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RPI-1 (human DCDC2) displays functional redundancy with Nephronophthisis 4 in regulating cilia biogenesis in C. elegans

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Abstract: Projecting from most cell surfaces, cilia serve as important hubs for sensory and signaling processes and have been linked to a variety of human disorders, including Bardet-Biedl Syndrome (BBS), Meckel-Gruber Syndrome (MKS), Nephronophthisis (NPHP), and Joubert Syndrome, and these diseases are collectively known as a ciliopathy. DCDC2 is a ciliopathy protein that localizes to cilia; nevertheless, our understanding of the role of DCDC2 in cilia is still limited. We employed C. elegans to investigate the function of C. elegans RPI-1, a Caenorhabditis elegans ortholog of human DCDC2, in cilia and found that C. elegans RPI-1 localizes to the entire ciliary axoneme, but is not present in the transition zone and basal body. We generated a null mutant of C. elegans rpi-1, and our analysis with a range of fluorescence-based ciliary markers revealed that DCDC2 and nephronophthisis 4 (NPHP-4/NPHP4) display functional redundant roles in regulating cilia length and cilia positions. Taken together, our analysis discovered a novel genetic interaction between two ciliopathy disease genes (RPI-1/DCDC2 and NPHP-4/NPHP4) in C. elegans.

Key words: DCDC2, cilia, NPHP4, rare diseases

1. Introduction
Cilia are cellular protrusions that extend outward from the majority of cells in the human body and are generally separated into three ciliary subcompartments: the basal body (BB), the transition zone (TZ), and the axoneme. The TZ at the base of the cilia serves as a barrier to govern the entry and exit of components into the cilia (Garcia-Gonzalo et al., 2011; Chih et al., 2012). The ciliary axoneme is a microtubule-based core that originates from the basal body and is surrounded by the ciliary membrane and is generally separated into two parts: proximal and distal, with the proximal part containing a microtubule doublet (both A and B tubules) and the distal part containing a microtubule singlet (only A tubule extensions). Cilia exist in two types: motile and nonmotile cilia, also known as primary cilia. Motile cilia use propelling forces to move cells or fluid flow (such as the motility of the unicellular green alga *Chlamydomonas reinhardtii*), whereas the primary cilium senses the extracellular environment (such as the sensation of urine flow by cilia in the kidney) and acts as a cellular hub for signaling pathways, including Hedgehog (Hh), Wnt, and platelet-derived growth factor receptor (PDGFRα) pathways (Anvarian et al., 2019). Cilia dysfunction or structural abnormalities cause cilia-related human conditions called ciliopathies, including Bardet-Biedl Syndrome (BBS), (Valente et al., 2014) Meckel-Gruber Syndrome (MKS) (Hartill et al., 2017), Nephronophthisis (NPHP) (Luo and Tao, 2018), and Joubert Syndrome (JBTS) (Paris, 2009). Ciliopathies possess a wide range of phenotypic manifestations, including polydactyly, developmental delay, obesity, polycystic kidneys, and retinal degeneration. Proteomics, genetics analysis, and protein localization studies indicated the presence of several functional modules encompassing several disease genes at the TZ: The MKS module and the NPHP module (Sang et al., 2011; Williams et al., 2011; Yee et al., 2015; Li et al., 2016).

Cilia have a dedicated and unique bidirectional intraciliary transport system termed intraflagellar transport (IFT) for cilia assembly, maintenance, and function (Kozminski et al., 1993; Rosenbaum and Witman, 2002). IFT is made up of large protein complexes comprising kinesins, dynein, IFT-B, and IFT-A components. Kinesin-2 motors mediate the distribution of IFT cargos throughout cilia with the help of the IFT-B and IFT-A complexes, whereas ciliary cargos are removed from cilia by cytoplasmic dynein-2 motors that collaborate with the IFT-A complex (Blacque, 2008; Rosenbaum and Witman, 2002).

Human DCDC2 (doublecortin domain containing 2) causes a range of human diseases, including

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Nephronophthisis-related ciliopathies (NPHP-RC), human recessive deafness DFNB66, and Neonatal sclerosing cholangitis (NSC). (Grati et al., 2015; Schueler et al., 2015; Girard et al., 2016; Grammatikopoulos et al., 2016). DCDC2 was connected to dyslexia in 2005, however, a recent study indicated that deletion of DCDC2 did not enhance a risk factor for dyslexia (Meng et al., 2005; Scerri et al., 2017). DCDC2 was found to localize to cilia in a range of human and mouse cells but is excluded from the basal body (Grati et al., 2015; Schueler et al., 2015; Girard et al., 2016). DCDC2 was discovered to be associated with KIF3A, the IFT kinesin motor (Massinen et al., 2011). DCDC2 overexpression lengths cilia and stimulates the Shh pathway, whereas DCDC2 downregulation has little effect on cilia length but impacts WNT signaling (Massinen et al., 2011). It is, however, uncertain why shRNA-mediated DCDC2 downregulation leads to no defect in cilia length, maybe because of partial downregulation.

Hence, we created a null mutant of RPI-1, the Caenorhabditis elegans ortholog of mammalian DCDC2, with CRISPR/Cas9 to investigate the exact role of DCDC2 in cilia (The Alliance of Genome Resources Consortium et al., 2020). In cilia, RPI-1/DCDC2 colocalizes with ciliary proteins such as CEPH-41 (the homolog of Joubert syndrome-associated CEP41) and IFT-140 (the homolog of human IFT140). While single rpi-1 mutants exhibit no severe ciliary abnormalities, rpi-1; nphp-4 (human nephrocystin-4) double mutants have a variety of ciliary structural defects, including short cilia, mispositioned cilia, and ectopic projections from the base of cilia. Our findings demonstrated the conserved role of C. elegans RPI-1/DCDC2 in cilia biogenesis and first time revealed a novel genetic interaction between RPI-1/DCDC2 and NPHP-4.

2. Materials and methods

2.1. C. elegans strains, maintenance, and genetic analysis

All worms were cultured using the previously established standard techniques (Brenner, 1974). For mutant genotyping, polymerase chain reaction (PCR) was utilized. A null mutant for rpi-1, namely rpi-1(syb722) (C. elegans W07G1.5) was kindly generated by SunyBiotech with CRISPR/Cas9, and to exclude any background mutations, rpi-1(syb722) was outcrossed four times to the wild-type. gpa-6prom::gfp, str-2prom::gfp, nphp-4(tm925) (a component of NPHP module) and bbs-5(gk537) (a component of the BBSome) mutants were obtained from the C. elegans Genetic Center (CGC), while the Japanese National Bioresource Project provided the mks-5(tm3100) mutant (NBRP). str-1prom::mCherry was a kind gift from Piali Sengupta, Brandeis University, USA. Both MKS-6 and IFT-140 strains (vuaSi24[pBP43; Pche-11::che-11::mCherry; cb-unc119(+)]) II; unc-119(ed3) III; che-11(tm3433)V were gift from Peterman Lab and Dammermann Lab, respectively. To generate mutants with transgenic strains and double mutants, standard genetic crosses were performed. Primers are listed below:

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>rpi-1(syb722)</td>
<td>rpi-1 null mutant</td>
<td>Cevik and Kaplan, 2021</td>
</tr>
<tr>
<td>bbs-5(gk537)</td>
<td>bbs-5 null mutant</td>
<td>Cevik and Kaplan, 2021</td>
</tr>
<tr>
<td>nphp-4(tm925)</td>
<td>Human IFT140 homolog</td>
<td>Cevik and Kaplan, 2021</td>
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2.2. Osmotic avoidance assay

For the osmotic avoidance assay, a freshly prepared 8M fructose solution containing bromophenol blue was used to generate a ring (using 1 mL pipette tips) (Culotti and Russell, 1978). Both wild-type and cilia-deficient mutant osm-5(p813) worms were used as controls, and wild-type worms are anticipated to stay in the ring, but cilia-defective mutant osm-5(p813) worms are expected to cross the 8M bromophenol blue labeled ring. Each osmotic avoidance experiment, which takes 5 min, included five worms. Five worms were placed in the ring for each experiment, and
the osmotic avoidance experiment was performed five times for each strain.

2.5. Dye-filling assay
A dye uptake assay was conducted as previously described (Perkins et al., 1986). Well-fed and healthy wild types and mutants were incubated in the lipophilic fluorescent dye for 45 min before being examined microscopically as previously reported (Kaplan et al., 2010).

2.6. Statistical analysis and plots
R was used to construct plots, and for the cilia length measurement, the Kruskal-Wallis test was used to evaluate whether or not there was a significant statistical distinction between wild type, single, and double mutants. The Chi-Square test was performed to calculate the statistical significance of osmotic avoidance between wild-type, single, and double mutants. The distinction between wild type, single, and double mutants. The measurement, the Kruskal-Wallis test was used to evaluate whether or not there was a significant statistical significance of osmotic avoidance between wild-type and mutants (osm-5 and rpi-1). The codes used to generate statistical analyses have already been published (Turan, MG, et al., 2022), and the GitHub link is available here: https://github.com/thekaplanlab/Protocol-for-determining-the-average-speed-and-frequency-of-kinesin-and-dynein-driven-IFT.

3. Results

3.1. C. elegans DCDC2 localizes to the entire cilium in the sensory neurons
To determine the subcellular distribution of RPI-1 (a mammalian DCDC2 orthologue) in C. elegans, we tagged RPI-1 proteins with a green fluorescent protein (GFP) and coexpress fluorescent-tagged RPI-1 with ciliary axoneme and basal body marker IFT-140, transition zone marker MKS-6 or middle segment-specific marker CEPH-41 (Figure 1) (Mijalkovic et al., 2018; Cevik and Kaplan, 2021; Garbrecht et al., 2021). Transgenic strains coexpressing RPI-1::GFP and a red fluorescent-tagged ciliary marker were examined using a fluorescent confocal microscope to establish where RPI-1 is localized in C. elegans (Figure 1A). C. elegans RPI-1/DCDC2 appears to preferentially localize to cilia, with no enrichment in other cellular compartments, including cell body, dendrite, and axon, in the sensory neurons (Figure 1A). Close examination of RPI-1 reveals that RPI-1 localizes to the middle and distal segments (MS and DS) of amphid (head) and phasmid (tail) channels, and it does not colocalize with the transition zone marker MKS-6, suggesting that RPI-1 is not mostly detected in the transition zone (TZ) and basal body (BB) (Figures 1B, C, D, E, F, and G). Our cilia localization findings are consistent with previous reports that show DCDC2 localizes to cilia in human cells and C. elegans (Schueler et al., 2015; Grati et al., 2015; Jensen et al., 2016; Girard et al., 2016), but extend the initial findings by revealing that C. elegans RPI-1/DCDC2 is excluded from the TZ and BB. Taken together, these results suggest that RPI-1 localizes to the ciliary axoneme, but is absent from the TZ and BB.

3.2. Cilia length and morphology are unaffected in CRISPR-generated DCDC2 loss of function mutant in C. elegans
Increases in ciliary length and activation of Shh signaling have been reported in over-expressed DCDC2 mammalian cells, but the shRNA-mediated reduction of DCDC2 did not affect ciliary length (Massinen et al., 2011). However, the lack of effect on cilia length might be due to incomplete DCDC2 downregulation by shRNA. To determine whether the unexplored C. elegans ortholog of DCDC2 has a function in cilia formation in C. elegans, we created a null RPI-1/DCDC2 mutant with CRISPR/Cas9 in C. elegans. C. elegans RPI-1/DCDC2 has four exons and our RPI-1/DCDC2 mutant (namely rpi-1(syb722)) possesses 1418 base pair deletion, removing entire exon II and III, as well as the majority of exon I and IV and it is a likely a null allele of RPI-1 (Figure 2A).

The next step is to conduct a dye-filling experiment to test if rpi-1 mutants display any structural defects in cilia. The dye-filling assay has been used to assess the presence of structural defects in cilia (Perkins et al., 1986; Starich et al., 1995). In summary, wild-type worms dye their ciliated sensory neurons by absorbing the lipophilic fluorescent dye through their cilia, but mutants with abnormal cilia structures, such as ift-140(c1810) (Intraflagellar transport 140), are unable to stain their ciliated sensory neurons due to structural defects in cilia. We found that similar to wild-type, rpi-1 mutants soak up the lipophilic fluorescent dye into cells, suggesting there are no gross structural defects in cilia (Figures 2B, C, and D). In C. elegans, there are different types of cilia in the head and tail. AWC and AW sensory neurons in the head have wing-like cilia at the dendrite end, whereas PHA/PHB sensory neurons in the tail have a joint extended cilia. We then picked AWA (marked with gpa-6promoter::gfp), PHA/PHB (marked with gpa-6promoter::gfp), and AWC (marked with str-2promoter::gfp) to examine individual cilia structure and morphology. These fluorescence-based markers illuminate specific cilia structures and we crossed them into rpi-1(syb722) mutants. Confocal microscopy analysis revealed that cilia morphology was generally unaffected in rpi-1 mutants, but we observed some structural abnormality in AWA cilia (Figure 2F, G and H, I). Specifically, wild-type AWA cilia project multiple branches, whereas some rpi-1 mutants have fewer branches in AWA cilia (Figure 2F and I). Our fluorescence-based marker analysis was consistent with normal dye uptake. In addition, our osmotic avoidance experiment, which measures cilia function, demonstrated that rpi-1(syb722) mutants possess functional cilia (Figure 2E).
3.3. DCDC2 and nephronophthisis 4 regulate cilia morphology in a functionally redundant manner in C. elegans

Because oligogenic inheritance has been reported for ciliopathies, and mutations in human DCDC2 have been linked to nephronophthisis-related ciliopathies (NPHP-RC), we generated double mutants of rpi-1 with ciliopathy gene ortholog mutants (Nephronophthisis, Bardet Biedl syndrome and Meckel–Gruber syndrome) to investigate the genetic interactions (Hoefele et al., 2007; Schueler et al., 2015). We picked the mutants bbs-5(gk537) (a component of BBSome), mks-5(tm3100) (a component of MKS module), and nphp-4(tm925) (a component of NPHP module) because they are all ciliopathy disease genes, they do not have severe defects in cilia structure, and structural enhancement in cilia would be visible if they do. BBS-5 is a BBSome component that is found in the ciliary axoneme, whereas MSK-5 and NPPH-4 are transition zone
proteins. We chose PHA/PHB cilia for genetic analysis and generated single and double mutants expressing IFT-74::GFP (an endogenously tagged fluorescence IFT protein) (Yi et al., 2017). Confocal microscopy analysis of fluorescence-based marker revealed that double mutants of rpi-1 with bbs-5(gk537) or mks-5(tm3100) showed no additive defects in cilia length and cilia morphology, however, rpi-1; nphp-4(tm925) C. elegans double mutant exhibited several structural abnormalities in cilia (short and mispositioned cilia, ectopic project) (Winkelbauer et al., 2005; Jauregui and Barr, 2005; Yee et al., 2015; Li et al., 2016). We found that the PHA/PHB cilia length of rpi-1; nphp-4 double mutant is 13% shorter than that of the wild type (wild type = 8.24 μm and rpi-1; nphp-4 = 7.22 μm)
(Figure 3A and B). Furthermore, we noticed that the rpi-1; nphp-4 doubled mutant has mispositioned PHA/PHB cilia (Figure 3A).

We sought to expand on the initial findings in PHA/PHB cilia, thus we created a rpi-1; nphp-4 double mutant expressing the AWB-specific fluorescence-based marker (str-1promoter::mCherry) to examine different cilia in this double mutant. Similar to the findings of PHA/PHB, our examination showed that the AWB cilia (long cilia) of the rpi-1; nphp-4 double mutant are shorter than the wild type or either single mutant (Figure 4A and B). Furthermore, we noticed that the rpi-1; nphp-4 double mutant has an ectopic projection from the base of AWB cilia. Taken together, our findings indicate that nphp-4 and rpi-1 act in parallel pathways to regulate cilia length and cilia positioning.

4. Discussion
DCDC2 is a Doublecortin Domain Containing 2 known to cause human diseases, including Nephronophthisis-related ciliopathies (NPHP-RC), human recessive deafness DFNB66, and Neonatal sclerosing cholangitis (NSC) (Grati et al., 2015; Schueler et al., 2015; Girard et al., 2016; Grammatikopoulos et al., 2016). DCDC2 has been reported to localize to cilia, and DCDC2 overexpression impacts cilia length in humans (Massinen et al., 2011; Grati et al., 2015; Schueler et al., 2015; Girard et al., 2016). Both the human and C. elegans DCDC2 protein
belong to a doublecortin domain-containing family, and the doublecortin domain has been linked to microtubule polymerization (Horesh et al., 1999). Here, we characterize a null mutant of rpi-1, a C. elegans ortholog of human DCDC2, in C. elegans. Genetic analysis is a powerful approach to revealing genetic interactions between two genes, and our genetic analysis helps us to uncover a novel genetic interaction between two ciliopathy disease genes (RPI-1/DCDC2 and NPHP-4/NPHP4) in C. elegans. This novel genetic interaction was revealed by analyzing cilia length and morphology in single and rpi-1; nphp-4 double mutants.

Normally, NPHP-4 and RPI-1 are single mutations that do not play essential roles in determining cilia length and positioning. Combination mutations, on the other hand, cause severe ciliary abnormalities, including cilia length and cilia positioning, highlighting the importance of these components for cilia length and cilia position. Such genetic interactions revealed previously undiscovered RPI-1 functions.

Similar ciliary abnormalities (short cilia and cilia positioning defects) were previously reported in mutants lacking two TZ components (mks-6 and nphp-4) from the MKS and NPHP modules (Williams et al., 2011). However, the genetic connection of DCDC2 with NPHP-4, a transition zone protein, is surprising because NPHP-4 is a component of the Y-links and the DCDC2 protein is found in the ciliary axoneme in both humans and C. elegans, excluded from the transition zone. How these two distinct localizing proteins interact with one another remains to be determined.

On the other hand, Reiter and colleagues previously reported genetic interactions between bbs-5(gk507) (a component of the BBSome and axoneme localizing protein) and nphp-4(tm925) in regulating cilia morphology, implying that nphp-4 may act in a parallel pathway with two different axonemal proteins (RPI-1 or BBS-5) to regulate cilia morphology (Yee et al., 2015). While bbs-5 and rpi-1 interact genetically with nphp-4, we demonstrated that simultaneous deletion of rpi-1 and bbs-5 did not disrupt ciliary structure in C. elegans.

Mutations in human DCDC2 were implicated in Nephronophthisis-related ciliopathies (NPHP-RC), human recessive deafness DFNB66, and Neonatal sclerosing cholangitis (NSC). The implications of our findings for these disorders are somewhat unclear, but the oligogenic inheritance was reported for human ciliopathies, including Bardet–Biedl syndrome (BBS).
Furthermore, the clinical characteristics were influenced by a variety of genetic modifiers (Perea-Romero et al., 2022). We believe our findings may offer some kind of clue regarding oligogenic gene combinations and modifiers. For example, could NPHP4 and DCDC2 genes act as genetic modifiers for one another in humans? We can speculate mutations in these two genes might lead to oligogenic inheritance in human ciliopathies. Consistent with this speculation, Yoder and colleagues demonstrated that genetic interactions between bbs-5 and nphp-4 in C. elegans are conserved in mammals (Bentley-Ford et al., 2022). Importantly, the conserved genetic interactions between bbs-5 and nphp-4 in mammals suggest that the observed genetic interactions between rpi-1 and nphp-4 may also be preserved in mammals. To fully comprehend the significance of these genetic connections, more research is required.

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Informed consent

There are no human samples used in the current work.

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