The Joubert syndrome protein ARL13B binds tubulin to maintain uniform distribution of proteins along the ciliary membrane

Ekaterina Revenkova1,6, Qing Liu1, G. Luca Gusella2,5, Carlo Iomini1,3,4,5*

Affiliations

1 Department of Ophthalmology, 2 Department of Medicine, 3 Department of Cell, Developmental and Regenerative Biology, 4 Friedman Brain Institute, 5 Graduate School of Biomedical Sciences, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, New York, NY 10029.

6 Current address: Grail, 1525 O'Brien Drive, Menlo Park, CA 94025

Contact Information

* Author for correspondence: carlo.iomini@mssm.edu

Keywords

Primary cilia, Arl13b, Ciliopathies, Joubert Syndrome, Ciliary membrane, Small GTPase, Tubulin, Axoneme
Abstract

Cilia-mediated signal transduction involves precise targeting and localization of selected molecules along the ciliary membrane. However, the molecular mechanism underlying these events is unclear. The Joubert syndrome protein ARL13B is a membrane-associated G-protein that localizes along the cilium and functions in protein transport and signaling. We identify tubulin as a direct interactor of ARL13B and demonstrate that the association occurs via the G-domain and independently from ARL13B’s GTPase activity. The G-domain is necessary for the interaction of ARL13B with the axoneme both in vitro and in vivo. We further show that exogenously expressed mutants lacking the tubulin-binding G-domain (ARL13B-ΔGD) or with inactivated GTPase domain (ARL13B-T35N) retain ciliary localization, but fail to rescue ciliogenesis defects of null Arl13b<sup>hnn</sup> mouse embryonic fibroblasts (MEFs). However, while ARL13B-ΔGD and the membrane proteins Smoothened (SMO) and Somatostatin receptor-3 (SSTR3) distribute unevenly along the cilium of Arl13b<sup>hnn</sup> MEFs, ARL13B-T35N distributes evenly along the cilium and enables the uniform distribution of SMO and SSTR3. Thus, we propose a novel function of ARL13B in anchoring ciliary membrane proteins to the axoneme through the direct interaction of its G-domain with tubulin.
**Introduction**

Primary cilia are elongated protrusions of the cellular membrane where components of multiple signal transduction pathways concentrate to enable the response to a range of extracellular stimuli including chemicals, light and mechanical forces. Defects of ciliary structure or function cause systemic developmental abnormalities that are collectively called ciliopathies. A cilium, is supported by a central core of nine or nine plus one microtubule doublets (A and B tubules), the axoneme, emanating from a modified centriole, the basal body. The axoneme is sheathed by a ciliary membrane which, despite its continuity with the plasma membrane, displays a distinct protein and lipid composition reviewed in (Emmer et al., 2010; Nachury et al., 2010).

The localization and arrangement of proteins along the ciliary membrane can vary greatly ranging from ordered functional clusters, which identify specific ciliary subdomains, to even distribution along the length of the cilium (Fujiu et al., 2009; Huang et al., 2016; Iomini et al., 2006; Watnick et al., 2003; Zhang et al., 2013). Signaling molecules and structural components of the cilium are transported along the axonemal microtubules by specialized protein complexes such as BBSome and the intraflagellar transport (IFT) motor proteins (Jin et al., 2010; Lechtreck, 2015; Piperno et al., 1996). Aberrant localization or accumulation of membrane proteins in the ciliary compartment can drastically interfere with the cilium signaling function (Dorn et al., 2012; Larkins et al., 2011). However, the mechanisms involved in the delivery, anchoring, and segregation of proteins along the ciliary membrane are poorly understood.

Small GTPases contain a guanine-nucleotide-binding (G) site that alternates between an off (GDP-bound) and on (GTP-bound) state and function as molecular switches to control a number of intracellular processes such as trafficking, cytoskeleton dynamics, and signal transduction.
ADP ribosylation factor (Arf)-like proteins (Arls) ARL3, ARL4, ARL6 and Arl13B are members of the small GTPase Ras superfamily that localize to cilia and function in cilia assembly and maintenance of axonemal architecture (Sung and Leroux, 2013). In particular, ARL13B originally identified in genetic screenings as a protein essential for the integrity of ciliated organs and neural tube patterning, is a membrane-associated protein implicated in ciliary protein transport and signaling (Caspari et al., 2007; Larkins et al., 2011; Sun et al., 2004). In humans, mutations of ARL13B cause Joubert syndrome, a ciliopathy characterized by brain malformations, combined with polydactyly and renal cyst formation (Cantagrel et al., 2008a; Thomas et al., 2015).

Although defective in size and structure, cilia persist in Arl13b mutants but, the compartmentalization of ciliary membrane proteins is disrupted often resulting in aberrant accumulations along the ciliary membrane (Cevik et al., 2010; Larkins et al., 2011). Smoothened (SMO), along with the Patched receptor and Sonic Hedgehog ligand (SHH), is a main component of the Hedgehog signaling pathway (HH). In wild-type cells, SMO concentrates into the cilium and distributes along the ciliary membrane only upon binding of SHH to Patched. In contrast, in mouse embryonic fibroblasts (MEFs) derived from the homozygote Arl13b<sup>hnn</sup> mouse allele, SMO is permanently present in the cilium and abnormally distributed in puncta along the ciliary membrane regardless the presence of SHH, thus impeding the proper HH signaling (Larkins et al., 2011; Mariani et al., 2016). Recently, it was shown that ARL13B acts as effector of ARL3 in binding to the carrier proteins UNC119a/b and prenyl-binding protein phosphodiesterase 6D (PDE6D) to release prenylated or myristoylated proteins such as Nephrocystin-3 (NPHP3) and Inositol polyphosphate-5-phosphatase E (INPP5E) specifically at the ciliary membrane (Gotthardt et al.,
Furthermore, absence of ARL13B leads to structural defects of axonemal microtubules (Caspary et al., 2007; Zhou and Anderson, 2010). Thus, the localization of ARL13B to cilia and its proper positioning along the cilium appear to be critical for normal trafficking and distribution of membrane and lipidated proteins to the cilium and for the maintenance of axonemal integrity. However, the factors that determine the proper localization of ARL13B within the cilium and the molecular interactions underlying its functions are currently unknown.

To gain mechanistic insight into ARL13B regulation and positioning along the ciliary membrane, we sought to identify interaction partners essential for its function in the cilium. Here we show that the G domain of ARL13B directly binds to tubulin and that this binding is required for the even distribution of ARL13B along the ciliary membrane. Our findings indicate that in addition to regulating IFT, ARL13B provides means for proper distribution of signaling proteins including SMO and Somatostatin Receptor 3 (SSTR3) along the ciliary membrane through interactions with the microtubules of the ciliary axoneme.

Results

The G-domain of ARL13B interacts with tubulin

ARL13B includes an N-terminal amphipathic helix, which is required for membrane insertion, followed by the G domain and an atypically elongated C-terminus that contains a coiled-coil domain and a proline-rich domain (PRD). The murine and human amino acid (aa) sequence of ARL13B show 80% identity, the most conserved regions spanning over the G-domain and the C-terminus. To enhance the discovery of ARL13B interactors with these regions, we generated an
antibiotic-selectable lentiviral vector that constitutively expresses the murine ARL13B fused with a tandem affinity purification (TAP) tag (GFP-TEV-S-tag) at its N-terminus in immortalized human retinal pigment epithelium (hTERT-RPE1) cell line (Fig. S1A). Following transduction of hTERT-RPE1 and antibiotic selection, we isolated a line, in which the recombinant GFP-S-mARL13B and the endogenous ARL13B were comparably expressed and similarly localized in the cilium (Fig. S1B, C). Cells of the selected line were grown to confluency, and ciliogenesis was induced by serum withdrawal. The cells were used to prepare a protein extract and isolate ARL13B-interacting proteins using a TAP procedure (Fig. 1A). The resulting set of proteins was resolved on a gel and the composition of the bands visible after silver staining was determined by mass-spectrometry (Fig. 1A).

In agreement with previous reports, we identified myosin and actin among the proteins interacting with the tagged ARL13B (Barral et al., 2012; Casalou et al., 2014). However, the most abundant proteins, based on band intensity and number of unique peptides, co-purifying with TAP-tagged ARL13B were α- and β-tubulin with 137 and 238 peptides recovered, respectively (Fig. 1A, B). Specifically, the analysis of a band excised from a preparative gel at approximately 50 kilodaltons included the following tubulin isotypes: tubulin beta chain (TUBB), 23 unique peptides; tubulin alpha-1B chain (TUBA1B), 17 unique peptides; tubulin beta-6 chain (TUBB6), 9 unique peptides; tubulin beta-2A chain (TUBB2A), 6 unique peptides; tubulin beta-4B chain (TUBB4B), 5 unique peptides; tubulin alpha-4A chain (TUBA4A), 3 unique peptides; tubulin beta-3 chain (TUBB3), 3 unique peptides. We corroborated the association of tubulin with ARL13B by immunoprecipitation of endogenous ARL13B from HEK293 cells using a specific anti human ARL13B antibody and detection of tubulin (Fig. 1C). These experiments not only confirmed the
interaction between ARL13B and tubulin but also indicated the overlapping activity of human and
murine ARL13B.

Next, we asked if the interaction with tubulin depends on ARL13B binding with GDP or GTP. GST-
ARL13B immobilized on GSH sepharose beads was loaded with GDP or the non-hydrolyzable
analog GTPγS and then incubated with hTERT-RPE1 cell lysate. Similar amounts of tubulin co-
precipitated with ARL13B regardless of its GDP or GTP bound form (Fig. 1D, lanes 2 and 3). To
further address this possibility, we analyzed tubulin binding using purified GST-ARL13B with the
point mutation R79Q. The diseases-causing mutation ARL13B R79Q was shown to reduce ARL13B
binding with GTP and to affect protein conformation in the GTP-bound state in the corresponding
Chlamydomonas ARL13B mutant (Cantagrel et al., 2008b; Miertzschke et al., 2014). Tubulin
binding of GST-ARL13B in the presence of GTPγS was not affected by the mutation (Fig. 1D, lanes
2 and 4), thus, confirming that the interaction of ARL13B with tubulins is not altered by its GTP
or GDP binding state. Moreover, tubulin molecules interacting with ARL13B appeared to be
acetylated (Fig. 1D). However, further investigation will be required to determine whether
tubulin acetylation is necessary for this interaction to occur.

To distinguish between a direct or indirect association of ARL13B with tubulin, we produced
recombinant GST-tagged human ARL13B from E. coli and incubated it in the presence of purified
bovine brain tubulin. The incubation of GST protein tag with tubulins was used as control.
Glutathione (GSH) sepharose beads were added to each reaction mix to purify fused GST-ARL13B
or control GST, and their interacting proteins were analyzed by western blotting. Tubulin was
found in the eluate from the beads loaded with GST-ARL13B, but not in that from control GST
(Fig. 2A and 2B, lanes "GST-ARL13B aa 1-428" and "GST"), thus indicating that ARL13B can directly
interact with tubulin dimers. To map the tubulin–interacting regions of ARL13B, we purified a series of GST fusion portions of ARL13B and a deletion mutant that lacked the G-domain while retaining the N-terminal amphipathic helix (Fig. 2A). GST pull-down of the mutants incubated with hTERT-RPE1 extract revealed that the G-domain and significantly truncated fragments of the G-domain (aa 20-119 and aa 20-79), but not the coiled-coil region or the amphipathic helix, mediated the interaction with soluble tubulin (Fig. 2A-E). We have also examined tubulin binding with GST-ARL13B carrying the point mutation T35N located in a motif referred to as P-loop, which is highly conserved among GTP-binding proteins. The mutation of the corresponding Ser17 in Ras proteins (Ras S17N) affects their GTPase activity and significantly interferes with Ras signaling by strongly altering Ras affinity to nucleotides and guanine-nucleotide exchange factors (GEFs) (Cool et al., 1999; Dascher and Balch, 1994; Feig, 1999). The T35N ARL13B mutation, which was previously shown to render ARL13B non-functional (Duldulao et al., 2009; Humbert et al., 2012), did not hinder binding to tubulin (Fig. 2F), thus confirming that, at least in vitro, the interaction with tubulin does not depend on ARL13B GTPase activity.

**ARL13B G-domain is not required for transport to cilium but is necessary for the control of the ciliary length and assembly**

To investigate the cellular function of the tubulin-binding region, we created lentiviral vectors to express GFP-tagged deletion mutants of ARL13B in MEFs obtained from wild-type and in protein null Arl13b<sup>hnn/hnn</sup> (Arl13b<sup>hnn</sup>) mutant mice (Fig. 3 and Fig. S2). Because the interaction with tubulin was mapped within the G domain of ARL13B, we first asked whether the G domain is required to translocate ARL13B to the ciliary compartment. As ARL13B interactions with the membrane
through palmitoylation or myristoylation at the N-terminus are critical for its compartmentalization (Cevik et al., 2010; Duldulao et al., 2009; Hori et al., 2008; Larkins et al., 2011; Zhou and Anderson, 2010), we maintained in our constructs the N-terminal amphipathic helix (aa 1-19) (Fig. 3A). In agreement with Nozaki et al. (Nozaki et al., 2017), we found that when the amphipathic N-terminal region (aa 1-19) was included in the chimeric proteins, both GFP-mARL13B∆GD, which lacks the GTPase domain (aa 20-198), and the GFP-mARL13B T35N, which contains the T35N mutation predicted to disrupt the GTP-binding site, efficiently translocated to the cilium of hTERT-RPE1 cells (Fig. S2) as well as wild-type and Arl13b hnn MEFs (Fig. 3D). In contrast, despite the presence of the complete G domain with the ciliary localization motif RVxP (Deretic et al., 2005; Geng et al., 2006; Ward et al., 2011), GFP-mARL13B∆CC lacking the coiled-coil domain (aa 199 - 353) failed to localize to the cilium of hTERT-RPE1 cells (Fig. S2). Thus, C-terminal domain, but not the G-domain, plays an essential role in ARL13B translocation into the ciliary compartment. However, the cilium-localized GFP-mARL13B∆GD could not restore the ciliary localization of the membrane-associated protein INPP5E in Arl13b hnn MEFs (Fig. S3) suggesting an active role of the G domain in INPP5E translocation into cilia (Nozaki et al., 2017). Several studies in different species and cell types have shown that the overexpression of ARL13B causes cilium elongation (Hori et al., 2008; Larkins et al., 2011). To determine whether this effect depends on the G domain we transduced wild-type MEFs with constructs expressing GFP-ARL13B with wild-type or mutated G domain. Cilia length in most non-transduced MEFs ranged between 2.0-2.9 µm (56.7% of cells) and 3.0-3.9 µm (33.3% of cells), whereas in either GFP-mARL13B- or GFP-huARL13B-transduced MEFs GFP-positive cilia measured 5.0-5.9 µm and extended over 8.0 µm in 30.2% and 3.8% of the cells, respectively (Fig. 3B, D and E). Expression of GFP-mARL13B∆GD
or GFP-ARL13B T35N led to a comparable increase in cilia length (Fig. 3D and E) indicating that abnormal elongation of cilia does not depend on the presence of the G domain.

Given that ectopic expression of the mARL13BΔGD mutant or wild-type mARL13B could similarly affect ciliary length, we asked whether the mARL13BΔGD mutant could rescue cilia length/assembly defect in protein null Arl13b<sup>hnn</sup> MEFS (Caspary et al., 2007; Larkins et al., 2012). For accurate cilia length evaluation, we measure cilia length of transduced Arl13b<sup>hnn</sup> MEFS by co-staining with IFT88, which concentrates at the base and at the tip of the cilium and in puncta along the cilia length, and γ-tubulin to mark the basal body (see Material and Methods) (Fig. 3C). Moreover, in untransduced or GFP-mARL13BΔGD-transduced Arl13b<sup>hnn</sup> MEFS, IFT subunits of either complex B (IFT88) or complex A (IFT140), accumulated at the ciliary tip, (Fig. S4), consistent with Nozaki et al. (Nozaki et al., 2017). The large majority of Arl13b<sup>hnn</sup> MEFS cilia (71.1 %) measured less than 2 μm (Fig. 3C and F), although some cilia longer than 4 μm (5.5%) were also present (Fig. 3F). However, the ectopic expression of GFP-tagged wild-type mARL13B or huARL13B re-established the cilia length of most Arl13b<sup>hnn</sup> MEFS to 2.0-4.0 μm (Fig. 3E and F) (Larkins et al., 2011). In contrast, GFP-mARL13BΔGD failed to rescue the blunted cilia phenotype of Arl13b<sup>hnn</sup> MEFS in the absence of the endogenous ARL13B (75% of cells with cilia shorter than 2μm and the remaining cilia > 2μm) (Fig. 3 E and F). The ARL13B mutant containing the T35N point mutation in the G-domain, which maintains it in the non-functional GDP-locked state without affecting ARL13B-tubulin binding, was similarly unable to fully rescue the short cilia of Arl13b<sup>hnn</sup> MEFS. However, 66% of cilia were longer than 2μm suggesting that in cilium elongation GFP-ARL13B T35N is more efficient than mARL13BΔGD (Fig. 3E and F). These results support the idea that although the GTPase activity is necessary for the normal assembly and maintenance of
the cilium, the G domain of ARL13B could harbor sequences with distinct functions important for cilia elongation.

The ARL13B G domain sequence, but not its GTPase activity, is required to establish and maintain uniform distribution of ARL13B and other membrane proteins along the ciliary membrane

Although the majority of the Arl13b<sup>hnn</sup> MEFs displayed a very short cilium we found that a small percentage of cells (~5%) were able to assemble a cilium with an axoneme that equaled or exceeded the length of cilia detected in wild-type MEFs (Fig. 3E). Because in Arl13b<sup>hnn</sup> MEFs the membrane protein SMO was reported to aberrantly accumulate in discrete regions of the cilium and predominantly at the ciliary tip (Larkins et al., 2011), we carefully analyzed the distribution of the GFP-mARL13B and the GFP-mARL13BΔGD along the cilium of normal and Arl13b<sup>hnn</sup> MEFs (Fig. 4A-J). In wild-type MEFs, both GFP fusion proteins were distributed uniformly along the full length of cilia overlapping the distribution of the endogenous ARL13B protein (Fig. 4A-C). In contrast, in Arl13b<sup>hnn</sup> MEFs, GFP-mARL13BΔGD localized at the very distal tip of the cilium and accumulated in expansions of the ciliary membrane but was undetectable or barely visible along the rest of the ciliary length (Fig. 4E). Occasionally, in shorter cilia we also observed accumulation of GFP-mARL13BΔGD at the ciliary base but, GFP signal remained very low along the central region of the cilium (Fig. 4F). Finally, GFP-mARL13BΔGD concentrated in stump-like cilia of Arl13b<sup>hnn</sup> MEFs (Fig. 4G). Cilia of variable length were also observed in non-transduced MEFs as shown by staining with an anti-acetylated tubulin antibody (Fig. 4H-J). Ultrastructural analysis of clonal mutant GFP-mARL13BΔGD–expressing Arl13b<sup>hnn</sup> MEFs revealed that electron-dense
material accumulated within the ciliary matrix, the space encompassed by the axoneme and the ciliary membrane, of the entire ciliary length and in stump-like cilia (Fig. 4K). This accumulation was not observed in the ciliary matrix of clonal Arl13b<sup>hnn</sup> MEFs expressing the wild-type GFP-mARL13B (Fig. 4K). Interestingly, the distribution of electron-dense material observed along the entire cilium of Arl13b<sup>hnn</sup> MEFs did not parallel the distribution of the membrane-associated GFP-mARL13BΔGD observed by IF at the distal or proximal tip in the Arl13b<sup>hnn</sup> MEFs cilia (Fig. 4E, F and L). These observations suggested that in addition to its role in cilia assembly, the G domain of ARL13B could play a role in the distribution of membrane proteins along the ciliary membrane.

To test this function, we analyzed the distribution along the cilium of ARL13B variants predicted or shown to interfere with the GTP-GDP exchange activity of ARL13B including the GFP-mARL13B T35N variant and the Joubert syndrome-causing mutation GFP-mARL13B R79Q. In wild-type MEFs, the wild-type and all the mutant GFP fusion proteins tested distributed evenly along the cilium (Fig. 3D and 4M). In contrast, in Arl13b<sup>hnn</sup> MEFs, while GFP-ARL13B-ΔGD distribution was visibly affected in more than 80% of the cilia both, GFP-mARL13B T35N and GFP-mARL13B R79Q distributed evenly along the cilium (Fig 4L and M). Thus, the even distribution of ARL13B along the cilium depends on ARL13B interactions that occur within the G domain, but are independent of its GTPase activity.

To determine whether the localization of other ciliary membrane proteins was affected by the absence of ARL13B, we analyzed the distribution of Smoothened (SMO) and somatostatin receptor 3 (SSTR3) along the cilium of Arl13b<sup>hnn</sup> and wild-type MEFs (Fig. 5). Consistent with previous studies, we found SMO evenly distributed along the cilium of SHH-stimulated wild-type MEFs, although in a slightly higher concentration at the ciliary base (Fig. 5A). Similarly, the
ectopically expressed mCherry-tagged SSTR3 fusion protein also displayed uniform distribution along all length of the ciliary membrane of wild-type MEFs (Fig. 5G). In contrast, in Arl13b<sup>hnn</sup> MEFs SMO concentrated in discrete regions of the cilium localized mostly at the distal and/or proximal extremities of cilia regardless the presence of SHH in the medium (Fig. 5B). However, we noticed that SMO in cilia of SHH-stimulated Arl13b<sup>hnn</sup> MEFs was almost exclusively detected at the distal tip of the cilium (Fig. 5B and E). Similarly, the Cherry-tagged SSTR3 also failed to distribute evenly along the cilium and accumulated at the distal tip of Arl13b<sup>hnn</sup> MEFs cilia (Fig. 5H). These results are consistent with previously reported observations (Larkins et al., 2011). Importantly, SMO and mCherry-tagged SSTR3 aberrant distribution along the ciliary membrane in Arl13b<sup>hnn</sup> MEFs could be rescued by wild-type GFP-ARL13B and by the GDP-locked variant ARL13 T35N, but not by GFP-mARL13BΔGD (Fig. 5C-F, I-L, and M). These results indicate that ARL13B plays an important role in localizing and maintaining the distribution of membrane proteins along the ciliary membrane independently of its GTPase activity.

**ARL13B interacts with the axoneme through its tubulin-binding domain**

To explain how the membrane-associated ARL13B distributed evenly along the cilium length we hypothesized that the capacity of ARL13B of binding to tubulin may provide means for physical interaction with microtubules and thus retention along the axoneme. To test whether ARL13B G-domain is required for anchoring ARL13B to the ciliary axoneme, we performed in vitro pull-down of GST-ARL13B fragments using demembranated axonemes purified from isolated *Chlamydomonas* flagella (Alper et al., 2013). The assay showed that both full-length ARL13B and the G-domain bind isolated axonemes while the ARL13B coil-coil domain does not (Fig. 6A).
Moreover, deletion of the G-domain drastically decreased the ability of ARL13B to bind to axonemes (Fig. 6B).

To confirm the binding of ARL13B to the ciliary axoneme in vivo we exposed distinct clones of ciliated wild-type MEFs stably expressing comparable amount of GFP-ARL13B or GFP-ARL13B-ΔGD (Fig. S5A) to different concentrations of Triton X-100 ranging from 0% to 0.05% prior to fixation and analyzed the retention of the GFP-fused proteins along the cilium. In absence of detergent, GFP signal was observed evenly distributed along the full length of all cilia of MEFs clones expressing either GFP-ARL13B or GFP-ARL13B-ΔGD. In contrast, after treatment with 0.025% Triton X-100 GFP signal was undetectable along cilia of MEF expressing GFP-ARL13B-ΔGD while it remained visible on >50% of wild-type MEFs expressing GFP-ARL13B (Fig. 6C-D and S5B). Finally, 0.05% Triton X-100 removed both GFP constructs from the large majority of MEFs (Fig. 6D). Similar experiments conducted on Arl13b
hnn MEFs expressing wild-type or mutant versions of GFP-ARL13B corroborated the importance of the G domain involvement in ARL13B interaction with the axoneme. Upon treatment with 0.1% Triton X-100 prior to fixation, IF signal was lost in the cilia of Arl13b
hnn MEFs expressing GFP-ARL13B-ΔGD but not completely in those expressing wild-type version of GFP-ARL13B or GFP-ARL13BΔT35N mutant (Fig. S5C). Taken together, in vitro as well as in vivo evidence revealed that ARL13B binds to the ciliary axoneme through residues located within the G domain.
Discussion

In this study, we have uncovered a functional interaction between the Joubert syndrome causing protein ARL13B and tubulin. We demonstrate that this interaction is direct, occurs within the ARL13B G domain, and is independent from the predicted ARL13B GTPase activity required for ciliary assembly. We also provide evidence that the ARL13B-tubulin interaction is required for uniform distribution of ARL13B and other membrane proteins along the ciliary membrane. Hence, we propose that ARL13B plays a key role in mediating interactions between ciliary membrane components and the axoneme.

GTPases and Tubulin Binding domains

Our TAP approach led to the identification of tubulin as one of the major interactors of ARL13B. Because tubulin is one of the most abundant proteins in cells we have carefully analyzed the specificity of this interaction using multiple approaches including immunoprecipitation of native ARL13B from different cell lines and in vitro binding assays of purified tubulin or whole cell extracts. Consistent with our finding, a proteomic approach using stable isotope labeling affinity chromatography, identified 424 ARL13B-associated proteins, including several tubulin isotypes (Cevik et al., 2013). TUBB, TUBB6, TUBA4A, TUBB4B, TUBB3 tubulin isotypes were in common with our study. Small discrepancies between the two studies could be due to differences in sensitivity of the analytic approaches or by the use of different cell lines. Pull-down experiments using GST fused fragments of ARL13B allowed to map the tubulin binding region within its G domain. Despite this physical overlapping however, our data indicate that nucleotide binding does not modify the status of the ARL13B-tubulin interaction. In support of this
conclusion, we found that the amount of tubulin interacting with ARL13B in vitro remains unchanged regardless the presence of GDP or the non-hydrolyzable analog GTPγS. Moreover, point mutations in the ARL13B G domain that reduce ARL13B binding with GTP (R79Q) or abolish ARL13B predicted GTPase activity (T35N) did not alter the binding of ARL13B with soluble tubulin or polymeric tubulin-based structures including microtubules and demembranated axonemes. The use of the huARL13B T35N mutant exogenously expressed in Arl13b

MEFs or in tubulin binding assays allowed us to uncouple the GTPase activity from the interaction of ARL13B with tubulin. Our results indicate that the interaction with tubulin and ARL13B is pivotal to maintain a normal distribution of proteins along the ciliary membrane but it is not sufficient to rescue cilia assembly and protein localization defects detected in absence of the wild-type ARL13B. Recent studies have shown that ARL13B is active as a GEF for ARL3 (Gotthardt et al., 2015; Zhang et al., 2016). Moreover, T35N and Joubert syndrome causing mutations nearly abolish or reduce this activity, respectively (Ivanova et al., 2017). Thus, ARL13B tubulin binding appears dispensable for the ARL13B GEF activity on ARL3. Interestingly, other members of the Arl family including ARL7 and ARL8 were found to interact with tubulin and interfere with microtubule-driven cellular processes including intracellular vesicular transport and chromosome segregation, respectively (Okai et al., 2004; Wei et al., 2009). Moreover, ARL2 binds to the tubulin cofactor D and with ARL3 modulates microtubules dynamics and polymerization (Bhamidipati et al., 2000) (Zhou et al., 2006). Although the mammalian ARL6, a Bardet-Biedl Syndrome causative protein also known as BBS3, does not contain the amphipathic N-terminal domain, its Trypanosoma brucei orthologue protein interacts with the ciliary membrane and with tubulin (Price et al., 2012). Intriguingly, ultrastructural analysis revealed an evolutionarily conserved role of ARL13B in
maintaining the structural integrity of the cilium. The protein-null mutation hnn and a deletion (169-342) of the Arl13b gene in mouse and C. elegans, respectively, lead to an open B-tubule of the axonemal doublets, further supporting a role for ARL13B on axonemal microtubules integrity (Caspary et al., 2007; Zhou and Anderson, 2010).

However, because ARL13B is also required for cilia localization of other components of the ciliary membrane such as INPP5E, it cannot be excluded that these axoneme-related structural defects are an indirect consequence of ARL13B dysfunction in protein translocation into the ciliary compartment. Moreover, consistent with previous studies, levels of acetylated and glutamylated axonemal tubulin as revealed by immunolabeling, appear lower in cells lacking ARL13B compared to control cells (Larkins et al., 2011; Nozaki et al., 2017). However, we found that absence or presence of wild-type or mutant ARL13B variants had no effect on the overall cellular levels of acetylated or polyglutamylated tubulin (Fig. S6). Thus, whether ARL13B has an effect on tubulin polymerization/depolymerization, on tubulin posttranslational modification levels in the axoneme, or in shaping axonemal architecture remains to be established.

The G-domain is dispensable for ciliary translocation of ARL13B to cilia

Although it was shown that GFP-tagged N-terminal or C-terminal domains of ARL13B fail to localize to cilia it was also demonstrated that ARL13B interactions with the membrane through palmitoylation or myristoylation at the N-terminus are critical for its compartmentalization (Cevik et al., 2010; Duldulao et al., 2009; Hori et al., 2008; Larkins et al., 2011; Zhou and Anderson, 2010). To preserve such interactions, we maintained the N-terminal amphipathic helix (aa 1-19) in all our constructs. In agreement with (Nozaki et al., 2017) GFP-ARL13B-ΔGD maintained cilia
localization although it was unable to rescue the defective cilia phenotype of Arl13b<sup>hnn</sup> MEFs. Thus, these observations strongly suggest that the G domain of ARL13B plays a crucial role in ciliary assembly and membrane protein distribution along the ciliary membrane, while it is dispensable for ARL13B trafficking to the ciliary compartment. These data confirm previous results for the T35N mutation and are in agreement with the findings that other point mutations that also affect the active site of ARL13B G-domain, R79Q or T38N, do not interfere with ARL13B trafficking to the cilium (Cantagrel et al., 2008a; Cevik et al., 2013; Duldulao et al., 2009; Hori et al., 2008; Mariani et al., 2016; Miertzschke et al., 2014). Furthermore, our findings strongly suggest that the G-domain and its interaction with tubulin are dispensable for targeting ARL13B to cilia, although it cannot be excluded that trafficking may be regulated by conformational changes in the G-domain, which may, in turn affect, for example, the exposure of the amphipathic helix as shown for ARF1 (Levi et al., 2008).

**ARL13B mediates ciliary membrane-axoneme interactions to maintain uniform distribution of proteins along the ciliary membrane**

We have shown that, although GFP-ARL13B-ΔGD can enter the cilium of Arl13b<sup>hnn</sup> MEFs it accumulates preferentially at the distal ciliary tip or, less frequently, at both, proximal and distal ciliary extremities. Moreover, other ciliary membrane proteins including SMO and SSTR3 display a similar non-uniform distribution along the cilium of Arl13b<sup>hnn</sup> MEFs. This is in striking contrast with the uniform distribution along the ciliary membrane of the wild-type GFP-ARL13B fusion protein that also rescues the short-cilia phenotype of the Arl13b<sup>hnn</sup> MEFs. Importantly, we have shown that the GFP-mARL13B T35N mutant, which allowed to uncouple ARL13B tubulin
interaction from its GTPase activity, can restore the uniform distribution of ARL13b, SMO and SSTR3 but not the short-cilia phenotype of Arl13b<sup>hnn</sup> MEFs. Consistent with these findings, the ciliary protein GPR161 that negatively regulates Hh signaling displays a non-uniform ciliary distribution (and mostly accumulated at the tip of the cilium) in ARL113B deficient cells under basal and Hedgehog-stimulated conditions (Nozaki et al., 2017). The discrete localization and accumulation of GFP-ARL13B-ΔGD at the ciliary tip could be linked to defective localization of IFT components that in Arl13b<sup>hnn</sup> MEFs accumulate mostly at the ciliary tip (Fig. S4) and (Nozaki et al., 2017). It was reported that ARL13B interacts with IFT-46 and IFT56 dimer, components of the IFT complex B. Although this interaction is not necessary for ARL13B localization to cilia, it could be required to move ARL13B along the cilium during anterograde IFT. Consistent with this possibility, GFP-ARL13B-ΔGD maintains its interaction with IFT complex B (Nozaki et al., 2017). In addition, the lack of the G domain has been suggested to interference with the function of IFT complex A in retrograde movement and further explain the accumulation at the ciliary tip and depletion in other ciliary regions of the GFP-ARL13B-ΔGD (Iomini et al., 2009; Mukhopadhyay et al., 2010; Nozaki et al., 2017). How can the ARL13B interaction with tubulin mediate protein distribution along the ciliary membrane? Based on our results we speculate that ARL13B plays a pivotal role in mediating interactions between the ciliary membrane and the axoneme. Interestingly, we found that GFP-ARL13B-ΔGD distributes uniformly along the cilium of wild-type MEFs. Thus, the presence of wild-type endogenous ARL13B allows the proper localization of exogenous GFP-ARL13B-ΔGD. Nevertheless, in vitro and in vivo binding assays to the axoneme revealed that GFP-ARL13B-ΔGD interaction with the axoneme is weakened even in presence of the wild-type endogenous ARL13B. This could be due to the existence of ARL13B homophilic
interactions with other ARL13B molecules along the axoneme. It was shown that ARL13B can be simultaneously localized at the membrane or move together with IFT particles (Cevik et al., 2013). Another pool of ARL13B molecules could bind to the axoneme and thus anchor other ARL13B molecules by direct binding. In support of this possibility, it was shown that ARL113B self-associates, as shown by immunoprecipitation (Hori et al., 2008). In our model, a pool of ARL13B molecule binds to the axoneme via regions of the G domain, however, other molecules of ARL13B could form protein complexes connecting the ciliary membrane and the axoneme. Our data showing the acetylation of α-tubulin molecules interacting with ARL13B is consistent with this possibility since axonemal tubulin is highly acetylated (Fig. 1). Elegant ultrastructural studies of cilia in different model systems have clearly documented the presence of a variety of electron-dense bridges that link the ciliary membrane with the axonemal microtubules along the length of the cilium/flagellum as well as at the ciliary extremities reviewed in (Dentler, 1990). Moreover, biochemical fractionation of *Chlamydomonas* flagella combined with proteomic analysis has revealed that, surprisingly, axonemal fractions of detergent-extracted flagella contained a large number of transmembrane proteins in addition to axonemal proteins (Pazour et al., 2005). This finding strongly supports the existence of interactions (direct or indirect) between ciliary membrane proteins and the axoneme. Our data suggest that ARL13B may play a critical role in mediating/regulating these interactions. Further studies specifically tailored to uncover molecular components and mechanisms underlying axoneme-membrane interactions will broaden our understanding of cilia-mediated signal transduction.
Materials and Methods

Plasmids and lentiviral vectors

The TAP-tag plasmid vectors pIC112 and pIC113 (Cheeseman and Desai, 2005) were provided by Dr. Iain Cheeseman, Whitehead Institute for Biomedical Research, Cambridge, MA. To express TAP-tagged ARL13B in mammalian cells, the sequence 5'- gcg ata tcg gcc tag gaa ggt acc aag gat ccc gga att cag acg agc tgg gcg c - 3' containing various cloning sites between the Nhel and NotI sites of the VVEW/BB lentivector (Battini et al., 2008) to generate VEB2. The Nhel-Spel fragment encoding the GFP-TEV-S tag was excised from pIC113 and inserted into the Nhel site of VEB2 to generate VEB113. Alternatively, VEB2 was digested with PmlI and NotI and the ends were filled-in using PfuUltra DNA polymerase (Agilent Technologies, Santa Clara, CA). The EcoRV-Pmel fragment encoding the 6xHis-PreScission-mRFP tag was excised from pIC112 and inserted into VEB2 to generate VEB112. To express GFP-TEV-S-tagged mouse ARL13B, wild-type mouse Arl13b cDNA (NCBI Reference Sequence: NM_026577.3) was amplified by PCR using PrimeSTAR HS DNA polymerase (Clontech), plasmid pMEmArl13bGFP as a template and primers Arl13/113/Kpn/5 (5'- gtg gta cca aat gtt cag tct gat ggc caa CTG CTG CAA ctt g-3') and Arl13/113/Bam/3 (5'- gag gat cct att atg aga tcg tgt cct gag cat cac cgt tag g - 3').

Plasmids for expression of wild-type human ARL13B or the mutant protein carrying the mutation R79Q, pGEX-ARL13B and pGEX-ARL13B R79Q (Cantagrel et al., 2008b), were a gift from Dr. Joseph Gleeson, UCSD, La Jolla, CA. To express human GFP-TEV-S-tagged ARL13B, cDNA was amplified by PCR using pGEX-ARL13B or pGEX-ARL13B R79Q and primers ARL13BHu-113/Kpn5 (5'- GTG GTA CCA AAT GTT CAG TCT GAT GGC CAG TTG C- 3') and ARL13BHu-113/Bam3 (5'-GAG GAT CCT ATT ATG AGA TCA CAT CAT GAG CAT CAC TGT TAG-3'). The PCR fragments were digested with restriction endonucleases KpnI and BamHI and inserted between the KpnI and BamHI sites of VEB113 to generate VEB113-GFP-TEV-S-mArl13b, VEB113-GFP-TEV-S-huARL13B, or VEB113-GFP-TEV-S-huARL13B-R79Q. To express GFP-TEV-S-tagged mouse ARL13B lacking the coiled-coil domain or the G-domain, the corresponding cDNA fragments were joined by PCR and inserted into VEB113-GFP-TEV-S-mArl13b between the AvrII and BamHI sites or between the AvrII and BstXI sites, respectively. Plasmid for expression of human ARL13B with the point mutations pSSN-huARL13B T35N (Humbert et al., 2012) was a gift from Dr. Seongjin Seo, University of Iowa, Iowa
City, IA. To express GFP-TEV-S-tagged human ARL13B with the point mutations, cDNAs were amplified by PCR and inserted between the KpnI and BamHI sites of VEB113.

To express 6XHis-PreScission-mRFP -tagged mouse ARL13B, wild-type mouse Arl13b cDNA (NCBI Reference Sequence: NM_026577.3) was amplified by PCR using the plasmid pME mARL13b GFP and primers Arl13/112/Kpn/5 (5'- gag gta ccg cca tgt tca gtc tga tgg cca act gct gca act tgt tc -3') and Arl13/112/Bam/3 (5'- gag gat cct gag atc gtg tcc tga gca tca ccc tta ggt ctc tg - 3'). To express 6XHis-PreScission-mRFP -tagged human ARL13B, cDNA was amplified using pGEX-ARL13B or pGEX-ARL13B R79Q (Cantagrel et al., 2008b) and primers ARL13BHu-112-Kpn-5 (5'- GAG GTA CCG CCA TGT TCA GTC TGA TGG CCA GTT GC-3') and ARL13BHu-112-Bam-3 (5'- GAG GAT CCT GAG ATC ACA TCA TGA GCA TCA CTG TTA G-3'). The fragments were digested with restriction endonucleases KpnI and BamHI and inserted between the KpnI and BamHI sites of VEB112 to generate VEB112-mArl13b-6xHis-PreScission-mRFP or VEB112-huARL13B-6xHis-PreScission-mRFP. All the constructs were confirmed by sequencing. Lentivirus-containing supernatants were produced by transient transfection of Lenti-X 293T cells (Clontech) as described previously (Fedorova et al., 2006).

**Cell culture, transfection and lentivirus transduction and HH activation**

Immortalized human retinal pigment epithelium hTERT RPE-1 (ATCC® CRL-400™) were grown in high-glucose DMEM , 10% fetal bovine serum (FBS). Immortalized wild-type and Arl13b<sup>hnn</sup> MEFs were a gift of Dr. Tamara Caspary, Emory University, Atlanta, GA. For immunofluorescence, MEFs were grown on gelatinized glass coverslips in high-glucose DMEM, 10% FBS. Transfection with plasmid SSTR3-mCherry (Addgene) was performed using X-treme GENE 9 DNA transfection reagent (Roche Diagnostics) according to the manufacturer's instructions. For transduction, lentiviral particles were resuspended in serum-free DMEM and added to cells grown in high-glucose DMEM, 10% FBS. Medium was replaced after 24 h. Treatment with SHH was performed as described (Larkins et al., 2011) using human recombinant SHH (StemRD) at 5 ng/ml in high glucose DMEM, 0.5% FBS.
TAP procedure, mass-spectrometry and database searching

A stable cell line hTERT RPE-1 expressing GFP-TEV-Stag-ARL13B (VEB113) was generated using a lentiviral expression vector as previously described (Nachury, 2008). Cells were selected using blasticidin at 10 ug/ml and cloned by FACS. For protein purification cells from a selected clone or wild-type control were grown on 15-cm dishes to confluency and serum-starved for 48 h to induce ciliogenesis. Cell lysates were prepared from 1.5 ml of packed cell pellets and ARL13B-interacting proteins were purified as described by (Nachury, 2008; Torres et al., 2009). Proteins were separated on a 4-15% polyacrylamide TGX gel (Bio-Rad) and analyzed by staining using SilverQuest Silver Staining kit (Life Technologies) and Western blotting. For mass-spectrometry gels were stained with Colloidal Blue staining kit (Life Technologies).

Individual bands were excised manually. Samples were prepared and processed at the Proteomics and Microchemistry Core facility, Memorial Sloan Kettering Cancer Center, New York, NY, as described previously (Schwer et al., 2011). All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.3.02) and X! Tandem (The GPM, thegpm.org; version CYCLONE 2010.12.01.1). Mascot was set up to search the Uniprot_sprot_20120815 database (selected for Homo sapiens, 20306 entries) assuming the digestion enzyme trypsin. X!Tandem was set up to search a subset of the uniprot_sprot_20120815 database also assuming trypsin digestion. Mascot and X! Tandem were searched with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 10.0 PPM. Deamidated of asparagine and glutamine, oxidation of methionine, acetyl of the N-terminus and propionamide of cysteine were specified in Mascot and X! Tandem as variable modifications.

Scaffold (version Scaffold_3.6.4, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 1 identified peptides. Protein probabilities were assigned by the PeptideProphet algorithm (Nesvizhskii et al., 2003).
Immunoprecipitation, protein purification and GST pull-down assay

Cell extracts were prepared as described (Miserey-Lenkei et al., 2010). 500 µg of total cell protein in the extraction buffer supplemented with protease inhibitor cocktail cOmplete mini, EDTA-free (Roche Diagnostics), 10 mM MgCl₂ and 0.1% NP-40 (Igepal CA-630, Sigma) was incubated with 1 µg of antibody (rabbit anti ARL13B pAb or rabbit IgG (Bethyl) at 4°C for 16 h. 10 µl of Pierce protein A agarose beads (ThermoFisher) was added and the mixture was incubated for 2 h. Beads were washed five times with 25 mM Tris pH 7.5, 10 mM MgCl₂, 50 mM NaCl, 0.1% NP40, eluted with 2x Laemmli loading buffer at 95°C, and proteins were loaded onto 4-15% TGX gel (Bio-Rad). Proteins were detected by Western blotting.

For expression of GST-tagged wild-type human ARL13B and the mutant protein carrying the mutation R79Q plasmids pGEX-ARL13B and pGEX-ARL13B R79Q (Cantagrel et al., 2008b) were used. For analysis of truncated domains of ARL13B, fragments of its cDNA were amplified by PCR using PrimeSTAR HS DNA polymerase (Clontech), and inserted into pGEX-6P-1 (GE Healthcare) between the BamHI and NotI sites. To express GST-huARL13B T35N the cDNA was amplified using pSSN-huARL13B T35N (Humbert et al., 2012). To express human ARL13B lacking the G-domain, the cDNA fragments flanking the deletion were joined by PCR and the resulting fragment was inserted into pGEX-ARL13B between the BamHI and SpeI sites. Control GST protein was expressed using pGEX-4T-2 (GE Healthcare). All the constructs were confirmed by sequencing. GST-tagged wild-type human ARL13B and its mutated variants were expressed in *Escherichia coli* strain BL21 –CodonPlus (DE3)-RIL (Agilent Technologies). Protein purification was performed as described previously (Hori et al., 2008). To remove glutathione, eluted proteins were dialyzed against Buffer A (Hori et al., 2008) using Slide-A-Lyzer dialysis cassettes with molecular weight cutoff of 10 kDa (Thermo Scientific).

For GST pull-down assay 20 µl of Glutathione Sepharose 4B beads (GE Healthcare) was equilibrated with Buffer A (after (Hori et al., 2008); 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 0.1% (w/v) Triton X-100, protease inhibitor cocktail cOmplete mini, EDTA-free (Roche Diagnostics)) and incubated with 20 pmoles of GST fusion protein in 200 µl Buffer A, 1 mM DTT, 0.1% triton X-100, for 2h at 4°C with rotation. Beads were washed and incubated in the same buffer supplemented with 200 µM of GTPγS or GDP and 5 mM EDTA for 1 hour at room
temperature (24°C). Buffer was replaced with cell extract containing 100 µg of total protein in LAP100 buffer supplemented with protease inhibitor cocktail cOmplete mini, EDTA-free (Roche Diagnostics), 200 µM of GTPγS or GDP (Sigma), 10 mM MgCl₂, and 0.3% (w/v) NP-40 (Igepal CA-630, Sigma, I3021). In the assays that tested binding to purified tubulin, 100 pmoles of bovine tubulin (>99% pure, Cytoskeleton, Cat. # T240) in 100 µl of LAP100 buffer supplemented with 1 mg/ml of bovine serum albumin (New England Biolabs), protease inhibitor cocktail cOmplete mini, EDTA-free, 200 µM of GTPγS or GDP, 10 mM MgCl₂, and 0.3% (w/v) NP-40 (Igepal CA-630, Sigma, I3021). Beads were incubated with rotation at room temperature for 90 min, washed four times with Buffer A, 10 mM MgCl₂, 0.1% triton X-100, and bound proteins were eluted with 2x Laemmli loading buffer at 95°C.

**Immunostaining and electron microscopy**

Cells were seeded on coverslips and grown in high-glucose DMEM, 10% FBS. To induce ciliogenesis, medium was replaced with serum-free high-glucose DMEM for 24-48 h. To induce accumulation of SMO in the ciliary compartment, cells were treated with 5ng/ml recombinant Sonic Hedgehog (StemRD, Burlingame, CA) during the last 14-16 h of treatment with serum-free medium. For electron microscopy, cells were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer for 16 hours at 4 °C. TEM was carried out as described (Grisanti et al., 2016). For immunostaining, cells were fixed with 4% PFA in PBS at 4 °C or cold methanol for 10-15 minutes and permeabilized with 0.1% triton X-100 in PBS for 10 minutes at room temperature. After blocking for 1 h with 2% bovine serum albumin (BSA) in PBS the primary antibodies diluted in 2% BSA in PBS were applied for 2 h. Secondary antibodies diluted in PBS were applied for 1 h after 3 washes of 10 min. Coverslips were mounted in Vectashield with or without DAPI (Vector Laboratories, Burlingame, CA) and analyzed using a Zeiss LSM510 and LSM880 confocal microscopes. Confocal Images were captured with Plan-Neofuar 40x/1.3 Oil and a Plan-Apochromat 63x/1.4 Oil objectives and the acquisition software ZEN Black (Zeiss). The images were exported, analyzed with ImageJ and processed in Photoshop 7.0.1 and Illustrator CS6 (Adobe Systems). Levels of fluorescence intensity of GFP tagged ARL13B constructs were
measured using line-scan-based analysis in ImageJ. The average intensity over a three-pixel-wide line along the axoneme were measured and plotted against normalized cilium length.

The following primary antibodies were used: rabbit anti-α-tubulin polyclonal antibody (pAb) (Abcam, ab18251), mouse anti-β-tubulin monoclonal antibody (mAb) (Sigma, T4026), goat anti γ-tubulin pAb (Santa Cruz Biotechnology, sc-7396), mouse anti-acetylated tubulin mAb (Sigma, T7451), mouse anti-polyglutamylated tubulin mAb (Enzo Life Sciences, ALX-804-885), mouse anti-ARL13B mAb (UC Davis/NIH NeuroMab Facility, clone N295B/66), rabbit anti-ARL13B pAb (Proteintech, 17711-1-AP), mouse anti-GFP mAb (Life Technologies, A11120), rabbit anti-GFP pAb (Life Technologies, A11122), mouse anti-GST mAb (GenScript, A00865), rabbit anti-S-tag pAb (GenScript, A00625), rabbit anti-IFT88 pAb (Proteintech, 13967-1-AP), mouse anti-dykddekkk (anti-FLAG) mAb (Sigma, F3165), mouse anti-actin mAb (EMD Millipore, MAB1501), rabbit anti-SMO pAb (1:500) was a gift of Dr. Kathryn V. Anderson, Memorial Sloan Kettering Cancer Center, New York, NY. Secondary antibodies used for immunofluorescence were FITC-conjugated AffiniPure donkey anti-mouse or anti-rabbit IgG (Jackson Immunoresearch, 715-095-150, 711-095-152), TRITC-conjugated AffiniPure donkey anti-mouse or anti-rabbit IgG (Jackson Immunoresearch, 715-025-150, 711-025-152), Alexa Fluor 555 donkey anti-rabbit IgG (Life Technologies, A31572), Alexa Fluor 647 donkey anti-goat (Life Technologies, A21447). Secondary antibodies used for Western blotting were HRP-conjugated goat anti-mouse or anti-rabbit IgG (1:500 - 1:1000, Pierce, 1858413, 1858415). Antibodies were diluted as suggested by vendor recommendation when not specified.

**Axoneme isolation and binding assays**

*C. reinhardtii* wild-type cells were cultured and flagella isolated as previously described (Iomini et al., 2001). Axonemes were demembranated and isolated as described in (Alper et al., 2013). Demembranated axonemes were resuspended in 200 µl of HMDEKP buffer with proteinase inhibitor cocktail cComplete mini, EDTA-free (Roche Diagnostics). For binding assay, 20 µl of the axoneme suspension was mixed with 2-20 pmoles of GST fusion protein in 200 µl Buffer A (Hori et al., 2008), 1 mM DTT, 0.1% triton X-100, and incubated for 2 h at room temperature with rotation. Axonemes were precipitated by centrifugation at 15996 x g for 10
min, washed five times in Buffer A, and dissolved together with bound proteins in 2x Laemmli loading buffer at 95°C.

Acknowledgements
We thank Iain Cheeseman for providing the TAP tag plasmid vectors pIC112 and pIC113. Plasmids for protein expression were a gift of Joseph Gleeson, Tamara Caspary and Seongjin Seo. Antibody anti-SM0 were kindly provided by Kathryn V. Anderson. We are grateful to Yasseris Rosario-Peralta and Ron Gordon for help with ultrastructural analysis and all Mlodzik and Iomini lab members for very helpful input and discussion. Confocal microscopy and cell sorting were performed at the Microscopy and the Flow Cytometry CoREs at the Icahn School of Medicine at Mount Sinai, respectively.

Competing interests: No competing interests declared.
Funding: This work was supported by National Institutes of Health grants R01DK106035 to G.L.G. and R01EY022639 to C.I. and the Research to Prevent Blindness Dolly Green Special Scholar Award to C.I.
References


Figure 1. ARL13B interacts with tubulin. (A, B) Identification of tubulin as an ARL13B-interacting protein by tandem affinity purification and mass spectrometry. (A) silver-stained gel, and (B) Western blot analysis of purified fractions. (C) Co-immunoprecipitation of tubulin from HEK293 cell lysate after pull down with anti-ARL13B antibody. (D) GST pull-down of recombinant GST-tagged human ARL13Bwt or GST-ARL13B R79Q after incubation with hTERT-RPE1 cell lysates. Note that ARL13B-interacting tubulin includes acetylated tubulin.
Figure 2. The G-domain of ARL13B contains a tubulin-interacting region. (A) Schematic representation of the main domains of the ARL13B protein and GST-fused fragments of ARL13B purified from *E. coli*. (B) GST pull-down from a solution of purified tubulin dimer. (C, D, E, F) GST pull-down from hTERT-RPE1 cell lysate. Bands corresponding to fusion proteins of the expected size are marked with red dots; degradation products run below.
Figure 3. ARL13B G-domain is dispensable for cilium localization but essential for cilium length regulation. (A) Domain structure of GFP-tagged wild-type and mutated ARL13B proteins expressed in mammalian cells as GFP fusions. (B-E) Cilia length in wild type MEFs (B, D) and *Arl13b* 

$hnn$ MEFs (C, E) untransduced (B, C) or transduced with the indicated lentiviral expression vectors (D, E) was determined following immunofluorescence for (B) ARL13B, acetylated tubulin and $\gamma$-tubulin in wild-type MEFs; (C) IFT88, acetylated tubulin and $\gamma$-tubulin; and (D, E) GFP, glutamylated tubulin and $\gamma$-tubulin. Nuclei were stained with DAPI. (F) Quantification of changes in cilia length distribution in wild-type and *Arl13b* 

$hnn$ MEFs expressing GFP-tagged ARL13B constructs compared to non-transduced controls. * indicates statistically significant changes ($\chi^2$-test, $P<0.0001$) from non-transduced wild-type MEFs. $n=$number of cilia measured in two independent experiments. Scale bars= 5µm.
Figure 4. The G-domain regulates ARL13B distribution along cilia. (A-J) Immunofluorescence of clonal Arl13b<sup>hnn</sup> and wild-type MEFs untrasduced or expressing GFP-mARL13B, GFP-mARL13BΔGD (see S4A for expression levels of GFP-tagged variants of Arl13b). GFP is in green, acetylated tubulin in red and γ-tubulin in white. (A and B) GFP-mARL13B and GFP-mARL13BΔGD uniformly distribute along cilia of wild-type MEFs. (C) Cilium of untransduced MEFs. (D) Ectopic GFP-mARL13B rescues the ciliary length defect and distributes uniformly along the ciliary membrane of Arl13b<sup>hnn</sup> MEFs. (E and G) Ectopic GFP-mARL13BΔGD accumulates at the distal end (E) or at both ends (F) of cilia and in stump-like structures (G) of Arl13b<sup>hnn</sup> MEFs but, it is dramatically reduced between cilia extremities (E and F). (K) Ultrastructural analysis of longitudinal sections of cilia of Arl13b<sup>hnn</sup> MEFs. Clonal Arl13b<sup>hnn</sup> MEFs transduced with GFP-mARL13BΔGD show accumulation of electron-dense deposit along the length of cilia (K middle panel) and in stump-like structures (K right panels). In contrast, clonal Arl13b<sup>hnn</sup> MEFs transduced with GFP-mARL13B show no electron-dense accumulation along the cilia (K left panel). Insets in K are gradient maps of red-delimited area. Note that axonemal microtubules (vertical yellow streaks) are visible in the cilium of Arl13b<sup>hnn</sup> MEFs transduced with GFP-mARL13B, but not in the cilium of Arl13b<sup>hnn</sup> MEFs transduced with GFP-mARL13BΔGD mostly filled with electron-dense material (blue granules). (L) Color-coded examples of variable distribution of GFP-tagged ARL13B mutants along cilia of transduced Arl13b<sup>hnn</sup> MEFs quantified in (M). Only the mGFP-ARL13BΔGD mutant lacking the G domain aa sequence fails to distribute uniformly along the ciliary membrane of Arl13b<sup>hnn</sup> MEFs. Wild-type and mutant ARLS13B carrying single aa substitution affecting the GTPase active site uniformly distribute along the ciliary length. (M) Quantification of the distribution of GFP-tagged proteins in cilia of wild-type and Arl13b<sup>hnn</sup> MEFs expressing GFP-
tagged ARL13B constructs as indicated below the graph. Bars show percentage of cilia with even distribution of GFP-ARL13B proteins along the cilium (mean of two independent experiments, total number of cilia per experiment per construct was between 70 and 385, error bars represent standard error of the mean). In Arl13b<sup>hnn</sup> MEFs, the difference between mARL13BdeltaGD and all other groups is significant (***, P< 0.001, one-way ANOVA with Tukey post-test for pairwise comparisons). Scale bars in (J) and (L)= 2µm. Scale bar in K= 0.5µm.
Figure 5. The ARL13B G domain sequence but not its GTPase activity is required for normal distribution of transmembrane proteins along the ciliary membrane. (A-E) Immunofluorescence signal for SMO (red), GFP (green) and ϒ-tubulin (white) in wild-type and Arl13b<sup>hnn</sup> MEFs untransduced (A and B) and Arl13b<sup>hnn</sup> MEFs stably expressing GFP-tagged wild-type or mutant ARL13B (C-E). MEFs shown in (A-E) were treated with recombinant SHH. (F) Average of fluorescence intensity (arbitrary units) ± standard deviation of Smo along cilium length. n=20 cilia were evaluated for each construct and pooled from three independent experiments. Only cilia longer than 2µm were analyzed. Student’s t test analysis of the differences between Smo signal intensity from MEFs expressing wild-type and mutant ARL13B, the error bar represents the s. d. For ARL13B-ΔGD, P<0.0001 for the proximal 80% of the cilia. For ARL13B-T35N, not significant along the entire length of the cilium. (G-K) Immunofluorescence signal for mCherry (red), GFP (green) and ϒ-tubulin (white) in wild-type and Arl13b<sup>hnn</sup> MEFs transduced with mCherry-SSTR3 lentivector (G-k) and in Arl13b<sup>hnn</sup> MEFs stably expressing GFP-tagged wild-type or mutant ARL13B (I-K). (L) Average of fluorescence intensity of mCherry-SSTR3 as calculated in (F). Student’s t test analysis of the differences between mCherry-SSTR3 signal intensity from MEFs expressing wild-type and mutant ARL13B, the error bar represents the s. d. For ARL13B-ΔGD, P<0.001 for the proximal 60% of the cilia. For ARL13B-T35N, not significant along the entire length of the cilium. (M) Quantification of percentage of cilia with even distribution of mCherry-SSTR3. The distribution was considered uneven if the fluorescence signal in any region of the cilium decreased more than 50% compare to the signal intensity of the remaining portion of the cilium. Dots show the results of three independent experiments, lines show means and standard
deviations. Cell lines and GFP-tagged ARL13b constructs are indicated below the graph. Scale bar= 2µm.
Figure 6. Deletion of the G-domain weakens ARL13B interaction with axonemes and association with cilia. (A and B) Arl13b G domain is required for strong interaction with tubulin. Purified fragments of ARL13B containing the G-domain co-sediment with isolated axonemes. Purified axonemes were incubated with solutions of GST fusion proteins and sedimented by centrifugation. Pellets and supernatants were analyzed by Western blot. Only fragments containing the G domain sequence bind to axonemes. (B) Progressively decreasing amounts of GST-ARL13B and GST-ARL13BΔGD independently incubated with equal amount of demembranated axoneme, sedimented by centrifugation and processed for WB (left lanes). Supernatant fractions containing free GST-tagged proteins not bound to the axonemes analyzed by WB (right lanes). Note that the amount of GST-ARL13B bound to the axoneme remains constant despite the drop of concentration of the GST-tagged protein loaded in the assay. In contrast, GST-ARL13BΔGD loses the interaction with the axoneme as the GST-tagged protein concentration decreases. (C) Confocal IF of cilia of clonal wild-type MEFs stably expressing similar levels of GFP-ARL13B (upper panels, cell line 8A11) or GFP-ARL13BΔGD (lower panels, cell line 7A11) treated with PBS (control) or low concentrations of Triton X-100 in PBS. See also Fig. S6A for comparison of levels of expression of transgenes in MEFs. Single channels for GFP (ARL13B fusion proteins) and acetylated tubulin (axoneme) are shown in monochrome images. Wider microscope fields of images shown in right panels of C are shown in color in Fig. S5. (D) Quantification of GFP fusion proteins localized to cilia. Cilium areas were defined based on acetylated tubulin immunofluorescence signal. Original 12-bit images were used for quantifications of mean GFP immunofluorescence intensity and standard deviations shown in D. Values were normalized to signals from non-treated cilia. Total number of quantified cilia per
clone per each Triton X-100 concentration in two independent experiments ranged from 17 to 26. GFP-ARL13B and GFP-ARL13BΔGD showed significant difference in Triton-dependent ciliary attachment (two-way ANOVA, p = 0.0277).
**Figure S1.** Characterization of the cell line used for the study of ARl13B interactions by the tandem affinity purification (TAP) procedure. (A) Schematic of the TAP-tagged mouse ARl13B expressed in transduced hTERT-RPE1 cells. The TAP-tag includes pEGFP, tobacco etch virus (TEV) protease cleavage site and S peptide. (B) Live EGFP fluorescence of stable hTERT-RPE1 cell clone expressing TAP-mARl13B in cilia. (C) Western blot of cell protein extracts (input) and anti-GFP immunoprecipitates (IPs) from the parental cell line hTERT-RPE1 and the stable cell clone expressing TAP-mARl13B. The blot was probed with rabbit polyclonal antibodies specific for ARl13B. For loading control, the same blot was probed with anti-actin antibodies. Positions corresponding to the endogenous human ARl13B and TAP-mARl13B are marked on the right.
diffuse band in the IPs is a non-specific signal from rabbit immunoglobulins. Note that this stable cell clone was selected for TAP procedure because the exogenously expressed TAP-mARL13B fusion protein parallels the localization and levels of expression of the endogenous ARL13B. Scale bar= 5µm
Figure S2.

**Figure S2. Localization of GFP-tagged wild-type mARL13B or mARL13B with deletion mutants in hTERT-RPE1 cells.** hTERT-RPE1 Cells were transduced with lentiviral expression vectors shown in Fig. 3A. Immunofluorescence signals for GFP (green) and ARL13B (red) in fixed hTERT-RPE1 cells. Nuclei were stained with DAPI (blue). Note that while the full length fusion protein GFP-mARL13B and the G domain-deficient mARL13BΔGD enter the cilia compartment, the C terminus-deficient mARL13BΔGD did not. Scale bar= 5µm.
Figure S3. INPP5E cilia localization requires the activity of the ARL13B G domain. (Upper panels) Immunofluorescence signal of endogenous INPP5E (red) in Arl13b<sup>hnn</sup> and parental wild-type MEFs stably transduced with lentivectors expressing GFP tagged wild-type or mutant ARL13B variants as indicated. (Lower panel) INPP5E (red) localized to the cilium (acetylated tubulin, green) in wild type but not in Arl13<sup>hnn</sup> MEFs.
Figure S4. Accumulation of IFT subunits at the ciliary tip allows accurate measurement of cilia length in Arl13b<sup>hnn</sup> MEFs before and after the expression of ARL13B mutants. Accurate measure of cilia length was achieved in wild-type and Arl13b<sup>hnn</sup> MEFs using overlapping immunofluorescence signals from acetylated tubulin (green) and IFT88 (red) in merged confocal maximal projections. Components of the IFT machinery’s complex A (IFT140) and B (IFT88) accumulate at the tip (yellow arrow) of Arl13b<sup>hnn</sup> MEFs. Importantly, this accumulation was not rescued in Arl13b<sup>hnn</sup> MEFs expressing GFP-mARL13BΔGD. Scale bar= 5µm.
Figure S5. The tubulin binding domain is required for anchoring ARL13B to the ciliary axoneme.

(A, left panel) Western blot analysis of GFP-ARL13B fusion protein expression in wild-type and Arl13b<sup>hnn</sup> MEFs. Cell lysates were prepared from selected clones that stably express GFP-mARL13B or GFP-mARL13B<sub>ΔGD</sub> (left panel) or cell populations that express GFP-mARL13B, GFP-mARL13B<sub>ΔGD</sub>, GFP-huARL13B or GFP-huARL13B<sub>T35N</sub> after transduction with lentivectors as indicated. (B) Wild-type MEFs were transduced with lentivector expressing comparable amount of GFP-ARL13B (left panels) or GFP-ARL13B-ΔGD (right panels). The two images shown here are
full microscope fields of cropped confocal images shown in the right panels of Fig. 6C. Confluent ciliated cells were treated with mild detergent immediately prior fixation. GFP signal was strongly reduced in MEFs expressing GFP-ARL13B construct but still visible in MEFs expressing the wild-type GFP-ARL13B. GFP signal is in green, ciliary axoneme (Acetylated tubulin) in red, basal bodies (γ-tubulin) in white and nuclei (DAPI) in blue. (C) Loss of GFP-ARL13B after treatment with Triton X-100. Immunofluorescence for GFP (ARL13B fusion proteins) in green, acetylated tubulin (axoneme) and γ-tubulin (basal body) in red. Nuclear DNA was stained with DAPI in blue.
Figure S6. Levels of ARL13B expression in wild-type or mutant cell lines do not modify overall extent of acetylated and glutamylated tubulin. (A) Western blot for the expression of ARL13B, polyglutamylated tubulins, acetylated tubulin, and α tubulin in cell protein extracts from parental hTERT-RPE1 and hTERT-RPE1 stably transduced with a lentiviral vector constitutively expressing ARL13B-cherry (E4 line). (B) Western blot for the expression of GFP tagged proteins, polyglutamylated tubulins, acetylated tubulin, and α tubulin in whole extracts from parental Arl13b<sup>hnn</sup> and wild-type (wt) MEFs and Arl13b<sup>hnn</sup> MEFs expressing wild-type or GFP-ARL13B mutants.