RESEARCH ARTICLE

CFAP157 is a murine downstream effector of FOXJ1 that is specifically required for flagellum morphogenesis and sperm motility

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ABSTRACT
Motile cilia move extracellular fluids or mediate cellular motility. Their function is essential for embryonic development, adult tissue homeostasis and reproduction throughout vertebrates. FOXJ1 is a key transcription factor for the formation of motile cilia but its downstream genetic programme is only partially understood. Here, we characterise a novel FOXJ1 target, Cfap157, that is specifically expressed in motile ciliated tissues in mouse and Xenopus in a FOXJ1-dependent manner. Cfap157 protein localises to basal bodies and interacts with tubulin and the centrosomal protein CEP350. Cfap157 knockout mice appear normal but homozygous males are infertile. Spermatozoa display impaired motility and a novel phenotype: Cfap157-deficient sperm exhibit axonemal loops, supernumerary axonal profiles with ectopic accessory structures, excess cytoplasm and clustered mitochondria in the midpiece regions, and defective axonemes along the flagella. Our study thus demonstrates an essential sperm-specific function for CFAP157 and suggests that this novel FOXJ1 effector is part of a mechanism that acts during spermiogenesis to suppress the formation of supernumerary axonemes and ensures a correct ultrastructure.

KEY WORDS: Cfap157, Ciliogenesis, Axoneme, Spermiogenesis, Male infertility

INTRODUCTION
Motile cilia are slender protrusions of the surface of eukaryotic cells. Their core, the axoneme, consists of nine peripheral microtubular doublets and typically two central microtubules. The axoneme carries dynein arms and originates at the basal body, a centriolar structure anchored to the cell cortex. Motile cilia move extracellular fluids, mediate cellular motility and play essential roles during development, tissue homeostasis and reproduction (reviewed by Choksi et al., 2014b; Gerdes et al., 2009; Nigg and Raff, 2009). In vertebrate embryos, motile cilia in the left-right organiser rotate and generate a leftward flow of the extracellular fluid, which is translated into left-right asymmetry of visceral organs (reviewed by Blum et al., 2014; Nonaka et al., 1998; Takeda et al., 1999). Coordinated beating of motile cilia on epithelia of the lung is required for airway clearance (Jain et al., 2010; Stannard and O’Callaghan, 2006). Motile cilia on ependymal cells lining the brain ventricles secure normal cerebrospinal fluid flow (Banizs et al., 2005; Jacquet et al., 2009; Lee, 2013; Spassky et al., 2005) and motile cilia in the fallopian tube contribute to movement of eggs (Lyons et al., 2006). Motility of the sperm flagellum is essential for sperm function (Afzelius and Eliasson, 1983). Consequently, human primary ciliary dyskinesia (PCD), which is caused by dysfunction of motile cilia, is associated with situs randomisation, respiratory problems, male infertility and, less frequently, with female infertility and hydrocephalus (reviewed by Praveen et al., 2015).

Whereas cells of respiratory epithelia and ependyma form multiple motile cilia, spermatozoa carry one flagellum, a specialised motile cilium with specific accessory structures. The flagellum consists of: the connecting piece, which links it to the sperm head and holds the centriole from which the axoneme nucleates; the midpiece, which contains nine outer dense fibres (ODFs) and peripheral mitochondria; the principal piece, which possesses both ODFs and a peripheral fibrous sheath; and the end piece (reviewed by Eddy, 2006). The flagellum develops during differentiation of spermatids in seminiferous tubules of testes. Concomitantly, the sperm head reshapes and the acrosome, which is a sperm-specific secretory vesicle, forms (reviewed by Fléchon, 2016). Most of the cytoplasm is removed via a residual body that detaches from the neck region and, via a cytoplasmic droplet that is shed at the midpiece-principal piece boundary, the annulus (reviewed by Hermo et al., 2010). Spermatozoa are stored in the cauda epididymis, where they undergo further maturation (reviewed by Buffone et al., 2012; Cornwall, 2009).

FOXJ1 is a key transcription factor for the formation of motile cilia throughout vertebrates (Alten et al., 2012; Brody et al., 2000; Chen et al., 1998; Stubbs et al., 2008; Vij et al., 2012; Yu et al., 2008). FOXJ1 targets include: structural, motor and accessory proteins of the axoneme; intraflagellar and vesicular transport proteins; and basal body components (reviewed by Santos and Reiter, 2008). Additional proteins are thought to be involved in the function of cilia (Andersen et al., 2003; Gerdes et al., 2009; Gherman et al., 2006; McClintock et al., 2008). Comparing the transcriptomes of fetal respiratory epithelia before and after motile ciliogenesis, and heterozygous with homozygous Foxj1 mutant
lungs, we identified a few hundred candidates for FOXJ1 effectors during ciliogenesis. Here, we describe the analysis of one candidate, \textit{Cfap157}, that encodes a basal body protein of unknown function. \textit{Cfap157} transcription correlates with the presence of motile cilia and depends on FOXJ1. Despite its expression in many cell types with motile cilia, loss of \textit{Cfap157} function in mice specifically affects flagellum morphogenesis and sperm motility.

**RESULTS**

**Expression and localisation of \textit{Cfap157}**

\textit{Cfap157} (GeneID 227736) encodes a 523 amino acid protein that lacks known motifs, except for a PEST domain (http://bioinf.cs.ucl.ac.uk/psipred/), is conserved among chordates and shares sequence identity of variable extent with proteins of various other eukaryotes, including the green algae \textit{Chlamydomonas} (Table S1). Mouse \textit{Cfap157} expression detected by RT-PCR was essentially confined...
to tissues containing motile cilia correlating with the expression of Foxj1 (Fig. 1A, boxes). Section in situ hybridisation showed Cfap157 expression in epithelia known to carry motile cilia and spermatogenic cells in testes (Fig. 1B-d, arrows). Cfap157 was downregulated in airway epithelial cells of Foxj1-null mutants (Fig. 1B-e, f, arrows), confirming that Cfap157 transcription depends on FOXJ1 in these cells. Whole-mount in situ hybridisation of Xenopus embryos revealed cfap157 (GeneID 780234) expression in cells carrying motile cilia (Fig. 1C). Consistent with the transcriptional dependence of Cfap157 on FOXJ1 in mice, overexpression of foxj1 induced ectopic expression of cfap157 in Xenopus embryos (Fig. 1D).

We analysed the subcellular localisation of CFAP157 in inner medullary collecting duct 3 (IMCD3) cells, which express endogenous Cfap157 and form non-motile cilia, by expressing GFP fusions (because our anti-CFAP157 antibodies did not detect endogenous CFAP157). In IMCD3 cells induced to form cilia CFAP157-GFP was enriched in two adjacent dots near the ciliary base (Fig. 1E-a-c) that co-stained with γ-tubulin (Fig. 1E-d-f), indicating localisation at the basal body. Consistently, GFP-labelled CFAP157 expressed in Xenopus embryos showed multiple dots subapical to the membrane that overlapped with γ-tubulin in multiciliated epithelial cells, also indicating localisation to basal bodies of motile cilia (Fig. 1F).

Identification of potential protein interaction partners of CFAP157

To identify interaction partners of CFAP157, SF-TAP-tagged (Gloeckner et al., 2007) full-length CFAP157 or CFAP157ΔPEST (lacking the C-terminal PEST domain) was expressed in HEK293T cells and purified by Strep-FLAG tandem affinity purification. The eluates were analysed by liquid chromatography fractionation and mass spectrometry (LC-MS). Interesting candidate interaction partners in HEK293T cells included the two tubulins TUBA4A and TUBB5, basigin, and the centrosomal proteins CEP350 and SSNA1 (Table S2). SSNA1 was also significantly enriched among proteins pulled down from testis lysates using purified SF-TAP-tagged CFAP157ΔPEST, in addition to various other proteins (Table S3). Co-immunoprecipitation assays using CHO cells co-expressing full-length HA-tagged CFAP157ΔPEST and myc- or flag-tagged versions of potential interaction partners thus far confirmed the interaction of CFAP157 with TUBA4A, TUBB5 and CEP350 (Fig. 2A, boxes) but not with basigin and SSNA1. Assays using HA-tagged full-length CFAP157, which is expressed at lower levels than the ΔPEST-version, produced similar results (data not shown). A direct or indirect interaction of CFAP157 with tubulins and CEP350 is consistent with the localisation of CFAP157 at ciliary basal bodies and with the co-expression of Cfap157 and Cep350 in brain, lung and testes (Fig. 2B).

Generation of Cfap157<sup>ΔlacZ</sup> and Cfap157<sup>Δex2</sup> knockout mice

To analyse the physiological function of CFAP157, we generated mutant mice (Fig. 3A) using the EUCOMM ESC clone G05 (1700019L03Rik<sup>ΔlacZ</sup>UCCOMM<sup>ΔΔmg</sup>) and validated them using PCR and Southern blot analysis (Fig. 3B, C). In these mice, a lacZ gene and a neo cassette were inserted into intron 1, and exon 2 was flanked by loxp sites (Cfap157<sup>ΔlacZ</sup>Δ<sub>ex2</sub>). Additional loxp and FRT sites allowed for removal of neo and exon 2 to generate a null allele with a lacZ-reporter (Cfap157<sup>ΔlacZ</sup>Δ<sub>ex2</sub>) or for removal of lacZ and neo to generate a conditional allele (Cfap157<sup>Δex2</sup>Δ<sub>ex3</sub>). Deletion of exon 2 (Cfap157<sup>Δex2</sup>) causes a frameshift and termination of translation in exon 3 and/or nonsense-mediated mRNA decay. These alleles were generated using Zp3-Cre (de Vries et al., 2000) or flp-deleter mice (Rodríguez et al., 2000) and verified by using PCR.

Homozygous Cfap157<sup>ΔlacZ</sup> or Cfap157<sup>Δex2</sup> mice were born at Mendelian ratios (Table S4) and were indistinguishable from wild-type mice. Cfap157 transcripts were not detected in RNA from homozygous brain and lung (Fig. 3D, Fig. S1). However, RT-PCR on testis RNA of homozygous Cfap157<sup>ΔlacZ</sup> mice amplified fragments of transcripts lacking either exon 2 or both exon 2 and exon 3 (Fig. 3E, asterisk and arrowhead), which is probably caused by alternative splicing and stabilisation of mRNA that can occur during spermatogenesis due to removal of destabilising 3′UTR sequences (Kleene, 2005; Li et al., 2016). The latter transcript could be translated into a CFAP157 protein lacking 142 amino acids. qRT-PCR suggested significant expression of this transcript (Fig. S2). However, northern blot analysis with a full-length Cfap157 cDNA probe detected no transcripts in Cfap157<sup>ΔlacZ</sup> and Cfap157<sup>Δex2</sup> testis RNA (Fig. 3F). Importantly, no CFAP157 protein could be precipitated from homozygous Cfap157<sup>ΔlacZ</sup> and Cfap157<sup>Δex2</sup> testis lysates (Fig. 3G, arrowheads and bracket; direct detection of CFAP157 in lysates using western blots was precluded by high background) using antibodies raised against peptides encoded by exons 1 and 7. These results demonstrated that Cfap157<sup>ΔlacZ</sup> and Cfap157<sup>Δex2</sup> are null alleles.

Analysis of reporter expression in Cfap157<sup>ΔlacZ</sup> mice and dependence of Cfap157 transcription on FOXJ1

β-Galactosidase staining of Cfap157<sup>ΔlacZ</sup> embryonic and fetal tissues revealed lacZ expression in the node of E8 embryos, in the fourth ventricle of the brain at E12.5 and in the airways at E18.5 (Fig. 4A, arrows). In adults, lacZ was expressed in the airways of the lung, the ependymal layer of the ventricles and the seminiferous tubules of testis (Fig. 4B). On a Foxj1-null background (Brody et al., 2000), reporter expression from the Cfap157<sup>ΔlacZ</sup> allele was lost (e.g. fetal
lungs, Fig. 4Ce,f; node, Fig. S4), corroborating the observation that FOXJ1 is an essential transcriptional activator of *Cfap157*.

**Essential function of *Cfap157* restricted to male fertility**

Analysis of inner organs revealed no situs defects in heterozygous and homozygous *Cfap157lacZ* (n=177 and 76) and *Cfap157Δex2* mice (n=37 and n=22). In addition, we observed no hydrocephalus, renal cysts (Fig. 5A) or mucus accumulation in nasal cavities (Fig. 5Ba,b, circles) in *Cfap157* mutants. Light and transmission electron microscopy (TEM) showed no differences between cilia in homozygous *Cfap157lacZ* and wild-type lung epithelia (Fig. 5Bc-h). Likewise, histological analysis of testis RNA probed with full-length *Cfap157* cDNA; box, signal at the expected size. The faint wild-type signal obtained for *Cfap157*Δex2neo/Δex2neo suggested read-through transcription and splicing around the lacZ/neocassette (Fig. S3). Homozygous *Cfap157lacZ* neo mice displayed both lacZ reporter expression and full male fertility. Bottom, same northern blot re-probed with β-actin. (G) Immunoprecipitation of CFAP157 from wild-type or *Cfap157* mutant testis lysates using polyclonal (PAb) or monoclonal (MAb) antibodies. IP w/o Ab, negative control without antibody; arrowheads, CFAP157; bracket, expected position of CFAP157Δex2,3 (44 kDa) (all CFAP157 signals were detected slightly below the expected size); asterisks, immunoglobulin heavy chain.

**Fig. 3. Generation and verification of *Cfap157* knockout mice.** (A) Targeting strategy. (B) Validation of targeting by LR-PCR at the 5′ and 3′ end of the integrated transgene performed with wild-type (wt) and *Cfap157Δex2neo* genomic DNA. (C) Southern blot analysis with external 5′ and 3′ probes on wild-type and *Cfap157lacZ* genomic DNA; arrowheads, expected transgenic signals. (D) RT-PCR with primers binding in *Cfap157* exons 1 and 3, *Cfap157* exons 4 and 7, and *Hprt* exons 7 and 9 of on RNA from adult tissues (for complete gel photos, see Fig. S4). (E) RT-PCR with primer pairs as in D and a pair binding in *Cfap157* exons 1 and 7 on adult testes RNA (asterisk and arrowhead, sequenced Δex2 and Δex2,3 products, respectively). H2O, control without cDNA. (F) Northern blot analysis of testis RNA probed with full-length *Cfap157* cDNA; box, signal at the expected size. The faint wild-type signal obtained for *Cfap157*Δex2neo/Δex2neo suggested read-through transcription and splicing around the lacZ/neocassette (Fig. S3). Homozygous *Cfap157lacZ*neo mice displayed both lacZ reporter expression and full male fertility. Bottom, same northern blot re-probed with β-actin. (G) Immunoprecipitation of CFAP157 from wild-type or *Cfap157* mutant testis lysates using polyclonal (PAb) or monoclonal (MAb) antibodies. IP w/o Ab, negative control without antibody; arrowheads, CFAP157; bracket, expected position of CFAP157Δex2,3 (44 kDa) (all CFAP157 signals were detected slightly below the expected size); asterisks, immunoglobulin heavy chain.
Cfap157 homozygous males) but never generated pregnancies. In addition, continuous matings (3-5 months) with homozygous Cfap157lacZ (n=16) or Cfap157Δex2 (n=7) males produced no offspring. Collectively, these analyses indicated a CFAP157 function that is distinctly required for sperm function.

Cfap157 functions in sperm motility and flagellum morphogenesis

Cfap157 mutant sperm dissected from the cauda epididymis ('cauda sperm') displayed a reduced and abnormal motility, often resulting in twitching or local circular movement (Movies 1 and 2). Most Cfap157 spermatozoa contained a bulge in the proximal flagellum next to a dislocated head (see below). Computer assisted sperm analysis (CASA; Yániz et al., 2015) of Cfap157 mutant cauda sperm showed a severely reduced motility rate (Fig. 6A, Table S6) and a significantly lower concentration (Fig. 6B, Table S6).

Cauda sperm of 4- to 6-month-old Cfap157 mutant males bound eggs inefficiently and hardly moved them, in contrast to wild-type sperm (Fig. 6C, n>12 eggs; Movies 3 and 4). In vitro fertilisation (IVF) assays showed that Cfap157 mutant sperm are essentially unable to fertilise eggs even when in close contact: 143/211 (68%) wild-type eggs incubated with sperm from fertile heterozygous Cfap157lacZ/+ males developed pronuclei, indicating fertilisation, whereas only 13/488 (2.7%) wild-type eggs incubated with sperm from homozygous Cfap157lacZlacZ males were fertilised (Table S7). Likewise, the development of blastocysts occurred in only 4/1111 (0.4%) and 1/810 (0.1%) wild-type eggs incubated with cauda sperm from Cfap157lacZlacZ and Cfap157Δex2/Δex2 males, respectively, whereas incubation with wild-type sperm produced blastocysts in 260/437 (58%) eggs (Fig. 6D, Table S8). As IVF with both wild-type and mutant sperm resulted in a lower percentage of blastocysts than fertilised eggs, the loss of embryos appears unrelated to the CFAP157 mutation.

Staining various structures in cauda sperm of ~6-month-old Cfap157 mutant males revealed morphological abnormalities in the midpiece region in 95% of sperm cells that were identical for both alleles (n=125 Cfap157lacZ; n=129 Cfap157Δex2 spermatozoa; Fig. 7A-E). Ninety-three percent (235/254) of sperm cells showed an axonemal loop of the midpiece (Fig. 7Ac,d,g,h, Bc,d,g,h) and 6% (14/254, some also with a loop) contained a bulge (Fig. 7Ae,f). Seventy-five percent (191/254) of sperm heads were bent abnormally (Fig. 7Ac,g, Bc,g) and 12% (31/254) were missing (Fig. 7Be). The loop appeared to contain excess cytoplasm (Fig. 7Ac,g, Bc,g). Haematoxylin and Eosin staining of Cfap157 sperm cells confirmed that surplus cytoplasm incorporated the loop completely in some, and partially in other spermatozoa (not shown). Co-staining of the annulus at the midpiece–principal piece boundary using anti-septin 7 antibodies revealed that the axonemal loop was restricted to the midpiece (n=45; Fig. 7Bd,f,h). Staining of acetylated tubulin showed that 76/180 spermatozoa contained a connecting microtubular link that appeared to fix the loop (Fig. 7Bd,h, arrows). The rest did not show such a link, perhaps obscured by twisted or narrow loops (Fig. 7Ad,h). Staining with AKAP3 antibodies showed the presence of ectopic fibrous sheath in the midpiece region close to the sperm head in 111/139 spermatozoa (arrows and arrowheads in Fig. 7Cd,f,h) and much less in the principal piece. Ten out of 139 mutant sperm cells had no AKAP3-
labelled fibrous sheath, and 18/139 looked like wild-type sperm, which contained fibrous sheath exclusively in the principal piece (n=34; Fig. 7Cb). Staining with MitoTracker revealed an accumulation of mitochondria near the axonemal link but not along the axoneme of the looped midpiece (n=75; Fig. 7Dd). The length of the looped midpiece (Cfap157lacZ/lacZ, 23±1 µm, n=6; Cfap157Δex2/Δex2, 23±1 µm, n=17; Cfap157lacZ/+ , 23±1 µm, n=8) and of the remaining flagellum (Cfap157lacZ/lacZ, 94±7 µm, n=16; Cfap157Δex2/Δex2, 93±4 µm, n=13; Cfap157lacZ/+ , 93±4 µm, n=6) was similar to wild type and consistent with previous wild-type measurements (Cummins and Woodall, 1985). Nuclei (n=147) and acrosomes (n=65) of Cfap157 sperm stained with DAPI and lectin appeared normal (Fig. 7Ab,d,f,h). Collectively, these analyses show that CFAP157 is required for the normal organisation of the midpiece axoneme, for the transport/localisation of the fibrous sheath and mitochondria, as well as for elimination of excess cytoplasm, processes that occur in the seminiferous tubules prior to spermiation. CFAP157 is absent from epididymal sperm (Fig. S5), perhaps reflecting the disintegration of centrioles in mature sperm (Eddy, 2006) or the overall loss of proteins during sperm maturation (Skerget et al., 2015). This indicates that CFAP157 is no longer present in mature sperm and supports a function exclusively in the testis.

To monitor the development of the Cfap157 phenotype in testes, we analysed sections of seminiferous tubules at all stages of spermatogenesis, which we identified by the size and shape of acrosomes and nuclei (Ahmed and de Rooij, 2009; Meistrich and Hess, 2013) that were indistinguishable between wild type and Cfap157 mutants (n=3) at all stages (total of 246 mutant tubule sections). Consistent with previous observations (Zhang et al., 2012), mitochondria (stained using anti-COX IV antibodies) aligned in wild-type midpieces of spermatids beginning at stage 14/15 (arrowheads in Fig. 8Aa,a′,b,b′; Fig. S6Ac,c′) and formed slender threads projecting from the sperm heads into the lumen at stage 16 (arrowheads in Fig. 8Ac,c′; Fig. S6Ad-g,d′-g′). As in wild type, Cfap157-null tubules displayed aligned mitochondria beginning at stage 14/15 (arrowheads in Fig. 8Ad-f′; Fig. S6An,n′). At stage 16, mitochondrial threads appeared less regular, partly kinked and bulky (arrows Fig. 8Af,f′; Fig. S6Ar,r′), and sperm heads lining the lumen were not well oriented (Fig. 8Af,f′; Fig. S6Ao-r, o′-r′). There was no evidence for the strong accumulation of mitochondria observed in cauda sperm (Fig. 7), suggesting that clustering of mitochondria occurs after spermiation. Prior to stage 12, the fibrous sheath marker AKAP3 was diffusely distributed in the cytoplasm of developing spermatids. As previously described (Sakai et al., 1986), fibre-like structures appeared first in spermatids at stage 13 (arrows in Fig. S6Ba,a′,l,l′) and were prominent thereafter (arrows in Fig. 8Ba,a′,c,c′). Fibres were present at a distance from the sperm heads in mature wild-type sperm (Fig. 8Bc,c′; Fig. S6Bg,g′), resulting in a zone devoid of AKAP3 staining between the sperm heads lining the lumen and the principal pieces (arrowheads in Fig. 8B; Fig. S6Bf,f′,g,g′).
contrast, fibrous, dotted and diffuse AKAP3 staining in Cfap157 mutant sperm was found in abnormal positions and in the vicinity of sperm heads (asterisks in Fig. 8Bd,f; Fig. S6Bn-r), suggesting that ectopic accumulation of fibrous sheaths develops during late stages of spermiogenesis.

Ultrastructural defects in CFAP157-deficient sperm

TEM of Cfap157lacZ/lacZ cauda epididymis sections showed fewer sperm in the mutant than in the wild type (Fig. 9A) and expanded structures probably representing the abnormal Cfap157-null midpieces observed by light microscopy (Fig. 9Ab, blue arrows). Cell membranes around some of these structures were fragmented (Fig. 9Ab, yellow arrow), indicating reduced stability.

Longitudinal sections of Cfap157lacZ/lacZ sperm (Fig. 9Ca,b,g) revealed a dislocated sperm head (bent by 180° in Fig. 9Ca) and a large cytoplasmic remnant (arrow). Although microtubule fibres were not visible in this section, ODFs continuous in midpiece branches and the principal piece (Fig. 9Cb, arrows; Fig. S7 Ae,f) suggested the fusion of two midpiece axonemes to form a single principal piece axoneme, i.e. an axonemal fork. The axonemal loop, visible by the ODFs, ran along the edge of the cell, encircling the excess cytoplasm (Fig. 9Cg, arrow).

Cfap157lacZ/lacZ midpiece regions contained irregular arrangements of mitochondria clustered around a central axonemal profile (Fig. 9Cc; stars, red arrow) and additional peripheral axoneme profiles (Fig. 9Cc, yellow arrows) that were surrounded by a fibrous sheath (Fig. 9Cd, hexagon) consistent with the enrichment of fibrous sheath in the midpieces of isolated cauda sperm (Fig. 7C). Peripheral axonemes were not surrounded by individual plasma membranes and are therefore unlikely to represent sections through folded or clustered flagella. The presence of similar groups of peripheral axonemes on opposite sides of the cell implied that bundles of a varying number of axonemes formed the axonemal loops, consistent with the lack of mitochondria attached to them (Fig. 7Dd, Fig. 9Cc,d).

Finally, we observed defective axonemes in Cfap157 mutant sperm missing or displaying dislocated microtubule doublets at various flagellar levels [compare Fig. 9Bc,d with 9Cc,d,f (arrowheads) and Fig. 9Be with 9Ce (arrowheads); Fig. S7] in about one out of three mutant axoneme profiles. Profiles of coupled axonemes confirmed the presence of axoneme bundles in Cfap157 mutant sperm (Fig. 9Cf). The mirror image of the incomplete axonemes and ODFs of this example (Fig. 9Cf, arrowheads and asterisks) suggested that both profiles belonged to the same folded axoneme. Defective axonemes typically possessed a central microtubule pair, suggesting that they were assembled incompletely rather than split after assembly, although we cannot exclude other mechanisms. Microtubules of mutant axonemes possessed inner and outer dynein arms (Fig. S8), indicating that Cfap157 is not required for dynein arm assembly.

DISCUSSION

We identified Cfap157 from microarray screens of FOXJ1-dependent genes in the mouse fetal lung (Stauber et al., 2016). Cfap157 also appeared in several other screens that suggested activation by FOXJ1 (Choksi et al., 2014a), involvement in ciliogenesis (Hoh et al., 2012; Ivliev et al., 2012; McClintock et al., 2008) and association with human PCD (Geremek et al., 2011, 2014). Here, we show that CFAP157 is a novel FOXJ1 effector that is expressed in various motile ciliated tissues but essential only during spermatogenesis.

Cfap157: a FOXJ1 target with a sperm-specific function

Cfap157 expression correlated with Foxj1 expression and the presence of motile cilia in mouse and frog, and endogenous Cfap157 expression in mice depended on FOXJ1. Despite its
expression in several tissues, both mutant Cfap157 alleles specifically affected only the formation and function of the sperm flagellum, although we cannot rule out minor defects in other motile cilia without obvious functional consequences. Possibly, CFAP157 function is compensated for in some motile cilia after gene disruption, as described in Cep131 mutant mice (Hall et al., 2013) and in zebrafish mutants (Rossi et al., 2015). However, the identity of any compensating factor remains undetermined and it appears plausible that the sperm flagellum with its unique accessory structures has requirements that differ from other motile cilia. This presumption is supported by mutations of other ciliogenesis factors that distinctly affect sperm cells: e.g. disruption of Spef2 causes disorganised flagellar axonemes and male infertility but normal tracheal axonemes (Sironen et al., 2011). In contrast to Cfap157, Spef2-deficient mice still exhibit sinusitis and hydrocephalus, putatively due to a reduction of the beat frequency of the respective motile cilia (Sironen et al., 2011). Conservation of mouse and human CFAP157 protein (Table S1) suggests that human CFAP157 may play a similar role in male fertility and that mutations would result in a similar phenotype.

**A unique sperm phenotype in Cfap157 mutants**

Impaired sperm motility, reduced sperm numbers and inefficient attachment of mutant spermatozoa to the zona pellucida (collectively observed in all 13 Cfap157lacZ/lacZ and 8 Cfap157Δex2/Δex2 males analysed) can be explained by the expanded and disorganised midpiece and dislocated head, supernumerary axonemes and a disrupted axonemal arrangement.
Cfap157-deficient sperm heads are bent with respect to the flagellar axis; consequently, flagellar beating does not drive spermatozoa into the egg but along the surface, away from the egg. This and sterical hindrance may have caused the reduced binding to eggs and the failure to penetrate the zona pellucida that requires the impetus of the sperm (Buffone et al., 2012). The reduced sperm concentration is unlikely to contribute to infertility because a similar reduction in sperm numbers in Arl4-null mice did not affect fertility (Schürmann et al., 2002).

The most peculiar aspect of the phenotype was the looped axoneme in the disorganised midpiece region containing multiple axonemal profiles. The additional axonemal profiles could be derived either from ectopic nucleation of several axonemes or from fragmented or broken axonemes. However, tubulin staining showed continuous axonemes throughout the flagellum (e.g. Fig. 7A,B,D) and localisation of CFAP157 to basal bodies would be consistent with a centriolar function, arguing in favour of ectopic nucleation. Fibrous sheath normally occurs only in the principal piece and its formation progresses from distal to proximal up to the annulus (Irons and Clermont, 1982). The presence of fibrous sheath around Cfap157-deficient midpiece axonemes suggests that an apparently normal annulus may not be sufficient to prevent the formation of fibrous sheath in the midpiece. Immunocytochemical analysis suggested that the abnormal distribution of fibrous sheath occurs during late stages of spermiogenesis and that mitochondrial clustering develops...
even after spermiation (Fig. 8). Formation of the sperm tail is essentially complete at spermiogenesis stage 12 (Vernet et al., 2016), preceding the alignment of mitochondria and formation of fibrous sheaths. This implies that the axonemal defects, including supernumerary axonemes develop prior to stage 12 and thus are the primary phenotype of \textit{Cfap157}-null sperm cells. As differentiation of the sperm tail is difficult to assess in testis sections (Vernet et al., 2012), further ultrastructural analyses of testes and epididymis will clarify this issue.

Various mouse mutations that affect sperm morphology and motility have been identified (de Boer et al., 2015; reviewed by Escalier, 2006; Naz et al., 2009) but none of them resemble the \textit{Cfap157} phenotype. Superficially, the structural defects of \textit{Cfap157} mutant sperm showed some similarity to \textit{Spem1}. In spermatozoa lacking \textit{Spem1}, the midpiece wraps around the head, which is bent backwards and kept in this position by remnants of the cytoplasm (Zheng et al., 2007). \textit{Cfap157} mutant sperm also contains excess cytoplasm; however, the axonemal loop of the midpiece appears to

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**Fig. 9.** Electron microscopy analysis of wild-type and \textit{Cfap157\textsuperscript{lacZ/lacZ}} sperm in the cauda epididymis. (A) Overviews of wild-type (a) and \textit{Cfap157\textsuperscript{lacZ/lacZ}} sections (b): yellow stars, sperm heads; red arrows, flagella. (B,C) Detailed images for wild type (B) and mutant (C). (a) Longitudinal section of a sperm cell: yellow stars, head; blue arrow, expanded midpiece. (b) Magnification of area boxed in a: yellow arrows, ODFs; hexagon, fibrous sheath. (c) Cross-section at the midpiece level; the wild-type section contains a cytoplasmic droplet. Red arrow indicates central axoneme; yellow arrows indicate peripheral axonemes; pink stars indicate mitochondria. (d) Magnification of the boxed area in c: pink star, mitochondria; asterisks, ODFs; blue arrowheads, missing microtubule doublets; hexagon, fibrous sheath. (e) Cross-sections of an axial filament: blue arrowhead, missing microtubule doublets. (f) Cross-section of a paired \textit{Cfap157\textsuperscript{lacZ}} axoneme: pink star, mitochondria; asterisks, ODFs; blue arrowheads, missing microtubule doublets; red circles, central pairs. (g) Longitudinal section of a \textit{Cfap157\textsuperscript{lacZ}} spermatozoon at the level of the axonemal loop: pink star, mitochondria. Scale bars: 2 µm in A; 2 µm in Ba,Ca; 0.2 µm in Bb,Cb; 1 µm in Bc,Cc; 0.2 µm in Bd,Cd; 0.2 µm in Be,Ce; 0.2 µm in Cf; 1 µm in Cg.
be fixed by a small axonemal link that may emerge from an axonemal fork, the flagellum is not wrapped around the head, and the head is bent in smaller angles in isolated sperm. The presence of multiple flagellar axonomes in the proximal tail region and looped midpiece axonomal bundles has to the best of our knowledge not been previously described.

The localisation of CFAP157 in spermatozoa is currently unknown because we were unable to detect endogenous CFAP157 with our mouse antibodies and commercially available antibodies applying various fixation and staining protocols. Two anti-C9ORF117 antibodies produced and applied by the Human Protein Atlas both detect ciliary protein in human bronchus and nasopharynx (Ivliev et al., 2012) but show no or low diffuse cytoplasmic staining in human testis and epidydymis (http://www.proteinatlas.org). This may indicate distinct localisation in different tissues or species, or be due to the inability of these antibodies to detect CFAP157 in developing spermatozoa.

Our data suggest CFAP157 localisation and function at basal bodies. An entry point into the biochemical characterisation of CFAP157 may be the validated interaction with CEP350, a centrosomal protein that binds microtubules (Hoppeler-Lebel et al., 2007; Patel et al., 2005). It controls microtubule stability and thereby plays a role in procentriole assembly, centriole elongation and Golgi organisation (Hoppeler-Lebel et al., 2007; Le Clech, 2008). Requirement of CEP350 for microtubule docking to the centrosome is controversial (Hoppeler-Lebel et al., 2007; Yan et al., 2006). CFAP157 could be involved in any of these processes. Intriguingly, CEP350 also targets proteins to the centrosome and is therefore required for ciliogenesis. An example is CYLD, a deubiquitinating enzyme that is anchored to the centrosome by CEP350, where it is required for migration and docking of basal bodies to the plasma membrane (Eguether et al., 2014).

In conclusion, we have identified a gene, Cfap157, that is expressed downstream of FOXJ1 in cell types carrying motile cilia with a pivotal function only in male germ cells. Our analyses suggest that CFAP157 is part of a novel mechanism that acts specifically in developing mammalian spermatozoa to ensure the formation of a single ultrastructurally correct flagellar axoneme and of a functional midpiece.

**MATERIALS AND METHODS**

**Ethics statement, generation and husbandry of mice**

Mouse and *Xenopus laevis* handling was in accordance with the German laws (Tierschutzgesetz), and was approved by the ethics committee of Lower Saxony for care and use of laboratory animals (LAVES) and by the Regional Government Stuttgart, Germany (A379/12 Zo ‘Molekular Embryologie’). Mice were housed in the animal facility of Hannover Medical School (ZTL) as approved by the responsible Veterinary Officer of the City of Hannover. Animal welfare was supervised and approved by the Institutional Animal Welfare Officer.

**Cfap157**

Cfap157 mice were generated with EUCOMM ESC clone G05 [1700019L03Rik, https://www.eumcr.org/]. Other mouse strains were Zp3:Cre (de Vries et al., 2000), FLPe (Rodríguez et al., 2004), Foxj1 (Brody et al., 2000) and CD-1 or CD-1/129/Sv hybrid as wild type. Mice were genotyped by PCR as described in the supplementary Materials and Methods.

**Computer-assisted sperm analysis (CASA) and in vitro fertilisation (IVF)**

For CASA, sperm were isolated from the cauda epididymis in 150 µl HTF medium and capacitated for 90 min at 37°C. Sperm suspension (3 µl) were analysed in a Leja Standard Count 4 Chamber Slide under an Olympus CX41 (Zuber Optik) using the QualiSperm (Biophos optimised for human sperm) software that automatically measures motility rates and concentration of sperm. For IVF, sperm isolated from the cauda epididymis in HTF was capacitated for 2 h at 37°C and 5% CO2. Eggs were isolated from superovulated wild-type females, incubated in HTF in groups of 40 with sperm for 6 h at 37°C under oil, washed and incubated in HTF. The presence of pronuclei was checked between 10 and 12 h after incubation with sperm. Development of blastocysts was checked after culture in KSOM from day 3.5 to 7.5 after IVF. Statistical analysis was performed using Prism (GraphPad) and one-way ANOVA.

**Xenopus experiments**

*Xenopus* microinjections, histological analyses and RNA in situ hybridisation were carried out essentially as described previously (Belo et al., 1997; Tingler et al., 2014). Full details are provided in the supplementary Materials and Methods.

**Analysis of subcellular localisation of CFAP157-GFP in IMCD3 cells**

CFAP157-GFP fusion proteins were transiently expressed in IMCD3 cells and analysed for GFP fluorescence. IMCD3 cells were also stained using acetylated and γ-tubulin. For further details, including the antibodies used, see the supplementary Materials and Methods.

**Identification and validation of protein interaction partners**

Tandem StreptII-Flag-tagged CFAP157 was transiently expressed in HEK293T cells and CFAP157 complexes were isolated (Gloeckner et al., 2007). Moreover, proteins from testis lysates were pulled down using purified StreptII-Flag-tagged CFAP157. Isolated CFAP157 complexes were analysed by LC-MS/MS as described previously (Boldt et al., 2011). Selected candidate interaction partners identified by MS were further analysed by co-immunoprecipitation. Details of constructs, SF-TAG purification, MS analysis and coimmunoprecipitation are provided in the supplementary Materials and Methods.

**Histological methods**

Immunofluorescence staining of testis sections and isolated sperm, section in situ hybridisation, β-galactosidase and Hematoxylin and Eosin staining were performed using standard procedures. Further details, including the antibodies used, can be found in the supplementary Materials and Methods.

**Transmission electron microscopy (TEM)**

Caudal epididymis and lungs were dissected from adult, 3-month-old wild-type and homozygous *Cfap157*–/– littermates, fixed, embedded and analysed for GFP fluorescence. IMCD3 cells were also stained using acetylated and γ-tubulin. For further details, including the antibodies used, see the supplementary Materials and Methods.

**Southern and northern blot analysis**

Southern and northern blot hybridisations were carried out according to standard procedures. Details on the probes and conditions used can be found in the supplementary Materials and Methods.

**LR-PCR and (q)RT-PCR**

Correct targeting of the *Cfap157* locus was confirmed by PCR on genomic DNA isolated from liver with the Expand Long Range Kit (Roche) according to the manufacturer’s instructions. RT-PCR was performed on cDNA generated from total RNA isolated from various tissues of different genotypes. Details of PCR conditions, primers and fragment lengths can be found in the supplementary Materials and Methods.

**Immunoprecipitation of CFAP157 from testis lysates**

The generation of the monoclonal and polyclonal antibodies against CFAP157 is described in detail in the supplementary Materials and Methods. Further details, including the antibodies used, can be found in the supplementary Materials and Methods.

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Precipitated proteins were detected using monoclonal antibody 3B9 (1:100) or polyclonal antibody (1:5000).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
M.W. designed and performed in vivo and in vitro experiments, validated knockout mice and antibodies, performed (co)immunoprecipitations, analysed data and wrote the paper; K.S.-G. established knockout mouse lines and performed in vitro fertilisations; M.S. participated in design and performance of some mouse experiments, analysed data and wrote the paper; C.W. and J.H. designed and performed electron microscopy, and analysed data; T.O. and M.B. designed and performed Xenopus experiments, and analysed data; K.B., T.B. and M.U. designed and performed tandem affinity purification and mass spectrometry, and analysed data; K.S. performed some immunoprecipitations, and stained, staged and analysed testis sections and mutant sperm; E.K. designed peptides and generated monoclonal antibodies; A.G. designed experiments, analysed data and wrote the paper; all authors read and approved the manuscript prior to submission.

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