and UNC119b share with RhoGDI we call them GDI like solubilising factors (GSFs) [*20*].

The release factors

Arl2 and Arl3 are small G proteins that belong to the Arf like small G protein subfamily, have a 52% sequence identity and share several interactors [*26*]. Arl2 and Arl3 have a number of known interactors including PDE6D, UNC119a and UNC119b whereby the interactions are GTP dependent and do not involve lipid moieties [*26*]. Arl3 is a ciliary protein and although has not been reported to be mutated in ciliopathies, knockout mice show retinal and renal defects that mimic that of ciliopathies [*27*]. The crystal structure of Arl2 in complex with PDE6D was reported and compared to the crystal structure of a farnesylated Rheb in complex with PDE6D [*9*]. The two complexes superimpose with no steric clashes, and Arl2 binding seemed to be taking place through a different interaction interface (Fig 1c). Nevertheless the conformation of PDE6D is different in complex with Arl2 from that with Rheb. The hydrophobic pocket of PDE6D in complex with Arl2 seems to be closed and several of the residues lining the pocket would clash with a prenyl group. Using polarisation based fluorescence assays and expression of fluorescently tagged proteins; it was shown that Arl2 and Arl3 allosterically release the prenylated cargo from PDE6D [*9*].

The interplay between UNC119, myristoylated cargo and Arl3 has been reported to be similar to that in case of PDE6D [*14,22*]. Although Arl3 releases the acylated cargo bound to UNC119a and UNC119b, the crystal structure of Arl3 in complex with UNC119a shows that Arl3 widens the acyl-binding pocket rather than closing the pocket. The most intriguing observation was the specific ability of Arl3, and not Arl2, to release the tightly bound acylated cargo. The structural basis for the release by Arl3 and not Arl2 is due to the N-terminal amphipathic helix of Arl3 that folds over the surface of protein stabilising a conformation that is competent in widening the hydrophobic pocket. In case of Arl2 this helix is exposed to the solution and not folded on the protein and hence is not competent in releasing the cargo (Fig1) [*14*].

Based on these studies a model has been proposed where GSFs solubilise prenylated or myristoylated proteins aiding them to diffuse through the cytosol and at the point of destination Arl2 or Arl3 release the cargos [*9,22,28*]. For examples of GSF-cargo binding affinities and their release factors see table1.

Regulating the releasing factors

The GTPase cycle of small G proteins, which have slow intrinsic GTPase activity and bind to nucleotides with high affinities, is usually regulated by GTPase activating proteins (GAPs) and Guanosine exchange factors (GEFs).

XRP2, the product of the retinitis pigmentosa 2 gene (RP2), is so far the only known GAP protein for Arl3 [*29*]. XRP2 is mutated in retinitis pigmentosa patients primarily due to a disruption of protein trafficking to the cilium including photoreceptor protein Opsin and Intraflagellar Transport protein IFT20 [*30-34*]. For Arl2 it has been shown that the tubulin cofactor TBCC and ELMOD proteins can function as GAPs for Arl2 [*35,36*]. There are no known GEF proteins for Arl2, nevertheless due to the relatively fast dissociation rate of nucleotides bound to Arl2 it is possible that Arl2 does not need a GEF.

Using an Arl3 mutant with low nucleotide affinity, Gotthardt and colleagues used a yeast-two-hybrid system to identify the classic ciliary marker and the Joubert syndrome disease gene, Arl13b, as the GEF for Arl3. The GEF activity of Arl13b is higher in the GTP bound form and crystal structure of chlamydomonas Arl13b.GppNHp in complex with Arl3GppNHp shows Arl13b switch regions being involved in the interaction with Arl3, which would explain the nucleotide dependency of the GEF activity [*15*]. The central role for Arl13b in ciliary release means that it is perhaps unsurprising that mutations in Arl13b have been linked with Joubert syndrome and nephronopthsis [*13,37*].

Model for sorting lipid modified cargo

PDE6D is involved in shuttling both ciliary and non-ciliary cargo which begs the question of how does one shuttling protein target different cargoes to different destinations. The mechanism of PDE6D mediated sorting of farnesylated cargo between the cilia and the cell body was proposed recently by Fansa and colleagues to be dependent on the affinity of the cargo to PDE6D and the specific release of the ciliary cargo by Arl3GTP [*38*].

The affinity of PDE6D to a fully modified farnesylated INPP5E peptide is 100 fold higher than that of PDE6D to fully modified farnesylated Rheb [*38*]. By comparing the crystal structures of PDE6D in complex with farnesylated INPP5E peptide to PDE6D in complex with farnesylated Rheb, the difference in affinities was found to be due to residues at positions -1 and -3 upstream of the farnesylated cysteine. Furthermore, the ciliary INPP5E is released from its complex with PDE6D only by Arl3GTP and not Arl2GTP whereas the Rheb-PDE6D is disrupted by both Arl2GTP and Arl3GTP. The high binding affinity of INPP5E to PDE6D is the basis of the selective release by Arl3GTP and was proposed to be the basis of the exclusive ciliary localisation of INPP5E. Indeed swapping of INNP5E Ile at -1 and Ser at -3 with the Rheb Ser -1 and Lys -3 results in reduced affinity of INPP5E to PDE6D and loss of the exclusive ciliary localisation of INPP5E. It`s worth mentioning that mutant INPP5E is still able to localise, although not exclusively, to the primary cilia. This could be due to the solubility of the PDE6D-INPP5E and its diffusion into the cilia or due to the presence of retention signals in cilia or both.

Based on this study and other studies and the presence of the Arl13b GEF and Arl3GAP in cilia a simplified model for shuttling and sorting lipid-modified cargo into cilia is shown in figure 2. The lipid modified protein is solubilised by binding GSFs in the cytosol. if cargo bind to GSFs with a low affinity the complex will be disrupted by active Arl2GTP in the cell body. In case of ciliary proteins, binding to GSFs with strong binding affinities, the soluble complex can diffuse into the cilia. Inside cilia Arl3 is activated by Arl13b and can release the cargo into cilia. The released cargo is then retained in the cilia by associating with the ciliary membrane. Most likely, additional factors are involved in retaining cargo in cilia, for example it has been shown that Arl13b interacts with INPP5E and is important for its ciliary targeting [*10*]. Arl3GTP bound to GSFs will be recycled by XRP2 hydrolysing GTP and the GSF is now ready for a new cargo (Fig2).

Open questions and future perspectives

Arl13b is a key regulator of ciliary trafficking [*10,15,39*], but thus far details of its own regulation are not known. By further understanding of Arl13b regulation we will have a far greater understanding of the mechanics of cilia signalling, whilst potentially identifying new families of proteins in the study of ciliopathies. Similarly, trafficking of proteins by Unc119 to cilia membranes and non-ciliary membranes appear to be very similar[*22*], the key differentiating factor being the specificity of Arl3 as a release factor specific to cilia, whilst Arl2 acts elsewhere [*14*]. Our understanding of the GSF model could be greatly illuminated by understanding the GEF responsible for activation of Arl2 at non-ciliary membranes.

The Intraflagellar transport system is a system that transports proteins within the cilia via the motor proteins dynein and kinesin. Dynein is responsible for moving cargo from the tip of the cilia to the base (retrograde) and kinesin from the ciliary base toward the tip (anterograde)[*40*]. Interactions with cargo take place via the multi-subunit IFT complexes [*41*]. Several ciliary membrane proteins are targeted to the base of the cilia via vesicle trafficking, where targeting involves other small GTPases such as Arf and Rab proteins, or lateral diffusion in the plasma membrane. At the base of the cilia the membrane proteins are handed over to the IFT where they are transported within the cilia. Whilst we understand that release of ciliary cargo is GTP dependent, we do not yet understand how those cargoes are recruited to the cilium in the first place. Furthermore, how do GSFs-cargo cross the ciliary diffusion barrier and whether it's an active process involving the IFT transport system and its motor proteins or rather a passive diffusion process? [*41*]

Cargo is released from Unc119 by interaction with activated Arl3 [*22*], this interaction causes a conformational change which allows for the release of proteins to the ciliary membranes [*14*]. The N- terminal amphipathic helix of Arl3 is critical for releasing high binding affinity ciliary cargo. Nevertheless this amphipathic helix, similar to Arf proteins, is predicted to interact with membranes. Interaction of amphipathic helices with membranes relies on the geometry and composition of membranes. We suggest that the geometry and composition of the cilium may play a vital role in the targeting and sorting of ciliary cargo. Further investigation of protein and membrane structure could open many new avenues of research in ciliary biology.

The role of GSFs in spatiotemporal regulation of several ciliary and non-ciliary proteins is an emerging field with many exciting implications. So far the system is controlled by three GTPases which represent possible regulation points. By determining what cues regulate Arl2, Arl3 and Arl13b we will have better and deeper understanding of the function of GSFs and their role in regulating signalling pathways. It seems likely that as our understanding of this field grows, we are seeing that GSF targeting is as vital to the function of the mature cilium as IFT, with many ciliopathies the result of abnormalities within the GSF pathway. At this exciting time for cilia biology GSFs may hold many more answers.

Figure 1.GSFs share structural fold with RhoGDI and bind to lipid modified cargo and Arl2 via two different interfaces a)superimposition of PDE6D (cyan), UNC119(grey) and RhoGDI (yellow) showing the common immunoglobulin-like beta-sandwich fold [*42*] b) PDE6D in cyan in complex with C-terminal carboxy methylated and farnesylated peptide (indicated by an arrow) from INPP5E (farnesyl in red and peptide in orange) c) UNC119a in grey in complex with lauroylated (red) N-terminal peptide from transducin alpha subunit (orange) d) superimposition of the crystal structures of PDE6D (purple) in complex with Arl2 (green) on that of PDE6D(cyan) in complex with farnesylated fully modified Rheb (orange). Arl2 N-terminal helix exposed to solution is shown in red.

Figure 2. A model for sorting and shuttling of prenylated/myristoylated ciliary cargo into the cilia. The GSF (blue; e.g. Unc119b) binds to the lipid modified tail of the ciliary cargo (green). This is transported to the cilium, whereby Arl3, maintained in a GTP bound state by Arl13b, binds the GSF, forcing a conformational shift, which releases the ciliary cargo to the ciliary membrane. Binding of Arl3 to the GAP RP2 results in an inactive, GDP bound state. Non-ciliary cargo (grey) is also solubilised by the GSF in the same manner before being released to endomembranes by binding with Arl2GTP. The GEF required to maintain Arl2 in an active, GTP bound state is not known, similarly the GAP required for Arl2 inactivation is unknown.

Table1. Examples of GSFs affinities to cargo and release by Arl2 and Arl3. GSF=GDI solubilising factor, NA=not available and text in red indicates that the prediction has not been verified experimentally yet. References are [*8,9,22,38,43,44*].

Acknowledgments

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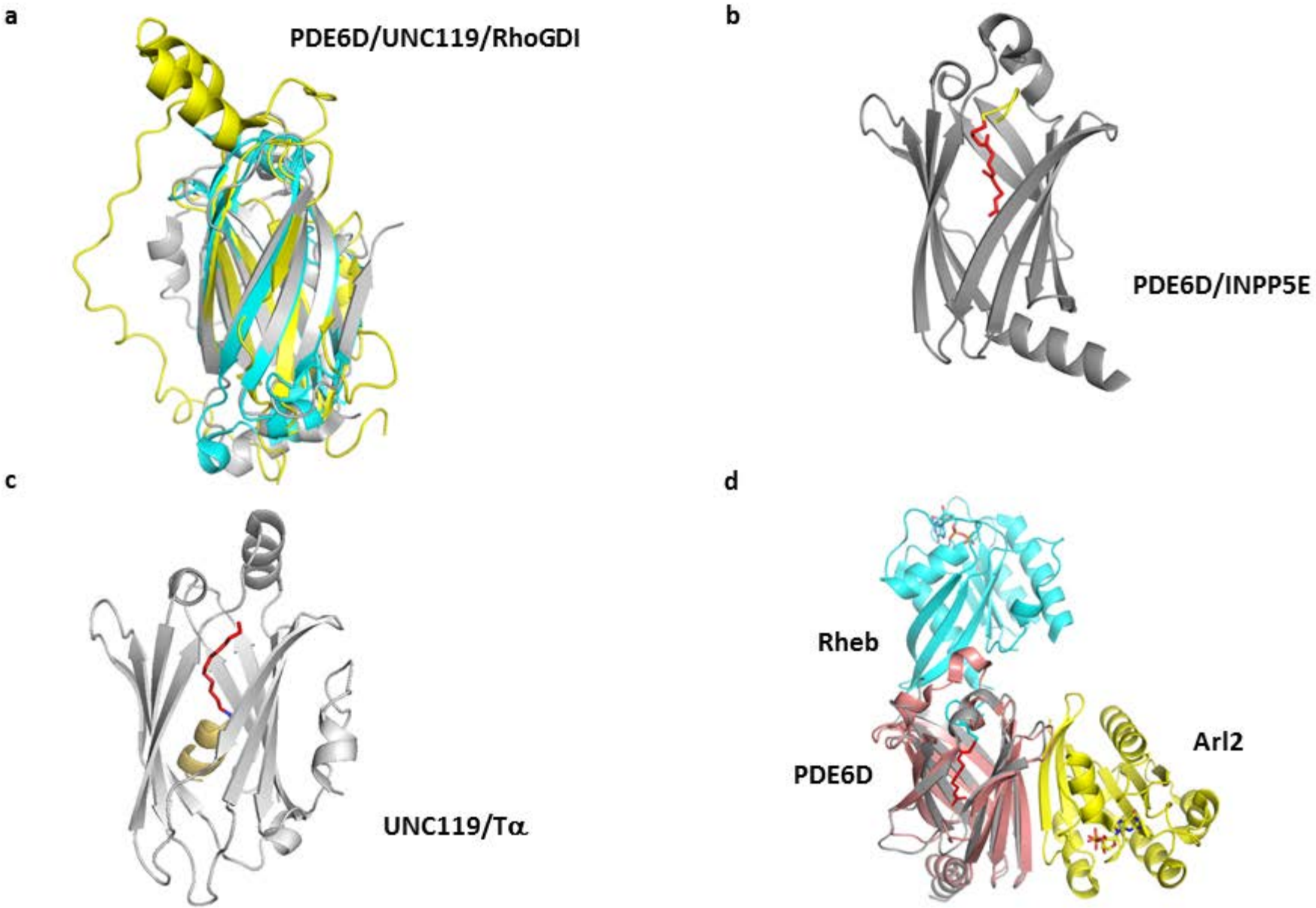
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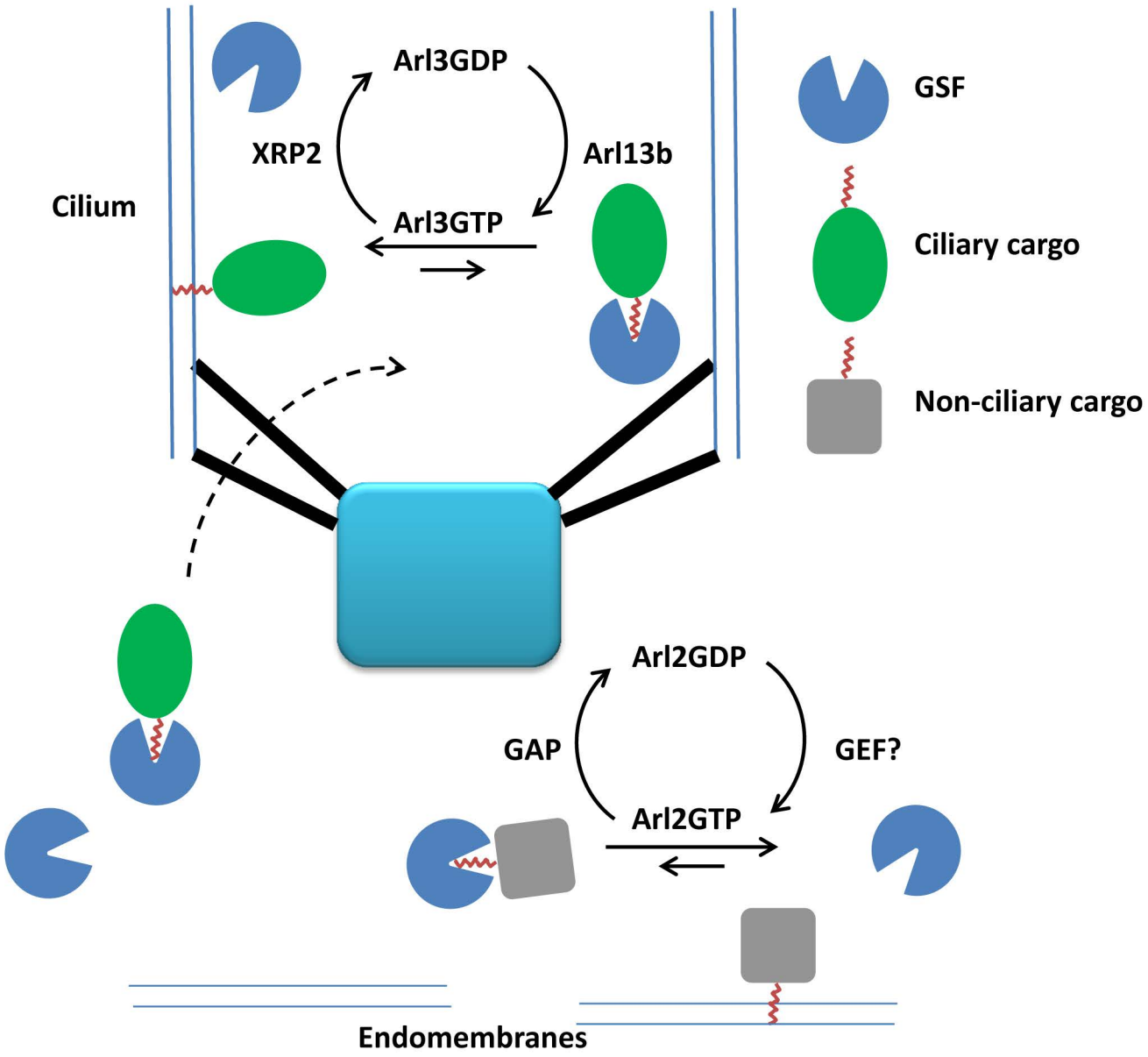
b
PDE6D/INPP5E
c
UNC119/Tα
d
Rheb
PDE6D
Arl2


Arl3GDP
XRP2
Arl13b
Cilium
Arl3GTP
GSF
Ciliary cargo
Non-ciliary cargo
Arl2GDP
GAP
GEF?
Arl2GTP
Endomembranes

GSF	Cargo	Affinity	Release factors
PDE6D	INPP5E	4nM	Arl3
	PDE6a	2nM	Arl3
	GRK1	7nM	Arl3
	Rheb	150nM	Arl3&Arl2
	Kras	225nM	Arl3&Arl2
UNC119	GNAT-1	7nM	Arl3
	NPHP3	14nM	Arl3
	Cystin	NA	Arl3