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Article

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WDR31 is a novel ciliopathy protein displaying functional redundancy with GTPase-activating proteins ELMOD and RP2 in recruiting BBSome to cilium

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Abstract

The term “ciliopathy” refers to a group of over 35 rare disorders characterized by defective cilia and many overlapping clinical features, such as hydrocephalus, cerebellar vermis hypoplasia, polydactyly, and retinopathy. Even though many genes have been implicated in ciliopathies, the genetic pathogenesis in certain cases remains still undisclosed. Here, we identified a homozygous truncating variant in *WDR31* in a patient with a typical ciliopathy phenotype encompassing congenital hydrocephalus, polydactyly, and renal agenesis. WDR31 is an evolutionarily conserved protein that localizes to the cilium and cilia-related compartment. Analysis from zebrafish supports the role of *WDR31* in regulating the cilia morphology. The CRISPR/Cas9 knock-in (p.Arg261del) *C. elegans* model of the patient variant (p.Arg268*) reproduced several cilia-related defects observed in *wdr-31* null mutants. Mechanistic analysis from *C. elegans* revealed that WDR-31 functions redundantly with ELDM-1 (ELMOD protein) and RPI-2 (RP2) to regulate the IFT trafficking through controlling the cilia entry of the BBosome. This work revealed WDR31 as a new ciliopathy protein that regulates IFT and BBosome trafficking.

Introduction

Cilia are structurally and functionally distinct cellular projections, consisting of a microtubule-based axoneme extending from a centriole-derived basal body anchored at the plasma membrane. Cilia consist of multiple sub-compartments (basal body, transition zone, and ciliary tip) that display different protein compositions and structure (Blacque and Sanders, 2014; Rosenbaum and Witman, 2002; Satir and Christensen, 2007). Motile cilia mediate the movement of unicellular organisms such as *Chlamydomonas reinhardtii* or are involved in fluid movement across a tissue surface (Silflow and Lefebvre, 2001; Sleight, 1989). Non-motile cilia, also known as primary cilia, possess mechanosensory, chemosensory, and osmosensory functions, and coordinate a range of extrinsic signaling pathways involved in cellular behavior, tissue development, and homeostasis such as those mediated by Hedgehog (Hh), Wnt, and receptor tyrosine kinase ligands (e.g. PDGF α) (Anvarian et al., 2019; Bloodgood, 2009; Nachury, 2014; Scholey, 2007).

The relation between cilia and human disorders has led to a greater understanding of their importance for human health. Both motile and primary cilia have been linked to the heterogeneous class of diseases known as ciliopathies, including Joubert Syndrome, Meckel Syndrome (MKS), and Nephronophthisis (NPHP). Owing to the presence of cilia on most cell types, ciliary defects result in varying multiorgan phenotypes such as kidney defects, retinitis pigmentosa, pancreatic cysts, hearing loss, congenital heart disease, and polydactyly (Reiter and Leroux, 2017; Wheway et al., 2019).

Over the last twenty years, there has been a large effort to reveal the molecular composition of cilia and its sub-compartments using several independent approaches, including clinical genomics, proteomics, functional genomics, and bioinformatics (Arnaiz et al., 2009; Avidor-Reiss et al.,

2004; Blacque et al., 2005; Breslow et al., 2018; Choksi et al., 2014; Jensen et al., 2016, p. 26595381; Lambacher et al., 2016; Li et al., 2004; Mick et al., 2015; Piasecki et al., 2010; Ruiz García et al., 2019; Shaheen et al., 2016; Shamseldin et al., 2020; Sigg et al., 2017; UK10K Rare Diseases Group et al., 2016; van Dam et al., 2019). Many proteins that make up the cilium, as well as many proteins that regulate cilia biology, have been identified. These collective efforts have resulted in the identification of 302 genes that are certain to be involved in cilia biogenesis, as well as over 180 ciliopathy genes. CiliaCarta estimates the total number of the ciliary genes to be about 1200 genes, implying that many more ciliary proteins and ciliopathy genes are yet to be discovered. Indeed, the genetic diagnosis of many ciliopathy disorders is still unknown (Shamseldin et al., 2020, 2020; SYSCILIA Study Group et al., 2013; van Dam et al., 2019; Wheway et al., 2019).

Owing to the lack of protein-synthesizing machinery in cilia, structural components of cilia and cilia cargos must be transported to cilia in order to construct and sustain cilia, and mutations in genes involved in ciliary trafficking are commonly seen in ciliopathies. Cilia has a one-of-a-kind protein delivery system called intraflagellar transport (IFT). IFT is made up of multisubunit protein complexes that travel bidirectionally along the cilia. The IFT complex contains two sub-complexes, IFT-A and IFT-B, consisting of 6 and 16 protein subunits, respectively (Prevo et al., 2017). The IFT-B sub-complex and Kinesin-2 motors mediate the motility of IFT and IFT cargos from the base of the cilia to the tip of the cilia (anterograde IFT), while the IFT-A sub-complex and the cytoplasmic dynein-2 motor facilitate the retrograde IFT transport (from the cilia tip to the base of the cilia) (Blacque, 2008; Rosenbaum and Witman, 2002). The IFT-A is involved in the transport of certain membrane proteins into cilia (Lee et al., 2008; Liem et al., 2012; Mukhopadhyay et al., 2010). Mutations in genes encoding IFT components lead to defects in cilia

formation in all examined organisms, indicating the importance of IFT for cilia assembly (Pazour et al., 2000; Prevo et al., 2017).

Bardet–Biedl syndrome (BBS) was classified as a ciliopathy in 2003, and eight of the highly conserved Bardet-Biedl syndrome proteins (BBS1, BBS2, BBS4, BBS5, BBS7, BBS8, BBS9, and BBIP10) establish a stable protein complex called the BBSome that undergoes IFT. Work from a range of organisms implicates the BBSome in a variety of cilia related process including acting as a cargo adaptor for removing proteins from cilia and as a regulator of the assembly and stability of IFT trains (Ansley et al., 2003; Lechtreck et al., 2009; Loktev et al., 2008; Nachury et al., 2007; Nozaki et al., 2019; Ou et al., 2007, 2005; Wei et al., 2012; Williams et al., 2014; Xu et al., 2015; Ye et al., 2018). For example, in the nematode *C. elegans* mutants lacking *bbs-7* or *bbs-8*, detachment of IFT-A and IFT-B in the amphid cilia was reported in the anterograde direction (Ou et al., 2005). Analysis with a hypomorphic mutant (*bbs-1*) revealed that BBSome is involved in attaching IFT-B components to the retrograde IFT machinery at the ciliary tips in the channel cilia of *C. elegans* (Wei et al., 2012).

To identify new ciliopathy genes, we focused on our single-cell RNA-seq data that compared expression profiles of ciliated cells with those of non-ciliated cells in the nematode *C. elegans*, and this work revealed novel cilia genes, including *WDR31* (manuscript in preparation). Our gene discovery approach in combination with the use of clinical genetic testing and experimental validations identified *WDR31* (WD repeat domain 31) as a novel ciliopathy gene encoding a highly conserved cilia-associated protein. Our work from Zebrafish and *C. elegans* provides significant insight into the function of WDR31 in cilia biogenesis. First, our work from *C. elegans* revealed that WDR-31 functions redundantly with two GTPase activating proteins (GAPs) ELMD-1 (the sole ortholog of the human ELMOD proteins) and RPI-2 (human retinitis pigmentosa 2

orthologue) to control the cilia morphology. Second, knocking out *wdr-31* along with *elmd-1* or *elmd-1;rpi-2* causes IFT trafficking to be disrupted, resulting in ciliary tip accumulations of IFT-B components and the OSM-3/KIF17 motor, as well as significantly reduced BBSome recruitment to cilia. Taken together, WDR31 is a new ciliopathy-associated protein that exhibits functional redundancy with two GAP proteins (ELMOD and RP2) in regulating cilia morphology and IFT trafficking.

Material and Methods

C. elegans strains, maintenance and genetic crossing

For strain maintenance and genetic crosses, a standard procedure was followed, as described by Sidney Brenner in 1974 (Brenner, 1974). After genetic cross with a marker to generate single, double and triple mutants, we used the PCR strategy to trace the mutations in following mutants: *wdr-31(T05A8.5)(tm10423)II.*; *wdr-31(tur003)II.*; *wdr-31(syb1568)II.*; *nphp-4(tm925)V.*; *mks-6(gk674)I.*; *elmd-1(syb630) III.*; *RB1550 rpi-2(K08D12.2)(ok1863) IV.*; and *bbs-8(nx77) V.* Primers can be found in Table S1.

Lipophilic fluorescent dye-uptake assay and rescue analysis

Healthy mixed-staged animals were collected using M9 buffer (3 g/L KH₂PO₄, 6 g/L Na₂HPO₄, 5 g/L NaCl, 1 mM MgSO₄), centrifuged for 1 minute at 2000 rpm, and washed twice with M9 buffer to remove any bacterial contaminations. Worms were incubated for 45-60 minutes at room temperature in an M9 buffer containing lipophilic dye (1:200 dilution in M9, Invitrogen™ Vybrant™ DiI Cell-Labeling Solution) (Herman and Hedgecock, 1990). The worms were then washed twice with M9 before being moved to a new NGM plate. Wild type was always included

in Dye filling assay, and the dye uptake control for the wild type was performed under a stereotyped fluorescence microscope, followed by imaging with the fluorescence upright microscope. For the rescue experiment, *N2; turEx24[arl-13p::GFP::elmd-1 (C56G7.3)::unc-54 3'UTR +rol-6}* (OIK1045) were crossed into *wdr-31(T05A8.5)(tm10423)II.*, *elmd-1(syb630) II.*, double and *wdr-31(T05A8.5)(tm10423)II.*, *elmd-1(syb630) II.*, *rpi-2(K08D12.2)(ok1863) IV.* triple mutants. *N2; turEx21[arl-13p::wdr-31 (T05A8.5)::GFP::unc-54 3'UTR +rol-6}* (1 ng) (OIK1042) were mated with *T05A8.5(syb1568)II.*, *elmd-1(syb630) II.*, *rpi-2(K08D12.2)(ok1863) IV.* triple mutants. Plasmid (*arl-13p::wdr-31 (T05A8.5)::GFP::unc-54 3'UTR*) was directly microinjected into triple mutant *T05A8.5(syb1568)II.*, *elmd-1(syb630) II.*, *rpi-2(K08D12.2)(ok1863) IV* (1 ng). *dpy-5(e907); nxEx386[rpi-2::gfp + dpy-5(+)]* (MX352) were crossed into *wdr-31(T05A8.5)(tm10423)II.*, *elmd-1(syb630) II.*, *rpi-2(K08D12.2)(ok1863) IV.* triple mutants. Three independent Dye uptake assays were performed, and fluorescence filters were set for GFP and Texas Red, followed by fluorescence imaging. Dye uptake of mutants with corresponding transgenic strains was compared to that of non-transgenic strains in the same rescue plates (**Fig. S2C, D**).

Generation of mutants using CRISPR/Cas9 in the nematode *Caenorhabditis elegans*

To generate *wdr-31(T05A8.5)(tur003)* allele, three sgRNAs targeting *C. elegans* T05A8.5 (human WDR-31) were chosen using an online tool, Benchling [Biology Software] (2019), followed by ordering complementary oligonucleotides (Macrogen, South Korea), and cloning of sgRNAs into an empty sgRNA vector pRB1017. The successful sgRNA insert was confirmed with colony PCR, followed by plasmid isolation. Three sgRNAs (each 50 ng/μl) were injected into the gonads of wild type together with pDD162 (*Peft-3::Cas9*; 15 ng/ μl plasmid pRF4) and pRF4 (50 ng/ μl

plasmid pRF4) (Dickinson et al., 2013). F1s with the roller phenotype were identified, and after they generated enough progenies, the PCR technique was used to identify the F1 generation bearing the predicted deletion, as well as the homozygosity of the allele mutation. The PCR products from knockout animals were then sent to the Sanger sequencing (Macrogen, South Korea). *wdr-31(tur003)II*. mutants have 1276-bp deletion covering a huge part of exon II (297 bp out of 359 bp) and whole exon III, IV, and exon V. This is likely a null allele of *wdr-31*. sgRNA sequences can be found in Table S1.

Generation of transgenic strains and strain list for *C. elegans*

To generate transgenic lines for localization, rescue experiments, and expression patterns, we generated the following transgenic animals via microinjections.

OIK1042 turEx21[arl-13p::wdr-31 (T05A8.5)::GFP::unc-54 3'UTR +rol-6};
T05A8.5(syb1568)II., elmd-1(syb630) II, rpi-2(K08D12.2)(ok1863) IV. (1 ng)
OIK1044 N2;turEx23[elmd-1p::GFP::elmd-1 (C56G7.3)::unc-54 3'UTR +rol-6} (5 ng)
OIK1045 N2;turEx24[arl-13p::GFP::elmd-1 (C56G7.3)::unc-54 3'UTR +rol-6} (5 ng)
OIK1046 N2;turEx25[elmd-1p(C56G7.3)::GFP::unc-54 3'UTR +rol-6} (50 ng)
OIK1047 N2;turEx26[wdr-31 (T05A8.5)p::GFP::unc-54 3'UTR +rol-6} (50 ng)

The *rol-6* plasmid (50 ng/ μ l plasmid pRF4) was co-injected as the co-transformation marker. In brief, the plasmids were delivered by microinjections into the gonads of 1-day adult worms. Worms were initially transferred onto a 2.5% agarose pad prior (Halocarbon oil, Sigma: 9002-83-9), followed by microinjection. The microinjection was done using a Zeiss Axio Vert.A1 inverted microscope with DIC optics coupled with a Narishige Micromanipulator MMO-4. We next manually inspected the plates to find successful transgenics animals.

Wild Type and mutant alleles: N2; *FX30333*; *wdr-31(T05A8.5)(tm10423)II.*; *OIK393*
T05A8.5(tur003)II.; *PHX1568 T05A8.5(syb1568)II.*; *nphp-4(tm925)V.*; *mks-6(gk674)I.*;
PHX630, elmd-1(syb630) III.; *RB1550 rpi-2(K08D12.2)(ok1863) IV.*; *MX52, bbs-8(nx77) V.* We
 obtained *wdr-31(T05A8.5)(tm10423)II. (FX30333)* mutant allele, which has a 160-bp deletion,
 causing a frameshift, from the National Bioresource Project, Japan. The *wdr-*
31(T05A8.5)(tm10423)II. were outcrossed to wild type four times. The Caenorhabditis Genetics
 Center (CGC), Minnesota, USA, provided the *RB1550 rpi-2(K08D12.2)(ok1863)* mutant, and the
rpi-2(ok1863) allele contains 1143-bp deletion that removes a large segment of exon III, exon IV,
 and some portion of exon V. *T05A8.5(syb1568)II.* has 1888-bp deletion, deleting all exons except
 exon I (**Fig. S3B**). Sunybiotech created an independent null allele of *elmd-1* via CRISPR–Cas9.
 The *PHX630, elmd-1(syb630) III.* mutant contains 1784-bp deletion, where except for exon I, all
 exons were removed (**Supplementary Fig. 2B**). *elmd-1(syb630) III.* were outcrossed to wild type
 two times.

196

Fluorescent transgenes for IFT proteins: *GOU2162 che-3(cas443[gfp::che-3]) I;* *xbx-*
1(cas502[xbx-1::tagRFP]) V; *GOU2362 ift-74(cas499[ift-74::gfp]) II.*; *EJP76 vuaSi15*
[pBP36; Posm-6::osm-6::eGFP; cb-unc119(+)] I; *unc-119(ed3) III;* *osm-6(p811) V;*
N2;lqIs2[osm-6::gfp], N2;ejEx[osm-3::GFP + pRF4]; N2;ejEx[kap-1::gfp+pRF4]; EJP81
vuaSi24 [pBP43; Pift-140::ift-140::mCherry; cb-unc-119(+)]II; *unc-119(ed3) III;* *ift-*
140(tm3433) V; *jhuEx [ift-140::GFP+pRF4]; Ex[rpi-2::GFP+ xbx-1::tdTomato+pRF4]; MX76*
dpy-5(e907); nxEx(bbs-7::gfp+dpy-5 (+)).

Fluorescent transgenes for ciliary proteins: *PHX1180, wdr-31(syb1180 [wdr-*
31(T05A8.5)::GFP]); PHX1328, wdr-31(syb1328[wdr-31(^{R261-de})::GFP]) (Disease mimicking

206 variant); *OIK1042* *N2; turEx21[arl-13p::wdr-31 (T05A8.5)::GFP::unc-54 3'UTR +rol-6} (5 ng);*
 207 *OIK1044* *N2; turEx23[elmd-1p::GFP::elmd-1 (C56G7.3)::unc-54 3'UTR +rol-6} (5 ng);*
 208 *OIK1045* *N2; turEx24[arl-13p::GFP::elmd-1 (C56G7.3)::unc-54 3'UTR +rol-6} (5 ng);*
 209 *OIK1046* *N2; turEx25[elmd-1p(C56G7.3)::GFP::unc-54 3'UTR +rol-6} (50 ng); OIK1047*
 210 *N2; turEx26[wdr-31 (T05A8.5)p::GFP::unc-54 3'UTR +rol-6} (50 ng); N2; Ex[mks-2::GFP +*
 211 *tram-1::tdTOMATO + pRF4]; MX1409* *N2; nxEx785[tax-4:gfp+ Posm-5::xbx-1::tdTomato +*
 212 *rol-6(su1006)]; vuaSi21[pBP39; Pmks-6::mks-6::mCherry; cb-unc-119(+)]II; MX2418*
 213 *N2; nxEx1259[pbbs-8::PLC-delta PH::GFP; MKSR-2::tdTomato; coel::GFP]; PY8847*
 214 *oyIs65[str-1p::mcherry]; Ex[str-1p::nphp-4::gfp, unc122p::dsRed].* All PHX strains were
 215 generated using CRISPR/Cas9 by Sunybiotech. The list of extensive transgenic and mutant strains
 216 was provided in Table S2.

217

218 **Fluorescence and Confocal microscopy for *C. elegans***

219 Fluorescence images (dye assay) and time-lapse movies (IFT movies) were captured using an epi-
 220 fluorescence upright microscope (Leica DM6 B) (3 frames per second, and up to 120 frames). The
 221 epi-fluorescence upright microscope was controlled with iQ3.6.2 Andor software and was attached
 222 with an Andor iXon Ultra 897 EMCCD (an electron-multiplying charge-coupled device camera).

223

224 The LSM900 confocal microscope with Airyscan 2 (ZEN 3 Blue edition software) was used to
 225 capture the high-resolution Z-stack images. On microscope slides, a drop of 2-3% agarose was
 226 used to create an agarose pad, and worms were then moved to the agarose pad containing 1-3 μ l
 227 of 10 mM levamisole (an anesthetic agent). Images were collected at 0.14 μ m intervals with a Plan

ApoChromat 63x/1.40 NA target, then analyzed with Blue edition software ZEN 3 to create Z-stacks, and processed with ImageJ (NIH) software (Schneider et al., 2012).

***In vivo* intraflagellar transport assay for IFT frequency**

The time-lapse movies of GFP-labelled IFT proteins were analyzed with the automatic kymograph analyzing tools KymographClear and KymographDirect, both of which are ImageJ based (Mangeol et al., 2016). KymographClear was used to produce kymographs from time-lapse movies. We examined each produced kymograph and calculated IFT frequency for IFT-74::GFP (a single copy transgene), OSM-6::GFP (a single copy transgene), OSM-3::GFP, and IFT-140::GFP in wild type, *wdr-31;elmd-1* double and *wdr-31;elmd-1;rpi-2* triple mutants.

Osmotic Avoidance Assay for assessing cilia functions in *C. elegans*

Right before each experiment, we freshly prepared an 8M fructose solution containing bromophenol blue and made a ring of 8M bromophenol blue solution using 1 ml pipette tips. 1-day adult worms of wild type and *osm-5(p813)* mutants were always tested as positive and negative controls before evaluating the mutant strains (Haycraft et al., 2001). Wild-type worms are supposed to remain in the ring under normal conditions, while *osm-5(p813)* mutants may be able to escape due to a defect in the cilia structure. Every assay lasted 5 minutes, and five worms were placed in the ring. At least three separate experiments (on different days) were conducted for each strain, and the total number of worms was more than 40 for wild type and *osm-5(p813)*, and more than 80 for the rest of mutants. Fig. S4A was a combined representation of the last two independent experiments.

Whole-mount *in situ* hybridization in zebrafish

Whole-mount *in situ* hybridization was performed as previously described (Thisse et al., 2004). The *wdr31* cDNA with T7 promoter was PCR amplified, with forward primer: ATGGGGAAGCTACAGAGCAAGTTC and reverse primer: TAATACGACTCACTATAGAAGCGAGCCACTTCAGTGATACTG, from a homemade cDNA library of zebrafish embryos at 24 hpf. The antisense probe for *wdr31* was then transcribed with digoxigenin-labeled UTPs and T7 RNA polymerases (Roche, Basel, Switzerland). The stained embryos were dehydrated in glycerol and photographed with a Nikon SMZ1500 stereomicroscope (Nikon, Tokyo, Japan).

Mutagenesis of *wdr31* in zebrafish using the CRISPR/Cas9 technology

Generation of zebrafish mutants using the CRISPR/Cas9 system was carried out as previously described (Chang et al., 2013). Briefly, two gRNAs targeting sequences in *wdr31* were chosen as following: 5'-ACCCATGTGTGTTGGGTACC-3' and 5'-GAAGCCATCCAGGAGTTCAG-3'. gRNA templates were PCR amplified and gRNAs were *in vitro* transcribed with T7 transcriptase (NEB, cat# M0251S). gRNAs and Cas9 protein (NEB, cat# M0251S) were simultaneously injected into the embryos at one-cell stage.

Immunofluorescence staining and microscopy

Immunostaining was performed as previously described (Xu et al., 2017). Briefly, embryos were fixed in cold Dent's fixative (80% methanol: 20% dimethyl sulfoxide) at -20°C overnight and then stored in methanol. Samples were permeabilized with 0.005% (m/v) trypsin for 30 minutes. Samples were blocked with blocking buffer (10% [v/v] goat serum in PBST), followed by incubation with primary antibody anti-acetylated tubulin (1:2000; Sigma-Aldrich, St. Louis, MO)

and then secondary antibodies conjugated with Alexa Fluor 568 (1:500; Invitrogen, Carlsbad, CA). Samples were mounted on ProLong Gold Antifade Mountant with DAPI (Invitrogen) and images were taken with a Nikon A1R confocal microscope.

Software and algorithms

Zen Blue	Zeiss	https://www.zeiss.com/corporate/int/home.html
Andor iQ3	Andor	https://andor.oxinst.com/
Fiji	ImageJ	https://fiji.sc
ImageJ	ImageJ	https://imagej.nih.gov/ij/
Illustrator (CS5.1)	Adobe, USA	https://www.adobe.com/
KymographClear	Peterman Lab	https://sites.google.com/site/kymographanalysis
R	R Core Team	https://www.r-project.org/

Statistical analysis and Generating Figures

For statistical analysis of dye assay, Fisher's exact test was performed. For data involving continuous variables like IFT speed and cilia length, Mann-Whitney U-test or Welch's t-test was used depending on the distribution of data. All statistical tests were performed using R software. The codes and files needed to generate figures and perform statistical analysis were openly shared, and the files and codes for making figures and performing statistical analysis may be accessed at <https://github.com/thekaplanlab/WDR31-ELMOD-RP2>.

Gateway Cloning (mammalian constructs)

Constructs for ELMOD3 (HsCD00288286) and WDR31 (HsCD00045652) were purchased

from Harvard Medical School. LR Reaction (Invitrogen, USA) was performed to transfer the target sequence into destination vectors ((N)RFP, (N)CFP plasmids) with a subsequent transformation into E.coli DH5 α . DNA was isolated according to Monarch® Plasmid Miniprep Kit (BioLabs, USA) and the PureYield® Plasmid Midiprep Protocol System (Promega, USA). Verification of successful cloning was done by sequencing (Eurofins, Luxembourg).

Immunofluorescence Staining (Mammals)

hTERT-RPE1 cells were transiently transfected according to Lipofectamine™ 3000 Reagent Protocol with ELMOD3-CFP and WDR31-RFP constructs followed by serum starvation for three days to induce cilia formation. Cells were fixed with 4% PFA for 45min at 4°C, permeabilized for 5min with 0.3% PBST and blocked with 10% goat serum in PBST at 4°C overnight. A primary antibody for ARL13B (1:50; Proteintech, Germany) and a secondary antibody conjugated to Alexa Fluor 647 (1:350; Invitrogen, USA) were used. Cells were mounted using Fluoromount-G (Invitrogen, USA).

Microscopy Setup (Mammals)

Images were captured using a Leica TCS SP8 scanning microscope (Leica Microsystems IR GmbH, Germany). The setup includes 488 nm, 532 nm and 635 nm pulsed excitation lasers as well as 100x oil immersion objective lens (NA 1.4) and a hybrid detector (HyD). Pixel number was 1024 x 1024 and optimal pixel size was determined by Nyquist calculation resulting in a size of 43 × 43 nm in XY. Additionally, Z-steps should not exceed 131 nm when acquiring a stack. Laser intensity was adjusted for each sample and images were recorded with 2x frame averaging.

Image Processing (Mammals)

Acquired images were processed (LasX, Leica Microsystems), deconvoluted (Huygens Software, SVI, Netherlands) and edited (FIJI software). The deconvolution was performed with a Classical Maximum Likelihood Estimation (CMLE) algorithm under experimentally defined settings. The background level was software estimated, the quality threshold was 0.001, the number of iterations was 50 and signal-to-noise ratio was set to 20. All images were brightness-corrected for the purpose of presentation.

Exome sequencing analysis.

Informed written consent was obtained from all participants under an IRB approved protocol (KFSHRC RAC# 2080006). Whole exome sequencing was performed on the DNA sample collected from proband as previously described (Saudi Mendeliome Group, 2015). Variant classification followed the recommendations of the American College of Medical Genetics (Richards et al., 2015).

Sequence analysis

Sanger sequencing and segregation analysis were done for the proband and family members.

Results

WDR31 and ELMOD are evolutionarily conserved constituents of cilia

As part of our ongoing effort to reveal novel cilia genes, using single-cell RNA-seq data, we conducted comparative expression analysis in *C. elegans* and discovered that the WDR31 and ELMOD orthologues, WDR-31 (T05A8.5) and ELMD-1 (C56G7.3), are likely expressed exclusively in ciliated sensory neurons (manuscript in preparation). The head (amphid) and tails

(phasmid) of *C. elegans* contain a total of 60 ciliated sensory neurons, and the expression patterns of genes can be reliably analyzed using fluorescence tagged promoter markers. Consistent with our predictions, promoter-based GFP reporters revealed that both WDR-31 and ELMD-1 are expressed in most of the ciliated sensory neurons (**Fig. S1A**). To explore if both proteins are concentrated within cilia, we investigated their subcellular localization in *C. elegans* and created CRISPR/Cas9-mediated knock-in of WDR-31::GFP and a transgenic animal expressing GFP-tagged ELMD-1. The endogenously expressed WDR-31::GFP is concentrated at the ciliary base in both head and tails, where it colocalizes with the basal body marker γ -tubulin (TBG-1, the ortholog of human TUBG1), and the IFT-140 (human IFT140) basal body signal (**Fig. 1A, B, C, and D**), so our co-localization data suggests that WDR-31 is a new cilia-associated protein. ELMD-1 is the sole orthologue of the three human ELMOD proteins (ELMOD1-3) that function as GTPase-activating proteins (GAP). We found that GFP::ELMD-1 localizes to the periciliary membrane compartment (PCMC) and the basal body (BB) (marked with IFT-140), but is proximal to the MKS-6-labeled transition zone that is adjacent to the BB (**Fig. 1E, F, and G**) (Kaplan et al., 2012).

To determine if both human WDR31 and ELMOD3 (also known RBED1, the top blastp hit for *elmd-1*) are concentrated within cilia in mammalian cell lines, we generated hTERT-RPE1 cells co-expressing WDR31-RFP and ELMOD3-CFP and stained them with a ciliary marker ARL13B. Furthermore, WDR31-RFP or ELMOD3-CFP were transiently transfected into hTERT-RPE1 cells, and they were additionally stained for ARL13B. Our super-resolution microscopy analysis revealed that both WDR31 and ELMOD3 are enriched in the cilium (**Fig. 2A, B and Fig. S2B and C**). Taken together, our complementary approach demonstrates that both WDR31 and ELMOD3 are evolutionarily conserved proteins associated with cilia.

A homozygous truncating WDR31 variant leads to a ciliopathy

WDR31 is a poorly characterized gene encoding a 367 amino acid long conserved protein with four WD domains, but with our WDR31 and ELMOD3 localization data, WDR31 and ELMOD3 emerge as a strong gene candidate for ciliopathy (**Fig. 3A**). Our ciliopathy patient database was scanned for the presence of mutations in *WDR31* and *ELMOD3*, and we identified a homozygous nonsense variant (NM_001012361.2: c.802C>T; p.Arg268*, rs ID: rs764141336) in a ciliopathy male patient. The conversion of the arginine residue at position 268 to a stop codon (R268*) in WDR31 prevents the translation of the last 99 amino acids with a WD domain (**Fig. 3A**). This variant is very rare in gnomAD (MAF 0.0000239) and in our database of 2,379 ethnically matched exomes (MAF 0.001). The patient possesses renal agenesis, congenital hydrocephalus, right microtia, right preaxial polydactyly, and congenital heart disease (**Fig. 3B, C D, and E**). Sanger sequencing confirmed the existence of a homozygous nonsense variant in the affected male while parents and two unaffected sisters (II:3, II:5) are heterozygotes for the mutation and unaffected brother and sister (II:4) had the wildtype genotype (**Fig. 3F, G**). Taken together, our clinical data linked a novel cilia gene *WDR31* to a typical ciliopathy.

Wdr31 regulates ciliogenesis in the Zebrafish ear

We turned our interest into zebrafish to develop an *in vivo* model for WDR31 to examine the role of WDR31. We first used the whole-mount *in situ* hybridization (ISH) within zebrafish to visualize the expression pattern of *wdr31* during zebrafish embryo development up to 1-day post-fertilization. The dynamic and wide expression of *wdr31* during zebrafish embryonic development (2 cell and 8 somite stages (SS)) has become restricted to the otic vesicle and brain region at 24 hours post-fertilization (24 hpf) (**Fig. 4A**). We next knocked out (KO) *wdr31* in zebrafish with the CRISPR/Cas9 system and the embryos showed heart edema and otolith malformation. As *wdr31*

is expressed in the otic vesicle and the otolith development requires cilia, we checked the cilia in the otic vesicle. The cilia bundle in the lateral crista (LC) of the otic vesicle was stained with acetylated tubulin in wild type and *wdr31* KO, and although we found that the length of LC cilia was comparable with wild type, the width of the cilia bundle was reduced by 33% in LC of the otic vesicle compared to that in the control embryos, suggesting the cilia number is decreased (**Fig. 4B, C, and D**). Our zebrafish work indicates that WDR31 plays a role in ciliogenesis in the zebrafish ear.

Functional redundancy of WDR31, ELMOD, and RP2 for determining cilia morphology

We next wanted to gain mechanistic insight into the functions of *wdr-31* in cilia biogenesis. To this end, we employed the nematode *C. elegans* and obtained/generated three *wdr-31* alleles: tm10423 (160-bp frameshift causing deletion), syb1568 (1888-bp deletion removing all exons except exon I), and tur003 (1276-bp deletion removing a large portion of exon II and exon III, IV, and exon V) (**Fig. S3B**). In *C. elegans*, the lipophilic fluorescent dye-uptake assay is employed to indirectly evaluate the cilia structure, and our analysis revealed *wdr-31* mutants display wild type level dye-uptake in both head (amphid) and tail (phasmid), suggesting cilia structures are likely unaffected in these mutants (**Fig. 4E**). To address whether loss of *wdr-31* leads to subtle defects in cilia morphology, we expressed the fluorescence-based marker *str-1pro::mCherry* that labels the AWB cilia in *wdr-31* mutants. The AWB dendritic tip extends the fork-like cilia in the wild type, and our confocal microscopy analysis showed that the structure and length of AWB cilia are comparable with that of wild type AWB cilia, suggesting, contrary to the importance of WDR31 for ciliogenesis in the zebrafish ear, the loss of *wdr-31* alone does not result in a severe defect in cilia structure (**Fig. 4B, D, and F**). The lack of an apparent cilia phenotype in *C. elegans* may be

413 attributed to a functional redundancy for WDR-31 in cilia biogenesis, and functional redundancy
414 is indeed a common phenomenon in ciliopathy-related genes. For example, while the absence of
415 individual ciliopathy genes encoding MKS/MKSR proteins or NPHP proteins (NPHP-1, NPHP-
416 4) does not result in serious cilia defect, the loss of *nphp-4* (the ortholog of human NPHP4) in
417 combination with *mks-6* (the ortholog of human CC2D2A) causes more severe cilia related defects
418 (transition zone membrane association defects) (Williams et al., 2011). We next explored genetic
419 interaction between *wdr-31* and *elmd-1*, since our results showed that both protein products are at
420 the ciliary base (**Fig. 1C, D, E, and F**). We discovered that the *wdr-31(tm10423);elmd-1(syb603)*
421 double mutant (hereinafter referred to as “double mutant”) has a partial Dyf defect in both head
422 and tail neurons, which was rescued by expression of a transgene containing the wild type *elmd-1*
423 sequence (**Fig. 4E and Fig. S3C**, $p < 0.0001$, Fisher's exact test). Using the AWB cilia
424 fluorescence marker, we subsequently set out to visualize the ciliary structures in the double
425 mutant and single mutants. The AWB cilia in the double mutants have an extra projection in the
426 middle part of the cilia, and the cilia are of normal length (7% (N: 100) and 52 % (N: 134) of extra
427 projections in WT and double mutant, respectively) (**Fig. 4F and G, and Fig. S4B, C and D**).
428 We explored genetic interaction between *wdr-31*, *elmd-1*, and *rpi-2* (the X-linked retinitis
429 pigmentosa protein RP2 and a GTPase-activating protein) since ELMD-1 and WDR-31
430 localizations are reminiscent of RPI-2 localisation in *C. elegans* (**Fig. S5B**) (Williams et al., 2011).
431 We, therefore, first generated an *elmd-1;rpi-2* double mutant, which revealed no Dyf phenotype.
432 However, when we created *wdr-31;elmd-1;rpi-2* triple mutants (hereinafter referred to as “triple
433 mutant”), we observed severe synthetic Dyf phenotype, which is significantly rescued by the
434 introduction of a wild-type copy of *wdr-31* or *elmd-1* or *rpi-2* (**Fig. 4E, Fig. S3C, D**, $p < 0.0001$,
435 Fisher's exact test).

We then sought to examine AWB cilia morphology in triple mutants and compared it with the *wdr-31;elmd-1* and *rpi-2;elmd-1* double mutants. The Dyf defect of triple mutants was indeed accompanied by significant changes in the AWB cilia that displayed the ectopic projections, including a backward projection from the base of AWB cilia in all three independent *wdr-31* alleles and as well as ectopic projections from the middle part of AWB cilia (0% backward projection in wild type vs over 30% backward projection in triple mutants; $p < 0.0001$, Fisher's exact test), but the length of AWB cilia (long) in triple mutants was similar to that of wild type (**Fig. 4F, F, and Fig. S4B, C, D**).

Finally, we investigated the effect of *wdr-31*, *rpi-2* and *elmd-1* loss on osmotic avoidance behaviour which is mediated by sensory cilia. Our analysis showed that double and triple mutants display defective osmotic avoidance (Osm) behaviour, suggesting cilia are not functioning properly (**Fig. S4A**).

Loss of WDR-31 and ELMD-1 leads to the ciliary accumulations of OSM-3/KIF17 Kinesin motor and Complex B components

We next aimed to investigate the mechanism by which WDR-31, ELMD-1, and RPI-2 regulate cilia morphology in *C. elegans*. To this end, we investigated whether these genes affect the localization of IFT proteins and IFT motors, including kinesin motors (Kinesin II and OSM-3/KIF17), dyneins, and IFT components (IFT-A and IFT-B). The IFT complex and motor proteins are critical for cilia construction and maintenance because they deliver ciliary constituents from the cell body to the cilia. In *C. elegans*, two kinesin motors Kinesin II and OSM-3/KIF17 (heterotrimeric kinesin-II and homodimeric OSM-3) work cooperatively to carry the IFT complex in the middle segment of the channel cilia in an anterograde direction, while OSM-3/KIF17

transports the entire IFT complex (IFT-A, IFT-B, and BBSome) in the distal part of channel cilia. We generated single, double, and triple mutants expressing fluorescence tagged IFT and motor proteins. Using single-copy transgenes, we found that IFT-B components accumulate at cilia tips and/or middle of cilia in double mutants (96% ciliary tip accumulations for IFT-74/IFT74, N: 26 and 82% ciliary tip accumulations for OSM-6/IFT52, N: 46) but the localization of GFP::CHE-3 (human dynein heavy chain DYNC2H1) remains less affected in double mutants (27% minor middle cilia accumulations for CHE-3; N: 48, see supplementary Movie S1) (**Fig. 5A, C and D and Supplementary Movie S1, 2, 4**). Furthermore, we next investigated the localization of GFP::CHE-3 in triple mutants because the cilia morphology of triple mutants is more severe than that of *wdr-31;elmd-1* or *wdr-31;rpi-2* or *elmd-1;rpi-2* double mutants (**Fig. 5F**). Compared with wild type and double mutants, we noticed additional IFT abnormalities in two independent triple mutants, including dim cilia staining with GFP::CHE-3 and accumulations of GFP::CHE-3 in the middle/distal part of cilia (81% middle cilia accumulations, N: 96; 6.6% ciliary tip accumulations, N: 90) (**Fig. 5A and Supplementary Movie S1**). Motor protein OSM-3/KIF17 accumulation within the ciliary tips was detected in double and triple mutants. The size of OSM-3 ciliary tip accumulations appeared to be larger in triple mutants than in double mutants (**Fig. 5B, and Supplementary Movie S3**). Furthermore, in triple mutants, the dim distal cilia staining was observed for IFT-140::GFP (IFT-A component) (63% dim distal cilia, N: 47) and XBX-1::mCherry (a dynein light intermediate chain) (**Fig. 5E and F and Supplementary Movie S5**), but the localization and motility of KAP-1::GFP (Kinesin II) remain unaffected (**Fig. 5G**). Our fluorescence microscopy analysis suggests defects in the return of IFT and OSM-3/KIF17 kinesin motors from the cilia in double and triple mutants. We hypothesized that certain IFT particles would be unable to return from the ciliary tips due to potential defects in the IFT returning, so we measured IFT particle frequency in wild type and affected mutants to explore if the number of IFT

particles entering into cilia is affected and/or if the return of IFT particles from the ciliary tips is altered. Using time-lapse video coupled with kymography, for a subset of IFT components, we discovered a substantial reduction in the number of IFT particles traveling along the cilia in both directions in double and triple mutants (**Fig. 6A, and B**). In wild type, the average of IFT-74 particles moving in anterograde and retrograde directions (33 seconds and 7.5 μm) is 19 and 17, respectively, while in triple mutants, the anterograde and retrograde IFT-74 particles are 9 and 3. Comparable reductions were observed for IFT-74::GFP (a single copy transgene) in *wdr-31;eldm-1* (the anterograde and retrograde: 19 and 17 in wild type vs 12 and 7 in *wdr-31;eldm-1*; $p < 0.0001$; the Mann–Whitney U test). However, for OSM-6::GFP, the retrograde IFT particles statistically differ between wild type and triple mutants (the retrograde: 19 in wild type, 13 in double mutants; and 11 in triple mutants; $p < 0.0001$; the Mann–Whitney U test) (**Fig. 6B**). Furthermore, we found significant decline in the IFT transport for OSM-3::GFP in double and triple mutants in both anterograde and retrograde directions, but the decrease in IFT transport was more severe for triple mutants (the anterograde and retrograde: 23 and 20 in wild type; 16 and 11 in double mutants; 11 and 8 in triple mutants, $p < 0.0001$; the Mann–Whitney U test) (**Fig. 6B**). On the other hand, the flux of cytoplasmic dynein motor protein CHE-3 along the cilium was unchanged in both directions in both double and triple mutants, while FT-140 transport declined in both directions (**Fig. 6B**). Our findings suggest that with the exception of heterotrimeric Kinesin II, all IFT proteins studied, including dynein CHE-3, OSM-3 kinesin, IFT-B, and IFT-A components, display certain defects in *wdr-31* related mutants, either ciliary accumulations for dynein CHE-3, OSM-3 and IFT-B component or dim distal cilia staining for IFT-A component and dynein motors (CHE-3 and XBX-1).

The ciliopathy associated variant in WDR-31 displays cilia defects in *C. elegans*

Like human WDR31, *C. elegans* WDR-31 has WD-repeat domains (**Fig. 3A, and Fig. S2A**). In the ciliopathy patient, we found the arginine-to-a termination codon substitution at residue 268 of human WDR31 that removes the 99 amino acids containing a WD domain, but the functional significance of this change is currently unknown in the context of cilia biogenesis. To investigate whether the last 99 amino acids in WDR-31 is essential for the subcellular localization of WDR-31 to the cilia base and the function of WDR-31, the CRISPR/Cas9 was used to engineer the *C. elegans* model harboring the corresponding WDR31 deletion (named WDR-31^{R261-del}) (**Fig. 7A**). Using confocal microscopy, we determined the subcellular localization of WDR-31^{R261-del}, which revealed that, similar to wild type WDR-31::GFP (Cas9/CRISPR GFP knock-in), WDR-31^{R261-del}::GFP is situated at the base of cilia proximal to the MKS-6 marked transition zone (**Fig. 7B, and Fig. S5A**), suggesting that the last WD repeat within WDR-31 are not necessary for the ciliary base localization. In contrast, we found several cilia abnormalities when the *wdr-31*^{R261-del} variant was combined with *elmd-1* and *rpi-2*. First, similar to *wdr-31;elmd-1;rpi-2* triple mutants, the triple *wdr-31*^{R261-del};*elmd-1;rpi-2* mutants exhibit a synthetic Dyf phenotype, suggesting structural defects in cilia in this triple mutant (**Fig. 4E, 7C**). Consistent with this expectation, we find that the AWB cilia structural abnormalities in *wdr-31*^{R261-del};*elmd-1;rpi-2*, including backward projections from the cilia base, were comparable to three separate *wdr-31* mutants (**Fig. 4G, 7D, E**, over 30% backward projection in triple mutants; $p < 0.0001$, Fisher's exact test). As expected, we observe no Dyf phenotype and cilia structural defects in *wdr-31*^{R261-del} single mutants (**Fig. 7C, D**).

Finally, because WDR-31, ELMD-1, and RPI-2 localize at the base of cilia, we wanted to examine the role of these proteins in the ciliary gate, and we chose the TRAM-1 protein (the ortholog of human TRAM1), which surrounds the PCMC in *C. elegans* but stays outside of cilia (Williams et

al., 2011). Our confocal microscopy analysis revealed that the TRAM-1 protein enters into cilia in all examined *wdr-31* triple mutants, including *wdr-31^{R261-del};elmd-1;rpi-2* but not in *wdr-31^{R261-del}* single mutants (**Fig. 7E**). Taken together, all these data from *C. elegans* indicates the human R268* mutation (an equivalent of R261-del in *C. elegans*) in *WDR31* observed in the ciliopathy patient is likely a pathogenic variant.

WDR-31 and ELMD-1 are required for recruitment of BBS-7, a BBSome component, to cilia

To find out the underlying reason for why IFT-B components and OSM-3/KIF17 specifically accumulate at the ciliary tip in double and triple mutants, we hypothesized that the IFT complex destabilization might be due to defects in the BBSome assembly. The IFT-B components were previously reported to accumulate at the ciliary tip in BBSome mutants (Blacque, 2004; Wei et al., 2012; Xu et al., 2015). We found that ciliary tip accumulations of IFT-74:GFP, an IFT-B component, in *bbs-8(nx77)* mutants were comparably similar to that of double and triple mutants (**Fig. 8A**). We next explored the localization of BBS-7::GFP, a core member of the BBSome, in double and triple mutants. BBS-7::GFP is lost or significantly diminished in the cilia of double and triple mutants, but BBS-7::GFP localization persists at the ciliary base in these mutants (**Fig. 8B**). The time-lapse movie analysis of BBS-7::GFP together with kymography reveals that BBS-7::GFP movements were undetectable in 27% and 42% of cilia in the head in double and triple mutants, respectively (**Supplementary Movie S6**). We were able to quantify the frequency of BBS-7::GFP even though the density of trajectories (IFT particles) on kymographs was weak in double and triple mutants relative to wild type (**Fig. 8C**). Our analysis revealed a substantial decrease in the average of BBS-7::GFP particles translocating in the anterograde and retrograde directions in the remaining worms that display IFT (average anterograde and retrograde IFT

particles: 18 and 19 for wild type; 10 and 11 for double mutant; 2 and 2 for triple mutant, $p < 0.0001$; the Mann–Whitney U test) (**Fig. 8C, D and Supplementary Movie S6**). We next explored the localization of transition fiber protein FBF-1 and TAX-4 (a membrane protein) in the triple mutants because FBF-1 was implicated in the ciliary entry of IFT machinery (Wei et al., 2013). Our data reveals that the localization of these proteins remains unaffected in the triple mutants (**Fig. 5SB and D**). Taken together, our results suggest that because the BBSome complex is unable to gain access to cilia in double and triple mutants, IFT-B components and OSM-3/KIF17 likely accumulate excessively in the distal portion of the cilia while IFT Complex A component staining the distal segment cilia become dim (**Fig. 8E**).

Discussion

WDR31 is a new ciliopathy gene required for cilia biogenesis

The list of both the primary and motile ciliopathy genes has been increasingly expanding with the large-scale adoption of next-generation sequencing technologies in the exploration of genetic causes for these diseases. So far, over 190 ciliopathy genes have been discovered (Wheway et al., 2019). Despite substantial advances in identifying many ciliopathy genes, elucidating the underlying genetic mutations and their functions in ciliopathies represent a major challenge. In the current work, we identified *WDR31* as a novel cilia protein and relate it to ciliopathy. We found a truncating variant in *WDR31* in a family of nine, with the only affected child harboring a homozygous truncating variant. The patient displayed ciliopathy-related clinical features, including congenital hydrocephalus, polydactyly, and congenital heart disease. The human counterpart variant results in an abnormal cilia structure in *C. elegans*, confirming the conclusion that this variant likely disrupts WDR-31 functions.

Human WDR31 is a member of the WD40-repeat protein (WDR) family, and it was previously understudied even though several members of the WD40-repeat protein family, including WDR34, WDR35, and WDR60, have been linked to cilia (Blacque et al., 2006; Patel-King et al., 2013; Rompolas et al., 2007). Strikingly, we found that WDR31 is a ciliary protein, and knocking it out in zebrafish using CRISPR/Cas9 causes ciliary defects, including a decrease in the number of cilia in the LC. Furthermore, the link between WDR31 and cilia was significantly strengthened by findings from *C. elegans*. First, the expression of the *WDR31* orthologue (WDR-31) is restricted to the ciliated sensory neurons, and WDR-31 localizes to the base of cilia. The exclusive cilia expression pattern of *wdr-31* in *C. elegans* is similar to that of ciliary and ciliopathy genes like ARL-13/ALR13b, IFT, and BBS (Blacque et al., 2005). Second, CRISPR/Cas9 mediated knock out of *WDR-31* combination with the ELMOD orthologue (ELMD-1) and RPI2 orthologue (retinitis pigmentosa 2; RPI-2) results in an abnormality in cilia functions and structure in *C. elegans*. Our findings reveal that WDR31 is a new ciliopathy protein required for cilia morphology in both zebrafish and *C. elegans*, suggesting the evolutionarily conserved role of WDR31 in cilia biology.

Functional redundancy between WDR-31, ELMD-1, and RPI-2

Our genetic analysis in *C. elegans* provides evidence for partial functional redundancy for *wdr-31*, *elmd-1*, and *rpi-2* in regulating cilia morphology and the trafficking of kinesin-IFT-BBSome complexes. Though cilia morphology is disrupted in *wdr-31;elmd-1;rpi-2* triple mutants, with altered recruitment of kinesin, IFT, and BBS proteins, simultaneous elimination of *elmd-1* and *rpi-2* did not result in severe defects in cilia and IFT. Our interpretation for these data is that the additive defects in the triple mutants were likely due to *wdr-31*, and WDR-31 is a central player in controlling cilia morphology and IFT machinery. However, we cannot rule out the possibility of microtubule defects in cilia ultrastructure in *elmd-1;rpi-2* double mutants. Additional work is

needed, and a transmission electron microscope (TEM) may be used to reveal further details in the ultrastructures of cilia in these double mutants.

How do these proteins work together to regulate cilia-related phenotypes? While WDR31 is a poorly characterized gene, the roles of human RP2 and ELMOD proteins in cilia biology are better understood. Human RP2 localizes to the cilium and the basal body and displays GAP activity toward two ARF family members, ARL2 and ARL3, both of which were linked to cilia biology (Evans et al., 2010; Schwarz et al., 2017; Wright et al., 2011), while the ELMOD protein family (ELMOD1-3) has GAP activity for ARL2 (ELMOD1 and ELMOD3) and ARF6 (ELMOD1) (Ivanova et al., 2014; Jaworek et al., 2013; Johnson et al., 2012; Miryounesi et al., 2019). ELMOD2 localizes to the basal body, and our study showed ELMOD3 localizes to cilium (Turn et al., 2021). Taking into account the fact that RPI-2 and ELMD-1 are GAPs, and loss of these two GAP proteins likely result in the overactivation of their target G proteins, how does WDR-31 function with these GAP proteins? One possibility is that WDR31 may function downstream of regulatory GTPases, including ARL2, ARL3, or unidentified GTPases, activated by these GAP proteins. One downside of this explanation is that *wdr-31* single mutants did not have significant ciliary and IFT defects; but, if this were valid, we would expect to observe further anomalies in cilia and IFT in *wdr-31* single mutants, close to the removal of these three genes. Alternatively, the activity/function of overactive GTPases can be somehow regulated by WDR-31, maybe WDR-31 may have a GAP activity for the regulatory GTPases or it may have a GTPase activity. While WDR-31 does not seem to have a GAP domain, HHMER search revealed that human WDR31 has a distant sequence resemblance to a nucleoside-triphosphatase (NTPase) domain (NACHT) containing protein in *Penicillium camemberti* (Gabler et al., 2020). PSI-BLAST search confirmed this result (unpublished data).

627

628 Furthermore, the ciliary roles of these two GAP proteins might be independent of their GAP
629 activities, and consistent with this idea, the recent study showed the ciliary roles of ELMOD2 are
630 partially independent of its GAP activity (Turn et al., 2021). However, a variety of evidence
631 suggests that WDR31 and ELMOD3 do not form a complex. First, mass spectrometry-based
632 proteomic analysis for either WDR31 or ELMOD3 showed that neither WDR31 nor ELMOD3
633 contains each other or RP2 (unpublished data). Second, the proper localization of any of these
634 three proteins was independent of each other (unpublished data). Further studies are needed to
635 understand a mechanistic link between WDR31, ELMOD, and RP2, and figure out the independent
636 contributions of WDR31 and both GAP proteins in cilia biology.

637

638 **How does the WDR31-ELMOD-RP2 regulate the IFT trafficking in cilia?**

639 Based on our results, which involve a decrease in moving IFT particles in anterograde transport,
640 ciliary tip aggregation of OSM-3/KIF17 and IFT-B, and docking of BBSome to the IFT machinery,
641 we propose that these defects likely stem from the failure of BBSome to associate with moving
642 IFT in cilia in double and triple mutants. Consistent with our proposal, the almost comparable
643 IFT/BBSome phenotypes were reported with hypomorphic mutations in *dyf-2* (WDR19
644 orthologue, an IFT-A component) and *bbs-1* (a BBSome component), where they showed that
645 while BBSome remains at the base of cilia and does not undergo IFT, the failure of IFT-B
646 reassociation with IFT-A at the ciliary tips results in IFT-B ciliary tip accumulations, despite the
647 fact the association of IFT-A and IFT-B components in anterograde transport persists (Wei et al.,
648 2012). They proposed that the BBSome controls the IFT assembly at the ciliary base and IFT
649 turnover at the ciliary tip. However, the IFT defects in the triple mutant are unlikely to be due to
650 mislocalization of transition fiber proteins such as DYF-19 (the FBF1 orthologue), which was

implicated in regulating the ciliary entry of IFT and BBSome, since our data showed that the localization of DYF-19 was not impaired in the triple mutants (Wei et al., 2013). On the other hand, the effect on IFT might be direct since RP2 was previously proposed to control the intraflagellar transport protein IFT20 pool in the peri-basal body and trafficking of Kif17 and Kif7 to the ciliary tip (Schwarz et al., 2017). However, several interesting questions have yet to be resolved: what is the mechanism by which WDR-31-ELMD-1-RPI-2 regulates the ciliary entry of the BBSome entry? How does WDR-31-ELMD-1-RPI-2 interact with proteins, including ARL6, that regulate the BBSome recruitment (Jin et al., 2010)? Future study research will help us to understand how these proteins regulate BBSome/IFT trafficking. In summary, this study provides a mechanistic explanation of the function of WDR-31, a new ciliopathy protein, in the regulation of cilia biogenesis and also contributes to our knowledge of the molecular etiology of WDR31 mediated disease.

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Figure 1: WDR-31/WDR31 and ELMD-1/ELMOD proteins are evolutionary conserved

677 **ciliary proteins A, B)** Shown are the representative drawing of the PHA/PHB sensory neuron
678 (phasmid neurons located in the tail). Cilia, dendrite, axon and cell soma (cell body) are depicted
679 in the drawing. Fluorescence images from the transgenic strain carrying WDR-31::GFP or
680 GFP::ELMD-1 were displayed in the PHA/PHB sensory neurons. Scale bars: 3 μ m **C, D)** Co-
681 localization of WDR-31::GFP (Green) with the IFT-140::mCherry (Red, an IFT-A component, a
682 ciliary marker) or TBG-1:mKate (Red, γ -Tubulin, the basal body) in the tail (phasmids) and head
683 (amphid) sensory neurons. TZ and BB denote the transition zone and the basal body, respectively
684 **E, F)** GFP::ELMD-1 (Green) localizes to the BB and PCMC (the periciliary membrane
685 compartment) proximal to the transition zone. Co-labelling of GFP::ELMD-1 with MKS-
686 6:mCherry marker (transition zone) or IFT-140::mCherry in the tail (phasmids) and head (amphid)
687 neurons. Scale bars: 2 μ m **G)**

688

689

690 **Figure 2: ELMOD3 and WDR31 localize to the primary cilium.** A, B) Shown are the staining
 691 of WDR31 (tagged with cyan fluorescent protein) and ELMOD3 (tagged with red fluorescent
 692 protein) together with a ciliary marker ARL13B and DAPI (nucleus) in hTERT-RPE1 cells.
 693 hTERT-RPE1 cells were transiently transfected with 100 ng of WDR31:CFP and ELMOD3:RFP.
 694 Scale bars: 10 μ m (A) and 3 μ m (B)

Figure 3: A homozygous p.R268* variant in WDR31 leads to a ciliopathy **A)** Shown is the schematic of WDR31 (NP_660284.1), with 367 amino acids in length and WD domains as tandem repeats. The position of disease associated p.R268* (rs ID: rs764141336) variant is depicted in human WDR31, resulting in a 268 amino acid protein in length. **B and C)** Clinical images and molecular analysis of the proband. Facial images of the proband (14DG0977) with hydrocephalus and right microtia, **D and E)** X-ray imaging highlighting right preaxial polydactyly, and hydrocephaly, respectively. **F)** schematic representation of the pedigree showing consanguinity between the parents (1st degree cousins). The proband is marked with an arrow. **G)** Sequence chromatogram confirming the variant segregation in the index (homozygote c.802C>T; p.(Arg268*)). The parents and two unaffected sisters (II:3, II:5) are carriers (heterozygotes), and the unaffected brother and sister (II:4) are wild types.

Figure 4: WDR31 regulates ciliogenesis in zebrafish **A)** Shown are the expression pattern analysis of WDR31 in zebrafish embryos. *Wdr31* is ubiquitously expressed before the segmentation stage (2 cell and 8 somite stages (SS)). The expression of *Wdr31* becomes limited to the otic vesicle and brain region at 24 hours post fertilization (24 hpf). Shown are cilia of lateral crista (LC) of the otic vesicle, stained with acetylated-tubulin, in wild type and *Wdr31* knockout, generated via CRISPR/Cas9. **C and D)** The length of cilia in the lateral crista (LC) of the otic vesicle remains unaffected in zebrafish *Wdr31* knockout while the cilia number is decreased, as shown with the measurement of width of cilia lateral crista (LC) of the otic vesicle. **E)** Shown are the schematic representations of lipophilic fluorescent dye uptake in the head (amphid) and tail (phasmid) sensory neurons in *C. elegans*. Red labelling indicates the normal dye uptake while the failure of Dye uptake was shown in white. Fluorescence images show the dye uptake in the head and tail neurons in the wild type and indicated mutant strains. No dye uptake was observed in both head (amphid) and tail (phasmid) of *wdr-31(tm10423);elmd-1* double mutants, *wdr-31(tm10423);elmd-1;rpi-2* and *wdr-31(syb1568);elmd-1;rpi-2* triple mutants. *Ex[elmd-1(+)]* rescues the dye uptake defects of *wdr-31(tm10423);elmd-1* double and *wdr-31(tm10423);elmd-1;rpi-2* triple mutants, while *Ex[wdr-31(+)]* rescues the Dye uptake defects of *wdr-31(syb1568);elmd-1;rpi-2* triple mutants. Scale bars: 10 μ m. **F)** Fluorescence images show the morphology of AWB cilia (fork-like structure located in the head) in wild type and indicated mutant backgrounds. The backward projection from the cilia is shown with asterisks (*) while p indicates the ectopic projections from the middle parts of cilia. The AWB cilia in wild type and affected mutants are shown in schematic sketches.

Figure 5: IFT-B proteins and OSM-3/KIF17 accumulate at the ciliary tip. Shown are fluorescent images of PHA/PHB cilia (phasmid tail). **A)** Fluorescent images from a single copy GFP::CHE-3 (human dynein heavy chain DYNC2H1) in wild type and indicated mutant backgrounds are displayed. GFP::CHE-3 accumulations within cilia and dim distal cilia staining were observed in two distinct triple mutants (*wdr-31(tm10423);elmd-1;rpi-2* and *wdr-31(syb1568);elmd-1;rpi-2*). Scale bars: 3 μ m **B, C, D, E, F)** Confocal microscopy analysis of IFT-A (IFT-140::GFP) and IFT-B complex components (OSM-6/IFT52::GFP and IFT-74::GFP) revealed differential abnormalities in the transport of IFT-A and IFT-B components in double

739 (*wdr-31;elmd-1*) and triple mutants. The localization of XBX-1::mCherry (Dynein subunit) in
740 *wdr-31(syb1568);elmd-1;rpi-2* triple mutants phenocopies the dim distal cilia staining of IFT-A
741 (IFT-140::GFP) in the *wdr-31(tm10423);elmd-1;rpi-2* triple mutants. OSM-3/KIF17 Kinesin
742 motor accumulates at the ciliary tips in *wdr-31(tm10423);elmd-1* double and *wdr-*
743 *31(tm10423);elmd-1;rpi-2* triple mutants. Compared to double mutants, the ciliary tip staining is
744 more strong in the triple mutants. Scale bars: 3 μ m **G**) Fluorescent images from Kinesin II motor
745 (KAP-1::GFP) revealed that the restricted middle segment localization of KAP-1 remains
746 unchanged in *wdr-31(tm10423);elmd-1;rpi-2* triple mutants. Scale bars: 3 μ m **H**) Shown are the
747 drawings of phasmid cilia (PHA/PHB sensory neurons in the tail) in wild type and mutants
748 showing ciliary accumulations.

Figure 6: Measurement of anterograde and retrograde IFT transport frequency A) Shown are representative kymographs of GFP tagged IFT proteins translocating in the tail cilia (PHA/PHB sensory neurons) of wild type and indicated mutants. Kymographs for anterograde, retrogrades and merged (Red & Green) were generated with ImageJ equipped with KymographClear. The trajectory represents a moving IFT particle, and the average number of moving IFT particles in

755 wild type and indicated double and triple mutants was calculated by counting all trajectories in
756 each kymograph. Travel time and distance are shown on kymographs. **B)** Box-and-Whisker charts
757 with error bars were created to visualize the average number of IFT anterograde and retrograde
758 particles between wild type and indicated mutants. The Mann–Whitney U test was used to measure
759 statistical analysis and significance. The four and three asterisks (**** and ***) at the top of the
760 brackets indicate that the p value between the two strains is less than 0.0001 and 0.001,
761 respectively, suggesting statistical significance. Ns stands for "not significant."

A

Human SCSNGFGGE GCEATLWDLRQTNR I CEYKGHFQTVA 282 NP_001012361.1
Chimp SCSNGFGGE GCEATLWDLRQTNR I CEYKGHFQTVA 281 XP_003312284.1
Macaca SCSNGFGGE GCEATLWDLRQTNR I CEYKGHFQTVA 282 XP_001102129.1
Rat SCSNGFGGE GCEATLWDLRQTNR I CEYKGHFQTVA 282 NP_001011976.2
C. elegans SCSNGFNNDGCETSIYDVRNPRQS-REARGHEGNTV 274 NP_494344.1

B

C

D

E

F

Figure 7: The CRISPR/Cas9 knock-in (p.Arg261del) *C. elegans* model of the human patient variant phenocopies the cilia related anomalies in the null *wdr-31* *C. elegans* mutants. **A)** Shown are multiple sequence alignments of WDR31 between human, chimp, macaca, rat and *C. elegans*. The arginine residue at 268 was converted to a stop codon (R268*) in the ciliopathy patient, causing the last almost 100 amino acids not to be translated, and the arginine residue at 268 of human WDR31 was conserved between humans and other organisms included. To mimic a comparable human patient variant in *C. elegans*, the CRISPR/Cas9 was used to create the deletion starting from the arginine residue at 261 of *C. elegans* WDR-31. **B)** Shown are fluorescence images of WDR-31::GFP and WDR-31^(R261-del)::GFP with the transition zone marker MKS-6::mCherry in the tail. Both WDR-31 markers are located directly underneath the TZ. **C)** Shown are fluorescence images together with cartoon representations. *wdr-31*^(R261-del); *elmd-1*; *rpi-2* triple mutants exhibit dye-filling (Dyf) phenotype. **D)** Fluorescence images and the representative cartons display the fork-like structure of the AWB cilia in wild type and indicated mutant backgrounds. In all examined *wdr-31* triple mutants, including *wdr-31*^(R261-del); *elmd-1*; *rpi-2*, the backward projections from the base of AWB cilia were observed. The ectopic projection from the cilia was shown in *wdr-31*^(R261-del); *elmd-1* double mutant. Asterisks (*) points the backward projection while p indicates ectopic projections from the middle parts of cilia. **E)** The percentage distribution of AWB cilia morphology in wild type and indicated mutants is displayed in the graph. **F)** Confocal fluorescent images exhibit the localization of tdTomato tagged TRAM-1 (a PCMC marker) and MSK-2::GFP (a TZ marker) in wild type and indicated mutants. TRAM-1 leaks into cilia in all four *wdr-31* triple mutants, including the disease phenocopying mutant. Cilia, PCMC, and TZ are depicted in the fluorescent image. Scale bars: 2 μ m

Figure 8: WDR-31 and ELMD-1 regulate the recruitment of BBSome to cilia.

A) Shown are fluorescence images from the transgenic strain carrying IFT-74::GFP, an IFT-B component, in wild type, *wdr-31;elmd-1* double mutants, *wdr-31;elmd-1;rpi-2* triple mutants, and *bbs-8(nx77)*. The IFT-B subunit IFT-74:GFP accumulates at the ciliary tips and cilia in the tail of

791 all three mutants. **B)** Confocal fluorescence images showing the localization of BBS-7::GFP, a
 792 BBSome subunit, in the heads and tails of wild type and *wdr-31(tm10423); wdr-31;elmd-1; rpi-*
 793 *2;elmd-1* double and *wdr-31;elmd-1;rpi-2* triple mutants. Fluorescence images showed absent or
 794 weak cilia staining of BBS-7::GFP in both the head and tails of *wdr-31;elmd-1* and *wdr-31;elmd-*
 795 *1;rpi-2* triple mutants. **C)** Kymographs were created from time-lapse BBS-7::GFP movies
 796 (PHA/PHB cilia) using KymographClear integrated into ImageJ. Shown are representative
 797 kymographs for BBS-7::GFP translocating in wild type and indicated mutants. Each trajectory in
 798 kymographs was counted. Travel time and distance are included on kymograph **D)** The graph
 799 depicts the average number of BBS-7::GFP particles traveling around cilia in both directions for
 800 wild type and indicated mutants. The Mann–Whitney U test revealed statistical significance
 801 between the compared strains and that the p value was less than 0.0001 shown by the four asterisks
 802 (****) at the top of the brackets **E)** In wild type, the assembly of the Kinesin-IFT-BBSome
 803 complex (Kinesin-II and OSM-3, IFT-B , IFT-A and BBSome) happens at the base of cilia. In the
 804 middle segment of amphid and phasmid cilia in *C. elegans*, both heterotrimeric Kinesin II and
 805 homodimeric OSM-3 transport the IFT-A- IFT-B- BBSome complex in an anterograde direction.
 806 Heterotrimeric Kinesin II returns to the ciliary base when it reaches the tip of the middle segment
 807 of amphid and phasmid cilia, whereas homodimeric OSM-3 is responsible for the anterograde
 808 translocation of the IFT-BBSome complex in the distal segment of amphid and phasmid cilia.
 809 When the OSM-3-IFT-BBSome complex reaches the ciliary tip, cytoplasmic dynein transports
 810 them back to the ciliary base. In *wdr-31;elmd-1* double *wdr-31;elmd-1;rpi-2* triple mutants, the
 811 BBSome failed to enter into cilia, thus leading to accumulations of OSM-3 and IFT-B components
 812 in the ciliary tips.

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815 **Supplementary Figure 1: Expression patterns of WDR-31 and ELMD-1.** A) A total of 60
 816 sensory neurons are distributed in the head (amphid) and tails (phasmid) of *C. elegans*. The ciliated
 817 sensory neurons are displayed in the schematic of *C. elegans*. The expression of *elmd-*
 818 *lpromoter::gfp* (1000 kb) and *wdr-31promoter::gfp* (1000 kb) were shown in the fluorescence
 819 images. The cell of ciliated sensory neurons (cell soma) in the head and tails were displayed in
 820 brackets.

Supplementary Figure 2: Ciliary localization of WDR31 and ELMOD3 in hTERT-RPE1 cells

A) Images showing immunostaining of a ciliary marker ARL13B and DAPI (nucleus) in hTERT-RPE1 cells. NT represents no transfection of hTERT-RPE1 with WDR31 (tagged with cyan fluorescent protein) and ELMOD3 (tagged with red fluorescent protein). Scale bars: 10 μm.

B) Images showing immunostaining of hTERT-RPE1 cells transfected with ELMOD3-CFP (cyan) and also stained for ARL13B (yellow). The magenta channel only shows some background but no localization to the cilium. Green regions in the “merge” picture show co-localization of both ELMOD3 and ARL13B proteins. Scale bars: 3 μm.

C) Fluorescence images displaying hTERT-RPE1 cells transfected with WDR31-RFP (cyan). The cyan channel shows some

832 background, with no cilium localisation. WDR31 and ARL1B proteins are co-localized in the pink
833 sections of the "merge" image. Scale bars: 3 μm .

Supplementary Figure 3: Rescue of dye uptake defects by ELMD-1 or RPI-2.

A) Shown are representative schematics of human and *C. elegans* WDR-31/WDR31. Human WDR31 (NP_660284.1) has four WD domains while *C. elegans* WDR-31 (NP_494344.1) has three WD domains (<https://prosite.expasy.org/>; Sigrist CJA et al 2012). **B)** Schematic diagrams of *wdr-31*, *elmd-1* and *rpi-2* together with corresponding deletions are shown. Scale bars: 250 bp. **C** and **D)** Fluorescence microscope images of mutant worms (head and tail) with red-fluorescent dye uptake (texas red filter) and GFP-tagged ELMD-1 and RPI-2 (fluorescence filter set for GFP) are shown. The head or tail in the dotted lines is indicated by numbers in the combined fluorescence images. GFP expression in combination with RED fluorescence indicates the rescue of dye uptake defects.

846 **Supplementary Figure 4:** **A)** 100% stacked column chart displays the proportion of *C. elegans*
847 wild type and indicated mutants cross the osmotic barrier containing an 8M fructose solution with
848 bromophenol blue. Stay represents not crossing the osmotic barrier while pass means that animal
849 crossed the osmotic barrier. The number of animals used in osmotic avoidance assays for each
850 strain are shown on the bar charts. **B and C)** The percentage of dye uptake in the head and tail in
851 wild type and mutants, as well as the defect in AWB cilia morphology, are displayed in bar charts.
852 Dyf represents dye uptake defects while the partial means some cells in the head or tail take up the
853 fluorescence dye but others could not fill up their cells. Fisher's exact test was performed for
854 statistical analysis between indicated triple mutants and a rescue gene for Dye assay or wild type
855 and indicated mutants for AWB cilia morphology. Brackets show statistical significance between
856 two strains compared ($p < 0.0001$) in B and **** indicates statistical significance in C. **D)** Shown
857 is the jitter plot for AWB long cilia length (μm) for wild type and indicated mutant strains.

859 **Supplementary Figure 5: A)** Shown is the co-localization of WDR-31(R261del)::GFP (a *C.*
860 *elegans* variant mimicking disease-associated human R268* variant) and MKS-6::mCherry
861 (transition zone protein) in the tail (PHA/PHB sensory neuron) of *C. elegans*. Shown is a schematic
862 drawing of the PHA/PHB sensory neuron. CB denotes the cell body, axon dendrite and transition
863 zone are shown in the images and schematic drawing. **B)** Shown is co-localization of RPI-2::GFP
864 (human RP2) and XBX-1::mCherry (human DYNC2LI1) in the head and tail sensory neurons.
865 XBX-1 stains the entire cilium and is used as a ciliary marker while RPI-2 is located at PCMC.
866 PCMC denotes the periciliary membrane compartment.

Supplementary Figure 6: **A)** Confocal images (Z-stack) display the localization of OSM-6::GFP (a single copy transgene, huma IFT52) in the head (a bunch of cilia) of wild type and indicated mutants. Arrow points the ciliary tips while asterisks indicate the ciliary base. **B, C and D)** Confocal images show colocalization of TAX-4 (a ciliary membrane protein) and XBX-1::tdTomato (a cilia marker) or NPHP-1::GFP (transition zone protein) and FBF-1::mCherry (transition fiber protein) or PLCδ1-PH::GFP (a marker for monitoring phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) in the plasma membrane) and MKSR-2 (a TZ marker). The

localization of TAX-4::GFP in *wdr-31;elmd-1;rpi-2* triple mutants was similar to wild type. The PLC δ 1-PH::GFP decorates the membranes of PCMC and does not enter into cilia in wild type. The PLC δ 1-PH::GFP stays outside of cilia in the *wdr-31;elmd-1;rpi-2* triple mutants.

Supplementary Movies

For the IFT assay, time-lapse movies (3 frames per second) were generated with Leica DM6, and were processed with Image J to generate GIFs (10 fps).

Supplementary Movies:

Supplementary Movie 1: IFT-74::GFP in wild type and indicated mutant backgrounds.

Supplementary Movie 2: OSM-6::GFP in wild type and indicated mutant backgrounds.

Supplementary Movie 3: OSM-3::GFP in wild type and indicated mutant backgrounds

Supplementary Movie 4: GFP::CHE-3 in wild type and indicated mutant backgrounds.

Supplementary Movie 5: IFT-140::GFP in wild type and indicated mutant backgrounds.

Supplementary Movie 6: BBS-7::GFP in wild type and indicated mutant backgrounds.

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