Pih1d3 is required for cytoplasmic preassembly of axonemal dynein in mouse sperm

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Introduction

Cilia are long appendages that extend from the cell body. There are two types of cilia: motile cilia and immotile cilia (also known as primary cilia). In mammals, motile cilia are present in the respiratory epithelium, reproductive system (for example, the oviduct), and central nervous system (for example, the ependyma). Motile cilia that are present singly or in small numbers per cell are referred to as flagella, as in single-cell protozoa such as Chlamydomonas and in mammalian sperm. Primary cilia are found in most mammalian cell types, including those of the skin, kidney (renal tubular epithelial cells), and blood vessels (endothelial cells; Wheatley et al., 1996).

Cilia contain a microtubule-based axoneme covered by a specialized ciliary membrane that is continuous with the plasma membrane of the cell. The axoneme is composed of nine peripheral microtubule doublets surrounding a central core that may or may not contain two central microtubules (9+2 for motile cilia and 9+0 for primary cilia, respectively). The axoneme of motile cilia specifically contains other associated structures such as outer dynein arms (ODAs) and inner dynein arms (IDAs), radial spokes, and nexin links.

Given that cilia do not contain DNA or any machinery necessary for protein synthesis, all ciliary proteins are synthesized in the cytoplasm and then transported to the site of ciliation assembly by a process known as intraflagellar transport (Rosenbaum and Witman, 2002). The history of ciliary proteins before such transport, including their state in the cytoplasm and how they are sorted, has remained unclear, however.

In Chlamydomonas, ciliary proteins are stocked in a cytoplasmic pool before they are sorted (Rosenbaum and Child, 1967; Rosenbaum et al., 1969). In the cytoplasm, components of multisubunit structures such as ODAs, IDAs, and radial spokes have been detected in preassembled complexes, rather than individually (Fok et al., 1994; Piperno and Mead, 1997; Fowkes and Mitchell, 1998; Qin et al., 2004), as the result of a process known as cytoplasmic preassembly (Kobayashi and Takeda, 2012).

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Abbreviations used in this paper: DNAAF, dynein axonemal assembly factor; H&E, hematoxylin-eosin; HC, heavy chain; IC, intermediate chain; IDA, inner dynein arm; LC, light chain; ODA, outer dynein arm; TEM, transmission electron microscopy; WT, wild type; X-gal, 5-bromo-4-chloro-3-indolyly-b-galactopyranoside.

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Figure 1. Sperm of Pih1d3<sup>−/−</sup> mice are immotile and morphologically abnormal. (A) Structural representation of four proteins that contain a PIH1 domain. The numbers indicate the amino acid residues that correspond to predicted PIH1 domain regions. (B) Motility of sperm from adult wild-type (WT) and Pih1d3<sup>−/−</sup> mice as evaluated by determination of the percentage of motile sperm at 1, 2, and 4 h after release from the cauda epididymis. Two batches of at least 100 sperm were examined for each mouse. Data are means ± SEM (n = 2 batches from one mice). *, P < 0.01 versus the corresponding value for WT sperm (Student’s t test). (C) Movement of 10 WT or Pih1d3<sup>−/−</sup> sperm was traced with TEMA software over 5 s. The movement of individual sperm cells is indicated by the different colored lines. Bars, 50 µm. (D) Light microscopy of WT and Pih1d3<sup>−/−</sup> spermatozoa collected from the cauda epididymis. Whereas the head of sperm from Pih1d3<sup>−/−</sup> males appeared normal, the tail was thinner, shorter, and more susceptible to breakage (arrows) compared with that of WT sperm. Images were obtained with a 40x objective. Bars, 50 µm. (E) Original data for B. (F) Sperm length, the width of the midpiece region, and the frequency of broken sperm are shown for WT and Pih1d3<sup>−/−</sup> sperm. The number of samples examined is shown as n.
The molecular components of the axoneme and mechanism of cytoplasmic preassembly have also been investigated in *Chlamydomonas*. ODAs have thus been found to be composed of three heavy chains (HCs; α, β, and γ, each of ~500 kD), two intermediate chains (IC1, 78 kD; IC2, 69 kD), 10 light chains (LC1 to LC10, 8 to 22 kD), and a 7S factor comprising three proteins that form an ODA attachment site or docking complex (DC105, DC62.5, and DC25). IDAs are structurally more divergent. Immunoprecipitation analysis has revealed that the three HCs and two ICs of ODAs exist in the cytoplasm as a complex (Fowkes and Mitchell, 1998).

Analysis of dynein-deficient mutants of *Chlamydomonas* has identified four proteins that are required for cytoplasmic preassembly of dynein complexes: PF13 (also known as Ktu or DNAAF2; Omran et al., 2008), ODA7 (also known as LLRRC50 or DNAAF1; Duquesnoy et al., 2009; Loges et al., 2009), MOT48 (Yamamoto et al., 2010), and PF22 (also known as DNAAF3; Mitchison et al., 2012). PF13 and ODA7 are required for stability of the three ODA HCs in the cytoplasm, PF22 for preassembly of ODAs and IDAs, and MOT48 for preassembly of IDAs. Two of these four proteins, DNAAF1 and MOT48, contain a PIH1 domain, a motif that was first identified in the *Saccharomyces cerevisiae* protein PIH1 (also known as Nop17p; Gonzales et al., 2005). This yeast protein functions to maintain or promote the assembly of the BOX C/D small nucleolar RNA, a prerRNA-processing complex, by interacting with the molecular chaperone HSP90 (Zhao et al., 2008).

Twister is another protein that contains a PIH1 domain. The Twister gene was first identified by genetic screening in *zebrafish* as one of the genes whose mutation resulted in the formation of kidney cysts, a phenotype reminiscent of polycystic kidney disease (PKD) in humans (Sun et al., 2004). The precise function of Twister has remained unknown, however. We have now generated a mutant mouse that lacks the *Twister*-like gene *Pih1d3*. Analysis of the mutant animals suggests that *Pih1d3* is required for the motility of sperm and is a dynein axonemal assembly factor (DNAAF) that promotes the stability and assembly of HCs and ICs of axonemal ODAs and IDAs in the cytoplasm of mouse sperm. *Pih1d3* is the first DNAF found to be required for the cytoplasmic preassembly of ICs and for the 9+2 organization of microtubules.

**Results**

**Pih1d3<sup>−/−</sup>** male mice are sterile

Two genes in the mouse genome show homology to *Twister* of *zebrafish*: RIKEN 4930521A18 (referred to as *Pih1d3* in this study; its human homologue is called PIH1D3) and E230019M04 (Twister2; Fig. 1 A). *Pih1d3* and Twister2 possess highly similar coding regions, but they differ in their 3′ untranslated regions. We focused on *Pih1d3* in the present study. To analyze the function of *Pih1d3* in mouse, we generated a mutant allele (*Pih1d3<sup>neo</sup>*) with a standard gene-targeting approach (Fig. S1). *Pih1d3<sup>−/−</sup>* and *Pih1d3<sup>fl<sup>ox</sup></sup>* alleles were subsequently generated from *Pih1d3<sup>neo</sup>* with the use of Cre and Flp recombinases, respectively. An Ires-lacZ cassette present in the 3′ untranslated region of the *Pih1d3<sup>fl<sup>ox</sup></sup>* allele would allow monitoring of *Pih1d3* expression by staining of embryos with the LacZ substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

Intercrossing of *Pih1d3<sup>fl<sup>ox</sup></sup>* mice yielded *Pih1d3<sup>−/−</sup>* offspring at the expected Mendelian frequency at birth, and the mutant animals showed no obvious phenotypic abnormalities. The knockout mice thus did not manifest kidney cysts or other abnormalities related to ciliary defects (such as situs inversus and respiratory infection), with the exception that *Pih1d3<sup>−/−</sup>* males proved to be infertile. Mating of *Pih1d3<sup>−/−</sup>* or *Pih1d3<sup>fl<sup>ox</sup></sup>* females thus yielded no offspring, whereas *Pih1d3<sup>−/−</sup>* females showed normal fertility.

**Sperm of Pih1d3<sup>−/−</sup>** mice are immotile and morphologically abnormal

To investigate the cause of the sterility of *Pih1d3<sup>−/−</sup>* males, we examined the mature spermatozoa stocked in the cauda epididymis of adult mice. Light microscopy revealed that sperm from *Pih1d3<sup>−/−</sup>* males failed to move any distance (Fig. 1, B–E; and Table S1). Although a small proportion of the sperm cells shook their tails weakly, they were not able to move forward (Videos 1 and 2). Tracking of sperm movement with software confirmed the immotility of the *Pih1d3*-deficient sperm (Fig. 1 C). Light microscopy also showed that, whereas the head of sperm from *Pih1d3<sup>−/−</sup>* males appeared normal, the tail was frequently broken (Fig. 1, D and F). The mutant sperm was slightly shorter and thinner than wild-type (WT) sperm (Fig. 1 F).

We also examined other motile cilia of the *Pih1d3<sup>−/−</sup>* mouse. Cilia of the respiratory tract were normal in their motility (Videos 3 and 4) and ultrastructure (Fig. S2 A). Adult *Pih1d3<sup>−/−</sup>* mouse did not show hydrocephalus (Fig. S2 B), suggesting that cilia of ependymal cells are also normal.

**Structural defects of Pih1d3-deficient sperm**

Sperm motility is generated by the sliding of microtubules forced by ODAs and IDAs. Given the loss of motility and fragile tail of *Pih1d3*-deficient sperm, we examined the ultrastructure of the mutant cells by transmission electron microscopy (TEM). Although the 9+2 organization of microtubules was apparent in WT sperm, this structural pattern was disrupted in the axoneme of the mutant sperm (Fig. 2, A–J). ODAs were thus almost entirely missing from the 19 *Pih1d3<sup>−/−</sup>* sperm examined, whereas IDAs were lost in some but not all of the mutant sperm. We counted 0.89 ODAs and 2.05 IDAs per mutant sperm, compared with values of 8.07 and 6.00 per WT sperm (Fig. 2 K). Furthermore, in 22 of 39 *Pih1d3*-null sperm examined, peripheral doublet microtubules were either reduced in number or all present but disorganized (Fig. 2).

**Pih1d3 is expressed in spermatogenic cells and encodes a cytoplasmic protein**

We next examined the expression of *Pih1d3* at various stages of development. Staining of *Pih1d3<sup>fl<sup>ox</sup></sup>* embryos with X-gal at embryonic day (E) 8.0, when breaking of left-right symmetry takes place in the node, did not reveal expression of the Ires-lacZ cassette present in the 3′ untranslated region of the *Pih1d3<sup>fl<sup>ox</sup></sup>* allele, suggesting that *Pih1d3* is not expressed at this time (Fig. 3 A).
of Pih1d3 flox/+ mice by X-gal staining but not in those of WT mice by in situ hybridization suggested that Pih1d3 mRNA may undergo posttranscriptional regulation in the testis. Immunofluorescence staining of the testis of adult WT mice revealed that Pih1d3 is localized to the cytoplasm of spermatogenic cells, ranging from late pachytene spermatocytes to round spermatids, with the abundance of the protein being highest in pachytene and diplotene spermatocytes (Fig. 4, A and B). Such staining was not detected in the testis of Pih1d3−/− males (Fig. 4 A), indicating the high specificity of the antibodies. Like Pih1d3 mRNA (Fig. 3 D), Pih1d3 protein was not detected in elongating or elongated spermatids, suggesting that Pih1d3 is not involved in intramanchette transport. Immunoblot analysis also showed that Pih1d3 is expressed in the testis of adult WT mice, but it was not detected in mature sperm (Fig. 5 A).
Figure 3. *Pih1d3* expression during sperm development. (A) WT (control) and *Pih1d3*lox/lox embryos at E8.0 were stained with X-gal. *Pih1d3* is not expressed in mouse embryos at E8.0. Bars, 0.5 mm. (B) Investigation of *Pih1d3* and *E230019M04Rik* expression in the indicated visceral organs from adult WT mouse and adult *Pih1d3*lox/lox testis (negative control) by RT-PCR. β-Actin serves as a loading control. (C) X-gal staining of the testis of adult *Pih1d3*lox/lox or WT (control) mice. The sections were counterstained with nuclear fast red. Roman numerals indicate stages of the cycle of the seminiferous epithelium. ps, pachytene spermatocyte; rs, round spermatids; es, elongated spermatids. The arrowheads indicate elongating spermatids. Bars, 50 µm. (D) In situ hybridization analysis of *Pih1d3* mRNA in the testis of newborn, 5-wk-old, and adult WT mice with an antisense probe. Insets show staining with the corresponding sense probe as a control. All sections were counterstained with nuclear fast red. Stages of the cycle of the seminiferous epithelium are indicated with roman numerals. Bars, 50 µm.
Figure 4. Subcellular localization of Pih1d3 in adult mouse testis. (A) Immunofluorescence staining of the testis of adult WT or Pih1d3−/− mice with antibodies to Pih1d3. Pih1d3 (green fluorescence) was detected in the cytoplasm of WT sperm cells from late pachytene spermatocytes to round spermatids (rs), with its abundance being highest at the diplotene (Di) and pachytene stages. Nuclei were stained with DAPI (blue fluorescence). Stages of the cycle of the seminiferous epithelium are indicated with roman numerals. Images were obtained with a 40× objective lens. Bars, 50 µm. (B) Staging diagram for Pih1d3 protein expression in the adult testis. Circles around the nucleus indicate cytoplasm, with the intensity of the blue color representing the expression level of Pih1d3.
Cytoplasmic preassembly of dynein arms requires Pih1d3

Sperm are produced in the seminiferous tubules of the testis. Given that Pih1d3 protein was detected in the cytoplasm of spermatogenic cells and that Pih1d3-deficient sperm have structural defects, we next examined whether spermatogenesis proceeds normally in the testis of Pih1d3<sup>−/−</sup> mice. Hematoxylin-eosin (H&E) staining revealed that most seminiferous tubules and the cauda epididymis of adult Pih1d3<sup>−/−</sup> males were normal, although a small number of seminiferous tubules showed the absence of spermatogenic cells, with the accumulation of elongated spermatids in the lumen (Fig. S3, A–C; and Table S2).

Immunohistochemical staining failed to detect the cleaved form of caspase-3 in the Pih1d3<sup>−/−</sup> testes (Fig. S3 E), suggesting that the frequency of apoptosis is not increased in the mutant.

SMART and Pfam programs predicted that mouse Pih1d3 contains a PIH1 domain spanning amino acid residues 70 to 209 (Fig. 1 A; Fig. S4). PF13 and MOT48 both contain a PIH1 domain (Fig. 1 A) and are required for cytoplasmic preassembly of dynein arm components in Chlamydomonas. PF13 is thus responsible together with ODA7 for the folding and stability of ODA HCs as well as for HC–IC complex assembly (Omran et al., 2008; Mitchison et al., 2012), whereas MOT48 contributes to IDA assembly (Yamamoto et al., 2010).
Furthermore, Pih1d3 was found to form a complex with Dnaic2 (Fig. 6 A), suggesting that it may function as a co-chaperone to help maintain the stability of dynein HCs and ICs. However, Pih1d3 was not found to be associated with Dnaaf1, Dnaaf2, and Dnaaf3 (Fig. 6 A), suggesting that Pih1d3 functions at a different step during the assembly of dynein complex. Pih1d3 may be required for assembly of the Dnaic1–Dnaic2 complex, given that formation of this complex is the first step of ODA assembly and that the levels of IC1 and IC2 are reduced in *Chlamydomonas* IC2 and IC1 mutants, respectively, indicating that IC1 and IC2 molecules unable to form the IC1–IC2 complex are unstable (Fowkes and Mitchell, 1998). However, Dnaic1 was found to coprecipitate with Dnaic2 in the testis of WT and *Pih1d3*−/− mice (Fig. 6 B), suggesting that the IC1–IC2 complex can be formed in the absence of Pih1d3. While Pih1d3 formed a complex with Dnaic2, Hsp70, and Hsp90 (Fig. 6, A and B), Hsp70/90 was not coprecipitated with Dnaic2 (Fig. 6 B). These results suggest that Pih1d3 may be stabilized by interaction with Hsp70/90, which in turn promotes efficient formation of IC–HC complex (Fig. 6 C).

**Discussion**

Absence of Pih1d3 protein in mature sperm, its localization in the cytoplasm of spermatogenic cells, and the phenotype of *Pih1d3* mutant mice collectively suggest that Pih1d3 is an essential...
factor for cytoplasmic preassembly of axonemal dyneins. Cytoplasmic preassembly of axonemal dyneins has been most well characterized in Chlamydomonas, with four DNAAFs having been identified to date: DNAAF1, DNAAF2, DNAAF3, and MOT48 (Omran et al., 2008; Yamamoto et al., 2010; Mitchison et al., 2012). Our data now suggest that Pih1d3 is a novel Dnaaf with unique features. Cytoplasmic preassembly of dynein HC's has been found to require at least two steps (Yamamoto et al., 2010). The first step involves the folding of the globular dynein head domain, which is required for HC stability. DNAAF1 and DNAAF2 contribute to this step together with HSP70. The second step involves formation of the HC–IC complex, which requires DNAAF3. Pih1d3 may be involved in a different step during axonemal dynein assembly because it does not interact with the known DNAfs (Fig. 6 A).

Unlike other DNAAFs, however, we have now shown that Pih1d3 interacts with DNAc2 (ODA IC2) as well as with HSP70 and HSP90, and the lack of Pih1d3 resulted in down-regulation of DNAc1 and DNAc2 in mouse testes. The formation of IC1–IC2 complex is required for maintenance of the stability of these two factors in the cytoplasm. Mutation of Chlamydomonas IC70 (IC1) thus renders IC78 (IC2) unstable, whereas mutation of IC78 results in a reduction in the abundance of IC70 in the cytoplasm (Fowkes and Mitchell, 1998). However, the IC1–IC2 complex was formed normally in the absence of Pih1d3 (Fig. 6 B), whereas the complex was rendered unstable in the mutant testis. Pih1d3 may be required to maintain the stability of the IC1–IC2 complex by directly interacting with the complex. Alternatively, Pih1d3 may promote the formation of the IC–HC complex, and the absence of Pih1d3 may indirectly reduce the level of Dnaic and DNAhc proteins in the testis. Because HSP70/90 was not co-immunoprecipitated with DNAc2, the role of HSP70/90 may be to maintain the stability and integrity of Pih1d3.

The level of DNAh9 and DNAh7 was severely reduced in the testis and sperm from the Pih1d3−/− mouse (Fig. 5 B). Given that formation of the IC1–IC2 complex precedes formation of the IC–HC complex, the reduced levels of DNAc1 and DNAc2 in sperm cells of Pih1d3−/− mice may be responsible for the associated down-regulation of dynein HC's. Alternatively, Pih1d3 may also directly regulate the stability of ODA and IDA HC's as well as that of ODA IC's in the cytoplasm by serving as a co-chaperone of HSP70 and HSP90 (Fig. 6 C).

The phenotype of mouse Dnaaf mutants or humans deficient in DNAAFs shows substantial variation. Humans that lack DNAAF1 or DNAAF2 show a typical primary ciliary dyskinesia (PCD) phenotype characterized by recurrent pneumonia, situs inversus, and immotile sperm (Omran et al., 2008; Loges et al., 2009), whereas those lacking DNAAF3 manifest a spectrum of PCD phenotypes (Mitchison et al., 2012). On the other hand, mutation of Pih1d3 appears to affect only the motility of sperm, with other motile cilia such as those in the trachea remaining unaffected. This relatively mild phenotype might be due to the presence of Twister2. Twister2 transcripts (RIKEN E230019M04) were indeed detected in various organs with motile cilia including the lung, brain, and oviduct (Fig. 3 B). Interestingly, Twister2 mRNA was also found in the testes of WT and Pih1d3−/− mice. These observations suggest that Pih1d3 and Twister2 may have distinct roles in the testis although their amino acid sequences are similar, or that Twister2 transcripts may be translationally arrested in the testis. Alternatively, there may be functional redundancy between Pih1d3 and other DNAAFs such as PF13/Dnaaf2. However, this possibility is also unlikely given that humans that lack PF13 and Pih1d3−/− mice show distinct ciliary defects. A more likely explanation may be that Pih1d3 contributes to a mechanism of dynein arm assembly that is required specifically for formation of the motile flagellum of sperm, but not for that of other motile cilia. In this regard, the structure of the sperm flagellum shows differences from that of motile cilia in the trachea. For example, DNAHS is localized only to the proximal region of sperm flagella but is present throughout the axoneme of respiratory cilia in humans (Fliegauf et al., 2005). The precise mechanism of ODA and IDA preassembly, as well as the composition of ODAs and IDAs, may thus differ between sperm flagella and motile cilia of the trachea.

Unlike Twister-null zebrafish, Pih1d3−/− mice do not manifest polycystic kidney disease. This difference may be attributable to structural differences in kidney cilia between these two species. Whereas kidney cilia in mammals are 9+0 immotile cilia, those in zebrafish are 9+2 motile cilia with ODAs and IDAs (Kramer-Zucker et al., 2005). Alternatively, Twister 2 may compensate the absence of Pih1d3 in the kidney.

Some DNAAF mutants show a loss of dynein arms but maintain an intact 9+2 microtubular organization. However, Pih1d3−/− sperm exhibit a disorganized pattern of axonemal microtubules, with some of the pairs in the 9+2 arrangement being absent. Given that Pih1d3 was found to interact with both HSP70 and HSP90, it is possible that it may perform other functions in addition to serving as a DNAaf. HSP90 also regulates the stability of axonemal β-tubulin in multiciliated cells of the airway (Takei et al., 2007). Pih1d3, together with Hsp90, may therefore also play a role in other aspects of axonemal assembly, such as in the cytoplasmic preassembly of intramanchette transport factors or their association with axonemal components.

### Materials and methods

#### Generation of Pih1d3 mutant mice

A targeting vector for Pih1d3 was constructed with a strategy based on flanking LoxP sites (Fig. S1 A). A DNA sequence for the Myc epitope tag, an internal ribosome entry site (Ires) and lacZ expression cassette, and an FRT-flanked phosphoglycerate kinase gene (Pkg) promoter–driven neomycin resistance gene (Neo) cassette were positioned downstream of the Pih1d3 exon, and the entire region was flanked by LoxP sites. The targeting vector was introduced into embryonic stem (ES) cells, and clones that had undergone homologous recombination were identified by Southern blot analysis and introduced into ICR morulas at the 4- to 16-cell stage. Chimeraic mice were distinguished on the basis of their coat color, with skin cells derived from the ES clones and ICR cells being black and white, respectively. Chimeras with a coat that was 80–100% black were crossed with CAG-Flp mice to generate Pih1d3+/− offspring or with CAG-Cre mice to generate Pih1d3−/− offspring. Pih1d3−/− mice were generated by intercrossing of Pih1d3+/− heterozygotes.

#### Southern blot and PCR analyses

Genomic DNA of ES clones or mice was digested with KpnI and EcoRV, and the resulting fragments were subjected to Southern blot analysis with an external probe (Fig. S1, A and B). The WT allele generated a hybridizing

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fragment of 9.4 kb, whereas the neo, flox, and null alleles yielded a
hybridizing fragment of 8.7 kb. PCR analysis was also performed to detect
the WT, flox, and null alleles (Fig. 5A, A, and C). The forward primer P1
(5'-CTGAGGACGTATCCCTAGGAA-3') and reverse primer P2 (5'-GACCA-
CACACTAATGTTGAC-3') were used to detect the WT allele, the forward
primer P3 (5'-GCTACCATGTACGTTGTC-3') and reverse primer P2 were
used to detect the flox allele, and the forward primer P4 (5'-TGAGT-
GATCCACAGGCTA-3') and reverse primer P2 were used to detect the
null allele.

Histological analysis
The testis and epididymis of adult WT or Pih1d3<sup>-/-</sup> mice were dissec-
ted, fixed overnight in Bouin’s solution at 4°C, dehydrated, and embed-
ded in paraffin wax. Serial sections (7-µm thickness) were then prepared from
the tissue and stained with H&E according to standard procedures. To
examine the cerebrum, the cerebrum of adult WT or Pih1d3<sup>-/-</sup> mice were
fixed in 4% paraformaldehyde in PBS at 4°C for 48 h, washed in PBS for
30 min three times, exposed consecutively to glucose solutions of 10, 15,
20, and 30%, and embedded in OCT compound for preparation of frozen
sections (7-µm thickness). Sections were then stained with H&E according
to standard procedures. Histological photos were taken using a micro-
scope (Diaphot; Nikon) and camera (model DP21; Olympus).

Assessment of sperm motility and sperm count
Cauda epididymis of adult WT or Pih1d3<sup>-/-</sup> mice were recovered, immu-
nized, prepared with HTF medium (52 mg/mL CaCl<sub>2</sub>-2H<sub>2</sub>O, 2.5 mM
lactose, 5.0 mM KCl, 0.4 mM KH<sub>2</sub>PO<sub>4</sub>, 0.2 mM MgSO<sub>4</sub>-7H<sub>2</sub>O, 100 mM NaCl,
25 mM NaCHO<sub>3</sub>, 18.5 mM sodium lactate, 0.3 mM sodium pyruvate,
0.2 mM penicillin G sodium salt, 0.3 mM streptomycin sulfate, 4.00 g/liter
BSA, and 2 mg/l phenol red [Jin et al., 2007]), and cut into several pieces
for 2 min three times, exposed consecutively to glucose solutions of 10, 15,
20, and 30%, and embedded in OCT compound for preparation of frozen
sections (7-µm thickness). Sections were then stained with H&E according
to standard procedures. Histological photos were taken using a micro-
scope (Diaphot; Nikon) and camera (model DP21; Olympus).

In situ hybridization
Tests were fixed overnight at 4°C with 4% paraformaldehyde, exposed con-
secutively to glucose solutions of 10, 15, 20, and 30%, and embedded in
OCT compound for preparation of frozen sections. A plasmid containing a
partial fragment of mouse Pih1d3 cDNA including the 3' untranslated region
was used to produce digoxigenin-labeled sense and antisense cRNA probes
with the use of T3 and T7 RNA polymerases in separate reactions. Hybrid-
ization was performed at 55°C for 16 h, after which the slides were washed,
incubated overnight at 4°C with alkaline phosphatase–conjugated anti-
bodies to digoxigenin (Roche), and exposed to nitroblue tetrazolium and
5-bromo-4-chloro-3-indolyl phosphate for detection of the immune complexes.
The sections were counterstained with nuclear fast red and mounted under
celloidin with 2.5% 1,4-diazabicyclo[2.2.2]octane, 0.02% Na<sub>2</sub>N<sub>3</sub>, and
50% glycerol in PBS. All photos were taken using a microscope (Dia-
phot; Nikon) and camera (model DP21; Olympus).

Generation of antibodies to Pih1d3 and immunostaining
Antibodies to Pih1d3 were prepared by injection of rabbits with a peptide
corresponding to amino acids 201–2018 [ETLEVRMTVORDLDFNIS] of the
mouse protein. For immunofluorescence staining of the tests of Pih1d3
and Dnaic2, frozen sections were prepared as described above for in situ
hybridization. Antigen retrieval was performed by boiling sections for 5 min
in 10 mM citrate buffer (pH 6.0) for staining of Dnaic2. The sections were
washed three times in PBS containing 0.1% Triton X-100, exposed to 20% sheep
serum for 1 h at room temperature, incubated overnight at 4°C with
mouse antibody to Dnaic2 (1:100 dilution; M01, clone IC8; Abnova) or
rabbit antibody to Pih1d3 (1:100 dilution), and washed again before incu-
bation for 1 h at room temperature with Alexa Fluor 568–conjugated goat
antibody to mouse IgG (Molecular Probes) or Alexa Fluor 488–conjugated
goat antibody to rabbit IgG (Molecular Probes) counterstaining with DAPI.
For immunofluorescence staining of sperm, cells collected from the cauda
epididymis were fixed with 4% paraformaldehyde for 30 min at room tem-
perature, transferred to slides, dried, and stored at −80°C until further analysis.
The sperm were then treated with 0.1% Triton X-100 for 10 min at room
temperature, exposed to 0.5% dried skim milk for 1 h at room tem-
perature, and incubated consecutively overnight at 4°C with antibodies to
Dnaic2 (1:100 dilution) and for 1 h at room temperature with Alexa Fluor 568–
conjugated goat antibodies to rabbit IgG. All images were acquired with a
colfocal microscope (Fluoview FV 1000; Olympus). For immuno-
histochemical analysis of cleaved caspase-3, paraffin-embedded sections
of the tests were prepared, subjected to antigen retrieval by boiling in 10 mM
citrate buffer (pH 6.0) for 5 min, and treated consecutively at room tem-
perature with 0.1% Triton X-100 for 10 min, 3% H<sub>2</sub>O for 30 min, and
5% BSA for 1 h. The sections were then incubated overnight at 4°C with
rabbit antibodies to the cleaved form of caspase-3 (1:100 dilution; Cell
Signaling Technology), after which immune complexes were detected with
HRP-conjugated goat antibodies to rabbit IgG (ImmPRESS reagent kit;
Vector Laboratories) and DAB (Dijindo).

Immunoblot analysis and immunoprecipitation
For immunoblot analysis and immunoprecipitation, the tissue or sperm
extracts were prepared from sperm or visceral organs with radioimmunoprecipitation (RIPA) buffer and subjected to SDS-PAGE. The separated proteins were transferred to a polyvinylidene difluoride membrane (GE Healthcare) and exposed to primary antibodies, and images complexes were detected with the use of HRP-conjugated sec-
dary antibodies (Jackson ImmunoResearch Laboratories, Inc.; or R&D
Systems) and ECL reagents (GE Healthcare). The primary antibodies in-
cluded those to Pih1d3, DNA2 (MO1, clone IC8; Abnova), DNA1
(SAB4501181; Sigma-Aldrich), Dna11/DNA11 (sc-160296; Santa Cruz
Biotechnol., Inc.), Dna7/DNA7 (sc-167567; Santa Cruz Biotechnol-
ology, Inc.), Dna9/DNA9 (Omran et al., 2008; Matsu et al., 2013),
and α-tubulin (DM1A; Sigma-Aldrich) and β-actin (sc-130656; Santa
Cruz Biotechnology, Inc.). The antibody to Dna9 was raised in rabbits against a peptide corresponding to amino acids 1003–1352 of the mouse
Dna9 protein. For coinmunoprecipitation analysis, protein extracts of tests
were prepared in a solution containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 0.8% NP-40, 1 mM PMSF, and a protease inhibitor cocktail (Roche). The extracts were incubated overnight with rotation at 4°C with antibodies to Pih1d3 or control rabbit IgG, after which Dynabeads G (Invitrogen) were added and the mixtures were incubated for an additional 4 h. The resulting immunoprecipitates were subjected to SDS-PAGE and subjected to immunoblot analysis with antibodies to Pih1d3, HSP90 (AP11356c; Abgent), DNAI1, DNAI2 (rabbit anti-mouse antibody raised against a full-length IC8; Abnova). Co-immunoprecipitation of Dnaic2 with Dnaic1, Hsp70, and Hsp90 was performed using the same method and same antibodies except that the anti-DNAIC1 antibody (12756–1-AP; Proteintech) was used.

Online supplemental material

Fig. S1 shows strategy for generation of mouse Ktu protein; Omran et al., 2008; Matsuo et al., 2013), and DNAAF3–(AP11356c; Abgent). Co-immunoprecipitation of Dnaic2 with Dnaic1, Pih1d3, and Hsp90 was performed using the same method and same antibodies except that the anti-DNAIC1 antibody (12756–1-AP; Proteintech) was used.

References


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Supplemental material

Figure S1. **Strategy for generation of Pih1d3−/− mice.** (A) Structure of the targeting vector as well as of the WT, neo, flox, and null alleles. The Pih1d3 gene contains one exon (black box). In the targeting vector, the Pih1d3 exon is followed by a Myc epitope tag sequence, an internal ribosome entry site (Ires)-lacZ ORF expression cassette, and an FRT-flanked phosphoglycerate kinase gene (Pgk) promoter–neomycin resistance gene (neo) cassette, all of which are flanked by LoxP sites. The targeting vector was integrated into the mouse genome by homologous recombination to generate the neo allele, the neo cassette of which was removed with Flp recombinase to yield the flox allele or the LoxP-flanked cassette of which was removed with Cre recombinase to yield the null allele. Diagnostic restriction enzymes (KpnI, EcoRV), a probe for Southern blot analysis (5′ probe), and primers for PCR analysis are indicated. (B) Southern blot analysis of Pih1d3 mutant mice. Genomic DNA isolated from mice of the indicated genotypes was digested with KpnI and EcoRV and subjected to hybridization with the 5′ probe. The 9.4- and 8.7-kb bands correspond to the WT allele and to the neo, flox, or null alleles, respectively. (C) PCR analysis of genomic DNA from Pih1d3 mutant mice. Primers P1 and P2 were used for detection of the WT allele (555 bp) in the left top and right top panels. Primers P3 and P2 were used to detect the flox allele (600 bp) in the bottom left panel. Primers P4 and P2 detected the null allele (444 bp) and the WT allele (1272 bp) in the bottom right panel. The 555-, 600-, and 444-bp PCR products correspond to the WT, flox, and null alleles, respectively. White lines indicate the removal of intervening lanes for presentation purposes.
Figure S2. Motile cilia in the trachea and brain of Pih1d3^−/− mouse. (A) TEM of cross section of trachea cilia recovered from adult WT and Pih1d3^−/− mice. Arrows, outer dynein arms; arrowheads, inner dynein arms. Bar, 100 nm. (B) H&E-stained frozen sections of the cerebrum from adult WT and Pih1d3^−/− mice. Arrow, the lateral ventricle. Bar, 1 mm.
Figure S3.  
**Spermatogenesis in seminiferous tubules of Pih1d3<sup>−/−</sup> mice.** [A] Gross appearance of the testis of adult WT and Pih1d3<sup>−/−</sup> mice. A few seminiferous tubules of the Pih1d3<sup>−/−</sup> testis appear abnormal (arrow).  
(B and C) H&E staining of the testis of adult WT and Pih1d3<sup>−/−</sup> mice. Stages of the cycle of the seminiferous epithelium are indicated with roman numerals. Whereas spermatogenesis appeared to proceed normally in most seminiferous tubules of the mutant (B), some seminiferous tubules (C, red arrows) appeared abnormal. Higher magnification (40×) of the boxed region in the left panel of C is shown in the right panel. Spermatogenic cells, which are normally located near the basal lamina of the seminiferous tubules, are lost in the tubules (black arrow), while elongated spermatids accumulate in the lumen (white arrow). Bars, 50 µm.  
(D) H&E staining of the cauda epididymis of adult WT and Pih1d3<sup>−/−</sup> mice. The cauda epididymis of the mutant appeared normal. Bars, 50 µm.  
(E) Immunohistochemical staining of the testis of adult WT and Pih1d3<sup>−/−</sup> mice with antibodies to the cleaved form of caspase-3. The arrow indicates an apoptotic cell (brown immunoreactivity), whereas the asterisk denotes abnormal seminiferous tubules in the mutant. Bar, 50 µm.
Figure S4. Amino acid sequence alignment of four PIH1 proteins. Protein sequence of four PIH1 proteins, Pih1d3 (mouse), E230019M04Rik (mouse), Dnaaf1/Pf13 (mouse), and MOT48 (Chlamydomonas), were aligned using Clustal X software.

Video 1. Movie of WT sperm. Motility of WT sperm was examined by EMCCD camera (iXon+; Andor Technology) with a halogen lamp via an intravital microscope (AF DMI 6000B; Leica). The images were captured at 30 frames/s during 5 s. WT sperm were motile.

Video 2. Movie of Pih1d3−/− sperm. Motility of Pih1d3−/− sperm was examined by EMCCD camera (iXon+; Andor Technology) with a halogen lamp via an intravital microscope (AF DMI 6000B; Leica). The images were captured at 30 frames/s during 5 s. The Pih1d3−/− sperm were immotile.
Video 3. Movie of WT trachea cilia. Motility of trachea cilia in WT mice was examined by a high speed camera (HAS-500; Detect) with a halogen lamp via an upright microscope (AxioPlan2; Carl Zeiss). The images were captured at 100 frames/s during 2 s. Note that trachea cilia were motile in WT mice.

Video 4. Movie of Pih1d3−/− trachea cilia. Motility of trachea cilia in Pih1d3−/− mice was examined by a high speed camera (HAS-500; Detect) with a halogen lamp via an upright microscope (AxioPlan2; Carl Zeiss). The images were captured at 100 frames/s during 2 s. Note that trachea cilia were motile in Pih1d3−/− mice.

Table S1. Testis weight and sperm numbers in WT and Pih1d3−/− mice

<table>
<thead>
<tr>
<th>Testis weight (mg)</th>
<th>Sperm number (× 10^6, per cauda epididymis)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>86.9</td>
<td>84.8</td>
</tr>
<tr>
<td>87.6</td>
<td>82.1</td>
</tr>
<tr>
<td>86.8</td>
<td>85.5</td>
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<tr>
<td>83.1</td>
<td>84.6</td>
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<tr>
<td>83.3</td>
<td>84.8</td>
</tr>
<tr>
<td>86.2</td>
<td>89.7</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>86.00 ± 0.46</strong></td>
</tr>
</tbody>
</table>

P value: >0.05 (WT) <0.01 (Pih1d3−/−)

Three WT mice (8 wk) and three Pih1d3−/− mice (8 wk) were examined for the weight of the testis and the number of sperm stored in the cauda epididymis. For each mouse, the two testes were measured for both. There was no significant difference in the weight between WT and Pih1d3−/− testes (P > 0.05). The number of mature sperm stored in the cauda epididymis was reduced in Pih1d3−/− mice (P < 0.01). Data are means ± SEM (n = 3).

Table S2. Percentage of abnormal seminiferous tubules in Pih1d3−/− mice

<table>
<thead>
<tr>
<th>Pih1d3−/−</th>
<th>Abnormal</th>
<th>Total</th>
<th>Percentage</th>
<th>Average percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1°</td>
<td>7</td>
<td>189</td>
<td>3.70</td>
<td>3.84</td>
</tr>
<tr>
<td>6</td>
<td>161</td>
<td></td>
<td>3.73</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>147</td>
<td></td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>176</td>
<td></td>
<td>4.54</td>
<td></td>
</tr>
<tr>
<td>2°</td>
<td>8</td>
<td>183</td>
<td>4.37</td>
<td>4.22</td>
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<tr>
<td>8</td>
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<td></td>
<td>3.74</td>
<td></td>
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<tr>
<td></td>
<td>10</td>
<td>212</td>
<td>4.32</td>
<td></td>
</tr>
<tr>
<td>3°</td>
<td>8</td>
<td>174</td>
<td>4.59</td>
<td>4.41</td>
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<tr>
<td>7</td>
<td>179</td>
<td></td>
<td>3.91</td>
<td></td>
</tr>
</tbody>
</table>

Abnormal/total (%) = 4.16 ± 0.10

Numbers of the abnormal seminiferous tubules in Pih1d3−/− mice were counted by observing H&E-stained cross sections of Pih1d3−/− testis. Sections of three testes from three independent Pih1d3−/− mice were observed. For one Pih1d3−/− testis, three sections from different regions of the testis were analyzed. Data are means ± SEM (n = 3).