NPHP4 controls ciliary trafficking of membrane proteins and large soluble proteins at the transition zone

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ABSTRACT
The protein nephrocystin-4 (NPHP4) is widespread in ciliated organisms, and defects in NPHP4 cause nephronophthisis and blindness in humans. To learn more about the function of NPHP4, we have studied it in Chlamydomonas reinhardtii. NPHP4 is stably incorporated into the distal part of the flagellar transition zone, close to the membrane and distal to CEP290, another transition zone protein. Therefore, these two proteins, which are incorporated into the transition zone independently of each other, define different domains of the transition zone. An nphp4-null mutant forms flagella with nearly normal length, ultrastructure and intraflagellar transport. When fractions from isolated wild-type and nphp4 flagella were compared, few differences were observed between the axonemes, but the amounts of certain membrane proteins were greatly reduced compared. Few differences were observed between the axonemes, but the amounts of certain membrane proteins were greatly reduced compared, few differences were observed between the axonemes, but the amounts of certain membrane proteins were greatly reduced.

INTRODUCTION
Nephronophthisis (NPHP) is a recessive form of cystic kidney disease that is the most frequent genetic cause of chronic renal failure in children (Wolf and Hildebrandt, 2011). To date, 18 causative human genes for NPHP have been reported (Wolf and Hildebrandt, 2011; Failler et al., 2014; Renkema et al., 2014); one of these is NPHP4, which codes for nephrocystin-4 (NPHP4). Mutations in NPHP4 lead to juvenile NPHP type 4 as well as retinitis pigmentosa; both disease features occur together in Senior–Løken syndrome (Hoefele et al., 2005; Mollet et al., 2002; Otto et al., 2002; Schuermann et al., 2002).

NPHP and Senior–Løken syndrome are ciliopathies (diseases caused by defects in cilia), and there is abundant evidence from model systems that loss of NPHP4 causes a ciliary phenotype. A study of Nphp4-mutant mice has shown that Nphp4 is necessary for normal photoreceptor formation and maintenance as well as sperm development (Won et al., 2011). In zebrafish, nphp4 morphants exhibit classic ciliary phenotypes, including abnormal body curvature and pronephric cysts (Burklé et al., 2011; Slanchev et al., 2011). In Caenorhabditis elegans, RNA interference (RNAi)-mediated knockdown of NPHP-4 causes defects in male mating behavior, which is mediated by sensory cilia (Wolf et al., 2005), and a mutant null for NPHP-4 has mild defects in cilia-dependent sensory functions (Winkelbauer et al., 2005). Cilia of the same mutant are stunted, misshapen and have anomalous ultrastructural defects (Jauregui et al., 2008).

These phenotypes most likely result from defective function of NPHP4 at the ciliary transition zone, a specialized region between the basal body and the cilium proper (Shiba and Yokoyama, 2012; Czarnecki and Shah, 2012; Reiter et al., 2012). Several studies have reported that NPHP4 localizes to the transition zone. LAP-tagged NPHP4 in IMCD3 cells is present at sites of cell–cell contact and at the base of the primary cilium, specifically in the transition zone (Sang et al., 2011). Others have reported the presence of NPHP4 in the transition zone of cultured mouse renal epithelial cells (Shiba et al., 2010) and in the connecting cilium – a greatly elongated transition zone – of mouse photoreceptor cells (Roepman et al., 2005; Patil et al., 2012; Won et al., 2011). In Chlamydomonas reinhardtii, NPHP4 has been identified in a proteomic study of basal bodies, which included the transition zone (Keller et al., 2005). In C. elegans, NPHP4 has been found to localize to the transition zone of the sensory cilia (Winkelbauer et al., 2005; Jauregui et al., 2008; Williams et al., 2011).

Evidence is accumulating that the transition zone functions as the ‘pore’ or gate proposed to exist at the base of the cilium to control access to the ciliary compartment (Rosenbaum and Witman, 2002). A highly conserved feature of the transition zone is the presence of Y-shaped links (Y-links) that connect the transition zone doublet microtubules to the overlying membrane (Fisch and Dupuis-Williams, 2011). In C. reinhardtii, these links are partially disrupted in the absence of CEP290, another transition zone protein, resulting in abnormal flagellar protein composition (Craigie et al., 2010). The mutant also has defects in intraflagellar transport (IFT), which is used by nearly all organisms to build cilia and flagella (Pedersen and Rosenbaum, 2008; Ishikawa and Marshall, 2011). These results provide experimental evidence that the transition zone functions to control protein and IFT particle entry into the cilium, and they indicate that CEP290 is important for this function. NPHP4 also might be involved in this activity; cilia of the C. elegans nphp-4 mutant accumulate the membrane-associated proteins RGI-2 and TRAM-1a, which normally are excluded from the cilium (Williams et al., 2011).
Despite this progress, we still do not know the precise location of NPHP4 in the transition zone nor how its loss affects overall ciliary composition. In this study, we have used *C. reinhardtii* to learn more about NPHP4. *C. reinhardtii* has many advantages for studying cilia and ciliary components. Particularly relevant for this study, its flagella can be isolated to determine the biochemical consequences of loss of NPHP4. NPHP4 is highly conserved (*C. reinhardtii* to human BLASTP E value = $1e^{-85}$), so conclusions from studying *C. reinhardtii* NPHP4 are likely to be applicable to humans and other organisms. We found that NPHP4 is located at the periphery of the distal transition zone, close to the membrane and distal to CEP290. In contrast to CEP290, NPHP4 at the transition zone does not undergo rapid turnover. We identified an NPHP4-null mutant; the mutant has full-length flagella and generally has normal transition zone and axonemal ultrastructure. We isolated the flagella of the *nphp4* mutant and found that a subset of the membrane-associated proteins that are present in wild-type flagella were greatly decreased in amount; conversely, the flagella contained many large cytosolic housekeeping proteins that normally are excluded from wild-type flagella. The results indicate that NPHP4 is a crucial component of the selective gate that functions at the transition zone to control the movement of both soluble and membrane-associated proteins between the flagellar and cytoplasmic compartments. It is likely that the various phenotypic consequences of NPHP4 mutations in humans and other organisms all follow from protein mislocalization due to defects in the transition zone barrier.

**RESULTS**

**NPHP4 loss has minor effects on cell motility but slows flagellar assembly**

To identify a *C. reinhardtii* *nphp4* mutant, genomic DNA from our collection of insertional mutants (Pazour et al., 1995; Pazour et al., 1998) was screened by real-time PCR with primer pairs specific to NPHP4. In one strain (B1179), no product was amplified. Further analysis by PCR revealed that the mutation, termed *nphp4*-1, deleted ~40 kbp around NPHP4, including the entire NPHP4 gene, several predicted genes that have no known association with the flagellum, and a part of DRC3 (Fig. 1A). DRC3 was first identified in our flagellar proteomic study as FAP134 (Pazour et al., 2005) and was later shown to be a component of the nexin–dynein regulatory complex (Lin et al., 2011). To ensure that the phenotype being analyzed in the studies that follow was not compromised by the absence of DRC3, B1179 was backcrossed twice to a wild-type strain, and some of the mutant progeny were then transformed with a DNA fragment amplifying. Further analysis by PCR revealed that the mutation, termed *nphp4*-1, deleted ~40 kbp around NPHP4, including the entire NPHP4 gene, several predicted genes that have no known association with the flagellum, and a part of DRC3 (Fig. 1A). DRC3 was first identified in our flagellar proteomic study as FAP134 (Pazour et al., 2005) and was later shown to be a component of the nexin–dynein regulatory complex (Lin et al., 2011). To ensure that the phenotype being analyzed in the studies that follow was not compromised by the absence of DRC3, B1179 was backcrossed twice to a wild-type strain, and some of the mutant progeny were then transformed with a DNA fragment containing the DRC3 gene (Fig. 1A). One of the resulting transformants, rescued for DRC3, was used in this study as the *nphp4* strain.

The *nphp4*-mutant cells are motile and can undergo phototaxis (data not shown), but their swimming paths are slightly more erratic than those of wild-type cells (Fig. 1B). To assess this objectively, motility was analyzed using computer-aided sperm analysis (CASA) (Fig. 1C) (Mortimer, 2000). Swimming speeds of wild-type cells and the *nphp4* mutant were similar (supplementary material Fig. S1B, VCL), but linearity was much lower for the mutant (Fig. 1C; see supplementary material Fig. S1A for definition of terms). Transformation of the *nphp4* mutant with the wild-type NPHP4 gene to generate strain NPHP4-R almost completely restored linearity (Fig. 1C), confirming that the phenotype is due to the absence of NPHP4.

Consistent with the ability to swim nearly normally, steady-state flagella of *nphp4* mutant cells are of near-normal length and appear to be ultrastructurally normal (Fig. 1D; Fig. 2A; data not shown). Following flagellar amputation, the *nphp4* mutant regenerated new flagella more slowly than did wild-type cells; normal flagellar regeneration was completely restored in the NPHP4-R strain (Fig. 1D). Therefore, NPHP4 is needed to build the flagella with normal kinetics.

**NPHP4 is located in the transition zone**

To determine where NPHP4 is located in *C. reinhardtii*, we made an antibody against the C-terminus of NPHP4. The antibody recognized a band of the expected size in western blots of wild-type but not *nphp4* whole cells; the band was restored in the NPHP4-R strain (Fig. 1E). These results confirmed that NPHP4 is missing in the mutant. We also rescued the mutant with constructs expressing a 3xHA tag at either the N- or C-terminus of NPHP4 to generate NPHP4–HAN and NPHP4–HAC cells, respectively; the anti-HA antibody recognized the tagged protein with high specificity in the rescued cells (Fig. 1F). In immunofluorescence microscopy, the anti-NPHP4 antibody strongly labeled the bases of the flagella of wild-type cells but not *nphp4* cells (Fig. 2A), indicating that this localization is specific to NPHP4. Similarly, the anti-HA antibody localized specifically to the bases of flagella in both NPHP4–HAN and NPHP4–HAC cells but not in wild-type cells, indicating that the localization is specific to HA-tagged NPHP4 (Fig. 2B).

NPHP4 has been found by immunofluorescence microscopy to localize to primary cilia of MDCK and mouse kidney cells (Mollet et al., 2005; Patil et al., 2012). Therefore, we carefully determined the distribution of NPHP4 in *C. reinhardtii* flagella versus cell bodies. When cells are deflagellated, the flagella detach at the junction of the transition zone and axoneme proper (Sandars and Salisbury, 1989). NPHP4–HAC cells were deflagellated with dibucaine and the resulting cell bodies and detached flagella were stained with anti-HA; NPHP4–HAC was found to stay with the cell bodies (Fig. 2C). To confirm this result, whole cells, cell bodies and isolated flagella were compared by western blotting; all of the NPHP4–HAC remained within the cell body and none could be detected in the isolated flagella (Fig. 2D). The results indicate that *C. reinhardtii* NPHP4 is located in either the transition zone or the basal body, but not in the flagella.

To further narrow down the location of NPHP4, both nucleoflagellar apparatuses and whole cells of the NPHP4–HAC strain were labeled with antibodies against the HA peptide and CEP290, which is located in the transition zone (Craigie et al., 2010). By both wide-field immunofluorescence microscopy (Fig. 3A,B) and total internal reflection fluorescence/epi-fluorescence structured light microscopy (TESM) (Fig. 3C), the NPHP4–HAC label was observed to be distal to the CEP290 label, indicating that NPHP4 is located in the transition zone. Analysis of TESM images indicated that the mean peak-to-peak distance between CEP290 and the C-terminus of NPHP4–HAC is ~137 nm ($n=130$).

**NPHP4 is located at the periphery of the distal transition zone**

To determine the location of NPHP4 within the transition zone, we carried out pre-embedding immunoelectron microscopy of nucleoflagellar apparatuses from wild-type, NPHP4–HAN and NPHP4–HAC cells. As expected from the immunofluorescence
Fig. 1. Characterization of the *C. reinhardtii* *nphp4* mutant. (A) Map of *C. reinhardtii* genome near the *NPHP4* locus. Numbers above each locus correspond to gene IDs in Phytozome version 9.1 (http://www.phytozome.net). Arrows indicate the positions of PCR products used to delimit the deleted region; plus and minus marks indicate whether the PCR products were amplified. Genome fragments from bacterial artificial chromosome (BAC) clones used for knock-in of full-length *NPHP4* and *DRC3* are indicated by white rectangles. WT, wild type. (B) Representative images showing swimming paths of wild-type and *nphp4* cells. White dots show cells where CASA began to monitor their tracks; green lines are the swimming paths analyzed. Blue tracks were not analyzed because the cells swam outside the microscope field before recording was completed. Cells marked with red dots were immobilized by attachment to the coverslip. The swimming paths of *nphp4* cells are more erratic than those of wild-type cells. Scale bar: 50 μm. (C) Bar graph (left) showing the mean ± s.d. of linearity and histogram (right) summarizing the distribution of linearity in swimming paths of populations of wild-type, *nphp4* and NPHP4-R cells (see supplementary material Fig. S1). For each strain, the population was calculated from the sum of values from a total of ten fields in each of five independent experiments. Statistical significance was determined by the Tukey–Kramer method: **P < 0.01; ***P < 0.001. (D) Flagella length prior to deflagellation and as a function of time after deflagellation of wild-type, *nphp4* and NPHP4-R cells. Flagellar length at each time-point is the mean ± s.d. of 30 flagella. (E) Western blot of whole cells probed with an antibody against the C-terminus of NPHP4. The antibody recognized a band (arrowhead) of the predicted size for NPHP4 (210 kDa) in wild-type cells and the NPHP4-R strain. (F) Western blot of wild-type, *nphp4*, NPHP4–HAN and NPHP4–HAC whole cells probed with a monoclonal antibody against the HA peptide. βF1-ATPase was used as a loading control.
microscopy results (Fig. 2B), gold particles were associated with the transition zone in both NPHP4–HAN and NPHP4–HAC specimens (Fig. 4A,B). Most gold particles in longitudinal sections and all particles in cross-sections of the transition zone were located at the transition zone periphery, in close association with remnants of the transition zone membrane, which is resistant to treatment with nonionic detergent (Kamiya and Witman, 1984). We observed no gold particles associated with the wild-type transition zones, indicating that the immunogold labeling was specific to HA-tagged NPHP4.

The locations, relative to the proximal end of the transition zone, of a total of 50 gold particles from longitudinal sections of both NPHP4–HAN and NPHP4–HAC specimens were measured and compared to that determined for CEP290 by Craige et al. (Craige et al., 2010) (Fig. 4C). Consistent with our immunofluorescence microscopy observations (Fig. 3), the distribution of both NPHP4–HAN and NPHP4–HAC gold particles had sharp peaks in the distal transition zone. The average distance of gold particles from the proximal end of the transition zone was 173±37 nm for NPHP4–HAC and 155±31 nm for NPHP4–HAN, compared to 61±38 nm for CEP290; the average distance between NPHP4–HAC and CEP290 gold particles was 111 nm, in good agreement with the separation (~137 nm) measured by TESM.
NPHP4 and CEP290 localize to the transition zone independently of each other
To explore if *C. reinhardtii* NPHP4 can be assembled at the transition zone independently of CEP290 and vice versa, we carried out immunofluorescence microscopy of *cep290*- and *nphp4*-mutant cells with antibodies against NPHP4 and CEP290, respectively. Localization of NPHP4 appeared to be normal in the *cep290* cells, and localization of CEP290 was normal in the *nphp4* cells (supplementary material Fig. S2A,B). Therefore, these two proteins are transported to and assemble into the transition zone independently of each other.

NPHP4 does not turn over rapidly
We reported previously that CEP290 at the transition zone is highly dynamic (Craige et al., 2010). To determine if NPHP4 is similarly dynamic, we mixed wild-type gametes with NPHP4–HAN or NPHP4–HAC gametes to form quadriflagellated zygotes in which two transition zones contained untagged NPHP4 and two contained HA-tagged NPHP4 in a common cytoplasm. Nucleoflagellar apparatuses were prepared at 20, 40 and 60 min after initiation of the mating reaction and labeled with anti-HA (Fig. 5). Even after 60 min, only two transition zones were labeled by the antibody, indicating that little, if any, HA-tagged NPHP4 was incorporated into the wild-type transition zones over this time period. Moreover, the label was as bright at 60 min as at 20 min, indicating that little, if any, HA-tagged NPHP4 was replaced with wild-type NPHP4. Therefore, in contrast to CEP290, NPHP4 is static at the transition zone.

Ultrastructure of the transition zone in the *nphp4* mutant
To determine whether loss of NPHP4 caused structural abnormalities in the transition zone, we compared the transition zones of wild-type and *nphp4*-mutant cells by electron microscopy. In cross sections, the Y-links between the transition zone doublet microtubules and membrane, which are disrupted in the *cep290* mutant (Craige et al., 2010), were present and appeared normal (Fig. 6A). In longitudinal sections, the wedge-shaped connectors, which extend between the transition zone doublets and membrane in wild-type cells, appeared to be missing or collapsed in the *nphp4* mutant (Fig. 6B). However, the wedge-shaped structures are similarly altered in transition zones of cells lacking the IFT protein IFT46 (Hou et al., 2007). To clarify whether disruption of this structure is correlated with lack of NPHP4, we performed immunofluorescence microscopy of *ift46* cells and found that NPHP4 was normally located at the base of their flagella (supplemental material Fig. S2C). Thus, the morphological variation in this structure is not specifically related to the loss of NPHP4. Moreover, because the Y-links are present in the *nphp4* mutant, we can conclude that they and the wedge-shaped structures are not the same. Finally, in ~30% of longitudinal sections of *nphp4* flagella, we observed, just distal to the transition zone, the apparently ectopic localization of dense material resembling the central cylinders of the *C. reinhardtii* transition zone (Fig. 6C).

Protein composition of the flagellar membrane and matrix is drastically altered by loss of NPHP4
To compare the protein compositions of wild-type versus *nphp4* flagella, flagella were isolated and treated with NP-40 to separate proteins into a detergent-soluble membrane-plus-matrix fraction and an axonemal fraction. In SDS-polyacrylamide gels of the axoneme fractions, very few differences were observed between wild-type and *nphp4* samples (Fig. 7A,B). However, numerous differences were observed in the membrane-plus-matrix fractions.
The levels of some proteins were decreased in the npihp4 mutant (arrowheads, Fig. 7A), whereas others were increased (arrows, Fig. 7A). Normal flagellar protein composition was restored in NPHP4-R (supplementary material Fig. S3), confirming that the differences were due to loss of NPHP4. Importantly, most differences between the wild-type and npihp4 membrane-plus-matrix fractions were observed in the mass range above 50 kDa; few differences were observed below 50 kDa (Fig. 7B). To identify the proteins that were decreased or increased in the npihp4 flagella, sets of slices containing the discordant bands were excised from gels of the membrane-plus-matrix fraction of wild-type, npihp4 and NPHP4-R flagella (supplementary material Fig. S3). The slices were then subjected to analysis by mass spectrometry. Multiple proteins were identified in all slices (supplementary material Table S1). To conclude that the amount of a protein was altered in the mutant flagella, we used the following criteria: (1) the number of peptides recovered had to be increased or decreased by more than ten compared to wild type, or the number of peptides recovered had to be greater than five when the protein was not found in the other slice (wild type or npihp4) being compared; and (2) the change was specifically rescued in the NPHP4-R cells. Based on these criteria, the proteins that were found to be increased or decreased in amount in the npihp4-mutant flagella are listed in Table 1 and supplementary material Table S1 (where blue or red font indicates increased or decreased proteins, respectively). Except for ATPase V1 complex-subunit A, all proteins that increased in amount were housekeeping proteins that presumably are abundant in the cell bodies. Valyl- and phenylalanyl-tRNA synthetase, ADP ribosylglycohydrolase and RNA-binding protein were not found in the C. reinhardtii flagellar proteome (Pazour et al., 2005). All increased proteins are predicted to be larger than 50 kDa (Table 1). We conclude that large housekeeping proteins are specifically increased in npihp4-mutant flagella.

By contrast, all proteins that decreased in amount (other than FAP208) are predicted to be membrane associated – the calcium ATPase is predicted to have a transmembrane domain, and flagellar adenylate kinase, FAP33, FAP295 and FAP12 have N-myristoylation sites. Additionally, when we examined other proteins also predicted to be membrane associated but not meeting the above criteria (these proteins were identified by less than five peptides in wild-type flagella, were not detected at all in
Fig. 5. **NPHP4 at the transition zone is static.** Wild-type (WT) gametes were mixed with NPHP4–HAN (A) or NPHP4–HAC (B) gametes to initiate the mating reaction. At the indicated times after mixing, nucleoflagellar apparatuses of the resulting quadriflagellated zygotes were prepared, fixed and stained with anti-acetylated tubulin, anti-HA and DAPI. The right panels are enlargements of the transition zones shown in the merged images. Scale bars: 1 μm.

**Table 1. Proteins decreased or increased in nphp4 flagella**

<table>
<thead>
<tr>
<th>Decreased in nphp4 flagella</th>
<th>Increased in nphp4 flagella</th>
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<tbody>
<tr>
<td>Ankyrin-repeat protein (FAP33)</td>
<td>213 kDa</td>
</tr>
<tr>
<td>Flagellar adenylate kinase (FAK1)</td>
<td>173 kDa</td>
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<tr>
<td>CaATPase</td>
<td>123 kDa</td>
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<tr>
<td>Ankyrin-repeat protein (FAP208)</td>
<td>99 kDa</td>
</tr>
<tr>
<td>cA(G)MP protein kinase (FAP295)</td>
<td>81 kDa</td>
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<tr>
<td>Triglyceride lipase (FAP12)</td>
<td>52 kDa</td>
</tr>
<tr>
<td>Valyl-tRNA synthetase</td>
<td>141 kDa</td>
</tr>
<tr>
<td>Elongation factor 3</td>
<td>135 kDa</td>
</tr>
<tr>
<td>ADP ribosylglycohydrolase</td>
<td>94 kDa</td>
</tr>
<tr>
<td>Methionine synthase</td>
<td>87 kDa</td>
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<td>Heat shock protein 90</td>
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<td>RNA binding protein</td>
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<tr>
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<td>76 kDa</td>
</tr>
<tr>
<td>ATPase, V1 complex, subunit A</td>
<td>68 kDa</td>
</tr>
<tr>
<td>Formate-tetrahydrofolate ligase</td>
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</tr>
<tr>
<td>Lysyl-tRNA synthetase</td>
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</tr>
<tr>
<td>Phenylalanyl-tRNA synthetase</td>
<td>59 kDa</td>
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</table>

Protein bands that were drastically changed in amount in SDS-polyacrylamide gels comparing wild-type versus nphp4 flagellar membrane-plus-matrix fractions were excised and subjected to mass spectrometry analysis. The listed proteins were selected based on differences in the number of peptides identified in the wild-type versus the nphp4-mutant samples, using criteria described in the text. aProteins with predicted N-myristoylation site; bprotein with predicted transmembrane domains; cproteins not found in the flagellar proteome study (Pazour et al., 2005).
the nphp4 mutant and were restored in the rescued strain), all were decreased in the mutant flagella. These included the large flagellar membrane glycoprotein FMG1-B, a predicted ion channel, another calcium transport ATPase, an ABC transporter and FAP29 – all of which have transmembrane domains – and ankyrin-repeat protein and apyrase, which contain N-myristoylation sites (yellow font in supplementary material Table S1). Nearly all proteins that decreased in amount were previously identified in the *C. reinhardtii* flagellar proteome (Pazour et al., 2005). Thus, proteins normally associated with the flagellar membrane are either not transported into or are not retained in the flagella in the absence of NPHP4.

To determine whether all flagellum-associated membrane proteins are reduced in amount in nphp4 flagella, we probed western blots of isolated flagella from wild-type and the nphp4-mutant cells with antibodies against two well-characterized flagellar membrane proteins not identified in our dataset. The flagellar mastigoneme protein (Nakamura et al., 1996) and PKD2 (a homolog of human polycystin-2; Huang et al., 2007) were not decreased in the nphp4 flagella (Fig. 7C). We also probed for FMG-1B, which appeared to be decreased in nphp4 flagella in our mass spectrometry analysis. The western blot confirmed that it was reduced in the mutant flagella. We conclude that only a subset of proteins normally associated with the flagellar membrane are decreased in the absence of NPHP4.

**IFT in the nphp4 mutant**

Several IFT proteins were identified by mass spectrometry, and the levels of most of these proteins did not seem to be altered in the mutant flagella (supplementary material Table S1). To confirm this, we examined the amounts of IFT components in isolated flagella of wild-type and nphp4 cells by western blotting. The levels of IFT motors, of IFT proteins of both complex A and B, and of BBS4, a subunit of the BBSome, were unaltered or slightly elevated in the nphp4-mutant flagella (supplementary material Fig. S4A). To more directly assess whether IFT is affected by lack of NPHP4, IFT-particle movement in steady-state flagella was analyzed (supplementary material Fig. S4B). IFT frequency was not affected, whereas IFT velocity was slightly decreased in the absence of NPHP4 (supplementary material Fig. S4C).

**DISCUSSION**

**NPHP4 and CEP290 define distinct regions of the transition zone**

In agreement with previous studies in *C. elegans* and mammals (Winkelbauer et al., 2005; Jauregui et al., 2008; Shiba et al., 2010; Sang et al., 2011; Williams et al., 2011), we found that *C. reinhardtii* NPHP4 is located in the transition zone. However, our studies provide much more precise information on the distribution and localization of NPHP4. Using both immunofluorescence microscopy and immunoelectron microscopy, we determined that NPHP4 is located in the distal part of the transition zone, distal to CEP290. The NPHP4 and CEP290 peaks observed by TESM were separated by a minimum of ~140 nm, and immunoelectron microscopy revealed that there is little overlap between the two proteins. Thus, NPHP4 and CEP290 define distinct structural domains of the transition zone (Fig. 7D). Immunoelectron
microscopy also indicated that the N- and C-termini of NPHP4 are located at the periphery of the transition zone, in close association with the transition zone membrane. Thus, NPHP4 is well positioned to control entry and/or exit of membrane proteins from the flagellum. This apparent localization of NPHP4 to the extreme periphery of the transition zone contrasts with that of CEP290, which is distributed throughout the space between the doublet microtubules and the membrane (Craige et al., 2010); however, we cannot rule out that the middle part of NPHP4 extends into the space between the membrane and microtubules. Finally, our biochemical studies of isolated flagella indicate that there is no detectable NPHP4 in the flagellum proper.

Consistent with NPHP4 and CEP290 being located in different regions of the transition zone, we found that NPHP4 and CEP290 each assembled normally into the transition zone in the absence of the other. Both the localization and assembly results indicate that *C. reinhardtii* NPHP4 and CEP290 are integrated into different complexes in the transition zone. These findings agree with those of Sang et al. (Sang et al., 2011), who concluded that mammalian NPHP4 and CEP290 were components of distinct modules, based on immunoprecipitation of LAP-tagged proteins from whole-cell lysates.

We also observed that NPHP4 at the transition zone undergoes little or no turnover during a 1-h period; this is in sharp contrast to CEP290, which is at least 50% replaced in the same time span (Craige et al., 2010). Therefore, NPHP4 is static at the transition zone, as might be expected for a structural protein. By contrast, CEP290 appears to be much more dynamic. Although highly speculative, the results raise the possibility that CEP290 has a role in shuttling IFT or other ciliary proteins into the transition zone.

**NPHP4 and CEP290 loss have different consequences for transition zone structure**

A highly conserved and defining feature of the transition zone is the occurrence of Y-links between the doublet microtubules and membrane as observed in electron microscopy cross-sections of various organisms (Gilula and Satir, 1972; Perkins et al., 1986; Ringo, 1967). Craige et al. (Craige et al., 2010) observed that the...
Y-links were missing or disrupted in *C. reinhardtii* CEP290 mutants. We found that loss of NPHP4, by contrast, did not affect the Y-links. Therefore, NPHP4 is not required for Y-link formation. The same is true in *C. elegans*, where loss of NPHP4 alone did not cause disruption of the Y-links (Jauregui et al., 2008). However, when mutations in NPHP4 and members of the MKS/B9 module were combined in *C. elegans*, the Y-links were lost (Williams et al., 2011).

Although no specific structural abnormalities were observed in nphp4 transition zones, the flagella often contained, distal to the transition zone, what appeared to be a partial duplication of the central cylinder that normally occupies the lumen of the transition zone of *C. reinhardtii*. Interestingly, a similar duplication of the central cylinder has been observed in unflagellar mutants of this organism (Huang et al., 1982; Piasecki et al., 2008). The duplication might occur because the cell detects abnormalities associated with the absence of NPHP4 and responds by moving additional central cylinder precursors into the flagellum.

**A size-dependent barrier to ciliary entry of large soluble housekeeping proteins is breached in nphp4 cells**

Because of the ease with which *C. reinhardtii* flagella can be purified, we were able to assess the biochemical consequences of loss of NPHP4 for the flagella. Strikingly, nearly all of the differences observed were in the membrane-plus-matrix fraction, not the axonemal fraction. The amounts of many proteins were increased in the nphp4 flagella, and, with the possible exception of subunit A of the V1-ATPase, all of these proteins were soluble housekeeping proteins. Most of these proteins were not detected at all in the membrane-plus-matrix fraction from wild-type flagella, suggesting that their presence in the nphp4 flagellum resulted from the breakdown of a mechanism that normally keeps them out of the organelle.

Importantly, abnormal entry of soluble cytoplasmic proteins into the nphp4 flagellum was observed only for proteins that migrated above 50 kDa in SDS-PAGE, indicating that there is an NPHP4-dependent barrier at the transition zone that excludes soluble proteins of ~50 kDa or larger. Consistent with this, Kee et al. (Kee et al., 2012) observed that when fluorescently labeled protein A (41 kDa) and bovine serum albumin (BSA) (67 kDa) were microinjected into hTERT RPE cells, the former entered the ciliary compartment, whereas the latter did not. Similarly, Breslow et al. (Breslow et al., 2013), working with permeabilized IMCD3 cells, found that the rate of entry of soluble proteins into the cilium progressively slowed as the proteins increased in size from a Stokes radius of 3.1 nm to 4.3 nm (corresponding to ~30 to ~70 kDa); no entry was observed for larger proteins. Taken together, these results indicate that there is a size-dependent barrier to diffusion of soluble proteins into the cilia of organisms ranging from protists to humans and that this barrier is dependent upon NPHP4 in the transition zone.

Using a chemically inducible diffusion trap approach, Lin et al. (Lin et al., 2013) also obtained evidence for a size-dependent diffusion barrier at the base of primary cilia. However, they observed slow entry of proteins as large as ~600–650 kDa, and suggested that “the steady-state distribution of proteins in cilia is unlikely to be regulated by the diffusion barrier.” Because the differences that we observed were in steady-state flagella that were at least 3 h old, the *C. reinhardtii* NPHP4-dependent diffusion barrier appears to be more impermeable to the diffusion-based entry of large soluble proteins.

**NPHP4 is essential to a transition zone barrier that retains a subset of membrane proteins in the flagella**

Although the levels of large soluble housekeeping proteins increased in *nphp4* flagella, levels of many membrane-associated proteins decreased. However, the levels of some other flagellar membrane proteins, such as PKD2 and the mastigoneme protein, were not affected in *nphp4* flagella. *C. reinhardtii* PKD2 is likely to be anchored to the axoneme because most PKD2 peptides were found in axonemal fractions in the flagellar proteome (Pazour et al., 2005) and ~90% of GFP-tagged PKD2 is stationary in the flagella (Huang et al., 2007). Similarly, 75% of peptides from the mastigoneme protein identified in the flagellar proteome were found in the fraction resistant to detergent, implying that this protein also is anchored to the axoneme (Pazour et al., 2005). These results agree well with reports that PKD2 and an odorant receptor are normally located in *C. elegans* nphp-4 cilia (Jauregui et al., 2008; Williams et al., 2011). We further observed that the level of FMG-1B was decreased; in the *C. reinhardtii* flagellar proteome, peptides from FMG-1B were evenly distributed between the membrane and axonemal fractions (Pazour et al., 2005), and at least some of this protein is moved in the plane of the flagellar membrane by IFT (Guilford and Bloodgood, 2013). Taken together, these results suggest that a subset of flagellar membrane proteins – specifically those presumed to be mobile in the plane of the membrane – are present at lower levels in nphp4 flagella, whereas those that are anchored to the axoneme exhibit no reduction. Because PKD2 and the mastigoneme protein that bind to the axoneme appear to be delivered normally to the nphp4 flagella, it is possible that most or all flagellar membrane proteins are targeted normally to the flagella, but those that are free to move in the plane of the membrane subsequently diffuse out of the organelle in the absence of an NPHP4-dependent barrier at the transition zone.

Previous studies have provided evidence for a membrane protein barrier at the base of the cilium that is consistent with our findings. Studies with mammalian cells showed that disruption of members of the MKS/B9 complex, which localizes to the transition zone, resulted in a decrease in the amount of several GFP-tagged membrane proteins in primary cilia (Garcia-Gonzalo et al., 2011; Chih et al., 2012). NPHP4 is not part of the MKS/B9 complex, but studies in *C. elegans* indicate that NPHP4 and proteins of the MKS/B9 complex genetically interact in functionally redundant pathways that are important for ciliary development (Williams et al., 2008; Williams et al., 2010; Williams et al., 2011; Huang et al., 2011; Warburton-Pitt et al., 2012). Therefore, NPHP4 and the MKS/B9 proteins are likely to function together to prevent ciliary membrane proteins from diffusing out of the cilium. NPHP4 is located close to or at the transition zone membrane and several of the MKS/B9 proteins are predicted to be transmembrane proteins, so it will be of interest to determine precisely where the MKS/B9 proteins are located relative to NPHP4 and CEP290 in the transition zone.

**Loss of NPHP4 has mild effects on flagellar motility**

Interestingly, the only behavioral defect that we observed in the nphp4 mutant was erratic swimming. The lack of a stronger motility defect is consistent with our finding that there is little, if any, change in the protein composition of the flagellar axoneme, which constitutes the motile machinery. However, changes in intraflagellar Ca2+ are crucial for controlling flagellar waveform (Bessen et al., 1980; Kamiya and Witman, 1984), so the erratic swimming that we observed is entirely consistent with the loss of
NPHP4 and CEP290 define different structural and functional domains in the transition zone

Although nphp4 cells are able to assemble full-length flagella, the kinetics of flagellar regeneration following flagellar amputation are significantly impaired. Presently, the reason for this is unclear. IFT frequency is normal and velocity is only slightly reduced in steady-state nphp4 flagella, suggesting that loss of NPHP4 does not directly affect the IFT machinery. These results are generally consistent with those in C. elegans, where mutation of NPHP-4 has modest (Jauregui et al., 2008) or no (Williams et al., 2011) effect on IFT. The slower regeneration kinetics that we observed might reflect reduced IFT cargo loading, impaired flagellar signaling necessary for normal regeneration or altered intraflagellar ion concentrations (see above section). In any case, the very slight effect of NPHP4 loss on IFT is in contrast to the effect of CEP290 loss, which results in severe IFT abnormalities and greatly impaired flagellar formation (Craigie et al., 2010).

Therefore, although NPHP4 and CEP290 are both essential to the flagellar signaling necessary for normal regeneration or altered cytoskeletons were attached to a coverslip coated with poly-L-lysine and 24 m–deep chamber for observation of the cells. 22 m-deep No. 1 coverslips were glued to opposite ends of a 25 slide with ProLong Gold Antifade Reagent (Life Technologies).

Secondary antibodies diluted in the blocking buffer were then applied to the sample overnight at 4˚C, followed by four washes with PBS. Primary antibody diluted in the blocking solution was applied to the coverslip for 1 h at room temperature. Primary antibody diluted in the blocking solution was applied to the sample overnight at 4°C, followed by four washes with PBS. Secondary antibodies diluted in the blocking buffer were then added to the coverslip for 1 h at room temperature. For double staining, secondary antibodies conjugated to Alexa Fluor 488, 568 or 594 (Life Technologies) were used. For tricolor labeling, secondary antibodies conjugated to Alexa Fluor 350 or 488 and 594 were used. Finally, the coverslip was given three 10-min rinses with PBS and mounted on a glass slide with ProLong Gold Antifade Reagent (Life Technologies).

To prepare nucleoflagellar apparatuses, NPHP4–HAC cells were treated with autolysin, resuspended in ice-cold microtubule-stabilizing (MTSB) buffer (30 mM HEPES-KOH pH 7.4, 5 mM MgSO4, 15 mM KC1, 2 mM EGTA) and mixed with an equal volume of 1% NP-40 (Calbiochem) in MTSB buffer. The solution was left on ice for 15 min and then mixed with an equal volume of 6% paraformaldehyde in MTSB buffer. After incubation on ice for 60 min, the nucleoflagellar apparatuses were collected by centrifugation at 1100 g for 10 min. The cytoskeletons were attached to a coverslip coated with poly-L-lysine and dried. The samples were blocked, labeled and mounted as described above for whole cells.

All transformations were performed by the glass beads method (Kindle, 1990). For transformation with wild-type and HA-tagged NPHP4, cells were co-transformed with the ble gene as a selectable marker (Stevens et al., 1996). Colonies on TAP medium plates containing zeomycin were randomly selected and screened by immunofluorescence microscopy with anti-NPHP4 or anti-HA antibodies. For transformation with DRC3, the aph7″ gene was used as the selectable marker (Berthold et al., 2002).
The microscope, camera and software used to take fluorescence images were as described previously (Claige et al., 2010). Gamma adjustment and creation of merged images were performed with Photoshop CS2; all images mounted together were processed similarly.

TESM
TESM images were taken using the custom-built TESM system of our Biomedical Imaging Group (Navaroli et al., 2012). Cells triple labeled with Alexa Fluor 488, 594 and 647 and mounted in ProLong Gold Antifade Reagent were imaged using three color epi-fluorescence illumination. The pixel size corresponded to 0.083 μm. Z-stacks consisted of 100 images with a z separation of 100 nm. At each z-plane, an exposure of 25 ms was taken in each emission bandpass. Tetraspec beads (200 nm) were used to measure chromatic aberration in the microscope system. Point spread functions fitted to the beads in each color revealed a chromatic aberration between the green and the red and far red channels of 1.6 pixels in the z direction. This measured offset was used to correct the experimental data.

Electron microscopy
Whole cells were pre-fixed in 1% glutaraldehyde in modified M medium for 15 min at room temperature, collected by centrifugation, resuspended with 1% glutaraldehyde in 100 mM sodium cacodylate-NaOH pH 7.2 (cacodylate buffer) and left for 2 h at room temperature. Specimens were then processed for ultra-thin sectioning as described previously (Claige et al., 2010).

For pre-embedding immunogold electron microscopy, nucleoflagellar apparatuses of 137c, NPHP4–HAN and NPHP4–HAC cells were prepared as described for immunofluorescence microscopy. After fixation with 3% paraformaldehyde, the samples were washed three times with PBS+0.02% Tween-20 (PBST) and blocked with 5% skimmed milk, 4% BSA in PBST for 1 h at room temperature. The cytoskeletons were then resuspended with the anti-HA antibody diluted 1:400 in PBST and incubated overnight at 4°C. After three washes with PBST, the samples were next incubated with 1% BSA in PBST containing anti-rat-IgG conjugated to 10-nm gold particles (Aurion). The cytoskeletons were then washed three times in MTSB buffer and fixed with 1% OsO4 in MTSB buffer for 30 min at room temperature. The pellets were then washed once with MTSB buffer, rinsed three times with distilled water and stained with 1% uranyl acetate overnight at 4°C. Dehydration and embedding was performed for whole cells, except that post-sectioning staining was omitted.

Generation of gametes and dikaryons
To produce gametes, 30 ml of 137c, CC124, NPHP4–HAN and NPHP4–HAC cultures grown to 105 cells/ml were collected and resuspended twice with nitrogen-free M medium. The second suspensions were transferred to 250-ml flasks and agitated under constant light overnight. Quadriflagellated zygotes were generated by mixing 137c with NPHP4–HAC and CC124 with NPHP4–HAN gametes. At various times after initiation of the mating reaction, aliquots of the mixture were removed and nucleoflagellar apparatuses were prepared as described for immunofluorescence microscopy.

Flagella isolation, SDS-PAGE and western blotting
Flagella were isolated by the dibucaine method (Witman, 1986) with the following modifications: DTT was omitted from the HMDS solution. Flagella were isolated by the dibucaine method (Witman, 1986) with the following modifications: DTT was omitted from the HMDS solution. The mixture was then incubated at 75°C for 20 min. Protein bands were visualized with silver stain (Silver Stain Plus; Bio-Rad). To prepare whole flagella for western blotting, a suspension of isolated flagella was mixed with SDS-sample solution and DTT as described above. For whole cells, cells were collected by centrifugation, lysed with the 5× SDS-sample solution and incubated at 75°C for 20 min in the presence of 50 mM DTT. Proteins in SDS-polyacrylamide gels were transferred onto Immobilon-P (Millipore). For western blots of whole cells, antibodies were diluted with HIKARI (Nacalai Tesque). The chemiluminescence or fluorophore signals from the secondary antibodies were captured with a FluorChem Q equipped with a CCD camera (ProteinSimple).

Mass spectrometry
Protein bands of interest were excised from silver-stained gels and subjected to trypsin digestion as described previously (Spiess et al., 2011). Mass spectrometry analysis was performed as described previously (Wagner et al., 2014) with the following modifications: the length of the LC column was 15 cm, and the flow rate of sample injection was 250 nl/min. The resulting spectra were searched against the C. reinhardtii Augustus 5 protein database (Erik Hom, personal communication; based on Assembly 4 of the C. reinhardtii genome from the Joint Genome Institute: http://genome.jgi-psf.org/Chlre4/Chlre4.home.html) using SEQUEST (Bioworks software, v3.3.1; Thermo Electron).

Flagella regeneration
To determine the kinetics of flagellar regeneration, the pH of mid-log phase cultures of 137c, nphp4 and NPHP4-R cells was lowered to 4.3 with 0.5 M acetic acid. As soon as deflagellation was confirmed by microscopy, the pH was raised to 6.9 with 0.5 M KOH. To fix cells at each time-point, a small aliquot of the cell suspension was mixed with an equal volume of M medium containing 2% glutaraldehyde. Flagella lengths were measured with the Segmented Line tool in ImageJ (National Institutes of Health) and mean values at each point were obtained from 30 cells.

Observation of IFT
137c and nphp4 cells at −104 cells/ml were used to observe IFT as described previously (Claige et al., 2010). Kymographs were made by ImageJ with the MultipleKymograph plug-in.

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Competing interests
The authors declare no competing interests.

Author contributions
J.A. designed and performed most experiments. S.T. performed the initial interpretation of the data. J.A. and G.B.W. prepared the manuscript. G.J.P and G.B.W. supervised and funded this study and helped to interpret the data.

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Supplementary material
Supplementary material available online at http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.155275/-/DC1
References


Fig. S1. Motility of *C. reinhardtii* cells analyzed by CASA. (A) Diagram indicating the two parameters used to calculate percent linearity (VSL/VCL x 100). VCL (velocity curvilinear) is “instantaneous” velocity determined point to point along the actual swimming path (d). VSL (velocity straight line) is calculated from the linear distance between the start and end points of swimming paths recorded by CASA (d’). (B) Means ± s.d. of VCL and VSL were calculated from values obtained from a total of 10 fields in each of 5 independent experiments. Statistical significance was determined by the Tukey-Kramer method: n.s., ≥0.05; *, <0.05-0.01; ***, <0.001.
Fig. S2. NPHP4 and CEP290 localize to the TZ independently of each other. (A) Four cep290 cells still within the mother cell wall were labeled with antibodies to acetylated tubulin and NPHP4; a merged image is on the right. (B) A nphp4 cell was labeled with anti-acetylated tubulin and anti-CEP290; a merged image is on the right. NPHP4 is localized normally in the TZ of the cep290 mutant, and CEP290 is localized normally in the TZ of the nphp4 mutant. (C) Immunofluorescence microscopy of ift46 cells labeled with antibodies against acetylated tubulin and NPHP4. The merged image is on the right. Bars are 2 µm.
Fig. S3. *nphp4* cells have abnormal flagellar protein composition that is rescued by transformation with wild-type NPHP4. Proteins of isolated membrane-plus-matrix of wild-type (WT), *nphp4*, and NPHP4-R flagella were separated in a 6% SDS-polyacrylamide gel. Rectangles with dashed lines indicate slices excised for analysis by mass spectrometry (see text, Table I, and Table S1).
Fig. S4. The absence of NPHP4 has little or no effect on IFT. (A) Western blots of isolated wild-type and nphp4 flagella probed with antibodies against IFT motor subunits (KAP and DHC1b); IFT complex-A protein IFT139; IFT complex-B proteins (IFT172, IFT81, IFT57, IFT52 and IFT46); and a subunit (BBS4) of the BBSome, an IFT cargo adaptor. The outer arm dynein intermediate chain IC2 was used as a loading control. (B) Kymographs of IFT in wild-type (WT) and nphp4 flagella. IFT was recorded by DIC microscopy. Bars are 1 μm. (C) Velocity and frequency of IFT particles in wild-type and nphp4 flagella. The values were determined from kymographs of eight movies analyzed for 10 s each. Anterograde and retrograde IFT are indicated by open and solid bars, respectively. P values between wild-type and nphp4: 0.0018 for anterograde velocity, 0.0016 for retrograde velocity, 0.0541 for anterograde frequency, and 0.5667 for retrograde frequency. Values were obtained by Student’s t-test.
Table S1. Complete list of proteins identified by mass spectrometry analysis of selected slices of SDS-polyacrylamide gel of isolated membrane-plus-matrix fractions of wild-type, nphp4 (Mutant), and NPHP4-R (Rescued) flagella. All proteins identified from slices A-T labeled in Figure S4 are listed with total number of peptides found for each. Protein IDs are from the Augustus 5 database. Proteins drastically decreased in nphp4 flagella are in red and those increased are in blue (see text for criteria). Membrane-associated proteins identified by less than 5 peptides in wild-type flagella and not detected at all in the nphp4 mutant flagella are in yellow. The protein ID for FMG-1B is not available (N/A). The gel slice from the rescued strain corresponding to slices S and T from wild type and the nphp4 mutant respectively was lost; FAP12 was considered decreased in the mutant flagella based on the many fewer peptides found for it in the mutant flagella as compared to wild-type flagella.

Download Table S1.
Table S2. Primers used in this study

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