Murine germ cell-specific disruption of *Ift172* causes defects in spermiogenesis and male fertility

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Abstract

Intraflagellar transport (IFT) is a conserved mechanism essential for the assembly and maintenance of most eukaryotic cilia and flagella. IFT172 is a component of the IFT complex. Global disruption of mouse *Ift172* gene caused typical phenotypes of ciliopathy. Mouse *Ift172* gene appears to translate two major proteins; the full-length protein is highly expressed in the tissues enriched in cilia and the smaller 130 kDa one is only abundant in the testis. In male germ cells, IFT172 is highly expressed in the manchette of elongating spermatids. A germ cell-specific *Ift172* mutant mice were generated, and the mutant mice did not show gross abnormalities. There was no difference in testis/body weight between the control and mutant mice, but more than half of the adult homozygous mutant males were infertile and associated with abnormally developed germ cells in the spermiogenesis phase. The cauda epididymides in mutant mice contained less developed sperm that showed significantly reduced motility, and these sperm had multiple defects in ultrastructure and bent tails. In the mutant mice, testicular expression levels of some IFT components, including IFT20, IFT27, IFT74, IFT81 and IFT140, and a central apparatus protein SPAG16L were not changed. However, expression levels of ODF2, a component of the outer dense fiber, and AKAP4, a component of fibrous sheath, and two IFT components IFT25 and IFT57 were dramatically reduced. Our findings demonstrate that IFT172 is essential for normal male fertility and spermiogenesis in mice, probably by modulating specific IFT proteins and transporting/assembling unique accessory structural proteins into spermatozoa.

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Introduction

Cilia and flagella are microtubule-based cellular appendages and play essential roles in mediating cell motility and/or sensing and transducing environmental stimuli (Carvalho-Santos *et al.* 2011, Ishikawa & Marshall 2011). Intraflagellar transport (IFT) machinery, which was originally described in *Chlamydomonas reinhardtii*, is a motor-driven cargo trafficking system and is required for formation and maintenance of ciliary/flagellar integrity and functions (Kozminski *et al.* 1993, Ishikawa & Marshall 2011). IFT involves bidirectional transport of granule-like large protein complexes, named IFT particles, along the axoneme of cilia and flagella (Kozminski *et al.* 1993). IFT is responsible for anterograde transport of essential precursor proteins from the basal body to the assembly site at the axonemal tip and retrograde transport of turnover products back to the cell body. Kinesin-2 and cytoplasmic dynein-2 motor

proteins regulate anterograde and retrograde trafficking in the IFT system, respectively (Cole *et al.* 1998, Pazour *et al.* 1999, Porter *et al.* 1999, Pedersen *et al.* 2006, Scholey 2013, Prevo *et al.* 2017).

Study of the composition of IFT particles isolated from *C. reinhardtii* found that they comprise of IFT-A and IFT-B complexes and are thought to mediate contacts between motors and axonemal precursor/turnover proteins (Piperno & Mead 1997, Cole *et al.* 1998, Ishikawa & Marshall 2011). The IFT-A complex is composed of six proteins, while the IFT-B complex contains 16 proteins (Prevo *et al.* 2017). The interaction between the different IFT subunits is mediated by protein-protein binding domains such as tryptophan/aspartic acid repeats (WD40 repeats), coiled-coil domains, or tetratricopeptide repeats (TPRs) (Piperno & Mead 1997, Cole *et al.* 1998, Prevo *et al.* 2017). Mutation in the subunit of the IFT-A or IFT-B complex impedes the structure and function of cilia and flagella and thus leads

to ciliopathies with distinct phenotypes, such as retinal degeneration, obesity, skeletal abnormalities, and cystic kidney (Beales *et al.* 2007, Keady *et al.* 2011, Waters & Beales 2011, Aldahmesh *et al.* 2014, Perrault *et al.* 2015, Schaefer *et al.* 2016).

IFT172 was originally shown to be a component of the IFT-B complex in *C. reinhardtii* and is not only enriched at the basal body but also present along the axoneme of cilia or flagella (Cole *et al.* 1998, Pedersen *et al.* 2005, Tsao & Gorovsky 2008). Although it is not required for formation of the IFT-B complex core, IFT172 is indispensable for cilia or flagella function. In the temperature-sensitive *C. reinhardtii* *fla11* strain, a single point missense mutation in *ift172* caused a defect in flagellar assembly at a non-permissive temperature (Adams *et al.* 1982, Pedersen *et al.* 2005). Further analysis of *fla11* mutant flagella showed accumulation of subunits of the IFT-B complex and abnormalities in the anterograde-to-retrograde particle turnover at the distal end of flagella, suggesting that IFT172 is critical for regulation of anterograde-to-retrograde IFT transition at the tip of flagella (Iomini *et al.* 2001, Pedersen *et al.* 2005). IFT172 is also conserved in a range of organisms possessing cilia or flagella. Mutation in OSM-1, the IFT172 ortholog in *Caenorhabditis elegans*, resulted in shortened axonemes of amphid cilia and defective chemosensory responses (Perkins *et al.* 1986, Bell *et al.* 2006). Insertional mutagenesis screen of zebrafish identified *ift172* mutation as a cause of kidney cysts, presumably involving disruption of renal cilia biogenesis (Sun *et al.* 2004). This gene is also required for photoreceptor outer segment development in zebrafish embryos (Gross *et al.* 2005, Sukumaran & Perkins 2009). Moreover, inactivation of IFT172 leads to a wide range of diseases associated with ciliary dysfunction in mammals. Germline disruption of mouse homologue of IFT172 (*wimble*) resulted in abnormal phenotypes, characteristic of defects in the hedgehog signaling pathway and embryonic lethality, suggesting that IFT172 is essential for embryonic development (Huangfu *et al.* 2003, Gorivodsky *et al.* 2009, Burnett *et al.* 2017). Wide tissue distribution of IFT172 and polydactyly and shortened limbs in conditional *ift172* knockout (KO) mice suggest that the protein is implicated in the proper tissue development (McClintock *et al.* 2008, Howard *et al.* 2010). More recent study also demonstrated that IFT172 deficiency was associated retinal degeneration (Gupta *et al.* 2018). In human, mutation of IFT172 has been associated with pituitary hypoplasia in childhood, Mainzer-Saldino syndrome, asphyxiating thoracic dystrophy, and Bardet-Biedl syndrome (Halbritter *et al.* 2013, Lucas-Herald *et al.* 2015, Schaefer *et al.* 2016).

Although IFT172 contributes to the assembly and function of cilia and flagella in somatic cells, it is unclear whether it plays a role in the formation of mammalian sperm tail. IFT172 is highly expressed in testis and sperm possess a long flagellum (Howard &

Maurer 2000), suggesting that it might be involved in the proliferation and development of male germ cells. In the present study, we investigated the function of IFT172 in spermatogenesis and male fertility. We found that mouse *ift172* gene translates two major proteins. The 130 kDa protein is only expressed in male germ cells and is abundant in the manchette of elongating spermatids, while the full-length protein is likely to be expressed in both somatic cells and germ cells in the testis. Male germ cell-specific knockout of *ift172* resulted in significantly decreased sperm count and motility, which were the causative factors for reduced male infertility. Sperm that completed spermatogenesis displayed aberrant sperm morphology, including bent tails. The levels of two major sperm cytoskeletal proteins ODF2 and AKAP4 were largely decreased in the mutant mice. However, inactivation of *ift172* had no effect on the testicular expression of most selected proteins of the IFT-B complex, except IFT25 and IFT57. This study demonstrates that IFT172 is essential for normal sperm function, probably through the transport of a set of unique cytoskeletal proteins for assembling into the sperm flagellum.

Materials and methods

Ethics statement

All animal research was approved by Wayne State University Institutional Animal Care and Use Program Advisory Committee (Protocol number: IACUC-18-02-0534).

Generation of *Ift172* conditional knockout (cKO) mice

Ift172^{fllox/fllox} mice (exons 2, 3 were floxed) were purchased from the Mutant Mouse Resource and Research Center—University of North Carolina (MMRRC Strain ID: 32829), and *Stra8-iCre* mice were purchased from the Jackson Laboratory (Stock No:008208). Cre recombinase is only active in male germ cells (Sadate-Ngatchou *et al.* 2008). To generate the germ cell-specific *Ift172* KO (cKO) mouse model, 3–4-month-old *Stra8-cre* males were crossed with 3–4-month-old *Ift172*^{fllox/fllox} females to obtain *Stra8-iCre; Ift172*^{fllox/+} mice. The 3–4-month-old *Stra8-iCre; Ift172*^{fllox/+} males were crossed back with 3–4-month-old *Ift172*^{fllox/fllox} females again, and the *Stra8-iCre; Ift172*^{fllox/fllox} were considered to be the homozygous cKO mice, and *Stra8-iCre; Ift172*^{fllox/+} mice were used as the controls. Genomic DNA was isolated and used for genotyping the offspring as described previously (Zhang *et al.* 2018). The following primers were used for genotyping: *Stra8-iCre* forward: 5'-GTGCAAGCTGAACAACAGGA-3'; *Stra8-iCre* reverse: 5'-AGGGACACAGCATTGGAGTC-3', and *Ift172* genotypes were determined as described previously (Howard *et al.* 2010).

RT-PCR and Real-time PCR

Total testicular RNA was isolated from the control and conditional *Ift172* KO mice with TRIzol (Invitrogen). The

RNA was reversely transcribed, and cDNAs were used for RT-PCR or real-time PCR. For RT-PCR, two sets of primers were used. Two RT-PCRs shared the same forward primer that is located in exon 1 (5'-GGAGTTGTCTCGGTCACTGAG-3'). One reverse primer is located in the floxed exon 3 (5'-GTAGACATAGATGATGTTGTC-3'); the other reverse primer is in exon 4 (5'-GATGAACCTGTTGCAGATGAC-3'). Real-time PCRs were performed using 2× SYBR Green master mix (Bio-Rad). The primer set for Real-time PCR was designed with GeneScript tools (forward primer: 5'-TGCCCTGGACCATTCAGATT-3' located in exon 45, and reverse primer: 5'-CCAGTCTCGGACCTCTTCTC-3' located in exon 46). The primer set amplifying exon 1 to exon 3 (the same as RT-PCR as described previously) was also used for real-time PCR. 18S rRNA level was used to normalize expression level of target genes (Silva et al. 2009).

Western blot analysis

Tissue samples were collected from 3–4 month-old mice, and Western blot was conducted as previously described (Zhang et al. 2016). The following antibodies were used: anti-AKAP4 (1:4000, from Dr George L Gerton at University of Pennsylvania); anti-ODF2 (1:800, Cat No: 12058-1AP, Proteintech); anti-IFT81 (1:2000, Cat No: 11744-1-AP, Proteintech); anti-SPAG16L (1:1000, generated in our laboratory); β -actin (1:2000, Cat No: 4967S, Cell Signaling); and GAPDH (1:500, Cat No: SC-47724, Santa Cruz). Antibodies against IFT20, IFT25, IFT27, IFT57, and IFT140 (1:2000) were provided by Dr Pazour's laboratory at University of Massachusetts Medical School. Four commercial anti-IFT172 antibodies were used: LifeSpan BioSciences (Cat No: LS-B8206); Santa Cruz Biotechnology (A-11: sc398393, 1:1000); ThermoFisher Scientific (Cat Nos: pA5-43702 and pA5-25305, 1:1000 for both); and one from Dr Kinga M Bujakowska at Harvard University (1:1000) (Gupta et al. 2018).

Spermatozoa counting

Sperm cells were collected from cauda epididymides in warm PBS solution and fixed for 10 min at room temperature with 2% formaldehyde. Counts were made using a hemocytometer chamber under a light microscope, and sperm number was calculated by standard methods (Zhang et al. 2006).

Spermatozoa motility assay

Sperm were collected after swimming out from the cauda epididymides in warm PBS. Sperm motility was observed using an inverted microscope (Nikon) equipped with 10× objective. Movies were recorded at 15 frames/s with a SANYO (Osaka, Japan) color charge-coupled device, high-resolution camera (VCC-3972), and Pinnacle Studio HD (version 14.0) software. For each sperm sample, ten fields were analyzed. Individual spermatozoa were tracked using Image J (National Institutes of Health, Bethesda, MD) and the plug-in MTrackJ. Sperm motility was calculated as curvilinear velocity (VCL), which is equivalent to the curvilinear distance (DCL) traveled by each individual spermatozoon in 1 s ($VCL = DCL/t$).

Histology on tissue sections

Testes and epididymides of adult mice were fixed in 4% formaldehyde and PBS solution, paraffin embedded, and sectioned for histology into 5 μ m. Sections were stained with hematoxylin and eosin and examined using a BX51 Olympus microscope and photographed with a ProgRes C14 camera (Jenoptik Laser, Germany).

Isolation of spermatogenic cells and epididymal sperm for immunofluorescence analysis

Spermatogenic cells were prepared as previously described (Zhang et al. 2016). Briefly, testes from an adult WT mouse were decapsulated and placed in 5 mL DMEM containing 0.5 mg/mL collagenase IV (Sigma-Aldrich) and 1.0 mg/mL DNase I (Sigma-Aldrich), and was incubated for 30 min at 32°C to dissociate testicular cells, then centrifuged for 5 min at 200 g. Dispersed mixed testicular cells were fixed by 15 min incubation in 4% paraformaldehyde/PBS (containing 4% sucrose) at room temperature, then washed three times with PBS. Prior to plating, cells were re-suspended in PBS and 100 μ L of cell suspension was spread on SuperFrost/Plus microscope slides (Fisher Scientific) and allowed to air-dry. For immunofluorescence staining, anti-IFT172 antibodies (1:100, Cat No: LS-B8206, LifeSpan BioSciences; 1:100 from Dr Kinga M Bujakowska; or ThermoFisher Scientific (1:100, Cat No: pA5-25305) and other primary antibodies were used, including anti- α -tubulin antibody (1:200, Cat No: T9026, Sigma) and anti-Sp56 antibody (1:200, Cat No: sc-373726, Santa Cruz Biotechnology). Images were captured by confocal laser-scanning microscopy (Zeiss LSM 700). For immunofluorescence on sperm samples, sperm were obtained from cauda epididymis of control and conditional *Ift172* knockout mice, centrifuged at 3000g, washed and resuspended in PBS, and layered onto polylysine-coated slides. The preparations were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. The rest of the procedure was the same as described in the testicular cell staining.

Immunofluorescence analysis on testis sections

Fresh testes from control and conditional *Ift172* KO mice were fixed with 4% paraformaldehyde in 0.1 M PBS (pH 7.4) and 5 μ m paraffin sections were made. For the immunofluorescence, the method described by Tsuneoka was used (Tsuneoka et al. 2006). Briefly, the sections were incubated with an anti-IFT172 antibody (1:100, pA5-25305, ThermoFisher Scientific) at 4°C overnight. Slides were washed with PBS and incubated for 1 h at room temperature with Cy3-conjugated anti-rabbit IgG secondary antibody (1:5000; Jackson ImmunoResearch Laboratories). Following secondary antibody incubation, the slides were washed with PBS three times, mounted using VectaMount with DAPI (Vector Laboratories, Burlingame, CA), and sealed with a cover slip. Images were captured by a confocal microscopy at the Wayne State University Microscopy Core facility.

Transmission electron microscopy

Mouse testes and epididymal sperm were fixed with 3% glutaraldehyde and 1% paraformaldehyde in 0.1 M sodium

cacodylate, pH 7.4, at 4°C overnight, and processed for electron microscopy as reported (Zhang *et al.* 2016). Images were taken with a Jeol JEM-1230 transmission electron microscope at Virginia Commonwealth University.

Results

Mouse *Ift172* gene translates two major proteins

To examine mouse IFT172 tissues distribution, Western blots were conducted using multiple commercial anti-IFT172 antibodies. Among the four commercial anti-IFT172 antibodies used, only the antibody from ThermoFisher Scientific (pA5-25305) detected the protein at the predicted size in the mouse tissues examined. The full length IFT172 was highly expressed in the testis and was also detected in the brain, lung, and liver. Besides the full length IFT172, the antibody also detected a 130 kDa protein only in testis (Fig. 1A). In addition, some smaller proteins were also detected in the examined tissues and these may represent non-specific binding using the antibody. The antibody was further used to examine IFT172 expression in the mouse testes collected during the first wave of spermatogenesis. The full length IFT172 protein was detected at a similar level throughout the whole spermatogenesis process. However, the 130 kDa protein was not detected until day 12 after birth and then was present throughout the experimental period (Fig. 1B). The two IFT172 proteins were also detected using another anti-IFT172 antibody generated recently by Dr Kinga M Bujakowska at Harvard University (Supplementary Fig. 1, see section on [supplementary materials](#) given at the end of this article).

Localization of IFT172 in male germ cells

Immunofluorescence staining was conducted to determine localization of IFT172 in isolated male germ cells. Three different anti-IFT172 antibodies were used. Strong IFT172 signal was detected in the elongating spermatids from these antibodies, and the signal was co-localized with a marker of the manchette, α -tubulin, indicating that IFT172 is a component of the manchette (Fig. 2).

Generation of male germ cell-specific *Ift172* cKO mice

To study the role of IFT172 in spermatogenesis, the *Cre/loxP* system was used to generate male germ cell-specific *Ift172* cKO mice as described previously (Howard *et al.* 2010, Zhang *et al.* 2016, Liu *et al.* 2017) (Supplementary Fig. 2). Disruption of *Ift172* gene was first evaluated by RT-PCR and Real-time PCR using the primers as illustrated in Supplementary Fig. 3A. When the primer set that amplified exons 1 to 3 was used, a strong 321-bp PCR product was observed only in the control mouse as predicted, and only a trace amount of

Figure 1 IFT172 tissue distribution and testicular expression during the first wave of spermatogenesis examined by Western blot analysis. Four commercial IFT172 antibodies were used and only the one from ThermoFisher Scientific (pA5-25305) cross-reacted with the protein corresponding to the full-length mouse IFT172 protein. (A) Expression of IFT172 in multiple mouse tissues extracts. The full length IFT172 was detected in the testis, brain, lung, and liver. In addition to the full length IFT172 protein, a 130 kDa protein was also detected only in the testis. Some smaller proteins were also detected in all tissues examined and these may represent nonspecific binding using the antibody. (B) Dynamic expression of IFT172 during the first wave of spermatogenesis. The full length IFT172 was expressed at a similar level throughout the whole process. However, the 130 kDa protein was not expressed until day 12 after birth, when germ cells became the predominant cell population in the testis; and the level appeared to be increased after day 24, when spermiogenesis occurred.

PCR product was detected in the cKO mouse. When the primer set that amplified exons 1 to 4 was used, a PCR product as predicted size (372 bps) was observed in the control mouse; however, in the cKO mouse, the signal of the same PCR product was dramatically reduced; instead, a smaller PCR product (about 115 bps) was amplified (Supplementary Fig. 3B). To compare testicular *Ift172* mRNA expression levels between control and *Ift172* cKO mice, real-time PCR was conducted using two primer sets. The first set was the same as the RT-PCR primer set that amplified exons 1 to 3, and the second set was to amplify the region closed to the 3' end of *Ift172* cDNA. Results from both sets demonstrated that testicular *Ift172* mRNA expression level was significantly reduced in the cKO (Supplementary Fig. 3C).

Germ-cell specific disruption of *Ift172* was further confirmed by Western blot analysis. Both the full-length and the 130 kDa IFT172 proteins were present in the control mice. Either none or only trace amounts of the 130 kDa protein was detected in the conditional *Ift172* KO mice, and the full length IFT172 level was also

Figure 2 Examination of IFT172 localization in isolated male germ cells by immunofluorescence staining. Isolated germ cells were double-stained with three IFT172 antibodies (A, from LifeSpan BioSciences; B, from ThermoFisher Scientific: pA5-25305; and C, from Dr Bujakowska at Harvard University) and a manchette marker, α -tubulin. IFT172 was stained in red, and α -tubulin was stained in green. The two proteins were co-localized (yellow) in the elongating spermatids (white arrows).

reduced in the conditional *Ift172* KO mice (Fig. 3A). Immunofluorescence staining was also conducted on the isolated testicular cells. Strong IFT172 signal was detected in the manchette of isolated male germ cells from the control mice, whereas the signal was greatly diminished in the mutant mice (Fig. 3B).

Disruption of *Ift172* in germ cells was also examined on testicular sections. Using the same antibody as the one used for Western blot analysis (ThermoFisher Scientific, Cat No: pA5-25305), specific IFT172 signal was detected in the manchette of elongating spermatids of control mice, but the signal was missing in the elongating spermatids of conditional *Ift172* KO mice (Supplementary Fig. 4). All other commercial anti-IFT172 antibodies tested did not result in any specific signal.

Knockout of *Ift172* specifically in male germ cells results in reduced male fertility

Homozygous *Ift172*-deficient mice survived to adulthood and did not exhibit any gross abnormalities in the ratio of testis weight to body weight (Table 1). To examine the effect of disruption of *Ift172* gene on male fertility, *Ift172* mutant or control mature males were mated with 3 to 4 months old WT female mice for over 2 months. All ten control males displayed normal fertility and

produced normal-sized litters. However, less than 50% of the conditional *Ift172* KO mice were fertile and they produced offspring with reduced litter sizes (Table 1).

Disruption of *Ift172* results in aberrant sperm morphology, reduced sperm counts and motility

To determine the causative factors of male infertility, morphology of cauda epididymal sperm was examined by light microscopy. Sperm density in the control mice was significantly higher than those observed in the *Ift172* cKO mice under the same dilution, as examined by light microscopy (Supplementary Fig. 5). Under high magnification, most spermatozoa from the *Ift172* cKO mice displayed bent tails. Vacuoles were present in the flagella of some mutant sperm (Fig. 4A). These abnormal sperm made up about 80% of the total sperm. *Ift172* disruption also resulted in a reduction in sperm counts and sperm motility, compared to the control mice (Fig. 4B and C).

Inactivation of *Ift172* leads to spermatogenesis defect

Aberrant sperm morphology and reduced sperm counts and motility may be caused by spermatogenesis defects. Thus, testicular histology was examined in control

Figure 3 IFT172 protein expression is dramatically reduced in the germ cells of conditional *lft172* KO mice. (A) Examination of testicular IFT172 expression in the control and conditional *lft172* KO mice by Western blot analysis. Notice that the full length IFT172 was still present in the conditional *lft172* KO mice but the level was reduced; however, no or only trace amount of the 130 kDa protein was detected in the cKO mice. (B) Examination of IFT172 expression in germ cells by immunofluorescence staining. Isolated germ cells were stained with the IFT172 antibody (red). IFT172 signal was observed in the manchette (arrows) of elongating spermatids from the control mice, but it was almost absent in the *lft172* cKO mice (dashed arrows). Remaining IFT172 signal was detected in some cells of the *lft172* cKO mice (arrowhead). The nuclei were stained in blue (DAPI).

and *lft172* cKO mice. H&E staining revealed that all stages of spermatogenesis in the control mice were present, and spermatozoa were found to be present in the lumen of the seminiferous tubules (Fig. 5A, upper panels). However, spermatogenesis was affected in the spermiogenesis phase in *lft172* cKO mice, with fewer sperm tails being observed in the lumen of seminiferous tubules (Fig. 5A, lower panels). Histological evaluation of the cauda epididymides revealed well-developed sperm and normal sperm concentrations in the control mice (Fig. 5B, upper panels), but in *lft172* cKO mice, fewer sperm were present in the lumen and round bodies and cytoplasmic debris were also present (Fig. 5B, lower panels).

Abnormally developed manchette and centrosomes, but normal acrosome biogenesis in *lft172*-deficient germ cells

Given that IFT172 is present in the manchette of elongating spermatids, we examined the effect of the depletion of IFT172 on manchette formation. Immunofluorescence staining was conducted on isolated testicular germ cells using an anti- α -tubulin antibody that labels the manchette. The signals were present in both the control and cKO mice. However, the manchette length in the cKO mice appeared to be longer than that in the control (Fig. 6A).

Basal bodies/centrosomes are the template for cilia formation (Ishikawa & Marshall 2011). It has been proposed that the centrosome may function as the nucleation site for initiation of the manchette (Lehti & Sironen 2016). Therefore, an immunofluorescence study was conducted to determine whether disruption of *lft172* could affect the formation of centrosomes in male germ cells. In control mice, IFT172 was detected in the manchette of elongating spermatids and a dot-like fluorescence signal for γ -tubulin, a centrosome marker, was detected in the caudal region of the spermatid nucleus. However, multiple centrosomal signals were observed in about 22.5% of the *lft172*-null male germ cells (Fig. 6B) and the number was less than 1% in the control mice.

Anti-Sp56 antibody was also used to evaluate the effect of *lft172* disruption on the formation of the acrosome. Sp56 was present in the acrosomal region of round spermatids in both the conditional *lft172* KO and control mice. However, in the elongating spermatids of *lft172* cKO mice, Sp56 showed abnormal aggregation in some cells (Fig. 6C).

Ultrastructural defects in the testis and epididymal sperm of conditional *lft172* KO mice

Transmission electron microscopy (TEM) was performed to study the impact of *lft172* disruption on germ cell ultrastructure (Fig. 7A) and epididymal sperm (Fig. 7B).

Table 1 Fertility, fecundity and testis weight of control and *lft172* mutant mice.

Genotype	Male fertility	Litter size	Testis/body weight (n = 10, mg/g)	Control/HO testis weight (n = 10)
Control	10/10	8.67 $\pm$ 1.2 (n = 10)	8.56 $\pm$ 0.34	1.09 $\pm$ 0.11
HO	6/13	4.57 $\pm$ 2.5 (n = 6)	8.32 $\pm$ 0.46	

Sexually mature males were bred to WT females for at least two months. Litter size was recorded for each mating. Testis weight and body weight of adult mice were measured.

Figure 4 Knockout of *Ift172* results in aberrant sperm morphology with reduced sperm count and motility. (A) Morphological examination of epididymal sperm by light microscopy at high magnification. Most *Ift172*-null sperm displayed bent flagella at the midpiece. Vacuoles were present in the flagellum of some mutant sperm (arrows). (B) The number of sperm was significantly reduced in the *Ift172* cKO mice; * $P < 0.05$; (C) Sperm motility measured as curvilinear velocity (VCL) was significantly decreased in the *Ift172* cKO mice, compared to the control. * $P < 0.05$.

In control mice, germ cells developed normally. Well-organized manchettes were observed and properly shaped nuclei and acrosomes were present (Fig. 7A, a). However, in the *Ift172* cKO mice, the manchette was formed, but appeared to be longer and mislocalized. The acrosome appeared to be normal in round spermatids, but elongating spermatids with abnormally developed heads also showed distorted acrosomes (Fig. 7A, b–f). A normal ‘9+2’ central microtubule axonemal complex and accessory structures were observed in the epididymal sperm of the control mice (Fig. 7B, a and b). However, some sperm of *Ift172* cKO mice displayed abnormal core axonemes and accessory structures, including missing or disorganized ‘9+2’ arrangement of the microtubules, missing outer dense fibers (ODFs). Failure of mitochondria and ODFs to assemble properly into the flagellum was also detected (Fig. 7B, c–j). The fibrous sheath structure seemed to be normal in the *Ift172* cKO mice.

Effect of IFT172 disruption on expression of sperm structural proteins and other IFT proteins

The impact of IFT172 inactivation on expression of components of the sperm flagellum was explored by

Figure 5 Histology of the testis and epididymis from the control and *Ift172* KO mice. (A) Representative histology of the testis from the control and conditional *Ift172* KO mouse. Upper panels: Low and high magnifications of the testis from control mouse. At low magnification, seminiferous tubules show wide lumens (L) with elongated spermatid (E) tails. The seminiferous epithelium contains normal layers of germ cells: spermatocytes (S), round spermatids (Rd), and elongated

spermatids (E). At higher magnification, specific stages of spermatogenesis are shown. In stages II–III, tails of step 14 spermatids are seen extending into the lumen. In late stage VII, step 16 spermatid heads line the lumen with their long tails (T) extending into the lumen. Cytoplasmic lobes (Cl), which later form residual bodies to be phagocytized by Sertoli cells, are seen forming near the heads of step 16 spermatids. At stage XII, step 12 spermatids have long tails (T) extending into the lumen. Lower panels: Low and high magnifications of the testis from *Ift172* cKO mouse. At low magnification, seminiferous tubules show lumens (L) that are very small and contain few spermatid tails. Spermatogenesis appears normal until the formation of elongated spermatids (E), with normal layers spermatocytes (S) and round spermatids (Rd). In some tubules, abnormal spermatids with attached cytoplasm are found in the epithelium (Ab). At higher magnification, specific stages of spermatogenesis are shown. In stages II–III, abnormal step 14 spermatid heads are present in the seminiferous epithelium, but tails are not observed extending into the lumen. Sloughed abnormal spermatids with attached cytoplasm (Sl) and round cytoplasmic bodies (Cy) are found in the lumen. The head of a step 16 spermatid is seen near the basement membrane where it has been phagocytized by the Sertoli cell. In late stage VII, round bodies of abnormal cytoplasmic lobes (AbCl) are seen extending into the lumen. Abnormal spermatid 16 heads are also identified, as well as phagocytized (Ph) step 16 spermatid heads. At stage XII, abnormal step 12 spermatids are seen in the epithelium, along with abnormal cytoplasmic lobes attached to the spermatids (AbCl). (B) Representative histology of the epididymis from the control and conditional *Ift172* KO mouse. Upper panels: Cauda epididymis from control mouse showing highly concentrated sperm, with alignment of sperm heads (H) and tails (T). Normal sperm heads and long attached tails are highly compacted in the lumen. Lower panel: Cauda epididymis from *Ift172* cKO mouse showing a low concentration of sperm in the lumen, with non-aligned sperm and numerous abnormalities. Abnormal sperm heads (AbH) and tails (AbT) are present, along with sloughed round bodies of residual spermatid cytoplasm (R). An occasional sloughed spermatocyte nucleus (S) was observed.

Figure 6 Knockout of *Ift172* leads to abnormal manchette morphology and centrosomes but normal acrosome biogenesis in germ cells. Isolated germ cells from control mice and conditional *Ift172* KO mice were stained with indicated antibodies. (A) IF was conducted with an anti- α -tubulin antibody that labeled manchette of elongating spermatids (in green). Nuclei were stained in blue (DAPI). Notice that the manchette of cKO mice (right panel) appeared to be longer than that in the control mice (left panel); (B) IF was conducted with the IFT172 antibody (in red) and a centrosome marker, γ -tubulin (in green). Notice that multiple centrosome signals were discovered in the *Ift172* cKO male germ cells (white arrows). (C) IF was conducted on germ cells isolated from the control (a) and *Ift172* cKO mice (b) with the anti-IFT172 antibody (in red) and an acrosome marker, Sp56 (in green). Notice that Sp56 localization was normal in round spermatids (white arrows), but appeared to be abnormal in some elongating spermatids in the *Ift172* cKO mice (white dashed arrows).

Western blotting and immunofluorescence staining analysis. The testicular expression levels of both ODF2 (outer dense fiber protein 2), a major component of outer dense fibers of sperm flagella (Donkor *et al.* 2004), and AKAP4 (A-kinase anchoring protein 4), a scaffold protein required for the formation of the fibrous sheath (Miki *et al.* 2002), was significantly reduced in the conditional *Ift172* KO mice, compared to the control mice (Fig. 8A). Consistently, immunofluorescence staining showed that these two proteins were present in the sperm flagellum of the control mice, while their signals were dramatically reduced in the *Ift172* cKO mice (Fig. 8D, E and Supplementary Fig. 6). Expression

levels of a central apparatus protein SPAG16L and three IFT proteins (IFT20, IFT27 and IFT81) were also examined by Western blotting. There was no significant difference in their expression levels between the control and mutant mice (Fig. 8A). We next examined expression of other IFT proteins, including IFT25, IFT57, IFT74, and IFT140. Only the expression levels of IFT25 and IFT57 were found to be significantly reduced in the cKO mice (Fig. 8B and C).

Discussion

Using two anti-IFT172 antibodies for Western blot analysis, we discovered two major cross-reacted proteins, one about 170 kDa and the other one about 130 kDa. The former is likely the full-length mouse IFT172 protein. In the testis, this protein was expressed at a stable level during the first wave of spermatogenesis, and it was still expressed in the conditional KO mice; however, the level was reduced. Thus, the full-length IFT172 is likely expressed in both somatic cells and germ cells. Instead, the 130 kDa protein was present only in the testis. Interestingly, this protein was not detected in the early phase of spermatogenesis, when germ cells are not the predominant cell population (Bellve *et al.* 1977). It was missing in the germ cell specific conditional KO mice. Thus, the 130 kDa protein is likely a germ cell specific IFT172 protein. Given that both antibodies recognize the region near the N-terminal end, it is likely the new isoform is generated through a germ cell-specific splicing mechanism in the C-terminus of the full length protein. Our laboratory is currently characterizing the new isoform. It is yet to be determined if the full-length IFT172 is required for normal male fertility. Other transgenic Cre mice should be used to study the role of the full-length IFT172 in the somatic cells of the testis.

Data presented in this study demonstrated that IFT172 is involved in male germ cell development and male fertility. The number of sperm collected from the cauda epididymis of conditional *Ift172* KO mice was significantly reduced, compared to the control mice. Although some *Ift172* cKO sperm could progress through all phases of spermatogenesis, most of them displayed abnormal morphology and reduced motility. Histological analysis of testes from the mutant mice revealed all germ cell types, including spermatogonia, spermatocytes, round and elongating spermatids; however, very few mature spermatozoa with normal flagella were detected. Thus, inactivation of *Ift172* appears to have no effect on mitosis and meiosis, but hinders spermiogenesis. These observations are consistent with the role of the IFT system in assembly and maintenance of flagella and cilia (Prevo *et al.* 2017). Although reduced sperm counts and motility would account for male infertility, about 40% of *Ift172*-null male mice were fertile. Therefore, some normal spermatozoa are being released. The decreased male fertility could be explained by at least two possibilities.

Figure 7 Analysis of testis and epididymal sperm of the control and *lft172* cKO mice by transmission electron microscopy (TEM). (A) Representative TEM images of testes from control (a) and conditional *lft172* KO mice (b–f). Normally formed manchette (arrows), heads (dashed arrows), and acrosome (arrow heads) were present in the control mice. However, in the cKO mice, a number of abnormalities were observed, including abnormal head formation (white * in c–f), mislocalized and longer manchette (black *). The acrosome was normal in the round spermatid (b), but distorted in elongating spermatids, probably secondary to the abnormal heads (# in d–f). (B) Representative TEM images of epididymal sperm. Sperm from the control mice (a, b) have normal ‘9+2’ axonemal microtubule cores (arrows) and accessory structures (dashed arrows and arrow heads). (c–j) TEM of sperm from the *lft172* cKO demonstrate multiple abnormalities in both the core axonemes and accessory structures. (c) The left and right lower inserts show two flagella with disrupted core structures; the right upper insert shows a flagellum with almost no internal structure. (d) The arrows point to mitochondria, and the dashed arrows point to disrupted unit fibers of the outer dense fiber (ODF) that are not assembled into the flagellum. The insert illustrates another flagellum with disrupted internal structure. (e) This image represents a flagellum with only six outer microtubules. (f) This photo shows a flagellum with ten outer microtubules. (g) In this cross section of the midpiece, the flagellum has normal mitochondria and ODF, as well as nine outer doublet microtubules in the axonemal complex, but the central apparatus (arrow) is missing the two microtubules and associated linking proteins. (h) This is the head of a spermatozoon (arrow) with an abnormal nuclear shape. (i) The flagellum shows a normal fibrous sheath, but a disorganized axonemal core structure. (j) The flagellum shows a normal fibrous sheath and core axoneme structure, but there is a reduction in the ODF subunits.

First, knockout of IFT172 was not complete in this mouse model, and thus the residual IFT172 may be sufficient for some germ cells to complete spermiogenesis. This is supported by the fact that the non-deleted RT-PCR product was still amplified in the cKO mice, and trace amounts of the 130 kDa IFT172 protein was detected in some cKO mice by either Western blot analysis and immunofluorescence staining. Second, as the IFT system consists of at least 22 subunits (Prevo *et al.* 2017), other IFT proteins may partially compensate for the loss of function when IFT172 is inactivated.

Immunofluorescence staining on the isolated male germ cells revealed that IFT172 is exclusively located in the manchette of elongating spermatids, a unique and transient structure required for spermiogenesis (Hermo *et al.* 2010). The manchette plays a role in shaping the

spermatid head and delivering proteins important for development of the functional sperm flagellum via an intra-manchette transport (IMT) system (Meistrich *et al.* 1990, Kierszenbaum 2002). It is believed that some cargo proteins are temporarily stored in the manchette and transported to the basal body of sperm by IMT and the developing sperm flagellum by IFT (Kierszenbaum 2002, Lehti & Sironen 2016). The presence of IFT172 in the manchette suggests that the protein might be implicated in the process of IMT. However, the fact that sperm flagella were still made in the cKO mice indicates that IFT172 might not be a key regulator for core axoneme formation in the flagellum. Given that most *lft172*-null sperm displayed bent flagella at the midpiece and vacuoles were present in the flagellum of some mutant sperm, it appears that IFT172 has a primary

Figure 8 Expression of ODF2, AKAP4, SPAG16L, and selective IFT proteins in the conditional *lft172* KO mice. (A) Representative Western blotting images showing testicular expression levels of three major components of sperm tail (ODF2, AKAP4, and SPAG16L), and three subunits of the IFT system (IFT20, IFT27, and IFT81) using specific antibodies in three control and three conditional *lft172* KO mice. Disruption of *lft172* expression decreased the level of ODF2 and AKAP4, while it had little effect on the expression levels of SPAG16L and three IFT proteins. β -Actin was used as an internal control. (B) Representative Western blotting images of another four IFT proteins. Notice that expression levels of IFT25 and IFT57 in the cKO mice were lower than that in the control mice. (C) Statistical analysis of relative expression of the IFT proteins normalized by β -actin or GAPDH. There is no difference in expression levels of IFT20, IFT27, IFT74, IFT81, and IFT140 between the control and the *lft172* cKO mice. IFT25 and IFT57 expression levels are significantly reduced in the *lft172* cKO mice. (D and E) Representative images of localization of ODF2 and AKAP4 in the epididymis sperm of a control mouse and an *lft172* cKO mouse, respectively. Both ODF2 and AKAP4 signals were reduced in the *lft172*-null sperm flagella.

role in sperm accessory structure formation. Such cargo proteins might include ODF2 and AKAP4, whose signals were significantly reduced in the epididymal sperm of the *lft172* cKO mice. Structural defects in the axoneme and reduced motility may be due to a reduction in accessory structures. Thus, different IFT components might play different roles for sperm flagella formation, which is supported by our recent finding that IFT74 is likely involved in core axoneme assembly (Shi *et al.* 2019). It has been reported that IFT172 is critical for the regulation of anterograde-to-retrograde IFT transition at the tip of *C. reinhardtii* flagella (Iomini *et al.* 2001, Pedersen *et al.* 2005). Thus, it is likely that IFT172 directly participates in the formation of developing sperm tails. Disruption of *lft172* gene in germ cells did not affect manchette formation, as both immunofluorescence staining and TEM revealed that an intact manchette was still present in the cKO mice, indicating that IFT172 is not a structural component responsible for manchette formation. However, the manchette appeared to be longer when *lft172* was disrupted. Thus, IFT172 might be a key protein that modulates manchette function and plays a role in manchette development. Besides cargo transport, other proposed manchette functions are for shaping sperm heads (Kierszenbaum 2002). Different sperm head shapes were discovered in the *lft172* cKO mice. The abnormal head formation might be caused by manchette dysfunction due to the disruption of *lft172*. However, the *lft172* gene was not completely depleted, and individual germ cells might express different amounts of residual IFT172 protein, which could result in the observed variations in germ cell phenotypes. IFT172 does not seem to be essential for acrosome biogenesis. The acrosome morphology looks normal in round spermatids as examined by both TEM and IF studies. The distorted acrosomes in the elongating spermatids might be a secondary effect due to the misshapen heads in the absence of IFT172.

TEM analysis of *lft172*-null sperm flagella revealed a missing central apparatus and/or disorganization of the '9+2' configuration of microtubules in some axoneme, the key structure required for the regulation of motility in cilia and flagella (Mitchell 2004). The abnormal organization of the axoneme likely leads to the immotility of *lft172*-null sperm. However, not all sperm had a bent tail, suggesting that the axoneme might not be as severely disrupted in some cells. The direct role of IFT172 on axoneme formation needs intensive TEM studies in the future through analyzing early step spermatids in the *lft172* cKO mice. It has been reported that the defects of the core axoneme structure might be secondary to the defects of the accessory structures (Young *et al.* 2016). It is also possible that the abnormal axoneme discovered in some *lft172*-null sperm is caused by disrupted accessory structures in the mutant mice. In addition, since the spermatid centrosome is a nucleation site for the axonemal microtubules and the

manchette is responsible for sorting a large numbers of structural proteins to the centrosome using the IMT machinery (Manandhar *et al.* 1998, Kierszenbaum 2002, Kierszenbaum *et al.* 2002), we speculate that dysfunction of the centrosome in the *Ift172*-null sperm could result in the missing and disrupted central apparatus of some flagella, in addition to the possible disruption in the accessory structure formation as discussed previously. Indeed, Immunofluorescence staining of γ -tubulin in germ cells of the *Ift172*-null mice showed multiple spots in the neck region of the elongating spermatid, which would be indicative of centrosome fragmentation. Even though IFT172 is highly abundant in the manchette, the 130 kDa protein was detected from day 12 after birth, when germ cells begin to enter the meiosis phase. Therefore, IFT172 might play other functions during spermatogenesis, including normal centrosome division and migration, which are currently under investigation in the laboratory.

IFT proteins usually function as adaptors for the transport of essential proteins in cilia and flagella (Prevo *et al.* 2017). To test the possibility that the abnormal sperm morphology is caused by altered expression of other flagella proteins in the *Ift172*-null mice, we examined the expression levels of selected IFT subunits, including IFT20, IFT25, IFT27, IFT57, IFT74, IFT81, IFT140, and three structural proteins. Most are components of the IFT-B complex (Luckner *et al.* 2005). Testicular IFT25 and IFT57 expression levels were significantly reduced in the *Ift172* cKO mice. This is consistent with the previous finding that IFT57 is a binding partner of IFT172 (Taschner *et al.* 2016). Our laboratory has generated male germ cell-specific *Ift20* KO mice and the adult males were completely infertile (Zhang *et al.* 2016); however, some of *Ift172* adult males were still fertile. Although both conditional KO mice displayed similar abnormal sperm morphology and ultrastructure, some *Ift20*-null germ cells retained redundant cytoplasm, which was not detected in the *Ift172*-null germ cells. In addition, it is interesting to find that disruption of *Ift172* resulted in reduced expression of AKAP4, a scaffold protein required for formation of the fibrous sheath (Miki *et al.* 2002). Fibrous sheath mediates diverse signaling/metabolic pathways important for sperm production and function (Eddy *et al.* 2003). However, fibrous sheath structure seemed to be normal in the mutant mice. The remaining AKAP4 might be sufficient to maintain normal fibrous sheath assembly. Given that the fibrous sheath is only present in the sperm flagellum but not in other cilia or flagella, it is likely that IFT172 has a unique function in regulating spermiogenesis that is different from the IFT20. IFT20 seems to play a more important role in core axoneme assembly in addition to being involved in the removal of excess cytoplasmic components (Zhang *et al.* 2016).

IFT25 and IFT27 are two other IFT proteins essential for spermiogenesis and sperm function (Liu *et al.* 2017,

Zhang *et al.* 2017). Besides bent tails, sperm from the two respective cKO mice had no motility or reduced motility, round heads, short tails, and abnormal thickness of sperm tails in some areas. Swollen tail tips in some sperm were also discovered in the *Ift25* and *Ift27* cKO mice (Liu *et al.* 2017, Zhang *et al.* 2017). However, these abnormalities were not found in the *Ift172* cKO mice, suggesting that IFT172 is less important in sperm function. Reduced IFT25 and IFT57 levels in the *Ift172* cKO mice suggest that IFT172 modulates some IFT proteins in male germ cells. It is not yet known if the reduction is functional to the phenotype described here. There are a total of 22 IFT proteins identified, and it is not known if other IFT proteins are affected when *Ift172* is disrupted. This will largely be dependent on the availability of specific antibodies. Recent studies demonstrated that IFT172 is a membrane-interacting protein with the ability to remodel large membranes into small vesicles. IFT172 adopts two different conformations that can be manipulated by lipids. The association of IFT172 with membranes is mutually exclusive with IFT57, implicating multiple functions for IFT172 within IFT (Wang *et al.* 2018).

In summary, the present study demonstrates that mouse *Ift172* gene translates two major proteins, the full-length IFT172, which might be expressed in the somatic cells of the testis, and a 130 kDa IFT172, which is only present in the germ cells. The germ-cell specific IFT172 is indispensable for the completion of normal spermatogenesis. This protein is abundant in the manchette of elongating spermatids and is believed to mediate delivery of essential proteins for the formation of sperm accessory structure and head shaping. The protein plays a role in spermiogenesis for normal centrosome and axoneme formation. The potential multiple functions of IFT172 and the molecular mechanisms involved in spermiogenesis require further investigation.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-17-0789>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

S Z, Y L, and Q H performed the experiments and wrote the manuscript. S Y, H L, L S, Y T Y, W L, and J Z participated in experimental work and data analysis. L Z and R A H contributed to the conception of study design and revised the manuscript. Z Z designed the study, interpreted the data, and revised the manuscript.

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