MKS and NPHP modules cooperate to establish basal body/transition zone membrane associations and ciliary gate function during ciliogenesis

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decel-Gruber syndrome (MKS), nephronophthisis (NPHP), and related ciliopathies present with overlapping phenotypes and display considerable allelism between at least twelve different genes of largely unexplained function. We demonstrate that the conserved C. elegans B9 domain (MKS-1, MKSR-1, and MKSR-2), MKS-3/TMEM67, MKS-5/RPGRIP1L, MKS-6/CC2D2A, NPHP-1, and NPHP-4 proteins exhibit essential, collective functions at the transition zone (TZ), an under-appreciated region at the base of all cilia characterized by Y-shaped assemblages that link axoneme microtubules to surrounding membrane. These TZ proteins functionally interact as members of two distinct modules, which together contribute to an early ciliogenic event. Specifically, MKS/MKSR/NPHP proteins establish basal body/TZ membrane attachments before or coinciding with intraflagellar transport–dependent axoneme extension and subsequently restrict accumulation of nonciliary components within the ciliary compartment. Together, our findings uncover a unified role for eight TZ-localized proteins in basal body anchoring and establishing a ciliary gate during ciliogenesis, and suggest that disrupting ciliary gate function contributes to phenotypic features of the MKS/NPHP disease spectrum.

Introduction

Primary cilia protrude from most mammalian cells and modulate sensory processes, including chemo-, mechano-, and photoreception (Fliegauf et al., 2007). Cilia regulate various signaling pathways during embryonic development and are needed for normal postnatal tissue homeostasis (Gerdes et al., 2009). Mutations disrupting ciliary functions cause human disorders (ciliopathies) that collectively affect nearly all tissues/organs (Sharma et al., 2008). A nonexhaustive list of ciliopathies includes Meckel-Gruber syndrome (MKS), nephronophthisis (NPHP), Bardet-Biedl syndrome (BBS), Joubert syndrome (JBTS), Senior-Løken syndrome (SLSN), Leber congenital amaurosis (LCA), polycystic kidney disease (PKD), and oral-facial-digital syndrome (OFD). These disorders present with variable but overlapping clinical phenotypes that encompass polycystic kidneys, liver fibrosis, skeletal anomalies, sensory impairment, and brain/nervous system deformities (Fliegauf et al., 2007).

At least 35 loci have been identified in ciliopathy patients, some of which contribute to multiple seemingly distinct syndromes (Baker and Beales, 2009). Many of these genes encode proteins that localize to the basal body (BB)—a centriolar structure universally required for extending the microtubule-based ciliary axoneme—or to an adjacent domain, termed “transition zone” (TZ) in most cilia, or “connecting cilia” in photoreceptors (Horst et al., 1990; see schematic of BB-TZ-cilia structures in Fig. 1 A and relevant disease proteins in Table S1 A). Within the BB-TZ region are subdomains that include transitional fibers (TFs) and Y-links. TFs form a pinwheel-like structure, of unknown

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Supplemental material can be found at:
http://doi.org/10.1083/jcb.201012116
Figure 1. *C. elegans* B9 and C2 domain ciliopathy proteins are found at the transition zone, adjacent to the basal body/transition fiber region. (A) Schematic of a prototypical basal body (BB)/transition zone (TZ)/cilium, highlighting the microtubule (MT) backbone of the organelle, the docking of vesicles at the base of a “ciliary gate,” and its intraflagellar transport (IFT) trafficking machinery. In *C. elegans*, the BB lacks discernable MTs (shown dashed) and consists mainly of transition fibers (TFs). The TZ is demarcated by Y-links spanning the axoneme to the membrane and likely organizes the “ciliary necklace” on the ciliary membrane. The TFs and TZ are proposed to form a gate that restricts entry of vesicles and potentially also nonciliary proteins. TEM cross sections from *C. elegans* show relevant substructures (TFs, TZ, middle and distal cilia segments; Bars, 100 nm). IFT particles carry cargo from the BB into cilia; kinesins transport two multi-protein complexes (IFT subcomplexes A and B) and a BBS protein complex [S] along with cargo, and dynein recycles.
Results

MKS/MKSR and NPHP proteins localize specifically to the ciliary TZ

To gain insights into MKS/MKSR/NPHP protein functions, we first defined their respective subcellular localization in C. elegans sensory neurons. Using fluorescently tagged proteins, we detect MKS/MKSR/NPHP proteins in a region corresponding to the TZ (adjacent to where IFT proteins concentrate at the TFs/BB). This is evident for MKS-1, MKS-1 related-1 (MKSR-1)/B9D1, MKS-1 related-2 (MKSR-2)/B9D2, MKS-3/mecellen, NPHP-1, and NPHP-4 (Winkelbauer et al., 2005; Williams et al., 2008, 2010; Bialas et al., 2009), as well as MKS-5/RPGRIP1L and MKS-6/CC2D2A, previously uncharacterized in C. elegans (Fig. 1, B–D; Fig. 2). By transmission electron microscopy (TEM), the ∼0.8-μm long TZ region containing Y-links is distal to the TFs, which sit at the ciliary base just inside the dendritic tip (Fig. 1 A; Perkins et al., 1986). These data suggest that the TZ represents a common site of dysfunction in MKS/NPHP ciliopathy patients; however, the functions of MKS/MKSR/NPHP proteins at the TZ remain undetermined. Notably, based on Hidden Markov Model profiling and structure/fold predictions, several TZ proteins (MKS-1, MKSR-1, MKSR-2, MKS-5, and MKS-6) share a related C2/B9 motif (Fig. 1, E–G; Table S1 C). This motif is predicted to bind Ca²⁺/lipids and participate—similar to synaptogamin—in membrane/vesicle trafficking and fusion (Nalefski and Falke, 1996). The presence of this motif in multiple-ciliopathy TZ proteins raises the possibility that they perform a shared function at the TZ.

Functional interactions between NPHP proteins and MKS-5 or MKS-6 are required for ciliogenesis

To examine potential ciliary roles of the uncharacterized C2 domain–containing MKS-5 and MKS-6 proteins, we first analyzed mks-5 and mks-6 mutants (Fig. 3 A). Similar to mutations disrupting B9 domain genes (mks-1, mkrsr-1, and mkrsr-2; Williams et al., 2008; Bialas et al., 2009), mks-6 (gk674) mutants have no overt cilia or IFT defects, based on: (1) normal uptake of
ultrastructure anomalies in mks-6(gk674) mutants, except for occasional loss of axoneme distal segments (Fig. 6). mks-6(gk674) mutants have a weak osmotic avoidance phenotype comparable to nphp-4(tm925) mutants (Fig. 4 D) but do not exhibit increased lifespan, unlike other cilia gene (e.g., IFT) mutants (Table S1 E). Analysis of mks-5(tm3100) mutants uncovered

hydrophobic fluorescent dye (DiI), indicating cilia are present and environmentally exposed (Fig. 3, B and D); (2) normal cilia length, observed by visualizing cilia with GFP-tagged IFT proteins (Fig. 4, B and C; Fig. 5, A, A’, B, and B’); and finally, (3) normal IFT rates, based on in vivo time-lapse microscopy (Table S1 D). TEM analysis verifies the absence of gross cilia...
between MKS-5 and MKS-6 and the MKS/MKSR or NPHP modules, we examined mks-5; mks-6, mks-5; nphp-1, mks-5; nphp-4, and mks-5; mksr-1 double-mutant combinations show little or no dye filling. Expressing GFP-tagged MKS-6 rescues the mks-6 (double) mutant phenotype. (C) mks-5(tm3100) mutants have reduced dye filling, whereas mks-5; mksr-2, mks-5; mks-6, and mks-5; nphp-4 double mutants show little to no dye filling. Bars, 17.5 µm. (D) Summary of dye-filling phenotypes in single and double mks/mksr/nphp mutants. NT, not tested; red, present study; black, data from Jauregui et al. (2008) and Williams et al. (2008, 2010).

Previously, we found that B9 domain proteins (of the MKS/MKSR module) functionally interact with NPHP-1 and NPHP-4 (NPHP module) to facilitate ciliogenesis (Williams et al., 2008). To determine if similar genetic interactions occur between MKS-5 and MKS-6 and the MKS/MKSR or NPHP modules, we examined mks-5 and mks-6 mutations in combination with mks/mksr and nphp mutations. Compared with wild-type, mks-3; mks-6, or nphp-4 single mutants, both mks-5; nphp-4 and mks-6; nphp-4 double mutants exhibit strong dye-filling (Dyf) and osmotic avoidance (Osm) phenotypes (Fig. 3, B–D; Fig. 4 D). However, dye filling is normal in mks-6; mks-1, mks-6; mksr-1, mks-6; mksr-2, and mks-6; mksr-2 double mutants (Fig. 3, B and D). These data suggest that mks-6 genetically

Figure 3. Functional interactions between MKS-5/MKS-6 and other TZ proteins are required for environmental exposure of cilia to a fluorescent dye, consistent with a role in ciliogenesis. (A) Gene structures of C. elegans mks-5 (C09G5.8) and mks-6 (K07G5.3) and the nature of three deletion alleles, tm3100, gk674, and nx105. (B and C) Representative images of fluorescence staining of environmentally exposed sensory neurons via Dil uptake through cilia in head (ampid) neurons (left panels) and tail (phasmid) neurons (right panels). Dye filling is noted as normal (+++), reduced (+), or absent (–). (B) Wild-type [N2] and mks-6 strains exhibit normal dye filling; nphp-1 and nphp-4 show normal or slightly reduced (+++) dye filling (see D; Jauregui et al., 2008). mks-6; mksr-2, mks-6; mksr-4, and mks-6; nphp-4 double-mutant combinations show little or no dye filling. Expressing GFP-tagged MKS-6 rescues the mks-6 (double) mutant phenotype. (C) mks-5(tm3100) mutants have reduced dye filling, whereas mks-5; mksr-2, mks-5; mks-6, and mks-5; nphp-4 double mutants show little to no dye filling. Bars, 17.5 µm. (D) Summary of dye-filling phenotypes in single and double mks/mksr/nphp mutants. NT, not tested; red, present study; black, data from Jauregui et al. (2008) and Williams et al. (2008, 2010).
respects to the phasmid cell body (Fig. 4 C; Fig. 5, C, C’, D, and D’; Williams et al., 2008). Although there is a low penetrance of these phenotypes in nphp-4 single mutants, they were consistently observed in mks-6;nphp-4, mks-5;nphp-4, and mks-6;nphp-1 double mutants (Fig. 4 C; Fig. 5).

Joint disruption of an MKS/MKSR protein and NPHP-4 results in BB/TZ membrane association defects

To study in greater detail the ciliary defects of single and double mks/mksr/nphp mutants, we used GFP-tagged ciliary (IFT) markers. We uncovered in double mutants multiple phenotypes, including missing or shorter cilia (Fig. 4 C; Fig. 5, A, A’, B, and B’), misoriented cilia (not depicted), and improperly positioned BB/TF regions within the amphid channel and with respect to the phasmid cell body (Fig. 4 C; Fig. 5, C, C’, D, and D’; Williams et al., 2008). Although there is a low penetrance of these phenotypes in nphp-4 single mutants, they were consistently observed in mks-6;nphp-4, mks-5;nphp-4, and mks-6;nphp-1 double mutants (Fig. 4 C; Fig. 5).

Figure 4. Functional interactions between TZ proteins are required for correct cilium length and positioning of basal bodies/TZs, as well as chemosensation. (A and B) Disruption of mks-5 (A) or mks-6 (B) has no major effect on the gross morphology/presence of amphid (head) and phasmid (tail) cilia, as revealed by GFP-tagged IFT markers (OSM-6 and CHE-2, respectively). BB, basal body. Bars, 5 µm. (C) Analyses of amphid and phasmid cilia length (white and light gray bars), spatial distribution of BB/TZ in amphid channel neurons (black bars), and position of the BB with respect to the cell body in tail neurons (dark gray bars) are summarized based on phenotypes and statistical analyses shown in Fig. 5. Mean values obtained for wild-type (N2) are shown; *, statistically significant difference (P < 0.01) vs. N2; §, statistically significant variance (P < 0.01) vs. N2. Schematics illustrate how measurements were made for amphid and phasmid cilia lengths, the distribution of BBs within amphid neurons (some BBs in mutants do not form a tight cluster as in wild type), and distance of the cilia from the phasmid neuron cell body (some mutants have abnormally short dendrites, indicating lack of anchoring of upon retrograde extension of the cell body during development). (D) Graph of cilium-dependent osmotic avoidance of N2 and TZ mutant strains. osm-5 (control IFT mutant) fails to avoid high osmolarity solution. Two mks-6 alleles show osmosensation defects in an nphp-4 mutant background, as do mks-2 and mks-5 mutants. *, significant difference compared with N2 [P < 0.05]; **, significant difference compared with either single mutant for the indicated double mutant.
Figure 5. **Detailed analysis of cilia length and positioning in strains with disrupted TZ proteins.** (A–D) Example of correct (wild type; top panels) or defective (for TZ gene mutants; bottom two panels) amphid (A) and phasmid (B) cilia length, distribution of BBs in amphid ciliated neurons (C), and positioning of the BB with respect phasmid neuron cell body (D). Using a CHE-11::GFP IFT marker (with the exception of the mks-5 data, which was obtained using OSM-6::GFP). CB, cell body; cil, cilia; BB, basal body. Bar, 5 µm. (A–D) Individual data points (n > 40) of the measurements in A–D are shown with a bar graph showing average and standard error. Statistically significant differences (P < 0.01) in the mean (*) or variance (§) are indicated for all combinations.
Figure 6. **MKS-6 and NPHP-4 are collectively required for BB/TZ attachments to membrane.** Shown are low and high magnification images from TEM serial cross sections of amphid channel cilia from wild-type (N2), mks-6, and mks-6;nphp-4 worms. Top row, distal segments (DS); middle row, middle segments (MS); bottom row, transition zone (TZ) and distal dendrites (DD). Below bottom row are magnified images of representative TZs. Boxed number denotes proximal positioning of section relative to top section. Schematics (longitudinal, transverse views) summarize the major ultrastructural observations. BB, basal body; TFs, transition fibers. Bars, 100 nm. (A) N2 worms showing 10 singlet microtubule (MT)-containing axonemes in DS, 10 doublets in MS (+3 µm), and TZs (constriction of MT doublets surrounding apical ring) with intact Y-links (connecting doublets with ciliary membrane) anchored at distal dendrite tips (+7 µm). TFs are also observed below this region. (B) Apart from rare axonemes lacking MTs in DS, mks-6 mutant cilia possess normal DS,
all double mutants analyzed. Unlike wild-type animals and mks-6 or nphp-4 single mutants, which show normal TZs (Fig. 6, A and B; Fig. 7; Jauregui et al., 2008), mks-6; nphp-4 and mks-5; nphp-4 double mutants exhibit mispositioned and disrupted TZ regions, with clear disconnections between the BB/TZ region and membrane, accompanied by missing Y-links (Fig. 6 C; Fig. 7; Fig. S1, Fig. S2); hence, their BB/TZ regions are not properly anchored at the distal end of the dendritic membrane. We also observed missing, misplaced, or shorter axonemes, and variably missing TFs. In perhaps the most severe instance of morphological abnormality, mks-5; nphp-4 double mutants had incomplete TZ microtubule rings (Fig. S2). Phenotypes nearly identical to mks-6; nphp-4 mutants were observed in the mksr-1; nphp-4 strain (Fig. 7; Fig. S3). The various cilia defects uncovered by TEM are quantified in Table I. Notably, all TZ/cilia anomalies are in addition to open tubules observed in the nphp-4 mutant (Jauregui et al., 2008) and are distinct from those of IFT and BBS mutants, in which TZ regions are intact (Perkins et al., 1986).

Given the severe ciliary phenotypes of mks-5; nphp-4 and mks-6; nphp-4 strains, we wondered if mks-1; nphp-4 and mks-3; nphp-4 mutants, which have milder Dyf phenotypes (Williams et al., 2008, 2010), would present with similar—yet partial or more specific—ultrastructure defects. Although similar ciliary defects were observed in the latter mutants (Fig. 7; Fig. S4, Fig. S5), some of their TZ regions are in normal proximity to the ciliary membrane but often lack connecting Y-links; this raises the possibility that MKS/MKSR/NPHP proteins comprise components of these structures or are required for their stability.

**TZ proteins participate in early ciliogenesis independent of IFT-associated proteins**

Our data indicate that MKS/MKSR/NPHP proteins are important for BB/TZ interactions with the membrane, which likely occur during ciliogenesis before IFT-dependent axoneme extension, suggesting a role in an early step of the ciliogenic pathway (Sorokin, 1968). To test if this early step depends on IFT, we looked for IFT defects using kymograph analyses in mks/mksr/nphp double mutants where a small proportion of cilia still form. Remarkably, IFT was largely unaffected in these double (as well as single) mutants (Table S1 D; Bialas et al., 2009), in striking contrast to C. elegans IFT mutants where disruption of anterograde or retrograde IFT occurs (Hao and Scholey, 2009), or BBS mutants (bbs-1/7/8), where stability/association of IFT subcomplexes A and B is compromised (Blacque et al., 2004; Ou et al., 2007). Because no TZ phenotypes observed in our study are apparent in IFT or BBS mutants (Perkins et al., 1986; unpublished data), and because IFT is largely unaffected, we propose that NPHP and MKS protein modules act before IFT-driven axoneme formation. From this model we predict that the TZ should be established properly when IFT is disrupted. This was validated in IFT and BBS mutants, where MKS-6 (Fig. 8 F) and other MKS/NPHP proteins (unpublished data) localize normally to the TZs of cilia lacking fully formed axonemes. Together, these data support a model where TZ proteins function in an early ciliogenic step (BB/TZ association with membrane) followed by a subsequent IFT/BBS-dependent step in axoneme elongation/trafficking.

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**Table I. Quantification of various ciliary ultrastructure defects in TZ protein–disrupted strains**

<table>
<thead>
<tr>
<th>Region</th>
<th>Feature/defect</th>
<th>N2</th>
<th>mks-6; nphp-4</th>
<th>mksr-1; nphp-4</th>
<th>mks-1; nphp-4</th>
<th>mks-5; nphp-4</th>
<th>mks-3; nphp-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS*</td>
<td>Ave. axoneme no./pore (n = 2–4)</td>
<td>10</td>
<td>10</td>
<td>3</td>
<td>2</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Ave. MT singlet no./axoneme (n = 3–44)</td>
<td>8</td>
<td>7</td>
<td>5</td>
<td>7</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>No. of axonemes with no MTs*</td>
<td>0</td>
<td>4</td>
<td>9</td>
<td>3</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>MS*</td>
<td>Ave. axoneme no./pore (n = 2–4)</td>
<td>12</td>
<td>12</td>
<td>5</td>
<td>4</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Ave. MT doublet no./axoneme (n = 12–100)</td>
<td>9</td>
<td>8.9</td>
<td>7.7</td>
<td>6.0</td>
<td>8.3</td>
<td>8.0</td>
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<tr>
<td></td>
<td>Axonemes with abnormal accumulations*</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>TZ*</td>
<td>Ave. diameter of surrounding membrane (nm) (n’ = 2–34)</td>
<td>260</td>
<td>262</td>
<td>564</td>
<td>436</td>
<td>286</td>
<td>337</td>
</tr>
<tr>
<td></td>
<td>Percentage with enlarged membrane diameters (n’ = 2–34)</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Percentage of TZs with Y-links (n’ = 2–14)</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>50</td>
<td>0</td>
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<tr>
<td>DD</td>
<td>Abnormal vesicle or membrane accumulations*</td>
<td>0</td>
<td>0</td>
<td>23</td>
<td>4</td>
<td>2</td>
<td>5</td>
</tr>
</tbody>
</table>

* DS, distal segment; MS, middle segment; TZ, transition zone; DD, distal dendrite; N, number of amphid pores analyzed; n, number of axonemes measured; n’, number of TZs analyzed.

* Singlet microtubule (MT) axoneme region.

* Doublet MT axoneme region.

* Drawing together of doublet and singlet MTs by the apical membrane; doublets linked to ciliary membrane via Y-links.

* As analyses were performed on serial sections, the same axoneme may have been counted multiple times.

* Electron-dense accumulations, often vesicular (30–60 nm; often tubular; clear or dense core), sometimes non vesicular (<20 nm).
MKS-5 is a central component required for docking/anchoring MKS and NPHP protein modules

Our TEM analyses provide fundamental insights into defects caused when TZ proteins (MKS-1, MKSR-1, MKS-3, MKS-5, and MKS-6) are disrupted jointly with NPHP-4 (Figs. 6 and 7; and Figs. S1–S5). The ultrastructural defects overlap greatly between mutants, implying a shared function for MKS/MKSR/NPHP proteins. Further support comes from biochemical data linking many ciliopathy proteins in shared macromolecular complexes (Table S1 A). To provide further evidence for shared/modular functions, we queried whether disrupting particular TZ proteins affected the localization of others. Using this approach, we rule out MKS-1, MKS-3, MKS-6, and NPHP-4 as critical docking proteins, as their disruption had no effect on localization of other TZ proteins (Fig. 8, B–F; Winkelbauer et al., 2005; Williams et al., 2008). In contrast, disrupting MKSR-1, MKSR-2, or MKS-5 results in TZ delocalization of MKS-6 (Fig. 8 B) and MKS-3 (Fig. 8 C, Fig. 9 L). Furthermore, mks-5 mutants failed to properly localize MKS-1, MKSR-1, and MKSR-2, suggesting a key role of MKS-5 in docking proteins at the TZ (Fig. 8 C). Notably, NPHP-1 and NPHP-4 localization was also altered in mks-5 mutants (Fig. 8 C), albeit differently; instead of failing to anchor at the TZ, NPHP-1 and NPHP-4 occupied a smaller region than wild-type animals (TZ length of 0.65 µm, n = 19 in mks-5 mutants vs. 1.05 µm, n = 23 in controls; t test P < 0.0001). MKS-5 was unaffected upon disruption of MKSR-2 or NPHP-4, which are otherwise required for TZ docking most other MKS/MKSR proteins and NPHP-1, respectively (Fig. 8 D; Winkelbauer et al., 2005; Williams et al., 2008). Moreover, MKS-5 is still TZ localized in mks-6;npkh-4 double mutants in which ciliary microtubule–membrane attachments are disrupted (compare Fig. 8 D with Fig. 8 E, in which transmembrane MKS-3 is predictably lost in the same double mutant). Thus, we conclude that MKS-5 localizes to the TZ independently of other TZ proteins tested and performs a central role as a scaffold for anchoring other MKS/MKSR and NPHP module proteins.
Figure 8. MKS/MKSR and NPHP proteins are organized hierarchically as modules, and independently of IFT/BBS proteins, at the TZ. (A) Schematic representing the region of phasmid (tail) cilia analyzed in B–E. Entire cilia/cilia regions are shown in F–K. (B–E) Co-dependent localization of fluorophore-tagged TZ proteins. TZs lacking normal localization of a particular protein are circled. (B) MKS-6::GFP localizes normally in wild-type (N2) and mks-1, mks-3, and nphp-4 mutants, but mislocalizes in mks-5, mksr-1, and mksr-2 mutants. (C) mks-5 mutants fail to localize MKS-1, MKSR-1, MKSR-2, and MKS-3, the latter dispersing along the cilium axoneme (see also Fig. 9 L). NPHP-1 and NPHP-4 localize to the TZ in mks-5 mutants, but to a subregion smaller than that occupied in N2 (compare brackets). (D) MKS-5::tdTomato localization at the TZ is not perturbed in mksr-2, nphp-4, or mks-6/nphp-4 double mutants. (E) MKS-3::tdTomato localization correctly to the TZ in nphp-4 and mks-6 single mutants but is mislocalized in the double mutant. (F) MKS-6::GFP localizes normally in bbs mutants (bbs-7, bbs-8) and ift mutants (osm-5, che-11), indicating the presence of an intact TZ. (G) tdTomato-tagged NPHP-1 (top) and MKSR-1 (bottom) localize normally at the TZ in mks-6(gk674) mutants. (H–K) CFP- or YFP-tagged MKS-1, MKSR-2, NPHP-1, and NPHP-4 localize normally at the TZ in mks-3 mutants. Bars, 2.5 µm.
Figure 9. MKS/NPHP proteins are required for ciliary gate function but not for ODR-10 trafficking. (A and B) GFP-tagged ODR-10 odorant receptor concentrates specifically at the AWB cilium in wild-type (N2) animals, showing a typical branched ciliary structure. Bars: (A) 25 µm; (B–K) 8 µm. (C–G) Disruption of the indicated TZ genes does not overtly affect the structure of the AWB cilium or presence of ODR-10::GFP. (H–K) Various double TZ mutants reveal abnormal AWB ciliary structures but otherwise normal localization of ODR-10::GFP to the ciliary membrane. (L) MKS-3::GFP localizes normally at the TZ in nphp-1 and mks-1 mutants but accumulates abnormally inside cilia (cil) and at dendritic tips (DT) in mks-5, mksr-1, and mksr-2 mutants. Bar, 2.5 µm. (M) GFP-tagged RPI-2 is found at dendritic tips but not inside cilia, marked by XBX-1::tdTomato in wild-type worms. RPI-2 localizes normally in mks-1, mks-3,
TZ proteins are not required for trafficking of a cilium-targeted protein

Given the presence of C2 domains in MKS-5 and MKS-6 and related B9 domains in MKS-1, MKSR-1, and MKSR-2, we hypothesized that these proteins may be required for vesicle trafficking and/or docking/fusion of vesicles harboring ciliary cargo at the TZ (Nalefski and Falke, 1996). Thus, we used a well-established odorant receptor (ODR-10; Sengupta et al., 1996) as a functional marker for vesicular trafficking to cilia. In wild-type animals, ODR-10 (expressed under the str-1 promoter) concentrates at the branched AWB cilium membrane (Fig. 9, A and B). In single TZ mutants, no apparent defects in cilia structure or ODR-10 localization are seen (Fig. 9, C–G). Although AWB cilium morphology in TZ double mutants is compromised, ODR-10 localization to its ciliary membrane appears normal (Fig. 9, H–K). These findings suggest that TZ proteins do not play an essential role in ciliary cargo-associated vesicle trafficking to, and docking/fusion steps at the base of cilia.

TZ proteins establish a gate that modulates ciliary composition

Our analyses uncovered important roles for MKSR-1, MKSR-2, and MKS-5 in TZ localization of the transmembrane protein MKS-3 (Fig. 8 C; Fig. 9 L). Interestingly, in addition to being lost from the TZ in mksr-1, mksr-2, and mks-5 mutants, MKS-3 accumulates along ciliary axonemes (and at nearby dendritic tips; Fig. 9 L). This indicates that MKS-1, MKSR-2, and MKS-5 restrict MKS-3 to the TZ membrane. Based on these observations, we wondered if other membrane-associated proteins (in particular, non-TZ-associated proteins) also accumulate in cilia when TZ proteins are disrupted. We examined RPI-2, the particular, non-TZ-associated protein) also accumulate in cilia.

Discussion

Modular function reduces the perceived complexity of heterogeneous disorders

There are at least 35 genes associated with primary cilia disorders (Baker and Beales, 2009). Several, including BBS genes, are linked to IFT (Blacque and Leroux, 2006; Beales et al., 2007), but most lack clearly assigned molecular functions. Many of these components display genetic and/or physical interactions and are associated with overlapping clinical ailments, suggesting involvement in a common cellular process.

Our findings indicate that C. elegans proteins implicated in many ciliopathies, including MKS, NPHP, JBTS, and LCA, function at the TZ and are required for BB and TZ attachment to the membrane and establishing a ciliary gate early in ciliogenesis (Fig. 10 D). Assignment of individual proteins to a particular TZ module (MKS/MKSR or NPHP) simplifies our understanding of the associated ciliopathies. Although previously seen as diseases of complex and seemingly unrelated molecular etiology, our data indicate that MKS and NPHP are likely disorders of macromolecular complexes sharing a common biological function. This is similar to the finding that several BBS proteins are constituents of the multimeric BBSome, which functions as a ciliary coat complex (Nachury et al., 2007; Jin et al., 2010). Indeed, a collective function of TZ proteins is supported by fragmented but increasing biochemical data in mammalian systems (Fig. 10 A; Table S1 A). Other functional modules/complexes may have disparate yet essential ciliary functions that are associated with equivalent ciliopathies; one likely includes NPHP2/invensin, NPHP3, and NEK8, which localize at the Inv ciliary compartment just distal to the TZ (Shiba et al., 2010).

A hierarchical, modular interaction network involving TZ proteins

We propose that MKS/MKSR and NPHP proteins form a hierarchical network comprised of distinct modules with partial functional redundancy (Fig. 10, B and C). The rationale for this modular hypothesis stems from our findings that in C. elegans, synthetic ciliary defects result from mutations in at least two TZ genes—but not any two genes (Fig. 3, B–D; Figs. 4–7). For example, even triple B9 gene (mks-1, mksr-1, and mksr-2) or nphp-1; nphp-4 double mutants lack prominent (additive) ciliary defects (Jauregui et al., 2008; Bialas et al., 2009). In contrast, disrupting any B9 gene together with nphp-1 or nphp-4 results in severe cilia anomalies (Williams et al., 2008). Other mutant combinations such as mks-1; mks-3, or mks-6; mksr-2, do not noticeably disrupt cilia, whereas the same four individual mutants show strong genetic interactions with nphp-1 or nphp-4 (Fig. 3, B–D; Figs. 4–7). Thus, we group MKS-1, MKSR-1, MKSR-2, MKS-3, and MKS-6 into an MKS/MKSR module and NPHP-1 and NPHP-4 into an NPHP module (Fig. 10 C). Importantly, MKS-5 represents an exception to this module assignment. Disrupting mks-5 together with mksr-1, mksr-2, mks-6 (MKS/MKSR module), or nphp-4 (NPHP module) compromises cilia morphology (Fig. 3, B–D). Moreover, mks-5 mutants fail to localize MKS-1, MKSR-1, MKSR-2, MKS-3, and MKS-6 (i.e., the entire known MKS/MKSR module) to the TZ;
Figure 10. MKS/MKSR and NPHP proteins form part of a functional interaction network required for an early stage of ciliogenesis and formation of an intact ciliary gate. (A) Previously identified physical interactions between C. elegans or mammalian TZ proteins (see also Table S1 A). Interactions between MKS1 and MKS3 and between NPHP1, NPHP4, and MKS5 were identified in mammals. The interaction between MKSR1 and MKSR2 was identified in C. elegans. (B) Summary of hierarchy analysis uncovering the requirement of some TZ proteins for normal localization of others. Black arrows represent previously known requirements (arrows point away from the protein required for localization of the other). Red arrows represent novel requirements uncovered in this study. (C) Summary of functional (genetic) interactions between C. elegans TZ components influencing ciliogenesis. Black lines represent previously known functional interactions, and red lines represent genetic interactions uncovered in this study (dye-filling/ciliogenesis and/or BB docking/anchoring and ciliary structures, as revealed by TEM). Modular assignments based on these functional interactions and hierarchy analysis data are represented by

**Stages of ciliogenesis**

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<td>MKS/MKSR/NPHP protein-dependent</td>
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the mks-5 mutation also partially alters NPHP-1 and NPHP-4 distribution at the TZ (summarized in Fig. 10 B). Based on our data, we propose that the MKS/MKSR and NPHP modules are functionally linked through MKS-5 (Fig. 10 C). Whether the modules are physically associated together at the TZ remains to be examined; however, the established biochemical interaction between mammalian MKS5/RPGRI1P1 and NPHP4 (Roepman et al., 2005) indicates this is likely. Further, the likelihood that MKS-5 links multiple functional modules is supported by the finding that human RPGRI1 mutations contribute to at least six distinct ciliopathies, including MKS and NPHP (Zaghloul and Katsanis, 2010).

The genetic interactions we observe in C. elegans also likely exist between TZ genes in mammals. For example, RPGRI1P1 and CEP290 heterozygous variants are linked to increased phenotypic pleiotropy in some NPHP patients in which primary disease symptoms are caused by mutations in other genes (Tory et al., 2007; Khanna et al., 2009). Our findings may also help explain data reported by Hosfele et al. (2005), who screened human NPHP patients and determined that single heterozygous mutations in NPHP4 were over three times more prevalent than two recessive mutations. Based on our genetic interaction studies in C. elegans, we predict that the ailments in these patients result from interactions between mutations in other TZ module genes. With the growing number of available MKS and NPHP rodent models, such possibilities could be formally examined by assessing the consequences of combining mutant alleles of multiple TZ genes.

Intriguingly, proteins seemingly absent in C. elegans or Drosophila have recently been implicated in BB- or TZ-associated functions in other organisms. These include the Ahi1 ciliopathy protein, which binds RAB8 and is proposed to play a role in polarized membrane trafficking (Hsiao et al., 2009), as well as OFD1 and Talpid3, which may assist with docking the ciliary vesicle onto the centriole (Yin et al., 2009; Singla et al., 2010). Another such protein is CEP290, which binds MKS6 (Gorden et al., 2008) and thus likely functions in the MKS or NPHP module. CEP290 was assigned as a component of Y-links and ciliary gate in Chlamydomonas (Croaire et al., 2010), and mutations in this gene occur in several ciliopathies. Whether CEP290 functionally interacts with other TZ proteins to facilitate BB/TZ membrane attachments in an early ciliogenic event—as we have shown in this study for several evolutionarily conserved TZ proteins—reinforces the idea that NPHP and MKS are diseases of BB dysfunction.

Confusion regarding differences between the C. elegans BB and TZ is also apparent. As C. elegans lacks classical BB microtubule architecture at the base of cilia (Perkins et al., 1986), similar to that observed in mature murine sperm (Manandhar et al., 1998), it has long been speculated that the nematode TZ is “analogous” to the BB. However, several observations suggest this is inaccurate. First, in daf-19 mutants, which lack Tzs and cilia, typical centriolar pairs are observed unattached to distal dendritic membranes where cilia would normally form (Perkins et al., 1986). Second, Tfs present at the base of C. elegans cilia (Fig. 1 A; Perkins et al., 1986) are evolutionarily conserved structures present at the distal end of all BBs (Silverman and Leroux, 2009). Third, the conserved centriolar BB protein HYLS-1 localizes in C. elegans within the TF region, just proximal to NPHP-4 at the TZ (Dammermann et al., 2009). Importantly, HYLS-1 in C. elegans and vertebrates is dispensable for centriole function during cell division but critical for ciliogenesis (Dammermann et al., 2009). We propose that the C. elegans TF region, where different IFT proteins concentrate—as shown for IFT52 in Chlamydomonas (Deane et al., 2001)—functions as a bona fide BB that is adjacent to but distinct from the TZ, where MKS/MKSR/NPHP proteins localize (Fig. 1 A, B–D; Fig. 2). Further, our finding that Y-links are completely, or even selectively, lost in MKS/MKSR/NPHP double mutants leaves open the possibility that at least some of these proteins represent structural elements (e.g., Y-links) of the TZ. Indeed, structural and biochemical properties of various MKS/MKSR/NPHP proteins support this possibility; NPHP1 binds microtubules (Otto et al., 2003), the C2/B9 domain proteins are predicted to associate with the inner leaflet of the plasma membrane, and MKS3 is membrane spanning. Taken together, one begins to envision two or more MKS/MKSR/NPHP modules—likely joined by MKS5—as components of the Y-link/ciliary necklace structures that connect axonemal microtubules to the surrounding membrane.

TZ-dependent establishment of the ciliary gate

Our analyses revealed that MKS/MKSR and NPHP modules are collectively required for two essential aspects of ciliogenesis, namely membrane anchoring of the BB/TZ and formation of an intact TZ region. Through these processes, the ciliary gate is
established (Fig. 10 D). The observed axoneme extension defects in TZ double mutants probably arise as a secondary consequence of anomalies in these early ciliogenic events.

The TZ in early ciliogenesis. Largely normal IFT rates in TZ mutant strains, and the fact that IFT mutants do not display phenotypes comparable to those of TZ mutants by TEM analysis (Perkins et al., 1986), support the notion that IFT proteins play a role in ciliogenesis distinct from that of TZ proteins, namely building the axoneme and delivering ciliary cargo vs. microtubule-membrane association/stabilization (Fig. 10 D). This difference is reflected in rodent models in which IFT perturbation (cilia ablation) causes early embryonic lethality (at ~E8.5–10.5), whereas disrupting MKS/MKSR/NPHP proteins leads to less severe ciliogenesis defects and developmental outcomes (Murcia et al., 2000; Jiang et al., 2009; Tamachote et al., 2009). TEM studies of mammalian cells forming cilia (Sorokin, 1962) are consistent with a possible role for the TZ in early ciliogenesis (Fig. 10 D). Specifically, an early ciliogenic step involves the interaction of a Golgi-derived “ciliary” vesicle (CV) with the distal end of the mother centriole/nascent BB to establish microtubule–membrane associations. A “ciliary bud” grows from the centriole, invaginating the CV, which itself appears to grow by fusion with secondary vesicles. In all likelihood, the ciliary bud, visible before BB docking/anchoring with the plasma membrane (Sorokin, 1962; Moser et al., 2010), represents a developing TZ; i.e., the first section of the ciliary axoneme (Fig. 10 D; Rohatgi and Snell, 2010). After docking and fusion with the membrane, the ciliary bud/TZ further extends to form the ciliary axoneme, a step dependent on IFT proteins. Alternatively, in other cell types, the centriole/TZ may dock directly with the plasma membrane, forgoing association with a CV; in this instance, microtubule–membrane associations mediated through the TZ also occur before axoneme extension (Fig. 10 D).

The TZ ciliary gate. The ciliary gate was long thought to be important in cilia function (Rosenbaum and Witman, 2002), but is only now being studied at the molecular level (Jauregui et al., 2008; Craig et al., 2010). The BB/TZ region is thought to facilitate ciliary gate function as a docking site for proteins destined for the cilium, as a region of selective active transport, and as a diffusion barrier. We demonstrate that individual MKS/MKSR/NPHP-disrupted strains lack a normal ciliary barrier, as evidenced by accumulation of nonciliary proteins within cilia. Alternatively, these proteins may enter cilia at low levels normally and instead require NPHP and MKS proteins for efficient removal from the compartment. Regardless, our data indicate that MKS/MKSR/NPHP proteins establish the TZ as a ciliary gate, and we predict that these TZ proteins likely function in coordination with other mechanisms—including IFT, the Ran-importin system, and BBSome (Bae et al., 2006; Jauregui et al., 2008; Craig et al., 2010; Dishinger et al., 2010; Jin et al., 2010)—to control cilium composition and thus function.

Concluding remarks

Ciliopathies are genetically heterogeneous but have overlapping phenotypic presentations, suggesting a common cellular mechanism as the basis of their etiology. In this study, we show that eight C. elegans proteins jointly function in establishing connections between the ciliary membrane and axoneme at the TZ, and in formation of the ciliary gate to regulate ciliary membrane composition. These TZ proteins are highly conserved in ciliated organisms (Hodges et al., 2010), suggesting that our model will be widely applicable.

Materials and methods

Strains and general methods

All strains (Table S1 B) used were maintained and cultured at 20°C using standard techniques (Brenner, 1974). Many procedures used in this study are summarized in Inglis et al. (2009). K07G5.3(gk674) and C09G5.8(tm3100) worms were obtained from the knockout consortium (http://celeganskoconsortium.omrf.org) and the National Bioresource Project [Japan], respectively, and outcrossed 5x to wild type. Because the gk674 deletion removes some neighboring xpa-1 gene sequence (Fig. 3 A), we confirmed that the nonciliary function of this DNA repair gene was not abrogated. First, the lifespan of the available xpa-1(nm157) mutant is shorter (P<X) than N2 worms, whereas gk674 mutants are wild type for lifespan (Table S1 E). Second, xpa-1 animals, but not gk674 or N2 worms, possess defective DNA repair as observed by UV irradiation dose-killing curves.

To generate a null allele for K07G5.3, we used PCR to screen for imprecise excision events from ttt17821 worms, which contain a Mos transposon inserted in K07G5.3 (Fig. 3 A). The mutagenesis protocol, modified from Boulin and Bessereau (2007), is as follows. Strain EG1642 (lin-15b[n765]X; oxEx166[HSP::MosTransposase + cc::gfp]) carrying the extrachromosomal Mos transposase under a heat-shock promoter was crossed into strain ttt17821. 100 young adult worms (ttt17821; oxEx166[HSP::MosTransposase + cc::gfp] [PO]) were heat-shocked (33°C; 1 h), allowed to recover for 1 h at 20°C, and heat-shocked again before removal to 20°C for 2 h. PO worms were individually propagated and allowed to lay eggs (F1) for 24 h at 20°C. After removal of F0 worms, F1 worms were allowed to produce F2 progeny. DNA lysates from ~50% of the F1–F2 worm mixture from each plate were prepared and PCR was performed using primers 5’-GCTACACGAAGACTAGTACTGTTC-3’ and 5’-GCTTGITGATGAGAAGACGAATG-3’ flanking the Mos insertion site. 100 worms from plates containing a K07G5.3 deletion were cloned and screened for the deletion and homozygosed. Using this scheme, we isolated five K07G5.3 alleles, including ox105 (1828-bp deletion + 10-bp insertion; Fig. 3 A). Before analysis, nx105 worms were outcrossed 5x to N2.

Imaging and subcellular localization of proteins

Live animals were anesthetized using 10 mM levamisole (diluted in M9 buffer), mounted on 2% agar pads, and observed using epifluorescence or spinning-disc confocal microscopy performed on an inverted microscope (model 2000U; Nikon) outfitted with a spinning-disc laser apparatus [UltraVIEW ERS 6F-ES; PerkinElmer]. Whole-mount immunostaining experiments were performed essentially as described previously (Bobinnex et al., 2000). In brief, gravid adults were cut in M9 buffer containing 15 mM levamisole to release gonads, intestine, and embryos. A coverslip was gently applied and the slide immersed in ~20°C methanol for 5 min and air-dried for 5 min. Worms were rehydrated in PBS-BSA (1% for 30 min, and incubated in 3% PBS-BSA with polyglutamylated tubulin antibody GT335 (1:2,300 dilution; a gift from Carsten Janke, Université Montpellier, Montpellier, France) for 1 h. After 3 x 10 min washes with PBS, animals were mounted in ProLong gold (Invitrogen), and observed by confocal microscopy. All images were captured using OpenLab or Velocity (PerkinElmer).

Intrflagellar transport (IFT) assays

Anterograde IFT rate analyses were performed as described previously (Bialas et al., 2009; Inglis et al., 2009). Individual worms containing GFP-tagged IFT-associated proteins were picked onto 1% agar pads and immobilized using 10–20 mM levamisole. Amphid/phasmid cilia were examined with a 100x 1.35 NA objective and an ORCA AG CCD camera mounted on a Zeiss Axioskop 2 mot+ microscope. Images were acquired using Openlab version 5.0.2 (PerkinElmer), with exposure rates ranging from 130 ms/frame (for OSM-6::GFP-containing strains) to 250 ms/frame (for CHE-1::GFP-containing strains). Openlab TIFF files were imported, with relevant metadata, into ImageJ (http://rsb.info.nih.gov/ij/) using the
Bio-formats importer plug-in (Laboratory for Optical and Computational Instrumentation, University of Wisconsin-Madison, Madison, WI). Kymographs for IFT rate analyses were generated using the MultiplexKymograph ImageJ plug-in (Inglis et al., 2009).

Phenotypic characterization

Ciliary analyses included dye-filling, lifespan, and osmotic avoidance assays. Cells were located, in brief, fluorescent dye uptake was performed as described previously (Jauregui et al., 2008). L4 larvae were incubated in Vybrant DiD (Invitrogen; 1:1,000-fold dilution of 1 mM stock in M9 buffer) for ~30 min, allowed to roam on a plate with bacteria for 1 h to clear intestinal dye, and observed by fluorescence microscopy.

Lifespan assays. Lifespan assays were performed exactly as described in Bialas et al. (2009). Each assay was performed twice with statistically significant results.

Osmoscope assays. Osmolarity avoidance assays were performed as described in Culotti and Russell (1978), testing for the crossing of worms over a ring of high osmorality solution (8 M glycerol); 50 worms/strain were assayed, and experiments were repeated three times with statistically significant results.

UV sensitivity assays. The mks-6(gk674) deletion mutant removes a small portion of the xpa-1 gene. We therefore tested if the mks-6 mutant has lifespan and UV sensitivity phenotypes indicative of xpa-1 dysfunction. Table S1 E reveals no lifespan defect for the mks-6 mutant; and UV assay, detailed below reveal no statistically significant increase in UV sensitivity of the mks-6 mutant compared with wild-type. UV assays on N2, mks-6(gk674), and xpa-1(mh157) worms were based on a protocol by Astin et al. (2008). Staged 1-d-old adults were placed onto NGM plates containing no food, then irradiated with a 254-nm UV Stratallinker 2400 (Agilent Technologies). 1 h after UV irradiation (100 J/m²), worms were transferred to NGM plates containing OP50 bacteria and survival was scored thereafter every 24 h for 4 d. Each assay consisted of ~90 worms and was repeated three times.

Analysis of basal body, ciliary, and dendritic characteristics using IFT markers

Analyses of phasmid and amphid cilium lengths, dendrite lengths, and clustering of basal bodies was facilitated by visualizing the GFP-tagged IFT markers CHE-11 (IFT140) or OSPM6 (IFT52) in wild-type and mutant strains and quantitated using Imagej; schematics depicting each of the four analyses are shown in Fig. 4, A–C, F; Fig. 5. In brief, ciliary axoneme lengths represent the distance between the distal end of the basal body and the tip of the cilium; dendrite lengths represent the distance between the beginning of a phasmid neuron cell body and its respective basal body; basal body clustering/distribution is the distance between the anterior-most and posterior-most basal bodies for each amphid channel. All measurements are repeated to statistical significance and reported in micrometers.

Transmission electron microscopy

Wild-type or mutant worms were washed directly into a primary fixative of 2.5% glutaraldehyde in 0.1M Sorenson phosphate buffer. To facilitate rapid ingress of fixative, worms were cut in half under a dissection microscope using a razor blade, transferred to Eppendorf tubes, and fixed for 1 h. After fixation, specimens were processed for electron microscopy by standard methods; in brief, specimens were dehydrated in ascending grades of alcohol to 100%, infiltrated with epon, and placed in aluminum plantcassettes orientated in a longiudinal axis and positioned at 15°C for 24 h. Utracatch, Leica UC6 ultramicrotome, individual worms were sectioned either in cross or longitudinal aspect and allowed to polymerize at 60°C for 24 h. Ultrathin sections of 80 nm were taken for electron microscopic examination. These were picked up onto 100 mesh copper grids and stained with uranyl acetate and lead citrate. Using a Tecnai Twin (FEI) electron microscope, sections were examined to locate, in the first instance, the most distal region of the ciliary region and subsequently from that point to the more proximal regions of the ciliary apparatus. At each strategic point, distal segment, middle segment, and transition zone/transition fiber regions were tilted using the Computag of the Tecnai microscope to ensure that the axonemal microtubules were imaged in an exact geometrical normalcy to the imaging system. All images were recorded at an accelerating voltage (120 kV) and objective aperture of 10 μm using a MegaView 3 digital recording system.

HMM analyses and structural predictions of C2/B9 domains

HMMer profile hidden Markov model software suite (http://hmmer.janelia.org) was used to identify all C2 and B9 domains in the C. elegans genome and query their possible evolutionary relatedness. C. elegans C2 domains were first extracted from known C. elegans genes containing C2 domains (Table S1 C), and their alignment (obtained with ClustalW) was used as input for the HMMER program hmmbuild. Resulting profile was calibrated using hmmbuild and used to search the C. elegans proteome (version WS180) with hmmsearch. Using the same approach, we searched for B9 domain-containing genes in C. elegans using as input the three known B9 domains from MKS1–1, MKS1–1, and MKS2–2 derived from three nematode species (C. elegans, C. briggsae, and C. remanei; Table S1 C). Structure predictions of the B9 domains from the human proteins MKS1, B9D1, and B9D2 were performed using the mGenTHREADER fold recognition program (McGu Finn and Jones, 2003; http://bioinf.cs.ucl.ac.uk/psipred).

Online supplemental material

Table S1 [excel file] contains (A) conserved transition zone proteins and reported physical interactions, (B) C. elegans strains used in this study, (C) genome-wide HMM search for C2 and C2-related domains, (D) intrflagellar transport rate analyses for wild-type and T2 protein-disrupted strains, and (E) lifespan measurements. Figs. S1–S5 show ultratransmission (TEM) analysis of (S1) mks-6;np-hnp-4, (S2) mks-5; np-hnp-4, (S3) mks-1;np-hnp-4, (S4) mks-1;np-hnp-4, and (S5) mks-3;np-hnp-4 amphid channel cilia. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201012116/DC1.

We thank N. Mahendran and N. Bebari for their assistance with the project, and the C. elegans gene knockout consortium, National Bioresource Project for C. elegans, and Caenorhabditis Genetics Center (CGC) for strains. This work was funded by grants from the March of Dimes (to M.R. Leroux), Canadian Institutes of Health Research (CIHR; MOP 828790 to M.R. Leroux), Science Foundation Ireland (to O.E. Blacque), European Commission FP7 framework program (to O.E. Blacque), NIH (DK207438; Hepato/Renal Fibrosis, Diseases Core Center; RO1 DK056555; to B.D. Vorderfeld; and NSEC to N. Chen). C.L. Williams was supported by NIH T32 DK075452/22. P.N. Inglis was supported by a scholarship from MSFHR. N. Chen holds a scholar award from MSFHR and is a CIHR New Investigator. M.R. Leroux holds a senior scholar award from the Michael Smith Foundation for Health Research (MSFHR). O.E. Blacque is funded by the European Community’s Seventh Framework Programme FP7/2009 under grant agreement no. 241955, SYSCILIA.

Submitted: 20 December 2010
Accepted: 17 February 2011

References


Figure S1. **Ultrastructural analysis of mks-6;nphp-4 amphid channel cilia.** (A) Low and high magnification images of amphid channel cilia from TEM serial cross sections showing ciliary distal segments (DS), middle segments (MS), transition zones (TZ), and distal dendrite region (DD). Boxed number denotes proximal positioning of section relative to the top left section. Phenotypes observed include a reduced number of axonemes in distal region of amphid pore (3–4, instead of 10; +2, +4), axonemes lacking microtubules (MTs) altogether (+4; bottom inset) or possessing reduced MT doublet number (+6, bottom inset), unzipped microtubules (+4, +6; arrowheads), and no properly formed TZs at the distal tip of dendrites. Instead the TZs are misplaced in more proximal regions of the DD region (+9, +11) and no membrane-connecting Y-links are observed. In addition, the DD regions frequently contain abnormal accumulations of membranous-type material, some of which is highly electron dense and/or vesicular in nature (+9, +11; arrows). Bars, 100 nm. (B) High magnification images of misplaced TZs. These misplaced structures exhibit most of the features that define *C. elegans* TZs such as an apical ring membrane (+6; open arrow), which draws together outer doublet MTs with inner singlet MTs (+6; arrowhead) to form the characteristic constricted MT arrangement; however, Y-links and TFs are never observed (indicated in +17). In some cases, the TZ ring is incomplete, lacking 1–2 MT doublets (+7, and +11 in part A above). In most cases, large amounts of membranous-type material such as vesicles surround the misplaced TZs (indicated in +10). Dendritic plasma membrane (PM) indicated in +14. Bars, 100 nm. (C) Schematics of amphid channel cilia (longitudinal and transverse views) from N2 and mks-6;nphp-4 worms showing the major ultrastructural defects observed (not to scale; e.g., TZs mispositioned even deeper than indicated).
Figure S2. **Ultrastructural analysis of mks-5; nphp-4 amphid channel cilia.** (A) Low and high magnification images of amphid channel cilia from TEM serial cross sections showing ciliary distal segments (DS), middle segments (MS), transition zones (TZ), and distal dendrite region (DD). Boxed number denotes proximal positioning of section relative to the top left section. Phenotypes observed include a reduced number of axonemes in distal region of amphid pore (5 instead of 10; top left panel), 1–2 axonemes missing in more proximal regions of pore (+3, +4), axonemes lacking microtubules (MTs; +4; bottom inset) or possessing abnormal MT doublet numbers (+3, both insets; +5, top inset; +6, bottom inset), or unzipped microtubules (arrowheads in +3, bottom inset; and +4, top inset). No properly formed TZs are observed at the distal tip of dendrites. Instead TZs are misplaced in more proximal regions of the DD region.
(±5, bottom inset; ±6 top inset) and there is evidence of incomplete formation of the MT ring (±5, bottom inset). In addition, DD regions sometimes contain abnormal accumulations of membranous-type material, some of which is highly electron dense and/or vesicular in nature (±5, bottom inset; ±6, top inset). Bars, 100 nm. (B) High magnification images of misplaced TZs. These misplaced structures exhibit most of the features that define C. elegans TZs such as an apical ring membrane (indicated in ±4, top middle image; open arrow), which draws together outer doublet MTs with inner singlet MTs (indicated in ±4; top middle image; arrowhead) to form the characteristic constricted MT arrangement; however, Y-links and TFs are never observed (indicated in ±4, top middle and ±5, bottom right). In some cases, the TZ ring is incomplete, lacking MT doublets (±4, top middle). In other cases, MT doublets are not organized into symmetrical rings, indicating that these structures may not be TZs (±4, left; ±5 top right image; ±5, bottom left image). In many cases, large amounts of membranous-type material such as vesicles surround the misplaced TZs (indicated in ±4, top left image and ±5, bottom left image). Dendritic plasma membrane (PM) indicated in ±4 (top middle image). Bars, 100 nm. (C) Schematics of amphid channel cilia (longitudinal and transverse views) from N2 and mks-5; nphp-4 worms showing the major ultrastructural defects observed (not to scale; e.g., TZs mispositioned even deeper than indicated).
**Ultrastructural analysis of mksr-1;nphp-4 amphid channel cilia.** (A) Low and high magnification images of amphid channel cilia from TEM serial cross sections showing ciliary distal segments (DS), middle segments (MS), transition zones (TZ), and distal dendrite region (DD). Boxed number denotes proximal positioning of section relative to the top left section. Phenotypes observed include a highly reduced number of axonemes in distal region [2–3, instead of 10; top left panel and +1] and proximal region of amphid pore (+2 to +4), axonemes lacking microtubules (MTs; +2), unzipped microtubules (+1; arrowheads), and TZs with no membrane connecting Y-links (+3, inset). Bars, 100 nm. (B) High magnification images showing TZ misplacement in more proximal regions of DD (+13, +16). Misplaced structures exhibit most of the features that define *C. elegans* TZs such as an apical ring membrane (+3; open arrow), which draws together outer doublet MTs with inner singlet MTs (+3; arrowhead) to form the characteristic constricted MT arrangement; however, Y-links and TFs are not observed (indicated in +3 and +13). Sometimes, abnormal accumulations of membranous-type material surround the misplaced TZs (indicated in +13). Dendritic plasma membrane (PM) indicated in +16. Bars, 100 nm. (C) Schematics of amphid channel cilia (longitudinal and transverse views) from N2 and mksr-1;nphp-4 worms showing the major ultrastructural defects observed (not to scale; e.g., TZs mispositioned even deeper than indicated).
Figure S4. **Ultrastructural analysis of mks-1;nphp-4 amphid channel cilia.** (A) Low and high magnification images of amphid channel cilia from TEM serial cross sections showing ciliary distal segments (DS), middle segments (MS), transition zones (TZ), and distal dendrite region (DD). Boxed number denotes proximal positioning of section relative to the top left section. Phenotypes observed include a moderate reduction in the number of axonemes in distal amphid pore (6, instead of 10; top left panel and +2), 1–2 axonemes missing in more proximal regions of pore (+4, +5), axonemes missing one or two outer MT doublets (+5; insets), and TZs that are sometimes misplaced in more proximal regions of DD (+8, bottom inset). Bars, 100 nm. (B) High magnification images of TZ structures. As indicated in +8 (top right image), and similar to wt worms, the TZ structures of mks-1;nphp-4 animals consist of an apical ring membrane (open arrow) which draws together outer doublet MTs with inner singlet MTs (arrowhead) to form the characteristic constricted MT arrangement. However, although Y-links are sometimes observed (+5), they frequently are absent (indicated in +8; top right image and +12). Also, some TZs are misplaced in more proximal regions of DDs (+8; bottom left image and +12). Dendritic plasma membrane (PM) indicated in +12. (C) Schematics of amphid channel cilia (longitudinal and transverse views) from N2 and mks-1;nphp-4 worms showing the major ultrastructural defects observed (not to scale; e.g., TZs mispositioned even deeper than indicated).
Figure S5. **Ultrastructural analysis of mks-3;nphp-4 amphid channel cilia.** (A) Low and high magnification images of amphid channel cilia from TEM serial cross sections showing ciliary distal segments (DS), middle segments (MS), transition zones (TZ), and distal dendrite region (DD). Boxed number denotes proximal positioning of section relative to the top left section. Phenotypes observed include a moderate reduction in axoneme number in distal amphid pore (+5, instead of 10; top left panel and +2), 2–3 axonemes missing in more proximal regions of pore (+3, +5), axonemes missing outer MT doublets (+3, +5; insets), and TZs misplaced in more proximal regions of DD (+7, top inset). Bars, 100 nm. (B) High magnification images showing that most TZs are misplaced in more proximal regions of DD. Misplaced structures exhibit characteristic features of *C. elegans* TZs such as an apical ring membrane (indicated in +6; top middle image; open arrow), which draws together outer doublet MTs with inner singlet MTs (indicated in +6; top middle image; arrowhead) to form the characteristic constricted MT arrangement; however, Y-links and TFs are typically never observed (indicated in +6; top middle image; and +12). In rarer cases where the TZ is positioned at distal dendrite tip (+6, top left image), intact Y-links are not clearly evident, although remnants may be present. Also, some TZs may be missing at least one doublet MT (+6; top middle image; +7; both images; +12). Dendritic plasma membrane (PM) indicated in +6 (top middle image). Bars, 100 nm. (C) Schematics of amphid channel cilia (longitudinal and transverse views) from N2 and mks-3;nphp-4 worms showing the major ultrastructural defects observed (not to scale; e.g., TZs mispositioned even deeper than indicated).
Table S1 is provided as an Excel file and shows (A) conserved transition zone proteins and reported physical interactions, (B) C. elegans strains used in this study, (C) genome-wide HMM search for C. elegans proteins containing B9- and C2-related domains, (D) intraflagellar transport rate analyses for wild-type and TZ protein-disrupted strains, and (E) lifespan measurements.