KIF1-binding protein interacts with KIF3A in haploid male germ cells

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Abstract

Male fertility relies on the production of functional spermatozoa. Spermatogenesis is a complex differentiation process that is characterized by meiosis and dramatic morphogenesis of haploid cells. Spermatogenesis involves active changes in the microtubular network to support meiotic divisions, cell polarization, the reshaping of the nucleus, and the formation of a flagellum. Previously, we have demonstrated that a microtubule-based anterograde transport motor protein KIF3A is required for the sperm tail formation and nuclear shaping during spermatogenesis. In this study, we show that KIF3A interacts with a KIF1-binding protein (KBP) in the mouse testis. We have characterized the expression and localization pattern of KBP during spermatogenesis and localized both KIF3A and KBP in the cytoplasm of round spermatids and manchette of elongating spermatids. Interestingly, KBP localized also in the late chromatoid body (CB) of elongating spermatids, whose function involves intracellular movement and association with the microtubular network. Altogether our results suggest a role for KBP in spermatid elongation and in the function of the late CB.

Introduction

Spermatogenesis is a complex process that begins with mitotic proliferation of spermatogonia, after which spermatocytes undergo meiotic divisions to give rise to haploid round spermatids (Ahmed & de Rooij 2009, Hess & Franca 2009). Thereafter, round spermatids begin dramatic morphogenesis during which the sperm head is elongated and reshaped, chromatin is tightly compacted, and the acrosome and sperm tail are formed. The differentiation of spermatids, spermiogenesis, can be divided into 16 steps in the mouse, including eight steps (1–8) for round spermatid differentiation and the remaining eight steps (9–16) for spermatid elongation. The formation of the axoneme begins already in early spermiogenesis when the basal body migrates into the cell periphery and the axoneme begins to elongate toward extracellular space. Basal body-axoneme complex invaginates and anchors itself to an implantation fossa at the nuclear envelope opposite to the acrosome. At step 8, the axoneme has been formed and the remaining spermiogenesis is spent to construct the accessory structures of the flagellum, at step 9 fibrous sheath structures appear, and at step 12 outer dense fibers start to compose. Finally, during steps 13–14 the mitochondrial layer is constructed behind the migrating annulus (Turner 2003). At the time when the spermatid elongation begins during steps 8–9, a microtubular structure called manchette appears to surround the condensing head. It has an important role in the head shaping and delivery of particles to the developing head and tail (Kierszenbaum & Tres 2004, Hermo et al 2010). The manchette is dismantled after spermiogenic step 12.

We have previously shown that KIF3A, a motor protein in a heterotrimeric Kinesin II protein complex, is required for normal spermatogenesis and male fertility (Lehti et al 2013). Depletion of KIF3A resulted in a malformed head shape due to problems in the manchette organization and defects in sperm tail formation (Lehti et al 2013). In somatic cells, KIF3A has an important role in intraflagellar transport during cilia formation and function (Scholey 2008).

Mass spectrometric analysis of the anti-KIF3A immunoprecipitate from a mouse testis revealed several potential interaction partners, including KIF1-binding protein/KIAA1279 (KBP) (Lehti et al 2013). KBP was initially identified as an interaction partner for KIF1Bα, which is a motor protein for the intracellular transport of mitochondria. It was reported to colocalize with the mitochondria in NIH3T3 cells and to affect mitochondrial distribution by binding to the carboxyl-terminal region of the motor domain of KIF1B and improving its motility (Wozniak et al 2005). However, mitochondrial localization was not observed in human fibroblasts (Alves et al 2010, Drevillon et al 2013) or Hela cells (Alves et al 2010), therefore leaving the mitochondrial role of KBP to be confirmed. Mutations in
the Kbp gene have been shown to cause defects in the enteric and central nervous system and to cause the Goldberg–Shprintzen syndrome (GOSH) in humans (Brooks et al. 2005). Zebrafish kbp was shown to be required for axonal outgrowth in the central and peripheral nervous system. In the kbp mutant fish, axonal microtubules were disrupted indicating neuronal cytoskeletal defects as a cause for GOSH (Lyons et al. 2008). In addition to KIF1B, KBP binds other proteins associated with microtubules, including several kinesin motor proteins and the microtubule destabilizing protein SCG10 (Alves et al. 2010, Lehti et al. 2013).

All of the evidence supports the important role of KBP in kinesin-mediated microtubular transport and microtubule organization, the processes that are also of critical importance for the normal development of spermatozoa. However, the function of KPB during spermatogenesis has not been investigated. In this study, we elucidated the potential role of KPB in spermatogenesis by characterizing its expression and localization pattern and its interaction with KIF3A in the testis.

Materials and methods

Generation of germ-cell specific Kif3a knockout mice

Kif3a fl/fl mice were bought from the Mutant Mouse Resource and Research Centers (MMRRC) (The Jackson Laboratory, Bar Harbor, ME, USA) and the mice expressing transgenic Cre under the Ngn3 promoter were kindly provided by Dr P L Herrera (University of Geneva, Switzerland.) The Cre+ Kif3a fl/fl mice were produced as described previously (Lehti et al. 2013). Cre expression and the floxed Kif3a allele were confirmed by PCR as described previously (Korhonen et al. 2011, Lehti et al. 2013). Animal experiments were approved by the Finnish ethical committee, and mice were maintained in a specific pathogen-free stage at the Central Animal Laboratory of the University of Turku.

RT-qPCR

For analysis of gene expression with qPCR RNA of the testes from WT mice was extracted (RNeasy Midi kit, Qiagen) and total RNA was reverse transcribed with random primers and an RT-PCR kit (Invitrogen™ Reverse Transcription System, Promega) according to the manufacturer’s instructions. Produced cDNA was amplified by using gene specific primers (Kbp_001 forward AGGTGAGACCGTTTACAT, Kbp_001 reverse AAAAACT-CCGTGCTTCTTC and Kbp_004 forward ATGGCCAGAGCA-CACTGAAA, Kbp_004 reverse CATATACGCCCCCAAACC). The housekeeping gene Rpl13a (forward AGGGG-CAGGTCTGTGATTG, reverse CCGAAACACTTGGAGAG-CAG) was used as a reference gene to calculate the relative expression. cDNA samples were diluted to 20 ng/µl prior to use. The qPCR was performed with a Viia7 Real-Time PCR System in 96-well microtiter plates using Absolute qPCR SYBR Green ROX Mix (VWR, Radnor, PA, USA). Amplification by qPCR contained 12.5 µl of Absolute qPCR SYBR Green Mix, 100 ng of cDNA, and 70 nM of each primer in a final volume of 25 µl. Amplifications were initiated with 15 min enzyme activation at 95 °C followed by 40 cycles of denaturation at 95 °C for 15 s, primer annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. All samples were amplified in triplicate, and the mean value was used for further calculations. Raw data were analyzed with the sequence detection software (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) and relative quantitation was performed with GenEx Software (MultiD, Göteborg, Sweden). Ratios between the target and reference gene were calculated by using the mean of these measurements. A standard curve for each primer pair was produced by serially diluting a control cDNA and used to correct the differences in amplification. A melting curve analysis was performed allowing single product-specific melting temperatures to be determined. No primer–dimer formations were generated during the application of 40 real-time PCR amplification cycles.

Co-immunoprecipitation and western blotting

Testis samples of adult mice were collected and homogenized in lysis buffer (50 mM Tris-HCl pH 8.0, 170 mM NaCl, 1% NP40, 5 mM EDTA, 1 mM DTT, and protease inhibitors; Complete mini, Roche Diagnostic) using Ultra Turrax. Tissue lysates were centrifuged at 14 000 g for 20 min at +4 °C. Protein G Dynabeads (Life Technologies) were coupled with anti-KBP (5 µg, H00026128-B01P, Abnova, Taipei City, Taiwan) and anti-mouse IgG (5 µg, Sigma–Aldrich), and immunoprecipitation (IP) was performed according to the manufacturer’s protocol. Precipitated proteins were separated in 4–20% gradient gel (Bio-Rad) and blotted to a Hybond membrane. Membranes were blocked with 5% nonfat milk in 0.1% PBS-Triton X-100 at RT for 1 h and incubated with anti-KIF3A (1:4000, PAI-20240, Thermo Fisher Scientific, 1:4000,) anti-ptKBP (1:300, 25653-1-AP, Proteintech, Chicago, IL, USA) or anti-KPB (1:300) antibody at +4 °C overnight. Specific bands were detected using HRP-conjugated anti-rabbit/mouse IgG (1:4000, GEHENA934, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) as a secondary antibody, and the specific signal was developed using ECL Plus western blotting detection system (Amersham Pharmacia, Piscataway, NJ, USA) and imaged using LAS-4000 (Fujifilm, Tokyo, Japan).

Immunofluorescent staining

Drying down preparations

Kif3a knockout (KO) and WT mice testes were dissected and decapsulated in PBS and drying-down preparations were prepared as described previously (Kotaja et al. 2004). For immunofluorescence staining, slides were post-fixed in 4% paraformaldehyde (PFA) for 5 min and washed in PBS. Autofluorescence was quenched with 100 mM ammonium chloride for 2 min, and slides were subsequently washed in PBS. Preparations were treated with 0.2% Triton X-100 for 2 min. After PBS washes, the slides were blocked with 10% normal goat/donkey serum and 3% BSA in PBS with 0.05% Triton X-100 at RT for 1 h. Primary antibodies (anti-KIF3A 1:1000, anti-TSKS 1:500 (kind gift from Prof. J Anton Grootegoed (Shang et al. 2015)) via free access
2010); anti-KBP 1:100, anti-ptKBP (1:200) anti-α-tubulin (1:1000, MS-581-P0, Thermo Scientific); negative control antibodies anti-mouse IgG (1:100, NC-748-P, NeoMarkers, Fremont, CA, USA); and anti-rabbit IgG (1:200, NC-100-P1, NeoMarkers) were diluted in 3% normal goat/donkey serum and 1% BSA in PBS with 0.05% Triton X-100 and incubated at +4°C overnight. The slides were washed in 0.1% PBS-Triton X-100 and secondary antibodies were diluted in 1:500 (AlexaFluor goat/donkey anti-rabbit/mouse 594/488, Molecular Probes, Eugene, OR, USA) and incubated at RT for 1 h. Nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI, 1:5000, D9542, Sigma–Aldrich) at RT for 5 min. The slides were mounted using Mowiol 4-88 (Polysciences, Inc., Eppelheim, Germany).

Cryosections

The mouse testis was dissected and embedded directly to Tissue-Tek O.C.T. Compound (Sakura Finetek Europe B.V., Alphen aan den Rijn, Netherlands) for freezing or fixed with 4% PFA at RT for 4 h, washed with PBS and incubated in 20% sucrose at +4°C overnight. The testis was embedded in Tissue-Tek O.C.T. Compound and frozen in dry ice-chilled isopentane. Testis sections were cut and stored at −80°C. Sections were fixed for 5 min in 4% PFA, washed with 0.1% PBS-Triton X-100, and permeabilized in 0.2% PBS-Triton X-100 for 3 min. After washes, the sections were blocked in 10% BSA in PBS and primary antibodies were diluted in 3% BSA in PBS (anti-KBP 1:100, anti-TSKS 1:500, anti-KIF3A 1:2000, anti-mouse IgG 1:100) and incubated at +4°C overnight. Secondary antibodies were used as described before and slides were mounted with Mowiol 4–88.

**Results**

**Two isoforms of Kbp are expressed in the mouse testis**

*Kbp* contains seven exons and its full length transcript encodes a protein product of 621 amino acids.

![Image](https://www.reproduction-online.org)
According to the Ensembl database, Kbp has eight transcripts of which four are protein coding containing two tetratricopeptide domains located near the 5’ end of the Kbp (Brooks et al. 2005, Wozniak et al. 2005). Previously we have shown the differential expression of Kbp during the first wave of spermatogenesis based on RNASeq data (Lehti et al. 2013). To confirm and investigate the expression of different splicing variants of Kbp in the testis, we analysed the gene expression using RT-qPCR from testis samples of postnatal days (P) 7, 14, 17, 21, and 28. During the first wave of spermatogenesis, spermatogenic cells appear in the seminiferous tubules in a temporal manner. The newborn mouse testis contains only Sertoli cells and gonocytes. At P7, spermatogonia are also present in the tubules. At P14 and P17, early and late pachytene spermatocytes appear, respectively. Secondary spermatocytes and round spermatids appear after P20 (Bellve et al. 1977) and elongated spermatids are present at P28. A complete set of spermatogenic cells is present in the seminiferous tubules at P35 (McKinney & Desjardins 1973). We were able to detect the expression of two isoforms of Kbp (Kbp_001 and Kbp_004, primer positions are shown in Fig. 1A). The longest isoform, Kbp_001, showed a dramatic increase in expression during spermatid elongation (P28, Fig. 1B). Kbp_001 expression was also present in the lung, heart, and liver and at a very low level in the kidney (Fig. 1C). The Kbp_004 isoform appears to be testis specific in analyzed tissue samples (Fig. 1C). During the first wave of spermatogenesis, the overall expression of Kbp_004 was low with a slight increase at P21 (Fig. 1B).

At the protein level, the expression was investigated using two different KBP-specific antibodies. The immunogen for the first KBP antibody (referred to as ptKBP) contains amino acids 272–621 and the immunogen for the second KBP antibody (referred to as KBP) was the full length KBP protein. Both KBP antibodies recognized the full-length form of KBP (Fig. 1D). In addition, the antibodies identified two shorter bands, which may represent the KBP_002 and KBP_004 isoforms (42 and 45 kDa respectively). However, we were not able to detect specific RNA expression for KBP_002 and the expression for KBP_004 was consistent throughout the first wave of spermatogenesis. The full-length isoform of KFP first appeared at P21 consistent with RNA expression (Fig. 1B and E). A high expression of the KBP_001 at P42 and in the adult testis indicates a role for KFP during late steps of spermiogenesis (Fig. 1D and E).

**KBP is expressed in haploid spermatids**

To study the overall localization of KFP during spermatogenesis, we performed immunofluorescence staining of WT mouse testis cryosections. Consistently with the increase in the RNA expression of Kbp_001 during late spermatogenesis, the strongest KFP signal was found in haploid spermatids (Fig. 1F). The KFP signal was detected already in round spermatids cytoplasm, further increased at stage IX in step 9 early elongating spermatids, and was most prominent during stages XII–V in steps 12–15 elongating spermatids. In elongating spermatids, the KFP signal was concentrated in a cytoplasmic granule near the basal body and developing tail. During stages VII–VIII, prior to release of mature sperm in the tubule lumen, KFP expression in elongated spermatids decreased dramatically (Fig. 1F).

The identified KFP localization in elongating spermatids was studied in more detail using drying-down preparations and confirmed by both KFP-specific antibodies (Fig. 2). Both antibodies detected the specific dot-like staining near the developing tail (Fig. 2), and similar staining was also seen in cryosections (Supplementary Figure 1, see section on supplementary data given at the end of this article). Both antibodies labeled also the manchette. In addition, the ptKBP antibody gave a strong signal in the acrosomal region (Fig. 2).

**KFP localizes in the late chromatoid body in elongating spermatids**

On the basis of its location and appearance, the KFP positive granule in elongating spermatids resembles the late chromatoid body (CB), which is an electron dense...
structure that has been reported to be a functionally transformed form of the CB found in the cytoplasm of round spermatids (Shang et al. 2010). In step 9 spermatids, all of the proteins characteristic for the round spermatid’s CB disappear and a testis-specific serine/threonine kinase substrate TSKS, in turn, accumulates in the late CB. At the same time, the late CB divides to form two distinct structures: a ring-like structure that stays around the axoneme and a so-called satellite body, both of which are positive for TSKS. The ring first locates close to the centriole and moves down the tail behind the annulus during mid piece formation. The satellite is moving freely in the cytoplasm and is removed with the residual body just before spermiation (Shang et al. 2010). To characterize in more detail the KBP specific dot-like staining, we performed double staining with a late CB marker TSKS. Colocalization of the KBP and TSKS in elongating spermatids was detected in frozen testis sections (Fig. 3A). A more detailed analysis of KBP expression in drying-down preparations revealed that the KBP-specific dot was partially colocalized with TSKS beginning from steps 8 to 9 during the spermatid elongation. The KBP and TSKS signals were overlapping completely from the beginning of step 10, and the KBP specific signal appeared to be mainly present in the satellite body. Phase contrast microscopy further confirmed that the KBP localize in the late CB (Fig. 3B).

**KBP interacts with KIF3A**

We have previously identified KBP as a possible interacting candidate for KIF3A by mass spectrometric

![Figure 3](https://www.reproduction-online.org)

**Figure 3** KBP localizes in the late CB. (A) On mouse testis cryosections, KBP colocalized in the late CBs of elongating spermatids with a specific late CB marker: testis-specific serine/threonine kinase substrate (TSKS). ES, elongating spermatids. (B) Immunostaining of drying-down preparations with anti-KBP and anti-TSKS antibodies confirmed the colocalization, which mainly appeared to take place in the satellite body (white arrow). Scale bar = 10 μm.

![Figure 4](https://www.reproduction-online.org)

**Figure 4** KBP interacts with KIF3A in the mouse testis. (A) KIF3A was co-immunoprecipitated from the mouse testis lysate with anti-KBP antibody. A specific KIF3A band was also visible in the total testis lysate (input) and in the supernatant after immunoprecipitation (SN). Immunoprecipitation from the Kif3a KO testis lysate (KO) was used as a negative control. IgG shows the equal amount of IgG heavy chain in the immunoprecipitations from both WT and KO lysates. (B) KBP and KIF3A colocalized in the cytoplasm of late round spermatids in the mouse testis cryosections. (C) KBP colocalized with KIF3A in the manchette of elongating spermatids. (D) ptKBP colocalization with z-tubulin in the manchette. Scale bar = 10 μm.
Kif3a granules were identified in steps 11–14 spermatids of round spermatids in cryosections (Fig. 4B). Furthermore, a colocalization of KIF3A and KBP in the cytoplasm of and manchette (Lehti et al. 2013). We were able to show a colocalization of KIF3A and KBP in the cytoplasm of round spermatids in cryosections (Fig. 4B). Furthermore, immunofluorescence staining of WT mouse drying-down preparations revealed colocalization in the manchette (Fig. 4C). KBP localization in the manchette appeared fragmented (Fig. 5). Several TSKS-specific granules were identified in steps 11–14 spermatids of Kif3a KO mice and most of these were also positive for KBP.

Discussion

In this study, we investigated the expression and localization of KBP during mouse spermatogenesis for the first time. We found that KBP was specifically expressed during the late steps of spermatogenesis, the expression being first detected in round spermatids. The most prominent KBP staining was identified in the late CB during steps 10–12 at the time of spermatid elongation and manchette-assisted nuclear reshaping. The CB is an electron-dense large cytoplasmic ribonucleoprotein granule that is mostly characterized in round spermatids, where it accumulated PIWI-interacting RNAs (piRNAs) and piRNA pathway proteins, and is involved in haploid RNA regulation (Kotaja & Sassone-Corsi 2007). When spermatid elongation begins at steps 8–9, CB loses its characteristic proteins, e.g., MIWI/PWIL1 and MVH/DDX4 (Fawcett et al. 1970, Parvinen 2005, Grivna et al. 2006, Kotaja & Sassone-Corsi 2007). At the same time, the CB is functionally transformed (Shang et al. 2010), yet the specific function of the late CB has remained unclear. It has been suggested that after CB transition to the ring and satellite body, it might function in mitochondria assembly during flagellogenesis (Chuma et al. 2009, Shang et al. 2010). Our study identifies KBP as a novel marker for the late CB. Contradictory results have also been published about the direct interaction of KBP with the cytoskeleton (Alves et al. 2010, Drevillon et al. 2013). Drevillon et al. reports KBP interaction with both tubulin and F-actin, hypothesizing KBP contribution in cross-linking microtubules and F-actin cytoskeleton, but the interaction with tubulin was not detected by Alves et al. Whether the interaction with microtubules is direct or mediated by other proteins, KBP appears to have a central role in the microtubule-mediated functions and microtubule dynamics. The CB is a dynamic structure that is actively moving, especially in the cytoplasm of round spermatids in a microtubule-dependent manner (Parvinen et al. 1997, Ventela et al. 2003). When the centrioles and the base of the flagellum move inward and become attached to the nuclear envelope, the CB reaches this area and encircles the distal centriole in close association with the annulus (Fawcett et al. 1970). The CB ring remains associated with the annulus during the migration along the sperm tail to the junction of the mid and principal piece. All of these processes involve the communication with microtubules, and it is tempting to speculate that KBP would function to regulate the association of the late CB with the microtubular network. However, further studies are needed to clarify the specific role of the KBP during late spermiogenesis and in relation to the late CB.

The interaction between KBP and kinesins has been demonstrated consistently in several publications, which strongly suggest that KBP cooperates with kinesins in its cellular functions (Wozniak et al. 2005, Alves et al. 2010, Lehti et al. 2013). In line with these previous studies, we confirmed the interaction of KBP with KIF3A in the mouse testis. The co-immunostaining showed that KIF3A is not present in the late CB, but KIF3A and KBP colocalized in the manchette of steps 9–12 spermatids and cytoplasm of round spermatids. Kif3a KO spermatogenesis showed severe defects in both prominent microtubule-based structures in elongating spermatids.
the manchette and sperm tail (Lehti et al. 2013). We have shown that KIF3A has a role in the delivery of the sperm tail component MNS1 (Lehti et al. 2013). In the WT mice, MNS1 localized in the acrosomal region, perinuclear ring, manchette, and the principal piece of the sperm tail. In the Kif3a KO mice, MNS1 was retained in the manchette indicating a role for KIF3A in the intramanchette transport (IMT). In contrast to MNS1, the localization of KBP in the manchette and in the late CB was not affected in the Kif3a KO tests. Interestingly, the CB structure appeared fragmented in the Kif3a KO elongated spermatids. On the other hand, the sperm tail formation in the Kif3a KO mice is severely compromised, and it is also possible that the normal axoneme per se is important for the integrity of the late CB that remains to be closely associated with the developing flagellum.

Considering the reported functions of KBP, it is possible that in differentiating spermatids, it could be involved in the regulation of KIF3A-mediated transport, for example, by augmenting its movement along the microtubules or by targeting KIF3A-associated cargo to specific locations. The male germ cell-specific KO mouse model for KBP is not yet available, and therefore, the physiological importance of KBP in the KIF3A function during spermatogenesis remains to be characterized. Although the functional evidence on the role of KIF3A-KBP interaction in elongating spermatids is still missing, our study provides an important basis for the future mechanistic studies.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-15-0173.

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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