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Caenorhabditis elegans ciliary protein NPHP-8, the homologue of human RPGRIP1L, is required for ciliogenesis and chemosensation

Lin Liu [a,1], Mingshu Zhang [b,1], Zhiping Xia [b], Pingyong Xu [b], Liangyi Chen [b,*,2], Tao Xu [a,b,*]

[a] Joint Laboratory of the Institute of Biophysics & Huazhong University of Science and Technology, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan 430074, PR China
[b] National Key Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, PR China

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ABSTRACT

Nephronophthisis (NPHP) is the most frequent genetic cause of end-stage renal failure in children and young adults. *NPHP8/RPGRIP1L* is a novel ciliary gene that, when mutated, in addition to causing NPHP, also causes Joubert syndrome (JBTS) and Meckel syndrome (MKS). The exact function of NPHP8 and how defects in NPHP8 lead to human diseases are poorly understood. Here, we studied the *Caenorhabditis elegans* homolog *nphp-8* (*C09G5.8*) and explored the possible function of NPHP-8 in ciliated sensory neurons. We determined the gene structure of *nphp-8* through rapid amplification of cDNA ends (RACE) analysis and discovered an X-box motif that had been previously overlooked. Moreover, NPHP-8 co-localized with NPHP-4 at the transition zone at the base of cilia. Mutation of *nphp-8* led to abnormal dye filling (Dyf) and shorter cilia lengths in a subset of ciliary neurons. In addition, chemotaxis to several volatile attractants was significantly impaired in *nphp-8* mutants. Our data suggest that *NPHP-8/RPGRIP1L* plays an important role in cilia formation and cilia-mediated chemosensation in a cell type-specific manner.

1. Introduction

Cilia are highly conserved microtubule-based organelles that play an important role in motility, sensory perception, and signal transduction [1–5]. Generally, cilia are composed of nine parallel microtubule doublets that extend from a modified centriole, termed the basal body. Transition zone or basal body dysfunction has been shown to be involved in a number of rare syndromes, including Nephronophthisis (NPHP), Joubert syndrome (JBTS) and Meckel syndrome (MKS). Based on genomic analysis, ten NPHP genes (*NPHP1–9* and *NPHP11*), seven JBTS genes (*JBTS1–7*) and nine MKS genes (*MKS1–9*) have been associated with these disorders [6–8]. In mammals, RPGRIP1L was first identified in individuals with cerebello-oculo-renal syndrome (CORS) and MKS (MKS5), and mutation analysis suggested that RPGRIP1L was involved in JBTS (JBTS7) and NPHP (NPHP8). Domain analysis has also revealed that RPGRIP1L contains an N-terminal coiled-coil and two C2 domains [9,10]. At the single cell level, RPGRIP1L resides at the basal body and interacts with other NPHP proteins, such as NPHP4 and NPHP6 [9–12]. However, because a knockout of the *RPGRIP1L* gene in mice is lethal [9], the exact function of RPGRIP1L and how its dysfunction leads to disease are still unknown.

Caenorhabditis elegans (*C. elegans*) is a powerful model system that has been used to study the roles of highly conserved proteins in cilia formation and function. In worms, cilia are only present at the tips of the sensory neurons in the head and tail to sense environmental signals. In *C. elegans*, homologous proteins that are associated with human ciliopathies usually reside specifically in sensory neurons and have diverse functions [13–17]. All cilia genes have been shown to be regulated by the transcription factor DAF-19 in *C. elegans* through an X-box motif, a conserved 14-nucleotide regulatory element located approximately 100 bp upstream of the start codon [18].

In *C. elegans*, the sequence name of the RPGRIP1L ortholog is *C09G5.8*. Here, we renamed it *nphp-8* based on its homology to the mammalian protein NPHP8. In this paper, we analyzed the gene structure of *nphp-8*, including its 5′- and 3′-termini, for the first time. Consistent with its expression in ciliated sensory neurons, an X-box element in the predicted promoter region was identified based on our rapid amplification of cDNA ends (RACE) results. The NPHP-8 protein specifically co-localized with NPHP-4 at the transition zones at the bases of the cilia. Disruption of *nphp-8* resulted in reduced cilia lengths and defective dye filling (Dyf) in a subset of ciliated neurons. Functionally, the *nphp-8* null worm exhibited a reduced response to volatile chemical odors. We propose that NPHP-8 may interact with other ciliary proteins at the transition zone to regulate the delivery of receptors or signaling

* Corresponding authors. Address: Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, PR China. Fax: +86 10 64871293.
E-mail addresses: chen_liangyi@yahoo.com (L. Chen), xutao@ibp.ac.cn (T. Xu).
[1] These authors contributed equally to this work.
[2] Present address: Institute of Molecular Medicine, Peking University, Beijing 100871, PR China.

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proteins to the distal tips of cilia and modulate ciliogenesis and ciliary function.

2. Materials and methods

2.1. Strains

Worm strains were obtained from the Caenorhabditis Genetics Center (CGC) and the National BioResource Project in Japan and were grown using standard *C. elegans* growth methods at 20 °C [19]. The *C. elegans* strain N2 Bristol was used as the wildtype. The following other strains were also used: PT709 *nphp-4(tm925)*, *nphp-8(tm3100)*, GJ443 gjIs140[*gpa-4::gfp*; *dpy-20(+)*], Ex[NPHP-8::SL2-GFP] in N2 worms, Ex[NPHP-8::mCherry; NPHP-4::GFP; pRF4] in N2 worms, Ex[P*nphp-8::nph-8*; P*myo-3::gfp*] in *nphp-8(tm3100)* worms, Ex[NPHP-8::mCherry; pRF4] in N2 and PT709 worms, and Ex[NPHP-4::GFP; pRF4] in N2 and *nphp-8(tm3100)* worms. All of the mutants were outcrossed to the wildtype N2 strain at least five times.

2.2. Molecular cloning and transgenic worms

To construct the genomic plasmid pXTL80 (P*nphp-8::nphp-8*), a 13.5 kb fragment containing the 3.9 kb promoter and the 3'untranslated region were cloned into the pPD49.26 vector (Addgene plasmid 1686). Vector pXTL115 was obtained by replacing the GFP sequence in pPD95.75 (Addgene plasmid 1494) with the mCherry sequence. Two *nphp-8* gene fragments were obtained using PCR. A 5.9 kb fragment containing the 3.9 kb promoter and the genomic sequence (exons and introns) up to exon 6 and another 3.0 kb fragment containing all of the exons downstream of exon 6 were amplified from the total cDNA library. These two sequences were inserted into vector pXTL115 in the proper order to generate the translational fusion pXTL123 (NPHP-8::mCherry). To create the SL2 expression vector pXTL162 (NPHP-8::SL2-GFP), the two fragments (the 3.0 kb *nphp-8* fragment with a stop codon) were cloned into the pJG7 vector. The pXTL127 (NPHP-4::GFP) vector was generated by restriction digest to cut the *nphp-4* promoter and gene from the NPHP-4::YFP vector and inserting the 6.0 kb fragment into the pPD95.75 vector. The wild-type or mutant worms received injections of plasmid DNA at concentrations of 10–100 ng/ml using 50 ng/ml of pRF4 or 20 ng/ml of P*myo-3::gfp* as a transformation marker. The total cDNA library was obtained from reverse transcribed mRNA using oligo-dT (Omniscript Reverse Transcriptase, Qiagen). All PCRs were performed using the Expand Long Range DNA Polymerase (Roche, USA) according to the manufacturer's instructions. All products were confirmed by sequencing.

2.3. RACE analysis

Total RNA was extracted from mixed stage Bristol N2 animals with the RNeasy Mini Kit, as described by the manufacturer (Qiagen). One microgram of total RNA was used to perform first-strand cDNA synthesis and was incorporated into either the 5′- or 3′-RACE adaptor sequence, as described by the manufacturer (SMART RACE cDNA Amplification Kit, Clontech).

2.4. Confocal imaging

Young adult worms were anesthetized with 50 mM NaN_3 and immobilized on a 2% agar pad. Images were captured with either a confocal laser scanning microscope (FV500, Olympus Optical Co., Tokyo, Japan) or a Zeiss Observer Z1 inverted microscope with an Evolve EMCCD (Photometrics, AZ, USA) controlled by Sidebook (Intelligent Imaging innovations, Inc, Co, USA). Further image processing was performed using ImageJ (NIH).

2.5. Co-immunoprecipitation

FLAG-tagged NPHP-8 and HA-labeled NPHP-4 were co-transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions. Transfected cells were collected and lyzed using 1 ml of lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA pH 8.0, and 0.1% NP-40) containing 1% PMSF and 1% protease inhibitors (Sigma). The cell extracts were frozen and thawed three times, and the insoluble material was pelleted at 15,000 rpm for 20 min. For co-immunoprecipitation experiments, 1 ml of supernatant was added to 50 μl of a 1:1 slurry of anti-FLAG M2 affinity resin (Sigma) and incubated for 1 h at 4 °C. The beads were washed three times for 5 min with PBS buffer, and the proteins were removed from the beads with 50 μl of SDS-loading buffer. Protein (25 μl) was loaded onto 8% Tris–glycine SDS–PAGE gels and transferred onto PVDF membrane for Western blot analysis.

2.6. Dye filling assay

Standard dye-filling assays were performed as previously described [20]. Briefly, a stock solution of 2 mg/ml DiI (Molecular Probes; Carlsbad, CA, US) in dimethyl sulfoxide was diluted 1:200 in M9 buffer. Young adult worms were collected and washed three times with M9 buffer to remove bacteria, incubated for 1 h in the DiI solution at 20 °C, and washed another three times with M9 buffer. The worms were transferred to a freshly seeded NGM plate and incubated at 20 °C for 1 h before observation.

2.7. Behavior assays

Assays measuring chemotaxis to a volatile attractant were performed as described [21,22]. Briefly, two spots were marked at opposite sides of a plate, 0.5 cm from the edge, and 1 μl of 1 M sodium azide was placed on each spot. Worms were collected, washed three times with S buffer, and pipetted onto the center of the plate. Then, the S buffer was removed quickly with filter paper. In the chemoattractant spot, 1 μl of the attractant was added. On the opposite side, 1 μl of ethanol was added as a control. After 60 min, the index of chemotaxis was calculated as the number of worms at the attractant zone minus the number of worms at the control zone divided by the total number of worms. The different attractants employed benzaldehyde (diluted in ethanol at 1:200), isoamyl alcohol (1:100), pyrazine (1:100), diacetyl (1:1000), 2,3-pentanedione (1:10,000) and 2,4,5-trimethylthiazole (1:1000). All chemicals were obtained from Sigma (St. Louis, MO).

3. Results

3.1. RACE analysis of the C. elegans RPGRIP1L homolog nphp-8

The *nphp-8* gene was predicted to have 22 exons (WormBase, release version WS219); however, we failed to amplify the full-length cDNA sequence with RT-PCR. Using two pairs of primers designed to amplify both the N-terminal and C-terminal fragments joined at exon 13, we only obtained the C-terminal fragment. When 5′ forward primers for the N-terminal half were changed from the predicted start codon in exon 2 to downstream exons along the *nphp-8* gene, the fragments were amplified only when the forward primer appeared in exon 7 and exon 8 (Fig. S1). These results suggest that the start codon of *nphp-8* gene is somewhere between exon 6 and exon 7. As shown in Fig. 1B, the 5′ RACE analysis indicated a loss of the first six exons and a new start codon in

C

genes	location	X-box sequence
nphp-1	-77 to -63	GTTGCCAGGGGCAAC
nphp-4	-168 to -155	ATTTCCATGACAAC
osm-5	-129 to -115	GTTACTATGGCAAC
osm-6	-114 to -100	GTTACCATAGTAAC
bbs-7	-121 to -107	GTTGCCATAGTAAC
nphp-8	-119 to -104	GTTGCTCATGGCAAC

Fig. 1. Gene model and X-box sequence of *nphp-8*. (A) The predicted gene model from WormBase (www.wormbase.org, release version WS219) for *nphp-8* is shown. (B) The gene structure of *nphp-8* was identified by 5′- and 3′-RACE. The boxes and lines represent exon and intron sequences, respectively. The black triangle indicates the start codon for the RACE model. (C) The conserved X-box sequences in *nphp-8* and other *C. elegans* cilia genes.

intron 6, followed by a short exon and intron. After *trans*-splicing, an in-frame methionine residue was introduced into the new transcript (see Supplementary data). Therefore, the RACE analyses show that the *C. elegans* database incorrectly predicted the exons in the *nphp-8* gene.

In *C. elegans*, *nphp-8* expression in ciliated neurons is normally regulated by an X-box element in the promoter region, which is bound by the RFX-type transcription factor DAF-19. In the previously predicted gene structure of *nphp-8*, no X-box element was found in the promoter region. However, based on the new gene structure of *nphp-8* from our RACE analysis, an X-box motif was found 119 bp upstream of the start codon, similar to the X-box motifs found in other cilia genes (Fig. 1C).

3.2. NPHP-8 localizes to the transition zone in a subset of ciliated neurons in C. elegans

To verify the expression profile of *nphp-8*, we generated an expression vector in which *nphp-8* is driven by its own promoter. A specialized spliced leader (SL) site, SL2, was inserted between the full-length *nphp-8* and *gfp* genes. SL2 was first found in the *C. elegans* operon and facilitated the processing of polycistronic pre-mRNAs into monocistronic mRNAs by trans-splicing [23,24]. Using this method, we drove the expression of *nphp-8* and *gfp* in the same cells. Strong NPHP-8 expression was found in a subset of ciliated sensory neurons in hermaphrodites. The expression of *nphp-8* overlapped with but was not limited to, DiI loaded neurons, including six neurons in the head (ASI, ASH, AWB, ASJ, ASK, and ADL) and two neurons in the tail (PHA and PHB) (Fig. 2A) [25]. The expression of NPHP-8 in the ciliated sensory neurons of *C. elegans* is also consistent with the presence of an X-box motif.

To determine the subcellular localization of NPHP-8 in *C. elegans*, an expression construct containing NPHP-8 fused to red fluorescent protein mCherry, driven by its own promoter, was created. The mCherry-tagged NPHP-8 was only observed at the distal end of dendrites of amphid and phasmid neurons in hermaphrodites under a spin-disk confocal microscope (Fig. S2). Little or no fluorescence was found in the ciliary axonemes. Because NPHP-4 has been reported to be located at the transition zone in *C. elegans* [15], we co-expressed NPHP-8::mCherry with NPHP-4::GFP and determined their relative subcellular localizations. As shown in Fig. 2B, NPHP-8 co-localized with NPHP-4 at the transition zone at the base of the cilia.

3.3. Cilia formation is abnormal in the nphp-8 mutants

To determine the function of *nphp-8*, we first explored whether the *nphp-8* mutants exhibited any abnormalities associated with ciliogenesis or cilia morphology. The ability of worms to take up a fluorescent dye was used to detect the structural integrity of the sensory cilia. When loading the *nphp-8* mutants with DiI, we found that the ASI neuron failed to fill with the dye, and the AWB neuron filled with less dye than other neurons. The dye-filling (Dyf) phenotype could be rescued by reintroducing the *nphp-8* gene into the mutants (Fig. 3A). Defects in tail neurons were also observed. Compared to 100% normal DiI loading in the phasmid neurons of wildtype N2 worms, the *nphp-8* mutants exhibited 20% lower dye loading (Table S1).

To further evaluate possible defects in the cilia structure of the ASI neuron, we crossed *nphp-8(tm3100)* worms with GJ443 worms, which stably express GFP in ASI neuron, and calculated the cilia lengths. The cilia lengths of the *nphp-8(tm3100)* worms were significantly shorter than those of the N2 worms (Fig. 3B). These data suggest that NPHP-8 plays an important role in cilia formation in *C. elegans*.

3.4. Cilia sensory function is defective in the nphp-8 mutants

Next, we examined the functional consequence of the *nphp-8* mutation on ciliary function using a chemotaxis assay. Following a standard protocol, we characterized the responses of worms to six attractants. Compared to the wildtype N2 worms, the *nphp-8* mutants responded normally to pentanedione, diacetyl and trimethylthiazole but displayed a significantly reduced response to pyrazine, benzaldehyde and isoamyl alcohol (Fig. 4A). To confirm that the defect was caused by the loss of *nphp-8*, three independent rescue lines were generated, and benzaldehyde was chosen for the chemotaxis assay. As shown in Fig. 4B, the delay in response toward benzaldehyde was rescued in all of the lines. To address whether the *nphp-8* mutants exhibited any other behavioral defects, we measured the pumping rate, defecation, progeny number and locomotion. As shown in Fig. S4, no difference was found between the wild-type N2 worms and the *nphp-8* mutants. These results suggest that NPHP-8 is required for sensing the surrounding environment.

4. Discussion

The identification of genes that cause inherited cystic kidney diseases has triggered a major interest in the concept of "ciliopathies". However, the molecular mechanisms, potential connections and clinical variability of the various forms of ciliopathies remain poorly understood. The recent identification of *RPGRIP1L/NPHP8* as a gene associated with JBTS, MKS and NPHP offers a new opportunity for the elucidation of the mechanisms of ciliopathies. In this paper, we identified the *C. elegans* ortholog *C09G5.8* of human

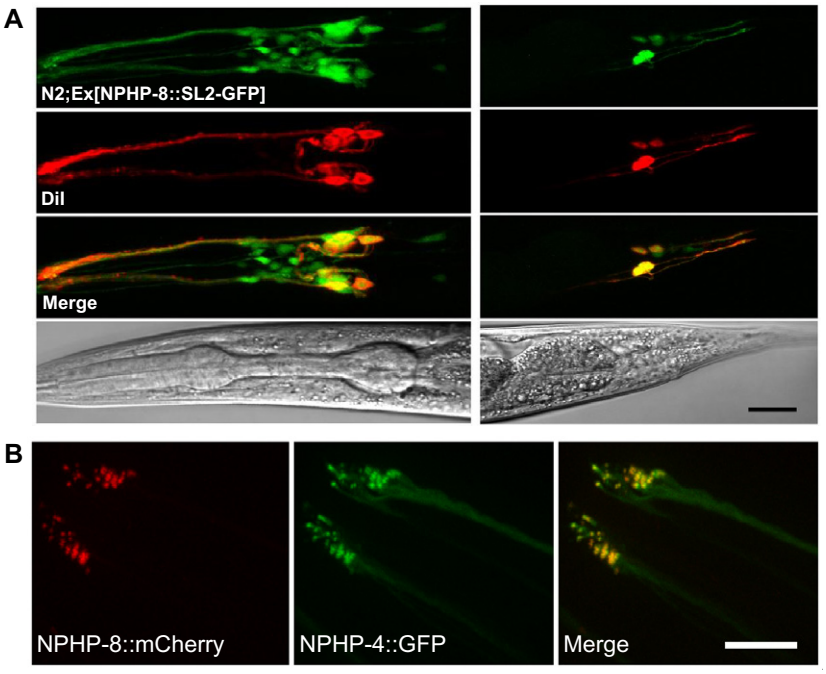

Fig. 2. Expression pattern of *nphp-8* in *C. elegans*. (A) NPHP-8::SL2-GFP was expressed in the ciliated sensory neurons, including the amphid neurons in the head (left panels) and the phasmid neurons in the tail (right panels). A subset of the GFP-expressing neurons overlaps with the DiI-loading neurons. (B) Transgenic lines expressing NPHP-8::mCherry and NPHP-4::GFP were generated under their endogenous promoters. The mCherry fluorescence was concentrated in the transition zone and co-localized with NPHP-4 in the amphid neurons. All images are compressions of a 3D deconvoluted z stack of amphid ciliated neurons. Scale bars, 20 μm (A) and 10 μm (B).

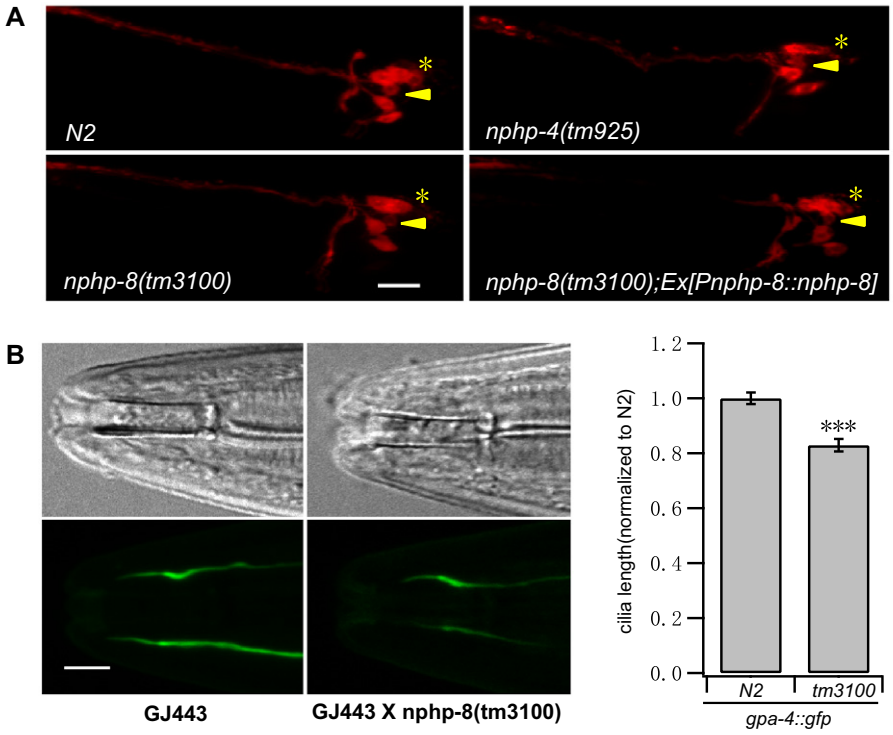

Fig. 3. Cilia formation analysis in the *nphp-8* mutant. (A) Cilia formation was analyzed using the DiI loading method in wildtype N2, *nphp-4* mutant, *nphp-8* mutant and *nphp-8(+)* rescued worms. The *nphp-4* and *nphp-8* mutant worms failed to take up DiI in the ASI neuron (marked by the yellow asterisk), which was rescued by expression of the *nphp-8* gene in the *nphp-8(tm3100)* worm. Arrowheads indicate the AWB neuron. (B) To evaluate the possible effects on cilia length, the cilia lengths were calculated in N2 and *nphp-8(tm3100)* worms expressing *gpa-4::gfp* carried by GJ443 worms. Data were normalized to the average length of the wild-type cilia (N2: 1, $n = 14$; tm3100 worms: 0.8293, $n = 14$). All images are compressions of 3D deconvoluted z stacks of amphid ciliated neurons. Statistical significance was evaluated using Student's *t*-test. *** denotes statistical significance with *p*-values less than 0.001. Scale bar, 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

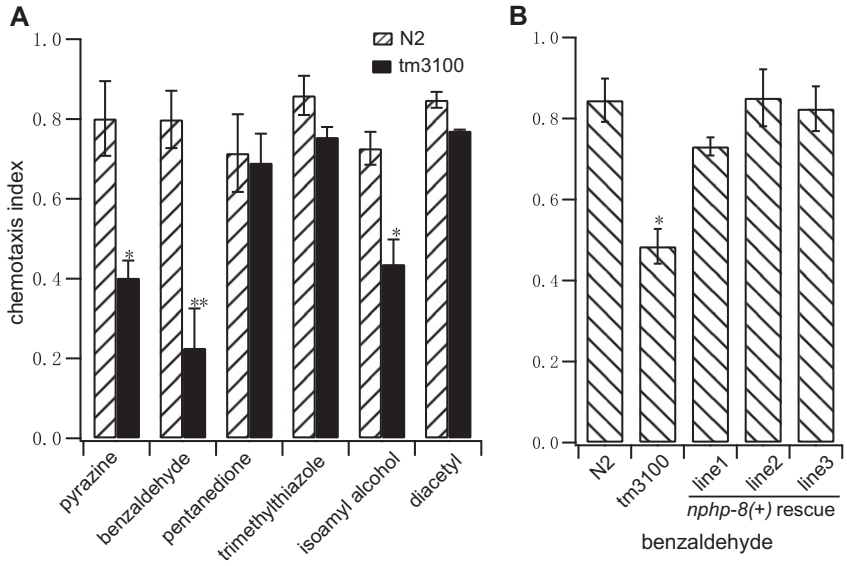

Fig. 4. Chemotaxis defects in the *nphp-8* mutants. (A) Comparison of the chemotaxis index for N2 and *nphp-8(tm3100)* worms in response to six different attractants. (B) The *nphp-8* transgenic lines significantly rescued the chemotaxis defect response to benzaldehyde. Data are presented as the means ± standard error of the mean (SEM). Statistical significance was evaluated using Student's *t*-test. * and ** denote statistical significance with *p*-values less than 0.05 and 0.01, respectively.

NPHP8 and characterized its function. Using RACE analysis, we showed that the transcript sequence of *nphp-8* is different from the originally predicted model. In addition, we identified an X-box sequence in the new promoter region of *nphp-8*, which was absent in the predicted model. These results demonstrate that the previously predicted gene model of *nphp-8* in WormBase may be incorrect. This may explain why the *nphp-8* gene was not identified as a significant ciliary gene by comparative genomics and serial analysis of gene expression (SAGE) in previous studies [7,26].

Our data show that NPHP-8 localizes to the transition zone in *C. elegans*, which agrees with the localization of the mammalian homolog RPGRIP1L to the basal body in MDCK-II cells and in the retina [9,10]. The co-localization and physical interaction between NPHP-8 and NPHP-4 (Fig. S3) suggest that these proteins form a complex forms the transition zone. The *nphp-8* mutants show a defect in Dyf and short cilia. Based on these findings, combined with the result that the *nphp-8* mutations affect the localization of B9 proteins and NPHP-4 [27], we conclude that the *nphp-8* mutations play a key role in the transition zone formation and result in axonemal defects in a cell type-specific manner.

We further found that the responses to different volatile odors were differentially affected in the *nphp-8* mutant. Because pyrazine and diacetyl are sensed by the AWA neuron, benzaldehyde, pentanedione and isoamyl alcohol are sensed by the AWC neuron, and trimethylthiazole can be detected by the AWA and AWC neurons [28,29], our results suggest that the *nphp*-8 mutations affect the responses of the AWA and AWC neurons to a subset of odors. Therefore, the different responses of the *nphp-8* mutants to odors indicate that NPHP-8 may regulate the subcellular localization or signaling of odor receptors in the sensory neurons of *C. elegans*, which may interrupt signal transduction from the environment to the cell interior. Taken together, we have characterized the *C. elegans* homologue of *nphp-8*, and proposed that NPHP-8 resides in a complex containing NPHP-4 at the transition zone and participates in the structural and functional assembly of cilia.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.06.041.

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