The role of transportin-1 in the ciliary targeting of membrane proteins

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ciliary targeting of membrane
proteins**

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School of Biological Sciences

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List of abbreviations

ACIII	adenyl Cyclase III
ALMS	Alström syndrome
Arf	ADP-ribosylation factor
Arl	Arf-like
ASAP1	Arf gap with SH3 domain, ankyrin repeat and PH domain
BBS	Bardet-Biedl syndrome
BFA	Brefeldin A
B9D1	B9 domain containing protein1
CC2D2A	coiled-coil and C2 domain-containing protein 2A
CD-M6PR	cation-dependent mannose 6-phosphate receptor
CDS	coding DNA sequence
CEACAM1	carcinoembryonic antigen-related cell adhesion molecule 1
CEP290	centrosomal protein of 290 kDa
CEP164	centrosomal protein of 164 kDa
CTS	ciliary targeting sequences
CPIR	cilium-to-the PM intensity ratio
CI-M6PR	cation-independent mannose 6-phosphate receptor
CLS	ciliary localization signal
CNGB1b	cyclic nucleotide-gated beta 1b
CO ₂	carbon dioxide
COP	coat protein
DMEM	Dulbecco's Modified Eagle Medium
EVC	Ellis-van Creveld syndrome
ER	endoplasmic reticulum
Evi5-like	ecotropic viral integration site 5-like
FDB	fluorescence Dilution Buffer

FRAP	fluorescence recovery after photobleaching
GPCR	G protein coupled receptor
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
G _{olf}	olfactory G protein
GPI.	glycosylphosphatidylinositol
GST	glutathione S-transferase
GTP	guanosine triphosphate
HEK 293T	human embryonic kidney HEK 293T
HeLa	Henrietta Lacks
HEPES	4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid
hnRNP	heterogeneous nuclear ribonucleoprotein
hTERT RPE-1	human telomerase-immortalised retinal pigmented epithelial-1
HTR6	5-hydroxytryptamine (serotonin) receptor 6
IFT	intraflagellar transport
IL-2R	Interleukin-2 receptor
IMCD3	inner medullary collecting duct
INM	inner nuclear membrane
INPP5E	inositol polyphosphate-5-phosphatase
IBDUs	isolated intrahepatic bile ducts
IPTG	isopropyl β -D-1-thiogalactopyranoside
JATD	Jeune asphyxiating thoracic dystrophy
JBTS	Joubert syndrome
Kif17	kinesin-like protein17
KIF3B	kinesin-like protein3B
MCHR1	melanin-concentrating hormone receptor1
MKS	Meckel-Gruber syndrome
MTOC	microtubule organizing center
NPHP	nephronophthisis
NES	nuclear export signal

NLS	nuclear localization signal
NPC	nuclear pore complex
NPY2R	neuropeptide Y receptor type2
NUP	nucleoporin
Ofd1	oral-facial-digital syndrome 1
ONM	outer nuclear envelope membrane
PBS	phosphate buffered saline
PC-1	polycystin-1
PC-2	polycystin-2
PFA	paraformaldehyde
PKHD1	polycystic kidney and hepatic disease 1
PDGFR	platelet derived growth factor receptor
PM	plasma membrane
PMSF	phenylmethanesulfonyl fluoride
PKD	polycystic kidney disease
PCD	primary ciliary dyskinesia
PVDF	polyvinylidene difluoride
Rab11-FIP3	Rab11 family interacting protein-3
RanBP1	Ran binding protein 1
RanGAP1	RanGTPase activating protein1
RCC1	regulator of chromosome condensation 1
RP2	retinal pigmentosa protein-2
RPGR	retinitis pigmentosa GTPase regulator
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SNARE	soluble N-ethylmaleimide-sensitive-factor attachment protein receptor
SSTR3	somatostatin receptor 3
TBC1D7	tre2-Bub2-Cdc16 (TBC) 1 domain family member 7
TMEM231	trans-membrane protein 231
TRAPPII	transport protein particle
VAMP	vesicle-associated membrane protein

Abstract

Cilia are microtubule based membrane-bound sub-cellular organelles which project from the surface of cells. Once considered as a vestige, cilia have been the focus of intensive studies in recent years due to its role in many genetic disorders, collectively termed as ciliopathies. Cilia play a role in motility and sensing extracellular signals. Though the ciliary membrane is contiguous with the plasma membrane (PM), many receptors and signalling factors are enriched in cilia, and are absent from the PM. The proper functioning of cilia depends upon the selective targeting of these receptors and signalling factors to the ciliary compartment, yet little is known about how cells achieve this highly polarized distribution.

Our study is focused on elucidating the ciliary targeting mechanism of membrane proteins. To that end, we established an image-based method to introduce an ensemble-averaged metric, called the cilium-to-PM intensity ratio (CPIR), to quantify the ciliary localization of a membrane protein in cultured mammalian cells. Using CD8a as a reporter in RPE1, BSC-1 and IMCD3 cells, we first established that, contrary to general belief, the native and chimeras of CD8a localize at cilia in addition to the PM. We found that PM-localized membrane proteins, which are conventionally considered non-residents of cilia, could enter the ciliary membrane. We demonstrated that CD8a uses lateral transport from the PM to enter cilia. To further characterize the diffusion barrier, we showed that clathrin-dependent endocytosis, as well as interactions with the cortical actin cytoskeleton, prevents the PM proteins from entering cilia. When the cytosolic domain of CD8a is artificially increased by fusing multiple units of GFP, the cilium-localized CD8a decreased sharply. Inspired by recent evidence that nucleocytoplasmic trafficking machinery could contribute to ciliary trafficking, we tested our hypothesis that importin- β binding motifs could be the ciliary targeting sequence (CTS) for membrane proteins. We found that the addition of the importin- β binding domain of importin- α , cNLS of SV40 and bPY-NLS of hnRNPM, increased ciliary localization of CD8a chimeras more than two-fold, demonstrating that, these signals are sufficient to drive non-cilia localized CD8a chimera to cilia. Screenings of known necessary and sufficient CTSs of ciliary membrane proteins revealed that the CTS of fibrocystin (autosomal recessive polycystic kidney disease protein) and retinol dehydrogenase interact specifically with

transportin-1 (TNPO1) but not β 1. We demonstrate that the fibrocystin-CTS (f-CTS) interaction with TNPO1 is essential for its ciliary localization. We further show that f-CTS interaction with TNPO1 is enhanced in the presence of Rab8-GDP bound form and all three form a tripartite complex. Our data suggest that TNPO1 could participate in ciliary trafficking as a receptor of membrane proteins. Considering that Rab8-GTP and β -GDP are enriched in cilia and cytosol respectively, our studies suggest that Rab8-GTP/GDP gradient and TNPO1 could play an important role in ciliary trafficking of membrane proteins.

CHAPTER 1: Introduction

A cell is the basic structural and functional unit of living organisms. It is compartmentalized into various membrane-bound organelles, which allow the cell to perform specific cellular activities and maintain proper function. The structurally similar cilia and flagella are membrane-bound sub-cellular organelles which project from the surface of certain eukaryotic cells. Primary cilia were once considered a vestige, but have since been transformed into an organelle of importance due to its role in various human disorders, termed as ciliopathies. Cilia are considered to function as “signalling antenna” of the cell, with a wide array of sensory functions. They play important roles in sensing extracellular signals and coordinating cellular functions [1].

In humans, motile cilia play important role in motility and are restricted to certain tissues. The roles include the clearing of mucus in the trachea by ciliary lining of respiratory cells, establishment of right-left symmetry in the embryo, transportation of ovum from the ovary to the uterus, and propulsion of sperm for fertilization in reproductive systems [2, 3]. Most cells contain a single non-motile cilium or primary cilium [3]. Primary cilia act as photo-sensors in rod and cone cells the retina, chemo-sensors by executing the role of olfaction in olfactory neurons, and as mechano-sensors by monitoring fluid flow in kidney epithelial cells [2, 3]. Additionally, the primary cilia contain components of many developmental and signalling pathways, such as Hedgehog, Wnt and platelet derived growth factor receptor (PDGFR) signalling, and respond to extracellular ligands [4-6]. Deficiency of cilia stalls transduction of Hedgehog pathway, and also causes defects in planar cell polarity [5, 7-9]. Cilia are known regulators of cell proliferation and ciliogenesis is dependent on the cell cycle [10].

1.1 Structural characteristics of cilia

The structure of cilia is highly conserved throughout eukaryotes, ranging from unicellular green algae *Chlamydomonas reinhardtii* to multicellular *Homo sapiens*. A single cilium is composed of the basal body, transition fibers (TFs), transition zone (TZ) and axoneme encompassed in a ciliary membrane (Fig. 1).

1.1.1 Characteristics of the axoneme

The central cytoskeleton of cilia is made up of a bundle of microtubules termed as axoneme.

Figure 1.1: Schematic representation of the cilium structure

Schematic representation of cilia indicating the axoneme, ciliary membrane, ciliary pocket, transition fibers, transition zone, 9+2 and 9+0 microtubule arrangement, of motile and non-motile cilia respectively. The inset of the dashed box from motile cilia indicates the radial spokes and dynein arms. The inset of the axonemal microtubule shows A-tubule, B-tubule and protofilaments within the tubules

(red dots). Centrosome with pericentriolar material (PCM) (yellow), mother (light gray) and daughter centriole are also represented. The dotted lines from the mother centriole to the basal body indicate the transformation of the mother centriole to the basal body. The magnified triplet microtubular arrangement of the basal body is shown in inset. Adapted from Hu and Nelson [11].

Due to the asymmetric polar structure the end of axoneme protruding outwards (tubulin assembly occurs) is designated as (+) end, and the end anchored in the centriole or basal body as (-) end [12]. Based on the arrangement of axonemal microtubules, the presence of dynein arms and radial spokes (essential for movement), cilia are categorized into motile and non-motile (primary cilia) variants. Motile cilia consist of nine outer doublet microtubules and a central pair of single microtubules in a 9+2 arrangement, along with dynein arms and radial spokes. Non-motile cilia contain a 9+0 arrangement and lack the 'central pair of microtubules' and 'dynein arms' (Fig. 1). However, there are some exceptions, such as the embryonic nodal motile cilia which have a (9+0) structure, or the cilia of olfactory sensory neurons (OSNs) that have a (9+2) structure but are immotile due to the lack of dynein arms [13-15]. Though the basic structure of cilia is conserved, it undergoes changes to fit its function. For example, photoreceptors of rod and cone cells are sensory neurons, compartmentalized into inner segment (site of protein synthesis) and outer segment (site of protein function). The axoneme of cilia connecting these two segments is present as a doublet of microtubules (9+0) in the inner segment, which later transforms to singlet microtubules in the outer segment [16-18].

1.1.2 The structural and functional features of the basal body

The basal body is a barrel-shaped structure made up of nine triplets (A, B and C) of microtubules. It is derived from the mother centriole, one of 2 centrioles present in a centrosome (Fig. 1) [19]. Centrosomes are the microtubule organizing centre (MTOC) of the cell, and they play a role in spindle pole formation during mitosis. The switching of centrioles between the basal body and MTOC is dependent on the cell cycle [20]. The biogenesis of cilia occurs when microtubules A and B from the basal body triplet (A, B and C) extend to form the ciliary axoneme during the G₀ phase. The resorption occurs during either the S or G₂/M phase of the cell cycle. After formation, the axoneme is marked by post-translational modifications such as acetylation, polyglutamylolation, detyrosinylation, and glycylation of tubulins [21]. Studies show that these post-translational modifications provide rigidity to the microtubules of growing axoneme, as well as play a role in their assembly and disassembly [22, 23].

Therefore, these tubulin modifications are generally used as markers to differentiate cilia from cytoplasmic microtubule cytoskeleton [24-26].

1.1.3 Organisation and functional roles of transition fibers and transition zone

The proximal region of cilia anchors the ‘ciliary gate’, which is composed of two distinct regions, namely, the TFs and TZ (Fig 1.2). The TFs are appendages that arise from the B-tubule of the basal body [27, 28]. TFs assist in anchoring the basal body to the PM, and act as docking sites for intraflagellar transport (IFT) complexes [29]. Electron microscopic studies showed that TFs form a mesh at the base of cilia. The space between TFs (highlighted with blue arrows in Fig. 1.2A) is too small for membrane vesicles to pass (Fig. 1.2A). This is consistent with reports that no vesicles were observed inside cilia [15, 30]. It was also reported that the basal body degenerates in the case of *Caenorhabditis elegans*, leaving the TFs undisturbed [31]. Hence, though the exact composition of TFs is unknown, it was proposed that they are part of a pore complex similar to the nuclear pore complex, which is required for regulating proteins in and out of cilia.

The TZ contains the ciliary necklace on the ciliary membrane and Y-shaped linkers (Fig. 1.2B). The ciliary necklace is a network of parallel strands of intra-membranous particles, where the number of strands depends on the cell type. As represented in the pictorial representation (see inset of Fig 1.2B), the Y-linkers start at a point where the triplet microtubules of the basal body are converted into doublets of ciliary axoneme [28]. Extensive studies have revealed the presence of proteins such as those from Meckel-Gruber syndrome (MKS) and nephronophthisis (NPHP), septin2, CC2D2A (coiled-coil and C2 domain-containing protein 2A), TMEM231 (trans-membrane protein 231) and B9D1 (B9 domain-containing protein 1), in the TZ. Experiments using *Caenorhabditis elegans* as a model organism showed that the disruption of MKS and NPHP family proteins results in genetic disorders related to cilia dysfunction [32]. Septins are GTPases involved in the formation of ordered structures such as rings and filaments etc. For example, in budding yeast, a septin ring forms at the budding neck during cytokinesis, causing proper segregation between the mother and daughter cells [33]. Septin2, a member of the septin family, was shown to form a ring at the base of cilia [34]. Using immunostaining, it was shown that septin2 exists between the ciliary axoneme and centriolar appendage proteins, such as CEP164. Fluorescence recovery after photobleaching (FRAP) of cilia demonstrated

that ciliary membrane proteins such as serotonin receptor 6 (Htr6) and somatostatin receptor 3 (SSTR3) cannot recover the fluorescence of bleached areas. However, depletion of septin2 resulted in rapid recovery of these proteins. This evidence suggests that septin2 acts as a diffusion barrier at the base to restrict the dynamic movement of membrane proteins across cilia and the PM [34, 35]. The localization of CC2D2A, TMEM231 and B9D1 to the TZ is dependent on each other and septin2-dependent. Through FRAP and knockdown experiments, it was shown that these three proteins together act as a diffusion barrier, similar to septin2 [32, 35].

Figure 1.2: Transition zone and transition fibers

Pictorial representation of cilia and its structural features, particularly the dashed box of the basal body indicate its degeneration in *Caenorhabditis elegans*. A) Transmission electron microscope [36] and simplified pictorial images of TFs (shown using blue arrows) of *Caenorhabditis elegans* sensory and human oviduct primary cilium show a mesh originating from the triplet microtubule (absent in *Caenorhabditis elegans*). The dashed box region is magnified to denote TFs and triplet microtubule. B)

TEM and simplified pictorial images of TZ of *Caenorhabditis elegans* sensory and human oviduct primary cilium show Y-links (shown using red arrows) connecting doublet microtubule and ciliary necklace in ciliary membrane. The dashed box region is magnified to denote the ciliary necklace, Y-link, microtubule doublet and apical ring (adapted from [37]).

Vieira et al. demonstrated the existence of lipid diffusion barrier using glycosylphosphatidylinositol (GPI) tagged fluorescent protein (FP). GPI-FP, localizing to the apical PM in Malin-Darby Canine Kidney (MDCK) cells, is excluded from the ciliary membrane. The exclusion area appears a hole of 1.2-1.8 μm , larger than the diameter of cilia (0.3 μm) [38]. Moreover, FRAP studies indicated that GPI-FP is highly mobile within the PM, similar to non-raft protein [39]. The lectin Galectin3 is absent from the PM and ciliary membrane but localizes precisely to the GPI-FP excluded region [38]. Furthermore, immunofluorescence studies reported the presence of densely-packed membrane lipids at the base of cilia, which could act as a barrier to prevent the diffusion of membrane proteins from PM [38, 40]. However, live cell imaging studies showed that GFP-GPI is found in the ciliary membrane of MDCK cells, but fixation can create the appearance of ciliary membrane exclusion [41]. Therefore, the nature and existence of lipid diffusion barrier at the base of cilia remains unclear.

1.1.4 Ciliary pocket or periciliary membrane

The region of the membrane present at the junction of the PM and ciliary membrane is called as periciliary membrane (Fig. 1.3). In cells such as fibroblasts or smooth muscle, the periciliary membrane appears as an invagination called as the ciliary pocket (CP). In these cells the cilium forms within the cell, by the fusion of a vesicle called the 'primary ciliary vesicle' with the appendages of the mother centriole. The axoneme growth takes place within this vesicle. This cilium emerges partially in the external environment through fusion with the PM, leaving tubular invagination of the PM at the base of the primary cilium. The CP appears to less extent in polarized epithelial cells, where cilia is formed on the apical membrane. In these cells the mother centriole docks directly at the PM, which could be the reason for the lack of PM invagination [42, 43]. Immunofluorescence, scanning electron microscope (SEM) and TEM studies disclosed that the CP is linked to the actin cytoskeleton and is enriched with dynamic clathrin-coated pits (well studied endocytic pits). However, knockdown studies reported that clathrin-mediated endocytosis is not

required for either the formation of cilia or CP. We predict that dynamic internalization of membrane proteins through clathrin-coated pits at the CP could be a mechanism for eliminating non-resident ciliary membrane proteins from cilia.

All these molecular evidence indicate the presence of a membrane diffusion barrier at the ciliary base. However, its mechanism and molecular components of the TFs, TZ, and CP remain uncharacterized.

Figure 1.3: TEM image of the cilium of serum-starved RPE1 cells

On the left: TEM image defining the organization of the ciliary pocket (CP), axoneme (Ax) and basal body [44] indicated by head-tail arrows. The cilium is denoted by an arrow head. On the right: the axoneme [45], basal body (red), PM (black) and ciliary pocket (blue) are highlighted using different colors [46].

1.2 Ciliary trafficking of proteins

1.2.1 Enrichment of specific proteins in the ciliary compartment

The ciliary membrane and intraciliary components are continuous with the PM and cytoplasm respectively [47]. Cilia are enriched with specific proteins, such as Hedgehog signalling components Gli2 (Glioblastoma), Gli3, Smoothened, Patched1 (Ptc1), PKD components cystin, polycystin-1 (PC-1), polycystin-2 (PC-2), polaris, and fibrocystin [8, 48-53]. Photoreceptor components such as rhodopsin, odorant receptors and components of the olfactory signalling cascade, such as adenylyl cyclase III (ACIII), G_{olf} and cyclic nucleotide-gated (CNG) channels, are present in cilia [54, 55]. Other receptors, such as SSTR3, Htr6, melanin concentrating hormone receptor1 (Mchr1) [4, 56], PDGFR α [5], dopamine receptor 1 [45] etc., are enriched in the primary cilia. Apart from proteins lipids such as ganglioside3 are highly concentrated

in cilia [57]. Proper functioning of cilia depends upon selective targeting of these receptors and signalling factors in the ciliary compartment. For example, binding of hedgehog (ligand) to Ptc1, causes Ptc1 to exit cilia and allows Smo to enter, consequently influencing the Gli2 transcription factor to proceed to the nucleus [8, 48, 50]. Similarly, retinal rod photoreceptors adapt to darkness when there is an increase in transducin (a G-protein activated by rhodopsin) and a decrease in arrestin (blocks transducin binding to rhodopsin) localization to outer segments of rods (specialized cilia of rod photoreceptors) [58]. However, the mechanism of trafficking of proteins to cilia is not well understood.

1.2.2 Trafficking of soluble proteins

Some soluble resident centriolar proteins reach the centrosome or basal body of cilia by diffusion. However, proteins such as centrin, pericentrin, γ -tubulin, PCM1 (pericentriolar material 1 protein) and Gli2 reach much faster than diffusion. These proteins reach the vicinity of the basal body ((-) end of the microtubule) by travelling on microtubules with help of the dynein–dynactin complex. The PCM1 is recruited at the centrosome by BBS4, an adaptor that binds to dynactin and PCM-1 [59, 60]. From the basal body, proteins enter cilia through different trafficking mechanisms, such as IFT [61-63].

The bidirectional movement of multiprotein complexes along the axonemal microtubule in cilia is termed IFT [64]. In vivo transport studies, as well as genetic studies, suggest that IFT is carried out by two subcomplexes, IFT-A and IFT-B [65]. Knockdown or knockout studies of the IFT-A subcomplex lead to the accumulation of proteins at ciliary tip, suggesting that IFT-A plays a role in retrograde transport. Depletion studies of IFT-B subcomplex showed that IFT-B causes defects in ciliogenesis, suggesting that IFT-B plays a role in the anterograde transport. The anterograde and retrograde trafficking of IFT transport depends on motor proteins kinesin (kinesin-II and KIF17) and dynein respectively. IFT transported cargos include radial spokes, dynein arms of the axoneme, and ciliary proteins such as opsin and arrestin [66, 67].

Using confocal microscopy, proteins of various molecular sizes, ranging from GFP (27 kDa), double GFP (54 kDa), to triple GFP (81 kDa), were shown to enter connecting cilia of *Xenopus* retinal rod photoreceptors [68, 69]. However, in hTERT

RPE1 cells, it was shown that fluorescently-labeled dextrans of 30 kDa diffused freely into and out of cilia, and those of 40 kDa failed to enter cilia [70]. This suggests that soluble proteins have a size limit for diffusion into cilia, similar to nuclear-cytoplasmic transport. Recently, it has been proposed by Kee et al. that the nucleocytoplasmic trafficking machinery is also involved in ciliary trafficking, and similar results are detailed in the following section [70].

1.2.3 Nuclear cytoplasmic transport

The nucleus segregates the genetic material and transcriptional machinery of a eukaryotic cell from the cytoplasm through the bi-layered nuclear membrane. In nucleocytoplasmic trafficking, proteins containing phenylalanine-glycine (FG) repeats at the centre of nuclear pores function as diffusion barriers to restrict the free movement of cargos into and out of nuclei [71]. The nuclear pore complex (NPC) is made of more than 30 different proteins called nucleoporins. NPCs allow the passive diffusion of proteins in a size-dependent manner [72-76].

1.2.3.1 Role of importin- β in nuclear trafficking

Soluble proteins of higher molecular weight are actively transported in and out of the nucleus by importin- β (Fig. 1.4)[77]. The importin- β family has more than 20 proteins which import and export cargo through the recognition of NLS (nuclear localization signal) and NES (nuclear export signal) respectively. Importin- β is organized into 20 HEAT repeats, with ~40 amino acids each. The structural feature of importin- β is that it contains conserved N-terminal Ran-GTP binding regions and C-terminal cargo binding regions [78]. Importin- β superfamily members, including importin- β 1 and importin- β 2/transportin-1 (TNPO1), are evolutionarily conserved nucleocytoplasmic trafficking receptors [79]. Importin- β 1, the first member of the family, works with the help of an adaptor protein importin- α to import cargos containing classical lysine-rich NLSs. However, it can also work by directly binding to cargo [80]. The weak and transient interactions between importin- β and FG-repeats facilitate the transport of the importin-cargo complex through the diffusion barrier at the NPC [73]. The directionality of this process is dependent on Ran-GTP gradient, which accumulates in the nucleus and causes the release of the cargo from importins, as well as the association of cargo to exportins (Fig. 1.4). The Ran-GTP gradient is maintained by the distribution of RCC1 (regulator of chromosome condensation 1)

and RanGAP (Ran GTPase activating protein). RCC1 is a Ran guanine nucleotide exchange factor (GEF) present in the nucleus, and it exchanges Ran-GDP with -GTP. RanGAP localizes to the cytosolic side of the NPC and activates the GTPase activity of Ran to hydrolyze GTP to GDP [78, 81].

Figure 1.4: Pictorial representation of nuclear import

1) The NLS of a cargo is recognised by importin- α , which in turn binds to importin- β , forming a cargo-importin- α - β complex. This complex helps the transport of cargo through NPC. 2) In the nucleus, the RCC1 converts Ran-GDP to Ran-GTP. The binding of Ran-GTP to importin- β causes the release of cargo-importin- α - β complex. 3) Importin- α , importin- β and Ran-GTP are recycled back to the cytoplasm, where Ran-GTP is converted into Ran-GDP by RanGAP (adapted from [82]).

TNPO1 recognizes PY-NLS sequence of the cargo and helps their transport [83]. Examples of proteins using the TNPO1 pathway include hnRNPs (heterogeneous nuclear ribonucleoproteins) such as A1, D, F, M and HuR. The most-studied NLS of TNPO1 binding is a 38 amino acid sequence present in the acidic loop

of hnRNPA1, termed as the M9 sequence. Apart from PY-NLS, TNPO1 also interacts with sequences that are highly basic [84-86]. Previous studies for characterizing NLS of TNPO1, using TNPO1 binding sequences of various cargo, suggest that NLS is structurally disordered, diverse and basic or hydrophobic [87].

1.2.3.2 Transport of inner nuclear membrane proteins

The outer nuclear envelope membrane (ONM) is continuous with the endoplasmic reticulum (ER). The ONM is connected to the inner nuclear envelope membrane (INM) at the region where NPCs are incorporated. Similar to cilia, the INM has a unique membrane protein composition which is maintained by either or the combination of the following two mechanisms. In the diffusion-retention mechanism, the chromatin and/or lamin binds to most INM proteins, and, therefore, immobilizes them at the INM [75, 88, 89]. In selective entry mechanism, NPCs assemble at the boundary between the INM and ONM and impose membrane diffusion barriers that allow selective entry of resident INM cargo [90].

1.2.4 Nucleocytoplasmic trafficking machinery involved in ciliary trafficking

Interestingly, nuclear transport proteins importin- β 1 and - β 2 are shown to localize in cilia [91-93]. TNPO1 interacts with KIF17 in a Ran-dependent manner; and with RP2 (retinal pigmentosa protein-2) in a Ran-independent manner [92, 94]. TNPO1 binds to KIF17 through CLS (ciliary localization signal), which is similar to NLS, and traffic it to cilia. Using domain swapping of CLS of KIF17 with importin- β 1, TNPO1 binding sequences or non-ciliary kinesin [94] showed that TNPO1 binding is necessary for KIF17 cilia localization. However, the fluorescent-tagged CLS motif of KIF17 (KRKK) is absent from cilia, indicating the requirement of additional sequences in kinesin for cilia localization. Taken together, either NLS or CLS with a kinesin motor domain can localize to cilia instead of the nucleus [94]. RP2 ciliary localization is dependent on the binding of TNPO1 to the M9-like sequence, which supports the role of NLS and TNPO1 [92]. Similarly, Crumbs3-CLPI (a protein that regulates ciliogenesis) interacts with importin- β 1 in a Ran-dependent manner; and the depletion of importin- β 1 abolishes its ciliary localization [91]. Using subcellular fractionation, immunofluorescence and electron microscopic studies, another important component of the nucleocytoplasmic trafficking machinery, Ran-

GTP, was shown to localize to basal bodies and cilia [36, 94]. RanBP1 (Ran-binding protein 1), which indirectly regulates Ran by stimulating RanGAP and inhibiting RCC1 activity, was also shown to localize to cilia [36]. Interestingly, the accumulation of RanG19V, a GTP-locked form (constitutively active), or the knockdown of RanBP1, can cause KIF17 to exit cilia, whereas the Ran-GDP has no effect [94]. This shows that the levels of Ran-GTP at cilia and centrosome may play a role in ciliary trafficking. Thus, ciliary trafficking could have similarities to nuclear transport [92, 94].

1.2.5 Trafficking of membrane proteins to cilia

The transport of integral membrane proteins from their sites of synthesis to their destination occurs in membrane-bound vesicles. It is a highly regulated process involving several different pathways, of which the secretory pathway is the best studied. In secretory pathway, an ER-synthesized protein passes through the Golgi and is sorted in membrane-bound vesicles at the trans-Golgi network (TGN) before reaching its destination. By default, most TGN sorted proteins reach the PM. Targeting of proteins to their specified destination depends upon distinct sorting signals and multiprotein complexes. Studies on some cilia localized transmembrane proteins revealed the presence of specialized targeting sequences that help in the targeting of the cargo to cilia. Such sequences are classified as ciliary targeting sequences (CTS) (Table 1). CTSs of transmembrane proteins facilitate in recruiting the trafficking machinery, which in turn guides the proteins to cilia. VxPx is a CTS motif of rhodopsin [44]. Similar to rhodopsin, PC-1, -2 and CNGB1 each has a VxPx CTS motif [95-97]. In GPCRs such as SSTR3, HTR6, and MCHR1, an AxxQ sequence in the 3rd intracellular loop was identified as the CTS [98]. Though a VxPx motif is present in these GPCRs, it was not verified experimentally. Moreover, the VxPx motif at the N-terminal of INPP5E was shown to be unnecessary for ciliary localization [99]. This suggests that there is no consensus in the CTSs of cilia localized proteins. Thus, the diversity of CTS sequences can lead to the involvement of multiple trafficking pathways, making ciliary transport a highly complex process.

Table 1: Summary of known CTSs

type	ciliary protein	sequence	role in ciliary localization
transmembrane	rhodopsin (human)	...FR...TTICCGKN...QVAPA-c	C-terminal 44 amino acids are sufficient and necessary [100].
	fibrocystin (mouse)	CLVCCWFKKSKTRK IKP.....-c	amino acids 1-15 in the C-terminal immediate to transmembrane domain are sufficient and necessary [101].
	peripherin (frog)	KSSWELVKSMGKLNKVE TAG....-c	amino acids 317–326 are sufficient in the presence of membrane targeting sequence [102].
	PC-1 (human)	KVHPSST-c	C-terminal 7 amino acids are sufficient when tagged to membrane targeting sequence [97].
	SSTR3 (mouse)	KVRSTTRRVRAPSCQW VQAPACQRRRSERRVTR....	3 rd intracellular loop (i3) amino acids 231-266 are necessary and sufficient when tagged to transmembrane [98].
	PC-2 (human)	n-MVNSSRVQPQPPGDA	N-terminal amino acids 1-15 are necessary and sufficient [95].
	neuropeptide Y receptor type 2 (NPY2R) (human)	RLW.....	3 rd intracellular loop (i3) amino acids are necessary [103].
	ODR-10 (<i>Caenorhabditis elegans</i>)	FR.....	amino acids in the C-terminal half are necessary [104].
	CNGβ1b [105]	RVSP.....	C-terminal amino acids are necessary [96].
peripheral	cystin (mouse)	n-...TASEGGTA....	amino acids 28–35 sufficient when fused to exogenous myristoylation sequence [106].
	retinol dehydrogenase (cattle)	LRCLACSCFRTPVWPR-c	C-terminal 16 amino acids are sufficient [105].
	RP2 (human)	n-MGCFFSKRRKADKES.....	N-terminal 1-15 amino acids are sufficient [92].
	NPHP3 (mouse)	n-MGTASSLVSP TG...amino acids 96–201.....	N-terminal amino acids 96–201 containing coiled-coil domain and the myristoylation site are necessary and sufficient for cilia targeting, VxP motifs are not necessary for cilia localization [107].
	INPP5E (human)	FDRELYL.....CSVs- c	C-terminal 66 amino acids are necessary [99].
cytoplasmic	Gli2, (human)	852–1183 amino acids.....	amino acids in central region are necessary but not sufficient [108].
	Kif17 (human)	KRKK.....-c	C-terminal 228 amino acids are necessary and sufficient to target non-ciliary kinesin [94].

The residues in blue are necessary, and the residues in green are presumptive palmitoylation, myristoylation or farnesylation sites. Dots indicates the presence of amino acids but not proportional to their number. If the sequence is near to the N-terminal it is indicated as ‘n-’ and if it is near to the C-terminal it is indicated as ‘c’.

For ciliary targeting, the membrane proteins follow one of two pathways after being sorted into membrane-bound vesicles at the Golgi [40]. Two pathways are known for the ciliary targeting of membrane cargos: 1) polarized exocytosis transport pathway 2) lateral transport pathway [40, 109]. In the polarized exocytosis pathway, a membrane protein is packed into a vesicle derived from either the secretory pathway or the endocytic pathway. The vesicle specifically fuses to the PM near the ciliary

base (periciliary membrane) and the membrane protein subsequently enters cilia (Fig. 1.5) [110].

1.2.5.1 Trafficking of secreted proteins from the Golgi directly to cilia

In general, for a membrane protein to traffic to the cilia, it must first be sorted into vesicles using signals. A well-studied example is the sorting of rhodopsin from the Golgi to cilia. VxPx of rhodopsin, binds to the active form of Arf4, a small GTPase, and Tctex-1, a subunit of the cytoplasmic dynein light chain [44, 111]. Arf4, with the help of a ternary protein complex, containing ASAP1, Rab11 and FIP3, regulates ciliary targeting. Similar to rhodopsin, PC-1 was shown to depend on the ASAP1 and Rab11 pathways [97]. It was shown that the BBS proteins form a complex called BBSome that interacts with ciliary proteins by binding to their CTSs. The BBSome complex is membrane-anchored via a small GTPase Arl6. The ciliary localization of GPCRs such as SSTR3, HTR6, MCHR1 and NPY2R depends on the BBSome complex proteins [112]. Though the mechanism of BBSome function is uncertain, it was anticipated that BBSome either coats vesicles carrying cilia-targeting proteins based on their analogy to COPI, COPII and clathrin-coated pits or it acts a planar coat and clusters cargos into a patch that enter cilia laterally from the PM [40, 113].

1.2.5.2 Trafficking of proteins to cilia via endocytic-recycling pathway

Recycling is defined as the inter-organelle movement of a molecule followed by its return to the original site, without intervening metabolic processing [114]. Recycling vesicles are heterogeneous tubular vesicles concentrated around the MTOC [115]. To support the role of recycling vesicles in ciliary targeting, several recycling pathway components such as the exocyst complex protein (Sec10) and TRAPP II (transport protein particle II) complex, Rab17, TBC1D7 (Rab 17 GAP), Evi5-like (Rab23 GAP), Rab8, Rabin8, and Rab11 were shown to be involved in cilia assembly [116-122]. Though many of these proteins are shown to be involved in ciliogenesis, their roles are not clear.

1.2.5.3 Trafficking of vesicles to cilia via lateral transport

In lateral transport, proteins can cross the ciliary barrier via diffusion from the PM to cilia (Fig 1.5). A known example is Smo, a seven-pass transmembrane protein involved in Shh [123]. To show that Smo is transported into cilia through lateral

diffusion from the PM, the surface and internal pools of SNAP-tagged Smo were labeled using either non-fluorescent or fluorescent SNAP substrates. Upon addition of Shh ligand, the initial pathway activated for ciliary transport of Smo was lateral transport, later the internal pool translocates to cilia through the recycling pathway [123]. Lateral transport has been reported to play a role in the transport of agglutinins to flagella in *Chlamydomonas reinhardtii*. To study this, the PM pool of non-flagellated *Chlamydomonas reinhardtii* was iodinated and then followed by the induction of flagella. It was found that the newly formed flagella had iodinated agglutinins [124]. This indicates that transmembrane proteins could be transported to cilia through the lateral transport pathway.

Figure 1.5: Routes for trafficking of membrane proteins to ciliary base

1) Polarized exocytosis pathway: 1a) Transmembrane protein carrying vesicle secreted from Golgi or endosomes could directly traffic to cilia base and fuse. 1b) Protein after reaching PM can be

internalized via recycling pathway and fuse at ciliary base. 2) Protein after reaching PM can diffuse laterally to ciliary membrane without vesicle fission and fusion [123].

1.2.6 Retention and exclusion of ciliary membrane proteins

Similar to INM proteins, ciliary membrane proteins can diffuse or be actively transported to cilia; however, their localization within cilia depends upon the retention of proteins by interactions in cilia. Domains/motifs that directly or indirectly bind to axonemal microtubule could serve as CTSs for ciliary retention of cargos. For example, when GFP-CEACAM1 is conjugated to tau (a tubulin binding protein), the resulting construct has a much higher concentration within cilia. This shows that proteins can be selectively retained in cilia by interaction with axoneme [41] (Fig. 1.6). BBSome could be the receptor for SSTR3 via binding to its CTS, which is the 3rd intracellular loop region (SSTR3i3) [113] and the ciliary localization of membrane protein smoothened and Crumbs could be attributed to their indirect binding to microtubules via KIF3a [125, 126].

Figure 1.6: Retention and exclusion of ciliary proteins

Soluble and membrane proteins are hypothesized to be retained inside the cilia either by directly or indirectly binding to microtubule linker. Proteins can be selectively excluded from cilia; for example, podocalyxin (blue) is excluded from the periciliary membrane domain by binding to cortical actin filaments below the PM [109].

Some proteins are selectively excluded from the cilia via crosslinking with either themselves or other proteins. Syntaxin3, a type IV transmembrane protein, does not

localize to the outer segment of photoreceptors (equivalent to primary cilia). However, deletion of the SNARE domain of Syntaxin3 results in the localization of Syntaxin-3-Δ-SNARE to the outer segment. It was also reported that GFP-CAAX could localize to cilia. However, the addition of the SNARE domain prevents it from localizing to cilia [127]. Podocalyxin is another example of a transmembrane protein excluded from the cilia due to its interaction with the actin cytoskeleton [41]. Another example is Glut1, a multi-pass transmembrane protein, which is excluded from the cilia due to the PDZ motif present in its cytoplasmic tail [128].

1.3. Rab8 and its functions

In vesicular trafficking, proper delivery of cargo to their destinations depends on small GTPases. Members of Rab and Arf family small GTPases are known regulators in membrane trafficking [129]. A small GTPase generally exists in two interchangeable forms: GTP and GDP-bound forms. The GTP and GDP forms of small GTPases are dependent on GEFs and GTPase activating proteins (GAPs). Upon receiving upstream signals, GEFs promote the exchange of GDP for GTP. A GAP activates the GTPase activity of small GTPase [130, 131]. Rab GTPases are shown to be involved in maintaining membrane identity, budding of vesicles, transport as well as their fusion to the destination. They perform these functions through the recruitment of effectors, such as adaptor proteins for sorting, motor proteins for transport and tethering factors for fusion. The vesicular trafficking is regulated spatially, through the crosstalk between different Rabs connected through their effectors [132, 133]. Among the Rabs tested, only Rab8 show cilia localization [63, 118, 121]. Previous studies showed that Rab8 is involved in membrane trafficking, cell migration, neuron differentiation and maintaining epithelial cell polarity. Immunofluorescence studies reported that Rab8 localizes to macropinosomes, centrosome, cilia and vesicles at the leading edge of cells [134, 135]. Rab8 has two GEFs: RPGR (retinitis pigmentosa GTPase regulator) and Rabin8 [135, 136]. Overexpression of Rab8-Q67L (a constitutively active form of Rab8) causes cytoskeletal reorganization and induces formation of the membrane protrusions, such as filopodia and lamellipodia. On the other hand, depletion of Rab8 or overexpression of Rab8-T22N (dominant negative form of Rab8) has opposite effect, i.e., increase in cell-cell adhesion and formation of actin stress fibers. Rab8-GTP localizes to cargo

sorted post-Golgi vesicles and mediates their transport to the PM [137, 138]. Among the Rab8 GEFs, RPGR localizes to cilia and Rabin8 localizes to the ciliary base. The N-terminus of RPGR contains an RCC1 (Ran-GTPase)-like domain, which interacts with Rab8 to exchange GDP for GTP. RPGR was shown to play a role in vesicular trafficking in photoreceptor cilia by regulating entry and retention of proteins [139]. Rabin8 localization to the ciliary base is transient and depends on cilia formation. Biochemical and immunofluorescence studies reported that Rab11-GTP interacts with Rabin8 and stimulates its GEF activity. Since Rab8 activation depends on Rabin8, which in turn depends on Rab11, it was proposed that Rab8/Rabin8/Rab11 together forms a complex [117, 140]. Similar to the non-ciliary roles of Rab8, overexpression Rab8-Q67L in RPE1 cells resulted in elongation of cilia, particularly the ciliary membrane; whereas Rab8-T22N had the opposite effect [63]. Moreover, FRAP and immunofluorescence studies demonstrated that overexpression of Rab8-T22N causes defects in the trafficking of ciliary membrane proteins such as Smo, kidney injury molecule-1, and fibrocystin [101, 141]. Consistent with the role of Rab8 in the recycling pathway, overexpression of Rab8-T22N in photoreceptor rod cells causes accumulation of rhodopsin-bearing vesicles at the ciliary base [142]. Interaction with Rab8 is essential for the basal body and cilia localization of cenexin-3/ODF2, a centriole appendage protein shown to be indispensable for ciliogenesis [143, 144]. CEP164, a centriolar appendage protein localized to the TZ zone, is essential for ciliogenesis and plays an important role in localizing Rabin8 to the centrosome, Rabin8, in turn, converts Rab8-GDP to Rab8-GTP [145, 146]. The Rabin8-Rab8 pathway is involved in vesicle trafficking through the exocyst complex (sec15 interacts with Rab8 and Rabin8), TRAPPII vesicle tethering complex and BBSome complex proteins (Rab8 interacts with BBSome through Rabin8) [63, 117, 120, 147]. Rab8 interacts with Rabaptin5, a regulator of endocytosis. Rabaptin5, in turn, interacts with Elipsa/IFT54, which is an IFT polypeptide. This establishes a link between Rab8 involved in the transport of membrane protein complex to cilia and IFT involved in the transport of membrane proteins within cilia [118]. Thus, Rab8 together with its effectors could regulate ciliogenesis, vesicle transport to the ciliary base, vesicle coat formation and tethering of vesicle to the ciliary base.

1.4 Ciliopathies

Heterogeneous groups of disorders, caused by mutations in cilium-centrosome complex localized proteins, are collectively termed as ciliopathies. They include Primary ciliary dyskinesia (PCD), Leber congenital amaurosis, polycystic kidney disease (PKD), Jeune asphyxiating thoracic dystrophy (JATD), Ellis-van Creveld syndrome [148], Oral-facial-digital syndrome type I (OFD1), NPHP, Joubert syndrome (JBTS), Bardet-Biedl syndrome (BBS), MKS, and Alström syndrome (ALMS) [149]. Since cilia are present in different cell types, ciliary defects affect a wide variety of tissues and organs. Patients with the above mentioned disorders can have one or combinations of phenotypes, such as polydactyly, infertility, cysts in the kidney, liver or pancreas, defects in the central nervous system, malformation of bones, mental retardation, retinal degeneration, obesity, genital malformations, craniofacial malformations, hyperphagia, ataxia and aneurysms [149-151].

1.4.1 Autosomal recessive polycystic kidney disease (ARPKD)

ARPKD is characterized by dilation and elongation of collecting ducts, along with interstitial fibrosis, which results in crowdedness and enlargement of kidneys and impairment of their functions. About 30-50% of infants affected with this disease eventually die at an early age. The disease is caused by mutations in the fibrocystin/PKHD1 (polycystic kidney and hepatic disease 1) gene [152].

1.4.2 Fibrocystin

The PKHD1 gene encodes one of the longest proteins in the human proteome. It has many splice variants with the longest splice variant of 4074 amino acids (447 kDa). It is a type I transmembrane protein, also known as fibrocystin [152]. More than 135 mutations that cause ARPKD disease have been identified. An immunofluorescence analysis of fibrocystin in MDCK cells showed cilia staining [51, 153]. Another study using knockdown and ARPKD patient samples showed that fibrocystin localizes to mitotic spindles, and plays an important role in maintaining spindle polarity and centrosome amplification [154]. Furthermore, knockdown of fibrocystin by siRNA in isolated intrahepatic bile ducts (IBDUs), or the disruption of the PKHD1 gene due to germline mutations in PKD, resulted in abnormalities of cilia morphology in rats [155]. This indicates that ARPKD arises due to dysfunction of cilia. The ciliary localization of fibrocystin is dependent on the 15 amino acid residues

immediate to the transmembrane domain in its cytoplasmic tail. These residues are necessary and sufficient for ciliary targeting of fibrocystin, in a Rab8-dependent manner [101]. Fibrocystin interacts indirectly with PC-2 (a protein involved in ADPKD) through KIF3B and regulates its functions [156]. Furthermore, immunofluorescence and knockdown studies showed that fibrocystin regulates calcium responses in the kidney epithelium similar to other PKD proteins PC-1 and PC-2 [157]. Cytosolic calcium concentration and activation of protein kinase C controls the proteolytic cleavage of fibrocystin releasing various fragments. Of these, the C-terminus containing NLS region localizes to the nucleus. From similarities with other proteins, it was suggested that fibrocystin could play a role in signalling [158]. Knockdown and overexpression studies show that fibrocystin negatively regulates cell proliferation and positively regulates apoptosis, consistent with its function as a defective gene in ARPKD, as the formation of cysts occurs due to abnormal proliferation and apoptosis of cells [159]. Arf4, a protein involved in ciliary trafficking, interacts with fibrocystin-CTS, but has no effect on its ciliary localization [101, 160]. This indicates that fibrocystin is important for proper cell proliferation, apoptosis and cilia function.

1.6 Objectives:

1. The nature of the diffusion barrier in the transport of membrane proteins remains uncharacterized. In this study we attempt to explore the mechanism on how it works.
2. A few recent studies have revealed a role of importins, which are nucleocytoplasmic trafficking factors, in targeting resident soluble cilia proteins. Defining the role of importins in trafficking of ciliary membrane proteins is the other aim of this study.
3. Fibrocystin is an ARPKD protein localized to cilia in Rab8 dependent manner. In this study, we showed that TNPO1 is essential for ciliary targeting of fibrocystin. Exploring the relation between Rab8 and TNPO1 in ciliary trafficking of fibrocystin is the 3rd aim of this study.

CHAPTER 2: Materials and Methods

2.1a General reagents and chemicals

The general reagents and chemicals used in this study and their corresponding sources are listed in alphabetical order in Table 2.

Table 2: List of reagents

Reagents	Source
Agarose	Bio-Rad
Ampicillin	Sigma Aldrich
Bradford Reagent	Sigma-Aldrich
Brefeldin A (BFA)	Merck
CO₂-independent medium	Life technologies
Coomassie Brilliant Blue	Bio-Rad
Deoxyribonucleotides (dNTPs)	New England Biolabs
Dithiothreitol (DTT)	Sigma-Aldrich
DMEM / F-12 (1:1 Mix of DMEM and Ham's F-12)	Life technologies
DNA ladder – 100bp	New England Biolabs
DNA ladder – 1kb	New England Biolabs
DNaseI	Sigma-Aldrich
Dulbecco's Modified Eagle Medium (DMEM), high glucose	Life technologies
Ethidium bromide	Bio-Rad
Ethylenediaminetetraacetic acid	Sigma Aldrich
Fetal bovine serum (FBS)	Hyclone
Geneticin ® Reagent (G418)	Invitrogen
Glutathione Sepharose™ 4B	GE Healthcare Bio-Sciences
Hoescht 33342	Invitrogen
Imidazole	Sigma Aldrich
Kanamycin	Sigma Aldrich
L-Glutamine	Life technologies
Luria Broth (LB) medium	Affymetrix
Ni-NTA beads	QIAGEN
Opti-MEM	Invitrogen
Paraformaldehyde (PFA)	Sigma Aldrich

Poly L- lysine	Sigma Aldrich
Polyethylenimine [PEI]	Polysciences
Polybrene	sigma
Polyvinyl difluoride	Bio-Rad
Protease inhibitor cocktail tablets (Complete EDTA free)	Roche
Protein Ladder (Precision Plus Protein™ Dual Color)	Bio-Rad
PuroMycin dihydrochloride	Santa Cruz
SnakeSkin™ Dialysis Tubing	Thermo scientific
Sodium bicarbonate	Sigma Aldrich
Sodium Dodecyl Sulfate (SDS)	Biorad
Sodium chloride	Sigma Aldrich
Sodium orthovanadate	Sigma Aldrich
TRIS- HCL	Sigma Aldrich
Triton ® X-100	Merck
Trypsin	Gibco
Tween ® 20	Sigma Aldrich
Wester Bright™ Quantum	advansta
β-mercaptoethanol	Merck

2.1b Recipes of buffers used

The composition of buffers used is listed in alphabetical order in Table 3.

Table 3: Recipe of buffers

Buffer	Composition
4X SDS sample buffer	250 mM Tris-Cl (pH 6.8), 400 mM DTT, 8% SDS, 0.4% bromophenol blue, 40% glycerol.
Acid wash buffer for Immunofluorescence	0.2 M glacial acetic acid, 0.5 M NaCl in PBS pH 2.
GST lysis buffer	1% Triton X-100, 50 mM Tris pH 8, 150 mM NaCl buffer, 5 mM MgCl ₂ , 10 μM DNaseI, 0.2 M lysozyme, 1mM PMSF and 1mM DTT.
Immunoprecipitation lysis buffer	1% Triton X-100, 50 mM HEPES pH 7.3, 150 mM NaCl buffer, 1mM PMSF, protease inhibitors tablet and 1 mM DTT.
Phosphate buffer saline (PBS)	137 mM NaCl, 2.7 mM KCl, 10 mM Na ₂ HPO ₄ , 1.8 mM KH ₂ PO ₄ , and MgCl ₂ 0.5 mM (optional). Adjust pH to 7.4.
Phosphate buffer saline with Tween ® 20 (PBST)	PBS with 0.1% Tween ® 20
Pull-down lysis buffer	0-1% Triton X-100, 50 mM Tris pH 7.3, 150 mM NaCl buffer, 1 mM PMSF, protease inhibitors tablet and 1 mM DTT.

RIPA buffer	50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS) and protease inhibitors tablet.
SDS running buffer	25 mM Tris and 190 mM glycine, 0.1% SDS.
Transfer buffer	25 mM Tris, 190 mM glycine and 10% methanol.
His-tag lysis buffer	100 mM Hepes pH7.3, 250 mM KCl, 5 mM MgCl ₂ , 10 µM DNaseI, 0.2 M lysozyme, 1mM PMSF and 1 mM DTT.
His-tag elution buffer	20 mM Hepes pH7.3, 250 mM KCl, 250 mM imidazole, 10% glycerol and 1 mM DTT.
His-denature lysis buffer	8 M urea in PBS.
Ponceau S staining buffer	0.2% (w/v) Ponceau S and 5% glacial acetic acid.

2.2 Mammalian expression constructs

2.2.1: GFP vector constructs

- **pEGFP×2-N1 vector**: The CDS of GFP was amplified by PCR using primers containing SalI/BamHI sites and ligated into SalI/BamHI digested pEGFP-N1 vector.
- **pEGFP×3-N1 vector**: The CDS of GFP was amplified by PCR using primers containing EcoRI/SalI sites and ligated with EcoRI/SalI digested pEGFP×2 vector.

2.2.2 CD8a constructs

2.2.2a Untagged CD8a constructs

- **CD8a**: The coding sequence (CDS) of CD8a [161] was amplified by PCR using primers containing EcoRI/XbaI sites and ligated into EcoRI/XbaI digested pCI-neo vector (Promega).
- **CD8a-furin**: Two PCR amplifications were performed. First PCR was performed using CDS of CD8a as template and CD8a-GFP-F/CD8a-furin-R as primers, second PCR using furin (IMAGE: 6579931; GenBank Acc No.: BU845991) as template and furin-F/furin-XbaI-R as primers. The two PCR fragments were purified, mixed and subjected to second round of PCR amplification using primers CD8a-GFP-F/Furin-XbaI-R as primers. The resulting PCR product was digested by XhoI/EcoRI and ligated into XhoI/EcoRI digested pCI-neo vector.
- **CD8a-CI-M6PR**: Two PCR amplifications were performed. First PCR was performed using CDS of CD8a as template and CD8a-GFP-F/CD8a-CI-M6PR-R as

primers, second PCR using CI-M6PR (IMAGE: 6722707; GenBank Acc No.: CA455182) as template and CI-M6PR-F/CI-M6PR-XbaI-R as primers. The two PCR fragments were purified, mixed and subjected to second round of PCR amplification using primers CD8a-GFP-F/CI-M6PR-XbaI-R as primers. The resulting PCR product was digested by XhoI/EcoRI and ligated into XhoI/EcoRI digested pCI-neo vector.

- **CD8a-CD-M6PR**: Two PCR amplifications were performed. First PCR was performed using CDS of CD8a as template and CD8a-GFP-F/CD8a-CD-M6PR-R as primers, second PCR using CD-M6PR (IMAGE: 3530887; GenBank Acc No.: BC024206) as template and CD-M6PR-F/CD-M6PR-XbaI-R as primers. The two PCR fragments were purified, mixed and subjected to second round of PCR amplification using primers CD8a-GFP-F/CD-M6PR-XbaI-R as primers. The resulting PCR product was digested by XhoI/EcoRI and ligated into XhoI/EcoRI digested pCI-neo vector.

- **CD8a-sortilin**: Two PCR amplifications were performed. First PCR was performed using CDS of CD8a as template and CD8a-GFP-F/CD8a-sortilin-R as primers, second PCR using sortilin (IMAGE: 6110988; GenBank Acc No.: BU196276) as template and sortilin-F/sortilin-XbaI-R as primers. The two PCR fragments were purified, mixed and subjected to second round of PCR amplification using primers CD8a-GFP-F/Sortilin-XbaI-R as primers. The resulting PCR product was digested by XhoI/EcoRI and ligated into XhoI/EcoRI digested pCI-neo vector.

2.2.2b GFP-tagged CD8a constructs

- **CD8a-GFP**: The CDS of CD8a was amplified by PCR using primers containing XhoI/EcoRI sites and ligated into XhoI/EcoRI digested pEGFP-N1 vector (Clontech).

- **CD8a-GFP×2**: The CDS of CD8a was released by digesting CD8a-GFP with XhoI/EcoRI and ligated into XhoI/EcoRI digested pEGFP×2-N1 vector.

- **CD8aGFP×3**: The CDS of CD8a was released by digesting CD8a-GFP with XhoI/EcoRI and ligated into XhoI/EcoRI digested pEGFP×3-N1 vector.

- **CD8a-NLS-GFP**: The CDS of CD8a was PCR amplified using CD8a-GFP as a template and the following oligonucleotides as primers CD8a-GFP-F/CD8aNLS-GFP-R. The resulting fragment was digested by XhoI/EcoRI and ligated to XhoI/EcoRI digested pEGFP-N1 vector.

- **CD8a-NLS-GFP×2**: The CD8a-NLS was released by digesting CD8a-NLS-GFP with XhoI/EcoRI and ligated into XhoI/EcoRI digested pEGFP×2-N1 vector.
- **CD8a-NLS-GFP×3**: The CD8a-NLS was released by digesting CD8a-NLS-GFP with XhoI/EcoRI and ligated into XhoI/EcoRI digested pEGFP×3-N1 vector.
- **CD8a-IBB-GFP**: The CDS of CD8a-IBB was PCR amplified using CD8a-IBB pCIneo as template and the following oligonucleotides CD8a-GFP-F/CD8aIBB-GFP-R as primers and the resulting fragment was digested by XhoI/EcoRI and ligated to XhoI/EcoRI digested pEGFP-N1 vector.
- **CD8a-IBB-GFP×2**: The CDS of CD8a-IBB was released by digesting CD8a-IBB-GFP with XhoI/EcoRI and ligated into XhoI/EcoRI digested pEGFP×2-N1 vector.
- **CD8a-IBB-GFP×3**: The CDS of CD8a-IBB was released by digesting CD8a-IBB-GFP with XhoI/EcoRI and ligated into XhoI/EcoRI digested pEGFP×3-N1 vector.
- **CD8a-hnRNPM-GFP**: The CDS of CD8a was PCR amplified using CD8a-GFP as template and hnRNPM was added stepwise using the following oligonucleotides as primers CD8a-GFP-F/CD8a-hnRNPM-GFP-R1. The first PCR fragment was purified and re-amplified with CD8a-GFP-F/CD8a-hnRNPM-GFP-R2. The second PCR product was purified and amplified with CD8a-GFP-F/CD8a-hnRNPM-GFP-R3. The resulting PCR product was digested by XhoI/EcoRI and ligated into XhoI/EcoRI digested pEGFP-N1 vector.
- **CD8a-hnRNPM-GFP×3**: The CDS of CD8a-hnRNPM was released by digesting CD8a-hnRNPM-GFP with XhoI/EcoRI and ligated into XhoI/EcoRI digested pEGFP×3-N1 vector.
- **CD8a-CD4-GFP**: Two PCR amplifications were performed. First PCR was performed using CD8a as template and CD8a-GFP-F/CD8a-CD4-R1 as primers, second PCR using CD4 (addgene plasmid: 51604) as template and CD8a-CD4-F2/CD8a-CD4-R2 as primers. The two PCR fragments were purified, mixed and subjected to second round of PCR amplification using CD8a-GFP-F/CD8a-CD4-R2 as primers. The resulting PCR product was digested by XhoI/EcoRI and ligated into XhoI/EcoRI digested pEGFP-N1 vector.
- **CD8a-VSVG-GFP**: Two PCR amplifications were performed. First PCR was performed using CD8a as template and CD8a-GFP-F/CD8a-VSVG-R1 as primers, second PCR using VSVG as template and CD8a-VSVG-F2/CD8a-VSVG-R2 as primers. The two PCR fragments were purified, mixed and subjected to second round of PCR amplification using CD8a-GFP-F/CD8a-VSVG-R2 as primers. The resulting

PCR product was digested by XhoI/EcoRI and ligated into XhoI/EcoRI digested pEGFP-N1 vector.

2.2.3 Fibrocystin constructs

2.2.3a GFP-tagged fibrocystin constructs

- **f-CTS-GFP**: The oligonucleotides f-CTS-GFP-F/f-CTS-GFP-R were annealed and ligated into EcoRI/BamHI digested f-CTS-pEGFP-N1 vector.
- **f-CTS-(KTRK)-GFP**: The CDS of f-CTS-KTRK was PCR amplified using f-CTS-GFP as template and the following oligonucleotides as primers f-CTS(KTRK)-GFP-F/f-CTS(KTRK)-GFP-R and the resulting fragment was digested with EcoRI/BamHI and ligated into EcoRI/BamHI digested pEGFP-N1 vector.
- **CD8a-f-CTS-GFP**: The CDS of CD8a-f-CTS was PCR amplified using primer pair CD8a-f-CTS-GFP-F/ CD8a-f-CTS-GFP-R. The resulting PCR product was digested by EcoRI/BamHI and ligated into EcoRI/BamHI digested pEGFP-N3 vector.
- **CD8a-CTS-(KTRK)-GFP**: The CDS of CD8a was amplified by PCR using primers CD8a-GFP-F/CD8a-f-CTS(KTRK)-GFP-R. The resulting PCR product was digested by NheI/EcoRI and ligated into NheI/EcoRI digested f-CTS-pEGFP-N1 vector.
- **CFFΔC-GFP**: The CDS of CD8a was PCR amplified using CD8a-GFP as template and transmembrane and fibrocystin CTS was added stepwise using the following oligonucleotides as primers CD8a-GFP-F/CFFΔC-GFP-R1. The first PCR fragment was purified and re-amplified with CD8a-GFP-F/CFFΔC-GFP-R2. The second PCR product was re-purified and amplified with CD8a-GFP-F/CFFΔC-GFP-R3. The resulting PCR product was digested by XhoI/EcoRI and ligated into XhoI/EcoRI digested pEGFP-N1 vector.
- **CFF-GFP**: The extracellular domain of CD8a and transmembrane domain of fibrocystin was PCR amplified using CFFΔC-GFP as template and following oligonucleotides as primers CD8a-GFP-F/CFF-GFP-R1. The cytosolic domain of fibrocystin was PCR amplified using an IMAGE clone (Genbank Accession No.:4238864) as template and the following oligonucleotides as primers CFF-GFP-F2/CFF-R. The mixture of two PCR fragments was subjected to PCR amplification by primers CD8a-GFP-F/CFF-R and resulting fragment were digested by XhoI/EcoRI and ligated into XhoI/EcoRI digested pEGFP-N1 vector.

- **CFF-(LV)-GFP**: Two PCR amplifications were performed by using CFF-GFP as template. PCR1 was performed using CD8a-GFP-F/CFF-LV-R, PCR2 using CFF-LV-F/pEGFPN1-R. The two PCR fragments were purified, mixed and subjected to second round of PCR amplification using primers CD8a-GFP-F/CFF-R. The resulting PCR product was digested by XhoI/EcoRI and ligated into XhoI/EcoRI digested pEGFP-N1 vector.
- **CFF-(WF)-GFP**: Two PCR amplifications were performed by using CFF-GFP as template. PCR1 was performed using CD8a-GFP-F/CFF-WF-R, PCR2 using CFF-WF-F/pEGFPN1-R. The two PCR fragments were purified, mixed and subjected to second round of PCR amplification using primers CD8a-GFP-F/CFF-R. The resulting PCR product was digested by XhoI/EcoRI and ligated into XhoI/EcoRI digested pEGFP-N1 vector.
- **CFF-(CCC)-GFP**: Two PCR amplifications were performed by using CFF-GFP as template. PCR1 was performed using CD8a-GFP-F/CFF-CCC-R, PCR2 using CFF-CCC-F/pEGFPN1-R as primers. The two PCR fragments were purified, mixed and subjected to second round of PCR amplification using primers CD8a-GFP-F/CFF-R. The resulting PCR product was digested by XhoI/EcoRI and ligated into XhoI/EcoRI digested pEGFP-N1 vector.
- **CFF-(KKS)-GFP**: Two PCR amplifications were performed by using CFF-GFP as template. PCR1 was performed using CD8a-GFP-F/CFF-KKS-R, PCR2 using CFF-KKS-GFP-F2/pEGFPN1-R. The two PCR fragments were purified, mixed and subjected to second round of PCR amplification using primers CD8a-GFP-F/CFF-R. The resulting PCR product was digested by XhoI/EcoRI and ligated into XhoI/EcoRI digested pEGFP-N1 vector.
- **CFF-(KTRK)-GFP**: Two PCR amplifications were performed by using CFF-GFP as template. PCR1 was performed using CD8a-GFP-F/CFF-KTRK-R, PCR2 using CFF-KTRK-F/pEGFPN1-R. The two PCR fragments were purified, mixed and subjected to second round of PCR amplification using primers CD8a-GFP-F/CFF-R. The resulting PCR product was digested by XhoI/EcoRI and ligated into XhoI/EcoRI digested pEGFP-N1 vector.

2.2.3b Myc-tagged fibrocystin constructs

- **CFF Δ C-Myc**: The CDS of CFF Δ C was released by digesting CFF Δ C-GFP with XhoI/EcoRI and ligated to XhoI/EcoRI digested pMyc-N1 vector.

- **CFF-Myc**: The CDS of CFF was released by digesting CFF-GFP with XhoI/EcoRI and ligated to XhoI/EcoRI digested pMyc-N1 vector.
- **CFF-(LV)-Myc**: The CDS of CFF was released by digesting CFF-(LV)-GFP with XhoI/EcoRI and ligated to XhoI/EcoRI digested pMyc-N1 vector.
- **CFF-(WF)-Myc**: The CDS of CFF was released by digesting CFF-(WF)-GFP with XhoI/EcoRI and ligated to XhoI/EcoRI digested pMyc-N1 vector.
- **CFF-(CCC)-Myc**: The CDS of CFF was released by digesting CFF-(CCC)-GFP with XhoI/EcoRI and ligated to XhoI/EcoRI digested pMyc-N1 vector.
- **CFF-(KKS)-Myc**: The CDS of CFF was released by digesting CFF-(KKS)-GFP with XhoI/EcoRI and ligated to XhoI/EcoRI digested pMyc-N1 vector.
- **CFF-(KTRK)-Myc**: The CDS of CFF was released by digesting CFF-(KTRK)-GFP with XhoI/EcoRI and ligated to XhoI/EcoRI digested pMyc-N1 vector.

2.2.3c GST-tagged fibrocystin constructs

- **GST-fibrocystin-CTS**: The CDS was released from GFP-f-CTS with EcoRI/BamHI and ligated into EcoRI/BamHI digested pGEB vector.
- **GST-fibrocystin-CTS-(KTRK-AAAA)**: The CDS was released from f-CTS-(KTRK)-GFP with EcoRI/BamHI and ligated into EcoRI/BamHI digested pGEB vector.

2.2.4 Gift plasmids

- mCherry-Flag-CD59 from addgene (50378), Vamp5-GFP, Vamp2-GFP, Vamp8-GFP, Vamp3-GFP.

2.2.5 GST-tagged constructs of CTSs

- **GST-f-CTS**: see GST tagged fibrocystin constructs.
- **GST-cystin**: The oligos GST-Cystin-F/CST-Cystin-R were annealed and ligated to EcoRI/BamHI digested pGEB.
- **GST-PC-1**: The oligos GST-PC-1-F/GST-PC-1-R were annealed and ligated to EcoRI/BamHI digested pGEB.
- **GST-PC-2**: The oligos GST-PC-2-F/GST-PC-2-R were annealed and ligated to EcoRI/BamHI digested pGEB.
- **GST-retinol dehydrogenase**: The oligos GST-rDH-F/GST-rDH-R were annealed and ligated to EcoRI/BamHI digested pGEB.

- **GST-SSTR3i3**: The CDS of SSTR3i3 was PCR amplified from CD8a-SSTR3i3-GFP using primers GST-SSTR3-F/GST-SSTR3-R. The resulting PCR product was digested by EcoRI/BamHI and ligated into EcoRI/BamHI digested pGEB vector.
- **GST-RP2-15aa**: The oligos GST-RP2-15aa-F/GST-RP2-15aa-R were annealed and ligated to EcoRI/BamHI digested pGEB.
- **GST-pheripherin**: The oligos GST-pheripherin-F/GST-pheripherin-R were annealed and ligated to EcoRI/BamHI digested pGEB.

2.2.6 IL2R constructs

- **IL2R α** : The CDS of IL2R α was amplified by PCR using primers IL2R-WT-F/IL2R-stop-R from IL2R α /hE-cadherin-cytotail (addgene plasmid: 45773). The resulting PCR product was digested by NheI/EcoRI and ligated into NheI/EcoRI digested pEGFP-N1 vector.
- **IL2R α -GFP**: The CDS of IL2R α was amplified by PCR using primers IL2R-WT-F/IL2R-WT-R from IL2R. The resulting PCR product was digested by NheI/EcoRI and ligated into NheI/EcoRI digested pEGFP-N1 vector.
- **IL2R α -cad-GFP**: The CDS of IL2R α -cad was amplified by PCR using primers IL2R-WT-F/IL2R-cad-R from IL2R/hE-cadherin-cytotail (addgene plasmid: 45773). The resulting PCR product was digested by NheI/EcoRI and ligated into NheI/EcoRI digested pEGFP-N1 vector.

2.2.7 Lentivirus constructs

- **TNPO1 shRNA**: The oligos TNPO1-sh-F/TNPO1-R were annealed and ligated in to AgeI/EcoRI digested pLKO.1-TRC vector.
- **GL2 shRNA**: The oligos GL2-sh-F/GL2-sh-R were annealed and ligated in to AgeI/EcoRI digested pLKO.1-TRC vector.

2.2.8 Rab8 constructs

2.2.8a GFP-tagged Rab8 constructs

- **GFP-Rab8-WT**: The CDS of Rab8 (IMAGE: 3547214, GenBank: BC002977.1) was PCR amplified using primer pair Rab8-F/Rab8-R. The resulting PCR product was digested by EcoRI/BamHI and ligated into EcoRI/BamHI digested pEGFP-C2 vector.
- **GFP-Rab8-Q67L**: Two PCR amplifications were performed by using Rab8 as template and primer pair 1(Rab8-F/Rab8-Q67L-R1) or primer pair 2 (Rab8-Q67L-F2/

Rab8-R). The two PCR fragments were purified, mixed and subjected to second round of PCR amplification using primers Rab8-F/Rab8-R. The resulting PCR product was digested by EcoRI/BamHI and ligated into EcoRI/BamHI digested pEGFP-C2 vector.

- **GFP-Rab8-T22N**: Two PCR amplifications were performed by using Rab8 as template and primer pair 1(Rab8-F/Rab8-T22N-R1) or primer pair 2 (Rab8-T22N-F2/Rab8-R). The two PCR fragments were purified, mixed and subjected to second round of PCR amplification using primers Rab8-F/Rab8-R. The resulting PCR product was digested by EcoRI/BamHI and ligated into EcoRI/BamHI digested pEGFP-C2 vector.

2.2.8b GST-tagged Rab8 constructs

- **GST-Rab8-WT**: The CDS of Rab8-WT was released by digesting Rab8-WT-GFP with EcoRI/BamHI and ligated to EcoRI/BamHI digested pGEX vector.

- **GST-Rab8-Q67L**: The CDS of Rab8-Q67L was released by digesting Rab8-Q67L-GFP with EcoRI/BamHI and ligated to EcoRI/BamHI digested pGEX vector.

- **GST-Rab8-T22N**: The CDS of Rab8-T22N was released by digesting Rab8-T22N-GFP with EcoRI/BamHI and ligated to EcoRI/BamHI digested pGEX vector.

2.2.8c His-Rab8 constructs

- **His-Rab8-WT**: The CDS of Rab8-WT was released by digesting Rab8-WT-GFP with EcoRI/BamHI and ligated to EcoRI/BamHI digested pET30ax vector.

- **His-Rab8-Q67L**: The CDS of Rab8-Q67L was released by digesting Rab8-Q67L-GFP with EcoRI/BamHI and ligated to EcoRI/BamHI digested pET30ax vector.

- **His-Rab8-T22N**: The CDS of Rab8-T22N was released by digesting Rab8-T22N-GFP with EcoRI/BamHI and ligated to EcoRI/BamHI digested pET30ax vector.

2.2.8d Myc-Rab8 constructs

- **pDMyc-Rab8-WT**: The CDS of Rab8-WT was released by digesting Rab8-WT-GFP with EcoRI/BamHI and ligated to EcoRI/BamHI digested pDMyc vector.

- **pDMyc-Rab8-Q67L**: The CDS of Rab8-Q67L was released by digesting Rab8-Q67L-GFP with EcoRI/BamHI and ligated to EcoRI/BamHI digested pDMyc vector.

- **pDMyc-Rab8-T22N**: The CDS of Rab8-T22N was released by digesting Rab8-T22N-GFP with EcoRI/BamHI and ligated to EcoRI/BamHI digested pDMyc vector.

2.2.9 Other constructs

- **GFP-CAAX**: The oligos CAAX-F and CAAX-R were annealed and ligated to EcoRI/BamHI digested pEGFP-C1.

- **GFPx3-CAAX**: The oligos CAAX-F and CAAX-R were annealed and ligated to EcoRI/BamHI digested pEGFPx3-C1.
- **Arl13b-mCherry**: The CDS of Arl13b was released from the CDS of Arl13b-GFP as template using XhoI/BamHI and ligated to XhoI/BamHI digested pm-CherryN1.
- **Dyn-1(K44A)-Myc**: The CDS of Dyn-1(K44A) was released from the CDS of Dyn-1(K44A)-GFP (addgene plasmid: 22197) as template using XhoI/BamHI and ligated to XhoI/BamHI digested pMyc-N1.

2.2.10 Vamp constructs

- **Vamp2-V43AM46A-GFP (Vamp2-MT-GFP)**: Two PCR amplifications were performed by using Vamp2 as template and primer pair 1 (Vamp2-V43AM46A-GFP-F1/Vamp2-V43AM46A-GFP-R1) or primer pair 2 (Vamp2-V43AM46A-GFP-F2/Vamp2-V43AM46A-GFP-R2). The two PCR fragments were purified, mixed and subjected to second round of PCR amplification using primers Vamp2-V43AM46A-GFP-F1/ Vamp2-V43AM46A-GFP-R2. The resulting PCR product was digested by XhoI/EcoRI and ligated into XhoI/EcoRI digested pEGFP-N1 vector.
- **Vamp8-V23PK24AM27A-GFP (Vamp8-MT-GFP)**: Two PCR amplifications were performed by using Vamp8 as template and primer pair 1 (Vamp8-V23PK24AM27A-GFP-F1/Vamp8-V23PK24AM27A-GFP-R1) or primer pair 2 (Vamp8-V23PK24AM27A-GFP-F2/Vamp8-V23PK24AM27A-GFP-R2). The two PCR fragments were purified, mixed and subjected to second round of PCR amplification using primers (Vamp8-V23PK24AM27A-GFP-F1/ Vamp8-V23PK24AM27A-GFP-R2). The resulting PCR product was digested by XhoI/EcoRI and ligated into XhoI/EcoRI digested pEGFP-N1 vector.

2.2.11 siRNA

The siRNAs used in this study, their corresponding sources and sequences are listed in Table 4

Table 4: siRNAs used for knockdown

siRNA	Source	Sequences
Luciferase GL2 Duplex	Dharmacon (D-001100-01-50)	CGUACGCGGAAUACUUCGA
ON-TARGET plus smart pool siRNA importin-β1	Dharmacon (L-017523-00-005)	GAACCAAGCUUGAUCUGUU, GCUCAAACCCACUAGUUAUA, GACGAGAAGUCAAGAACUA, GGGCGGGAGAUCGAAGACUA

ON-TARGET plus smart pool siRNA TNPO1	Dharmacon (L-011308-00-005)	GCAAAGAUGUACUCGUAAG, GUAUAGAGAUGCAGCCUUA, GUAAAUACCAGCAUAAGAA, GCAAAUGUGUAUCGUGAUG
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2.3 Antibodies

The antibodies used in this study are listed below (Table 5 and 6) and their respective dilutions used for the western blot and immunofluorescence experiments are specified. The secondary antibodies were conjugated to horseradish peroxidase (HRP) for western and Alexa Fluor fluorescent molecules in case of immunofluorescence.

2.3.1 Primary antibodies

The primary antibodies used in this study, their corresponding sources and dilutions for are listed in alphabetical order in Table 5.

Table 5: List of primary antibodies and their corresponding sources.

Primary antibodies	Source	Dilution
Acetylated α -tubulin antibody	Sigma-6-11B-1	1:5000 [162]
Arl13b polyclonal antibody	Home made using Arl13b-C-terminus (245-428aa)	1:1000 (WB, IF)
GAPDH antibody	Santa Cruz (sc25778)	1:1000 (WB)
GFP mouse monoclonal antibody	Santa Cruz (sc9996)	1:1000 (WB)
GFP rabbit polyclonal antibody	Santa Cruz (sc8334)	1:1000 (WB)
Importin- β 1 antibody	Abcam (ab2811)	1:5000 (WB)
TNPO1 antibody	Abcam (ab10303)	1:3000 (WB)
Myc antibody	Santa Cruz (sc40)	1:1000 (WB)
OKT8 antibody	Hybridoma culture	1:500 [IF]
Rab8	BD biosciences-610844	1:500 (WB)
α -tubulin antibody	SantaCruz (sc8035)	1:1000 (WB)
β -tubulin antibody	Santa Cruz (sc5274)	1:1000 (WB)

2.3.2 Secondary antibodies

The secondary antibodies used in this study, their corresponding sources and dilutions are listed in Table 6.

Table 6: List of secondary antibodies and their corresponding sources.

Secondary antibodies	Source	Dilution
Goat anti-mouse IgG-HRP	Bio-Rad (1721011)	1:10000 (WB)
Goat anti-rabbit IgG-HRP	Bio-Rad (1706515)	1:10000 (WB)
Anti-mouse Alexa Fluor 488	Molecular probe (A11001)	1:500 [IF]
Anti-mouse Alexa Fluor 594	Molecular probe (A11005)	1:500 [IF]
Anti-mouse Alexa Fluor 647	Molecular probe (A21235)	1:500 [IF]
Anti-rabbit Alexa Fluor 488	Molecular probe (A11008)	1:500 [IF]
Anti-rabbit Alexa Fluor 594	Molecular probe (A11012)	1:500 [IF]
Anti-rabbit Alexa Fluor 647	Molecular probe (A21245)	1:500 [IF]
Protein-A-HRP	Abcam (ab7245)	1:3000 (WB)

2.4 Cell culture

hTERT RPE-1 (Retinal pigmental epithelial cells immortalized with hTelomerase) and IMCD3 (inner medullary collecting duct) cells were maintained in DMEM Ham's F12 medium supplemented with 1.2 g/L sodium bicarbonate and 10% FBS at 37°C in 5% CO₂ humidified atmosphere. HeLa (Henrietta Lacks) cells, BSC-1 (derived from African green monkey) cells, HEK 293T (Human Embryonic kidney HEK 293T cells) and HEK 293FT were cultured in DMEM High glucose medium supplemented with 3.7 g/L sodium bicarbonate and 10% FBS at 37°C in 5% CO₂ humidified atmosphere.

2.4.1 Transfection

Transfection was performed once the cells reached 70-80% confluency. HEK 293T HeLa cells were transfected with PEI. For PEI transfection, DNA and PEI were used in the ratio of 1:3. RPE1, IMCD3, 293FT and BSC-1 cells were transfected using Lipofectamine 2000. The DNA and Lipofectamine 2000 were mixed in the ratio of 1:1 and transfected according to manufacturer's protocol.

2.4.2 Lentivirus production

293FT cells were seeded on 0.01% poly-L-lysine coated 6-well plate and incubated until it reaches 60-70% confluency. Cells were transfected with packaging plasmids: pLP1, pLP2, pLP/VSVG and lentiviral transfer construct in the ratio of

2:1:1:4 using Lipofectamine 2000. After 18 hours of transfection, the media was replaced with fresh media. After 36-48 hours of transfection, the virus-containing medium was collected and filtered using a 0.45 μm filter to remove any cell debris. The lentivirus filtrate was used immediately for transduction or aliquoted into cryovials and frozen at -80°C .

2.4.3 Lentiviral transduction

RPE1 cells were seeded on a 24-well (with or without glass coverslips) and allowed to grow till 60-70% confluency. 500 μl of lentivirus supernatant was added to the cells along with polybrene (8 $\mu\text{g}/\text{ml}$) and incubated overnight at 37°C . A second infection was performed after 24 hrs. Cells were incubated for additional 24 hours. The infected cells are used for assays such as transfection of other plasmids, immunofluorescence and western blotting. For generation of stable clones, cells were selected in DMEM Ham's F12 medium containing 12 $\mu\text{g}/\text{ml}$ puromycin.

2.5 Western blot

Cells were lysed in $1 \times$ SDS sample buffer. Cell lysates were boiled for 10 minutes and spun down at $14,000 \times g$ for 5 minutes. Equal volumes of protein samples were resolved on SDS-PAGE and transferred to PVDF membrane. The membrane was blocked with 5% non-fat milk in PBST containing 0.1% Tween 20 for 1 hour at room temperature. The membrane was incubated with primary antibody diluted in 5% milk in PBST for 1 hour at room temperature. After primary antibody incubation, membrane was washed with PBST, followed by incubation with HRP conjugated secondary antibody diluted in 5% milk in PBST for 30 minutes - 1 hour at room temperature. To observe the signal, the membrane was incubated with WesternBrightTM Quantum for 5 minutes at room temperature and detected using Image Quant LAS-4000 (GE health care life sciences).

2.6 Purification of His-tagged Rab8 constructs

A single BL21(DE3) colony containing the transformed plasmid was inoculated into LB media with kanamycin and allowed to grow at 37°C overnight at $250 \times g$. IPTG was added to the culture at a final concentration of 0.5 mM, and the culture was grown at 16°C overnight. The culture was centrifuged at $5000 \times g$ for 10 minutes. The pellet was resuspended in 30 ml His-tag lysis buffer (Table 3) and lysed

thrice by freeze-thaw method. Later the lysate was centrifuged at $20,000 \times g$ for 30 minutes. The supernatant was incubated for binding with pre-washed Ni-NTA agarose beads in the presence of 10 mM imidazole at 4°C for 2 hours. The beads were washed with wash buffer containing 10 mM, 20 mM, and 50 mM imidazole respectively. The protein was eluted with His-tag elution buffer containing 250 mM imidazole (Table 3). Imidazole in the elution buffer was removed by dialysis. The protein was concentrated using Amicon Ultra-15 centrifugal filter units and quantified by Bradford assay and Coomassie gel. Purified protein was stored at -80°C till further use.

2.7 Purification of His-tagged Arl13b-C-terminus under denaturing condition

A single BL21(DE3) colony containing the transformed plasmid was inoculated into LB media with kanamycin and allowed to grow at 37°C overnight at $250 \times g$. IPTG was added to the culture at a final concentration of 0.5 mM, and the culture was grown at 16°C overnight. The culture was centrifuged at $5000 \times g$ for 10 minutes. Cell pellet was suspended in 8M urea PBS (Table 3) and lysed by sonication. The lysate was incubated at room temperature for 90 minutes and later centrifuged at $20,000 \times g$ for 30 minutes. The supernatant was incubated for binding with pre-washed Ni-NTA agarose beads at room temperature for 2 hours. The beads were washed with 8M urea PBS containing 20 mM imidazole. The protein was eluted with 8M urea PBS containing 250 mM imidazole. The concentration of urea was reduced to 4M by gradual buffer replacement for antibody production. Protein was concentrated using Amicon Ultra-15 centrifugal filter units and quantified.

2.8 Immunoprecipitation

HEK 293T were transfected with desired constructs. After 24-36 hours of transfection, cells were scraped in lysis buffer and incubated on ice for 30 minutes followed by centrifugation at $16000 \times g$ (4°C). Supernatant was incubated with 1µg of antibody for 4 hours or overnight. The antigen-antibody complex was incubated with pre-washed 15 ul of A/G beads for 2-4 hours. The beads were washed 4 times with lysis buffer and the proteins were eluted using SDS sample buffer. Samples were resolved using 10-12% SDS-PAGE gel for western blot analysis.

2.9 GST pull-down

HEK 293T were scraped in lysis buffer and incubated on ice for 30 minutes. Samples were centrifuged at 4°C at 16000 × g. The supernatant was incubated for 4 hours or overnight with 10-40 µg of GST fusion proteins pre-washed with lysis buffer. The beads were washed 4 times with lysis buffer and the proteins were eluted using SDS sample buffer. The samples were resolved using 10-12% SDS-PAGE gel for western blot analysis.

2.10.1 Immunofluorescence

Cells were seeded on 12 mm coverslips in a 24-well plate. Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After 24 hours of transfection, cells were serum-starved to induce ciliogenesis. For hTERT RPE1, IMCD3 and BSC-1 cells, typical starvation time was 2, 2 and 5 days respectively. After induction of cilia, cells were fixed using 4% PFA in PBS at room temperature for 20 minutes. This was followed by neutralizing PFA with 100 mM ammonium chloride followed by washing with PBS. Primary and secondary antibodies were diluted in fluorescence dilution buffer (FDB) supplemented with 0.1% saponin. Cells were incubated with primary antibody, washed and followed by fluorescence conjugated secondary antibody. After washing, coverslips were mounted using Mowiol mounting medium. For surface labeling, cells grown on coverslips were cooled down on ice and incubated with OKT8 monoclonal antibody (mAb) for 1 hour on ice. After washing 3 × with ice-cold PBS, cells were fixed and subjected to immunofluorescence labeling as described above.

2.10.2 Microscopy

The inverted wide-field microscope (Olympus IX83) combined with MetaMorph software (Molecular devices) was used to capture the images. The basic configurations of microscope were as follows: 63× objectives (Plan-apochromatic and N.A. 1.42); Neo sCMOS camera (Andor technology) and a metal halide lamp (Lumen 200PRO, PRIOR 16 scientific). ImageJ software was used to analyze captured images.

2.11 CPIR (cilium-to-PM intensity ratio)

In this method, a line was drawn on the cilium using Arl13b as a marker and the ciliary intensity at that point was measured in specific channel using line plot profile option in ImageJ. PM intensity surrounding the cilium and the background devoid of cells was measured. The CPIR was measured using the following formula.

$$CPIR = \frac{(\text{maximum of line intensity profile}) - (\text{mean intensity of PM})}{(\text{mean intensity of PM}) - (\text{mean intensity of background})}$$

2.12 Knock down of importin-β1 and TNPO1

hTERT RPE1 cells were grown on 12mm coverslips in a 24-well plate. At ~85-90% confluency, the cells were transfected with GL2/importin-β1/TNPO1 using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol. After 24 hours, cells were transfected with f-CTS-GFP/CFF-GFP. After 24 hours, the coverslips were serum-starved for 48 hrs. In case of f-CTS-GFP, cells were PFA fixed and stained with Arl13b pAb, followed by incubation with AF594 secondary antibody and mounted. In case of CFF-GFP, the coverslips were chilled on ice to block endocytosis and incubated with OKT8 mAb for 1 hour, then washed 3× with ice cold PBS. Cells were fixed with PFA and incubated with Arl13b polyclonal antibody (pAb) for 1 hour and washed 3× with PBS. After washing, the cells were incubated with mouse AF594 for OKT8 and rabbit AF647 for Arl13b secondary antibody for 30 minutes. Cells were washed for 3× with PBS and mounted on a glass slide. Parallel to this for western blot analysis, one 24 well of cells were lysed with 2×SDS sample buffer and 50% lysate volume was resolved on a SDS PAGE gel.

2.13 Fluorescence recovery after photo bleaching (FRAP)

hTERT RPE1 cells were grown on 35mm glass bottomed dish. Using Lipofectamine™ 2000, cells were transfected with CD8a constructs along with Arl13b-mCherry as a marker for cilia. After 24 hours of transfection, cells were serum-starved for 48 hours to induce cilia. The media was changed to CO₂-independent medium. For capturing images, an inverted spinning disk confocal microscope in a 37°C heated stage, with a 63× oil objective and 3× gain was used.

Photobleaching experiments were carried out using the iLAS²-FRAP Roper scientific software. Cilia were imaged in a single focal plane. Images were collected every 5 seconds for 1 minute before photobleaching and 5 minute after photo bleaching. Image analysis was performed using ImageJ software. A mask for cilia was created with Arl13b-mCherry as a marker using threshold and pencil functions. Average fluorescence intensity changes of each frame were calculated. The t_1 of the fluorescence is measured from fitting in to single exponential decay equation

$$y = y_0 + A \times e^{-x/t_1}$$

Where Y_0 is final intensity plateau after recovery, A is first post bleach intensity, x is decay constant, and t_1 is time.

The immobile fraction was measured using the formula

$$\text{Immobile fraction} = \frac{y_b - y_0}{y_b - A}$$

Where Y_b is pre-bleach fluorescence intensity, Y_0 is final intensity plateau after recovery; A is first post-bleach intensity.

CHAPTER 3: Results

3.1 Characterization of the ciliogenesis of RPE1, BSC-1, HeLa, HEK 293T cells

Most vertebrate cells contain primary cilia, particularly those cells that are non-dividing and differentiated [163]. For this study, we characterized different cell lines to find the best condition for ciliogenesis. Immunofluorescence was used to characterize the cilia in HEK 293T, HeLa, BSC-1, and RPE1. Antibodies against the post-translational modifications of tubulin, such as acetylation, glutamylation, and glycylation are generally used to stain cilia. Under these conditions, the modified tubulin of cilia appears brighter than the cytoplasmic network. In this study, antibodies against Arl13b (cilia-localized protein) and acetylated tubulin were used to distinguish cilia. From previous studies, it was known that most cells have <20% cilia under normal culture conditions [163]. Therefore, cells need to be arrested in the G1 phase of their cell cycle, which is most commonly achieved by serum starvation.

3.1.1 Ciliogenesis of HEK 293T cells

Though HEK 293T cells have been reported to possess cilia [164], the cell line is not commonly used for studying cilia. To further characterize the ciliogenesis of HEK 293T, cells were seeded on poly-L-Lysine-coated glass coverslips, and serum-starved after reaching >90% confluency for 0, 1 and 2 days. Fixed cells were stained with Arl13b pAb to label cilia (Fig. 3.1.1A). The number of cells, the number of cilia and length of cilia was calculated using ImageJ. Under normal conditions, HEK 293T cells were found to have around ~40% ciliated cells. Serum starvation for 2 days increased the percentage of ciliated cells to ~69% (Fig. 3.1.1B). After starvation, cilia length was also found to increase threefold, from 1.4 to 3.6 μm , compared to serum fed cells (Fig. 3.1.1C).

Figure 3.1.1: Ciliogenesis of HEK 293T cells

A) Immunofluorescence images of HEK 293T cells serum-starved for 0, 1 and 2 days; fixed and stained with Arl13b pAb. Bar, 10 μm . (B) Quantification of percentage of ciliated HEK 293T cells under serum starvation for 0, 1 and 2 days. The data represents the mean of total cells counted (n is indicated in the graph). (C) Quantification of cilia length (μm) of HEK 293T cells under serum starvation for 0, 1 and 2 days (Mean (μm) $\pm$ SEM; n is indicated in the graph). Length in μm . The results are from a single experiment.

3.1.2 Ciliogenesis of HeLa cells

To characterize ciliation, HeLa cells were serum-starved after reaching >90% confluency for 0, 1, 2, 3, 4 and 5 days. Fixed cells were stained with Arl13b pAb to label cilia (Fig. 3.1.2A). HeLa cells showed a lower percentage of ciliated cells, i.e., 0.4% under normal conditions (Fig. 3.1.2B). Unlike HEK 293T, ciliation in HeLa cells was slow and only 50% of the cells were found to possess cilia after 5 days of serum starvation. Under serum starvation, the length of cilia was found to increase from 1.9 μm to 2.9 μm (Fig. 3.1.2C). This result indicates HeLa cells are poorly-ciliated, and, therefore, they are not used for this study.

Figure 3.1.2: Ciliogenesis of HeLa cells

A) Immunofluorescence images of HeLa cells serum-starved for 0, 1, 2, 3, 4 and 5 days, fixed and stained with Arl13b pAb. Scale bar, 10 µm. (B) Quantification of percentage of ciliated HeLa cells under serum starvation for 0, 1, 2, 3, 4 and 5 days. The data represents the mean of total cells counted (n is indicated in the graph). (C) Quantification of cilia length (µm) of HeLa cells under serum starvation for 1, 2, 3, 4 and 5 days (Mean ± SEM; n is indicated in the graph). Length in µm. The results are from a single experiment.

3.1.3 Ciliogenesis of BSC-1 cells

BSC-1 is a renal epithelium cell line derived from the African green monkey and is mostly used in centrosomal reproduction studies [165]. However, the ciliation capability of BSC-1 cells has not been reported according to our knowledge. To characterize ciliation, BSC-1 cells were serum-starved after reaching >90% confluency for 0, 1, 2, 3 and 4 days. Fixed cells were stained for acetylated tubulin to label cilia (Fig. 3.1.3A). BSC-1 cells showed about 22% of ciliated cells under fed conditions (Fig. 3.1.3B). The percentage of ciliated cells increased after 4 days of

starvation, from 22% to 70%. There was a significant increase in the length of the cilia upon starvation. Under starvation conditions, the length of cilia was found to increase from 2.0 μm to 3.9 μm (Fig. 3.1.3C). This indicates that BSC-1 cells could be suitable for this study.

Figure 3.1.3: Ciliogenesis of BSC-1 cells

A) Immunofluorescence images of BSC-1 cells serum-starved for 0, 1, 2, 3 and 4 days fixed and stained with anti-acetylated tubulin. Scale bar, 10 μm . (B) Quantification of percentage of ciliated BSC-1 cells under serum starvation for 0, 1, 2, 3 and 4 days. The data represents the mean of total cell counted (n is indicated in the graph). (C) Quantification of cilia length (μm) of BSC-1 cells under serum starvation for 0, 1, 2, 3 and 4 days (Mean $\pm$ SEM; n is indicated in the graph). Length in μm . The results are from a single experiment.

3.1.4 Ciliogenesis of hTERT RPE1 cells

The human retinal-pigmented epithelial (RPE1) cell line is commonly used to study cilia. To optimize ciliation conditions, RPE1 cells were serum-starved for 0, 1, 2 and 3 days. Fixed cells were stained with acetylated tubulin to label cilia. Consistent with previous reports [1], about 25% of RPE1 cells form cilia under normal conditions (Fig. 3.1.4A-B). Percentage of ciliated cells increased upon starvation, from 25% to ~90%, at the end of 2 days of induction. However, on 3rd day there was a reduction in the percentage of cells with cilia (Fig. 3.1.4B). Different from HeLa, BSC-1, and HEK cells, there was no significant increase in the length of cilia (Fig. 3.1.4C). Our study indicates that serum starvation for 2 days at >90% confluency is the optimal condition to induce cilia in RPE1 cells.

Figure 3.1.4: Ciliogenesis of RPE1 cells

A) Immunofluorescence images of RPE1 cells serum-starved for 0, 1, 2 and 3 days, fixed and stained with anti-acetylated tubulin. Scale bar, 10 μ m. (B) Quantification of percentage of ciliated cells of RPE1 under serum starvation for 0, 1, 2 and 3 days. The data represents the mean of total cells counted (n is indicated in the graph). (C) Quantification of cilia length (μ m) of RPE1 under serum starvation for 0, 1, 2 and 3 days (Mean $\pm$ SEM; n is indicated in the graph). Length in μ m. The results are from a single experiment.

Our results suggest 293T, BSC-1, and RPE1 cells which possess $\geq 50\%$ ciliated cells by the end of 2 days starvation, are suitable for cilia studies. Among these, RPE1

which possesses 90% of ciliated cells at the end of 2 day starvation were used in this study. BSC-1 cells which need longer time (4 days) to get ciliated (~70%) were used as an additional cell line to confirm localization. 293T cells which are ciliated well by the end of 2 days of starvation are used for co-immunoprecipitation and pull-down experiments for which they are widely used.

3.2 Quantification of ciliary localization by CPIR

A conventional approach to quantify ciliary localization is by counting the percentage of cells that are positive for cilia. However, this method unavoidably introduces bias, as the threshold for positive localization is set subjectively. The other method based on measurement of whole cilia intensity suffers from the expression levels of the testing proteins. To solve these issues, we established an image-based method to introduce an ensemble-averaged metric, called the CPIR (cilium-to-the PM intensity ratio). The CPIR is used to quantify the ciliary localization of a membrane protein in cultured mammalian cells. To that end, a PM-localized membrane protein is tagged with a fluorescent protein and imaged with conventional fluorescence microscopy. To calculate the CPIR using ImageJ, a line is drawn across the cilium and the maximum of the line intensity profile is subsequently obtained (Fig. 3.2). The CPIR of the protein is defined as

$$CPIR = \frac{(\text{maximum of line intensity profile}) - (\text{mean intensity of PM})}{(\text{mean intensity of PM}) - (\text{mean intensity of background})}$$

The line intensity profile across the cilium has a width of 1 μm ; therefore, the maximum of line intensity profile is an average of 1 μm length of the cilium. Since the diameter of the cilium (~200-300 nm) approaches the diffraction-limited size of conventional optical microscopes (~250 nm), the maximum of the line intensity profile is proportional, but not equal, to ciliary membrane intensity. When a membrane protein is excluded from the PM, the minimum of the line intensity profile is used; therefore, the CPIR value could be negative. Using this method, the CPIR of SSTR3-GFP, was found to be 3.14 ± 0.4 (mean $\pm$ SEM, n=50) (Fig. 3.3.1C). If there is a significant interference with the quantification of the PM intensity due to intracellular structures such as endosomes, surface fluorescence labeling could be

applied. The CPIR indicates the relative enrichment of a ciliary membrane protein in the cilium, by normalizing its expression level.

3.2.1 The CPIR value is independent of the expression level

To exclude the possibility that CPIR is dependent on the expression level of the testing protein, we measured the expression levels of SSTR3-GFP using total cellular fluorescence. The CPIR values were plotted against the total cellular fluorescence (Fig. 3.2C). We found that the CPIR value remains relatively constant within at least a 10-fold range of expression levels (Fig. 3.2C). Therefore, the CPIR reflects the intrinsic ciliary targeting property of a ciliary membrane protein, and we will make use of it to quantitatively compare ciliary localizations of membrane proteins.

Figure 3.2: Quantification of ciliary localization using CPIR value

A) Image showing line profile (orange) drawn on cilia of SSTR3-GFP and area selected to measure the PM and background intensity using dashed circles. Scale bar, 5 μ m. (B) Line profile showing the maximum intensity value at cilia. (C) Graph shows CPIR value plotted against total cellular fluorescence of SSTR3-GFP.

3.3 PM-localized membrane proteins could be found at cilia

3.3.1 CD8a localizes to cilia

The mechanism of the transport of membrane proteins to cilia is not clearly understood. Though there are reports showing sequences necessary for ciliary localization of some proteins, the machinery involved in their transport to cilia is not clear. In order to investigate the machinery and mechanism of trafficking, we chose CD8a as a reporter to study the ciliary targeting mechanism. CD8a, a type I

transmembrane protein (Fig. 3.3.1A), is commonly used in the membrane trafficking field for chimeric constructs, as it is natively expressed in only T cells, which have no cilia. CD8a localizes to the PM and is conventionally assumed to be a non-ciliary protein. It has been widely used as a membrane reporter to study ciliary targeting of membrane proteins [101, 113]. Hence, it can be used to validate our method of quantification and to study CTSs of ciliary membrane proteins. Surprisingly, our fluorescence imaging data always demonstrated a significant ciliary localization of CD8a and CD8a-GFP in RPE1 cells (Fig. 3.3.1B-D).

Figure 3.3.1: CD8a localizes to cilia

A) Schematic representation of the domain structure of CD8a showing signal peptide, an extracellular domain, transmembrane domain and cytosolic tail. B) Immunofluorescence images of RPE1 cells expressing SSTR3-GFP, CD8a (OKT8 Ab), CD8a-GFP and IL2R α -cad (2A3A11H mAb), starved for 2 days, fixed and stained with Arl13b pAb (red). Scale bar, 10 μ m. (C) Quantification of ciliary localization of SSTR3-GFP, CD8a, CD8a-GFP and IL2R α -cad using CPIR value (n=50 $\pm$ SEM). The results are from a single experiment. D) Immunofluorescence images of BSC-1 cells expressing CD8a

(OKT8 Ab) and CD8a-GFP, starved for 4 days, fixed and stained with Arl13b pAb (red). Immunofluorescence image of an IMCD3 cell expressing CD8a-GFP, starved for 2 days, fixed and stained for acetylated tubulin (red). Scale bar, 10 μ m. The results are from a single experiment.

Hence, we used another chimeric construct not expressed in ciliated cells, IL2R α -cad (interleukin-2 receptor alpha subunit with cadherin cytotail) to validate our quantification method. IL2R α -cad showed cilia no localization when expressed in RPE1 cells (Fig. 3.3.1B-C). In RPE1 cells, CPIRs of CD8a, CD8a-GFP and IL2R α -cad were 0.54, 0.28 and 0.04 respectively (Fig. 3.3.1C). In comparison to the CPIR of IL2R α -cad, which was 0.04 (Fig. 3.3.1C), the ciliary localization of CD8a seemed significant, but it was much lower than the known ciliary protein SSTR3 (Fig. 3.3.1C). To further confirm that CD8a localizes to cilia, CD8a was transfected in other cell lines such as BSC-1 and IMCD3. A pool of CD8a localized to cilia in both BSC-1 and IMCD3 cells, thus strengthening the above observation that CD8a localizes to cilia (Fig. 3.3.1D).

3.3.2 CD8a localizes to cilia non-selectively

Most of the cilia localized membrane proteins were shown to have a CTS, which is necessary for their localization to cilia [40]. Hence, we initially thought that the ciliary localization of CD8a could be specifically mediated by an unknown CTS in its cytosolic domain, which comprises of 32 amino acids. In order to test the presence of the CTS sequence, cytosolic domain of CD8a was truncated and the ciliary localization of cytosolic domain truncation (CD8a Δ cyto) was verified. Unfortunately, CD8a Δ cyto-GFP was arrested in the ER when expressed in RPE1 cells (Fig. 3.3.2).

Figure 3.3.2: CD8a Δ cyto was arrested in the ER

Immunofluorescence image of RPE1 cells expressing CD8a Δ cyto-GFP, the cells were starved for 2 days, surface-labeled with OKT8 mAb (red), fixed and stained with Arl13b pAb (blue). Scale bar, 10 μ m. The result is from a single experiment.

Therefore, instead of truncation, both cytosolic and transmembrane domains of CD8a were swapped with their corresponding domains from CD4 or VSVG (Fig. 3.3.3A). CD4 and VSVG are both type I transmembrane proteins similar to CD8a and are not endogenously present in RPE1 cells. Both CD8a-CD4 and CD8a-VSVG were found to have significant ciliary localization (Fig. 3.3.3B). The CPIR of CD8a-CD4-GFP (0.55) was similar to CD8a-GFP (0.44) (Fig. 3.3.3C). However, the CPIR of CD8a-VSVG is very high, similar to resident cilia membrane protein SSSTR3-GFP (Fig. 3.3.3C). This prompted us to test the ciliary localization of full-length CD4, VSVG, and other membrane reporters.

Figure 3.3.3: CD8a-CD4 and CD8a-VSVG localize to cilia

A) Schematic representation of the domain structure of CD8a-CD4 and CD8a-VSVG, showing the signal peptide, extracellular domain from CD8a, transmembrane domain and cytosolic tail from CD4 and VSVG respectively. B) Immunofluorescence images of RPE1 cells expressing CD8a-CD4-GFP and CD8a-VSVG-GFP. The cells were starved for 2 days, fixed and stained with Arl13b pAb. Scale bar, 10 μ m. C) Quantification of ciliary localization of CD8a-CD4-GFP, CD8a-VSVG-GFP and CD8a-GFP using CPIR value ($n=38 \pm$ SEM). The results are from a single experiment.

3.3.3 Diverse PM proteins could localize to cilia

We tested reporter membrane proteins for their ciliary localization. CD4, which is specific to immune cells and localizes to the PM, was found to localize to cilia (Fig. 3.3.4). This could be the reason for ciliary localization of CD8a-CD4. Similar to CD8a and CD4, another immune cell specific protein interleukin-2 receptor alpha subunit (IL2R α) was found to localize to cilia when expressed in RPE1 cells (Fig. 3.3.4). GPI-anchored protein mCherry-flag-CD59 also showed ciliary localization, when transfected in RPE1 cells (Fig. 3.3.4). These results were consistent with the previous findings that GPI-GFP, an apical membrane targeted protein, can localize to cilia [41]. CAAX is a farnesylation motif which helps proteins to localize to the PM [166]. A GFP construct with C-terminal CAAX motif, when expressed in RPE1 cells, was found to localize to cilia (Fig. 3.3.4). Vamp5 is a tail-anchored protein that is associated with the PM and intracellular vesicular structures. A pool of Vamp5-GFP co-localized with Arl13b pAb, indicating its ciliary localization (Fig. 3.3.4). VSVG, a glycoprotein of vesicular stomatitis virus, is used as a reporter to study the trafficking of the secretory pathway [167, 168]. When expressed in RPE1 cells VSVG-GFP showed very weak ciliary localization compared to others (Fig. 3.3.4). Hence, we do not have a convincing explanation for CD8a-VSVG concentration in cilia, as full-length VSVG showed very weak ciliary localization (Fig. 3.3.4). Our data demonstrated that these PM-localized membrane proteins can enter ciliary membrane. As they are considered non-residents of cilia, their cilia localizations may be achieved non-selectively.

Figure 3.3.4: PM-localized membrane proteins localize to cilia

Immunofluorescence images of RPE1 cells expressing GFP-CAAX, CD4-GFP, Vamp5-GFP, VSVG-GFP, IL-2R (2A3A11H mAb) or mCherry-flag-CD59. The cells were starved for 2 days and fixed. For Arl13b-mCherry untransfected constructs staining of cilia was performed with Arl13b pAb. Scale bar, 10 μ m. The results are from a single experiment.

3.4 Clathrin-coated pits trapped PM membrane proteins are not found in cilia

To test the hypothesis that CD8a localizes to cilia non-selectively, we performed a domain swapping of the CD8a cytoplasmic tail. The cytosolic tails of furin, sortilin, CD-M6PR (cation-dependent mannose 6-phosphate receptor) and CI-M6PR (cation-independent mannose 6-phosphate receptor) were used, as their cytoplasmic tails fused to CD8a chimeras are widely used to study trafficking pathways of membrane

proteins [161]. When the cytosolic tail of CD8a was replaced with the corresponding region of sortilin, furin, CD-M6PR or CI-M6PR, the resulting CD8a chimeras constitutively cycle between the PM and TGN via endosomes [169-172]. However, in ciliated RPE1 cells, surface labeling by an anti-CD8a monoclonal antibody (CD8a mAb/OKT8 mAb) revealed that they formed numerous puncta on the PM, but were absent at cilia (Fig. 3.4.1B). Quantification of ciliary localization of CD8a-furin, CD8a-sortilin, CD8a-CD-M6PR, CD8a-CI-M6PR and CD8a using CIPR values, also indicated that, compared to CD8a, these chimeras were absent at cilia (Fig. 3.4.1C). There are multiple endocytic signals in cytosolic tails of furin, sortilin, CD-M6PR and CI-M6PR but not in CD8a (Fig. 3.4.1A). These cytosolic tails are known to associate with clathrin adaptor proteins, so the puncta could be clathrin-coated pits. To confirm this, we used one of the clathrin light chains LCA (light chain A) as a marker. The puncta of CD8a-furin, CD8a-sortilin, and CD8a-CI-M6PR colocalized with LCA-mCherry (Fig. 3.4.1D). Collectively, our results suggest that clathrin-coated pits could trap these proteins and impede their cilium entry.

Previously, it was reported that under normal conditions, CD8a has no known endocytic signal; hence it has very low endocytosis efficiency and mostly resides at the PM [169]. To verify the endocytic trafficking of CD8a, HeLa cells were transfected with CD8a, with CD8a-furin transfected cells used as the positive control. Cells on a coverslip were surface-labeled with an antibody against the extracellular domain of CD8a and chased at 37°C for different time intervals. Surface-bound and non-internalized antibody was removed by a subsequent acid wash (see Materials and Methods) (Fig 3.4.2A). A boundary of the whole cell was manually drawn, and the intensity of CD8a labeled antibody was measured using ImageJ. The ratio of the total cellular intensity of CD8a-furin or CD8a-GFP, internalized during the course of endocytic trafficking to their respective initial surface-labeled antibody was quantified. ~60% of total CD8a-furin was internalized and reached Golgi after 80 min of chase. In the case of CD8a-GFP, the amount of internalized CD8a-GFP was less than ~22% of the total CD8a-GFP present on the surface (Fig. 3.4.2A-C). Thus, the major pool of CD8a-GFP is present at the PM. Collectively, our results suggest that clathrin-mediated endocytosis could be a regulating factor in the ciliary absence of CD8a-furin, -sortilin, -CD-M6PR and -CI-M6PR.

Figure 3.4.1: Clathrin-coated pits trapped PM membrane proteins are not found in cilia A) Amino acid sequence of the cytoplasmic tails of CD8a, furin, sortilin, CD-M6PR and CI-M6PR along with their putative endocytic sequence (red). The semi colon in the sequence represents presence of amino acid sequences but is not proportional to their numbers. B) Immunofluorescence images of RPE1 cells expressing CD8a, CD8a-furin, CD8a-sortilin, CD8a-CD-M6PR and CD8a-CI-M6PR. The cells were starved for 2 days surface-labeled with OKT8 mAb, fixed and stained with Arl13b pAb (red). Scale bar, 10 μ m. C) Quantification of ciliary localization of CD8a-furin, CD8a-sortilin, CD8a-CD-M6PR, CD8a-CI-M6PR and CD8a, using CPIR values (n=25 $\pm$ SEM). D) Immunofluorescence images of RPE1 cells co-expressing CD8a, CD8a-furin, CD8a-sortilin or CD8a-CI-M6PR with LCA-mCherry. The cells were surface-labeled with OKT8 mAb, fixed and stained with Arl13b pAb (red). Scale bar, 10 μ m. The results are from a single experiment.

Figure 3.4.2: Major pool of CD8a-GFP is present at the PM A) Time course images showing the endocytic trafficking of CD8a-furin and CD8a-GFP, expressing HeLa cells. The cells were surface-labeled by OKT8 mAb and chased for various time intervals before fixing. Scale bar, 10 μ m. (B-C) The ratio of total cell intensity of CD8a-furin and CD8a-GFP internalized during time course of endocytic trafficking to their respective initial surface-labeled antibody (Mean $\pm$ SEM). The results are from a single experiment.

To further test our hypothesis that clathrin-coated pit trapped PM proteins could be prevented from cilia entry, we used native cellular proteins such as Vamp2, Vamp3, and Vamp8, instead of CD8a chimeras. These proteins are SNAREs involved in docking and fusion of vesicles to the PM and are known to follow clathrin-dependent endocytosis [173, 174]. GFP-tagged Vamp2, Vamp3, and Vamp8 expressed in RPE1 cells (starved for 2 days after transfection to induce cilia) showed numerous puncta on the PM and were absent at cilia, similar to CD8a chimeras (Fig. 3.4.3B). To confirm the role of clathrin-dependent endocytosis in the ciliary localization of the transmembrane proteins, the endocytic motifs of Vamp2 and Vamp8 were mutated [173] (Fig. 3.4.3A). The Vamp2-V43AM46A-GFP (Vamp2-MT-GFP) and Vamp8-V23PK24AM27A-GFP (Vamp8-MT-GFP) displayed less puncta and localized to the PM, as well as cilia similar to CD8a-GFP (Fig. 3.4.3C).

Quantification of ciliary localization of Vamp2-GFP, Vamp2-MT-GFP, Vamp8-GFP and Vamp8-MT-GFP constructs using CPIR values, showed significant cilia localization of Vamp2-MT-GFP and Vamp8-MT-GFP, compared to their respective WT constructs (Fig. 3.4.3D).

Figure 3.4.3: Clathrin-coated pits trapped tail-anchored membrane proteins are not found at cilia

A) Sequence alignment of SNARE motifs of VAMP2, VAMP3 and VAMP8. Conserved residues are boxed in gray. The position of mutated residues in VAMP8: V23A (open triangle), K24A (black circle) and M27A (open circle) are indicated. The position of mutations in VAMP2: V43A (closed square) and M46A (open rectangle) are indicated. B) Immunofluorescence images of RPE1 cells expressing GFP-tagged Vamp2, 3, 8, Vamp2-MT-GFP, and Vamp8-MT-GFP, starved for 2 days, fixed and stained with Arl13b pAb (red). Scale bar 10 μ m. C) Immunofluorescence images of RPE1 cells co-expressing Vamp2, 2-MT, 8, 8-MT and Vamp3-GFP with LCA-mCherry. The cells were fixed and stained with Arl13b pAb (red). Scale bar, 10 μ m. D) Quantification of the ciliary localization of Vamp2-GFP, Vamp2-MT-GFP, Vamp8-GFP and Vamp8-MT-GFP using CPIR values ($n=25 \pm \text{SEM}$). The results are from a single experiment.

To confirm the puncta, we used one of the clathrin light chains LCA (light chain A) as a marker. The puncta of Vamp2, Vamp3, and Vamp8 colocalized with LCA-mCherry whereas Vamp2-MT-GFP and Vamp8-MT-GFP showed no colocalization (Fig. 3.4.2C). This further confirms that clathrin-coated pits can trap proteins and impede their entry into cilia. Therefore, dynamic internalization of membrane proteins through clathrin-coated pits could be a mechanism of excluding non-resident ciliary membrane proteins from cilia.

3.5 Association with actin could restrict IL2R α -cad from entering cilia

As observed in the Fig. 3.3.4, IL2R α -GFP which was generated by the replacement of the cytosolic tail of non-cilia localized IL2R α -cad-GFP (refer to Fig. 3.3.1) by replacing the cytosolic tail with that of IL2R α -WT, showed cilia localization (Fig. 3.5A). The CPIR value of IL2R α -GFP showed significant cilia localization as compared to that of IL2R α -cad-GFP ($p=2.7 \times 10^{-10}$) (Fig. 3.5B). Earlier, a study by Francis et al. showed that actin-binding could be employed by cells to retain a membrane protein on the PM and prevent it from entering cilia [41]. The cytosolic domain of E-cadherin is known to bind to the actin cytoskeleton through β -catenin [175]. To verify whether the interaction with actin is the difference between IL2R α -GFP and IL2R α -cad-GFP, HEK 293T cells expressing IL2R α -Cad-GFP or IL2R α -GFP were immunoprecipitated with GFP mAb. IL2R α -Cad-GFP was able to co-immunoprecipitate endogenous actin whereas IL2R α -GFP cannot (Fig. 3.5C). This supports the previous result that actin-binding can retain proteins and impede their cilia entry. In summary, our data suggests that mobile PM proteins, instead of those

retained or immobilized by clathrin-coated pits or cortical actin cytoskeleton, can have significant localization on the ciliary membrane.

Figure 3.5: Association with actin could restrict IL2Rα-cad from entering cilia

A) Immunofluorescence images of RPE1 expressing IL2Rα-GFP and IL2Rα-cad-GFP, starved for 2 days, fixed and stained with Arl13b pAb (red). Scale bar, 10 μm. B) Quantification of the ciliary localization of IL2Rα-GFP and IL2Rα-cad-GFP by CPIR value (n=25±SEM).C) HEK 293T cells expressing IL2Rα-Cad-GFP or IL2Rα-GFP were immunoprecipitated with GFP mAb. The immunoprecipitated sample was blotted with GFP and actin to detect GFP constructs and actin respectively. The results are from a single experiment.

3.6 Resident and non-resident ciliary membrane proteins have different properties

Our results seem to blunt the distinction between the resident and non-resident ciliary membrane proteins. However, our experiments showed that non-resident membrane proteins were different from ciliary resident membrane proteins. Ciliary residents, such as SSTR3 and Arl13b, had much higher CPIRs (>1) than non-residents (Table 7). It was reported that the dynamics of ciliary resident proteins within cilia

was very high [176]. In order to study the transport dynamics of ciliary resident and non-resident membrane proteins, we performed FRAP. The whole cilium was photobleached by high power laser and the recovery of fluorescence in the cilium was recorded under a spinning disk confocal microscope (Fig. 3.6).

Table 7: A list of CPIR values of various PM proteins

construct	CPIR value
CD8a-CI-M6PR	0.02
CD8a-CD-M6PR	0.03
IL2R α -cad-GFP	0.03
Vamp8-WT-GFP	0.05
IL2R α -cad	0.07
CD8a-sortilin	0.09
Vamp2-WT-GFP	0.10
CD8a-furin	0.11
GFPX3-CAAX	0.18
CD8a-GFP	0.28
GFP-CAAX	0.40
Vamp2-MT-GFP	0.43
CD8a-CD4-GFP	0.55
IL2R α	0.63
Vamp8-MT-GFP	0.66
IL2R α -GFP	0.70
CD8a	0.54
SSTR3-GFP	3.54
Arl13b	5.43

The dynamics of proteins was analyzed using 2 parameters: recovery at half-time ($t_{1/2}$) and the immobile fraction. $t_{1/2}$ reflects the speed of recovery while the immobile fraction is the measure of the percentage of protein in the bleached area that cannot be exchanged. The immobile fraction of resident ciliary membrane proteins, such as SSTR3-GFP (0.7) and Arl13b-mCherry (0.9), was very high, whereas CD8a-GFP was highly mobile, with a low immobile fraction of 0.3 (Fig. 3.5A). Though there is some recovery $t_{1/2}$ of resident cilia membrane protein SSTR3-GFP cannot be calculated, as the sample size is insufficient to get a conclusion (Fig. 3.6B). Arl13b-mCherry has no recovery as only newly synthesized protein can recover the fluorescence (Fig. 3.6C). The non-resident cilia membrane protein CD8a-GFP had a very low $t_{1/2}$, 54.1 seconds (Fig. 3.6D). Previous studies of SSTR3-GFP and Arl13b-mCherry reported that these 2 proteins are highly mobile within cilia, but not across cilia and PMs [176-178]. But their experiments face the same problem of insufficient sampling.

Figure 3.6: Resident and non-resident membrane proteins have different properties

A) Quantification of immobile fraction of CD8a-GFP (0.3), SSTR3-GFP (0.7) and Arl13b-mcherry (0.9) (n (5-18) $\pm$ SEM). B-D) Intensity vs time plot shows the recovery of SSTR3-GFP (~6%), CD8a-GFP (>60% recovery) and Arl13b-mCherry after FRAP. E) Time course images of SSTR3-GFP, CD8a-GFP and Arl13b-mCherry, showing their respective recovery time. Scale bar, 5 μ m. The results are from a single experiment.

Generally in the FRAP, a high immobile fraction usually means high retention, which could be due to the retention or selective entry mechanisms. For example, it has been reported that the interaction between BBSome and SSSTR3 could provide a retention mechanism [40]. But the highly mobility within the cilia cannot be explained by receptor binding. In the selective entry mechanism, transport receptors selectively facilitate the crossing of ciliary residents through the diffusion barrier by binding to their CTSs. The differences in CPIR values, immobile fractions, and $t_{1/2}$, therefore, suggest that resident ciliary proteins could have distinct transport receptors for their ciliary targeting and/or retained inside the cilia by receptor binding mechanisms.

3.7 Dynamin-1(K44A) blocks CD8a-GFP endocytosis

We subsequently addressed how a PM protein, such as CD8a, could enter the ciliary membrane. Two pathways are known for the ciliary targeting of membrane cargos: lateral and polarized exocytosis transport pathway [40, 109].

To find out the pathway through which CD8a localizes to cilia, it was necessary to verify the effect of each of two pathways on ciliary localization. In the polarized exocytosis pathway, a membrane protein is packed into a vesicle derived from either the secretory pathway or the endocytic pathway. The vesicle specifically fuses to the PM near the ciliary base (periciliary membrane) and the membrane protein subsequently enters cilia [179]. First we studied the effect of endocytic pathway on ciliary localization pathway of CD8a. Endocytosis is required for the recycling pathway but not for lateral transport. Examining the sequence of its cytosolic domain, CD8a has no recognizable endocytic motif. Dynamin is essential for clathrin or caveolae-mediated endocytosis. For example, Dynamin polymerizes around the neck of a clathrin-coated pit, and its GTP-hydrolysis-dependent structural reorganization triggers constriction or stretching of the membrane to promote fission [180]. In order to inhibit endocytosis, we used dynamin-1 harboring K44A mutation, which impairs GTP binding and or hydrolysis cycles and imposes dominant negative effects on endocytosis [181-183].

CD8a-furin was used as a positive control as it undergoes endocytosis through clathrin-dependent pathway [184]. CD8a-furin was co-transfected with dyn-1(K44A)-GFP in HeLa cells. After 24 hours, cells were surface-labeled with OKT8 mAb and

chased at 37°C for various length of time. As shown in Fig. 3.4.1, CD8a-furin has a very high endocytic rate and reaches the Golgi via endosomes. However, when co-transfected with dyn-1(K44A)-GFP, the internalization of OKT8 mAb bound to CD8a-furin was blocked (Fig. 3.7.1A). This suggests that co-transfection with dyn-1(K44A) successfully blocked endocytosis. After validating the effect of dyn-1(K44A), we co-transfected dyn-1(K44A)-Myc with CD8a-GFP and performed an acid wash assay (see Materials and Methods), and verified its effect on CD8a-GFP endocytosis. As shown in the Fig. 3.7.1B, the endocytosis of CD8a-GFP was blocked by dyn-1(K44A) (Fig. 3.7.1B). Here, we could not use anti-Myc antibody to show co-transfection, as the OKT8 antibody used for internalization assay was also of mouse origin. The high co-expression rate of CD8a-GFP with dyn-1(K44A)-Myc can be observed in Fig. 3.7.1C. Thus, dyn-1(K44A) can be used to block the endocytic recycling pathway of CD8a.

Figure 3.7: Dyn-1(K44A) blocks endocytosis of CD8a-furin and CD8a-GFP

A) Immunofluorescence images of HeLa cells co-expressing CD8a-furin and dyn-1(K44A)-GFP, surface-labeled with OKT8 mAb (red) against extracellular domain of CD8a-furin. The images are representative of OKT8 internalized for various time points 0, 10, 20, 40, and 80 minutes, fixed and stained with secondary antibody. Scale bar 10 μ m. B) Immunofluorescence images of HeLa cells co-expressing CD8a-GFP and dyn-1(K44A)-Myc, surface-labeled with OKT8 mAb against extracellular domain of CD8a-GFP. The top image of the panel shows the OKT8 mAb labeling of CD8a surface pool, fixed and stained with secondary antibody. The remaining images are representative of OKT8 internalized for various time points 0, 20, 40, and 80 minutes, followed by acid wash, fixed and stained with secondary antibody. Scale bar, 10 μ m. C) Immunofluorescence images of HeLa cells showing co-expression of CD8a-GFP and dyn-1(K44A)-Myc, fixed and stained with Myc mAb. Scale bar, 10 μ m. D) Immunofluorescence image of RPE1 cells expressing CD8a-GFP fixed and stained with Golgi marker giantin (mouse mAb). Scale bar, 10 μ m. E) Dyn-1(K44A) inhibited the endocytosis of CD8a-GFP. RPE1 cells co-expressing CD8a-GFP and dyn-1(K44A)-Myc were incubated with anti-CD8a antibody on ice for 60 min. After washing away unbound antibody, cells were warmed up to 37°C for indicated time before treatment with ice cold acid to remove surface bound antibody. The internalized antibody was fluorescence stained. The total intensity of the internalized antibody was divided by the total intensity of GFP and plotted against the incubation time before acid wash. Error bar, SEM. Mean values are indicated. n ranges from 36 to 100. The results are from a single experiment.

Second, membrane proteins packed into a vesicle derived the Golgi or endosomes. Staining CD8a-GFP expressing RPE1 cells with giantin (a Golgi marker) showed no Golgi co-localization (Fig. 3.7.1D). This in turn indicates that CD8a is secreted continuously and fuse with plasma membrane. This prompted us to suspect the role of secretory pathway, in CD8a-GFP ciliary localization.

3.8 CD8a accesses cilia by the lateral transport pathway

To rule out the contribution from endocytic pathway, we inhibited endocytosis by over-expressing dyn-1(K44A) [182]. Brefeldin A (BFA) is a fungal metabolite that is widely used to study membrane traffic. BFA treatment results in rapid inactivation and dissociation of Arf1 and subsequently causes the disassembly of the Golgi complex and leading to its collapse into the ER. This, in turn, leads to the inhibition of

membrane trafficking from the Golgi complex to the plasma membrane [162, 185, 186]. Therefore, treatment with BFA prevents the newly synthesized CD8a from reaching to cilia. Hence, BFA was used for 30 minutes, before fixing cells to block potential polarized exocytosis pathway (if it exists) [186]. Under such conditions, the whole cilium FRAP was conducted, and the $t_{1/2}$ of CD8a-GFP was measured. The $t_{1/2}$ of CD8a-GFP under 3 conditions 1) BFA; 2) dyn-1(K44A)-Myc; 3) dyn-1(K44A) together with BFA were found to be 46.0, 38.9, and 24.8 seconds respectively (Fig. 3.8.1A). The immobile fractions of CD8a-GFP under 1) BFA; 2) dyn-1(K44A)-Myc; 3) dyn-1(K44A) together with BFA were found to be 0.3, 0.3, and 0.4 respectively (Fig. 3.8.1B). A comparison of CD8a-GFP recovery with recoveries under inhibited endocytic or polarized exocytosis pathways showed no significant difference. The immobile fractions also showed no significant difference (Fig. 3.8.1B). However, under inhibition of both endocytic and polarized exocytosis pathways, where the lateral transport pathway would be expected to be the sole mechanism to access cilia, $t_{1/2}$ of CD8a-GFP was found to decrease to 24.8 ($n=5$, $p=4\times 10^{-3}$) (Fig. 3.8.1A). We could not find a satisfactory explanation for the significantly increased exchange dynamics. Currently, the approach to specifically block the lateral diffusion is not available; therefore, we could not directly demonstrate the necessary role of the lateral transport in ciliary localization of CD8a. Our data suggests the lateral transport pathway could be involved in the cilia entry of CD8a.

Figure 3.8.1: Exocytosis and recycling pathway having no effect on ciliary dynamics of CD8a

A) Quantification of $t_{1/2}$ of CD8a-GFP, CD8a-GFP with BFA, CD8a-GFP with dyn-1(K44A)-Myc and CD8a-GFP with dyn-1(K44A)-Myc and BFA in seconds (Mean $\pm$ SEM) (Note: CD8a-GFP from Fig. 3.6 used for comparison). B) Quantification of immobile fractions of CD8a-GFP, CD8a-GFP with BFA, CD8a-GFP with dyn-1(K44A)-Myc and CD8a-GFP with dyn-1(K44A)-Myc and BFA (Mean $\pm$ SEM) (Note: CD8a-GFP from Fig. 3.6 used for comparison). The results are from a single experiment.

An alternative approach was adopted to demonstrate that the lateral transport is sufficient for the cilium entry of CD8a. The assay took advantage of our discovery that incubation with a monoclonal antibody against CD8a, under live cell conditions, significantly reduced the ciliary localization of CD8a, probably due to the selective crosslinking and subsequent trapping of cell surface CD8a on the PM (note that CD8a forms a homodimer). Ciliated RPE1 cells co-expressing CD8a-GFP and dyn-1-(K44A) were treated with BFA at 37°C for 30 minutes and the CPIR was measured to be 0.37, similar to the pre-BFA treatment (0.41) (Fig. 3.8.2). After cells were incubated with OKT8 on ice for 60 minutes, there is a significant decrease in the CPIR of CD8a-GFP to 0.23 ($p=4 \times 10^{-2}$) compared to pre-OKT8 treatment (i.e., CD8a-GFP/dyn-1-(K44A) treated with BFA). After removing the surface-bound CD8a antibody by an ice cold acid wash, the ciliary localization rapidly recovered with a CPIR of 0.53 ($p=5 \times 10^{-4}$); and with further incubation at 37°C for 60 minutes, the

CPIR stabilized at 0.44, suggesting that CD8a could traffic from PM to cilia via lateral diffusion. In contrast, when warmed up to 37°C, cells with surface-bound antibody but without acid wash did not recover their ciliary localization and the CPIR of CD8a-GFP remained at 0.27 (Fig. 3.8.2). Our assay demonstrated the forward and reverse lateral transport of CD8a between the ciliary and PM membrane pool under conditions in which both polarized exocytotic and endocytic recycling pathways were inhibited, suggesting that PM proteins could laterally diffuse through the membrane barrier to the ciliary membrane.

Figure 3.8.2: CD8a accesses cilia by the lateral transport pathway

A) Quantification of CPIR values of CD8a-GFP under different conditions shown by pictorial representation (Mean $\pm$ SEM). B) IF images of RPE1 cells expressing CD8a-GFP were[45] starved for 2 days, treated with different conditions, fixed and stained with Arl13b pAb (blue). For time point 1, cells were stained with Myc mAb (red) and Arl13b pAb (blue). In case of time point 2 cells were stained with giantin (red) and acetylated α -tubulin (blue). Scale bar, 10 μ m. The results are from a single experiment.

3.9 Ciliary localization of non-resident PM proteins is size-dependent

We observed that the ciliary localization of CD8a was affected when tagged with GFP, which is evident from CPIR value and images (Fig. 3.3.1 and Table 7). To

study the effect of size on the ciliary localization of CD8a, the length of the cytosolic tail of CD8a was increased by fusing to 1, 2 and 3 GFP molecules (Fig.3.9A). RPE1 cells were transfected with CD8a, CD8a-GFP, CD8a-GFP×2, and CD8a-GFP×3, along with Arl13b-mCherry, and the CPIR values were measured. It was observed that the CPIR of CD8a sharply decreased when the size of cytosolic tail increased (Fig 3.9B-D). CD8a-GFP showed significant localization to cilia (but still lower compared to CD8a), whereas CD8a-GFP×2 and CD8a-GFP×3 were essentially undetectable in cilia (Fig 3.9B-D). A similar trend was observed in the cases of RP2-7aa tagged to GFP and GFP×3, CAAX domain with GFP and GFP×3 (Fig. 3.9E-G). Tagging the cytosolic tail of CD8a with 0-3 GFP molecules results in increased cytosolic volume or steric hindrance in crossing the membrane diffusion barrier at the ciliary base. Our data therefore suggests that ciliary diffusion barrier could be leaky and the diffusion of membrane proteins to cilia is size dependent. This is similar to the transport of inner nuclear membrane proteins where, the inner nuclear membrane proteins with cytosolic/nucleoplasmic domains greater than ~70 kDa fail to localize to the inner nuclear membrane [75, 76, 187-189].

Figure 3.9: Ciliary localization of non-resident PM proteins is dependent on size

A) Schematic representation of CD8a constructs with increase in size of the cytoplasmic tail using GFP, GFP×2, and GFP×3. B) Quantification of ciliary localization of CD8a, CD8a-GFP, CD8a-GFP×2 and CD8a-GFP×3 using CPIR values (Mean ± SEM). The results are from three independent experiments. C) Western blot showing the expression of CD8a-GFP, CD8a-GFP×2 and CD8a-GFP×3 constructs. D) Immunofluorescence images of RPE1 cells co-expressing CD8a, CD8a-GFP, CD8a-GFP×2 or CD8a-GFP×3 with Arl13b-mCherry, starved for 2 days and fixed. Scale bar, 10 μm. E) Quantification of ciliary localization of RP2-7aa-GFP and RP2-7aa-GFP×3 using CPIR values (Mean ± SEM). F) Quantification of ciliary localization of GFP-CAAX and GFP×3-CAAX using CPIR values (Mean ± SEM). G) Immunofluorescence images of RPE1 cells co-expressing GFP-CAAX or GFP×3-CAAX with Arl13b-mCherry were starved for 2 days and fixed. Scale bar, 10 μm. The results are from a single experiment.

3.10 Importin binding motifs or domains increase ciliary localization of membrane reporters

A few recent studies have revealed an interesting role of importins, which are nucleocytoplasmic trafficking factors, in targeting resident cilia proteins, such as KIF17 [94], Crumbs3 CLIP1 isoform [91] and RP2 [190]. Various importin binding motifs or domains are tested for their capabilities in targeting CD8a to cilia. To the C-terminal cytosolic tail of CD8a, we fused the following importin binding motifs or domains [79]: 1) SV40 large T antigen classical NLS, which binds to importin-α and importin-β1 heterodimer [73], 2) importin-β1 binding domain of importin-α [44], which binds to importin-β1 [191] and 3) the basic PY-NLS (bPY-NLS) motif of hnRNPM, which binds to TNPO1 [83]. Compared to CD8a-GFP×2 and GFP×3 (which cannot localize to cilia), chimeras tagged with SV40-cNLS, IBB, and bPY-NLS showed significantly increased ciliary localization (Fig. 3.10.1A and C). CPIRs of CD8a-GFP, -GFP×2 and GFP×3 increased significantly; in particular, CD8a-GFP×2 and GFP×3 increased by more than double when tagged with SV40-cNLS, IBB and bPY-NLS (Fig. 3.10.1B and D). Collectively, our data demonstrates that importin binding motifs/domains, such as SV40-cNLS, IBB, and bPY-NLS, are sufficient to drive the type I membrane reporter CD8a to cilia.

Figure 3.10.1: Importin binding motifs/domains are sufficient to drive CD8a to cilia

A) Immunofluorescence images of RPE1 cells co-expressing CD8a-GFP, CD8a-GFPx2, CD8a-GFPx3, CD8a-NLS-GFP, CD8a-NLS-GFPx2 or CD8a-NLS-GFPx3 with Arl13b-mCherry (red) were starved for 2 days and fixed. Scale bar, 10 μ m. B) Quantification of ciliary localization of CD8a-GFP, CD8a-GFPx2, CD8a-GFPx3, CD8a-NLS-GFP, CD8a-NLS-GFPx2 and CD8a-NLS-GFPx3 using CPIR values (Mean $\pm$ SEM). The results are from two independent experiments. C) Immunofluorescence images of RPE1 cells expressing CD8a-GFP, CD8a-IBB-GFP, CD8a-hnRNPM-GFP, CD8a-GFPx3, CD8a-IBB-GFPx3 or CD8a-hnRNPM-GFPx3, starved for 2 days, fixed and stained with Arl13b pAb. Scale bar, 10 μ m. D) Quantification of the ciliary localization of CD8a-GFP, CD8a-IBB-GFP, CD8a-hnRNPM-GFP, CD8a-GFPx3, CD8a-IBB-GFPx3 and CD8a-hnRNPM-GFPx3 using CPIR values (Mean $\pm$ SEM). The results are from a single experiment.

FRAP was employed to examine the role of importin binding motifs/domains in driving ciliary localization of CD8a (Fig. 3.10.1). FRAP $t_{1/2}$ of CD8a-GFP was observed to be 54 ± 8 ($n=18$) seconds. When fused to IBB or bPY-NLS, the resulting CD8a-GFP chimeric constructs had a significantly reduced $t_{1/2}$ of 29 ± 3 ($n=17$, $p=0.01$) seconds and 33 ± 3 ($n=13$, $p=0.02$) seconds respectively. However, there was

no significant difference in the immobile fractions of CD8a-hnRNPM-GFP and CD8a-IBB-GFP. Thus, analysis of FRAP results suggest that importin- β 1 and TNPO1 could facilitate membrane cargos in overcoming the membrane diffusion barrier of cilia.

Figure 3.10.2: Importin binding motifs/domains increase the dynamics of CD8a to cilia

A) Graph indicates quantification of the $t_{1/2}$ of CD8a-hnRNPM-GFP, CD8a-GFP and CD8a-IBB-GFP in seconds (Mean $\pm$ SEM). B). Graph indicates quantification of the immobile fractions of CD8a-hnRNPM-GFP, CD8a-GFP and CD8a-IBB-GFP (Mean $\pm$ SEM). C) Time course images of CD8a-hnRNPM-GFP, CD8a-IBB-GFP and CD8a-GFP representing their respective recovery time. Scale bar, 5 μ m. The results are from a single experiment.

3.11 CTSs of fibrocystin and retinol dehydrogenase interact with TNPO1

Since importin- β 1 or TNPO1 binding motifs/domains are sufficient to drive ciliary localization of membrane reporters, we hypothesized that there could be native ciliary membrane residents that utilize this mechanism for targeting. As discussed in the introduction section 1.2.5, CTSs of some ciliary transmembrane and peripheral membrane proteins are known. Of these, only ten CTSs were shown to be necessary and sufficient for their localization to cilia (see Table 1). Among those, rhodopsin and NPHP3 CTSs were not included, as their sequence consisted of more than 40 amino acids. The CTSs used were from fibrocystin, cystin, PC-1, PC-2, retinol dehydrogenase, peripherin, SSTR3, and RP2. To test our hypothesis, these eight CTSs of 7-30 residues long were screened for their interaction with importins. The CTSs were fused to the C-terminus of GST and the resulting fused proteins were used as baits to incubate with cell lysate during the GST pull-down assay. We found GST-fused fibrocystin-CTS (hereafter referred to as f-CTS) and retinol dehydrogenase-CTS specifically pulled down endogenous TNPO1. However, the CTSs of cystin, PC-1, PC-2, peripherin, SSTR3 and RP2 did not pull-down endogenous TNPO1 (Fig. 3.11). None of the eight sequences screened showed any interaction with importin- β 1. Amino acid sequences of fibrocystin and retinol dehydrogenase-CTSs do not conform to the PY-NLS consensus motif, which is known to be recognized by TNPO1 [83]. Since f-CTS showed more robust interactions with TNPO1 than retinol dehydrogenase-CTS, f-CTS was the focus for our subsequent studies.

Figure 3.11: CTSs of fibrocystin and retinol dehydrogenase interact with TNPO1

GST fusion proteins of fibrocystin, cystin, PC-1, PC-2, retinol dehydrogenase (RDH), peripherin, SSTR3 and RP2 were incubated with HEK 293T cell lysate and the pull-down sample was blotted with

TNPO1 and importin- β 1 antibodies. Loading of GST fusion proteins was shown by Coomassie staining. The results are from two independent experiments.

3.12 f-CTS specifically interacts with TNPO1

During GST pull-down experiment, GST fused f-CTS (Fig. 3.12A) specifically pulled down endogenous TNPO1, but not β 1 from HEK 293T cell lysate, while GST did not pull-down either of importins (Fig. 3.12A). Full-length fibrocystin is a type I transmembrane protein, hence, its cytoplasmic tail is available for binding to trafficking machinery. Lacking the full-length fibrocystin construct due to its unusually long coding sequence (~12 kbp), we constructed a fibrocystin mimetic fusion protein by replacing the transmembrane and cytosolic domain of CD8a with the corresponding domains of mouse fibrocystin (Fig. 3.12B). The resulting clone, named CFF, is expected to be more physiological than GST-f-CTS, as it has a native transmembrane and cytosolic domain. CFF Δ C was generated by deleting the C-terminus, and it contains only the transmembrane domain and f-CTS. CD8a-f-CTS was generated by replacing the cytosolic domain of CD8a by f-CTS.

Figure 3.12: f-CTS specifically interacts with TNPO1

A) For pulldown HEK 293T cell lysate was incubated with GST and GST-f-CTS, and the pull-down sample was blotted with importin-β1 and TNPO1. Loading of GST fusion proteins was shown by Coomassie staining. B) Schematic representation of various fibrocystin constructs. C) HEK 293T cells expressing CD8a-hnRNPM-GFP, CD8a-f-CTS-GFP, CFFΔC-GFP, CFF-GFP, IL2R-GFP and CD8a-GFP were immunoprecipitated with GFP-Trap. The immunoprecipitated sample was blotted with GFP and TNPO1 to detect GFP constructs and endogenous TNPO1 respectively. D) HEK 293T cells co-expressing f-CTS-GFP or GFP with untagged TNPO1 were immunoprecipitated with GFP-Trap. The immunoprecipitated sample was blotted with GFP and TNPO1 to detect GFP constructs and TNPO1 respectively. The results are from 3 independent experiments.

When expressed in HEK 293T cells, CD8a-hnRNPM-GFP (positive control), CD8a-f-CTS-GFP, CFF-GFP and CFFΔC-GFP co-immunoprecipitated with endogenous TNPO1, suggesting that f-CTS is sufficient for fibrocystin to interact with TNPO1 (Fig. 3.12C). The interaction between f-CTS and TNPO1 is specific since IL2R-GFP and CD8a-GFP did not pull-down TNPO1. f-CTS, when fused to the N-terminus of GFP, has been reported to be a palmitoylated membrane protein that localizes to cilia [101]. f-CTS-GFP, but not GFP could co-immunoprecipitate

endogenous TNPO1 (Fig. 3.12D). Collectively, our data demonstrates that fibrocystin interacts with TNPO1 via its CTS.

3.13 Fibrocystin chimeras behave similarly to resident ciliary membrane protein

To confirm that all the f-CTS constructs behave as resident cilia-localized proteins, we quantified their CPIR values. CD8a-f-CTS-GFP, CFF Δ C-GFP and CFF-GFP chimeric constructs have very high CPIR values (>8) (Fig. 3.13 D-E). FRAP analysis of CD8a-f-CTS-GFP and CFF-GFP showed that their $t_{1/2}$ were around 10.6 and 8.1 seconds respectively, with no significant difference between them (Fig. 3.13A). The CD8a-f-CTS and CFF-GFP immobile fraction was very high, in contrast to CD8a-GFP, which had a low immobile fraction. Consistent with very high immobile fractions of resident ciliary membrane proteins, the immobile fractions of CD8a-f-CTS-GFP and CFF-GFP were 0.7 and 0.8 respectively (Fig. 3.13B). These two factors suggest that the fibrocystin chimeras might behave as native cilia resident membrane proteins.

Figure 3.13: Fibrocystin chimeras behave similarly to resident ciliary membrane proteins

A) Quantification of $t_{1/2}$ of CD8a-f-CTS-GFP, CFF-GFP and its comparison with other resident and non-resident ciliary membrane proteins in seconds (Note: CD8a-hnRNPM-GFP, CD8a-GFP and CD8aIBB-GFP shown in Fig. 3.10.2 were used to show comparison) (Mean $\pm$ SEM). B) Quantification of immobile fractions of CD8a-f-CTS-GFP, CFF-GFP and its comparison to other resident and non-resident ciliary membrane proteins (Note: CD8a-hnRNPM-GFP, CD8a-GFP, CD8a-IBB-GFP and SSTR3-GFP shown in Fig. 3.6 & Fig. 3.10 were used for comparison) (Mean $\pm$ SEM). C) Representative images of CD8a-f-CTS-GFP recovery after FRAP. Scale bar, 5 μ m. D) Quantification of ciliary localization of CD8a-GFP, CD8a-f-CTS-GFP, CFF Δ C-GFP and CFF-GFP using CPIR values. E) Immunofluorescence image of RPE1 cells expressing CD8a-f-CTS-GFP, CFF Δ C-GFP and CFF-GFP, starved for 2 days, surface-labeled with OKT8 mAb, fixed and stained with Arl13b pAb (red). Scale bar, 10 μ m. The results are from a single experiment.

3.14 The interaction between f-CTS and TNPO1 is probably not regulated by Ran GTPase

In order to determine the region important for the interaction of TNPO1 with f-CTS, N-terminal deletion constructs of TNPO1 were constructed. The $\Delta 315$, $\Delta 539$ and $\Delta 699$ TNPO1 truncation constructs lack the Ran-GTP binding domain, acidic loop and M9 binding regions respectively (Fig. 3.14A). During GST pull-down assay, HEK 293T lysate expressing Myc-tagged N-terminal truncation constructs of TNPO1 and negative control, Myc-importin- $\beta 1$ were incubated with immobilized GST-f-CTS. Myc- $\Delta 315$ -TNPO1 construct which lack Ran-GTP binding domain can interact with GST-f-CTS. Though the Myc- $\Delta 539$ -TNPO1 construct had M9 binding region, its interaction with GST-f-CTS was abolished. Therefore, the region from 315-540 amino acids, which represents the acidic-loop of TNPO1, is important for the interaction with GST-f-CTS. Consistent with this result it was found that Myc- $\Delta 699$ -TNPO1 construct also lost interaction with GST-f-CTS. Furthermore, as shown in Fig. 3.1.2A, f-CTS did not interact with Myc-importin- $\beta 1$ (Fig. 3.14B). Our data indicates that the interaction of fibrocystin with TNPO1 is different from the classical interaction of bPY-NLS with TNPO1 [87]. It has been reported that the importin/cargo complex could be disassembled by Ran-GTP's binding to importin [79]. We prepared recombinant GST-Ran with wild type, T24N (GDP form) and Q69L (GTP form) mutations and observed that TNPO1 preferred to interact with GST-Ran-Q69L significantly more than GST-Ran-wild type (wt) and T24N, (Fig. 3.14C) [92, 192, 193]. However, we found that saturating amount of GST-Ran-Q69L did not reduce the interaction between CFF and endogenous TNPO1 (Fig. 3.14D). Furthermore, the CPIR of f-CTS-GFP was not affected by overexpressing Ran-Q69L as observed from CPIR of f-CTS-GFP vs Ran-Q69L expression (Fig. 3.14E-F). Our data, therefore, suggest that the interaction between f-CTS/TNPO1 is probably not regulated by Ran GTPase, in contrast to the reported binding and regulation of TNPO1 to RP2 [194].

Figure 3.14: The interaction between f-CTS and TNPO1 is probably not regulated by Ran GTPase

A) Schematic representation of the domain structure of TNPO1 indicating Ran-GTP binding domain, acidic loop (black square) and its truncations. On the right the positive and negative interactions were represented with (+) and (-) respectively. B) During GST pull-down assay HEK 293T lysate, expressing Myc-tagged N-terminal truncation constructs of TNPO1 and negative control, Myc-importin- β 1 were incubated with GST-f-CTS. The pull-down sample was blotted with Myc antibody to detect TNPO1, its truncations and importin- β 1. Loading of GST fusion protein was shown using Coomassie staining. C) During GST pull-down assay HEK 293T lysate, was incubated with GST-Ran-WT, GST-Ran-Q69L, GST-Ran-T24N and GST. The pull-down sample was blotted with TNPO1 antibody to detect TNPO1. Loading of GST fusion protein was shown using Coomassie staining. D) HEK 293T cells expressing CFF-Myc was immunoprecipitated with Myc mAb in the presence of GST-Ran-WT, GST-Ran-Q69L, GST-Ran-T24N and GST. The immunoprecipitated sample was blotted with Myc and TNPO1 to detect CFF-Myc and TNPO1 respectively. Loading of GST fusion protein

was shown using Coomassie staining. E) Immunofluorescence image of RPE1 cells co-expressing f-CTS-GFP and Ran-Q69L-mcherry, starved for 2 days, fixed and stained with Arl13b pAb (red). Scale bar, 10 μ m. The cells expressing various levels of Ran-Q69L-mcherry (high and low) are written on the side of images. F) Graph indicates the CPIR of f-CTS-GFP relative to the expression of Ran-Q69L-mcherry. The dotted line indicates the mean of f-CTS-GFP CPIR value. The results are from a single experiment.

3.15 The amino acid motif KTRK is essential for the ciliary targeting of fibrocystin

To characterize the interaction of f-CTS with TNPO1, mutational constructs of f-CTS were generated using alanine-scanning mutagenesis, as shown in Fig. 3.15A. We found that mutational construct CFF-(KTRK)-Myc generated by the mutation of four conserved residues—KTRK—to alanine (Fig. 3.15A), failed to interact with TNPO1 (Fig. 3.15B). HEK 293T lysate, expressing Myc-tagged CFF-WT, CFF Δ C-WT, mutational constructs of CFF and untransfected lysate (negative control), was immunoprecipitated with Myc antibody. All the constructs except CFF-(KTRK)-Myc and untransfected lysate, were able to co-immunoprecipitate endogenous TNPO1. To further confirm this interaction, a GST pull-down experiment was performed. During GST pull-down, HEK 293T lysate was incubated with immobilized GST-f-CTS, GST-f-CTS-(KTRK) and GST. GST-f-CTS wild type successfully pulled down TNPO1, whereas GST as well as GST-f-CTS-(KTRK), did not pull-down endogenous TNPO1 present in HEK 293T cell lysate (Fig. 3.15C). The corresponding mutation also disrupted the ciliary localization of the fibrocystin mimetic protein—CFF-GFP (Fig. 3.15 D-E.), therefore suggesting that the interaction with TNPO1 is essential for ciliary targeting of fibrocystin.

Figure 3.15: The amino acid motif KTRK is essential for the ciliary targeting of fibrocystin

A) Amino acid sequence of f-CTS and its alanine-scanning mutations, mutated residues were shown in red. B) HEK 293T expressing Myc-tagged WT and mutational constructs of CFF, CFFΔC-WT and untransfected cell lysate were immunoprecipitated with Myc antibody. The immunoprecipitated sample was blotted with TNPO1 and Myc mAb to detect endogenous TNPO1 and Myc-tagged constructs respectively. The region of TNPO1 IP blot corresponding to CFF-(KTRK)-Myc lane was surrounded by dashed rectangle. C) During pulldown the HEK 293T cell lysate was incubated with GST-f-CTS, GST and GST-f-CTS-(KTRK). The pulldown sample was blotted with TNPO1 to detect endogenous TNPO1. Loading of GST fusion proteins was shown using Coomassie staining. The results are from a single experiment. D) Immunofluorescence images of RPE1 cells, expressing CFF-GFP and mutation constructs, starved for 2 days, surface-labeled with OKT8, fixed and stained with Arl13b pAb (red). The KTRK-AAAA mutation which disrupted the localization was surrounded by a dashed rectangle. Scale bar, 10 μm. E) Quantification of ciliary localization of CFF-GFP (wild type) and its alanine mutational GFP constructs LV, WF, CLVCC, KKS, KTRK using CIPR values. The KTRK-AAAA

mutation which disrupted the localization was surrounded by a dashed rectangle. (Note: CFF-GFP-WT was from the earlier result 3.1.3E as the experiment was performed parallel). The results are from a single experiment.

3.16 TNPO1 is essential for ciliary targeting of fibrocystin

To further explore the essential role of TNPO1 in ciliary targeting of fibrocystin, the ciliary localization of f-CTS was quantitatively studied when endogenous importin- β 1 or TNPO1 were selectively knocked down. The endogenous importin- β 1 level could be significantly reduced to ~20% via its siRNAs. It was found that TNPO1 was reduced to just ~50% even though the knockdown approach was similar. However, when endogenous TNPO1 was knocked down by ~50%, the CPIR value of f-CTS-GFP was significantly reduced to ~50% of the control ($p=14\times 10^{-12}$). In contrast, the CPIR value remained the same as control upon the knockdown of importin- β 1. Thus, knockdown experiment indicated that TNPO1 is essential for the ciliary targeting of fibrocystin. These results were further confirmed using a shRNA against TNPO1 (Fig. 3.16D-F). In addition, the CPIR value of CFF-GFP showed significant decrease ($p=3\times 10^{-3}$), when measured under siRNA knockdown of TNPO1 (Fig. 3.16G-I). All these results demonstrate that TNPO1 is essential for the ciliary targeting of fibrocystin.

Figure 3.16: TNPO1 is essential for ciliary targeting of fibrocystin

A) Western blots showing the siRNA knockdown efficiency of TNPO1 and importin- β 1 respectively. β -tubulin was used as loading control. B) Representative images f-CTS-GFP ciliary localization in GL2, importin- β 1 and TNPO1 siRNA transfected RPE1 cells. The cilia were stained with Arl13b pAb (red). Scale bar, 10 μ m. C) Quantification of the effect of GL2, importin- β 1 and TNPO1 knockdown on ciliary localization of f-CTS-GFP using CPIR values. The results are from 3 independent experiments. D) Western blots showing the shRNA knockdown efficiency of TNPO1. α -tubulin was used as loading control. The results from three independent experiments. E) Representative images f-CTS-GFP ciliary localization in GL2 and TNPO1 shRNA transfected RPE1 cells. The cilia were stained with Arl13b pAb (red). Scale bar, 10 μ m. F) Quantification of the effect of GL2 and TNPO1 knockdown on ciliary localization of f-CTS-GFP using CPIR values. The results are from a single experiment. G) Western blot showing the siRNA knockdown efficiency of TNPO1. GAPDH was used as loading control. H) Representative immunofluorescence images of CFF-GFP ciliary localization in GL2 and TNPO1 siRNA transfected RPE1 cells. The cells were surface labelled with OKT8 mAb, fixed and stained with Arl13b pAb (red). Scale bar, 10 μ m. I) Quantification of the effect of GL2 and TNPO1 knockdown on ciliary localization of CFF-GFP using CPIR values. The results are from a single experiment.

3.17 Rab8 regulates f-CTS localization and its interaction with TNPO1

3.17.1 Rab8-GDP affects the ciliary localization of CFFAC

Previous reports showed that the ciliary localization of f-CTS is affected, in Rab8-T22N (GDP-bound form) overexpressing cell line [101]. It was also reported previously that some peripheral membrane proteins behave similarly to soluble proteins (the hydrophobic region is shielded by other proteins) and enter cilia [195, 196]. Hence, here we used CFFAC and expected it to be more physiological than GFP-f-CTS. Consistent with the previous reports GFP-Rab8-T22N did not localize to cilia, whereas GFP-Rab8-WT and GFP-Rab8-Q67L localized to cilia. Moreover, GFP-Rab8-T22N expressing cells had very low percentage of ciliated cells. Co-expressing GFP, GFP-Rab8-WT, GFP-Rab8-Q67L or GFP-Rab8-T22N with CFFAC-Myc showed that ciliary localization of CFFAC-Myc was inhibited when co-expressed with GFP-Rab8-T22N (Fig. 3.17.1A). The quantification of CPIR values indicated a significant decrease in the ciliary localization of CFFAC-Myc in the presence of GFP-Rab8-T22N (Fig. 3.17.1B). The result is consistent with the previously published result, that Rab8 is required for the ciliary localization of fibrocystin [101].

Figure 3.17.1: Rab8-GDP affects the ciliary localization of CFFΔC

A) Immunofluorescence images of RPE1 cells co-expressing GFP, GFP-Rab8-WT, GFP-Rab8-Q67L or GFP-Rab8-T22N with CFFΔC-Myc were starved for 2 days, surface-labeled with OKT8 (red), fixed and stained with Arl13b pAb (blue). Scale bar, 10 μ m. B) Quantification of the ciliary localization of CFFΔC-Myc in the presence of GFP, GFP-Rab8-WT, GFP-Rab8-Q67L or GFP-Rab8-T22N using CIPR values (Mean $\pm$ SEM). The results are from a single experiment.

3.17.2 GST-f-CTS binds more to Rab8-T22N than –WT and –Q67L form

It was previously reported that f-CTS interacts with Rab8, with a preference for its GDP-bound form [101]. We similarly found that GST-f-CTS was able to pull-down the Rab8-GDP (Rab8-T22N), more than the wild type (WT) and Rab8-GTP (Rab8-Q67L) (Figure 3.17.2). The observation suggested an unconventional Rab interaction module, in which inactive or GDP form, instead of active or GTP form, participates in the interaction.

Figure 3.17.2: GST-f-CTS binds more to Rab8-T22N than –WT and –Q67L form

HEK 293T cell lysate expressing GFP-Rab8-WT, GFP-Rab8-Q67L and GFP-Rab8-T22N and GFP was incubated with GST-f-CTS. The pull-down sample was blotted with GFP mAb. Loading of GST fusion proteins is shown by Ponceau S staining. The results are from two independent experiments.

3.17.3 F-CTS interacts directly with Rab8 and TNPO1

In the above experiments, direct interaction between f-CTS and TNPO1 could not be confirmed as the cell lysate contains endogenous proteins. Hence, we

performed *in vitro* pull-down assay by purifying His-Rab8-WT, -Q67L and -T22N, GST-f-CTS and rabbit reticulocyte for TNPO1. Rabbit reticulocyte lysate of TNT mixture, when tested using western blot, showed presence of endogenous TNPO1 but not Rab8 (Fig. 3.17.4B). Hence, rabbit reticulocyte lysate was used to test the direct interaction of TNPO1 and f-CTS, given that the lysate was deficient of Rab8. GST-f-CTS pulled down TNPO1, whereas GST did not, indicating that GST-f-CTS can directly interact with TNPO1 (Fig. 3.17.4C). Similarly, when His-Rab8-WT, -Q67L and -T22N was incubated with GST-f-CTS or GST, only GST-f-CTS pulled down His-Rab8 forms, particularly His-Rab8-T22N (Fig. 3.17.4D). This indicates that Rab8-T22N interacts with f-CTS directly and this interaction does not require TNPO1.

Figure 3.17.3: F-CTS interacts directly with Rab8 and TNPO1

A) Rabbit reticulocyte lysate with pMyc-TNPO1 or pMyc-Rab8 plasmids was translated at 30°C for 2hrs. The translated sample was blotted with TNPO1 and Rab8, to detect endogenous or translated

TNPO1 and Rab8 respectively. B) Rabbit reticulocyte lysate (for TNPO1) was incubated with GST-f-CTS or GST. The pull-down sample was blotted with TNPO1 to detect the direct interaction. Loading of GST proteins was indicated by Coomassie staining. C) Purified His-Rab8-WT, -Q67L and -T22N was incubated with GST-f-CTS or GST. The pull-down sample was blotted with Rab8 to detect direct interaction. Loading of GST proteins was indicated by Coomassie staining. The results are from two independent experiments.

3.17.4 Rab8-GDP/f-CTS/and TNPO1 form a ternary complex

We subsequently explored how the three proteins bind. There are two possibilities 1) f-CTS could simultaneously interact with both Rab8 and TNPO1 to form a tripartite complex. 2) Alternatively, it could bind exclusively to either of them. To resolve these two possibilities, HEK 293T cells were co-transfected to co-express three proteins: HA-TNPO1, CFF-Myc and Rab8-GFP (wild type, Q67L or T22N forms) or GFP (as negative control) (Fig.3.1.7.4A). Immunoprecipitation of CFF-Myc led to co-precipitation of Rab8-T22N-GFP (Fig. 3.17.4A). This was consistent with the GST-f-CTS pull-down result shown in Section 3.17.2. Although TNPO1 was detected in all lanes, which was consistent with the finding that TNPO1 interacts with f-CTS; it was repeatedly observed that a significantly larger amount of TNPO1 was pulled down in the presence of Rab8-GDP, instead of the wild type and Rab8-GTP (Fig.3.17.4A). This suggests that f-CTS could simultaneously interact with both Rab8 and TNPO1 to form a tripartite complex.

As f-CTS interact with Rab8-GDP as well as TNPO1, we explored whether Rab8 interacts with TNPO1. To test whether Rab8-GDP could directly interact with TNPO1, GST-tagged Rab8-WT, -Q67L and -T22N proteins were purified. During GST pull-down, HEK 293T cell lysate expressing CD8a-GFP or CFF-GFP was incubated with GST-tagged Rab8-WT, -Q67L and -T22N or GST (negative control). Only GST-Rab8-T22N could pull-down endogenous TNPO1, and in addition, only in the presence of CFF-GFP (Fig. 3.17.3A). This suggests that the Rab8-GDP form interacts with TNPO1, but only in the presence of fibrocystin. To validate this result, a co-immunoprecipitation experiment using TNPO1 as bait was performed (Fig. 3.17.3B). Myc-TNPO1 was immunoprecipitated from HEK 293T cell lysate co-expressing CFF-HA or CD8aΔCyto-HA with Myc-TNPO1 and Rab8-GFP (wild type, Q67L or T22N forms). Myc-TNPO1 could co-immunoprecipitate GFP-Rab8-T22N, and in addition, only in the presence of CFF-HA (Fig. 3.17.3B). This confirms that the Rab8-GDP form interacts with TNPO1, but only in the presence of fibrocystin. Though there should be some interaction of Rab8 with TNPO1 in all the lanes due to

endogenous fibrocystin, it was not detectable under the current experimental conditions.

To verify the formation of the tripartite complex *in vitro*, a GST pull-down was performed by incubating rabbit reticulocyte lysate (for TNPO1), and His-Rab8-WT, -Q67L and -T22N with GST-f-CTS or GST. GST-f-CTS could pull-down TNPO1, and in addition, particularly in the presence of His-Rab8-T22N. GST used as negative control could not interact with either His-Rab8-WT, -Q67L and -T22N or TNPO1 (Fig.3.17.4E). This demonstrates that f-CTS interacts strongly with TNPO1 in the presence of Rab8-GDP form. Collectively, the most plausible explanation of our data is that Rab8-GDP, but not -GTP, could promote f-CTS to bind to TNPO1 to form a tripartite complex.

Figure 3.17.4: Rab8-GDP/f-CTS/and TNPO1 form a ternary complex

A) HEK 293T cell lysate co-expressing HA-TNPO1, CFF-Myc and Rab8-GFP (wild type, Q67L or T22N forms) or GFP (negative control) was immunoprecipitated with Myc antibody. The immunoprecipitated sample was blotted with Myc, HA and GFP antibodies to detect CFF-Myc, TNPO1, Rab8-GFP (wild type, Q67L or T22N forms) or GFP respectively. B) HEK 293T expressing CD8a-GFP or CFF-GFP was incubated with GST-Rab8 (wild type, Q67L or T22N forms) or GST (negative control). The pull-down sample was blotted with GFP and TNPO1 to detect CD8a-GFP or CFF-GFP and endogenous TNPO1 respectively. Loading of GST proteins was indicated by Coomassie staining. C) HEK 293T cell lysate co-expressing Myc-TNPO1, Rab8-GFP (wild type, Q67L or T22N forms) and CFF-HA or CD8aΔCyto-HA (negative control) was immunoprecipitated with Myc antibody. The immunoprecipitated sample was blotted with Myc, HA and GFP antibodies to detect TNPO1, CFF-HA, CD8aΔCyto-HA and Rab8-GFP (wild type, Q67L or T22N forms) respectively. D) To test the formation of tripartite complex *in vitro*, rabbit reticulocyte lysate (for TNPO1), and purified His-Rab8-WT, -Q67L and -T22N were incubated with GST-f-CTS or GST. The pull-down sample was

blotted with TNPO1 and Rab8. Loading of GST proteins was indicated by Coomassie staining. The results are from two independent experiments.

3.18 Model for the ciliary targeting of membrane proteins

In summary our results suggest that mobile PM proteins cross the ciliary diffusion barrier by lateral diffusion (Section 3.8). In the selective entry or receptor-mediated translocation mechanism, ciliary cargos possess signals such as CTSs that selectively interact with importins (Section 3.10). The retention of mobile proteins inside the cilia depends on their interaction with ciliary receptors. Immobilized PM proteins, such as those anchored by the cortical actin network or trapped by clathrin-coated pits, are excluded from ciliary localization (Section 3.4-3.5) (Fig. 3.18A).

Fibrocystin laterally diffuses to the periciliary membrane via lateral diffusion on the PM. Since Rab8-GDP is the predominant form outside cilia [112], fibrocystin (via f-CTS), TNPO1 and Rab8-GDP cooperatively assemble a ternary import complex (Section 3.17.4). Facilitated by TNPO1, the ternary complex crosses the membrane diffusion barrier at the transition zone. Once inside the cilium, or simultaneously during the translocation, the GDP of Rab8 is exchanged to GTP by its GEF, Rabin8 and/or RPGR [135, 136]. When Rab8-GDP is converted to –GTP, the ternary complex weakens and disassembles, therefore releasing fibrocystin to the ciliary membrane (Fig. 3.18B).

Figure 3.18: Model for the ciliary targeting of membrane proteins

A) Mobile PM proteins cross the ciliary diffusion barrier by lateral diffusion. In the selective entry or receptor-mediated translocation mechanism, ciliary cargos possess signals such as CTSs that selectively interact with importins. The retention of mobile proteins inside the cilia depends on their interaction with ciliary receptors. Immobilized PM proteins, such as those anchored by the cortical actin network or trapped by clathrin-coated pits, are excluded from ciliary localization. B) Fibrocystin laterally diffuses to the periciliary membrane via lateral diffusion on the PM. Since Rab8-GDP is the predominant form outside cilia, fibrocystin (via f-CTS), TNPO1 and Rab8-GDP cooperatively assemble a ternary import complex. Facilitated by TNPO1, the ternary complex crosses the membrane diffusion barrier at the transition zone. Once inside the cilium, or simultaneously during the translocation, the GDP of Rab8 is exchanged to GTP by its GEF, Rabin8 and/or RPGR. When Rab8-GDP is converted to -GTP, the ternary complex weakens and disassembles, therefore releasing fibrocystin to the ciliary membrane.

Chapter 4: Discussion

The PM and ciliary membrane share the same membrane sheet, yet their proteins and lipids specifically enriched in cilia [40]. The unique identity of cilia is maintained by the membrane diffusion barrier at the ciliary base, which restricts lateral membrane diffusion. How the ciliary resident membrane proteins cross the membrane diffusion barrier and accumulate in cilia is still not yet understood. Similar scenarios could be found at the inner nuclear envelope membrane (INM), which is in direct continuity with the Endoplasmic Reticulum (ER), including the outer nuclear envelope membrane (ONM) [197]. Nuclear pore complexes assemble at the connecting boundary between the INM and ONM, and impose membrane diffusion barriers to impede the entry of non-INM proteins. Similar to cilia, the INM is a membrane domain, distinct from the ER and ONM. Resembling nuclear pores at the INM/ONM junction, a functionally equivalent membrane diffusion barrier at the ciliary base has been proposed [40]; but little is known about their role in ciliary trafficking. Here, we attempted to elucidate the molecular and cellular mechanism on how CTS and their transport receptors work in the ciliary trafficking of membrane cargo.

We introduced a novel metric to quantify ciliary localization (Fig. 3.2), and examined various PM-localized membrane proteins, which are conventionally considered non-ciliary, and found that they have significant pools localized at cilia (Fig. 3.3.3). The ciliary localization of such proteins is dependent on their cytosolic molecular weight. Tagging the cytosolic tail of CD8a with 0-3 GFP molecules resulted in increased cytosolic volume or the steric hindrance for crossing of the membrane diffusion barrier at the ciliary base. This is similar to the transport of inner nuclear membrane proteins where, the inner nuclear membrane proteins with cytosolic/nucleoplasmic domains greater than ~70 kDa fail to localize to the inner nuclear membrane [75, 76, 187-189] (Fig. 3.9); similar to previous findings on soluble cargo [69, 70, 148, 177]. The lateral mobility is required here as immobilized PM proteins, such as those anchored by the cortical actin network or trapped by clathrin-coated pits, do not have significant ciliary localization (Fig. 3.4 and Fig. 3.5), consistent with the reported effect of actin-binding on ciliary localization [198, 199]. Using FRAP and antibody-induced ciliary depletion of CD8a, we further found that

membrane proteins access cilia by the lateral transport instead of the polarized exocytosis pathway (Fig. 3.8). Similar to what is known about the soluble diffusion barrier of cilia, our data therefore demonstrated that the ciliary membrane diffusion barrier is leaky, and PM proteins could passively diffuse across the barrier to cilia in a cytosolic molecular weight-dependent manner. Although both ciliary resident and non-resident membrane proteins could localize to cilia, resident proteins were observed to have much higher CPIRs and FRAP immobile fractions (Fig. 3.6). Our results parallel what we know about the INM, as non-INM proteins could also localize on the INM; but resident INM proteins have stronger localization and higher FRAP immobile fractions than non-INM proteins, due to the presence of their binding receptors at INM [90]. High FRAP immobile fractions are consistent with the existence of unidentified ciliary receptors for ciliary residents, which would enrich ciliary residents within the confined ciliary compartment (diffusion-retention mechanism).

The role of nuclear trafficking machinery on ciliary trafficking has been recently reported [36, 91, 92, 94]. We sought to better understand if nuclear localization signals could target membrane reporters to cilia. We found that importin- α , - β 1 and TNPO1 binding motifs/domains, including SV40-NLS, IBB and the bPY-NLS motif of hnRNP M, were sufficient to promote the ciliary localization of non-resident membrane reporters such as CD8a. FRAP experiments performed on whole cilia demonstrated that the IBB and bPY-NLS motifs of hnRNPM significantly decreased the $t_{1/2}$ of the CD8a reporter, therefore suggesting that importin binding motifs/domains could facilitate crossing of the membrane diffusion barrier of cilia (Fig. 3.10). Our finding is consistent with the role of importins in helping nuclear cargo overcome the diffusion barrier at the central and peripheral channel of the nuclear pore complex [197].

Similar to cilia, two mechanisms have been proposed previously to explain the targeting of INM proteins and an INM protein could adopt either or both of them [53]. In the diffusion-retention mechanism, specific components at the immediate vicinity of the INM, such as the chromatin, lamin or KASH domain containing proteins, serve as receptors to immobilize membrane cargo at the INM. In the selective entry or receptor-mediated translocation mechanism, INM cargo possess signals such as NLSs or INM-sorting motifs that selectively interact with importins [200, 201], which are cargo receptors in facilitating the translocation across nuclear pores. We provided

experimental evidence that importins and their binding motifs/domains could be receptors and signals respectively, for selective entry across the ciliary diffusion barrier. FRAP $t_{1/2}$ s of membrane reporters with importin binding motifs/domains were found to be significantly less than those without; while FRAP immobile fractions showed no significant difference. Subsequently it was identified that CTSs of two native proteins, retinol dehydrogenase and fibrocystin, bind to TNPO1 (Fig. 3.11). Furthermore, TNPO1 was found to be required for the ciliary localization of fibrocystin (Fig. 3.12). Together with previous findings on the role of importins in ciliary targeting Crumb3 and RP2 [91, 92], our data further strengthened the view that, similar to their nuclear role, importins could facilitate membrane cargo in crossing the ciliary diffusion barrier. Ciliary resident membrane proteins could utilize both the diffusion-retention and selective entry mechanisms to ensure that they not only have a high ciliary concentration, but also fast exchange between the ciliary and non-ciliary pool. Indeed, the ciliary resident membrane protein CFF was measured to have high CPIR and FRAP immobile fraction, but low FRAP $t_{1/2}$ (Fig. 3.13).

The cellular role of TNPO1 in ciliary targeting was further studied using fibrocystin, a type I transmembrane ciliary resident protein. Fibrocystin is known to be subjected to regulated proteolytic cleavage at its transmembrane domain to generate a nuclear targeted intracellular fragment [158]. A stretch of residues downstream of f-CTS was found to be the NLS of the intracellular fragment, but the role of the f-CTS/TNPO1 interaction in nuclear targeting could not be completely ruled out. While f-CTS was previously shown to be sufficient for ciliary targeting [101], using fibrocystin mimetic clone CFF, we further demonstrated that f-CTS is necessary and sufficient for ciliary targeting of fibrocystin (Fig. 3.15 and Fig. 3.16). Together with our finding that the CPIR, FRAP $t_{1/2}$ and immobile fraction of f-CTS are similar to those of CFF, it seems that all properties for the ciliary targeting of fibrocystin could be attributed to f-CTS.

Previously, Follit and colleagues discovered that f-CTS binds to Rab8 with a preference for its GDP-bound form [101]. In our case, after the interaction was confirmed (Fig. 3.17.2), we further found that Rab8-GDP, but not -GTP, significantly promotes the interaction between f-CTS and TNPO1 by forming a novel ternary protein complex (Fig. 3.17.4). It has been reported that Rab8-GTP is enriched while -GDP is absent in cilia (Fig. 3.17.1) [63]. The Rab8-GTP/GDP gradient from cilia to cytosol is probably due to the centrosomal or TZ localization of Rabin8 [135] and

RPGR [136], both of which are GEFs for Rab8. From our findings, the following model could be conceivable for the ciliary trafficking of fibrocystin: Fibrocystin laterally diffuses to the periciliary membrane via lateral diffusion on the PM. Since Rab8-GDP is the predominant form outside cilia, fibrocystin (via f-CTS), TNPO1 and Rab8-GDP cooperatively assemble a ternary import complex. Facilitated by TNPO1, the ternary complex crosses the membrane diffusion barrier at the transition zone. Once inside the cilium, or simultaneously during the translocation, the GDP of Rab8 is exchanged to GTP by its GEF, Rabin8 and/or RPGR. When Rab8-GDP is converted to Rab8-GTP, the ternary complex weakens and disassembles, therefore releasing fibrocystin to the ciliary membrane (Fig. 3.18B). Whether or how Rab GDI and/or GDF participate(s) in the ternary import complex is currently unknown and remains to be determined. Our model provides a novel role of Rab8 in ciliary trafficking, in which the Rab8 guanine nucleotide cycle regulates the crossing of ciliary membrane diffusion barrier. The Rab8-GTP gradient would confer both the directionality and driving force to specifically enrich resident cargo within cilia. Our model bears similarity to the Ran dependent nucleocytoplasm transport, in which Ran-GTP disassembles the importin: cargo complex in the nucleus. The Ran-GTP gradient, as maintained by its nucleus localized GEF—RCC1 and cytoplasmic localized GTPase activating protein (RanGAP), drives the directionality of the nuclear trafficking of cargo [77]. Since TNPO1 can bind to Ran-GTP, which is concentrated in cilia [36], it could be possible that the Rab8-GDP:TNPO1:f-CTS ternary complex is also regulated by Ran-GTP within cilia. However, we found that over expression of the Ran-GTP mutant form did not affect the ciliary localization of f-CTS (Fig. 3.14). Therefore, the role of Ran in the ciliary trafficking of fibrocystin is still open. Together with previous work, our study supports the view that importins could participate in ciliary trafficking as transport receptors. There are more than 20 importins, and they have multiple repeats of armadillo or HEAT domains that are capable of forming diverse interfaces to engage with a large repertoire of cargo [79]. Our screening showed that retinol dehydrogenase-CTS interacts TNPO1 apart from fibrocystin-CTS. It is interesting to see whether retinol dehydrogenase also follows the same pathway. It is tempting to speculate that there could be many more ciliary membrane residents that are regulated by importins for their ciliary targeting. Thus, in the future, it will be important to systematically screen ciliary resident membrane proteins for their interaction with importins.

Chapter 5: Future directions

- 1) My data indicated that clathrin-dependent endocytosis could play a role in limiting the ciliary access by PM proteins. However, the actual role of clathrin-dependent endocytosis in ciliary trafficking is unclear. Previous studies showed that clathrin-coated pits are highly prevalent at the ciliary pocket but not required for the formation of either cilia or flagella [46]. The effect of AP2 knockdown on clathrin-mediated endocytic cargos such as Vamp2 and Vamp8 will give us the more direct evidence about the role of clathrin-coated vesicles in ciliary targeting.
- 2) Our model provides a novel role of Rab8 in ciliary trafficking, in which the Rab8 guanine nucleotide cycle regulates the crossing of ciliary membrane diffusion barrier. The Rab8-GTP gradient would confer both the directionality and driving force to specifically enrich resident cargo within cilia. Though supported by our biochemical data, imaging the dynamics of the ternary complex *in vivo* could help us to further define its role.
- 3) Together with work from other labs, our study supports the view that importins could participate in ciliary trafficking as transport receptors. Our screening showed that retinol dehydrogenase-CTS and rhodopsin-CTS interact with transportin-1 in addition to fibrocytin-CTS. It is tempting to speculate that there could be many more ciliary membrane residents that are regulated by importins for their ciliary targeting. Therefore, in the future, it will be important to systematically screen ciliary resident membrane proteins for their interaction with importins.

References

1. Plotnikova, O.V., E.N. Pugacheva, and E.A. Golemis, *Primary cilia and the cell cycle*. Methods Cell Biol, 2009. **94**: p. 137-60.
2. Ibanez-Tallon, I., N. Heintz, and H. Omran, *To beat or not to beat: roles of cilia in development and disease*. Hum Mol Genet, 2003. **12 Spec No 1**: p. R27-35.
3. Satir, P. and S.T. Christensen, *Overview of structure and function of mammalian cilia*. Annual Review of Physiology, 2007. **69**: p. 377-400.
4. Berbari, N.F., A.K. O'Connor, C.J. Haycraft, and B.K. Yoder, *The primary cilium as a complex signaling center*. Curr Biol, 2009. **19**(13): p. R526-35.
5. Schneider, L., C.A. Clement, S.C. Teilmann, G.J. Pazour, E.K. Hoffmann, P. Satir, and S.T. Christensen, *PDGFRalpha signaling is regulated through the primary cilium in fibroblasts*. Curr Biol, 2005. **15**(20): p. 1861-6.
6. Sharma, N., N.F. Berbari, and B.K. Yoder, *Ciliary dysfunction in developmental abnormalities and diseases*. Curr Top Dev Biol, 2008. **85**: p. 371-427.
7. Goetz, S.C. and K.V. Anderson, *The primary cilium: a signalling centre during vertebrate development*. Nat Rev Genet, 2010. **11**(5): p. 331-44.
8. Haycraft, C.J., B. Banizs, Y. Aydin-Son, Q. Zhang, E.J. Michaud, and B.K. Yoder, *Gli2 and Gli3 localize to cilia and require the intraflagellar transport protein polaris for processing and function*. PLoS Genet, 2005. **1**(4): p. e53.
9. Ross, A.J., H. May-Simera, E.R. Eichers, M. Kai, J. Hill, D.J. Jagger, C.C. Leitch, J.P. Chapple, P.M. Munro, S. Fisher, P.L. Tan, H.M. Phillips, M.R. Leroux, D.J. Henderson, J.N. Murdoch, A.J. Copp, M.M. Eliot, J.R. Lupski, D.T. Kemp, H. Dollfus, M. Tada, N. Katsanis, A. Forge, and P.L. Beales, *Disruption of Bardet-Biedl syndrome ciliary proteins perturbs planar cell polarity in vertebrates*. Nat Genet, 2005. **37**(10): p. 1135-40.
10. Delaval, B., A. Bright, N.D. Lawson, and S. Doxsey, *The cilia protein IFT88 is required for spindle orientation in mitosis*. Nat Cell Biol, 2011. **13**(4): p. 461-8.
11. Hu, Q. and W.J. Nelson, *Ciliary diffusion barrier: the gatekeeper for the primary cilium compartment*. Cytoskeleton (Hoboken), 2011. **68**(6): p. 313-24.
12. Desai, A. and T.J. Mitchison, *Microtubule polymerization dynamics*. Annu Rev Cell Dev Biol, 1997. **13**: p. 83-117.
13. Jenkins, P.M., D.P. McEwen, and J.R. Martens, *Olfactory cilia: linking sensory cilia function and human disease*. Chem Senses, 2009. **34**(5): p. 451-64.
14. Menco, B.P., *Ciliated and microvillous structures of rat olfactory and nasal respiratory epithelia. A study using ultra-rapid cryo-fixation followed by freeze-substitution or freeze-etching*. Cell Tissue Res, 1984. **235**(2): p. 225-41.
15. Reese, T.S., *Olfactory Cilia in the Frog*. J Cell Biol, 1965. **25**(2): p. 209-30.
16. Greiner, J.V., T.A. Weidman, H.D. Bodley, and C.A. Greiner, *Ciliogenesis in photoreceptor cells of the retina*. Exp Eye Res, 1981. **33**(4): p. 433-46.
17. Insinna, C. and J.C. Besharse, *Intraflagellar transport and the sensory outer segment of vertebrate photoreceptors*. Dev Dyn, 2008. **237**(8): p. 1982-92.
18. Knabe, W. and H.J. Kuhn, *Ciliogenesis in photoreceptor cells of the tree shrew retina*. Anat Embryol (Berl), 1997. **196**(2): p. 123-31.

19. Kobayashi, T. and B.D. Dynlacht, *Regulating the transition from centriole to basal body*. J Cell Biol, 2011. **193**(3): p. 435-44.
20. Tucker, R.W., A.B. Pardee, and K. Fujiwara, *Centriole Ciliation Is Related to Quiescence and DNA-Synthesis in 3t3-Cells*. Cell, 1979. **17**(3): p. 527-535.
21. Gaertig, J. and D. Wloga, *Ciliary Tubulin and Its Post-Translational Modifications*. Ciliary Function in Mammalian Development, 2008. **85**: p. 83-113.
22. Pathak, N., C.A. Austin, and I.A. Drummond, *Tubulin Tyrosine Ligase-like Genes *ttll3* and *ttll6* Maintain Zebrafish Cilia Structure and Motility*. Journal of Biological Chemistry, 2011. **286**(13): p. 11685-11695.
23. Snell, W.J., J.M. Pan, and Q. Wang, *Cilia and flagella revealed: From flagellar assembly in Chlamydomonas to human obesity disorders*. Cell, 2004. **117**(6): p. 693-697.
24. Pugacheva, E.N., S.A. Jablonski, T.R. Hartman, E.P. Henske, and E.A. Golemis, *HEF1-Dependent aurora a activation induces disassembly of the primary cilium*. Cell, 2007. **129**(7): p. 1351-1363.
25. Westermann, S. and K. Weber, *Post-translational modifications regulate microtubule function*. Nature Reviews Molecular Cell Biology, 2003. **4**(12): p. 938-947.
26. Wloga, D., D.M. Webster, K. Rogowski, M.H. Bre, N. Levilliers, M. Jerka-Dziadosz, C. Janke, S.T. Dougan, and J. Gaertig, *TTLL3 Is a Tubulin Glycine Ligase that Regulates the Assembly of Cilia*. Developmental Cell, 2009. **16**(6): p. 867-876.
27. Czarnecki, P.G. and J.V. Shah, *The ciliary transition zone: from morphology and molecules to medicine*. Trends Cell Biol, 2012. **22**(4): p. 201-10.
28. Szymanska, K. and C.A. Johnson, *The transition zone: an essential functional compartment of cilia*. Cilia, 2012. **1**(1): p. 10.
29. Rosenbaum, J.L. and G.B. Witman, *Intraflagellar transport*. Nat Rev Mol Cell Biol, 2002. **3**(11): p. 813-25.
30. Boquist, L., *Cilia and vesicular particles in the endocrine pancreas of the Mongolian gerbil*. J Cell Biol, 1970. **45**(3): p. 532-41.
31. Inglis, P.N., G. Ou, M.R. Leroux, and J.M. Scholey, *The sensory cilia of Caenorhabditis elegans*. WormBook, 2007: p. 1-22.
32. Williams, C.L., C. Li, K. Kida, P.N. Inglis, S. Mohan, L. Semenec, N.J. Bialas, R.M. Stupay, N. Chen, O.E. Blacque, B.K. Yoder, and M.R. Leroux, *MKS and NPHP modules cooperate to establish basal body/transition zone membrane associations and ciliary gate function during ciliogenesis*. J Cell Biol, 2011. **192**(6): p. 1023-41.
33. Luedeke, C., S.B. Frei, I. Sbalzarini, H. Schwarz, A. Spang, and Y. Barral, *Septin-dependent compartmentalization of the endoplasmic reticulum during yeast polarized growth*. J Cell Biol, 2005. **169**(6): p. 897-908.
34. Hu, Q.C., L. Milenkovic, H. Jin, M.P. Scott, M.V. Nachury, E.T. Spiliotis, and W.J. Nelson, *A Septin Diffusion Barrier at the Base of the Primary Cilium Maintains Ciliary Membrane Protein Distribution*. Science, 2010. **329**(5990): p. 436-439.
35. Chih, B., P. Liu, Y. Chinn, C. Chalouni, L.G. Komuves, P.E. Hass, W. Sandoval, and A.S. Peterson, *A ciliopathy complex at the transition zone protects the cilia as a privileged membrane domain*. Nature Cell Biology, 2012. **14**(1): p. 61-U97.

36. Fan, S., E.L. Whiteman, T.W. Hurd, J.C. McIntyre, J.F. Dishinger, C.J. Liu, J.R. Martens, K.J. Verhey, U. Sajjan, and B. Margolis, *Induction of Ran GTP drives ciliogenesis*. Mol Biol Cell, 2011. **22**(23): p. 4539-48.
37. Reiter, J.F., O.E. Blacque, and M.R. Leroux, *The base of the cilium: roles for transition fibres and the transition zone in ciliary formation, maintenance and compartmentalization*. EMBO Rep, 2012. **13**(7): p. 608-18.
38. Vieira, O.V., K. Gaus, P. Verkade, J. Fullekrug, W.L.C. Vaz, and K. Simons, *FAPP2, cilium formation, and compartmentalization of the apical membrane in polarized Madin-Darby canine kidney (MDCK) cells*. Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(49): p. 18556-18561.
39. Kenworthy, A.K., B.J. Nichols, C.L. Remmert, G.M. Hendrix, M. Kumar, J. Zimmerberg, and J. Lippincott-Schwartz, *Dynamics of putative raft-associated proteins at the cell surface*. Journal of Cell Biology, 2004. **165**(5): p. 735-746.
40. Nachury, M.V., E.S. Seeley, and H. Jin, *Trafficking to the Ciliary Membrane: How to Get Across the Periciliary Diffusion Barrier?* Annual Review of Cell and Developmental Biology, Vol 26, 2010. **26**: p. 59-87.
41. Francis, S.S., J. Sfakianos, B. Lo, and I. Mellman, *A hierarchy of signals regulates entry of membrane proteins into the ciliary membrane domain in epithelial cells*. Journal of Cell Biology, 2011. **193**(1): p. 219-233.
42. Molla-Herman, A., R. Ghossoub, T. Blisnick, A. Meunier, C. Serres, F. Silbermann, C. Emmerson, K. Romeo, P. Bourdoncle, A. Schmitt, S. Saunier, N. Spassky, P. Bastin, and A. Benmerah, *The ciliary pocket: an endocytic membrane domain at the base of primary and motile cilia*. Journal of Cell Science, 2010. **123**(10): p. 1785-1795.
43. Benmerah, A., *The ciliary pocket*. Current Opinion in Cell Biology, 2013. **25**(1): p. 78-84.
44. Mazelova, J., L. Astuto-Gribble, H. Inoue, B.M. Tam, E. Schonteich, R. Prekeris, O.L. Moritz, P.A. Randazzo, and D. Deretic, *Ciliary targeting motif VxPx directs assembly of a trafficking module through Arf4*. EMBO J, 2009. **28**(3): p. 183-92.
45. Domire, J.S., J.A. Green, K.G. Lee, A.D. Johnson, C.C. Askwith, and K. Mykityn, *Dopamine receptor 1 localizes to neuronal cilia in a dynamic process that requires the Bardet-Biedl syndrome proteins*. Cell Mol Life Sci, 2011. **68**(17): p. 2951-60.
46. Molla-Herman, *The ciliary pocket: an endocytic membrane domain at the base of primary and motile cilia*. JCS, 2010. **123**: p. 1785-1795.
47. Rohatgi, R. and W.J. Snell, *The ciliary membrane*. Curr Opin Cell Biol, 2010. **22**(4): p. 541-6.
48. Corbit, K.C., P. Aanstad, V. Singla, A.R. Norman, D.Y. Stainier, and J.F. Reiter, *Vertebrate Smoothed functions at the primary cilium*. Nature, 2005. **437**(7061): p. 1018-21.
49. Hou, X., M. Mrug, B.K. Yoder, E.J. Lefkowitz, G. Kremmidiotis, P. D'Eustachio, D.R. Beier, and L.M. Guay-Woodford, *Cystin, a novel cilia-associated protein, is disrupted in the cpk mouse model of polycystic kidney disease*. J Clin Invest, 2002. **109**(4): p. 533-40.
50. Rohatgi, R., L. Milenkovic, and M.P. Scott, *Patched1 regulates hedgehog signaling at the primary cilium*. Science, 2007. **317**(5836): p. 372-6.
51. Ward, C.J., D. Yuan, T.V. Masyuk, X. Wang, R. Punyashthiti, S. Whelan, R. Bacallao, R. Torra, N.F. LaRusso, V.E. Torres, and P.C. Harris, *Cellular and*

- subcellular localization of the ARPKD protein; fibrocystin is expressed on primary cilia.* Hum Mol Genet, 2003. **12**(20): p. 2703-10.
52. Yoder, B.K., X. Hou, and L.M. Guay-Woodford, *The polycystic kidney disease proteins, polycystin-1, polycystin-2, polaris, and cystin, are co-localized in renal cilia.* J Am Soc Nephrol, 2002. **13**(10): p. 2508-16.
53. Katta, S.S., C.J. Smoyer, and S.L. Jaspersen, *Destination: inner nuclear membrane.* Trends Cell Biol, 2014. **24**(4): p. 221-9.
54. McEwen, D.P., P.M. Jenkins, and J.R. Martens, *Olfactory cilia: our direct neuronal connection to the external world.* Curr Top Dev Biol, 2008. **85**: p. 333-70.
55. Wolfrum, U. and A. Schmitt, *Rhodopsin transport in the membrane of the connecting cilium of mammalian photoreceptor cells.* Cell Motil Cytoskeleton, 2000. **46**(2): p. 95-107.
56. Handel, M., S. Schulz, A. Stanarius, M. Schreff, M. Erdtmann-Vourliotis, H. Schmidt, G. Wolf, and V. Holtt, *Selective targeting of somatostatin receptor 3 to neuronal cilia.* Neuroscience, 1999. **89**(3): p. 909-26.
57. Janich, P. and D. Corbeil, *GM1 and GM3 gangliosides highlight distinct lipid microdomains within the apical domain of epithelial cells.* FEBS Lett, 2007. **581**(9): p. 1783-7.
58. Calvert, P.D., K.J. Strissel, W.E. Schiesser, E.N. Pugh, Jr., and V.Y. Arshavsky, *Light-driven translocation of signaling proteins in vertebrate photoreceptors.* Trends Cell Biol, 2006. **16**(11): p. 560-8.
59. Garcia-Gonzalo, F.R. and J.F. Reiter, *Scoring a backstage pass: mechanisms of ciliogenesis and ciliary access.* J Cell Biol, 2012. **197**(6): p. 697-709.
60. Young, A., J.B. Dichtenberg, A. Purohit, R. Tuft, and S.J. Doxsey, *Cytoplasmic dynein-mediated assembly of pericentrin and gamma tubulin onto centrosomes.* Mol Biol Cell, 2000. **11**(6): p. 2047-56.
61. Hou, Y., G.J. Pazour, and G.B. Witman, *A dynein light intermediate chain, D1bLIC, is required for retrograde intraflagellar transport.* Mol Biol Cell, 2004. **15**(10): p. 4382-94.
62. Hou, Y., H. Qin, J.A. Follit, G.J. Pazour, J.L. Rosenbaum, and G.B. Witman, *Functional analysis of an individual IFT protein: IFT46 is required for transport of outer dynein arms into flagella.* J Cell Biol, 2007. **176**(5): p. 653-65.
63. Nachury, M.V., A.V. Loktev, Q. Zhang, C.J. Westlake, J. Peranen, A. Merdes, D.C. Slusarski, R.H. Scheller, J.F. Bazan, V.C. Sheffield, and P.K. Jackson, *A core complex of BBS proteins cooperates with the GTPase Rab8 to promote ciliary membrane biogenesis.* Cell, 2007. **129**(6): p. 1201-13.
64. Kozminski, K.G., K.A. Johnson, P. Forscher, and J.L. Rosenbaum, *A motility in the eukaryotic flagellum unrelated to flagellar beating.* Proc Natl Acad Sci U S A, 1993. **90**(12): p. 5519-23.
65. Ou, G., O.E. Blacque, J.J. Snow, M.R. Leroux, and J.M. Scholey, *Functional coordination of intraflagellar transport motors.* Nature, 2005. **436**(7050): p. 583-7.
66. Marszalek, J.R., X. Liu, E.A. Roberts, D. Chui, J.D. Marth, D.S. Williams, and L.S. Goldstein, *Genetic evidence for selective transport of opsin and arrestin by kinesin-II in mammalian photoreceptors.* Cell, 2000. **102**(2): p. 175-87.

67. Qin, H., D.T. Burnette, Y.K. Bae, P. Forscher, M.M. Barr, and J.L. Rosenbaum, *Intraflagellar transport is required for the vectorial movement of TRPV channels in the ciliary membrane*. *Curr Biol*, 2005. **15**(18): p. 1695-9.
68. Calvert, P.D., W.E. Schiesser, and E.N. Pugh, Jr., *Diffusion of a soluble protein, photoactivatable GFP, through a sensory cilium*. *J Gen Physiol*, 2010. **135**(3): p. 173-96.
69. Najafi, M., N.A. Maza, and P.D. Calvert, *Steric volume exclusion sets soluble protein concentrations in photoreceptor sensory cilia*. *Proc Natl Acad Sci U S A*, 2012. **109**(1): p. 203-8.
70. Kee, H.L., J.F. Dishinger, T.L. Blasius, C.J. Liu, B. Margolis, and K.J. Verhey, *A size-exclusion permeability barrier and nucleoporins characterize a ciliary pore complex that regulates transport into cilia*. *Nat Cell Biol*, 2012. **14**(4): p. 431-7.
71. Denning, D.P., S.S. Patel, V. Uversky, A.L. Fink, and M. Rexach, *Disorder in the nuclear pore complex: the FG repeat regions of nucleoporins are natively unfolded*. *Proc Natl Acad Sci U S A*, 2003. **100**(5): p. 2450-5.
72. Boni, A., A.Z. Politi, P. Strnad, W. Xiang, M.J. Hossain, and J. Ellenberg, *Live imaging and modeling of inner nuclear membrane targeting reveals its molecular requirements in mammalian cells*. *J Cell Biol*, 2015. **209**(5): p. 705-20.
73. Lange, A., R.E. Mills, C.J. Lange, M. Stewart, S.E. Devine, and A.H. Corbett, *Classical nuclear localization signals: definition, function, and interaction with importin alpha*. *J Biol Chem*, 2007. **282**(8): p. 5101-5.
74. Popken, P., A. Ghavami, P.R. Onck, B. Poolman, and L.M. Veenhoff, *Size-dependent leak of soluble and membrane proteins through the yeast nuclear pore complex*. *Mol Biol Cell*, 2015. **26**(7): p. 1386-94.
75. Soullam, B. and H.J. Worman, *Signals and structural features involved in integral membrane protein targeting to the inner nuclear membrane*. *J Cell Biol*, 1995. **130**(1): p. 15-27.
76. Ungricht, R., M. Klann, P. Horvath, and U. Kutay, *Diffusion and retention are major determinants of protein targeting to the inner nuclear membrane*. *J Cell Biol*, 2015. **209**(5): p. 687-703.
77. Stewart, M., *Molecular mechanism of the nuclear protein import cycle*. *Nat Rev Mol Cell Biol*, 2007. **8**(3): p. 195-208.
78. Cook, A., F. Bono, M. Jinek, and E. Conti, *Structural biology of nucleocytoplasmic transport*. *Annu Rev Biochem*, 2007. **76**: p. 647-71.
79. Marfori, M., A. Mynott, J.J. Ellis, A.M. Mehdi, N.F. Saunders, P.M. Curmi, J.K. Forwood, M. Boden, and B. Kobe, *Molecular basis for specificity of nuclear import and prediction of nuclear localization*. *Biochim Biophys Acta*, 2011. **1813**(9): p. 1562-77.
80. Palmeri, D. and M.H. Malim, *Importin beta can mediate the nuclear import of an arginine-rich nuclear localization signal in the absence of importin alpha*. *Mol Cell Biol*, 1999. **19**(2): p. 1218-25.
81. Clarke, P.R., *Signaling to nuclear transport*. *Dev Cell*, 2008. **14**(3): p. 316-8.
82. Kutay, U., F.R. Bischoff, S. Kostka, R. Kraft, and D. Gorlich, *Export of importin alpha from the nucleus is mediated by a specific nuclear transport factor*. *Cell*, 1997. **90**(6): p. 1061-71.
83. Lee, B.J., A.E. Cansizoglu, K.E. Suel, T.H. Louis, Z. Zhang, and Y.M. Chook, *Rules for nuclear localization sequence recognition by karyopherin beta 2*. *Cell*, 2006. **126**(3): p. 543-58.

84. Arnold, M., A. Nath, J. Hauber, and R.H. Kehlenbach, *Multiple importins function as nuclear transport receptors for the Rev protein of human immunodeficiency virus type 1*. J Biol Chem, 2006. **281**(30): p. 20883-90.
85. Darshan, M.S., J. Lucchi, E. Harding, and J. Moroianu, *The l2 minor capsid protein of human papillomavirus type 16 interacts with a network of nuclear import receptors*. J Virol, 2004. **78**(22): p. 12179-88.
86. Klucsevsek, K., J. Daley, M.S. Darshan, J. Bordeaux, and J. Moroianu, *Nuclear import strategies of high-risk HPV18 L2 minor capsid protein*. Virology, 2006. **352**(1): p. 200-8.
87. Twyffels, L., C. Gueydan, and V. Kruys, *Transportin-1 and Transportin-2: protein nuclear import and beyond*. FEBS Lett, 2014. **588**(10): p. 1857-68.
88. Antonin, W., R. Ungricht, and U. Kutay, *Traversing the NPC along the pore membrane: targeting of membrane proteins to the INM*. Nucleus, 2011. **2**(2): p. 87-91.
89. Ellenberg, J., E.D. Siggia, J.E. Moreira, C.L. Smith, J.F. Presley, H.J. Worman, and J. Lippincott-Schwartz, *Nuclear membrane dynamics and reassembly in living cells: targeting of an inner nuclear membrane protein in interphase and mitosis*. J Cell Biol, 1997. **138**(6): p. 1193-206.
90. Zuleger, N., A.R. Kerr, and E.C. Schirmer, *Many mechanisms, one entrance: membrane protein translocation into the nucleus*. Cell Mol Life Sci, 2012. **69**(13): p. 2205-16.
91. Fan, S., V. Fogg, Q. Wang, X.W. Chen, C.J. Liu, and B. Margolis, *A novel Crumbs3 isoform regulates cell division and ciliogenesis via importin beta interactions*. J Cell Biol, 2007. **178**(3): p. 387-98.
92. Hurd, T., W. Zhou, P. Jenkins, C.J. Liu, A. Swaroop, H. Khanna, J. Martens, F. Hildebrandt, and B. Margolis, *The retinitis pigmentosa protein RP2 interacts with polycystin 2 and regulates cilia-mediated vertebrate development*. Hum Mol Genet, 2010. **19**(22): p. 4330-44.
93. Kee, H.L. and K.J. Verhey, *Molecular connections between nuclear and ciliary import processes*. Cilia, 2013. **2**(1): p. 11.
94. Dishinger, J.F., H.L. Kee, P.M. Jenkins, S. Fan, T.W. Hurd, J.W. Hammond, Y.N. Truong, B. Margolis, J.R. Martens, and K.J. Verhey, *Ciliary entry of the kinesin-2 motor KIF17 is regulated by importin-beta2 and RanGTP*. Nat Cell Biol, 2010. **12**(7): p. 703-10.
95. Geng, L., D. Okuhara, Z. Yu, X. Tian, Y. Cai, S. Shibazaki, and S. Somlo, *Polycystin-2 traffics to cilia independently of polycystin-1 by using an N-terminal RVxP motif*. J Cell Sci, 2006. **119**(Pt 7): p. 1383-95.
96. Jenkins, P.M., T.W. Hurd, L. Zhang, D.P. McEwen, R.L. Brown, B. Margolis, K.J. Verhey, and J.R. Martens, *Ciliary targeting of olfactory CNG channels requires the CNGB1b subunit and the kinesin-2 motor protein, KIF17*. Curr Biol, 2006. **16**(12): p. 1211-6.
97. Ward, H.H., U. Brown-Glaberman, J. Wang, Y. Morita, S.L. Alper, E.J. Bedrick, V.H. Gattone, 2nd, D. Deretic, and A. Wandinger-Ness, *A conserved signal and GTPase complex are required for the ciliary transport of polycystin-1*. Mol Biol Cell, 2011. **22**(18): p. 3289-305.
98. Berbari, N.F., A.D. Johnson, J.S. Lewis, C.C. Askwith, and K. Mykityn, *Identification of ciliary localization sequences within the third intracellular loop of G protein-coupled receptors*. Mol Biol Cell, 2008. **19**(4): p. 1540-7.
99. Humbert, M.C., K. Weihbrecht, C.C. Searby, Y. Li, R.M. Pope, V.C. Sheffield, and S. Seo, *ARL13B, PDE6D, and CEP164 form a functional*

- network for INPP5E ciliary targeting. *Proc Natl Acad Sci U S A*, 2012. **109**(48): p. 19691-6.
100. Tam, B.M., O.L. Moritz, L.B. Hurd, and D.S. Papermaster, *Identification of an outer segment targeting signal in the COOH terminus of rhodopsin using transgenic Xenopus laevis*. *J Cell Biol*, 2000. **151**(7): p. 1369-80.
 101. Follit, J.A., L. Li, Y. Vucica, and G.J. Pazour, *The cytoplasmic tail of fibrocystin contains a ciliary targeting sequence*. *J Cell Biol*, 2010. **188**(1): p. 21-8.
 102. Tam, B.M., O.L. Moritz, and D.S. Papermaster, *The C terminus of peripherin/rds participates in rod outer segment targeting and alignment of disk incisures*. *Mol Biol Cell*, 2004. **15**(4): p. 2027-37.
 103. Loktev, A.V. and P.K. Jackson, *Neuropeptide Y family receptors traffic via the Bardet-Biedl syndrome pathway to signal in neuronal primary cilia*. *Cell Rep*, 2013. **5**(5): p. 1316-29.
 104. Dwyer, N.D., E.R. Troemel, P. Sengupta, and C.I. Bargmann, *Odorant receptor localization to olfactory cilia is mediated by ODR-4, a novel membrane-associated protein*. *Cell*, 1998. **93**(3): p. 455-66.
 105. Luo, W., N. Marsh-Armstrong, A. Rattner, and J. Nathans, *An outer segment localization signal at the C terminus of the photoreceptor-specific retinol dehydrogenase*. *J Neurosci*, 2004. **24**(11): p. 2623-32.
 106. Tao, B., S. Bu, Z. Yang, B. Siroky, J.C. Kappes, A. Kispert, and L.M. Guay-Woodford, *Cystin localizes to primary cilia via membrane microdomains and a targeting motif*. *J Am Soc Nephrol*, 2009. **20**(12): p. 2570-80.
 107. Nakata, K., D. Shiba, D. Kobayashi, and T. Yokoyama, *Targeting of Nphp3 to the primary cilia is controlled by an N-terminal myristoylation site and coiled-coil domains*. *Cytoskeleton (Hoboken)*, 2012. **69**(4): p. 221-34.
 108. Santos, N. and J.F. Reiter, *A central region of Gli2 regulates its localization to the primary cilium and transcriptional activity*. *J Cell Sci*, 2014. **127**(Pt 7): p. 1500-10.
 109. Breslow, D.K. and M.V. Nachury, *Primary cilia: how to keep the riff-raff in the plasma membrane*. *Curr Biol*, 2011. **21**(11): p. R434-6.
 110. Papermaster, D.S., B.G. Schneider, and J.C. Besharse, *Vesicular Transport of Newly Synthesized Opsin from the Golgi-Apparatus toward the Rod Outer Segment - Ultrastructural Immunocytochemical and Autoradiographic Evidence in Xenopus Retinas*. *Investigative Ophthalmology & Visual Science*, 1985. **26**(10): p. 1386-1404.
 111. Tai, A.W., J.Z. Chuang, C. Bode, U. Wolfrum, and C.H. Sung, *Rhodopsin's carboxy-terminal cytoplasmic tail acts as a membrane receptor for cytoplasmic dynein by binding to the dynein light chain Tctex-1*. *Cell*, 1999. **97**(7): p. 877-87.
 112. Berbari, N.F., J.S. Lewis, G.A. Bishop, C.C. Askwith, and K. Mykityn, *Bardet-Biedl syndrome proteins are required for the localization of G protein-coupled receptors to primary cilia*. *Proc Natl Acad Sci U S A*, 2008. **105**(11): p. 4242-6.
 113. Jin, H., S.R. White, T. Shida, S. Schulz, M. Aguiar, S.P. Gygi, J.F. Bazan, and M.V. Nachury, *The conserved Bardet-Biedl syndrome proteins assemble a coat that traffics membrane proteins to cilia*. *Cell*, 2010. **141**(7): p. 1208-19.
 114. Kok, J.W., K. Hoekstra, S. Eskelinen, and D. Hoekstra, *Recycling pathways of glucosylceramide in BHK cells: distinct involvement of early and late endosomes*. *J Cell Sci*, 1992. **103** (Pt 4): p. 1139-52.

115. van Ijzendoorn, S.C., *Recycling endosomes*. J Cell Sci, 2006. **119**(Pt 9): p. 1679-81.
116. Hoffmeister, H., K. Babinger, S. Gurster, A. Cedzich, C. Meese, K. Schadendorf, L. Osten, U. de Vries, A. Rascle, and R. Witzgall, *Polycystin-2 takes different routes to the somatic and ciliary plasma membrane*. J Cell Biol, 2011. **192**(4): p. 631-45.
117. Knodler, A., S. Feng, J. Zhang, X. Zhang, A. Das, J. Peranen, and W. Guo, *Coordination of Rab8 and Rab11 in primary ciliogenesis*. Proc Natl Acad Sci U S A, 2010. **107**(14): p. 6346-51.
118. Omori, Y., C. Zhao, A. Saras, S. Mukhopadhyay, W. Kim, T. Furukawa, P. Sengupta, A. Veraksa, and J. Malicki, *Elipsa is an early determinant of ciliogenesis that links the IFT particle to membrane-associated small GTPase Rab8*. Nat Cell Biol, 2008. **10**(4): p. 437-44.
119. Prigent, M., T. Dubois, G. Raposo, V. Derrien, D. Tenza, C. Rosse, J. Camonis, and P. Chavrier, *ARF6 controls post-endocytic recycling through its downstream exocyst complex effector*. J Cell Biol, 2003. **163**(5): p. 1111-21.
120. Westlake, C.J., L.M. Baye, M.V. Nachury, K.J. Wright, K.E. Ervin, L. Phu, C. Chalouni, J.S. Beck, D.S. Kirkpatrick, D.C. Slusarski, V.C. Sheffield, R.H. Scheller, and P.K. Jackson, *Primary cilia membrane assembly is initiated by Rab11 and transport protein particle II (TRAPP II) complex-dependent trafficking of Rabin8 to the centrosome*. Proc Natl Acad Sci U S A, 2011. **108**(7): p. 2759-64.
121. Yoshimura, S., J. Egerer, E. Fuchs, A.K. Haas, and F.A. Barr, *Functional dissection of Rab GTPases involved in primary cilium formation*. J Cell Biol, 2007. **178**(3): p. 363-9.
122. Zuo, X., W. Guo, and J.H. Lipschutz, *The exocyst protein Sec10 is necessary for primary ciliogenesis and cystogenesis in vitro*. Mol Biol Cell, 2009. **20**(10): p. 2522-9.
123. Milenkovic, L., M.P. Scott, and R. Rohatgi, *Lateral transport of Smoothed from the plasma membrane to the membrane of the cilium*. J Cell Biol, 2009. **187**(3): p. 365-74.
124. Hunnicutt, G.R., M.G. Kosfisz, and W.J. Snell, *Cell body and flagellar agglutinins in Chlamydomonas reinhardtii: the cell body plasma membrane is a reservoir for agglutinins whose migration to the flagella is regulated by a functional barrier*. J Cell Biol, 1990. **111**(4): p. 1605-16.
125. Fan, S., T.W. Hurd, C.J. Liu, S.W. Straight, T. Weimbs, E.A. Hurd, S.E. Domino, and B. Margolis, *Polarity proteins control ciliogenesis via kinesin motor interactions*. Curr Biol, 2004. **14**(16): p. 1451-61.
126. Kovacs, J.J., E.J. Whalen, R. Liu, K. Xiao, J. Kim, M. Chen, J. Wang, W. Chen, and R.J. Lefkowitz, *Beta-arrestin-mediated localization of smoothed to the primary cilium*. Science, 2008. **320**(5884): p. 1777-81.
127. Baker, S.A., M. Haeri, P. Yoo, S.M. Gospe, 3rd, N.P. Skiba, B.E. Knox, and V.Y. Arshavsky, *The outer segment serves as a default destination for the trafficking of membrane proteins in photoreceptors*. J Cell Biol, 2008. **183**(3): p. 485-98.
128. Gospe, S.M., 3rd, S.A. Baker, and V.Y. Arshavsky, *Facilitative glucose transporter Glut1 is actively excluded from rod outer segments*. J Cell Sci, 2010. **123**(Pt 21): p. 3639-44.
129. Itzen, A. and R.S. Goody, *GTPases involved in vesicular trafficking: structures and mechanisms*. Semin Cell Dev Biol, 2011. **22**(1): p. 48-56.

130. Macara, I.G., K.M. Lounsbury, S.A. Richards, C. McKiernan, and D. Barsagi, *The Ras superfamily of GTPases*. FASEB J, 1996. **10**(5): p. 625-30.
131. Macara, *The Ras superfamily of GTPases.pdf*. 1996.
132. Zerial, M. and H. McBride, *Rab proteins as membrane organizers*. Nat Rev Mol Cell Biol, 2001. **2**(2): p. 107-17.
133. Stenmark, H., *Rab GTPases as coordinators of vesicle traffic*. Nat Rev Mol Cell Biol, 2009. **10**(8): p. 513-25.
134. Hattula, K., J. Furuhielm, J. Tikkanen, K. Tanhuanpaa, P. Laakkonen, and J. Peranen, *Characterization of the Rab8-specific membrane traffic route linked to protrusion formation*. J Cell Sci, 2006. **119**(Pt 23): p. 4866-77.
135. Hattula, K., J. Furuhielm, A. Arffman, and J. Peranen, *A Rab8-specific GDP/GTP exchange factor is involved in actin remodeling and polarized membrane transport*. Mol Biol Cell, 2002. **13**(9): p. 3268-80.
136. Murga-Zamalloa, C.A., S.J. Atkins, J. Peranen, A. Swaroop, and H. Khanna, *Interaction of retinitis pigmentosa GTPase regulator (RPGR) with RAB8A GTPase: implications for cilia dysfunction and photoreceptor degeneration*. Hum Mol Genet, 2010. **19**(18): p. 3591-8.
137. Huber, L.A., S. Pimplikar, R.G. Parton, H. Virta, M. Zerial, and K. Simons, *Rab8, a small GTPase involved in vesicular traffic between the TGN and the basolateral plasma membrane*. J Cell Biol, 1993. **123**(1): p. 35-45.
138. Peranen, J., *Rab8 GTPase as a regulator of cell shape*. Cytoskeleton (Hoboken), 2011. **68**(10): p. 527-39.
139. Rao, K.N., L. Li, M. Anand, and H. Khanna, *Ablation of retinal ciliopathy protein RPGR results in altered photoreceptor ciliary composition*. Sci Rep, 2015. **5**: p. 11137.
140. Chiba, S., Y. Amagai, Y. Homma, M. Fukuda, and K. Mizuno, *NDR2-mediated Rabin8 phosphorylation is crucial for ciliogenesis by switching binding specificity from phosphatidylserine to Sec15*. EMBO J, 2013. **32**(6): p. 874-85.
141. Boehlke, C., M. Bashkurov, A. Buescher, T. Krick, A.K. John, R. Nitschke, G. Walz, and E.W. Kuehn, *Differential role of Rab proteins in ciliary trafficking: Rab23 regulates smoothed levels*. J Cell Sci, 2010. **123**(Pt 9): p. 1460-7.
142. Moritz, O.L., B.M. Tam, L.L. Hurd, J. Peranen, D. Deretic, and D.S. Papermaster, *Mutant rab8 Impairs docking and fusion of rhodopsin-bearing post-Golgi membranes and causes cell death of transgenic Xenopus rods*. Mol Biol Cell, 2001. **12**(8): p. 2341-51.
143. Ishikawa, H., A. Kubo, S. Tsukita, and S. Tsukita, *Odf2-deficient mother centrioles lack distal/subdistal appendages and the ability to generate primary cilia*. Nat Cell Biol, 2005. **7**(5): p. 517-24.
144. Yoshimura, S., A.K. Haas, and F.A. Barr, *Analysis of Rab GTPase and GTPase-activating protein function at primary cilia*. Methods Enzymol, 2008. **439**: p. 353-64.
145. Kim, J., S.R. Krishnaswami, and J.G. Gleeson, *CEP290 interacts with the centriolar satellite component PCM-1 and is required for Rab8 localization to the primary cilium*. Hum Mol Genet, 2008. **17**(23): p. 3796-805.
146. Schmidt, K.N., S. Kuhns, A. Neuner, B. Hub, H. Zentgraf, and G. Pereira, *Cep164 mediates vesicular docking to the mother centriole during early steps of ciliogenesis*. J Cell Biol, 2012. **199**(7): p. 1083-101.
147. Feng, S., A. Knodler, J. Ren, J. Zhang, X. Zhang, Y. Hong, S. Huang, J. Peranen, and W. Guo, *A Rab8 guanine nucleotide exchange factor-effector*

- interaction network regulates primary ciliogenesis*. J Biol Chem, 2012. **287**(19): p. 15602-9.
148. Lin, Y.C., P. Niewiadomski, B. Lin, H. Nakamura, S.C. Phua, J. Jiao, A. Levchenko, T. Inoue, R. Rohatgi, and T. Inoue, *Chemically inducible diffusion trap at cilia reveals molecular sieve-like barrier*. Nat Chem Biol, 2013. **9**(7): p. 437-43.
149. Badano, J.L., N. Mitsuma, P.L. Beales, and N. Katsanis, *The ciliopathies: an emerging class of human genetic disorders*. Annu Rev Genomics Hum Genet, 2006. **7**: p. 125-48.
150. Lee, J.E. and J.G. Gleeson, *A systems-biology approach to understanding the ciliopathy disorders*. Genome Med, 2011. **3**(9): p. 59.
151. Tobin, J.L. and P.L. Beales, *The nonmotile ciliopathies*. Genet Med, 2009. **11**(6): p. 386-402.
152. Ward, C.J., M.C. Hogan, S. Rossetti, D. Walker, T. Sneddon, X. Wang, V. Kubly, J.M. Cunningham, R. Bacallao, M. Ishibashi, D.S. Milliner, V.E. Torres, and P.C. Harris, *The gene mutated in autosomal recessive polycystic kidney disease encodes a large, receptor-like protein*. Nat Genet, 2002. **30**(3): p. 259-69.
153. Zhang, M.Z., W. Mai, C. Li, S.Y. Cho, C. Hao, G. Moeckel, R. Zhao, I. Kim, J. Wang, H. Xiong, H. Wang, Y. Sato, Y. Wu, Y. Nakanuma, M. Lilova, Y. Pei, R.C. Harris, S. Li, R.J. Coffey, L. Sun, D. Wu, X.Z. Chen, M.D. Breyer, Z.J. Zhao, J.A. McKanna, and G. Wu, *PKHD1 protein encoded by the gene for autosomal recessive polycystic kidney disease associates with basal bodies and primary cilia in renal epithelial cells*. Proc Natl Acad Sci U S A, 2004. **101**(8): p. 2311-6.
154. Zhang, J., M. Wu, S. Wang, J.V. Shah, P.D. Wilson, and J. Zhou, *Polycystic kidney disease protein fibrocystin localizes to the mitotic spindle and regulates spindle bipolarity*. Hum Mol Genet, 2010. **19**(17): p. 3306-19.
155. Masyuk, T.V., B.Q. Huang, C.J. Ward, A.I. Masyuk, D. Yuan, P.L. Splinter, R. Punyashtiti, E.L. Ritman, V.E. Torres, P.C. Harris, and N.F. LaRusso, *Defects in cholangiocyte fibrocystin expression and ciliary structure in the PCK rat*. Gastroenterology, 2003. **125**(5): p. 1303-10.
156. Wu, Y., X.Q. Dai, Q. Li, C.X. Chen, W. Mai, Z. Hussain, W. Long, N. Montalbetti, G. Li, R. Glynn, S. Wang, H.F. Cantiello, G. Wu, and X.Z. Chen, *Kinesin-2 mediates physical and functional interactions between polycystin-2 and fibrocystin*. Hum Mol Genet, 2006. **15**(22): p. 3280-92.
157. Wang, S., J. Zhang, S.M. Nauli, X. Li, P.G. Starremans, Y. Luo, K.A. Roberts, and J. Zhou, *Fibrocystin/polyductin, found in the same protein complex with polycystin-2, regulates calcium responses in kidney epithelia*. Mol Cell Biol, 2007. **27**(8): p. 3241-52.
158. Hiesberger, T., E. Gourley, A. Erickson, P. Koulen, C.J. Ward, T.V. Masyuk, N.F. Larusso, P.C. Harris, and P. Igarashi, *Proteolytic cleavage and nuclear translocation of fibrocystin is regulated by intracellular Ca²⁺ and activation of protein kinase C*. J Biol Chem, 2006. **281**(45): p. 34357-64.
159. Sun, L., S. Wang, C. Hu, and X. Zhang, *Regulation of cell proliferation and apoptosis through fibrocystin-prosaposin interaction*. Arch Biochem Biophys, 2010. **502**(2): p. 130-6.
160. Follit, J.A., J.T. San Agustin, J.A. Jonassen, T. Huang, J.A. Rivera-Perez, K.D. Tremblay, and G.J. Pazour, *Arf4 is required for Mammalian development but dispensable for ciliary assembly*. PLoS Genet, 2014. **10**(2): p. e1004170.

161. Mahajan, D., B.K. Boh, Y. Zhou, L. Chen, T.C. Cornvik, W. Hong, and L. Lu, *Mammalian Mon2/Ysl2 regulates endosome-to-Golgi trafficking but possesses no guanine nucleotide exchange activity toward Arl1 GTPase*. Sci Rep, 2013. **3**: p. 3362.
162. Lippincott-Schwartz, J., L. Yuan, C. Tipper, M. Amherdt, L. Orci, and R.D. Klausner, *Brefeldin A's effects on endosomes, lysosomes, and the TGN suggest a general mechanism for regulating organelle structure and membrane traffic*. Cell, 1991. **67**(3): p. 601-616.
163. Alieva, I.B., L.A. Gorgidze, Y.A. Komarova, O.A. Chernobelskaya, and I.A. Vorobjev, *Experimental model for studying the primary cilia in tissue culture cells*. Membr Cell Biol, 1999. **12**(6): p. 895-905.
164. Gerdes, J.M., Y. Liu, N.A. Zaghoul, C.C. Leitch, S.S. Lawson, M. Kato, P.A. Beachy, P.L. Beales, G.N. DeMartino, S. Fisher, J.L. Badano, and N. Katsanis, *Disruption of the basal body compromises proteasomal function and perturbs intracellular Wnt response*. Nat Genet, 2007. **39**(11): p. 1350-60.
165. Euteneuer, U. and M. Schliwa, *Mechanism of centrosome positioning during the wound response in BSC-1 cells*. J Cell Biol, 1992. **116**(5): p. 1157-66.
166. Hancock, J.F., K. Cadwallader, H. Paterson, and C.J. Marshall, *A CAAX or a CAAL motif and a second signal are sufficient for plasma membrane targeting of ras proteins*. EMBO J, 1991. **10**(13): p. 4033-9.
167. Hirschberg, K., C.M. Miller, J. Ellenberg, J.F. Presley, E.D. Siggia, R.D. Phair, and J. Lippincott-Schwartz, *Kinetic analysis of secretory protein traffic and characterization of golgi to plasma membrane transport intermediates in living cells*. J Cell Biol, 1998. **143**(6): p. 1485-503.
168. Subathra, M., A. Qureshi, and C. Luberto, *Sphingomyelin synthases regulate protein trafficking and secretion*. PLoS One, 2011. **6**(9): p. e23644.
169. Kozik, P., R.W. Francis, M.N. Seaman, and M.S. Robinson, *A screen for endocytic motifs*. Traffic, 2010. **11**(6): p. 843-55.
170. Lin, S.X., W.G. Mallet, A.Y. Huang, and F.R. Maxfield, *Endocytosed cation-independent mannose 6-phosphate receptor traffics via the endocytic recycling compartment en route to the trans-Golgi network and a subpopulation of late endosomes*. Mol Biol Cell, 2004. **15**(2): p. 721-33.
171. Mallet, W.G. and F.R. Maxfield, *Chimeric forms of furin and TGN38 are transported with the plasma membrane in the trans-Golgi network via distinct endosomal pathways*. J Cell Biol, 1999. **146**(2): p. 345-59.
172. Schweizer, A., S. Kornfeld, and J. Rohrer, *Proper sorting of the cation-dependent mannose 6-phosphate receptor in endosomes depends on a pair of aromatic amino acids in its cytoplasmic tail*. Proc Natl Acad Sci U S A, 1997. **94**(26): p. 14471-6.
173. Miller, S.E., D.A. Sahlender, S.C. Graham, S. Honing, M.S. Robinson, A.A. Peden, and D.J. Owen, *The molecular basis for the endocytosis of small R-SNAREs by the clathrin adaptor CALM*. Cell, 2011. **147**(5): p. 1118-31.
174. Renard, H.F., M.D. Garcia-Castillo, V. Chambon, C. Lamaze, and L. Johannes, *Shiga toxin stimulates clathrin-independent endocytosis of the VAMP2, VAMP3 and VAMP8 SNARE proteins*. J Cell Sci, 2015. **128**(15): p. 2891-902.
175. Knudsen, K.A., A.P. Soler, K.R. Johnson, and M.J. Wheelock, *Interaction of alpha-actinin with the cadherin/catenin cell-cell adhesion complex via alpha-catenin*. J Cell Biol, 1995. **130**(1): p. 67-77.

176. Hu, Q., L. Milenkovic, H. Jin, M.P. Scott, M.V. Nachury, E.T. Spiliotis, and W.J. Nelson, *A septin diffusion barrier at the base of the primary cilium maintains ciliary membrane protein distribution*. Science, 2010. **329**(5990): p. 436-9.
177. Breslow, D.K., E.F. Koslover, F. Seydel, A.J. Spakowitz, and M.V. Nachury, *An in vitro assay for entry into cilia reveals unique properties of the soluble diffusion barrier*. J Cell Biol, 2013. **203**(1): p. 129-47.
178. Larkins, C.E., G.D. Aviles, M.P. East, R.A. Kahn, and T. Caspary, *Arl13b regulates ciliogenesis and the dynamic localization of Shh signaling proteins*. Mol Biol Cell, 2011. **22**(23): p. 4694-703.
179. Papermaster, D.S., B.G. Schneider, and J.C. Besharse, *Vesicular transport of newly synthesized opsin from the Golgi apparatus toward the rod outer segment. Ultrastructural immunocytochemical and autoradiographic evidence in Xenopus retinas*. Invest Ophthalmol Vis Sci, 1985. **26**(10): p. 1386-404.
180. Hinshaw, J.E., *Dynamin and its role in membrane fission*. Annu Rev Cell Dev Biol, 2000. **16**: p. 483-519.
181. Altschuler, Y., S.M. Barbas, L.J. Terlecky, K. Tang, S. Hardy, K.E. Mostov, and S.L. Schmid, *Redundant and distinct functions for dynamin-1 and dynamin-2 isoforms*. J Cell Biol, 1998. **143**(7): p. 1871-81.
182. van der Blik, A.M., T.E. Redelmeier, H. Damke, E.J. Tisdale, E.M. Meyerowitz, and S.L. Schmid, *Mutations in human dynamin block an intermediate stage in coated vesicle formation*. J Cell Biol, 1993. **122**(3): p. 553-63.
183. Ferguson, S.M. and P. De Camilli, *Dynamin, a membrane remodelling GTPase*. Nature reviews. Molecular cell biology, 2012. **13**(2): p. 75-88.
184. Teuchert, M., S. Berghofer, H.D. Klenk, and W. Garten, *Recycling of furin from the plasma membrane. Functional importance of the cytoplasmic tail sorting signals and interaction with the AP-2 adaptor medium chain subunit*. J Biol Chem, 1999. **274**(51): p. 36781-9.
185. Helms, J.B. and J.E. Rothman, *Inhibition by brefeldin A of a Golgi membrane enzyme that catalyses exchange of guanine nucleotide bound to ARF*. Nature, 1992. **360**(6402): p. 352-4.
186. Lippincott-Schwartz, J., T.H. Roberts, and K. Hirschberg, *Secretory protein trafficking and organelle dynamics in living cells*. Annu Rev Cell Dev Biol, 2000. **16**: p. 557-89.
187. Lusk, C.P., G. Blobel, and M.C. King, *Highway to the inner nuclear membrane: rules for the road*. Nat Rev Mol Cell Biol, 2007. **8**(5): p. 414-420.
188. Laba, J.K., A. Steen, P. Popken, A. Chernova, B. Poolman, and L.M. Veenhoff, *Active Nuclear Import of Membrane Proteins Revisited*. Cells, 2015. **4**(4): p. 653-73.
189. Popken, P., A. Ghavami, P.R. Onck, B. Poolman, and L.M. Veenhoff, *Size-dependent leak of soluble and membrane proteins through the yeast nuclear pore complex*. Molecular Biology of the Cell, 2015. **26**(7): p. 1386-1394.
190. Geng, *Polycystin-2 traffics to cilia independently of PC_1 by using N-terminal RVxP motif*. 2006.
191. Gorlich, D., P. Henklein, R.A. Laskey, and E. Hartmann, *A 41 amino acid motif in importin-alpha confers binding to importin-beta and hence transit into the nucleus*. EMBO J, 1996. **15**(8): p. 1810-7.

192. Izaurralde, E., A. Jarmolowski, C. Beisel, I.W. Mattaj, G. Dreyfuss, and U. Fischer, *A role for the M9 transport signal of hnRNP A1 in mRNA nuclear export*. J Cell Biol, 1997. **137**(1): p. 27-35.
193. Siomi, M.C., P.S. Eder, N. Kataoka, L. Wan, Q. Liu, and G. Dreyfuss, *Transportin-mediated nuclear import of heterogeneous nuclear RNP proteins*. J Cell Biol, 1997. **138**(6): p. 1181-92.
194. Hurd, T.W., S. Fan, and B.L. Margolis, *Localization of retinitis pigmentosa 2 to cilia is regulated by Importin beta2*. J Cell Sci, 2011. **124**(Pt 5): p. 718-26.
195. Wright, K.J., L.M. Baye, A. Olivier-Mason, S. Mukhopadhyay, L. Sang, M. Kwong, W. Wang, P.R. Pretorius, V.C. Sheffield, P. Sengupta, D.C. Slusarski, and P.K. Jackson, *An ARL3-UNC119-RP2 GTPase cycle targets myristoylated NPHP3 to the primary cilium*. Genes Dev, 2011. **25**(22): p. 2347-60.
196. Zhang, H., R. Constantine, S. Vorobiev, Y. Chen, J. Seetharaman, Y.J. Huang, R. Xiao, G.T. Montelione, C.D. Gerstner, M.W. Davis, G. Inana, F.G. Whitby, E.M. Jorgensen, C.P. Hill, L. Tong, and W. Baehr, *UNC119 is required for G protein trafficking in sensory neurons*. Nat Neurosci, 2011. **14**(7): p. 874-80.
197. Hetzer, M.W., T.C. Walther, and I.W. Mattaj, *Pushing the envelope: structure, function, and dynamics of the nuclear periphery*. Annu Rev Cell Dev Biol, 2005. **21**: p. 347-80.
198. Francis, S.S., J. Sfakianos, B. Lo, and I. Mellman, *A hierarchy of signals regulates entry of membrane proteins into the ciliary membrane domain in epithelial cells*. J Cell Biol, 2011. **193**(1): p. 219-33.
199. kim, H., *Planar Cell Polarity Acts Through Septins to Control Collective Cell Movement and Ciliogenesis*. 2010.
200. Meinema, A.C., J.K. Laba, R.A. Hapsari, R. Otten, F.A. Mulder, A. Kralt, G. van den Bogaart, C.P. Lusk, B. Poolman, and L.M. Veenhoff, *Long unfolded linkers facilitate membrane protein import through the nuclear pore complex*. Science, 2011. **333**(6038): p. 90-3.
201. Saksena, S., M.D. Summers, J.K. Burks, A.E. Johnson, and S.C. Braunagel, *Importin-alpha-16 is a translocon-associated protein involved in sorting membrane proteins to the nuclear envelope*. Nat Struct Mol Biol, 2006. **13**(6): p. 500-8.

Appendix 1

Primers used in this study (Arranged in alphabetical order)

Primers	Sequence
CAAX-F	5`AATTCAGGCTGCATGAGCTGCAAGTGTGTGCTCTC CTGAG3'
CAAX-R	5`GATCCTCAGGAGAGCACACACTTGCAGCTCATGC AGCCTG3`
CD8a-CD4-F2	5`CTG GAC TTC GCC TGT GAT ATG GCC CTG ATT GTG CTG3'
CD8a-CD4-R1	5`CAG CAC AAT CAG GGC CAT ATC ACA GGC GAA GTC CAG3'
CD8a-CD4-R2	5`CGA TCG GAA TTC TAA TGG GGC TAC ATG TCT TCT G3'
CD8a-CD-M6PR-R	5`CCTTTGGCTCCCACTACCAGTCGCTGGCAGTAAAG GGTGATAACCAAGTGAC3'
CD8a-CFF-R	CCAAGATGATAGTTGATCTCTCATCACAGGCGAAGT CCAG
CD8a-CI-M6PR-R	5`CATTGTTTCCCTCCTCTTCTTCTTGCAGTAAAGGG TGATAACCAAGTGAC3'
CD8a-f- CTS(KTRK)-GFP-R	5`CAC GTC GAA TTC GGA CGT ATC TCG CCG AAA GGC3'
CD8a-f-CTS-GFP-F	5`GTC TAG AAT TCA GCC ACC ATG GCC TTA CCA GTG ACC GCC TTG C3'
CD8a-f-CTS-GFP-R	5`AAT TCG TTC TGG CTT TAT CTT TCT GGT CTT GCT CTT CTT AAA CCA GCA GCA AAC GAG GCA CAT C3'
CD8a-furin-R	5`CGAAAACTAAAGCCAGAGCGCAGCTGGCAGTAA AGGGTGATAACCAAGTG3'
CD8a-GFP-F	5`GCA GTC CTC GAG ATG GCC TTA CCA GTG ACC'3

CD8a-hnRNPM-GFP-R1	5'CTC CTT CCT CTT CTC GTT CTG GGC GGG CCT CTC CAT GAC GTA TCT CGC CGA AAG3'
CD8a-hnRNPM-GFP-R2	5'GGG CTC GAA CCT GTT GCC GCC CCT CTT GAT GTT CTT CTC CTT CCT CTT CTC GTT C3'
CD8a-hnRNPM-GFP-R3	5'ACT GCA GAA TTC CCC TCT TGG TGG GGT TGG CGT AGG GCT CGA ACC TGT TGC C3'
CD8aIBB-GFP-R	5'CAC GTC GGA TCC GC TTC TAG ACT TTC TTC TTG GG3'
CD8a-NLS-GFP-R	5'GAA TTC CTA CCT TTC TCT TCT TTT TTG GAT CTA CCT TTC TCT TCT TTT TTG GAT CGA CGT ATC TCG CCG AAA GG3'
CD8a-sortilin-R	5'GAACCTTCCCCCACAGACATATTTCTTGCAGTAAA GGGTGATAACCAGTGAC3'
CD8a-VSVG-F2	5'CTG GAC TTC GCC TGT GAT TTT TTC TTT ATC ATA GGG3'
CD8a-VSVG-R1	5'CCC TAT GAT AAA GAA AAA ATC ACA GGC GAA GTC CAG3'
CD8a-VSVG-R2	5'ATC GCT GAA TTC TGT TTC CAA GTC GGT TCA TCT C3'
CD-M6PR-F	5'GTCACTGGTTATCACCCCTTTACTGCCAGCGACTGG TAGTGGGAGCCAAAGG3'
CD-M6PR-XbaI-R	5'GACCTGTCTAGACTACATTGGTAATAAATGGTCAT CC3'
CFF(KTRK)-GFP-F2	5'GCT GGT TTA AGA AAA GCG CAG CCG CAG CCA TAA AAC CAG AAG ACA TAT C3'
CFF(KTRK)-GFP-R1	5'GAT ATG TCT TCT GGT TTT ATG GCT GCG GCT GCG CTT TTC TTA AAC CAG C3'
CFF(KTRK)-GFP-R2	5'CCA AGA TGA TAG TTG ATC TCT CAT CAC AGG CGA AGT CCA G3'
CFF-CCC-F	5'CATGGGTGGCTCTGAGCGCTCTCGTTGCCGCCTGG TTTAAGAAAAGCAAAACC3'
CFF-CCC-R	5'GGTTTTGCTTTTCTTAAACCAGGCGGCAACGAGA GCGCTCAGAGCCACCCATG3'

CFF-F	5'CTGGACTTCGCCTGTGATGAGAGATCAACTATCAT CTTGG3'
CFF-GFP-F2	5'CTG GAC TTC GCC TGT GAT GAG AGA TCA ACT ATC ATC TTG G 3'
CFF-GFP-R1	5'CCA AGA TGA TAG TTG ATC TCT CAT CAC AGG CGA AGT CCA G3'
CFF-GFP-R2	5'ACT GCA GAA TTC GCT GGA TGG TTT CTG GTG G3'
CFF-KKS-F	5'CGTTTGCTGCTGGTTTGCCGCTGCCAAAACCAGAA AAATAAAACC3'
CFF-KKS-R	5'GGTTTTATTTTTCTGGTTTTGGCAGCGGCAAACCA GCAGCAAACG3'
CFF-KTRK-F	5'GCTGGTTTAAGAAAAGCGCAGCCGCAGCCATAAA ACCAGAAGACATATC3'
CFF-KTRK-R	5'GATATGTCTTCTGGTTTTATGGCTGCGGCTGCGCT TTTCTTAAACCAGC3'
CFF-LV-F	5'GGGTGGCTCTGAGCTGTGCCGCCTGCTGCTGGTTT AAGAAAAGC3'
CFF-LV-R	5'GCTTTTCTTAAACCAGCAGCAGGCGGCACAGCTC AGAGCCACCC3'
CFF-R	5'ACTGCAGAATTCGCTGGATGGTTTCTGGTGG3'
CFF-WF-F	5'GCTGTCTCGTTTGCTGCGCCGCCAAGAAAAGCAA AACCAG3'
CFF-WF-R	5'CTGGTTTTGCTTTTCTTGGCGGCGCAGCAAACGAG ACAGC3'
CFF Δ C-GFP-R1	5'CCA CGC TGC ACA GGC TCA GGG CCA GGA TGA TGG TGC TTC TCT CAT CAC AGG CGA AGT CC3'
CFF Δ C-GFP-R2	5'CTT AAA CCA GCA GCA AAC GAG GCA GCT CAG GGC CAC CCA GCT GGC CAC GCT GCA CAG G3'
CFF Δ C-GFP-R3	5'GCT CAT GAA TTC GTT CTG GCT TTA TCT TTC TGG TCT TGC TCT TCT TAA ACC AGC AGCA 3'

CI-M6PR-F	5'GTCACTGGTTATCACCCCTTTACTGCAAGAAGAAG AGGAGGGGAAACAATG3'
CI-M6PR-XbaI-R	5'GACCTGTCTAGATCAGATGTGTAAGAGGTCCTCG TC3'
f-CTS(KTRK)-GFP- F	5'GCA GTC GAA TTC ATG TGC CTC GTT TGC TGC TGG TTT AAG AAG AGC GCC GCC GCA GCC ATA AAG CC3'
f-CTS(KTRK)-GFP- R	5'GGC GAC CGG TGG ATC CCG TTC TGG CTT TAT CTT TCT G3'
f-CTS-GFP-F	5'AA TTC ATG TGC CTC GTT TGC TGC TGG TTT AAG AAGAGC AAG ACC AGA AAG ATA AAG CCA GAA CGG3'
f-CTS-GFP-R	5'GA TCC CGT TCT GGC TTT ATC TTT CTG GTC TTG CTC TTC TTA AAC CAG CAG CAA ACG AGGCAC ATG3'
Furin-F	5'CACTGGTTATCACCCCTTTACTGCCAGCTGCGCTCT GGCTTTAGTTTTTCG3'
Furin-XbaI-R	5'GACCTGTCTAGATCAGAGGGCGCTCTGGTCTTTGA TAAAGG3'
GL2-sh-F	5'CCGG AAC GTA CGC GGA ATA CTT CGA CTC GAG TCG AAG TAT TCC GCG TAC GTT TTT TTG 3'
GL2-sh-R	5'AATTC AAAAA AAC GTA CGC GGA ATA CTT CGA CTC GAG TCG AAG TAT TCC GCG TAC GTT 3'
GST-Cystin-F	5'AA TTC ACC GCC AGC GAG GGC GGC ACC GCC CGG3'
GST-Cystin-R	5'GA TCC CGG GCG GTG CCG CCC TCG CTG GCG GTG3'
GST-PC-1-F	5'AA TTC AAG GTG CAC CCC AGC AGC ACC CGG3'
GST-PC-1-R	5'GA TCC CGG GTG CTG CTG GGG TGC ACC TTG3'
GST-PC-2-F	5'AA TTC ATG GTG AAC AGC AGC AGG GTG CAG CCC CAG CAG CCC GGC GAC GCC CGG3'
GST-PC-2-R	5'GA TCC CGG GCG TCG CCG GGC TGC TGG GGC TGC ACC CTG CTG CTG TTC ACC ATG3'

GST-pheripherin-F	5' AATTC AAG AGC AGC TGG GAG CTG GTG AAG AGC ATG GGC AAG CTG AAC AAG GTG GAG ACC GCC GGC CGG3'
GST-pheripherin-R	5' GAT CCC GGC CGG CGG TCT CCA CCT TGT TCA GCT TGC CCA TGC TCT TCA CCA GCT CCC AGC TGC TCT TG3'
GST-rDH-F	5' AA TTC CTG AGG TGC CTG GCC TGC AGC TGC TTC AGG ACC CCC GTG TGG CCC AGG CGG3'
GST-rDH-R	5' GA TCC CGC CTG GGC CAC ACG GGG GTC CTG AAG CAG CTG CAG GCC AGG CAC CTC AGG3'
GST-RP2-15aa-F	5' AATTC ATG GGC TGC TTC TTC AGC AAG AGA AGA AAG GCC GAC AAG GAG AGC AG G3'
GST-RP2-15aa-R	5' GATCC CT GCT CTC CTT GTC GGC CTT TCT TCT CTT GCT GAA GAA GCA GCC CAT G3'
GST-SSTR3-F	5' GCA GTC GAA TTC ATG GTG GTG AAG GTG CGC TCA G3'
GST-SSTR3-R	5' ACG ACT GGA TCC TTA GCG CGT GAC CCT GCG TTC3'
IL2R-WT-F	5' GCA GTC CTC GAG ATG GAT TCA TAC CTG CTG ATG TGG3'
IL2R-WT-R	5' CAC TAC GAA TTC GGA TTG TTC TTC TAC TCT TCC TCT GTC TCC GCT GCC AGG TGA GCC CAC TCA GGA G3'
TNPO1-sh-F	5' CCGG CAA TGC TCA ACC AGA TCA ATA CTC GAG TAT TGA TCT GGT TGA GCA TTG TTT TTG3'
TNPO1-sh-R	5' AATTC AAAAA CAA TGC TCA ACC AGA TCA ATA CTC GAG TAT TGA TCT GGT TGA GCA TTG3'
pEGFPN1-R	5' GTCGTAACAACCTCCGCCCC3'
Rab8A(Q67L)-F2	5' GAC ACA GCC GGT CTG GAA CGG TTT CGG3'
Rab8A(Q67L)-R1	5' CCG AAA CCG TTC CAG ACC GGC TGT GTC3'
Rab8A(T22N)-F2	5' GGG GTG GGG AAG AAC TGT GTC CTG TTC C3'

Rab8A(T22N)-R1	5'GGA ACA GGA CAC AGT TCT TCC CCA CCC C3'
Rab8A-F	5'GTC TAG GAA TTC GCG AAG ACC TAC GAT TAC CTG TTC AAG3'
Rab8A-R	5'CAC TAC GGA TCC CTA CAG AAG AAC ACA TCG GAA AAA GCT GCT3'
Sortilin-F	5'GTCACTGGTTATCACCCCTTTACTGCAAGAAATATG TCTGTGGGGGAAGGTTC3'
Sortilin-XbaI-R	5'GACCTGTCTAGACTATTCCAAGAGGTCCTCATCTG AG3'
Vamp2-V43AM46A-GFP-F1	5'TCA GAT CTC GAG ATG TCG GCT ACC GCT GCC3'
Vamp2-V43AM46A-GFP-F2	5'GTG GCG GAC ATC GCC AGG GTG AAT GTG GAC AAG3'
Vamp2-V43AM46A-GFP-R1	5'CCT GGC GAT GTC CGC CAC CTC ATC CAC CTG GGC CTG3'
Vamp2-V43AM46A-GFP-R2	5'ACT GCA GAA TTC GAG AGC TGA AGT AAA CTA TG3'
Vamp8-V23PK24AM27A-GFP-F1	5'TCA GAT CTC GAG ATG GAG GAG GCC AGT GGG3'
Vamp8-V23PK24AM27A-GFP-F2	5'CCC GCC AAT ATT GCC ACC CAG AAT GTG GAG CGG3'
Vamp8-V23PK24AM27A-GFP-R1	5'GGT GGC AAT ATT GGC GGG TCC CTC CAC CTC ACT CTG3'
Vamp8-V23PK24AM27A-GFP-R2	5'ACT GCA GAA TTC GAG TGG GGA TGG TAC CAG TG3'