Expression of SPEF2 During Mouse Spermatogenesis and Identification of IFT20 as an Interacting Protein

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ABSTRACT

SPEF2 is expressed in all ciliated cells and is essential for correct sperm tail development and male fertility. We have previously identified a mutation within the SPEF2 gene as the cause for infertility because of immotile and malformed sperm tails in pigs. This mutation in pigs alters the testis-specific long SPEF2 isoform and exclusively affects the sperm tail development. In infertile boars, axonemal and all accessory structures of the sperm tail are affected; thus, SPEF2 seems to participate in the organization of these structures. In the present study, we have investigated the expression of SPEF2 during mouse spermatogenesis. SPEF2 mRNA and protein products appear to be localized both in germ cells and in Sertoli cells. In differentiating germ cells, SPEF2 protein is localized in the Golgi complex, manchette, basal body, and midpiece of the sperm tail. In mature murine sperm, SPEF2 is present in the distal part of the sperm tail midpiece. Using yeast two-hybrid assay and coimmunoprecipitation experiments, we identified an interaction between SPEF2 and the intraflagellar transport protein IFT20 in the testis. Furthermore, these two proteins colocalize in differentiating male germ cells. These results support the crucial importance of SPEF2 in sperm differentiation and involvement of SPEF2 in structuring of the sperm tail.

IFT20, SPEF2, sperm, sperm motility and transport, sperm tail, spermatid, spermatogenesis, testis

INTRODUCTION

Male infertility is becoming increasingly prevalent, partly because of the influence of environmental factors, but many defects in sperm development arise from a genetic cause. Problems in the production and maturation of sperm are the most common causes of male infertility, resulting in low sperm numbers, morphologically abnormal sperm, or low sperm motility [1–3]. Despite efforts to reveal the genes and their functions in spermatogenesis, little is known about the underlying causes of male infertility. Similarities in spermatogenesis and sperm structures among mammalian species [4] highlight the importance of animal models for research on the causes of human male infertility.

Mammalian spermatogenesis is a complex process in which diploid spermatogonia develop into haploid, highly specialized spermatozoa. Somatic Sertoli cells support germ cells at all spermatogenetic stages [5, 6]. Spermatogenesis includes many testis-specific processes that are controlled by complex regulatory mechanisms [7, 8]. In spermiogenesis, haploid round spermatids undergo dramatic biochemical and morphological changes that are governed by specialized gene expression and interactions between various genes and their protein products [9]. Some of these genes or their alternative transcripts are specifically expressed in the testis. Identification of these genes and their roles is important in understanding the mechanisms of spermatogenesis.

Correct sperm tail organization is essential for sperm motility. Recently, we identified a mutation within the SPEF2 gene as the cause for immotile short tail sperm (ISTS) defect in pigs [10]. ISTS-affected boars are infertile because of immotile and short sperm tails and have lower sperm counts. Histological examination indicates that the sperm tail accessory structures are disorganized and that the axonemal complex is severely compromised [11]. Most of the affected sperm tails lack one or both central microtubuli, and in many cases, less than nine doublets are present. In addition, the outer dense fibers and mitochondrial sheath are often disorganized, and, in most cases, the midpiece and principal piece of the sperm tail are reduced in length. Sperm heads appear to develop normally, but cytoplasmic droplets are abundant [12]. ISTS is manifested during spermiogenesis and appears to affect spermatogenesis at the spermatid elongation phase, because only the number of elongated spermatozoa is reduced in affected boars [12].

The causative mutation in ISTS-affected boars is a LINE-1 insertion within SPEF2 intron 30 [13], which alters the splicing pattern of SPEF2 and induces premature stop codons in the long variant 1. SPEF2 has been shown to be expressed mainly in tissues containing axonemal structures, and expression in the rat testis has been suggested to be phase-specific [14]. Our previous studies have indicated a differential expression pattern of SPEF2 in various pig tissues [10]. In humans, two different SPEF2 transcripts have been predicted, although only one has been identified in Northern blot analyses [14]. Because the ISTS defect seems to affect exclusively the development of spermatozoa [11], the mutation presumably only alters the splicing pattern of a testis-specific transcript, or the mutated transcript is only essential for spermatogenesis.

Several known functional domains with a possible function in sperm tail or its formation have been detected in the SPEF2 sequence [10]. The N-terminus contains a calponin homology domain, indicating potential actin-binding activity. Presence of a calcium-binding EF-hand motif indicates a possibility that SPEF2 activity could be regulated by calcium. Furthermore, a
domain of unknown function called DUF1042 in the N-terminal classifies SPEF2 together with other proteins implicated in flagella function, such as the human SPATA4 protein (spermatogenesis associate 4, NP_653245), the mouse sperm flagella protein SPEF1 (AY860964), and CPC1 (central pair complex 1, AAT40991) of the unicellular organism Chlamydomonas reinhardtii. In addition to the DUF1042-domain, CPC1 also contains an ADK domain and EF-hands, which suggests that SPEF2 and CPC1 serve similar functions. Mutations in CPC1 are known to disrupt assembly of the central pair microtubule-associated complex and to alter flagella beat frequency [15].

SPEF2 is clearly important for sperm tail organization, but the mechanisms of its function are unknown. To further elucidate the functions of SPEF2 in spermatogenesis, we have studied the expression and localization of SPEF2 in the testicular tissue of the mouse. SPEF2 was demonstrated to have a versatile expression pattern in seminiferous epithelium, and it appears to be localized in both germ cells and Sertoli cells. Strong SPEF2 protein localization was found in the manchette and in both the tail of elongating spermatids and the mature sperm tail. Interestingly, SPEF2 was shown to interact with the intraflagellar transport protein IFT20, and these two proteins colocalize in the Golgi complex and manchette of elongating spermatids. Current results support a crucial role of SPEF2 in sperm tail formation.

MATERIALS AND METHODS

Animal Material

Tissue samples from C57BL/6N Hsd mice (Mus musculus) were used for all studies in the mouse, and tissue samples for the pig (Sus scrofa) were collected from a local slaughterhouse. All mice were handled in accordance with the institutional animal care policies of the University of Turku, and the studies were approved by the Animal Ethics Committee.

Expression Analysis

Tissue samples from C57BL/6N Hsd mice were snap-frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted with RNeasy mini kit (Qiagen) and reverse transcribed with lmProm-ll Reverse Transcription System (Promega). Spef2 primers corresponding to exons 3–7 (forward, GAAAGTGGCTCTAAAGAGCATA; reverse, GTGAGCTGCTCCTGCGGATA), exons 37–43 (forward, ACGCACCATCCACCTGCAAC; reverse, GTGCATGCCTCCTCTTCTTTGTA), and exons 6–43 (forward, GACGGTGCC AAATGAGTAT; reverse, GTGCATGCCTCCTCTTCTTTGTA) were used for amplification of cDNA, and differences in transcript quantity were determined on 1% agarose gels with ethidium bromide staining. The exon content of fragments amplified with primers within exon 3 and exon 7 was confirmed by sequencing on a MegaBace 500 capillary DNA sequencer (Amersham Pharmacia) using DyEnamic ET Terminator kits with Thermo Sequenase ll DNA Polymerase (Amersham Pharmacia).

Antibody Generation

A polyclonal antibody was raised against the peptide CSQGERLPSRLLTDEKK corresponding to the C-terminal part of the region encoded by exon 43 of the mouse Spef2 (Medprobe). To verify the specificity of the partial C-terminal amino acid (aa) sequence of SPEF2, protein databases (Swiss-Prot and European Molecular Biology Laboratory [EMBL]) were searched. The specificity of serum to the peptide was confirmed by ELISA.

Immunoprecipitation and Western Blot Analysis

Tissue samples were homogenized in lysis buffer (50 mM Tris-HCl [pH 8.0], 170 mM NaCl, 5 mM ethylenediamine-tetra-acetic acid, 1 mM dithiothreitol, and protease inhibitors [Complete mini; Roche Diagnostics GmbH]) containing either 1% NP-40, 0.5% sodium deoxycholate, and 0.05% SDS (for Western blot analysis of adult tissues) or 1% Triton X-100 (for Western blot analysis of 7- to 50-day-old mouse testes and for immunoprecipitation [IP]) and then quantified using Bradford protein assay reagent [16]. For IP, the lysate was precleared with 10 µl of protein G-sepharose (GE Healthcare Bio-Sciences AB) for 30 min at 4°C. The precleared lysate was incubated with 0.5 µl of rabbit IgG (1 mg/ml; NeoMarkers) or 5 µl of SPEF2 antibody for 2 h at 4°C. Subsequently, 10 µl of protein-G sepharose were added, and mixtures were rotated for 1 h at 4°C. After three washes with lysis buffer, proteins were released in 30 µl of SDS sample buffer by boiling for 5 min at 95°C. IP samples were separated under denaturing conditions by 8% or 15% SDS-PAGE and electroblotted to polyvinyldene fluoride membrane. Non-specific sites were blocked with 5% nonfat dry milk in 0.3% Tween-20 in PBS for 1 h at room temperature, and the membrane was incubated overnight with anti-SPEF2 (1:500), anti-IFT20 (1:500), or anti-tubulin-α (1:1000; NeoMarkers) antibody. Antibody-antigen complexes were detected by incubation of the membranes with the anti-rabbit or anti-mouse secondary antibody (horseradish peroxidase-conjugated; Amersham) for 2 h at room temperature. The bound secondary antibodies were located with the ECL Plus Western blotting detection system (Amersham Pharmacia) according to the manufacturer’s instructions and exposed to a film thereafter.

Spermatozoa Preparations

Spermatozoa collected from caput, corpus, and cauda epididymis or ejaculates were washed three-times with PBS, centrifuged at 3000 × g for 5 min, and diluted in PBS for slide preparation. Slides were dried at room temperature and stored at −80°C.

Squash Preparations

Testes from an adult C57BL/6N Hsd mouse were dissected and decapsulated in PBS. After identification of the waves of the seminiferous epithelium by transfusion microscopy, stage-specific short tubule segments were cut [17]. Thereafter, the tubule segments were transferred with a pipette on microscope slides in 15 µl of PBS. A coverslip was placed carefully onto the tubule segment, and the excess fluid was removed by blotting, which allowed the cells to float out from the tubule. The exact stage was identified under phase-contrast microscopy, slides were snap-frozen in liquid nitrogen, and the cover slip was removed. Cells were fixed with 96% ethanol and air-dried at room temperature.

Drying-Down Preparations

Stage-specific segments of adult C57BL/6N Hsd mouse seminiferous tubules were isolated as described previously and then transferred in 20 µl of 100 mM sucrose solution in a small Petri dish. For pig drying-down preparations, wild-type and ISTS testis samples were collected and incubated with collagenase (1.5 mg/ml) at 37°C for 30 min in PBS, and short tubule segments were cut [17]. Cells were released from tubules by squeezing carefully with fine forceps and were then suspended by gentle up-and-down pipetting. The cell suspension was spread on a slide dipped in the fixing solution (1% paraformaldehyde [PFA], 0.15% Triton X-100 [pH 9.2]), and slides were dried overnight in a highly humified box.

Cryosections

Testes from adult C57BL/6N Hsd mice were collected embedded with Tissue-Tek OCT (Sakura Finetek) and frozen in liquid nitrogen. Thereafter, sections (thickness, 8 µm) were cut, dried at 40°C for 20 min, fixed with 4% PFA, and stored at −80°C.

Immunofluorescence

Sperm slides, cryosections, squash, and drying-down preparations were postfixed with 4% PFA for 10 min and permeabilized with 0.2% Triton X-100 for 2–5 min. Nonspecific sites were blocked by incubating slides in blocking solution containing 10% normal goat serum (NGS) and 3% bovine serum albumin or in 10% NGS for 2 h. The primary antibody incubation was carried out at 4°C in blocking solution or in 3% NGS with anti-SPEF2 polyclonal antibody (1:200), anti-AKAP82 monoclonal antibody (1:200, 611564; BD Biosciences), anti-IFT20 monoclonal antibody (1:300; a generous gift from Professor G. J. Pazour, University of Massachusetts Medical School), anti-glyceraldehyde-3-phosphate dehydrogenase, spermatogenic (GAPDH-S) polyclonal antibody (1:500; a generous gift from Professor G. Kamp, Molecular Physiology Section, Johannes Gutenberg-University), or anti-tubulin-α monoclonal antibody (1:150; NeoMarkers). Rabbit IgG was used at the same concentration as a negative control. Alexa Fluor 594 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-mouse IgG (1:500; Molecular Probes) were used as secondary antibodies. For detection of mitochondria, slides were incubated for...
were washed twice in PBS and once in water and then mounted with a Vectashield mounting medium before microscopic examination.

**Yeast Two-Hybrid Screen**

The open reading frame of IFT20 and a series of SPEF2 fragments—SPEF2-N (aa, 1–516), SPEF2-M (aa, 429–924), SPEF2-C (aa, 1324–1676), SPEF2-C2 (aa, 1462–1676), and SPEF2-C3 (aa, 1671–1823)—were obtained by PCR from human cerebellum (Stratagene) and were sequenced using BigDye Terminator v.3.1 Cycle Sequencing Kit (PE Applied Biosystems) and primers specific for pGAD10. The inserted sequences were identified by BLASTn searches against GenBank.

**RESULTS**

**Expression of Spef2 Gene Products in Mouse Tissues and During the First Wave of Spermatogenesis**

We analyzed expression of different regions of the murine Spef2 in various tissues using RT-PCR with primers homologous to specific exons encoded by the locus (exons 3–7, exons 37–43, and exons 6–43). Fragments were designed to represent known human SPEF2 transcripts (Fig. 1) or possible shorter transcript variants of 3' and 5' ends. All three PCR products spanning the regions between exons 3 and 7, exons 37 and 43, or exons 6 and 43 of Spef2 appeared to be tissue-specific and expressed in cilia-containing tissues (Fig. 2A). All studied exons were expressed in the testis, consistent with earlier studies showing that the porcine long SPEF2 variant is mainly expressed in the testis [10]. To support this observation, a fragment from exon 6 to exon 43 was demonstrated to be predominantly expressed in the murine testis, whereas the shorter form detected by the amplification from exon 37 to exon 43 was expressed in the lung, brain, kidney, and liver, but not in the heart. We also detected expression of the Spef2 variant covering the region from exon 3 to exon 7 in the lung, brain, kidney, and liver by using primers specific for exons 3 and 7 that were shorter than the testis fragment, and sequencing showed that the mRNA lacked exon 4 in these tissues (data not shown). Although exon 4 appeared to be predominantly expressed in the testis on the basis of our RT-PCR results, the BLAST search against National Center for Biotechnology Information database revealed expressed sequence tag sequences containing exon 4 from various tissues.

Spatiotemporal expression during spermatogenesis was detected for all examined Spef2 fragments. The mRNAs containing exons 37–43 were already weakly expressed at Postnatal Day 7. At this point, the first wave of spermatogenesis has already been initiated, and mitotically dividing spermatogonia are present together with somatic cells of the testis. More intense expression was detected at Postnatal Day 21, coinciding with the completion of meiosis and the appearance of round spermatids (Fig. 2A). A testis-specific fragment from exon 6 to exon 43 first appeared between Postnatal Days 14 and 21. Spef2 exons 3–7 were first detected in the testis collected at Postnatal Day 28, when the elongation of spermatids has just started, and the expression was significantly higher in the adult testis (Fig. 2A). The differences in the spatiotemporal expression patterns of these different regions of Spef2 mRNA may suggest that in addition to the long Spef2 transcript (containing exons 1–43), an mRNA variant lacking the 5' end (exons 6–43) and possibly shorter transcripts containing either the 5' or the 3' end (exons 3–7 or exons 37–43) are transcribed in the murine testis. Overall, the current data indicate that different transcript variants of Spef2 are present in a range of tissues, and in the testis, expressions are temporally regulated.
To localize Spef2 mRNA in the seminiferous epithelium, in situ hybridization was performed by using specific LNA probes designed to recognize either exon 7 or exon 43. Both probes gave similar expression patterns, suggesting that they both detected the testis-specific long Spef2 variant 1 and the possible variant containing exons 6–43. Spef2 mRNA was shown to be expressed predominantly in late spermatocytes and round spermatids in the mouse testis (Fig. 2, B–D). Male germ cell differentiation is organized in the seminiferous tubules, according to the cycle of the seminiferous epithelium that can be divided into 12 stages (I–XII) in the mouse, with each stage containing a defined collection of cell types [17] (Fig. 2D). Spef2 mRNA was first detected in the cytoplasm of stage VII pachytene spermatocytes with increasing intensity, reaching a peak in meiotically dividing spermatocytes at stage XII (Fig. 2B). The localization is consistent with the RT-PCR result demonstrating that expression of the transcript containing exons 6–43 is first visible in the testis collected at Postnatal Day 21 (stage VII pachytene spermatocytes appear between Postnatal Day 14 and Postnatal Day 21). Spef2 mRNA expression was also intense in step 1–7 spermatids but was absent beginning at step 9 (Fig. 2, B and C). No signal was detected with a scrambled probe, which was used as a negative control (data not shown).

Expression and Localization of SPEF2 Protein

A testis/sperm-specific, high-molecular-weight SPEF2 band was detected in immunoblotting with an antibody raised against a peptide corresponding to exon 43. This approximately 200-kDa band was detected in testis samples and at lower level in the epididymis (Fig. 3A). Additional lower-molecular-weight bands were also visible in the immunoblotting, and these might correspond to other possible SPEF2 isoforms, degradation products of the long SPEF2 isoforms, or even cross-reactive products. All bands were lost by neutralizing the antibody by peptide blocking (Fig. 3A). The testis-specific long isoform was first detected at Postnatal Day 21 (Fig. 3A), which correlates well with the mRNA expression results (Fig. 2A). Although we did not detect a clear expression on Postnatal Days 7 and 14, it is also possible that SPEF2 is weakly expressed throughout spermatogenesis. Antibody against α-tubulin was used to ensure equal protein content in each lane (Fig. 3A).

Localization of SPEF2 protein products was investigated by immunostaining experiments. In cryosections of the mouse testis, a weak, grainy, germ cell-specific signal of SPEF2 was detected in pachytene spermatocytes and round spermatids (Fig. 3B). SPEF2 protein was intensively visible in elongating spermatids and was detected in tails of step 15–16 spermatids (stages IV–VIII) (Fig. 3B). All SPEF2 staining patterns were lost by neutralizing the antibody by peptide blocking (Fig. 3B), and no staining was detected when rabbit IgG was used as a negative control (not shown).

Interestingly, SPEF2 immunoreactivity was also present in Sertoli cell cytoplasm and spermatid crypts when immunostaining was performed either on testis cryosections, staged squashes preparations (Fig. 4, A and B), or drying-down preparations (Fig. 4, C and D). Diffuse staining first appeared in Sertoli cell cytoplasm at stage VIII but was then concentrated to surround the heads of elongating spermatids in spermatid bundles (Fig. 4, A and B) until the bundles were dispersed at stage VI. The cytoplasmic SPEF2 staining in Sertoli cells was clearly visible in some of the drying-down cell preparations, in which the connection between long protrusions of Sertoli cell cytoplasms and elongating spermatid heads in the bundles remained intact (Fig. 4C). In situ hybridization results suggest that Spef2 mRNA is also expressed in stage VIII–XI Sertoli cells (Fig. 4E).

Localization of SPEF2 in the Developing Sperm Tail

To study the localization of SPEF2 in elongating spermatids in detail, immunostaining on stage-specific drying-down preparations was performed. SPEF2 protein was first observed in the manchette of step 10–12 elongating spermatids (Fig. 5A). In step 13–14 spermatids, SPEF2 localized in the basal body and neck region of elongating spermatids, and finally, at steps 15–16, a signal was detected in the midpiece of the sperm tail (Fig. 5A). Localization of SPEF2 in the sperm tail midpiece was confirmed by costaining with Mitotracker, which visualizes the mitochondrial sheath in the midpiece. SPEF2
accumulation in the sperm tail midpiece correlated with the mitochondrial sheath formation.

SPEF2 protein localization changed during epididymal passage of spermatozoa. In caput sperm, the staining pattern of SPEF2 was essentially the same as that of step 16 spermatids in the testis, showing the presence of SPEF2 over the whole length of the midpiece of the sperm tail (Fig. 5B). However, in the caput and more clearly in the corpus sperm, the staining pattern was already seen to have begun to scatter and concentrate to the distal part of the midpiece. Finally, in the cauda epididymis and vas deferens, the staining was observed only in the distal part of the midpiece (Fig. 5B). Mitotracker was used as a marker for the mitochondrial sheath.

Sperm Tail Malformations in Boars with ISTS Defect

The sperm tails of ISTS pigs were analyzed in greater detail to better understand the involvement of SPEF2 in sperm tail development. The fibrous sheath of the wild-type and ISTS-affected ejaculated sperm tails was visualized with the GAPDH-S antibody [22]. GAPDH-S staining was detected in the fibrous sheath and acrosome in normal boar spermatozoa (Fig. 6A). In ISTS sperm, the GAPDH-S localization in the acrosome was comparable to that in unaffected control.
samples. However, in the sperm tail, most of the staining was detected only in the proximal droplet (Fig. 6A). In a few ISTS sperm tails, GAPDH-S was also located along the sperm tail, but the staining was irregular and strands were found to radiate from the flagella. Based on two-dimensional electrophoresis and matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) analysis, GAPDH-S was substantially reduced in ISTS sperm (data not shown).

The presence of mitochondria in ISTS ejaculated sperm was demonstrated with Mitotracker and examined more closely using electron microscopy. In most cases, the mitochondrial sheath was present but disorganized (Fig. 6, A and B). In ISTS sperm tails, mitochondria were unevenly distributed in the midpiece, and the axonemal structure was disrupted (Fig. 6B). The α-tubulin staining showed that microtubules are present along the ISTS tail, but the tails are short and often curled (Fig. 6A). Most of the sperm tails contain only the midpiece, but some longer tails are also present. In some cases, the flagellar structure assembly is totally depleted (Fig. 6B).

A staining pattern similar to that seen in ejaculated sperm was also detected in testicular sperm. In germ cells isolated from the seminiferous tubules of ISTS boars, most of the GAPDH-S staining was detected only in the proximal droplet (not shown) and infrequently in short fragments of the tail of the elongating spermatids (Fig. 6C). The α-tubulin staining showed that microtubules are present along the ISTS tail, but the tails are short and often curled (Fig. 6A). Most of the sperm tails contain only the midpiece, but some longer tails are also present. In some cases, the flagellar structure assembly is totally depleted (Fig. 6B).

Next, we sought to elucidate the functions of SPEF2 by identifying proteins that interact with SPEF2 using the yeast two-hybrid system. Three different fragments of human SPEF2 were screened against a human brain cDNA library, which revealed an interaction between the C-terminal part of SPEF2 (SPEF2-C) and IFT20 (Fig. 7A). The interaction was confirmed using the porcine counterparts of SPEF2 and IFT20 (data not shown). Thereafter, deletion constructs of SPEF2-C were tested against IFT20 to further characterize the interaction. The data showed that only SPEF2-C and SPEF2-C1 were capable of interacting with IFT20 (Fig. 7A), indicating that the fragment consisting of aa 1324–1676 was sufficient to allow interaction between SPEF2 and IFT20. Furthermore, both of these constructs contained aa 1324–1462, highlighting the importance of this region in facilitating the protein-protein interaction.

The interaction between SPEF2 and IFT20 was validated by coimmunoprecipitation (co-IP) experiments from mouse testis extracts. After IP with SPEF2 antibody, the 15-kDa IFT20 band was clearly detected by immunoblotting with the antibody against IFT20 (Fig. 7B). The data showed that only SPEF2-C and SPEF2-C1 were capable of interacting with IFT20 (Fig. 7A), indicating that the fragment consisting of aa 1324–1676 was sufficient to allow interaction between SPEF2 and IFT20. Furthermore, both of these constructs contained aa 1324–1462, highlighting the importance of this region in facilitating the protein-protein interaction.

To support the protein-protein interaction data, the localization of IFT20 protein in the testis was investigated. It has been shown previously that IFT20 protein is present in the Golgi complex of ciliated cell lines [23]. This Golgi localization was also detected in mouse late spermatocytes and in round spermatids (Fig. 7D). During the spermatid elongation process, just like SPEF2, IFT20 was also present in the manchette of step 10–12 spermatids. Around step 14,
IFT20 was only detected in the basal body of the sperm tail and was depleted thereafter (Fig. 7C). SPEF2 seems to have a granulated localization pattern in the cytoplasm of late spermatocytes and round spermatids (Fig. 3B). Therefore, detailed investigation of the SPEF2 protein localization in stage-specific drying-down preparations was performed. It was shown that at least in part, the granulated staining is associated with the Golgi complex of late spermatocytes and round spermatids and was comparable with the IFT20 localizations (Fig. 7D). Thus, IFT20 and SPEF2 colocalize in the manchette of elongating spermatids and are both associated with the Golgi complex of late spermatocytes and round spermatids, supporting a possible functional interaction of these two proteins.

**DISCUSSION**

Truncation of the long SPEF2 isoform 1 in pigs causes the ISTS defect [10, 13], which clearly demonstrates the essential role of this testis-specific SPEF2 variant in sperm tail development. In some cases of ISTS-affected boars, development of the flagella is completely inhibited, whereas most of the ISTS sperm tails contain the midpiece part of the flagella. In all cases, however, the mitochondria and outer dense fibers are disorganized and at least the central pair of the axoneme is missing. In most cases, the short sperm tail lacks the fibrous sheath, and when present, the fibrous sheath fibers are disorganized. Both axonemal and accessory structures of the ISTS tail appear to be affected, but it is not yet clear whether all these defects are primarily a result of the lack of functional SPEF2 or if some of the defects are secondary disruptive changes (e.g., during the epididymal transit of sperm). Because most of the sperm analyses have been done with ejaculated sperm, it is also unclear at which step of sperm differentiation or maturation all these ISTS defects are generated. It is clear, however, that the ISTS phenotype originates already during spermatogenesis, because the short tail characteristics are observed in testicular sperm [12] (Fig. 6C) and in the caput epididymis (data not shown) before the epididymal transit. Our results reveal the dynamic localization pattern of SPEF2 during mouse spermatogenesis, which further supports the idea that the long form of SPEF2 participates in the initial organization of the tail structures.

Based on the current results, SPEF2 staining appears in the mouse sperm tail at a very late stage during spermiogenesis. The SPEF2 protein localizes in step 10–12 elongating spermatids in the manchette, then in the basal body at steps 13–14 and finally, in the midpiece of the sperm tail at steps 14–16. Sperm tail construction starts with the axoneme formation in round spermatids, and the accessory structures of the tail appear only later in elongating spermatids. The longitudinal columns of the fibrous sheath first appear during step 9 [24], and the ribs appear between steps 11 and 15 [25]. The midpiece of sperm tail originates when the annulus migrates distally to the proximal end of fibrous sheath (steps 14–15). Formation of the mitochondrial helical sheath starts after the migration of the annulus at approximately step 15 [26]. During these events,
SPEF2 localizes first in the manchette, when the fibrous sheath is formed, and thereafter into the midpiece, simultaneously with the mitochondrial sheath formation.

As demonstrated by the phenotype of ISTS boars, not only the organization of accessory structures but also the axonemal structure are affected by the mutation of SPEF2 [10]. Defining the exact step when the axonemal defects appear requires further studies. Previous studies have shown that SPEF2 is involved in the development of cilia [14], which supports a possible role of SPEF2 in axoneme formation. Based on the present study, the C-terminal part of SPEF2 is not a structural axonemal protein, because it localized in the accessory structures of the sperm tail and no axonemal staining was detected. The axonemal defects in ISTS sperm thus are probably generated by an indirect mechanism. It has been shown that defects of sperm accessory structure affect the axonemal structure, but the exact mechanism is not known [27].

We report here that SPEF2 interacts with the IFT20 protein both in the yeast two-hybrid assay and in co-IP from testis extracts. IFT20 has been shown previously to be involved in the intraflagellar transport system to traffic proteins into the cilium. IFT20 is also localized in the Golgi complex in ciliated cell lines. It has been shown to be anchored to the Golgi complex by the golgin GMAP210 (TRIP11), and mice defective in GMAP210 have ciliary defects [23, 28]. IFT20 together with GMAP210 are proposed to function in marking the vesicles that are destined to relocate to ciliary membrane and, thus, to play a role in delivery of ciliary proteins from the Golgi complex to the cilium [23, 28]. We show that SPEF2 colocalizes with IFT20 in the Golgi complex of differentiating male germ cells. Therefore, it can be speculated that SPEF2 together with IFT20 may function in an analogous way in male germ cells by delivering tail proteins from the Golgi complex to the elongating sperm tail. However, SPEF2 may also have a role in Golgi complex, which is totally unrelated to cilia or flagella formation.

Manchette is a male germ cell-specific microtubular platform that has an important role in shaping the head structure of the elongating sperm. Microtubules of manchette are involved in the transport of molecules via an intramanchette transport mechanism that shares similar molecular components and features with the intraflagellar transport mechanism [29]. Biochemical and structural evidence also suggests an intramanchette transport mechanism for the delivery of molecules to the centrosome and developing spermatid tail [29–33], but direct evidence for intramanchette transport of specific cargos remains to be experimentally shown. The colocalization of SPEF2 and its interaction partner, IFT20, in the manchette and basal body region of elongating spermatids raises an interesting possibility that SPEF2 could mediate sperm tail structuring via functions in the intramanchette transport/intraflagellar transport mechanism.

Interestingly, the SPEF2 antibody also detected a stage-specific protein product in the Sertoli cell cytoplasm. In the mouse, the staining of SPEF2 in the Sertoli cell cytoplasm seems to be concentrated around the elongating spermatid heads at the stages just before spermatid bundle formation and stages with existing spermatid bundles. This points to a role for SPEF2 in bundle formation, possibly via Sertoli cell-spermatid junctions called ectoplasmic specialization. SPEF2 is known to be expressed in parallel with dynein [14], and the N-terminal calponin homology domain (Fig. 1, CH-domain) suggests that it may interact with actin, which is involved in ectoplasmic specializations, crypt formation, and movement. Apical ectoplasmic specialization is only found in the testis and is involved in the translocation of spermatids during elongation and in spermiation [6]. The organization of germ cells in seminiferous tubules of ISTS boars appeared to be disrupted,
suggesting that one possible mechanism for SPEF2 action could be related to connections between Sertoli cells and germ cells. However, SPEF2 localization in Sertoli cells (and possible functions thereof) requires further investigation.

A Western blot of testis extract by the polyclonal SPEF2 antibody used in the present study resulted in not one but several bands that probably represent different isoforms of SPEF2. These bands disappeared when the antibody was

FIG. 7. Interaction studies of SPEF2 with IFT20. A) Yeast two-hybrid interaction assay with SPEF2. A schematic presentation of the fragments (SPEF2-N, SPEF2-M, and SPEF2-C) used for the initial brain cDNA library screen and deletion constructs used to map the interaction site between SPEF2-C and IFT20 is shown. The length of the fragments is shown in amino acids (aa). Interaction between SPEF2-C and IFT20 was detected in the initial library screen. The important site for interaction was further defined by deletion constructs to the SPEF2 region containing aa 1324–1462. Yeast cells were selected on synthetic complete triple dropout (-Trp, Leu, Ade). Each row on the plates contains five spots representing the same yeast cells in different dilutions to give an indication of the strength of the putative interaction. The constructs are outlined on the left. B) Western blot with IFT20 of the mouse testis samples immunoprecipitated with anti-SPEF2 or a negative control rabbit IgG and input samples. IFT20 protein was detected in input samples and samples precipitated with anti-SPEF2 but not with rabbit IgG. The IgG light-chain protein band was used as a loading control. C) IFT20 is located in the manchette of step 9–12 spermatids. The manchette localization is depleted after step 12 and detected in the basal body at steps 13–14. No staining was detected in step 15–16 spermatids. The nucleus is shown by blue DAPI staining. D) SPEF2 and IFT20 protein localizations in the association with the Golgi complex of round spermatids and late spermatocytes. SPEF2 and IFT20 (red fluorescence) are found concentrated in cytoplasmic areas that are associated with the Golgi-marker HPA (green fluorescence). SPEF2 (red) is retained in the sperm tail midpiece; however, it concentrates to the distal region during epididymal maturation. Bar = 10 μm.
neutralized by the peptide that was used as an antigen in immunization, suggesting that these are SPEF2-specific bands. Some of them may also be degradation products of the longer isoforms. Because of the possibility of unspecific staining, we used various methods to confirm the data presented. In addition, the localization of Spef2 mRNA as detected by in situ hybridization experiments supports the observed localization of SPEF2 protein.

The functional studies of SPEF2 are hampered because of the differential expression pattern of Spef2 in mammalian tissues. Previously, we have demonstrated distinct expression patterns for different exons of SPEF2 in porcine tissues [10]. In the current study, our RT-PCR results using primer sets amplifying three different regions of Spef2 indicate the presence of various SPEF2 isoforms in murine tissues. Some variants appear to be tissue-specific, and there seem to be differences in the expression pattern of SPEF2 between species. The variation in gene products complicates the interpretation of results and an overall understanding about the function of SPEF2. However, SPEF2 is highly conserved across species; therefore, it is reasonable to assume common functions of protein products in mammalian spermatogenesis, even though some differences may exist.

The SPEF2 isoform that most likely is affected in ISTS boars is the testis/sperm-specific long variant 1. In the mouse, the long form of SPEF2 has a dynamic expression pattern in the testis during the differentiation of spermatozoa, implying that SPEF2 controls the initial formation of sperm tail rather than being a structural component of the tail. This hypothesis is further supported by the finding that SPEF2 is not a stable component of the sperm tail; rather, its localization in the midpiece region of the tail is further condensed in the distal part of the midpiece during the epididymal transit. The mechanisms of SPEF2 action remain to be characterized. Our results demonstrating expression and localization of SPEF2 during murine spermatogenesis pave the way to functional studies of SPEF2 in mammalian testis. The interaction with the intraflagellar transport protein IFT20 provides novel insights regarding the possible functions for SPEF2 in sperm tail development through the delivery of structural or functional components to the sperm tail and organization of the developing tail structures.

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