TMEM231, mutated in orofaciodigital and Meckel syndromes, organizes the ciliary transition zone

Elle C. Roberson,1,* William E. Dowdle,1* Aysegul Ozanturk,2 Francesc R. Garcia-Gonzalo,1 Chunmei Li,5 Jan Halbritter,6 Nadia Elkhartoufi,9 Jonathan D. Porath,6 Heidi Cope,3 Allison Ashley-Koch,2,3 Simon Gregory,4 Sophie Thomas,7,8 John A. Sayer10,11 Sophie Saunier,7,8 Edgar A. Otto,12 Nicholas Katsanis,2 Erica E. Davis,2 Tania Attié-Bitach,7,8,9 Friedhelm Hildebrandt,6,13 Michel R. Leroux,5 and Jeremy F. Reiter1

Introduction

Primary cilia project from the surfaces of many cell types and communicate intercellular signals during development and adult tissue homeostasis (Goetz et al., 2009). Consistent with roles in cell–cell communication, membrane-associated proteins involved in signal transduction, such as Polycystic kidney disease 2 (Pkd2) and Smoothened (Smo), localize to cilia, resulting in phenotypes characteristic of MKS such as polydactyly and kidney cysts. Tmem231 and B9d1 are essential for each other and other complex components such as Mks1 to localize to the transition zone. As in mouse, the Caenorhabditis elegans orthologue of Tmem231 localizes to and controls transition zone formation and function, suggesting an evolutionarily conserved role for Tmem231.

We identified TMEM231 mutations in orofaciodigital syndrome type 3 (OFD3) and MKS patients that compromise transition zone function. Thus, Tmem231 is critical for organizing the MKS complex and controlling ciliary composition, defects in which cause OFD3 and MKS.

The Meckel syndrome (MKS) complex functions at the transition zone, located between the basal body and axoneme, to regulate the localization of ciliary membrane proteins. We investigated the role of Tmem231, a two-pass transmembrane protein, in MKS complex formation and function. Consistent with a role in transition zone function, mutation of mouse Tmem231 disrupts the localization of proteins including Arl13b and Inpp5e to cilia, resulting in phenotypes characteristic of MKS such as polydactyly and kidney cysts. Tmem231 and B9d1 are essential for each other and other complex components such as Mks1 to localize to the transition zone. In mouse, the Caenorhabditis elegans orthologue of Tmem231 localizes to and controls transition zone formation and function, suggesting an evolutionarily conserved role for Tmem231.

We identified TMEM231 mutations in orofaciodigital syndrome type 3 (OFD3) and MKS patients that compromise transition zone function. Thus, Tmem231 is critical for organizing the MKS complex and controlling ciliary composition, defects in which cause OFD3 and MKS.
with MKS (Hopp et al., 2011; Shaheen et al., 2013) as well as Joubert syndrome (Srour et al., 2012; Romani et al., 2014).

*B9D1* encodes one of three B9 domain–containing proteins in mammals, all of which are components of the MKS complex and are involved in MKS (Weatherbee et al., 2009; Dowdle et al., 2011; Garcia-Gonzalo et al., 2011; Hopp et al., 2011; Chih et al., 2012). Mouse embryos lacking *B9d1* function display MKS-like phenotypes (Dowdle et al., 2011). The genomes of diverse ciliated organisms encode B9 domain–containing proteins, suggesting that this protein family has an evolutionarily conserved role in ciliary function (Bialas et al., 2009; Dowdle et al., 2011). We detected *Tmem231*, a 36-kD two-pass transmembrane protein, as a potential *B9d1* interactor (Dowdle et al., 2011). *Tmem231* mutant mouse embryos display ciliopathy hallmarks including polydactyly, microphthalmia, and dorsalization of the neural tube (Chih et al., 2012).

Here, we demonstrate that *B9d1* and *Tmem231* are required to localize a subset of MKS complex components to the TZ and to maintain ciliary protein composition in diverse tissues and cilia types in vivo and in cultured cells. We show that the role of *Tmem231* in controlling TZ and ciliary membrane protein composition are conserved in *C. elegans*. Mouse embryos of mixed background and homozygous for a loss-of-function *Tmem231* mutation exhibit MKS-like phenotypes including polycystic kidneys, polydactyly, and hepatic ductal plate malformations. Additionally, we identify eight novel human *TMEM231* mutations that cause MKS and orofaciodigital syndrome type 3 (OFD3). The missense mutations identified in our ciliopathy cohorts compromise the ability of the TZ to control ciliary membrane-associated protein composition.

**Results**

**Tmem231 is a component of the MKS complex**

*Tmem231* was identified as an interaction partner of *B9d1* (Dowdle et al., 2011; Chih et al., 2012). To determine if *Tmem231* interacts with only *B9d1* or additional constituents of the TZ, we fused *Tmem231* to a localization and affinity purification (LAP) tag and subjected copurifying proteins to mass spectrometric identification. This experiment confirmed the interaction between *B9d1* and *Mks1*, as shown in the third and fourth lanes in the top blot.

Inherited defects in ciliary function underlie a diverse set of diseases called ciliopathies. Mutations in TZ complex genes cause a subset of ciliopathies, including NPHP, characterized by corticomedullary kidney cysts; Joubert syndrome, marked by cerebellar vermis aplasia; and MKS, distinguished by cystic kidneys, hepatic ductal plate malformations, polydactyly, and encephalocele (Smith et al., 2006; Arts et al., 2007; Baala et al., 2007; Gorden et al., 2008; Mougou-Zerelli et al., 2009; Otto et al., 2009; Dowdle et al., 2011; Garcia-Gonzalo et al., 2011; Hildebrandt et al., 2011; Huang et al., 2011; Sang et al., 2011; Otto et al., 2009; Dowdle et al., 2011; Garcia-Gonzalo et al., 2011; Hildebrandt et al., 2011; Huang et al., 2011; Sang et al., 2011; Shaheen et al., 2011; Chih et al., 2012; Davis and Katsanis, 2012; Srour et al., 2012; Shaheen et al., 2013; Tuz et al., 2013). For example, *B9D1* and *TMEM231* mutations are associated with MKS (Hopp et al., 2011; Shaheen et al., 2013) as well as Joubert syndrome (Srour et al., 2012; Romani et al., 2014).

**Tmem231 mutant mice exhibit ciliopathy phenotypes including kidney cysts, hepatic ductal plate malformations, and polydactyly**

Some mouse mutants affecting kidney cysts, hepatic ductal plate malformations, and polydactyly...
and Tmem231 are interacting partners (Fig. 1), we assessed whether loss of Tmem231 has similar effects on embryonic development using mutant mice in which the Tmem231 transcript is truncated at the second exon by an insertion cassette (Chih et al., 2012). On a C57BL/6 background, homozygous Tmem231 mutant embryos die at embryonic day (E) 15.5 and exhibit abrogated Hedgehog signaling, microphthalmia, and polydactyly (Chih et al., 2012). To analyze later stages of development, we generated a homozygous Tmem231 mutation on a C57BL/6-CD1 mixed background; these Tmem231−/− embryos survive until birth (unpublished data).

Human MKS fetuses and mouse B9d1−/− embryos display hepatic ductal plate malformations, where the portal vein fails to separate from the bile duct and the portal mesenchyme does not remodel (Salonen, 1984; Weatherbee et al., 2009; Dowdle et al., 2011). Similarly, Tmem231−/− embryos display hepatic ductal plate malformations at E15.5 (Fig. 2 A). Diverse ciliopathies manifest kidney cysts and polydactyly. At E18.5, Tmem231−/− embryos exhibit kidney cysts, predominantly at the corticomedullary border and hindlimb preaxial polydactyly (Fig. 2, B and C). Thus, loss of mouse Tmem231 function causes a spectrum of phenotypes similar to those observed in mice with mutations in some other MKS complex components and human MKS–affected individuals (Salonen, 1984; Weatherbee et al., 2009; Dowdle et al., 2011).

Given that Tmem231 is a biochemical component of the MKS complex, we tested whether Tmem231 participates with Tctn1 and B9d1 in MKS complex function. Like Tctn1−/− or B9d1−/− mouse embryonic fibroblasts (MEFs), Tmem231−/− MEFs fail to localize Adcy3 and Arl13b to cilia (Fig. 3 A and Fig. S1, A and C), indicating that Tmem231 is an essential component of the MKS complex required to control the composition of the ciliary membrane.

Arl13b is required for the ciliary localization of Inpp5e, a membrane-associated protein implicated in Joubert syndrome (Humbert et al., 2012). As the MKS complex was essential for the ciliary localization of Arl13b, we examined whether Inpp5e also fails to localize to cilia in B9d1−/− and Tmem231−/− MEFs and found that both B9d1 and Tmem231 are necessary for the ciliary localization of Inpp5e (Fig. 3, A and B; and Fig. S1, A and C). Additionally, B9d1 and Tmem231 are required for Pkd2 to localize to cilia (Fig. 3, A and B; and Fig. S1, A and C). In contrast, localization of intraflagellar transport protein Ift88 along the axoneme is similar between wild-type and Tmem231−/− or B9d1−/− MEFs; although there is a decrease in the fluorescence intensity of Ift88, the percentage of Ift88-positive cilia is unaffected by disruption of the TZ complex (Fig. 3, A and B; and Fig. S1, A and C). Furthermore, loss of Tmem231 or B9d1 in MEFs results in longer cilia but in a decrease in the proportion of cells possessing cilia (Fig. S1, D and E). Thus, Tmem231 and B9d1 are essential for controlling the composition of the ciliary membrane, but are not essential for ciliogenesis.

It is not clear if the function of the MKS complex and the TZ in MEFs is similar to their function in other cell types in vivo. Therefore, to determine if Tmem231 and B9d1 control ciliary composition in vivo, we analyzed ciliary membrane protein composition across diverse tissues in wild-type, Tmem231−/−, or B9d1−/− embryos. In wild-type embryos, Arl13b localizes to cilia in the pancreas, bile duct, trachea, and kidney (Fig. 3, C and D). In the absence of either Tmem231 or B9d1, Arl13b no longer localizes to cilia in these tissues (Fig. 3, C and D). Together with our in vitro data, these results demonstrate that the

Figure 2. Tmem231 mutant mice display MKS-like phenotypes. (A) Hematoxylin and eosin–stained sections of portal triads of E15.5 livers of wild-type and Tmem231−/− embryos. In Tmem231−/− embryos, the portal vein (P) and the bile duct (B) are surrounded by increased mesenchyme. Bar, 100 µm. (B) Hematoxylin and eosin–stained sections of E18.5 kidneys show cyst formation in Tmem231−/− embryos. (C) Postnatal day 0 hindlimb skeletons stained with alcian blue and alizarin red reveal an extra digit (*) in Tmem231−/− embryos. Bars, 1 mm.

Tmem231 and B9d1 control ciliary membrane composition

Other MKS components participate in developmental signaling, at least in part, by controlling the membrane composition of cilia. For example, Tctn1 and B9d1 are essential for Smo, Adenylyl cyclase 3 (Adcy3), and Arl13b to localize to cilia (Dowdle et al., 2011; Garcia-Gonzalo et al., 2011). Smo, Adcy3, and Arl13b are all associated with the membrane: Smo and Adcy3 are transmembrane proteins and Arl13b associates with the ciliary membrane through its palmitoyl moiety (Cevik et al., 2010).
MKS complex is essential for regulating the membrane composition of varied types of cilia in vivo.

**Tmem231 and B9d1 are required for assembly of the MKS complex at the TZ**

At least 20 proteins localize to the TZ, a subdomain at the base of cilia critical for controlling ciliary composition and function (Garcia-Gonzalo and Reiter, 2012; Reiter et al., 2012). To understand how Tmem231 and B9d1 control ciliary composition, we investigated whether Tmem231 and B9d1 are required to localize other MKS complex components to the TZ. In wild-type MEFs, Tmem231 and B9d1 are present at the TZ (Fig. 4, A–C; and Fig. S2, A–C). In Tmem231−/− MEFs, B9d1 fails to localize to the TZ (Fig. 4 A and Fig. S2, A and C). Similarly, in B9d1−/− MEFs, Tmem231 no longer concentrates at the TZ (Fig. 4 B and Fig. S2, B and C). Thus, B9d1 and Tmem231 are...
reciprocally required for their localization to the TZ. Moreover, two additional MKS complex components, Mks1 and Tmem67 (Mks3), are lost from the TZ in the absence of either B9d1 or Tmem231 (Fig. 4, A and B; and Fig. S2, A–C). In contrast, neither Rpgrip11 nor Nphp1 require Tmem231 or B9d1 to localize to the TZ (Fig. 4, A and B; and Fig. S2, A–C). Instead, loss of either Tmem231 or B9d1 results in increased accumulation of Nphp1 at the TZ (Fig. 4, A and B). These data indicate that B9d1 and Tmem231 are both indispensable for, and perform similar roles in, the assembly of the MKS complex.
Mammalian B9d1 was identified as an MKS complex component via LAP-tandem affinity purification and mass spectrometry analysis of Mks1 and Tctn1 (Garcia-Gonzalo et al., 2011; Sang et al., 2011). Like B9d1 and Tmem231, Tctn1 is necessary to localize Mks1 and Tmem67 to Tzs, suggesting that Tctn1 may function with B9d1 and Tmem231 to construct the TZ (Garcia-Gonzalo et al., 2011). To test this hypothesis, we analyzed B9d1 and Tmem231 localization in wild-type and Tctn1−/− MEFs. In the absence of Tctn1, B9d1 and Tmem231 are no longer at the TZ (Fig. 4 C and Fig. S2 C), demonstrating that Tctn1 participates with B9d1 and Tmem231 in TZ organization.

Cilia perform different roles in different mammalian cell types, raising the possibility that their Tzs may have different compositions. Moreover, loss of certain MKS complex components, including B9d1 or Tctn1, compromises ciliogenesis in some but not all tissues, which indicates that TZ components can have tissue-specific functions (Garcia-Gonzalo et al., 2011). We tested whether these differential requirements for ciliogenesis reflect tissue-specific differences in TZ composition and found that Tmem231 and B9d1 localize to the TZ of cilia in the kidney, bile duct, pancreas, trachea, and retina of mice (Fig. 4, D and E), suggesting that TZ composition is similar in many ciliated cell types.

Similar to our observations in MEFs, B9d1 fails to localize to the TZ in all Tmem231 mutant tissues examined (Fig. 4 D). Reciprocally, in all B9d1 mutant tissues examined, Tmem231 is absent from the TZ (Fig. 4 E). These data reveal that the requirements for MKS complex assembly are similar in diverse ciliated mammalian tissues.

**Tmem231 control of TZ and ciliary composition is conserved in C. elegans**

Given that the localization and function of Tmem231 in TZ assembly and control of ciliary membrane protein composition is conserved among multiple cell types in mouse, we explored whether the localization and function of Tmem231 is conserved in an evolutionarily distant organism. To examine the localization of the C. elegans orthologue of Tmem231, TMEM-231 (also called T26A8.2), we created a transgenic line that expresses a Tmem231 promoter, is expressed exclusively at the TZ, and localizes pericellularly in ciliated sensory neurons, similar to other TZ proteins (Fig. 5, A and B; Bialas et al., 2009; Williams et al., 2011). Also consistent with a role in the TZ, TMEM-231 localizes specifically to this ciliary region, just distal to the basal body (Fig. 5, A and B; basal body and axoneme marked with the IFT protein XBX-1).

We tested whether, as in mammals, Tmem231 localization to the TZ requires other TZ proteins. Tmem231 localization to the TZ depends on MKS-2 (the orthologue of mammalian Mks2/Tmem216; Huang et al., 2011) and MKS-5 (the orthologue of mammalian Nphp8/Rpgrp11), but not NPHP-4 (the orthologue of mammalian Nphp4, Fig. 5 B). Reciprocally, NPHP-1 and MKS-5 localize normally to the TZ in nematodes lacking Tmem231, but Tmem231 and MKS-2 no longer concentrate at the TZ in mutants lacking exons 2 and 3 of tmem-231 (Fig. 5 C). The mammalian orthologue of Tmem231 was identified as a biochemical interactor of Tmem231 (Fig. 1) and B9d1 and is required for B9d1 to localize to the TZ in MEFs (Chih et al., 2012). These data suggest that Tmem231 is a functional component of the MKS complex in both mammals and nematodes.

To ascertain whether Tmem231 also plays a role in restricting the entry of nonciliary proteins into the cilium, we examined the localization of the periciliary membrane component TRAM-1a, the orthologue of mammalian translation-associated membrane protein 1. TRAM-1a is excluded from cilia in wild-type animals (Fig. 5 C, bottom). Loss of Tmem231 allows TRAM-1a to inappropriately leak into cilia (Fig. 5 C). In accordance with observations of its mammalian orthologue, these experiments demonstrate that C. elegans Tmem231 is a TZ protein that interacts functionally with other TZ proteins to control the composition of the TZ and ciliary membrane.

**Mutation of Tmem231 is associated with OFD3**

To further assess whether mutations in Tmem231 contribute to human ciliopathies, we performed targeted exon and splice site sequencing of Tmem231 in 1,056 individuals with NPHP-related ciliopathies, applying a recently developed high-throughput mutation analysis approach (Halbritter et al., 2012). We identified compound heterozygous mutations of Tmem231 (A3472-21/-24: c.241C>T, p.Leu81Phe/c.373C>G, p.Pro125Ala; Table S1, Fig. 6 A, and Fig. S4 A) in two affected siblings with OFD3 (Sugarman et al., 1971; Smith and Gardner-Medwin, 1993). Both alleles were predicted in silico to be disease causing (PolyPhen2-HumVar: 0.976 and 0.998) and affect residues conserved among chordates (Fig. S3). Pro125 is even conserved beyond chordates, including among several ciliated protists. Tmem231 orthologues are not found in unciliated organisms, further suggesting that this protein has conserved roles in ciliary biology. The variants were either absent or exceedingly rare in publicly available single nucleotide polymorphism databases (1000 Genomes, Exome Variant Server: c.241C>T, p.Leu81Phe: TT = 0/TC = 1/CC = 6155) and segregated with the OFD3 phenotype within the family (Fig. S4 A).

Although overlapping phenotypically with Joubert syndrome and OFD6, OFD3 is thought to be a clinically distinct autosomal recessive disorder characterized by “metronome” eye movements, lingual hamartomas, and postaxial polydactyly in addition to cerebellar vermis hypoplasy and moderate intellectual disability, all of which were present in both affected siblings (Baraitser, 1986). The younger sister also exhibited a large Dandy-Walker malformation with cystic dilatation of the fourth ventricle. Notably, both affected individuals presented with normal renal morphology and function at birth, but developed end-stage renal failure at 13 and 24 years old (Table S1).

**Recurrent Tmem231 mutations are associated with MKS**

To further investigate the mutational contribution of Tmem231 in MKS, we conducted whole exome sequencing (MKS-1163, MKS-855, and MKS-43), targeted ciliary gene exome sequencing (MKS-947/MKS-948 and GEF1200207), or bidirectional Sanger sequencing (n = 95) of samples from individuals with MKS or MKS-like clinical features. We detected variants inherited under...
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hydrocephalus (MKS-947 and MKS-948), Dandy-Walker mal-
formation with either hydrocephalus (NTDMGS 80399) or cer-
ebellar vermis agenesis (MKS-79 and MKS-692), renal cysts,
and hepatic portal fibrosis (Table S1). Like the residues affected
in OFD3, Asn90 is a highly conserved residue possessed by
chordates including Ciona intestinalis (Fig. S3).

The intronic c.664+4A>G variant was not unique to the
MKS family with two affected individuals (MKS-947/MKS-
948), as three additional MKS pedigrees carried this putative
splice-altering variant (Table S1 and Fig. S4, F–H). Two con-
sanguineous families of Turkish and Libyan descent harbored
this change in homozygosity (MKS-1163 and MKS-855, respec-
tively). Further, we detected a maternally inherited heterozygous
c.664+4A>G variant in a fourth family of Turkish/European
origin (MKS-374), with a paternally inherited heterozygous
frameshift mutation (p.Ile232SerfsX). To test the functional con-
sequences of the c.644+4A>G change, we assessed TMEM231

a recessive model of disease and segregating with the disease
phenotype in nine pedigrees (Table S1, Fig. 6 A, and Fig. S4).
The MKS-associated variants were not present in the exomes of
6,503 control individuals (4,300 European American and 2,203
African American individuals; Exome Variant Server).

Four families of northern European descent harbored
the same c.373C>G, p.Pro125Ala change as the OFD3 family
(Table S1 and Fig. S4, B–E). In one nonsanguineous pedi-
gree (NTDMGS 80399), p.Pro125Ala was present in homozy-
gosity (Fig. S4 B). Three other families each had TMEM231
variants in trans with p.Pro125Ala. One pedigree (MKS-692) also
had a heterozygous nonsense mutation (c.544C>T, p.Gln182X);
a French proband (MKS-79) harbored a heterozygous c.270C>T,
p.Asn90Ile mutation; and another family (siblings MKS-947
and MKS-948) had a heterozygous c.664+4A>G intronic
change (Fig. 6 A and Fig. S4, C–E). All probands with p.Pro-
125Ala displayed postaxial polydactyly on at least three limbs,

hydrocephalus (MKS-947 and MKS-948), Dandy-Walker mal-
formation with either hydrocephalus (NTDMGS 80399) or cer-
ebellar vermis agenesis (MKS-79 and MKS-692), renal cysts,
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sequences of the c.644+4A>G change, we assessed TMEM231

Figure 5. C. elegans TMEM-231 functionally interacts with other TZ proteins and is required for ciliary gating. (A) Schematic of C. elegans phasmid ciliary
structure. The region delineated by the dashed line is depicted in subsequent panels. (B) TMEM-231::GFP (green) is enriched at the TZ of wild-type animals,
but is mislocalized in the mks-2 and mks-5 mutants, but not in the nphp-4 mutants. The Dynein 2 component XBX-1::tdTomato (red) marks the basal body
transition fibers and axonemes. (C) NPHP-1::CFP (blue) localizes to the TZ of wild-type and tmem-231 mutant animals. Similarly, MKS-5::tdTomato (red)
localizes to the TZ of wild-type and tmem-231 mutant animals. CHE-13::YFP (yellow), the C. elegans orthologue of Ift57, and DYF-11::GFP (green), the
orthologue of Ift54, mark the transition fibers and axonemes. In contrast, TMEM-17::GFP (green) fails to localize to the TZ (dotted line) in tmem-231 mutants.
MKS-2::GFP (green) fails to localize to the TZ in tmem-231 mutants and is instead within the more distal cilia (asterisks). TRAM-1a::tdTomato (red) localizes
to the periciliary membrane (pcm) in wild-type animals. However, in tmem-231 mutants, TRAM-1a::tdTomato enters cilia, indicating defects in ciliary gating
(asterisks). The dashed ovals delineate the region of the transition zone. Bars, 5 µm.
Figure 6. **TMEM231 mutations are associated with OFD3 and MKS.** (A) Schematic of the TMEM231 locus and mutations identified in OFD3- and MKS-affected individuals. Mutation locations are indicated with asterisks and mutation names correspond to GenBank accession no. NM_001077418. Blue boxes, exons; white boxes, untranslated regions; gray lines, introns; black numbers exons. (B) Arl13b localization in Tmem231−/− MEFs is rescued by transient transfection of wild-type Flag-tagged Tmem231. The mutant forms of Tmem231 associated with OFD3 and MKS partially restore Arl13b localization to cilia. Bars, 2.5 µm. (C) Quantitation of the normalized fluorescence intensity of Arl13b at wild-type and Tmem231−/− cilia (at least 10 cilia per condition) transfected with the indicated expression constructs. The data shown are from a single representative experiment out of three repeats. (D) Coimmunoprecipitation of FLAG-tagged Tmem231 constructs and V5-tagged B9d1. Despite two mutant forms of Tmem231 being expressed at lower levels (lanes 3–7, second blot), all four mutant proteins were able to immunoprecipitate B9d1 (third blot, third through seventh lanes). The data are shown are from a single representative experiment out of two repeats. (E) Transfection of wild-type Flag-tagged Tmem231 restores B9d1 localization to Tmem231−/− MEFs. Ciliopathy-associated point mutations restore B9d1 localization to a lesser extent. Arrowheads show the TZ. Bars, 2.5 µm. (F) Quantitation of the normalized fluorescence intensity of B9d1 at wild-type and Tmem231−/− cilia (at least 10 cilia per condition) transfected with the indicated expression constructs. The data shown are from a single representative experiment from two repeats. Error bars represent the 95% confidence interval.

*P < 0.05, as measured by Student’s t test with Welch’s correction.

Transcripts present in cultured primary skin fibroblasts from MKS-374. Direct sequencing of RT-PCR products using primers spanning exons 4–6 revealed the abolishment of the exon 5 splice donor site, utilization of a novel splice site in intron 5–6, and the retention of 47 bp of intronic sequence to produce a truncated message encoding p.Val222fsX21 (Fig. S4 K).
We identified two additional MKS pedigrees with TMEM231 mutations that segregated appropriately under a recessive model (Table S1, Fig. 6 A, and Fig. S4, I and J): a consanguineous Tunisian family with a homozygous c.664G>A, p.Asn90Ile mutation, reported previously to disrupt splicing of exons 5–6 (Shaheen et al., 2013), and a family with novel compound heterozygous splice acceptor (c.439-1G>C) and missense (c.646G>C; p.Ala216Pro) mutations (GEF1200207). In contrast to the affected individuals with p.Pro125Ala mutations who had cerebellar malformations and/or hydrocephalus, the other MKS individuals displayed severe neural tube defects including anencephaly (MKS-374) or encephalocele (MKS-1163, MKS-855, MKS43, and GEF1200207; Table S1).

**TMEM231 disease-associated alleles are hypomorphic**

To test the effect of the disease-associated nonsynonymous mutations on the ability of Tmem231 to function with the TZ complex, we created a cell-based assay to assess the membrane–protein–associated composition of cilia. In the absence of Tmem231, Arl13b fails to localize to cilia (Fig. 6, B and C; and Fig. S5 A). Expression of amino terminally Flag-tagged wild-type Tmem231 in Tmem231−/− MEFs restores Arl13b localization to cilia, as assessed by the fluorescence intensity of Arl13b at cilia (Fig. 6, B and C; and Fig. S5 A). Expression of ciliopathy-associated Tmem231 mutants in Tmem231−/− MEFs failed to restore ciliary Arl13b localization to the same extent as wild-type Tmem231 (Fig. 6, B and C; and Fig. S5 A).

Each of these mutant forms of Tmem231 (p.Leu81Phe, p.Pro125Ala, p.Asn90Ile, and p.Ala216Pro) rescue ciliary Arl13b to a modest extent, suggesting that the OFD3- and MKS-associated mutations are hypomorphic and partially compromise TMEM231 function.

To test how the disease-associated mutations affect Tmem231 function, we tested whether mutant forms of Flag-tagged Tmem231 could associate with V5-tagged B9d1 by co-immunoprecipitation (Fig. 6 D). The four disease-associated Tmem231 mutant proteins retain their ability to interact with B9d1, but two mutations, p.Asn90Ile and p.Pro125Ala, compromised Tmem231 protein levels (Fig. 6 D).

To explore further how the disease-associated mutations in Tmem231 affect its function, we tested if they were able to support MKS complex organization at the TZ of Tmem231−/− MEFs. In the absence of Tmem231, B9d1 fails to localize to the TZ (Fig. 4 A; Fig. 6, E and F; and Fig. S3 F). Expression of wild-type Flag-Tmem231 in Tmem231−/− MEFs restored B9d1 localization to the TZ (Fig. 6, E and F; and Fig. S5 F).

In contrast, all four disease-associated mutant forms of Tmem231 failed to restore B9d1 localization to the TZ to the same extent as wild-type Tmem231 (Fig. 6, E and F). Interestingly, p.Asn90Ile expression in wild-type MEFs mislocalized B9d1 away from the TZ to the centrosome, suggesting this mutant may interfere with the function of wild-type Tmem231 (Fig. 6, E and F; and Fig. S5 F). Thus, disease-associated mutations in Tmem231 disrupt formation of the MKS complex at the TZ, leading to defective ciliary membrane composition and ciliary signaling.

**Discussion**

We found that B9d1 and Tmem231 are evolutionarily conserved components of the TZ MKS complex required to control the composition of the ciliary membrane. In mice, B9d1 and Tmem231 are required for the TZ localization of each other and their interacting proteins, Tmem67 and Mk1. Similarly, in *C. elegans*, TMEM-231 localizes to the TZ and participates with other MKS complex proteins to build the TZ and prevent a non-ciliary protein, TRAM-1a, from entering the cilium. Like B9d1, Tmem231 is required for mammalian development: Tmem231 mutant mice have developmental defects reminiscent of MKS patients, including corticomedullary kidney cysts, polydactyly, and hepatic ductal plate malformation. Consistent with a role in human disease, we identified hypomorphic and truncating TMEM231 mutations that segregate with OFD3 and MKS.

B9d1 and Tmem231 are essential for the organization of the MKS complex at the TZ. Each is required for the other, and Mk1 and Tmem67, to localize to the TZ. Additionally, an extracytosolic protein, Tctn1, is required for the TZ localization of B9d1 and Tmem231. Thus, the MKS complex is comprised of functionally integrated components that span the membrane and depend on extracytosolic (e.g., Tctn1), transmembrane (e.g., Tmem231), and cytoplasmic (e.g., B9d1) constituents for its organization. However, none of these components are necessary for formation of the entire TZ. For example, Tmem231, B9d1, and Tctn1 are required for Mk1 and Tmem67 TZ localization, but not Rpgrip1l or Nphp1 localization (this study; Garcia-Gonzalo et al., 2011). Tctn1 is required for Mk1, Tmem67, Tmem231, and B9d1 to localize to the TZ, but not for Cc2d2a, Rpgrip1l, or Tmem216 (this study; Garcia-Gonzalo et al., 2011). Tmem231 and B9d1 are needed for TZ localization of each other, Mk1, and Tmem67, and also Cc2d2a and Tmem17 (this study; Chih et al., 2012). Reciprocally, Cc2d2a and Tmem17 are necessary for Tmem231 and B9d1 to localize to the TZ (Chih et al., 2012). These data are consistent with at least two possible models of MKS complex assembly.

In hierarchical assemblies, a core component recruits a set of interactors that, in turn, recruit the peripheral proteins of the complex. A candidate for the initiating TZ component in *C. elegans* is MKS-5, the orthologue of mammalian Rpgrip1l and Rpgrip1 (Williams et al., 2011). In the absence of MKS-5, all other MKS complex components tested fail to localize to the TZ (Huang et al., 2011; Williams et al., 2011). Moreover, MKS-5 localization to the TZ is not dependent on the MKS complex components MKS-6 (orthologue of Cc2d2a) or MKSR-2 (B9d2), raising the possibility that MKS-5 forms a core scaffold for TZ assembly in nematodes (Williams et al., 2011). Consistent with a central role for MKS-5 in TZ assembly, we found that it is critical for TMEM-231 localization to the TZ. One of the mammalian homologues of MKS-5, Rpgrip1l, behaves similarly in that Rpgrip1l does not require B9d1, Tmem231, or Tctn1 to localize to the TZ. However, whether Rpgrip1l is required for Tmem231 or B9d1 to localize to the TZ remains unclear. Furthermore, whether the second mammalian MKS-5 homologue, Rpgrip1, plays a semi-redundant role with Rpgrip1l in MKS complex formation remains unclear.
Alternatively, the mammalian TZ may not be assembled on any one protein, but might require multiple reciprocal interactions, as would occur if the complex were destabalized by the absence of any of several core constituents. Our finding that B9d1 and Tmem231 are reciprocally required for each other’s localization and mutually essential for TZ function suggests that this model may better describe mammalian TZ assembly. However, blended models involving both hierarchical and reciprocal dependencies can also account for the available data.

Components of the MKS complex are required to control the protein composition of the ciliary membrane (Garcia-Gonzalo et al., 2011; Dowdle et al., 2011; Chih et al., 2012). Consistent with their inclusion in the MKS complex, Tmem231 and B9d1 are necessary to localize certain membrane proteins to cilia, both in MEFs and in diverse mammalian tissues. For example, Arl13b, Adcy3, Pkd2, and Inpp5e fail to localize to cilia in Tmem231−/− MEFs, and Tmem231 and B9d1 are required for Arl13b ciliary localization in all mouse tissues examined. Interestingly, despite the varied functions of cilia in different tissues, we found that the primary cilia of varied cell types possess Tzs of similar composition and Tmem231 is required to control ciliary composition in all of the diverse tissues examined.

Similar to mammalian Tmem231, C. elegans TMEM-231 controls protein entry into cilia, as TRAM-1a, a protein normally excluded from cilia, exhibits ciliary localization in tmem-231 mutant nematodes. This defect is comparable to that observed upon disruption of other TZ proteins in C. elegans, including the MKS module components MKSR-1 (B9d1), MKSR-2 (B9d2), and MKS-6 (Ccd2a; Williams et al., 2011), and suggests that the TZ functions to promote the localization of certain membrane-associated proteins to the cilium and to prevent the ciliary entry of others.

Because of its roles in controlling ciliary composition, the TZ is essential for ciliary signaling and mammalian development (Reiter and Skarnes, 2006; Weatherbee et al., 2009; Dowdle et al., 2011; Garcia-Gonzalo et al., 2011; Sang et al., 2011; Chih et al., 2012). Similarly, Tmem231 is crucial for mouse development: Tmem231 mutant mice develop phenotypes that are hallmarks of MKS, including cystic kidneys, ductal plate malformations, and hindlimb polydactyly. The Tmem231 mutant phenocopies that of B9d1 mutants on a mixed genetic background, emphasizing their shared functions (Dowdle et al., 2011).

The two siblings that originally defined OFD3 are compound heterozygotes for missense mutations in TMEM231 predicted to produce p.Leu81Phe and p.Pro125Ala. The latter allele, encoding p.Pro125Ala, is homozygous in a MKS fetus and compound heterozygous with a separate heterozygous mutation (p.Asn90Ile, p.Gln182X, and c.664+4A>G) in three additional MKS families. Present in five different ciliopathy pedigrees (both OFD3 and MKS), it is unclear whether the hypomorphic p.Pro125Ala allele is a founder mutation that occurred in northern European populations or whether it is a mutational hotspot. Similarly, we identified a recurrent intronic c.664+4A>G mutation that disrupts the exon 5 splice donor site, resulting in aberrant splicing and premature termination. This change is present in four MKS pedigrees of varying ethnicity: two in homozygosity and two families with heterozygous c.664+4A>G changes with a second pathogenic heterozygous TMEM231 variant in trans. Together, our mutational data indicate that TMEM231 contributes to >5% of MKS cases, and the identification of two recurrent mutations will aid in the development of diagnostic tests for suspected ciliopathies.

To understand how nonsynonymous ciliopathy-associated TMEM231 mutations cause disease, we assessed if they disrupt the ability of Tmem231 to function with the MKS complex. The disease-associated mutant forms of Tmem231 are compromised in their ability to restore Arl13b to Tmem231−/− cilia. This phenotype is consistent with the failure of the disease-associated forms of Tmem231 to support formation of the MKS complex to the TZ: the mutant proteins fail to rescue B9d1 localization to the TZ in Tmem231−/− cilia. Interestingly, although two mutations destabilize Tmem231, all four mutant proteins interact with B9d1 similar to wild-type Tmem231, suggesting that the hypomorphic nature of these point mutants may be separate from their capacity to interact with other complex components.

We hypothesized that the OFD3-associated mutant proteins would be more functional than MKS-associated mutant proteins, given the greater severity of clinical ailments in MKS. However, one of the MKS-associated mutant proteins, p.Asn90Ile, displays more activity than the OFD3-associated mutant protein, p.Leu81Phe, in our cell-based assay. One possible explanation is that the two mutant TMEM231 alleles associated with OFD3 may partially complement each other. Partial intragenic complementation would indicate that Leu81 and Pro125 participate in separate aspects of TMEM231 function. Whereas p.Asn90Ile retains partial function in localizing Arl13b to cilia, this mutation interferes with other critical aspects of TMEM231 function such as B9d1 localization to the TZ. Given our inability to stratify TMEM231 allele pathogenicity by phenotype, we surmise that the MKS samples have an increased mutational burden in other ciliary or TZ genes in comparison to the OFD3-affected individuals, accounting, at least in part, for the different phenotypes of the two syndromes.

How might TMEM231 and the TZ control the composition of the ciliary membrane? The loss of ciliary proteins from Tmem231 mutant cells and the entrance of TRAM-1a into the cilia of C. elegans tmem-231 mutant animals suggest that the TZ acts as a membrane diffusion barrier, allowing ciliary proteins to enter and preventing entry of nonciliary proteins. Knockdown of B9d1 or Tmem231 can increase the entry rate of ciliary proteins into the cilium, suggesting that the MKS complex serves as an entry barrier (Chih et al., 2012).

Conversely, the MKS complex could act as an exit barrier, retaining certain membrane-associated proteins within cilia, while allowing exit of nonciliary membrane proteins. In this model, disruption of MKS complex function would not alter entry of ciliary membrane proteins, but would prevent their accumulation, resulting in decreased ciliary localization at steady state. We anticipate that assessing the role of the MKS complex in entry and exit of membrane proteins at the cilium will test these models.
Materials and methods

Plasmids

Mouse Tmem231 was cloned into the pGLAP destination vector (plasmid 19706, backbone: pEFS/FRT5V, promoter: EF1α; Addgene) using Clonase II (Invitrogen) to produce pGLAP-Tmem231, as previously described (Dowdle et al., 2011). Similarly, mouse B9d1 and Mks1 were cloned in pEFS/V5/Frt (Invitrogen) destination vector using Clonase II to produce pEFS/V5/Frt-B9d1 and pEFS/V5/Frt-Mks1, as described previously (Dowdle et al., 2011). Mouse Tmem231 was cloned into a pCMV-Flag vector, such that the resulting construct expressed N-terminally flag-tagged mouse Tmem231 (pCMV-Flag-Tmem231). Mutant alleles were constructed from pCMV-Flag-Tmem231 using QuickChange site-directed mutagenesis with Herculease II DNA polymerase (Agilent Technologies). The four mutations are as follows: Tmem231 P125A (c.373C>G), and Tmem231 568 (Alexa Fluor).

Immunoprecipitation and immunoblot

COS1 or 293T cells, grown on 15-cm-diameter plates, were transfected with pCMV-Flag-Tmem231 and pEFS/V5/Frt-Mks1 or B9d1 using Lipofectamine 2000 (Life technologies) and lysed after 48 h in a buffer consisting of 50 mM Hepes, pH 7.4, 300 mM KCl, 1 mM EGTA, 1 mM MgCl2, 10% glycerol, 200 ng/ml neomycin (Life Technologies) and lysed after 48 h in a buffer consisting of 50 mM Hepes, pH 7.4, 300 mM KCl, 1 mM EGTA, 1 mM MgCl2, 10% glycerol, 0.3% NP-40, 0.5 mM DTT, and protease and phosphatase inhibitors. Lysates were cleared via centrifugation at 3,500 g for 20 min and incubated with FLAG-M2 beads (Sigma-Aldrich) for 4 h at 4°C. After 4°C wash in lysis buffer, beads were resuspended in 6x SDS-PAGE loading buffer and denatured at 100°C for 5 min. Immunoprecipitations were resolved on 10% SDS-PAGE gels (Bio-Rad Laboratories) and transferred to PVDF membrane (EMD Millipore). Membranes were blocked with 5% nonfat dried milk in TBS with 0.1% Tween, and then incubated with primary antibodies followed by secondary antibodies in 5% nonfat dried milk in TBS with 0.1% Tween. Blots were developed with Western lightning enhanced chemiluminescence reagent (PerkinElmer). Apparent molecular weights were determined using full-range rainbow prestained protein standards (GE Healthcare).

Mass spectrometry

IMCD3 cell lines harboring stable, single integrations of pGLAP5 vector or pGLAP5-Tmem231 were created using IMCD3 Flp-In cells (gift of M. Nachury, Stanford University, Palo Alto, CA). The IMCD3 Flp-In cells were created based on the Invitrogen Flp-In system. In brief, transfection of FRT site-bearing pGLAP5-based vectors and a Flp recombinase expression plasmid recombines the pGLAP-based vector at a genomic FRT site within the IMCD3 Flp-In genome. 50 15-cm-diameter plates each of pGLAP5 (control) and pGLAP5-Tmem231 stable cells were used for LAP purification, as described previously (Cheeseman and Desai, 2005). Cell lysates were cleared via centrifugation, GFP trap beads (ChromoTek) were used in the first capture, and after TEV cleavage, samples were further purified with anti-FLAG-M2 antibodies (Sigma-Aldrich). The four mutants were outcrossed to wild type (N2) at least five times.

Immunohistochemistry and histology

For tissue analysis, organs were fixed in 10% formalin overnight at 4°C, sectioned, and stained with hematoxylin and eosin according to standard protocols. Immunofluorescent staining of paraffin sections was performed as described previously (van Eyll et al., 2004). In brief, paraffin sections were rehydrated, permabilized for 15 min with 0.3% Triton X-100 in PBS, blocked for 45 min with 3% milk/0.3% Triton X-100 in PBS at room temperature, and stained with primary and secondary antibodies. For B9d1 staining, an additional antigen retrieval was performed by incubating sections with 1% SDS dissolved in PBS for 5 min at room temperature followed by extensive rinses in PBS.

Antibodies

Primary antibodies used were mouse α-acetylated tubulin 6-11B-1 (TubAc; Sigma-Aldrich), rabbit α-Tmem231 (Sigma-Aldrich), rabbit α-Mks1 (ProteinTech), rabbit α-Mks5 (ProteinTech), rabbit α-Nphp1 (gift of G. Pazour, University of Massachusetts Medical School, Worcester, MA; amino acids 1–209 [Benzing et al., 2001; Fliegauf et al., 2006]), rabbit α-Arl13b (ProteinTech), mouse α-Arl3b (ProteinTech), rabbit α-NP5p5 (gift of S. Schurmans, Université de Liège, Liège, Belgium; antigen: N-terminal MGQGQPNTEKIL pepptide [Jacobov et al., 2009]), rabbit α-Ady3 (ProteinTech), rabbit α-IFB88 (ProteinTech), rabbit α-Pkd2 (JYCC; gift of S. Somlo, Yale School of Medicine, New Haven, CT), rabbit α-tubulin (Santa Cruz Biotechnology, Inc.), goat α-V5 (Abcam), and mouse α-FLAG-M2 (Sigma-Aldrich). Rabbit α-B9d1 antibody was generated by injecting a full-length mouse B9d1 protein fused to a His tag into rabbits (Pacific Immunogenetics) and affinity purifying the antibody with recombinant B9d1-6xHis. Secondary antibodies are as follows: donkey α-mouse 487, donkey α-mouse 647, donkey α-rabbit 488, donkey α-rabbit 647, and donkey α-goat 568 (Alexa Fluor).

Cell lines

MEFs were derived from E13.5 littermate-matched wild-type and B9d1 mutant or Tmem231 mutant embryos and grown in Advanced DMEM supplemented with 10% FBS, 1% GluMax, and 1% antibiotic-antimycotic (Invitrogen). COS-1 and 293T cells were grown in DMEM supplemented with 10% FBS and 1% GluMax.

C. elegans strains used and localization studies

All strains (Table S2) were maintained and cultured on NGM plates at 20°C. The tmem-231(m35963), mks-5(m3100), and nphp-4(m925) mutations were obtained from the National Bioresource Project (Japan) and we previously generated the mks-2(n1111) [Williams et al., 2011]. All mutants were outcrossed to wild type (N2) at least five times. The construct harboring GFP-tagged C. elegans Tmem231 was generated by creating a translational fusion construct with its native promoter (1,620-bp upstream of the start codon) and all exons and introns fused in-frame to EGFP at the C-terminal end of the coding region together with a 3’ UTR from unc-54. Multiple transgenic lines for this construct were generated by coinjection of the rol-6(su1006) and screening for roller worms as described previously (Williams et al., 2011). Standard mating procedures were used to introduce the GFP-tagged Tmem231 protein construct into mks-2, mks-5, and nphp-4 mutant backgrounds and fluorescently labeled protein constructs for Tmem-17, Mks-5, NPHP-1, and TRAM-1 into the tmem-231 mutant background. Single-worm PCR amplifications were used to genotype the various mutants. Information for all mutant strains used, namely mks-2(n1111) (5,373-bp deletion), mks-5(m3100) (540-bp

B9d1. These mice were outcrossed to CD1 mice (Charles River) and made conditionally ready by crossing to a mouse expressing to a ubiquitous FLP recombinase-expressing mouse. Subsequently, the B9d1+lox/lox mice were crossed to a ubiquitous Cre recombinase-expressing mouse to delete exon 3. The Tmem231+/− (B6N 129S5-Tmem2311025T353275Kv4.4/Mucumd) strain was obtained from the Mutant Mouse Regional Resource Center at the University of California, Davis (Tang et al., 2010). The Tmem231 gene trap mutation was made by oligo-directed embryonic stem (ES) cell injection. Chimeric animals were crossed to C57BL/6J albino mice (The Jackson Laboratory) to generate 1 F1 heterozygous animals. F1 mice were intercrossed to produce 2 heterozygotes, which were backcrossed to C57BL/6NCrl (Charles River) to N11 before cryopreservation of spermatozoa. The Tmem231+/− strain was recovered at University of California, San Francisco, in a C56BL/6NCrl background. The mice were maintained by crossing to CD1 mice (Charles River) for two generations.
RT-PCR to evaluate splice-site mutations

We cultured primary skin fibroblasts from MKS fetus MKS-374 according to standard procedures and harvested total RNA in Trizol according to the manufacturer's instructions. We generated oligo-dT primed cDNA using Superscript III reverse transcription (Life Technologies), and conducted PCR using primers placed in exons 4 and 6 (TMEM231-4R: 5'-ACCCCTCGGTA-TCACACGC-3'; TMEM231-6R: 5'-TGCGGATCCAGGACGCTG3'; TMEM231-6R2-R: 5'-GAATGCTTCCACGAGGAT-3'). Resulting PCR products were sequenced directly using bidirectional Sanger sequencing.

Immunofluorescence

For cilia and TZ staining, MEFs were plated on glass coverslips, grown to confluence, and starved for 24–72 h in OptiMEM. For Mtks1, Tmem67, and Tmem231 staining, cells were fixed in methanol for 3 min at −20°C. For B9d1, Tmem231, and Rgrip11, cells were fixed for 10 min at RT (20–25°C) in 4% methanol-free formaldehyde (FA) in PBS, followed by antigen retrieval for 5 min at RT in 1% SDS dissolved in PBS. After SDS antigen retrieval, cells were washed 3 x 10 min with PBS. Arl13b, Pkd2, Itib8, Nphp1, and Adcy3 cells were fixed for 10 min at RT with 5% FA in PBS, followed by methanol for 3 min at −20°C. After fixation, cells were blocked for 60 min at RT or overnight at 4°C in dPBS + 0.1% Triton X-100 + 2.5% BSA (IF block). Cells were incubated with primary antibodies diluted in IF block overnight at 4°C. Coverslips were washed with dPBS, and then incubated with Alexa-conjugated secondary antibodies raised in goat or donkey (Invitrogen) at RT. After three dPBS washes, coverslips were mounted with ProLong Gold antibody (Invitrogen) and sealed with nail polish. Cells were imaged with a TCS SP5 microscope (Leica). Images were processed using FIJI (ImageJ).

Mutation analysis of ciliopathy patients

Full ethics approval was obtained by the Northern and Yorkshire Research Ethics Committee (UK), the University of Michigan, Necker Hospital (France), and Duke University Medical Center, and informed written consent was obtained from parents and their relatives. DNA was extracted from whole blood or fetal tissue using standard techniques. All coding exons and exon–intron boundaries of TMEM231 were amplified in 1,056 patients with nephropathies-related ciliopathies in a large-scale microfluidic multiplex PCR by using a Fluidigm Access Array system (48-48). By primer pooling and microfluidics, this approach allows for simultaneous generation of >800 amplicons for each of 48 patient samples (Halbritter et al., 2012, 2013). In a second PCR reaction, amplicons were barcoded by adding a patient-specific 10-bp nucleotide sequence. Consequently, generated amplicons from 1,056 patients were pooled and submitted to next generation sequencing, performed on a Genome Analyzer II platform (Illumina). Whole exome sequencing and variant filtering was conducted as described previously (Gordon et al., 2013). In brief, SureSelect libraries (Agilent Technologies) were prepared from 3 µg of genomic DNA sheared with an S2 Ultrasonicator (Covaris) according to the manufacturers’ instructions. Exome capture was performed with the 51-Mb SureSelect Human All Exon kit V5 (Agilent Technologies) via a multiplex approach with molecular barcodes for traceable identity of samples. Sequencing was performed on pooled exome libraries with an HiSeq2500 (Illumina) machine. 76-bp paired-end reads were generated with the HiSeq2500. After demultiplexing, paired-end sequences were aligned to the reference human genome (hg19, UCSC Genome Browser) with the Burrows-Wheeler Aligner (Illumina). The mean depth of coverage obtained for each sample was >80x, and >90% of the exome was covered at least 15x.

Ciliary exome–targeted sequencing and bioinformatics filtering was conducted using custom SureSelect capture kits (Agilent Technologies) targeting 4.5 Mb of 20,168 exons (1,221 ciliary candidate genes), including TMEM231 (Thomas et al., 2012; Failler et al., 2014). In brief, SureSelect libraries were prepared from 3 µg of 300 genomic DNA samples sheared with an S2 Ultrasonicator, according to the manufacturers’ instructions. Precapture SOLID libraries were prepared without any barcode. The SOLID molecular barcodes for traceable ID of samples were added at the end of the capture step. The Ovation Ultralow System (NuGEN Technologies) was used to prepare HiSeq2500 precaptured barcode libraries. The ciliome capture by hybridization was performed on a pool of 10–16 barcoded precapture libraries. Sequencing was performed on pools of bar-coded ciliome libraries (64 barcoded ciliome libraries per SOLID Flow-Chip™) and 16 ciliome libraries per HiSeq FlowCell lane) using SOLIDSS500XL (Life Technologies) and HiSeq2500. Paired-end reads were generated (75 + 35 for SOLID and 100 + 100 for HiSeq) and mapped on the human genome reference (NCBI build37/hg19 version) using Burrows-Wheeler Aligner or mapred (SOLID). Downstream processing was performed with the Genome Analysis Toolkit, SAMtools, and Picard Tools, following documents published previously (Van der Auwera et al., 2013). All variants were annotated using a software system developed by the Paris Descartes University Bioinformatics platform. The mean depth of coverage obtained was >90x, and >90% of the exome was covered at least 15x. Different filters were applied to exclude all variants located in nonexonic regions, pseudogenes, UTRs, or known polymorphic variants with a frequency ~1% (i.e., present in databases such as dbSNP and 1000 Genome Projects, and all variants identified by whole exome sequencing of 5,150 exomes and 1,020 ciliomes). The functional consequence of missense variants was predicted using the SIFT (http://sift.jcvi.org/www/SIFT_enst_submit.html) and PolyPhen2 programs (http://genetics.bwh.harvard.edu/pph2/).

For candidate gene analysis of 95 MKS samples, we conducted bidirectional Sanger sequencing of exons and splice junctions according to standard methodology. Mutations identified in all studies were confirmed using Sanger sequencing in patients and their parents to confirm segregation.

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shows sequences of the TMEM231 mutations identified in the OFD3- and MKS-affected individuals described in this study. Fig. S5 displays quantification of the rescue assays (related to Fig. 6). Table S1 shows mutations of TMEM231 associated with OFD3 and MKS. Table S2 shows the C. elegans strains used. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201411087/DC1.

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