Essential role of nephrocystin in photoreceptor intraflagellar transport in mouse

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Nephrocystin mutations account for the vast majority of juvenile nephronophthisis, the most common inherited cause of renal failure in children. Nephrocystin has been localized to the ciliary transition zone of epithelial cells or its analogous structure, connecting cilium of retinal photoreceptors. Thus, the retinal degeneration associated with nephronophthisis may be explained by a functional ciliary defect. However, the function of nephrocystin in cilia assembly and maintenance of common epithelial cells and photoreceptors is still obscure. Here, we used Nphp1-targeted mutant mice and transgenic mice expressing EmGFP-tagged nephrocystin to demonstrate that nephrocystin located at connecting cilium axoneme can affect the sorting mechanism and transportation efficiency of the traffic machinery between inner and outer segments of photoreceptors. This traffic machinery is now recognized as intraflagellar transport (IFT); a microtubule-based transport system consisting of motors, IFT particles and associated cargo molecules. Nephrocystin seems to control some of the IFT particle components moving along the connecting cilia so as to regulate this inter-segmental traffic. Our novel findings provide a clue to unraveling the regulatory mechanism of nephrocystin in IFT machinery.

INTRODUCTION

Familial juvenile nephronophthisis (MIM 256100) is an inherited autosomal recessive cystic kidney disease. It is the most common genetic cause of end-stage renal disease in children and young adults. Linkage analysis identified the defective gene responsible for the vast majority of patients as NPHP1 (1). The prominent histological changes that occur in the kidney as a result of the disease are tubular basement membrane thickening, interstitial fibrosis, tubular atrophy and medullary cyst formation (2). Various extrarenal manifestations including ocular motor apraxia, retinal degeneration, cerebellar vermis aplasia, mental retardation, liver fibrosis and cone-shaped epiphyses are frequently associated with nephronophthisis (3). These multi-organ complaints indicate that the protein encoded by NPHP1 has distinct roles in diverse tissue types.

Since the identification of the NPHP1 gene in 1997 many advances have been made in the search for the possible function of its gene product nephrocystin (1). Initial studies suggested that nephrocystin might be involved in focal adhesion and/or adherens junction signaling, as reviewed by Hildebrandt and Otto (4). However, later work has suggested that nephrocystin is colocalized with inversin (the NPHP2 gene product responsible for infantile nephronophthisis) in the primary cilium of renal tubular cells (5). In addition, nephrocystin is also targeted to the ciliary base of respiratory epithelial cells by a PACS-1-mediated process involving phosphorylation by casein kinase 2 (6). Recent data have further demonstrated that nephrocystin specifically localizes to the transition zone of renal and respiratory cilia as well as to the photoreceptor-connecting cilia (7). These findings have led to the proposition that nephrocystin might be involved in cilia assembly and maintenance. Thus, the multi-organ
involvement in nephronophthisis might be explained by a functional ciliary defect in various tissues.

Cilia (and flagella) are microtubule-filled, hair-like cellular projections that extend from the surface of almost all cell types in the human body (see the Primary Cilia Resource web page: http://www.global2000.net/bowser/cilia.html). Although these highly conserved structures are also present across a broad range of species, they are found nearly ubiquitously only in vertebrates. The multiple, motile cilia on the cells lining the lumens of ducts in several tissues (e.g. respiratory epithelial cells) have been familiar for decades. But most ciliated cell types have only one non-motile cillum (a primary cilium), which has become a focus of research in recent years because of its central role in the pathogenesis of polycystic kidney diseases, one of the most common inherited diseases (8,9). The basic axonemal structure of most motile cilia is nine peripheral doublet microtubules surrounding two central single microtubules (9+2 structure). In most immotile primary cilia, the two central microtubules are absent (9+0 structure). Each cilium extends from a specialized centriole called a basal body. The centriolar triplet microtubular structure converts within the transition zone into the axonemal doublet microtubular structure. While the mechanical function of motile cilia is well-known (e.g. in epithelial cells of the respiratory tract), the functional role of immotile primary cilia is currently under investigation. Recent evidence suggests that immotile primary cilia functions as sensory organelles to detect environmental cues of a mechanical and/or chemical nature as well as osmotic, photonic, hormonal or olfactory signals (10). Consistent with the ubiquitous presence and the diversified functions of primary cilia in various tissues, their defects are associated with a range of human diseases such as primary ciliary dyskinesia, hydrocephalus, polycystic liver and kidney diseases, and some forms of retinal degeneration (11).

Vertebrate photoreceptors are specialized sensory neurons consisting of a photosensitive outer segment (OS) that develops from a modified non-motile primary cilium. During development of the OS, a large amount of membrane and phototransduction proteins are transported into the distal segment of the cilium and assembled into the membranous discs. Like other cilia, the OSs contain an axoneme, which begins in the basal body and passes through a transition zone (the so-called 'connecting cilium') and into the OS (12). The connecting cilium is the only direct link between the outer and inner segments of the cell, and all lipids and proteins synthesized in the inner segment must be transported through this connecting cilium to reach their functionally active sites in the OSs. Disruption of this intersegmental transport results in photoreceptor degeneration and blindness. The transport machinery that carries the cargo is thought to be intraflagellar transport (IFT) (13). IFT was first identified in Chlamydomonas (14) and has subsequently been shown to be essential for the assembly and maintenance of many types of cilia and flagella in many organisms including mice (15). Two types of microtubule-based motors, kinesin-2 and dynein 1b, drive the anterograde and retrograde movement of IFT particles, respectively, along the ciliary axoneme. IFT particles are multi-subunit complexes of proteins that are unique to this process and function as adaptors between various motors and cargo proteins (15).

Since nephrocystin localizes at the connecting cilium of pig photoreceptors (7), and retinal degeneration is frequently associated with nephronophthisis (3), the role of nephrocystin in photoreceptor ciliogenesis and IFT warrants further exploration. In our previous study, we generated a mouse line with targeted disruption of Nphp1, and delineated its role in spermatogenesis (16). Here, we used the targeted mutants to explore the role of nephrocystin in mouse photoreceptor development. We show that nephrocystin is specifically localized to the axoneme of the connecting cilium, and can regulate photoreceptor IFT machinery. Although the axoneme of the connecting cilium was still present in the mutant photoreceptors, some phototransduction proteins destined for the OS accumulated in the inner segment leading to severe disorganization of the OS with accompanying apoptotic degeneration. In contrast, some proteins destined for the inner segment leaked to the OS. These data indicate that nephrocystin depletion does not completely abolish photoreceptor ciliogenesis and IFT, but that the transportation efficiency and sorting mechanism of IFT are affected. Our novel findings shed new light on nephrocystin function in ciliary/flagellar IFT and pathogenesis of the retinopathy associated with nephronophthisis. This model system is a valuable tool for further investigation of the regulatory mechanism of nephrocystin on IFT at the molecular level.

RESULTS

Retinal degeneration in homozygotes of Nphp1-targeted mutants

In our previous study, we generated Nphp1-targeted mutants with exon 20 deletion (16). A general histological examination of the mutant homozygotes (Nphp1<sup>+/de20</sup>) at 8 months of age revealed that the photoreceptor cells had almost disappeared in the outer retina (Fig. 1N). The mutants showed dramatic degeneration of the outer and inner segments (OS and IS) as well as of nuclei at the outer nuclear layer (ONL) of the photoreceptor cells compared with their age-matched control littermates (Fig. 1M). The heterozygotes (Nphp1<sup>+/-de20</sup>) had shown similar phenotypes as their wild-type control littermates (data not shown). To explore the progression of the retinopathy, we serially examined mouse retinas at postnatal day (p) 1, 14, 21 and 28. Mouse OSs normally begin to develop around p4, achieving adult proportions with well-aligned disc membranes by p20 (17). Histological examination of retinas seemed normal in the homozygotes at p1 and p14 (Fig. 1A, B, D and E) but after p21, the homozygote retinas had significantly shorter OS and IS and a reduced ONL (Fig. 1G, J, H and K), suggesting a rapid degeneration of photoreceptor cells before p21. In this study, we mainly delineate the eye phenotype in the Nphp1-targeted mutant cognic for C57BL/6J strain (N10F1 progeny). The targeted mutant at N2F1 or N3F1 progeny of crosses to C57BL/6J also showed severe retinal degeneration (data not shown). In addition, the targeted mutant derived from intercrossing F1 hybrid of C57BL/6J and FVB/NJ as well as of C57BL/6J and 129/Sv showed likewise, suggesting the penetrance of the eye phenotype during the crossing of the targeted allele.
Nphp1del20/del20 mice mislocalize rhodopsin and display photoreceptor apoptotic cell loss

Rhodopsin, the most prominent membrane protein of OS membranes, is synthesized in the proximal inner segments and transported to the OSs through connecting cilia. Rhodopsin immunostaining of the retinas of Nphp1del20/del20 mice before significant loss of photoreceptor cells (p14) showed rhodopsin mainly in the ISs but not in the OSs. Wild-type controls showed rhodopsin mainly in the OSs (Fig. 2A and B). The connecting cilia visualized by acetyl-tubulin immunostaining looked normal in Nphp1del20/del20 mice at this stage (Fig. 2C and D), indicating that the rhodopsin mislocalization was not caused by an axoneme assembly defect in the connecting cilium.

We examined the nature of the photoreceptor degeneration by terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay. We found that at p14 TUNEL-positive photoreceptors were rarely observed in wild-type mice (Fig. 2E), but in Nphp1del20/del20 mice a large number of apoptotic photoreceptors had emerged (Fig. 2F). The apoptotic nuclei were mainly located in the inner portion of the ONL, but the TUNEL signal was also found in the IS and in the junction zone between the OS and the retinal pigment epithelium. In contrast, the TUNEL signal was rarely observed in undifferentiated

Figure 1. Haemotoxylin and eosin-stained retinal sections of age-matched wild-type (right column), homozygous Nphp1 mutant (middle column) and rescued (right column) mice. Mice were examined at postnatal day 1 (A–C), 14 (D–F), 21 (G–I), 28 (J–L) and 240 (M–O). Some degenerative changes in IS, OS and ONL were first noticed in p21 mice and progressively worsened with time as shown by arrows (H, K and N). At 8 months of age, the ONL was almost totally degenerated and no IS and OS were observed (N). The retinopathy induced by Nphp1 disruption was fully reversed by transgenic expression of EmGFP-tagged nephrocystin-1 in the rescued mice (right column). OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; G, ganglion cell layer. Scale bars: 100 μm in D–F; 50 μm in the rest. All results are representative of at least three mice per genotype in two independent experiments.
photoreceptors at p1 and p7 of either Nphp1<sup>del20/del20</sup> or control mice (data not shown). These findings suggest that apoptosis could be initiated in differentiated photoreceptors, in which the ISs and OSs are destined to develop.

**A failure of outer segment development in Nphp1<sup>del20/del20</sup> mice**

The fine structure of the photoreceptors was further characterized by transmission electron microscope (TEM). During OS development in wild-type mice, most OS discs were well-aligned in a stacked array (see p14, Fig. 3A and B; p21, Fig. 4A); however, normal OS structure was not found in Nphp1<sup>del20/del20</sup> mice at p14 (Fig. 3C), p21 (Fig. 4B) or p28 (data not shown). Instead, a few disc stacks could be seen arranged randomly in disorganized OSs at p14 (Fig. 3D) and p21 (Fig. 4C). OS development requires the synthesis of many lipids and proteins in the IS, which are then transported to the OS through the connecting cilium. This intersegmental traffic is known as IFT—a microtubule-based transport system along the ciliary/flagellar axoneme. The axonemal structure of connectin cilium was still present in Nphp1<sup>del20/del20</sup> mice (Fig. 3E), correlating well with the acetyl-tubulin immunostaining result. Therefore, we speculate that the underlying mechanism leading to the disorganized OSs and rhodopsin mislocalization could be attributed to IFT defects but not to defects in the axonemal construction of the connecting cilium.

**Sorting mechanism and transportation efficiency of the photoreceptor intraflagellar transport are abnormal in Nphp1<sup>del20/del20</sup> mice**

To further explore why rhodopsin was not transported to the OSs in the photoreceptors of our Nphp1<sup>del20/del20</sup> mutants,
we examined the distribution of myosin VIIa and retinitis pigmentosa GTPase regulator (RPGR), which are also known to be involved in rhodopsin-trafficking through the connecting cilia apart from the IFT system (18,19). We found no obvious differences in their localization between our mutant and control photoreceptors (see Supplementary data), increasing the possibility of IFT defects in \textit{Nphp1}^{del20/del20} photoreceptors. Although we do not know the underlying mechanism for the downregulation of transducin, its transport to the OS was also disrupted in \textit{Nphp1}^{del20/del20} photoreceptors. Light-dependent translocalization of transducin through the connecting cilium is known to be regulated by a ciliary microtubule-associated protein, centrin (20). Centrin content and localization in our mutant photoreceptors was similar to control photoreceptors (see Supplementary data), indicating that the failure of transducin transport to the OS could also be caused by IFT defects.

However, photoreceptor IFT is the major transport system by which phototransduction proteins and the other proteins destined for the OSs are moved between segments. In other words, all proteins targeted to the OS are candidates for IFT particle-associated cargo proteins. To briefly examine the activity of the photoreceptor IFT in \textit{Nphp1}^{del20/del20} mice, we immunolocalized several proteins (using antibodies already available in our laboratory) in the mutant photoreceptors. IFT57 and TCP-1 were mainly delivered to and resident in the OS of control photoreceptors at p14, similar to rhodopsin and transducin (see Supplementary data). In contrast, both of these two proteins were present in both IS and OS of the mutant photoreceptors (see Supplementary data), suggesting that IFT might be attenuated but not completely abolished in the mutants, delaying the delivery of proteins still in the IS. Another possibility is that the presence of these two proteins in the IS resulted from a leakage via retrograde IFT from the OS to the IS, suggesting a defect in the sorting mechanism of retrograde IFT. In contrast, clathrin and filamin 3 were retained in the IS of the control photoreceptors (see Supplementary data), indicating that they were not delivered by anterograde IFT; they had leaked to the OSs in our mutant photoreceptors (see Supplementary data), also suggesting defects in the sorting mechanism of anterograde IFT. To briefly examine the IFT machinery of the \textit{Nphp1}^{del20/del20} photoreceptors, we observed the distribution of anterograde IFT motors and IFT particles by immunohistochemistry at p14. Heterotrimeric kinesin-2 was first identified as one of the anterograde IFT motors, and conditional knockout of KIF3A, a subunit of kinesin-2, also causes rhodopsin mislocalization leading to retinal apoptotic degeneration (21). KIF3A was found to be distributed symmetrically along the connecting cilia in both the mutant and wild-type control photoreceptors (Fig. 5), suggesting that the IFT motor function in \textit{Nphp1}^{del20/del20} photoreceptors seems to be unimpaired. IFT particles are protein complexes that bridge IFT cargoes and motors. One of the IFT particle subunits, IFT88, is the ortholog of mouse Tg737, and a Tg737 hypomorphic mutant also exhibits rhodopsin mislocalization (22). In wild-type

\textbf{Figure 4.} Lack of well-organized OS in \textit{Nphp1}-targeted mutants at p21, and the rescued phenotype in the mutants with transgenic expression of EmGFP-tagged nephrocystin at p21. (A) An electron microscope image of typical well-organized OSs, ISs and ONL from wild-type mice. (B and C) ISs and ONL are obviously shrunk, and well-organized OSs are lacking in \textit{Nphp1}-targeted mutants. Severely disorganized OSs are seldom observed (arrows). The nuclear condensation of apoptotic photoreceptor is found (asterisks in B). (D) The retinopathy of the \textit{Nphp1}-targeted mutants is fully reversed in the rescued mice. Scale bars: 5 \(\mu m\) in (A, B and D); 2 \(\mu m\) in (C).
control photoreceptors, IFT88 was concentrated primarily at the base of the cilia (Fig. 5), where molecules targeted to the OS are loaded and then transported via IFT machinery. It is worth noting that only a few IFT88 signals were found along the connecting cilia of control photoreceptors. When compared with the symmetrical KIF3A signals along the connecting cilia, this finding suggests that only a few but not all IFT motors carry IFT88 and associated cargo proteins through the connecting cilia; the composition of each IFT particle complex carried by IFT motors is diverse. In Nphp1<sup>del20/del20</sup> photoreceptors IFT88 were evenly distributed at the ciliary base and the axoneme, indicating that the IFT particle complexes containing IFT88 were more abundant at the connecting cilia when compared with wild-type control (Fig. 5). Accumulation of IFT88 on the connecting cilia was also observed with another IFT particle—WDR19 (Fig. 6), whose Drosophila ortholog, OSEG6, was first characterized as a member of a novel family of proteins (OSEGs: OS) essential for ciliogenesis (23). The distribution of another mouse ortholog of OSEG1, IFT122, in Nphp1<sup>del20/del20</sup> photoreceptors was similar to the wild-type control, all concentrated at the ciliary base (Fig. 7). These findings along with the accumulation of rhodopsin in the IS of Nphp1<sup>del20/del20</sup> photoreceptors demonstrate an important aspect of nephrocystin function: nephrocystin may facilitate the movement of IFT particle complexes containing IFT88 and/or WDR19 but not IFT122 along the connecting cilia. Depletion of nephrocystin would slow the movement of these affected IFT particle complexes on the connecting cilia, leading to the situation observed in the Nphp1<sup>del20/del20</sup> photoreceptors.

**Rescue of retinopathy in Nphp1<sup>del20/del20</sup> mice by transgenic expression of EmGFP-tagged nephrocystin**

To verify that retinopathy in Nphp1<sup>del20/del20</sup> mice was caused by nephrocystin depletion, we generated transgenic mouse lines ubi-
quitously expressing EmGFP-tagged nephrocystin under the control of human ubiquitin B promoter. Nephrocystin localization was traced by the EmGFP tag under epifluorescence microscopy. The transgenic mice, Tg (Nphp1-EmGFP), were crossed with Nphp1 heterozygous mutants (Nphp1+/del20) to breed double transgenic mice (Nphp1-EmGFP/Tg/Nphp1+/del20). The double transgenic mice were then backcrossed with Nphp1+/del20 mice to generate homozygous (Nphp1del20/del20), rescued (Nphp1-EmGFP/Tg/Nphp1del20/del20) and wild-type (Nphp1+/+) mice for analysis. The transgene expression in the retina of these mice was analyzed by western blot, as shown in Figure 7B. An immunoreactive band of approximately 115 kDa was detected only in rescued mice by either GFP or nephrocystin antibodies, demonstrating that EmGFP-tagged nephrocystin (NC1-EmGFP) was expressed. The endogenous nephrocystin (NC1) was totally absent in Nphp1 homozygotes and rescued mice (Fig. 7B, lower panel). Both histological examinations (Fig. 1, right column) and TEM analysis (Figs 3F and G and 4D) showed that the retinopathy induced by Nphp1 targeting was fully reversed in the rescued mutants. The localization of the EmGFP-tagged nephrocystin in the rescued mouse retina was further examined by epifluorescence microscopy. The EmGFP signals appeared highly concentrated as separate bar-like structures distributed at the boundary between the IS and OS layers where the connecting cilia are located (Fig. 7C).

**Localization of EmGFP-tagged nephrocystin to the axoneme of connecting cilia in rescued mutants**

To further characterize the localization of EmGFP-tagged nephrocystin in rescued mouse retina, we immunolabeled the retinal cryosections with antibodies specific to γ-tubulin and Bbs2, that are known to target the ciliary proximal end and basal body, respectively (24). The EmGFP-tagged
nephrocystin appeared separated but adjoined to these two markers, and extended distally beyond them (Fig. 8A–F). In contrast, immunolabeling of the distal end of connecting cilium by IFT88 demonstrated the junction between EmGFP-tagged nephrocystin and IFT88 (Fig. 8G–I) (22). Its worth nothing that the localization of IFT88 in the well-developed photoreceptors as shown in Figure 5. In the developing photoreceptors, a relatively high abundance of newly synthesized proteins destined for the OSs is waiting to be transported through the connecting cilium by IFT machinery. In this regard, the turn-around and recycling of IFT88 responsible for the anterograde IFT machinery should be very rapid resulting to concentrate at the base of the predicted molecular weight. The endogenous nephrocystin was absent in the targeted mutants and the rescued mutants. (C) Localization of EmGFP-tagged nephrocystin in photoreceptors of the rescued mutants at p60 by direct EmGFP fluorescence imaging (green). DAPI was used to stain the nuclei (blue). RPE, retinal pigment epithelium; OS, outer segment; IS, inner segment; ONL, outer nuclear layer. Scale bars: 20 μm.

DISCUSSION

The connecting cilium is the only direct link between the ISs and OSs of photoreceptors. All components that are necessary for assembly, maintenance and continuous turnover of the OS are synthesized in the cell body and are moved through the connecting cilium by IFT. IFT is an intracellular motility system first described in Chlamydomonas. It has subsequently been found to be essential for the assembly of motile and sensory cilia in many organisms including mice (25). Large protein complexes, called IFT particles, are bidirectionally transported between the flagellar membrane and the outer doublet microtubules of cilia and flagella. The IFT particles are not components of the axoneme or flagellar membrane but are still an essential part of the IFT machinery. Significant information about the biological properties of IFT particles was obtained from the studies of motile flagella in Chlamydomonas and of sensory cilia in Caenorhabditis elegans. Biochemically defined in these model systems, IFT particle proteins fall into two different complexes based on ciliary phenotypes of their mutants (15). Complex B mutants typically show severely reduced cilia where other IFT particle components fail to enter and migrate from the base to the distal tip of the cilium, but rather they accumulate at transition zones, supporting a role of IFT-B components in anterograde transport. Cilia of IFT-A mutants are only slightly stunted and show a bulb-like structure at the distal tip with massive accumulation of IFT particle proteins along the axoneme, indicating the importance of IFT-A for retrograde transport (15). In mice, targeted disruption of IFT88, one subunit of the IFT complex B disrupted IFT and caused ectopic rhodopsin accumulation leading to severe retinal degeneration (22). In Nphp1^{pde20/del20} photoreceptors accumulation of IFT88 along the connecting cilium was noted indicating sluggish movement of the IFT complex B and associated cargo proteins, e.g. rhodopsin. This implies that the underlying mechanism of the retinopathy in the Nphp1-targeted mutants could be an effect on IFT particles. In addition to IFT88, the distribution of another component of IFT complex B, WDR19, was also shown to accumulate along the connecting cilia in Nphp1^{pde20/del20} photoreceptors. These phenotypes are somewhat like the IFT-B mutants mentioned above. In addition, the distribution of one component of IFT complex A, IFT122, was normal in Nphp1^{pde20/del20} photoreceptors. These results indicate that nephrocystin may influence the anterograde transport of IFT. To further verify this hypothesis, extensive examination of the distribution of the other known IFT particles is necessary. We will try to perform this investigation when suitable antibodies are available in the future.

Secondary structure predictions of the IFT particle proteins provided some interesting clues to IFT function. For example, many components contain TPR and WD40 repeats. TPR motifs occurring as 3–16 tandem repeats per protein are packed in a parallel manner and form a superhelical structure for interaction with a diverse range of target proteins. In IFT88, there are three closely spaced TPR repeats in the
N-terminal half of the protein and another seven TPR repeats in the C-terminal half of the protein (22). These two separate TPR domains might be involved in intersubunit interactions with the other IFT proteins or in the attachment of cargo to the IFT particle. WD40-containing proteins are to fold into a β-propeller structure and coordinate multiprotein complex assemblies. Notably, the N-terminal WD40 domains of COPI and clathrin have been implicated in cargo recognition and sorting along the endocytic or secretory pathways (26). The identification of some IFT proteins, including IFT80, IFT122, IFT140, IFT172, WDR19 and WDR35, with distinct N-terminal WD40 domains accompanied by various numbers of TPR repeats may provide the structural basis for selective cargo recognition within IFT machinery (23). They may play distinct roles, and different cargos are likely to be matched to specific IFT proteins. In this study, we found that the sorting mechanism and transportation efficiency of the photoreceptor IFT were hampered in \( Nphp1^{del20/del20} \) mice. The accumulation of IFT88- and/or WDR19-containing IFT complexes along the connecting cilium or the connecting cilium, respectively. The red/green merged images are shown in the right panel (C, F, I and L). The colocalization of these immunolabeled markers (red) with EmGFP-tagged nephrocystin (Green) indicates yellow. OS, outer segment; IS, inner segment. Scale bars: 5 μm.

Figure 8. Colocalization of EmGFP-tagged nephrocystin and acetylated α-tubulin to the photoreceptor connecting cilia of the rescued mutants at p60. Localization of EmGFP-tagged nephrocystin in the photoreceptor cryosection by direct EmGFP fluorescence imaging (green) is shown in (A, D, G and J). Cryosections were double-immunolabeled with anti-Bbs2 (red in B), anti-γ-tubulin (red in E), anti-IFT88 (red in H) or anti-acetylated α-tubulin (red in K) to reveal the position of basal body, microtubule organization center, distal end of connecting cilium or the connecting cilium, respectively. The red/green merged images are shown in the right panel (C, F, I and L). The colocalization of these immunolabeled markers (red) with EmGFP-tagged nephrocystin (Green) indicates yellow.
particles are accumulated at the start- and/or end-points of the IFT journey. Here we found that nephrocystin is also localized in the connecting cilium but is not concentrated at the ends as the IFT motors and particles are. Nephrocystin, instead, appears with a gradient distribution concentrated more at proximal site but still separate from the most proximal end. Because of this observation, we propose that nephrocystin is not a component of the IFT motors or particles. In addition, nephrocystin was shown to interact and to colocalize with β-tubulin in the primary cilia of renal tubular epithelial cells (5). Therefore, the nephrocystin in the connecting cilium might be directly associated with the axonemal tubulin. The results of immunogold electron microscopy also support this conclusion.

The intracellular motility machinery of kinesin- and dynein-mediated transportations in microtubule is well-documented. This machinery seems to be implicated in IFT with some modifications and has been under extensive investigation in recent years. The interaction between microtubule and microtubule-dependent motor proteins in general motility machinery has been investigated at the molecular level for decades. The corresponding interaction in IFT is still poorly understood although some specific motors and IFT particles have been identified. There is still much to learn about how the activity of these motors is regulated so as to generate the coherent pathways of bidirectional IFT. In addition, it is difficult to explain how over 200 individual components of cilia and flagella can all be recognized and carried by the IFT. In general, the sorting mechanism is thought to be mediated through vesicle trafficking at the trans-Golgi network (TGN) similar to the protein sorting for creation and maintenance of apical–basal polarity in epithelial cells. Recently, a TGN-resident protein, FAPP2, has been proved to be essential for ciliogenesis but not for apical–basal polarity of epithelial cells (28), suggesting that a cilium-specific sorting machinery is responsible for ciliogenesis. However, this sorting mechanism remains to be a poorly understood aspect of IFT. Here we demonstrated the essential role of nephrocystin in regulating IFT activity and sorting in photoreceptors; we do not, however, know whether these findings are applicable to IFT machinery in the other cell types. Interestingly, nephrocystin dock at the microtubular track of the connecting cilia rather than the TGN, strongly suggesting that IFT sorting could also operate at ciliary transition zone. Our findings provide a new insight into the IFT machinery, which has been spotlighted in recent years because of the myriad of disorders including renal cystic diseases, retinitis pigmentosa and Bardet–Biedl syndrome associated with cilia (11). Deciphering the molecular mechanism of IFT is crucial to understanding the pathogenesis and developing therapeutic strategies for these diseases.

MATERIALS AND METHODS

**Nphp1-targeted mutant mouse**

*Nphp1*-targeted mutants were established as described in our previous study (16). Briefly, exon 20 of *Nphp1* was deleted, and the heterozygotes (*Nphp1<sup>+/−</sup>del20<sup>β</sup>) were backcrossed with C57BL/6J mice for a further nine generations (N10 progeny). Heterozygotes (*Nphp1<sup>−/−</sup>del20<sup>β</sup>β*), heterozygotes (*Nphp1<sup>+/−</sup>del20<sup>β</sup>) and wild-type control littermates (*Nphp1<sup>+/−</sup>*) analyzed in this study were generated by intercrossing between congeneric heterozygotes. The *Nphp1* genotypes were determined by PCR and/or Southern blot analysis of tail genomic DNA as described previously (16). Most of the phenotypic analyses were performed with the clone no. 472-derived mouse line, and identical results were also obtained with the clone no. 483-derived line.

**Generation of transgenic mice expressing EmGFP-tagged nephrocystin**

The transgene construct is depicted in Figure 7A. A 1.1 kb human ubiquitin B promoter was digested from pDRIVE-hubib (Invitrogen, CA, USA) and subcloned into pBluescript (Stratagene, TX, USA). The bovine growth hormone polyadenylation sequence (bGHpA) was digested from pcDNA3.1 (Invitrogen) and subcloned behind the ubiquitin B promoter in pBluescript. The chicken β-globin insulator (a gift from Dr Julie Wallace, National Institute of Health) was digested from pJC13-1 and subcloned behind bGHpA. The full-length cDNA of *Nphp1* was amplified from an IMAGE clone 6447851 (Invitrogen) by PCR cloning, and the stop codon was deleted, Nphp1<sup>−/−</sup>del20<sup>β</sup>β<sup>−</sup>) were backcrossed with C57BL/6J mice for a further nine generations (N10 progeny). Heterozygotes (*Nphp1<sup>−/−</sup>del20<sup>β</sup>β*), heterozygotes (*Nphp1<sup>+/−</sup>del20<sup>β</sup>) and wild-type control littermates (*Nphp1<sup>+/−</sup>*) analyzed in this study were generated by intercrossing between congeneric heterozygotes. The *Nphp1* genotypes were determined by PCR and/or Southern blot analysis of tail genomic DNA as described previously (16). Most of the phenotypic analyses were performed with the clone no. 472-derived mouse line, and identical results were also obtained with the clone no. 483-derived line.

**Figure 9.** Immunoelectron microscopic localization of EmGFP-tagged nephrocystin in the connecting cilium of the rescued mutant photoreceptors. (A and B) Immunogold labeling of EmGFP-tagged nephrocystin in longitudinal sections of photoreceptors from the rescued mutants. EmGFP-tagged nephrocystin labeling was mainly localized to the axonemal microtubule doublets of the connecting cilium (arrowheads). Scale bar: 0.2 μm.
(lines 2 and 5) fluoresced green. The transgene expression pattern and intensity of these two lines were similar, and both could rescue the retinal and testicular defects caused by Nphp1 disruption. Most of the data shown in this study were derived from the line 2 transgenic founder.

**Histology and immunofluorescence studies**

For histological analysis, specimens were fixed in formalin, embedded in paraffin, sectioned at 4 μm and stained with haematoxylin and eosin (H&E) for light microscopic examination. For immunofluorescence staining procedures, the frozen sections were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), and then were rinsed in PBS. Non-specific binding sites were blocked with 2% bovine serum albumin (BSA), followed by incubation with an appropriately diluted primary antibody. After rinsing in PBS, samples were incubated in secondary antibodies conjugated with Alexa Fluor® 555 (Invitrogen). Samples were further rinsed in PBS, mounted in an antifade reagent with 4',6-Diamidino-2-phenylindole (Invitrogen) and observed with a Zeiss microscope with epifluorescence, and photographed in a Zeiss LSM-510 laser-scanning confocal imaging system.

**Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay**

For each genotype, 4-μm-thick sections of formalin-fixed eyes were deparaffinized and hydrated in graded alcohols. The staining procedure followed the instruction manual of the ApopTag Plus Peroxidase in situ Apoptosis Detection Kit (Chemicon, CA, USA).

**Electron microscopy analysis**

Eyes from the homozygotes (Nphp1<sup>del20/del20</sup>) and control littermates (Nphp1<sup>+/+</sup>) were fixed at 4°C with 4% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, post-fixed with 1% OsO<sub>4</sub> in cacodylate buffer, dehydrated in an ethanol series, equilibrated in propylene oxide and then embedded in epoxy resin. Thin sections were stained with uranyl acetate and lead citrate and examined in a Tecnai G2 Spirit TWIN electron microscope (FEI Company, the Netherlands). For immunoelectron microscopy, eyes from rescued mutants were fixed overnight in 4% paraformaldehyde, 0.1 M sodium phosphate buffer, pH 7.0. Fixed eyes were dehydrated to 98% ethanol, embedded in LR White resin. Ultrathin sections were cut and incubated overnight with a rabbit anti-GFP polyclonal antibody (Clontech Laboratories, Inc.) diluted 1:50 in 1% BSA. 0.1 M Tris buffer, pH 7.4, and labeled for 1 h with anti-rabbit Ig-gold (12 nm; Jackson ImmunoResearch) diluted 1:20 in 0.1 M Tris buffer, pH 7.4, 1% BSA. The sections were then washed in 0.1 M Tris buffer and stained with 2% ethanolic uranyl acetate for 10 min before examination.

**Antibodies**

Rabbit antibodies to nephrocystin and KIF3A, and a mouse monoclonal antibody against acetylated tubulin were pur-