The *Caenorhabditis elegans* nephrocystins act as global modifiers of cilium structure

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Nephronophthisis (NPHP) is the most common genetic cause of end-stage renal disease in children and young adults. In *Chlamydomonas reinhardtii*, *Caenorhabditis elegans*, and mammals, the NPHP1 and NPHP4 gene products nephrocystin-1 and nephrocystin-4 localize to basal bodies or ciliary transition zones (TZs), but their function in this location remains unknown. We show here that loss of *C. elegans* NPHP-1 and NPHP-4 from TZs is tolerated in developing cilia but causes changes in localization of specific ciliary components and a broad range of subtle axonemal ultrastructural defects. In amphid channel cilia, nphp-4 mutations cause B tubule defects that further disrupt intraflagellar transport (IFT). We propose that NPHP-1 and NPHP-4 act globally at the TZ to regulate ciliary access of the IFT machinery, axonemal structural components, and signaling molecules, and that perturbing this balance results in cell type–specific phenotypes.

Introduction

Nephronophthisis (NPHP) is a rare autosomal recessive tubulo-interstitial nephropathy characterized by cystic kidneys and renal fibrosis. NPHP extrarenal manifestations may include retinal degeneration, situs inversus, and neurological disorders. Mutations in one of nine genes (*NPHP1*-9) account for ~35% of all NPHP cases, which suggests that other disease-causing loci exist (Caridi et al., 2006; Arts et al., 2007; Attanasio et al., 2007; Delous et al., 2007; for review see Hildebrandt and Zhou, 2007; Otto et al., 2008). The NPHP gene products, the nephrocystins, have been localized to several regions of the cell including the cilium (Hildebrandt et al., 1997; Olbrich et al., 2003; Otto et al., 2003, 2005; Mollet et al., 2005; Sayer et al., 2006). Given that many cell types possess cilia, it is not surprising that NPHP presents with a variety of extrarenal pathologies that may correlate with ciliary dysfunction (Pazour and Rosenbaum, 2002).

Cilia are specialized microtubule-based organelles that function in motility or sensation. Nonmotile primary cilia are found on most nondividing vertebrate cells (Wheatley 1995). In general, primary cilia have a 9 + 0 axoneme (nine outer doublet microtubules with no central pair). In the kidney, primary cilia project from the apical surfaces of epithelial cells and act as flow mechanosensors (Praetorius and Spring, 2001, 2003). In the eye, connecting cilia regulate protein trafficking between inner and outer photoreceptor segments (Marszalek et al., 2000; Pazour et al., 2002). In the mammalian embryo, nodal cilia exhibit a unique rotary motion required for left–right asymmetry (McGrath et al., 2003). Defects in these types of cilia may result in polycystic kidneys, retinal degeneration, and situs inversus, respectively (Pazour and Rosenbaum, 2002). In humans, perturbation of cilia formation, maintenance, morphogenesis, or function may result in a “ciliopathy” (Badano et al., 2006).

The development of all cilia and flagella requires intraflagellar transport (IFT; Rosenbaum and Witman, 2002). IFT was first observed in the alga *Chlamydomonas reinhardtii* as a rapid bidirectional movement of particles along the flagellar length in a FLA10 kinesin-2–dependent manner (Kozminski et al., 1993, 1995). The IFT kinesin-2 motor consists of two different heterodimerized motor subunits and one accessory (KAP) subunit (Cole et al., 1993). The basic unicellular IFT machinery comprises heterotrimeric kinesin-2 and retrograde cytoplasmic dynein motors that move IFT particles and cargo to and from the distal tips of cilia. The IFT particle is composed of two subcomplexes (IFT-A and IFT-B) containing 16–18 polypeptides (Cole et al., 1998). In *C. reinhardtii* and the nematode *Caenorhabditis*
*C. elegans*, defects in IFT motors or polypeptides result in flagellar and ciliary defects. In mice, a mutation in the IFT88 polypeptide Polaris or the IFT kinesin-2 motor KIF3A causes autosomal recessive polycystic kidney disease (PKD; Pazour et al., 2000; Lin et al., 2003). In humans, IFT80 polypeptide mutations result in the ciliopathy Jeune asphyxiating thoracic dystrophy (Beales et al., 2007).

*C. elegans* is a powerful model system to study ciliogenesis and human ciliopathies (Scholey et al., 2004; Barr, 2005; Badano et al., 2006). In the worm, IFT builds and maintains cilia on dendritic endings of sensory neurons. The amphids, a pair of lateral sensilla in the head, are the principal sensory organs of nematodes. Amphid channel cilia are ∼7 μm in length and can be divided into three distinct segments (Ward et al., 1975; Perkins et al., 1986). The transition zone (TZ) is a modified basal body and the place where IFT proteins accumulate for transport into the axoneme. The axoneme has middle and distal segments, consisting of nine doublet (A tubule and B tubule) and nine singlet (A tubule only) microtubules, respectively. In addition to the amphids, four cuticular sensilla (cephalic, inner/outer labial quadrants, and outer labial lateral) are found in the head and the phasmid sensilla are located in the tail (Ward et al., 1975; Ware et al., 1975; Hall and Russell, 1991). In the male, each cephalic sensillum contains an additional exposed ciliated neuron (CEM) and the tail possesses sensilla required for sexual behaviors (Sulston et al., 1980; Liu and Sternberg, 1995; Barr and Sternberg, 1999). These cilia exhibit diverse morphologies and sensory properties (Sulston et al., 1980; Perkins et al., 1986; Bargmann, 2006).

In *C. elegans* amphid channel cilia, the IFT machinery has been extensively studied using mutant analysis combined with in vivo time-lapse fluorescence imaging (Orozco et al., 1999; Blacque et al., 2004; Snow et al., 2004; Ou et al., 2005, 2007; Pan et al., 2006). The following model has emerged. Amphid and phasmid channel cilia possess two IFT kinesin-2 motors: heterotrimeric kinesin-II and homodimeric OSM-3 (Shakir et al., 1993; Signor et al., 1999). The slow kinesin-II and fast OSM-3 act cooperatively to build the middle segment, whereas OSM-3 acts alone to drive IFT in the distal segment (Snow et al., 2004). Consistent with in vivo velocity measurements, purified mixtures of kinesin-II and OSM-3 produce intermediate motility rates in vitro (Pan et al., 2006). The Bardet-Biedl syndrome (BBS) proteins have been proposed to stabilize the association of the two kinesin-2 motors with their associated IFT subcomplexes (Ou et al., 2005, 2007). In a *bbs-1*, *bbs-7*, or *bbs-8* mutant background, IFT-A moves at the slow kinesin-II rate, whereas IFT-B moves at the fast OSM-3 rate. However, in a kinesin-II:*bbs* double mutant, IFT-A moves with OSM-3 and, likewise, in an *osm-3:*bbs double mutant, IFT-B moves with kinesin-II, which suggests that kinesin-II and OSM-3 mechanically compete for IFT-A and IFT-B in a BBS-dependent manner (Pan et al. 2006). Variations from the canonical slow, fast, and intermediate rates are observed in some mutant backgrounds, which indicates that there is an unappreciated complexity to IFT motor regulation (Burghoorn et al. 2007; Mukhopadhyay et al., 2007). Deviations observed from canonical rates in vivo may reflect changes in the normal molar ratio of active kinesin-II:OSM-3 bound to the IFT particles. Moreover, the basic IFT machinery is modified in a cell-specific manner to generate diversity in ciliary shape and function (Bae et al., 2006; Evans et al., 2006; Mukhopadhyay et al., 2007).

*C. elegans* nphp-1 and nphp-4 orthologues are expressed in the ciliated sensory nervous system and are coexpressed with the *C. elegans* autosomal dominant PKD genes *lov-1/*PKD1 and *pkd-2* (Jauregui and Barr 2005; Winkelbauer et al., 2005; Wolf et al., 2005). *nphp-1* and *nphp-4* deletion mutants form cilia as judged by lipophilic fluorescent dye filling and light microscopy, and are only slightly defective in several sensory behaviors. GFP-tagged NPHP-1 and NPHP-4 proteins localize to the ciliary TZ, with NPHP-1 requiring the presence of NPHP-4 for TZ localization (Winkelbauer et al., 2005). This TZ subcellular localization is evolutionarily conserved. Human nephrocystin-1 also localizes to the Tzs of renal, respiratory, and photoreceptor connecting cilia (Schmerer et al., 2005; Fliegauf et al., 2006). Moreover, both mammalian and *C. reinhardtii* nephrocystin-4 localize to ciliary basal bodies (Keller et al., 2005; Mollet et al., 2005). The TZ serves as a boundary between cytoplasmic and ciliary components and has been proposed to act as a “ciliary pore complex” (Rosenbaum and Witman, 2002). How the nephrocystins act at the TZ is not known.

In this paper, we address the functions of the nephrocystins in regulating ciliary receptor localization, ciliary shape, and ciliogenesis. *nphp-1* and *nphp-4* are not required for the ciliary localization of the transient receptor potential vanilloid (TRPV) channel OSM-9 (Colbert et al., 1997) or the TRP polycystin (TRPP) channel PKD-2 (Barr et al., 2001). Rather, *nphp-1* and *nphp-4* are important modulators of ciliary ultrastructure, with defects resulting in a broad phenotypic spectrum. Although not localizing to the axoneme, NPHP-1 and NPHP-4 indirectly regulate the velocity distributions of most IFT components examined. In addition, a subset of GFP-tagged IFT reporters (IFT52/OSM-6, OSM-3, BBS-7, and BBS-8) are not required for the interaction of the two kinesin-II motors: heterotrimeric kinesin-II and homodimeric OSM-3 (Shakir et al., 1993; Signor et al., 1999). The slow kinesin-II and fast OSM-3 act cooperatively to build the middle segment, whereas OSM-3 acts alone to drive IFT in the distal segment (Snow et al., 2004). Consistent with in vivo velocity measurements, purified mixtures of kinesin-II and OSM-3 produce intermediate motility rates in vitro (Pan et al., 2006). The Bardet-Biedl syndrome (BBS) proteins have been proposed to stabilize the association of the two kinesin-2 motors with their associated IFT subcomplexes (Ou et al., 2005, 2007). In a *bbs-1*, *bbs-7*, or *bbs-8* mutant background, IFT-A moves at the slow kinesin-II rate, whereas IFT-B moves at the fast OSM-3 rate. However, in a kinesin-II:*bbs* double mutant, IFT-A moves with OSM-3 and, likewise, in an *osm-3:*bbs double mutant, IFT-B moves with kinesin-II, which suggests that kinesin-II and OSM-3 mechanically compete for IFT-A and IFT-B in a BBS-dependent manner (Pan et al. 2006). Variations from the canonical slow, fast, and intermediate rates are observed in some mutant backgrounds, which indicates that there is an unappreciated complexity to IFT motor regulation (Burghoorn et al. 2007; Mukhopadhyay et al., 2007). Deviations observed from canonical rates in vivo may reflect changes in the normal molar ratio of active kinesin-II:OSM-3 bound to the IFT particles. Moreover, the basic IFT machinery is modified in a cell-specific manner to generate diversity in ciliary shape and function (Bae et al., 2006; Evans et al., 2006; Mukhopadhyay et al., 2007).

*Results*

**NPHP-1 and NPHP-4 are required for CEM cilia length and shape**

NPHP-1 and NPHP-4 colocalize with the polycystins LOV-1 and PKD-2 in male-specific sensory neurons (Jauregui and Barr, 2005). Mutations in the polycystin-encoding genes cause autosomal dominant PKD (Igarashi and Somlo, 2002). To determine the function of the nephrocystins in polycystin-expressing neurons, we expressed NPHP-1::GFP and NPHP-4::GFP using
nphp-4 mutants have ultrastructural defects in CEM, CEP, and OLQ cilia

We used transmission EM (TEM) to examine CEM ciliary ultrastructure of wild-type and nphp-4 males, the latter of which was missing both NPHP-1 and NPHP-4 at the TZ. Wild-type CEM TZs are similar to the CEP and OLQ TZs (Perkins et al., 1986), with 9 + 0 microtubule doublets and Y-linked membrane attachments (Fig. 4 e). The CEM and CEP TZs are enclosed within the same sheath and socket cells. The wild-type CEM middle segment swells in diameter and displays many peripheral microtubule singlets and a few dispersed central microtubule singlets (Fig. 4, c and d). This is different from amphid channel cilia middle segments, which remain narrow and contain nine microtubule doublets comprised of A and B tubules (see Fig. 8 i). More anteriorly, the wild-type CEM axoneme winds around and away from the CEP cilium and OLQ distal region. CEM distal segments are narrow and contain many dark filled microtubule singlets (Fig. 4 b) reminiscent of distal segments of ray RnB cilia (Sulston et al., 1980). The most distal region of the CEM curves outward and is exposed to the environment via a narrow cuticular opening (Ward et al., 1975). Here, the CEM cilium typically bends by 90° to open laterally; thus, its microtubules are seen lengthwise in a transverse section (Fig. 4 a). CEP and OLQ distal segments end embedded in the cuticle at the nose tip (Perkins et al., 1986).

nphp-4 CEM axonemes were also stunted or misshapen and long (Figs. 2 i, 3, and S1 c; and Video 3, available at http://www.jcb.org/cgi/content/full/jcb.200707090/DC1) but observed at a lower frequency compared with nphp-1 mutants (15% vs. 45%). The nphp-1;nphp-4 double mutant resembled the nphp-4 single mutant (Fig. 3). These data indicate that nphp-4 acts epistatically to nphp-1 in CEM cilia and are consistent with the observation that NPHP-1 requires NPHP-4 to localize to the ciliary TZ (Winkelbauer et al., 2005). We conclude that the ciliary TZ proteins NPHP-1 and NPHP-4 regulate ciliary length and morphology in the polycystin-expressing CEM neurons.

Figure 1. NPHP-1 and NPHP-4 localize to TZs of male-specific CEM cilia. 3D deconvolution images of NPHP-1::GFP (a) and NPHP-4::GFP (c) expressed only in male-specific CEM neurons and overlaid on Nomarski images (b and d). NPHP-1::GFP and NPHP-4::GFP localize to the CEM TZ but not the cilium proper (a–d). The CEM axoneme emanates from the TZ to the cuticle [territories of CEM cilium are indicated by brackets in b and d]. Lateral view is shown. Bar, 10 μm.
TZ and localizes along the ciliary membrane (Qin et al., 2005; Bae et al., 2006), and the underlying ciliary axoneme follows this pattern (Ppkd-2::tbb-4::tdTomato in Fig. 2, a–c).

In nphp-1 mutants, PKD-2::GFP localizes and slightly accumulates around misshaped cilia (Fig. 2, d–f). In nphp-4 mutants, PKD-2::GFP localizes to stunted cilia and accumulates at the TZ and distal dendrite (Fig. 2, g–i). We also examined the localization of the TRPV channel OSM-9, which localizes to OLQ, amphid, and phasmid cilia (Colbert et al., 1997; Tobin et al., 2002). In nphp-1 and nphp-4 mutants, OSM-9::GFP localizes to OLQ cilia that are occasionally misshaped or stunted (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200707090/DC1), which is consistent with TEM data (Fig. 4). We conclude that nphp-1 and nphp-4 are not essential for PKD-2 and OSM-9 ciliary localization.

nphp-4 regulates ciliary localization of a subset of IFT components

To determine the molecular basis for the stunted and deformed cilia, we examined the IFT machinery in nphp-1 and nphp-4 mutants. To measure IFT in CEM cilia, we used the pkd-2 promoter to express the IFT-B polypeptide OSM-5 and observed OSM-5::GFP motility in wild-type CEM cilia, nphp-4 stunted cilia, and nphp-1 misshaped/long cilia (Videos 4–6, available at http://www.jcb.org/cgi/content/full/jcb.200707090/DC1). However, we were unable to measure IFT velocities because the CEMs curve out of the microscopic plane. As IFT has been (Fig. 4 i). Misshaped CEM cilia wander circuitously while traversing the sheath channel but middle segments appear fairly normal and distal segments usually reach the cuticular opening (unpublished data).

Ultrastructural defects are also seen in the nearby CEP and OLQ cilia of nphp-4 animals. CEP cilia are enlarged and misshaped, and sometimes displaced posteriorly, elaborating their cilium within the sheath and socket without embedding in the cuticle (unpublished data). In wild-type CEP cilia, the majority of tubulin-associated material (TAM) is found in distal regions (Fig. 4, a and b). In nphp-4 CEP cilia, large amounts of TAM are displaced into middle regions (Fig. 4 j), with dense aggregations and reduced amounts of TAM in distal segments (Fig. 4, g and h). nphp-4 OLQ cilia are occasionally stunted (Fig. 4 f), distorted due to severed or missing microtubules (Fig. 4, g and h), or posteriorly misplaced (Fig. 4 i). In a few cases, the OLQ cilium may be truncated at or near the TZ. Although CEM and OLQ TZs appear normal, few CEP TZs were obtained in our slices, likely because of posterior displacement. We conclude that the TZ proteins NPHP-1 and NPHP-4 regulate ciliary length (OLQ and CEM), ciliary morphogenesis (CEM, CEP, and OLQ), and transport of axonomal components such as TAM (CEP).

NPHP-1 and NPHP-4 are not required for ciliary localization of TRP channels

NPHP-1 and/or NPHP-4 may regulate ciliary receptor localization. In wild-type CEMs, PKD-2::GFP accumulates at the TZ and localizes along the ciliary membrane (Qin et al., 2005; Bae et al., 2006), and the underlying ciliary axoneme follows this pattern (Ppkd-2::tbb-4::tdTomato in Fig. 2, a–c). In nphp-1 mutants, PKD-2::GFP localizes and slightly accumulates around misshaped cilia (Fig. 2, d–f). In nphp-4 mutants, PKD-2::GFP localizes to stunted cilia and accumulates at the TZ and distal dendrite (Fig. 2, g–i). We also examined the localization of the TRPV channel OSM-9, which localizes to OLQ, amphid, and phasmid cilia (Colbert et al., 1997; Tobin et al., 2002). In nphp-1 and nphp-4 mutants, OSM-9::GFP localizes to OLQ cilia that are occasionally misshaped or stunted (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200707090/DC1), which is consistent with TEM data (Fig. 4). We conclude that nphp-1 and nphp-4 are not essential for PKD-2 and OSM-9 ciliary localization.
extensively studied in amphid channel cilia (Scholey et al., 2004; Snow et al., 2004; Ou et al., 2005, 2007; Evans et al., 2006; Pan et al., 2006; Scholey and Anderson, 2006), we focused on these cilia types. We compared the localization patterns of GFP-tagged IFT motors, IFT-A and IFT-B polypeptides, and regulators in wild-type and nephrin-deficient cilia. In the nphp-1 mutant, all IFT components localized similarly to the wild type (Fig. 5). Strikingly, in nphp-4 single or nphp-1; nphp-4 double mutants, some but not all IFT components exhibited abnormal ciliary localization patterns (Fig. 5, b3, b4, d3, d4, e3, e4, f3, f4, i3, and i4).

In wild-type and nphp-1 animals, IFT-A and IFT-B polypeptides localize to the ciliary TZs, middle segments, and distal segments (Fig. 5, a1, b1, c1, and d1). In nphp-4 mutants, the complex B IFT52–OSM-6 polypeptide was noticeably dimmer in cilia, with weak a GFP signal in distal segments (Figs. 5 b3 and S3, available at http://www.jcb.org/cgi/content/full/jcb.200707090/DC1). In contrast, IFT-B polypeptides IFT88/Polaris/OSM-5 and IFT80/CHE-2 (not depicted) displayed normal localization patterns (Fig. 5 c). Occasionally, CHE-13::GFP was missing from distal segments of nphp-4 and nphp-1; nphp-4 animals (in 6 out of 23 animals; Fig. 5 d), although this has not been observed previously (Winkelbauer et al., 2005). IFT-A polypeptide CHE-11::GFP was normal in the single and double mutants (Fig. 5, a3 and a4).

We examined the IFT kinesin-2 motors and the BBS proteins, which are proposed to connect and stabilize kinesin-II and OSM-3. In the wild type, nphp-1, and nphp-4 single and double mutants, kinesin-II (visualized by KAP-1::GFP) localizes only to the middle segment and is not detectable in the distal region (Fig. 5 j). In wild-type and nphp-1 animals, OSM-3::GFP localizes to both the middle and distal segments (Fig. 5, i1 and i2). In a striking contrast, nphp-4 and nphp-1; nphp-4 mutants exhibited reduced ciliary levels and increased dendritic levels of OSM-3::GFP (Fig. 5, i3 and i4). In wild-type and nphp-1 animals, BBS-7::GFP and BBS-8::GFP localize to the TZ and along the middle and distal segments of the cilium (Fig. 5, e1, e2, f1, and f2). In nphp-4 and nphp-1; nphp-4 distal segments, BBS-7::GFP was dim or occasionally absent in distal segments (Fig. 5, e3 and e4), although this reduction was not as severe as observed for OSM-6::GFP. BBS-7::GFP occasionally aggregated between the middle and distal segments (Fig. 5 e3, arrow). Unlike BBS-7, BBS-8 did not appear to aggregate at the middle–distal segment boundary. However, BBS-8::GFP was extremely dim in middle and distal segments (Fig. 5, f3 and f4). Seven BBS proteins form a stable biochemical complex (the “BBSome”, Nachury et al., 2007). Our data suggests that in the absence of NPHP-1 and NPHP-4 at the TZ, the stoichiometry of the BBSome in the cilium may be altered.

We analyzed regulators of kinesin-2 activity. Dye filling defective (DYF)-1 has been proposed to activate OSM-3 (Ou et al., 2005) or regulate tubulin posttranslational modifications (Pathak et al., 2007). DYF-1::GFP did not have an overt mislocalization defect in nphp-1 or nphp-4 single or double mutants (Fig. 5 g), which suggests that OSM-3 dendritic accumulation in nphp-4 mutants is DYF-1 independent. The dyf-13 mutant, similar to nphp-4 animals, occasionally lacks OSM-6::GFP in amphid distal segments (Blaque et al., 2005; Ou et al., 2005). DYF-13::GFP localization is intact in nphp-1 and nphp-4 single and double mutants (Fig. 5, h1–h4).

In conclusion, loss of both NPHP-1 and NPHP-4 but not NPHP-1 alone leads to the abnormal ciliary localization of the IFT-B polypeptide OSM-6, the OSM-3–kinesin, and the BBS proteins BBS-7 and BBS-8. In contrast, NPHP-1 and NPHP-4 are not required for the localization of the IFT-B polypeptides OSM-5 and CHE-2, IFT-A polypeptide CHE-11, kinesin-II, and ciliogenic proteins DYF-1 and DYF-13.

Figure 3. nphp-1 and nphp-4 mutants have misshaped, long, and stunted cilia. (A) CEM cilia are visualized using Ppkd-2::tbb-4::GFP. Dark gray indicates the frequency of misshaped curly cilia; light gray indicates the frequency of stunted or mispositioned cilia. nphp-1 mutants have predominantly curly cilia with some stunted cilia. nphp-4 and nphp-1; nphp-4 mutants have equal numbers of stunted and curly cilia. At least 50 animals are scored per genotype (−200 individual CEM cilia are observed). Error bars indicate ± SEM. (B) CEM cilia were divided into wild-type, curly, and stunted categories. From z stack compressions of 3D wild-type and mutant CEM cilia; lengths were measured by tracing the cilium from the base of the TZ to the end of the axoneme. Curly cilia are significantly longer than the wild type. Stunted or misplaced cilia are slightly shorter than the wild type. The z distance of curls is not represented in this assay. p-values from a t test compare mutants to the wild type.

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Figure 4. CEM, CEP and OLQ ciliary ultrastructure is abnormal in nphp-4 mutants. TEM of wild type (a–e) and nphp-4 (f–j) mutant animals in transverse thin sections. Black arrows, OLQ; white arrows, CEP; arrowheads, CEM. As depicted in the top drawings, the distal-most region of the CEM axoneme is narrow, curved outward, and exposed to the environment. Microtubule singlets are seen longitudinally near the opening (a). Traveling posteriorly, the distal segment aligns with the body axis and nears the edge of CEP socket cell (b). The CEM middle segment widens and is filled with microtubule outer singlets and dispersed inner singlets (c). The middle segment twists around the CEP away from the OLQ such that the CEM axoneme is furthest away from OLQ (d). CEM TZs are similar to CEP and OLQ TZs with 9 + 0 microtubule doublets (e). In nphp-4 mutants, OLQ distal segments are occasionally missing (f) but middle segments are present (g) with missing microtubules (g and h). CEM and OLQ TZs appear normal (i and j). CEP axonemes are irregular with large unidentified accumulations (g–i). Panel j is from a different section of the same worm illustrating well-formed OLQ and CEM TZs but apparently missing a CEP TZ, replaced by a large mass of TAM surrounded by microtubule singlets. CEM axonemes can lack distal segments [empty channel in f with no microtubules]. Drawings at bottom illustrate nphp-4 defects. Bars: (top) 100 nm; (bottom) 0.5 μm.
**nphp-4** mutation perturbs the velocity distributions of most IFT components examined

We measured IFT rates of kinesin-II, OSM-3, representative IFT-A (CHE-11) and IFT-B polypeptides (OSM-5 and OSM-6), BBS-7, and DYF-1 (Table I). KAP-1 velocities were indistinguishable between the wild type and **nphp** mutants. OSM-3, OSM-5, CHE-11, BBS-7, and DYF-1 are not significantly different between wild-type and nephrocystin-deficient animals. However, their underlying population of velocities in the middle segment of **nphp-4** and **nphp-1:nphp-4** animals was significantly different from the wild type (Table I, t-test), indicating that these IFT components are traveling at a wider range of speeds. Noncanonical IFT velocities have been observed by genetic mutations in vivo or by varying concentrations of kinesin-II and OSM-3 in vitro (Pan et al., 2006; Mukhopadhyay et al., 2007). We propose that by genetically perturbing the composition of the IFT machinery, abnormal velocities may emerge.
detected in the middle segments (Fig. 6, k and o) and moved at 0.5 μm/s (Table I). These results are consistent with OSM-6::GFP association with endogenous kinesin-II in nphp-4 mutant cilia.

To determine if OSM-6::GFP was capable of associating with endogenous OSM-3, we examined OSM-6::GFP distribution in a kap-1 bbs-7;nphp-4 triple mutant (Fig. 6, l and p). In kap-1;bbs-7 double mutants, kinesin-II cargo is shifted to OSM-3 (Pan et al., 2006). kap-1 bbs-7;nphp-4 phenotypically resembles the kap-1;nphp-4 double mutant. OSM-6::GFP remains aggregated at the ciliary base and distal dendrite (Fig. 6, j and n), and no IFT is detected. Conversely, in an osm-3;nphp-4 double mutant, OSM-6::GFP was detected in the middle segments (Fig. 6, k and o) and moved at 0.5 μm/s (Table I). These results are consistent with OSM-6::GFP association with endogenous kinesin-II in nphp-4 mutant cilia.

Unexpectedly, OSM-6::GFP velocity was dramatically affected by nphp-4 mutation, slowing to 0.49 μm/s in the middle segment of nphp-4 and nphp-1;nphp-4 animals (Table I) and suggesting that overexpressed OSM-6::GFP is now traveling with endogenous kinesin-II alone. In dim distal segments of nphp-4 mutants, OSM-6::GFP moved at 1.1 μm/s, the normal and predicted velocity of the fast kinesin OSM-3 (Table I). To test the hypothesis that OSM-6::GFP was abnormally associated with endogenous kinesin-II in the absence of nphp-4, we examined OSM-6::GFP in a kap-1;nphp-4 double mutant. In these animals, OSM-6::GFP severely aggregates at ciliary bases and distal dendrites of amphid and phasmid sensory neurons (Fig. 6, j and n), and no IFT is detected. Conversely, in an osm-3;nphp-4 double mutant, OSM-6::GFP was detected in the middle segments (Fig. 6, k and o) and moved at 0.5 μm/s (Table I). These results are consistent with OSM-6::GFP association with endogenous kinesin-II in nphp-4 mutant cilia.

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### Table I. Transport velocities of GFP tagged IFT proteins in wild-type and nephrocystin mutant animals

| Reporters | Genotype | Middle segment | Distal segment |
|-----------|----------|----------------|---------------|
|           |          | Mean velocity (μm/s) | n | t test | f-test | Mean velocity (μm/s) | n |
| Kinesin-2 motors |          |               |               |               |               |               |               |
| KAP-1::GFP | Wild type | 0.69 ± 0.09 | 446 | None |          | None |          |
|           | nphp-1 | 0.69 ± 0.09 | 420 | 0.49 | 0.4 | None |          |
|           | nphp-4 | 0.68 ± 0.09 | 375 | 0.66 | 0.93 | None |          |
|           | nphp-1;nphp-4 | 0.68 ± 0.09 | 423 | 0.77 | 0.46 | None |          |
| OSM-3::GFP | Wild type | 0.72 ± 0.07 | 128 | None |          | None |          |
|           | nphp-1 | 0.72 ± 0.07 | 81 | 0.9 | 0.37 | None |          |
|           | nphp-4 | 0.83 ± 0.15 | 101 | <0.001 | <0.001 | None |          |
|           | nphp-1;nphp-4 | 0.81 ± 0.15 | 213 | <0.001 | <0.001 | None |          |
| IFT particle A | CHE-11::GFP | Wild type | 0.71 ± 0.09 | 119 | 1.13 ± 0.15 | 119 |          |
|           | nphp-1 | 0.72 ± 0.08 | 131 | 0.9 | 0.22 | None |          |
|           | nphp-4 | 0.74 ± 0.14 | 252 | 0.09 | <0.001 | None |          |
|           | nphp-1;nphp-4 | 0.74 ± 0.13 | 350 | 0.04 | <0.001 | None |          |
| IFT particle B | OSM-6::GFP | Wild type | 0.71 ± 0.08 | 219 | 1.14 ± 0.16 | 218 |          |
|           | nphp-1 | 0.70 ± 0.09 | 98 | 0.48 | 0.25 | 1.26 ± 0.14 | 98 |
|           | nphp-4 | 0.49 ± 0.06 | 132 | <0.001 | 0.02 | 1.07 ± 0.17 | 104 |
|           | nphp-1;nphp-4 | 0.47 ± 0.07 | 57 | <0.001 | 0.2 | ND |          |
|           | nphp-4;kap-1 | None | - | - | - | 1.11 ± 0.16 | 144 |
|           | kap-1 bbs-7;nphp-4 | None | - | - | - | 1.06 ± 0.18 | 125 |
|           | osm-3 | 0.50 ± 0.07 | 252 | <0.001 | 0.22 | None |          |
|           | osm-3;nphp-4 | 0.51 ± 0.06 | 221 | <0.001 | 0.006 | None |          |
|           | OSM-5::GFP | Wild type | 0.71 ± 0.06 | 125 | 1.06 ± 0.16 | 132 |          |
|           | nphp-1 | 0.72 ± 0.08 | 281 | 0.567 | 0.004 | 1.07 ± 0.12 | 163 |
|           | nphp-4 | 0.75 ± 0.13 | 336 | <0.001 | <0.001 | 1.03 ± 0.14 | 132 |
|           | nphp-1;nphp-4 | 0.76 ± 0.15 | 472 | <0.001 | <0.001 | 1.05 ± 0.13 | 233 |
| DYF proteins | DYF-1::GFP | Wild type | 0.72 ± 0.08 | 194 | 1.18 ± 0.14 | 139 |          |
|           | nphp-1 | 0.70 ± 0.09 | 83 | 0.17 | 0.15 | 1.07 ± 0.16 | 84 |
|           | nphp-4 | 0.77 ± 0.16 | 107 | <0.001 | <0.001 | 1.06 ± 0.16 | 80 |
|           | nphp-1;nphp-4 | 0.76 ± 0.16 | 167 | <0.001 | <0.001 | 1.04 ± 0.16 | 130 |
|           | BBS-7::GFP | Wild type | 0.68 ± 0.08 | 23 | 1.14 ± 0.16 | 56 |          |
|           | nphp-1 | 0.70 ± 0.07 | 110 | 0.19 | 0.63 | 1.15 ± 0.16 | 148 |
|           | nphp-4 | 0.82 ± 0.19 | 100 | <0.001 | <0.001 | 1.01 ± 0.23 | 56 |
|           | nphp-1;nphp-4 | 0.78 ± 0.17 | 66 | <0.001 | <0.001 | 1.02 ± 0.14 | 31 |

IFT velocities in wild type and nephrocystin mutants. The velocities of many IFT components are altered in nphp-4 and nphp-1;nphp-4 but not nphp-1 animals. Distal segment velocities were similar to the wild type except for the slow OSM-6::GFP rates. Mean difference was shown statistically by a t test, whereas statistical variance difference was shown by an analysis of variance test F test.

IFT velocities in wild type and nephrocystin mutants. The velocities of many IFT components are altered in nphp-4 and nphp-1;nphp-4 but not nphp-1 animals. Distal segment velocities were similar to the wild type except for the slow OSM-6::GFP rates. Mean difference was shown statistically by a t test, whereas statistical variance difference was shown by an analysis of variance test F test.
Overexpression of certain elements of the IFT machinery is toxic to nephrocystin-deficient cilia

Lipophilic fluorescent dye filling of amphid and phasmid neurons is routinely used to diagnose defects in ciliary structure (Hedgecock et al., 1985; Inglis et al., 2006). IFT-A, IFT-B, and osm-3 mutants are all Dyf (Fig. 7 c; Perkins et al., 1986). In contrast, kinesin-II mutants are non-Dyf (Fig. 7 b) and have subtle effects on cilium formation and function but obviously affect ciliary transport at the molecular level (Snow et al., 2004; Ou et al., 2005; Bae et al., 2006; Mukhopadhyay et al., 2007). Likewise, nphp-1 and nphp-4 single and double mutants have no obvious dye filling defects (Jauregui and Barr, 2005; Winkelbauer et al., 2005) but subtly affect cilia formation at the ultrastructural and molecular levels (this paper). It was puzzling that nphp-4 mutants perturbed the IFT machinery and yet had no overt dye filling defects. We therefore reexamined dye filling in nphp-4 mutants using a more sensitive assay with reduced Dil concentration and shorter incubation time and observed partial Dyf defects (Fig. 7, d and f).

Because OSM-6::GFP exhibited abnormal motility rates in middle segments and was largely absent from distal segment of nphp-4 mutants, we examined ciliary integrity of these strains via Dil labeling. In the absence of the OSM-6::GFP array, nphp-4 single and nphp-4;kap-1 double mutants were weakly Dyf (Fig. 7, d and e). To our surprise, the OSM-6::GFP array produced a strong Dyf phenotype in combination with the nphp-4 mutation (Fig. 7, j–l). In the nphp-4 background, we also observed more severe Dyf defects with BBS-7::GFP and very weak enhancement of Dyf defects with BBS-8::GFP and OSM-3::GFP. In contrast, properly localized IFT components (OSM-5::GFP and KAP-1::GFP) do not affect ciliogenesis in the nphp-4 background (unpublished data). This synthetic Dyf (SynDyf) defect was only observed in nphp-4 mutants, as OSM-6::GFP, BBS-7::GFP, BBS-8::GFP, and OSM-3::GFP are not reported to enhance ciliogenic defects in other genetic backgrounds (Collet et al., 1998; Snow et al., 2004; Ou et al., 2005, 2007). We propose that the nephrocystins functionally interact with OSM-6 and select IFT components at the TZ. When NPHP-1 and NPHP-4 are missing from the TZ, overexpression of certain elements of the IFT machinery is toxic to cilia (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200707090/DC1).

nphp-4 mutants have B tubule defects in amphid channel cilia

To correlate molecular and biophysical defects in IFT at the ultrastructural level, we used TEM to examine nphp-4 amphid
channel cilia (Fig. 8). Wild-type and nphp-4 TZs appeared similar, with the characteristic 9 + 0 microtubule doublet pattern drawn together by an apical ring and attached to the membrane by Y links (Fig. 8, c and f). Wild-type amphid channel middle and distal segments consist of nine doublet (A tubule and B tubule; Fig. 8 j) and nine singlet (A tubule only) microtubules, respectively. In nphp-4 middle segments, one or two doublet microtubules were occasionally missing entirely or deformed and more often prematurely reduced to singlets (Fig. 8 e). Some middle segments possessed abnormal microtubule singlets lacking the B tubule (Fig. 8, e and i, arrowhead), abnormal doublets with a truncated B tubule, or open B tubules (Fig. 8 i, arrows). In both wild-type and nphp-4 animals, ADL amphid cilia typically showed inner seam openings of all or most B tubules along most of the middle segment (Fig. 8, g–i). Openings at an outer seam of the B tubule were very rare, as were lateral openings of the B tubule (unpublished data). In the wild type, most surrounding amphid cilia showed few or no open B tubules, except for ASI, where more limited inner seam breaks occurred with regularity over short portions.

In nphp-4 mutant amphid channel cilia, B tubule inner seam breaks were increased in number and in linear dimension (compare Fig. 8, k–m, to the wild type in Fig. 8 j). In nphp-4 animals, ADL cilia still showed the highest degree of inner seam breaks (Fig. 8, g and m), and ASI seam breaks were more common and extended over much of the middle segment (Fig. 8 k). ASI is one of the two neurons that does not fill with Dil in nphp-4 animals (Fig. 7 f). In nphp-4 mutants, smaller, limited inner seam breaks also occurred in ASJ (Fig. 8, g and l) and to a lesser extent in ASG, ASH, and ASK cilia (Fig. 8 i). In affected cilia other than ADL and ASI, individual microtubules generally showed small seam breaks at random along the middle segment, with two to five microtubules interrupted per ciliary axoneme, often not at the same level. Occasionally, one middle segment was missing entirely, with 9 rather than 10 cilia in the amphid channel (Fig. 8 e). Because true serial thin sections along the entire amphid channel were not collected, we cannot always determine which cilia are truncated, but some truncations involved cilia with unusual B tubule seam breaks in nearby portions of the middle segment (including ASJ and ASH). Two to four amphid distal segments were often missing, likely because of premature truncation of the distal segments of some cilia (Fig. 8 d). Intact distal segments contained normal microtubule singlets. We conclude that nphp-4 mutations do not disrupt TZ formation but do result in axonemal defects in a cell type–specific manner.

Discussion

NPHP is one of several human diseases that share the features of ciliary localized gene products and cystic kidneys (for review see Hildebrandt and Zhou, 2007). C. elegans nephrocystin-1 and nephrocystin-4 homologues have been implicated in signal transduction (Jauregui and Barr, 2005; Winkelbauer et al., 2005; Wolf et al., 2005). Here, we rule out a role for the nephrocystins in ciliary receptor localization of TRPP channel PKD-2 and the TRPV channel OSM-9 (Figs. 2 and S2). Instead, we demonstrate for the first time that nephrocystin-1 and nephrocystin-4 act globally at the TZ to modulate ciliary development and morphogenesis in a cell type–specific manner. The human nephrocystins also localize to the TZ of renal and respiratory epithelial cells and photoreceptors (Mollet et al., 2005; Otto et al., 2003, 2005; Fliegauf et al., 2006), which suggests an evolutionarily conserved function.
that NPHP-1 and NPHP-4 contribute to ciliary development. In nphp-4 middle segments, microtubule axonemal defects include missing or incomplete B tubules, which suggests that the nephrocystins may be required for A–B microtubule stability (Stephens, 2000). Interestingly, similar B tubules defects have been observed in the hennin/ar13b GTPase mutant mouse, which has defects in ciliary development and sonic hedgehog signaling (Caspary et al., 2007).

Although nphp-4 ciliary ultrastructural defects vary depending on cell type, two generalities may be drawn. First, there is variable phenotypic expressivity within a type of cilium. However, the frequency of nphp-4 CEM ciliary defects (~30%; Fig. 3) is similar to the frequency of nphp-4 amphid channel defects (4 of 10 distal segments are missing; Fig. 8). Variable phenotypic expressivity is also observed in NPHP patients. Second, nphp-4 ultrastructural defects appear not in the TZ but occur principally in the middle segment, raising the question of how the NPHP-1...
and NPHP-4 TZ proteins act. Rosenbaum and Witman (2002) have proposed that the transition fibers at the base of the *C. reinhardtii* flagellum serve as a "flagellar pore complex" to restrict the access of proteins to the cilium. At the bioinformatics level, some IFT proteins are related by sequence to components of the nuclear pore complex (Jekely and Arendt, 2006). At the ultrastructural level, transition fibers form physical links between basal body microtubules and the flagellar membrane (Ringo, 1967; Weiss et al., 1977). At the molecular level, most of the IFT particles accumulate at the basal body (Cole et al., 1998; Pazour et al., 1999). The *C. elegans* ciliary TZ also possesses links from microtubules to the membrane and accumulates IFT motors and polypeptides (Figs. 4 e and 8 f; Perkins et al., 1986). Unlike IFT components that localize to the TZ and ciliary compartment, the *C. elegans* nephrocystins localize to the TZ and are not detected in the cillum proper (Fig. 1; Jauregui and Barr, 2005; Winkelbauer et al., 2005). Notably, immunoelectron microscopy suggests that IFT52 (homologous to *C. elegans* OSM-6) associates at the periphery of transition fibers, leading to the proposal that this region serves as an IFT docking site (Deane et al., 2001). Our data suggests that NPHP-4 and OSM-6 functionally interact to regulate ciliogenesis (Figs. 7 and S4).

In amphid channel neurons, *nphp-4* mutations (lacking both NPHP-1 and NPHP-4 at the TZ) increased B tubule breaks in the middle segment, which in turn likely affected IFT velocity distributions (Table I, f-test column). We propose that NPHP-1 and NPHP-4 may indirectly regulate the balance between the IFT motors, control the composition of individual IFT particles with a cilium, and/or modulate IFT in a cell type–specific manner that is masked by an average taken from 10 axonemes. In this respect, it is notable that a minority of cilia within the amphid bundle exhibit different assembly patterns even in the wild type, including a pronounced weakness at the inner seam junction between A and B tubules. These structural weaknesses become more general in the *nphp-4* mutant cilia, now affecting the majority of all cilia.

Surprisingly, IFT-B polypeptide OSM-6::GFP moved at the slow kinesin-II rate in *nphp-4* and *nphp-1;nphp-4* middle segments and produced the SynDyf phenotype, reflecting a severe defect in ciliogenesis. The former result is not predicted by the current IFT model, whereby the kinesin-II/IFT-A and OSM-3/IFT-B units are linked by the BBS proteins (Ou et al., 2005). With the exception of OSM-6/IFT52 and the GTPase IFT27, IFT polypeptides contain WD40, TRP, or coiled-coil protein–protein interaction domains (Jekely and Arendt 2006). OSM-6 contains a GIFT domain, for which a sugar-binding role is proposed (Beatson and Ponting, 2004), and a proline-rich region, which is predicted to bind SH3 domains (Collet et al., 1998). Interestingly, NPHP-1 contains an SH3 domain (Jauregui and Barr 2005; Wolf et al., 2005). Given that mammalian nephrocystin-1 and nephrocystin-4 physically associate (Mollet et al., 2002), it is tempting to speculate that they form a ternary complex with OSM-6/IFT52 at the TZ.

**Cilia length control and the nephrocystins**

The misshapen and long CEM cilia of *nphp-1* and *nphp-4* mutants are unique. The nature of this defect is not known, nor do we know the reason for the higher frequency of misshaped cilia in *nphp-1* animals. Marshall et al. (2005) have proposed that ciliary length is controlled by a balance between ciliary assembly and disassembly and that disrupting this balance could result in long, short, or no cilia (Wemmer and Marshall, 2007). Human nephrocystin interacts with nephrocystin-2/inversin, a protein that binds to β-tubulin (Otto et al., 2003; Nurnberger et al., 2004), and nephrocystin-3, a protein that contains a tubulin tyrosine ligase (TTL) domain (Olbrich et al., 2003). TTLs and other tubulin modification enzymes catalyze tubulin posttranslational modifications, which regulate axonemal microtubules and perhaps IFT (Janke et al., 2005; Redeker et al., 2005; Pathak et al., 2007; Stephan et al., 2007). In *C. elegans*, the ODR-3 Gα protein, kinesin-II, and the mitogen-activated protein kinase DYP-5 regulate ciliary morphogenesis and length in a cell type–specific manner (Roayaie et al., 1998; Bae et al., 2006; Evans et al., 2006; Burghoorn et al., 2007). In mammalian cells, overexpression or constitutive activation of the Rab8 GDP/GTP exchange factor results in abnormally long cilia (Nachtury et al., 2007; Yoshimura et al., 2007). In Bbs4<sup>−/−</sup> mice, primary cilia length is also increased (Mokrzan et al., 2007). The NIMA (never in mitosis A) kinase nekl8 is mutated in human NPHP9 patients and the juvenile cystic kidney (*jck*) mouse model, which has primary cilia that are significantly longer than in the wild type (Smith et al., 2006; Otto et al., 2008). Mislocalization or misregulation of these types of proteins may cause defects in ciliary length or shape in nephrocystin mutants (Fig. S4).

**Genetic modifiers in cilia development, maintenance, and function**

Loss of the nephrocystins may sensitize cells to ciliogenic defects, explaining why overexpression of some IFT components in the absence of *nphp-4* increases ciliary defects (Fig. 7, j–l; and Fig. S4). Genetic and functional redundancy is a mechanism to ensure normal development and protect against disease. Intriguingly, genetic modifier loci may account for extrarenal symptoms associated with NPHP (Paris et al., 2004; Tory et al., 2007), and oligoencephaly is observed in some NPHP patients (Hoefele et al., 2007). In the *pcy*NPHP3 mouse, genetic background affects the cystic phenotype (Guay-Woodford, 2003). In humans, NPHP has clinical phenotypes that overlap Joubert and Meckel syndromes (Delous et al., 2007).

Biochemical approaches have been used to study the physical interactions of IFT components (Cole et al., 1998; Cole, 2002; Mollet et al., 2002). In *C. elegans*, endogenous IFT complexes are visualized by immunoelectron microscopy (IEM) of segmented gonadal primary cilia (Stephan and Jekely, 2007). Two-dimensional IEM data reveal that the IFT polypeptides contain WD40, TRP, or coiled-coil protein–protein interaction domains (Jekely and Arendt 2006). OSM-6 contains a GIFT domain, for which a sugar-binding role is proposed (Beatson and Ponting, 2004), and a proline-rich region, which is predicted to bind SH3 domains (Collet et al., 1998). Interestingly, NPHP-1 contains an SH3 domain (Jauregui and Barr 2005; Wolf et al., 2005). Given that mammalian nephrocystin-1 and nephrocystin-4 physically associate (Mollet et al., 2002), it is tempting to speculate that they form a ternary complex with OSM-6/IFT52 at the TZ.
Materials and methods

C. elegans mutant alleles and strains

Nematodes were raised using standard conditions (Brenner, 1974). The following general strains were used. CB1490: him-5(e1490); PT1283: pha-1(e2123) and kap-1(ok676); LGII: nphp-4(tm925); bbs-7(ok1351) and mls17[OSM-6::GFP;unc-36(+)]. The following transgenic strains were used. CB1490: him-5(e1490); PT1283: pha-1(e2123) and kap-1(ok676); LGII: nphp-4(tm925); bbs-7(ok1351) and mls17[OSM-6::GFP;unc-36(+)]. The him-5(e1490) allele was crossed into all strains to increase the frequency of males and represents the wild type in all assays. Some strains were obtained from the Caenorhabditis Genetics Center and the Japanese Bioresource Project for the Nematode C. elegans. The mls17[OSM-6::GFP] reporter strain was provided by R. Herman and J. Collet (University of Minnesota, Minneapolis, MN) and some IFT fluorescent reporter strains were provided by J. Scholty and G. Ou (University of California, Davis, Davis, CA).

The following general strains were used. CB1490: him-5(e1490); PT9: pkd-2(ok500); him-5(e1490); PT10136: nphp-4(tm925); him-5(e1490); and PT1040: nphp-4(tm925); him-5(e1490).

The following transgenic strains were used. PT1283: pha-1(e2123); him-5(e1490); myx552[PKD-2::GFP;pBX]; PT1247: pha-1(e2123); him-5(e1490); myx552[PKD-2::GFP]; PT11018: nphp-4(tm925); him-5(e1490); PT11038: nphp-4(tm925); him-5(e1490); and PT1040: nphp-4(tm925); him-5(e1490).

Fluorescent reporters

GFP or tandem dimmer Tomato tdTomato, obtained from R. Tsien, University of California, San Diego, La Jolla, CA, was used as a reporter for all expression constructs. Ppkd2::NPHP-1::GFP [pA16] and Ppkd2::NPHP-4::GFP [pA17] was generated by PCR addition of Spel and KpnI restriction sites to nphp-1 and nphp-4 cDNAs followed by cloning into pPD95.75 containing the 1.3 kb p2 promoter (pKKS). Pkk2::ibb-4::GFP has been previously described (Baë et al., 2006). To generate Pkk2::ibb-4::tdTomato, tdTomato was PCR modified to add أجل and EcoRI restriction sites such that tdTomato replaces the GFP insert of Pkk2::ibb-4::GFP to create Pkk2::ibb-4::tdTomato [pA18].

Dye-filling assay

Standard dye-filling assays were performed (Perkins et al., 1985). Alternatively, reduced dye concentration and incubation time were used. A 2 mg/ml DiD or DiI (Invitrogen) in dimethyl formamide stock was diluted 1:1,000 in M9. Worms were incubated for 30 min in this solution and washed three times with M9.

Microscopy

Animals were raised at 20°C and imaged at 25°C using standard C. elegans slide mounts and Plan Apochromats 63x 1.4 oil differential interference contrast (DIC), 100x 1.3 oil DIC, or 100x 1.4 oil DIC objectives (Carl Zeiss, Inc.) on an imaging microscope (Axioplan 2, Carl Zeiss, Inc.). Fluorescent images were captured under a epifluorescence microscope using a Plan APOCHROMA 100 × 1.3 or 1.4 NA oil objective (Carl Zeiss, Inc.). Motility stacks were recorded using a camera (Photometrics Cascade 512B, Molecular Scientific, Inc.) and the particle movement rate was measured using Metamorph software (MDS Analytical Technologies) and the length was recorded and averaged.

IFT motility was observed with an Axioplan 2 imaging microscope using a Plan APOCHROMA 100x 1.3 or 1.4 NA oil objective (Carl Zeiss, Inc.). Motility stacks were recorded using a camera (Photometrics Cascade 512B, Molecular Scientific, Inc.) and the particle movement rate was measured using Metamorph software. Strains were synchronized by picking L4 worms, culturing at 15°C overnight, and imaging within 24 h. Worms were anesthetized in a drop of M9 containing 10 mM mesculin, transferred to an agarose mount slide, and imaged immediately.

“Dim in cilia” is defined as significantly reduced fluorescence of IFT::GFP reporters in cilia compared with the wild type (Fig. S3). For mutants with “dim cilia,” threshold adjustments were increased to visualize cilia. Additionally, “dim distal” is defined as amphi distal segment that have significantly reduced fluorescence of IFT::GFP reporters when compared with adjacent middle segments. The basis of the “dim” phenotype is likely a combination of reduced number of distal segments in the amphi channel and reduced frequency of IFT particles entering the distal segment.
overnight at 4°C. Animals were rinsed in buffer and stained with 1% osmium tetroxide in cacodylate buffer for 1 hr at 4°C. After embedding in small groups in agarose, the specimens were en bloc stained with 1% uranyl acetate in sodium acetate buffer, dehydrated, and embedded in Em- bed®812 resin according to the general procedures described by Hall (1995). Thin sections were collected on a diamond knife and post-stained before being viewed on a CM10 electron microscope (Philips; Hall, 1995).

Details in wild-type amphid cilia microtubule architecture were reconfirmed in additional adult animals fixed by high-pressure freezing followed by freeze substitution (Rostaghi et al., 2004). This analysis (not depicted) reconfirmed the strong tendency of ADL and ASI cilia to suffer inner seam breaks of the B tube, eliminating any chance that the microtubule defects were a fixation artifact.

Software for image processing with operations involved AutoDeblur 1.4.1 (Media Cybernetics, Inc.), which was used only for 3D blind deconvolution, and Photoshop (Adobe), which was used for image rotation, cropping, brightness/contrast, and filter “unsharp mask” for TEM and for generation of all figures.

Online supplemental material

Fig. S1 shows that CEM cilia are curled or stunted in nephrocystin mutant C. elegans. Fig. S2 shows that OLQ cilia are misshapen in nephrocystin mutants. Fig. S3 shows that OSM-5:GFP is dim in nphp-4 amphid and phasmid cilia. Fig. S4 shows a model for NPHP-1 and NPHP-4 function. Videos 1–3 show a 3D reconstruction of wild-type and nephrocystin mutant CEM cilia. Videos 4–6 show IPT of pkd-2:OSM-5;GFP in wildtype and nephrocystin mutant CEM cilia. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200707090/DC1.

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