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Elongin B is a binding partner of the male germ cell nuclear speckle protein sperm-associated antigen 16S (SPAG16S) and is regulated post-transcriptionally in the testis

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Abstract

In this study we identified Elongin B, a regulatory subunit of the trimeric elongation factor Elongin ABC, which increases the overall rate of elongation by RNA polymerase II, as a major binding partner of sperm-associated antigen 16S (SPAG16S), a component of nuclear speckles. Nuclear speckles are nuclear subcompartments involved in RNA maturation. Previously, we showed that SPAG16S is essential for spermatogenesis. In the present study, a specific antibody against mouse Elongin B was generated and reacted with a protein with the predicted size of Elongin B in the testis; immunofluorescence staining revealed that the Elongin B was located in the nuclei and residual bodies. In round spermatids, Elongin B is colocalised with splicing factor SC35 (SC35), a marker of nuclear speckles. During the first wave of spermatogenesis, *Elongin B*

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Conflicts of interest

The authors declare no conflicts of interest.

transcripts were initially detected at Postnatal Day (PND) 8, and levels were greatly increased afterwards. However, Elongin B protein was only found from PND30, when germ cells progressed through spermiogenesis. Polysomal gradient analysis of *Elongin B* transcripts isolated from adult mouse testes revealed that most of the *Elongin B* mRNA was associated with translationally inactive, non-polysomal ribonucleoproteins. An RNA electrophoretic mobility shift assay demonstrated that the 3′ untranslated region of the *Elongin B* transcript was bound by proteins present in testis but not liver extracts. These findings suggest that post-transcriptional regulation of Elongin B occurs in the testis, which is a common phenomenon in male germ cell development. As a major binding partner of SPAG16S, Elongin B may play an important role in spermatogenesis by modulating RNA maturation.

Keywords

3′ untranslated region; post-transcriptional regulation; spermiogenesis; transcription elongation factor B

Introduction

Mammalian sperm-associated antigen 16 (Spag16) encodes the orthologue of *Chlamydomona reinhardtii* PF20 (Smith and Lefebvre 1997; Pennarun *et al.* 2002), an axoneme central apparatus protein essential for flagellar function. Both the human and mouse *Spag16* genes are transcribed from alternative promoters to produce two major transcripts of 1.4 and 2.5 kb in the testis (Pennarun *et al.* 2002; Zhang *et al.* 2002). The two mouse *Spag16* transcripts exhibit different expression patterns and encode proteins of 35 kDa (SPAG16S) and 71 kDa (SPAG16L), with different localisations (Zhang *et al.* 2004, 2006). SPAG16L is expressed meiotically and is located in the axonemal central apparatus of the sperm tail, whereas SPAG16S is predominantly localised in the nucleus of round and condensing spermatids (Zhang *et al.* 2004; Nagarkatti-Gude *et al.* 2011). Targeted disruption of the *Spag16* gene showed that these two proteins function differently during spermatogenesis. SPAG16L is required for sperm flagellar motility, whereas SPAG16S seems to be crucial for viability of male germ cells (Zhang *et al.* 2004, 2006). Functional studies of SPAG16S revealed that the protein is able to elevate expression of SPAG16L, and thus may act as a transcriptional activator (Nagarkatti-Gude *et al.* 2011). Because SPAG16S does not contain a known DNA-binding motif, but has contiguous WD repeats required for protein-protein interaction (Zhang *et al.* 2002; Schapira *et al.* 2017), it could exert its regulatory effect on gene expression by binding to other protein partner(s).

Although SPAG16S contributes to normal spermatogenesis, the mechanisms underlying its function remain unknown. Immunofluorescence staining revealed that SPAG16S colocalised with splicing factor SC35 (SC35), a protein regulating RNA splicing (Bregman *et al.* 1995), and a marker for nuclear speckles, structures discovered as sites for splicing factor storage and RNA modification and metabolism (Galganski *et al.* 2017). To further study the function of SPAG16S in spermatogenesis, we performed a yeast-two hybrid screen and identified Elongin B as one of its major binding partners (Zhang *et al.* 2008).

Elongin B is an 18-kDa ubiquitin-like protein consisting of an 84-residue N-terminal ubiquitin homology domain and a 3 4-residue C-terminal tail (Garrett *et al.* 1995). It is a positive regulatory subunit of the trimeric elongation factor Elongin ABC, which increases the overall rate of elongation by RNA polymerase II through suppression of transient pauses of the polymerase at multiple sites along the DNA template (Aso *et al.* 1 995; Garrett *et al.* 1995; Allen and Taatjes 2015). In addition to stimulating transcript elongation, the Elongin BC complex functions as an adaptor in the proteasomal degradation of target proteins through distinct E3 ubiquitin ligase complexes (Okumura *et al.* 2012). Target proteins include hypoxia-inducible factor (HIF)-1α, p53, apolipoprotein B mRNA editing enzyme catalytic polypeptide-like editing complex 3G (APOBEC3G) and suppressor of cytokine signalling (SOCS) 1 (Kamura *et al.* 1998; Ohh *et al.* 2000; Querido *et al.* 2001; Kobayashi *et al.* 2005). These findings suggest that the Elongin BC complex participates in different cellular events via regulation of multiple key proteins.

In the present study we characterised the expression pattern of Elongin B in the testis. We found that the protein was localised in the nuclei of postmeiotic male germ cells and that protein expression occurred much later than mRNA expression, suggesting a translational delay. Like protamine 1, whose protein expression was also under translational control, most *Elongin B* mRNA was stored in ribonucleoprotein (RNP) granules. Testis extract rather than liver extract bound to the 3′ untranslated region (UTR) of *Elongin B* mRNA. These findings suggest that Elongin B participates in the mechanism of action of SPAG16S, probably by modulating RNA metabolism in nuclear speckles, and that Elongin B expression is subject to post-transcriptional regulation via the 3′ UTR of its mRNA.

Materials and methods

Source of tissues

Tissue samples were collected from C57BL6/J mice and stored at −80°C. Animal work was approved by the Institutional Animal Care and Use Committee of Wayne State University (Protocol IACUC-18–02-0534) and Virginia Commonwealth University (AD10000167) in accordance with federal and local regulations regarding the use of non-primate vertebrates in scientific research.

Yeast two-hybrid assays

The interaction between SPAG16S and Elongin B was confirmed using the yeast Matchmaker LexA two-hybrid system (Clontech). Briefly, a cDNA containing the full-length mouse *Elongin B* coding sequences were generated by reverse transcription-polymerase chain reaction (RT-PCR) using the following primer sets: ElonginB-F1, 5′-GAATTCATGGACGTGTTTCTCATG-3′; and ElonginB-R1, 5′-CTCGAGTCACTGCACAGCTTGTTC-3′. The PCR product was cloned into the pCR2.1 TOPO vector (Invitrogen) and verified by DNA sequencing. Then, the correct *Elongin B* cDNA was subcloned into a pB42AD vector (Clontech). The SPAG16S/pLexA plasmid was created previously (Zhang *et al.* 2004). Elongin B/pB42AD was cotransformed with either the SPAG16S/pLexA plasmid or the empty pLexA vector into yeast EGY48 strains containing p8op-lacZ plasmid. The yeast was grown on synthetic triple-dropout plates

lacking histidine, tryptophan and uracil, with or without galactose and raffinose as inducers. Two plasmids containing p53 in pLexA and simian virus (SV) 40 large T antigen in pB42D were cotransformed into EGY48 and used as a positive control.

Generation of a specific anti-Elongin B polyclonal antibody

A cDNA encoding full-length of *Elongin B* was amplified by PCR using the following primers: 5′-CATATGATGGACGTGTTTCTCATG-3′ (forward) and 5′-AAGCTTCCTGCACAGCTTGTTCATTG-3′ (reverse). The correct *Elongin B* cDNA was inserted into the pET28a expression vector (Novagen). The construct was transformed into *Escherichia coli* strain BL21 (DE3) cells, and the fusion protein was induced and purified as described previously (Zhang *et al.* 2002). The resulting fusion protein contains His tags at both the N and C termini. The purified recombinant protein was used to generate a rabbit polyclonal antibody (Rockland Immunochemicals).

Cell culture and transient transfection

COS-1 cells were cultured with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C. The cells were transfected with the indicated plasmids using Lipofectamine 2000 transfection reagent (Invitrogen). After 48 h transfection, the cells were processed for coimmunoprecipitation assays.

Coimmunoprecipitation assays

The generation of mouse SPAG16S/pTarget has been described elsewhere (Zhang *et al.* 2014). To make mouse Elongin B/pEGFP-N2 construct, *Elongin B* cDNA was amplified using the primer set ElonginB-F1 and ElonginB-R2 (5′-GGATCCCCTGCACAGCTTGTTCATTG-3′), and the correct *Elongin B* cDNA was ligated into pEGFP-N2 vector. Two SPAG16S/pTarget and Elongin B/pEGFP-N2 plasmids were cotransfected into COS-1 cells and coimmunoprecipitation assays were performed as described previously (Lee 2007). Briefly, after 48 h transfection, the cells were lysed with IP buffer (Catalogue no. P0013; Beyotime) for 5 min and centrifuged at 10 000*g* for 3–5 min. The supernatant of cell lysates was precleaned with protein A beads at 4°C for 30 min, and the precleared lysate was then incubated with antibodies against the N- or C-terminus of SPAG16 at 4°C for 2 h. Protein A beads were added, followed by another incubation at 4°C overnight. The beads were washed with IP buffer three times and then resuspended in 2 × Laemmli sample buffer and heated at 95°C for 5 min. The samples were centrifuged at 3000*g* for 30 s at 4°C and the supernatant was then subjected to western blot analysis with an anti-green fluorescent protein (GFP) antibody.

Coimmunoprecipitation studies were performed using the same procedure as described previously (Liu *et al.* 2017). Anti-SPAG16S antibody was used to pull down protein complexes and anti-Elongin B antibody was used for western blotting.

Northern blot analysis

A mouse multiple tissue RNA gel blot was purchased from Clontech. Total testicular RNA was extracted from mice at indicated ages using TRIzol reagent (Invitrogen). An equal amount of total RNA was separated on a denaturing gel and transferred to a nylon

membrane. The full-length Elongin B cDNA was released from the Elongin B/pEGFP-N2 plasmid and labelled with ^{32}P using DNA labelling beads (Amersham). Hybridisation was conducted as described previously (He and Green 2013).

Western blot analysis

Tissue samples were homogenised in T-PER protein extraction reagent (Thermo Scientific) with Halt protease inhibitor cocktail (Thermo Scientific) using an Ultra Turrax homogenizer (IKA) and centrifuged at 15 871*g* for 10 min at 4°C. Protein concentrations were quantified using a DC protein assay kit (Bio-Rad). An equal amount of protein was heated to 95°C for 10 min in 2 × Laemmli sample buffer. Samples were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene difluoride (PVDF) membranes (Millipore). The PVDF membranes were then blocked in a Tris-buffered saline solution containing 5% non-fat dry milk powder and 0.1% Tween 20 (TBST) for 1 h at room temperature and incubated with the anti-Elongin B antibody at 4°C for 16 h. After washing with TBST three times, the PVDF membranes were immersed in horseradish peroxidase-labelled goat anti-rabbit IgG (dilution 1: 2000) at room temperature for 2 h. Following three washes in TBST, the immunoreactive proteins were detected with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific).

Immunofluorescence staining of testis sections and isolated germ cells

Testes from adult mice were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C overnight and embedded in paraffin wax (Tissue-Tek). Then, 5-μm sections of paraffin-embedded tissue were deparaffinised in xylene and rehydrated with ethanol (100%, 90%, 70% and 50%) and phosphate- buffered saline (PBS). Microwave-stimulated antigen retrieval was performed by immersing the slides in boiled sodium citrate buffer (10 mM, pH 6.0) for 5 min. The slides were maintained at a sub-boiling temperature (~95°C) for 10 min and then cooled to room temperature. The slides were immersed in PBS for 5 min. Tissue sections were permeabilised in 0.1% TritonX-100 in PBS at 37°C for 5 min and then blocked with 3% (w/v) bovine serum albumin in PBS for 1 h, followed by incubation with anti-Elongin B antibody at 4°C overnight. The slides were washed with PBS and incubated with Cy3-conjugated anti-rabbit IgG secondary antibody (1:5000 dilution; Jackson ImmunoResearch Laboratories) at room temperature for 1 h. The slides were washed with PBS three times and mounted using VectaMount (Vector Laboratories, Inc) with 4′,6′-diamidino-2-phenylindole (DAPI). Images were taken by confocal laser-scanning microscopy (Zeiss LSM 700).

To stain isolated germ cells, testes from adult mice were dissected as described previously (Zhang *et al.* 2017), and the cells were double stained with anti-Elongin B antibody and anti-SC35 antibody. Images were captured by confocal laser-scanning microscopy (Zeiss 780).

Polysomal gradients and analysis

Fractionation of mouse testis extracts over sucrose gradients was performed as described previously (Yang *et al.* 2007). Each testis was dissected from an adult mouse and homogenised on ice in 1 mL lysis buffer containing 20 mM HEPES (pH 7.6), 1.5 mM $MgCl_2$, 40 mM KCl and 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate

was centrifuged at 2300*g* for 10 min at 4°C. The supernatant was layered over 5 mL of 10–30% linear sucrose gradients on a 0.25-mL cushion of 60% sucrose and then centrifuged at 100 000*g* for 90 min at 4°C in a Beckman SW28 Ti rotor. The gradients were fractionated into 17 tubes. RNA from each fraction was purified with TriReagent (Sigma) and subjected to northern blot analysis using *Elongin B, Protamine 1* and Clusterin (*Clu*) probes.

Electrophoretic mobility shift assays

RNA probes for electrophoretic mobility shift assays (EMSAs) were prepared as follows: the 3′-UTR of *Elongin B* mRNA was amplified by PCR using the primers 5′-TAATACGACTCACTATAGGGGGGCCTAGGCCTACCCCCT-3′ (forward; underline indicating the T7 promoter) and 5′-CTGTAGTCTTTACTTGGG-3′ (reverse) and an Expand High Fidelity PCR system (New England Biolabs Inc). The PCR product was cloned into pCR2.1 TOPO vector and verified by DNA sequencing. The correctly ligated plasmid was digested with *EcoRV* to linearise the DNA for the labelling. Radiolabelled RNA probe was synthesised *in vitro* using a MAXIscript T7 *In vitro* Transcription Kit (Ambian) according to the manufacturer's instructions. The full-length RNA probe was separated by electrophoresis of the transcription reaction mixture on a denaturing 6% polyacrylamide-7 M urea gel. The gel slice containing the band of interest was excised, cut into tiny pieces and recovered with 0.3 mL of 0.5 M sodium acetate (pH 5.0). The slurry was incubated at room temperature overnight with shaking. After centrifugation, the supernatant was added to 1 mL of 100% ethanol and kept at −80°C overnight. After centrifugation at 13 523*g* for 10 min at 4°C, the supernatant was discarded and the pellet was kept at room temperature for 5 min and resuspended in 20 μL RNAse-free water. Radioactivity was quantified using a scintillation counter with 1 μL probe (between 1×10^4 and 1×10^5 c.p.m. probe was used for each binding reaction).

To prepare the tissue extracts for binding assay, the testis or liver was homogenised in 1 × buffer containing 10 mM Tris (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 100 U mL^{-1} RNasin and 1 × proteinase cocktail. After centrifugation at 13 523*g* for 10 min at 4°C, the supernatant was centrifuged again at 100000*g* for 1 h at 4°C, after which the supernatant was collected and protein concentration was measured.

EMSAs were performed in 20-μL reaction mixtures containing radiolabelled RNA probe (between 1×10^4and1×10^5 c.p.m. for each reaction), 50 μg testis or liver extract, 1× binding buffer (20 mM HEPES (pH 7.6), 40 mM KCl, 3 mM $MgCl_2$, 2 mM DTT and 5% glycerol) and RNasin inhibitor. After incubation at room temperature for 40 min, reaction mixtures were mixed with 4 μL of 2× RNA loading buffer (60% glycerol, 0.025% bromophenol blue) and electrophoresed on a 5% non-denaturing polyacrylamide gel (60:1) in 0.5 × Tris-borate-EDTA (TBE) buffer. Gels were vacuum dried and visualised by autoradiography.

Results

Interaction between SPAG16S and Elongin B

In our initial yeast two-hybrid screen using SPAG16S as bait, Elongin B was identified to be one of its putative binding partners (Zhang *et al.* 2008). To confirm the interaction between SPAG16S and Elongin B, a direct yeast two-hybrid assay was performed in the absence or presence of galactose. Beta-galactosidose (LacZ) activity was induced when yeast cells were cotransformed with the plasmid pairs encoding the coding region of SPAG16S and Elongin B, or the p53/Large T antigen, which was used as a positive control (Fig. 1a). In addition, the interaction between SPAG16S and Elongin B was further verified by coimmunoprecipitation assays. COS-1 cells were transfected with Elongin B/pEGFP-N2 and SPAG16S/pTarget expression plasmids. The cells were immunoprecipitated with antibodies corresponding to the N- and C-termini of SPAG16 (SPAG16N and SPAG16C antibodies respectively) and then subjected to western blot analysis with an anti-GPF antibody. Because *Spag16S* is transcribed from an alternative transcriptional start site in front of exon 12 of full-length *Spag16* (Zhang *et al.* 2002) and Elongin B was pulled down by the SPAG16C antibody but not the SPAG16N antibody, which does not react with SPAG16S, the results indicate that SPAG16S interacts with Elongin B *in vitro* (Fig. 1b). The interaction between SPAG16S and Elongin B *in vivo* was further examined by coimmunoprecipitation studies using mouse testis extracts. Elongin B protein was pulled down using the specific anti-SPAG16S antibody (Fig. 1c).

Generation of a specific polyclonal anti-Elongin B antibody

To further study the function of Elongin B *in vivo,* we generated a polyclonal antibody against mouse Elongin B using purified full-length protein. To test the specificity of the antibody, COS-1 cells were transfected with Elongin B/pEGFP-N2 plasmid or an empty pEGFP-N2 plasmid. The cell lysates were used for western blot analysis using the anti-enhanced GFP (EGFP) antibody (Fig. 2a) or the anti-Elongin B antibody (Fig. 2b). The GFP antibody cross-reacted with both free GFP protein and GFP-tagged Elongin B fusion protein (Fig. 2a). However, the Elongin B antibody only reacted with the GFP-tagged Elongin B fusion protein, indicating that the Elongin B antibody was specific.

Expression pattern of Elongin B in mouse tissues

The expression profile of Elongin B in eight different mouse tissues was examined by northern and western blot analyses. Northern hybridisation showed an approximate 0.7-kb Elongin B transcript in the heart, brain, liver and kidney, whereas an approximate 0.8-kb transcript was detected in the testis (Fig. 3a). Western blotting using an anti-Elongin B antibody revealed protein in brain and testis at approximately 18 kDa, which corresponds to the predicted molecular weight of Elongin B. In addition to the 18-kDa protein, the antibody detected proteins with different molecular weights in the heart and liver (Fig. 3b). These large protein bands may represent Elongin B linked with other proteins or proteins translated from unidentified Elongin B transcripts.

Localisation of Elongin B in nuclei of postmeiotic germ cells and nuclear speckles of round spermatids

To examine localisation of Elongin B in the testis, immune-fluorescence was conducted. No specific signal was observed in spermatogonia and spermatocytes. A weak signal was observed in round spermatids, whereas a strong signal was only observed in elongating spermatids and residual bodies (Fig. 4; see Fig. S1, available as Supplementary Material to this paper).

We previously reported that SPAG16S was localised in the nuclear speckles of round spermatids (Nagarkatti-Gude *et al.* 2011). Given the interaction between SPAG16S and Elongin B, we next examined whether Elongin B is also present in nuclear speckles. Testicular germ cells were isolated from adult mice, and the cells were double stained with the specific polyclonal antibody against Elongin B and a monoclonal antibody against SC35, a protein localised in nuclear speckles (Bregman *et al.* 1995; Galganski *et al.* 2017). In round spermatids, Elongin B and SC35 proteins were colocalised in some nuclear speckles (Fig. 5).

Dynamic changes in Elongin B expression during male germ cell differentiation

To examine the expression profile of Elongin B at the transcriptional and protein levels during the first wave of spermatogenesis, total RNA and proteins were extracted from mouse testes at different times after birth. Northern blotting indicated that the *Elongin B* gene was first transcribed at Postnatal Day (PND) 8, and increased markedly from Day 16 after birth when germ cells reach the pachytene stage (Fig. 6a). However, western blotting analysis showed that the Elongin B protein was first detected at PND20, and levels were significantly increased at Days 30 and 42, when germ cells progress through spermiogenesis (Fig. 6b), much later than the appearance of mRNA expression. These findings suggest that expression of Elongin B is subject to post-transcriptional regulation.

Elongin B *mRNA in non-polysomal RNP fractions*

To examine the translational status of *Elongin B* mRNA, polysome fractionation coupled with northern blotting was performed. The purified RNAs from each fraction of sucrose gradients were hybridised to an *Elongin B*-specific probe prepared from the coding region. Most *Elongin B* mRNA was present in the RNP fractions, whereas a small portion of this mRNA cosedimented with polysomes (Fig. 7a). This pattern is similar to *Protamine 1* (Fig. 7b), which is also under translational control. *Clu* mRNA, which is expressed in Sertoli cells and is not under translational regulation (Iguchi *et al.* 2006), is primarily associated with polysomes (Fig. 7c).

Elongin B mRNA 3 UTR binding proteins in the testis

The eukaryotic mRNA 3′ UTR plays critical roles in the modulation of mRNA decay and translation (Geissler and Grimson 2016), and the data presented above suggest that the expression of Elongin B is under post-transcriptional control. To investigate the presence of putative *trans*-acting factors binding to the 3′-UTR of *Elongin B* mRNA, RNA EMSAs were performed. Liver or testis extracts from adult mice were incubated with a radiolabelled portion of the 3′-UTR of *Elongin B* mRNA. As shown in Fig. 8, several bands were detected

for the free RNA probe, likely due to the presence of different RNA conformations. Two shifted bands were observed with the testis but not liver extracts (Fig. 8, Lanes 2, 3). The mobility was further reduced and then abolished by adding excess of the same but unlabelled specific RNA competitors (Lanes 4, 5). The EMSA complexes were not affected when unspecific RNA competitor that was made from the *Elongin B* coding region was added (Lane 6). Thus, the data indicate the existence of trans-acting factors for the control of *Elongin B* translation in the mouse testis.

Notice that the shifted bands were observed with the testis extract but not with the liver extract. Adding excess specific probe but not the non-related probe abolished the binding.

Discussion

SPAG16S is exclusively expressed in the mouse testis and appears to have an essential role in spermatogenesis (Zhang *et al.* 2004). To elucidate the functions of SPAG16S in spermatogenesis, a yeast two-hybrid screen was initially performed using SPAG16S as bait, and we found several putative binding partners, including Elongin B (Zhang *et al.* 2008). The interaction between SPAG16S and Elongin B was verified by a direct yeast two-hybrid and coimmunoprecipitation assay from testis extracts. SPAG16S has seven WD repeat domains that mediate protein-protein interactions (Zhang *et al.* 2004; Nagarkatti-Gude *et al.* 2011; Schapira *et al.* 2017). Interestingly, the Elongin BC complex has been shown to bind to WD-40 repeat proteins other than SPAG16S (Kamura *et al.* 1998), and it is likely that the interaction between Elongin B and SPAG16S is mediated through the WD repeat domains. The interaction between SPAG16S and Elongin B suggests that SPAG16S may function in RNA elongation and proteasomal degradation of proteins through ubiquitination, or it may serve as a scaffold for proteins carrying these functions (Aso *et al.* 1995; Garrett *et al.* 1995; Okumura *et al.* 2012; Allen and Taatjes 2015).

Few studies have investigated the function of Elongin B in germ cell development. In *Caenorhabditis elegans,* Elongin B and C form a stable complex, and depletion of either gene product by RNA interference causes pronounced defects in the second meiotic division (Sasagawa *et al.* 2005). The present study is the first to characterise mouse Elongin B expression in the testis and suggests a unique function of the complex in mouse germ cell development. This is supported by the discovery of a testis-specific Elongin A2 (Aso *et al.* 2000). As a regulatory subunit of the trimeric elongation factor Elongin ABC complex, Elongin B may regulate transcriptional activity by modulating Elongin A2 activity in male germ cells (Aso *et al.* 1996). In addition, von Hippel-Lindau (VHL) protein, a tumour suppressor protein, has been shown to be a binding partner of the Elongin B/C complex (Duan *et al.* 1995; Kibel *et al.* 1995). Testes of male VHL (f/d)/Cre mice were small with a markedly reduced sperm count resulting in infertility (Ma *et al.* 2003).

Elongin B mRNA was only expressed in certain tissues, including the heart, brain, liver, kidney and testis, but the translated protein varied in these tissues. For example, low levels of *Elongin B* transcripts were observed in the kidneys, but no protein was detected by western blotting. This could possibly be due to low translational efficiency in the kidney. Using the antibody generated against purified full-length *Elongin B,* an immunoreactive

protein with the predicted size of Elongin B was only detected in the brain and testis. Multiple proteins of larger molecular weights were detected in the heart and liver. These proteins may be translated from an alternative transcript or unidentified transcripts of Elongin B, or they may be cross-reacting proteins.

Localisation of Elongin B in the testis supports the notion that the protein functions as a regulator of RNA elongation. It is present in the nuclei of rounds spermatids, and the signal was significantly increased in elongating spermatids, indicating that the protein plays a more important role in spermiogenesis, when germ cells undergo marked morphological changes (Hermo *et al.* 2010). The pattern localisation matched the western blot results from a study of the first wave of spermatogenesis. During the first wave of spermatogenesis, Elongin B was first detected at Day 20 after birth, and expression levels were markedly increased at Day 30 after birth, when germ cells differentiate into elongating spermatids (Laiho *et al.* 2013). This pattern is also similar to SPAG16S (Nagarkatti-Gude *et al.* 2011). In addition, SPAG16S was localised in speckles that have a role in pre-mRNA splicing (Ye *et al.* 1998; Zhang *et al.* 2004; Morimoto and Boerkoel 2013; Galganski *et al.* 2017). Because antibodies against SPAG16S and Elongin B were made in rabbits, we were not able to stain the two proteins simultaneously. However, we were able to double stain with Elongin B and SC35 in round spermatids. Even though the localisation of these two proteins was not identical, they were partially colocalised in nuclear speckles, which suggests that, like SPAG16S, Elongin B has a function in nuclear speckles. Even though C-terminal amino acid sequence of SPAG16L is identical to sperm-associated antigen 16S, it is unlikely Elongin B forms a complex with SPAG16L *in vivo* because SPAG16L is not expressed in the nuclei (Nagarkatti-Gude *et al.* 2011).

The interesting finding of the present study is that transcription of Elongin B was found to start at PND8 during the first wave of spermatogenesis, much earlier than the first appearance of Elongin B protein. The delay in protein expression indicates that *Elongin B* mRNA is subject to post-transcriptional regulation and is stored in a translationally repressed state during spermatogenesis. This hypothesis is supported, in part, by the observation that the *Elongin B* transcript from adult mouse testis is primarily associated with non-polysomal RNP particles. In common with translational repression of other genes in early haploid spermatids (Kleene 1993; Penttilö *et al.* 1995), *Elongin B* mRNA is primarily associated with the non-polysomal RNP particles to ensure availability for translation in the later stage of spermiogenesis when transcription ceases (Steger 2001). During spermiogenesis, several unique structures are formed in germ cells, including the acrosome and flagella. Several proteins are translated and assembled to these structures. Many of these genes are transcribed but not translated until the proteins are needed (Licatalosi 2016).

It has been reported that the eukaryotic 3′-UTR are involved in the regulation of mRNA decay and translation, and several RNA binding proteins have been identified to interact with the 3′-UTR of transcripts during spermatogenesis (Steger 2001; Geissler and Grimson 2016; Licatalosi 2016). Given the fact that testicular *Elongin B* mRNA was larger than that in somatic tissues, we speculate that the 3′-UTR of *Elongin B* may participate in translational repression of its expression in the testis. The results of RNA EMSA suggest the existence of a *trans-acting* factor(s) able to interact with the 3′-UTR of *Elongin B* mRNA in the testis.

These proteins may modulate the translation of *Elongin B* during spermatogenesis. In addition, analysis of the 3′-UTR of *Elongin B* mRNA revealed a putative polyadenylation consensus motif AATAAA. This suggests that *Elongin B* transcripts are polyadenylated and translationally regulated by interaction with poly(A)-binding proteins (Derry *et al.* 2006; Licatalosi 2016). Overall, our studies provide a foundation for further studies of how Elongin B functions as a partner with SPAG16S to regulate in spermatogenesis, and how Elongin B expression is controlled.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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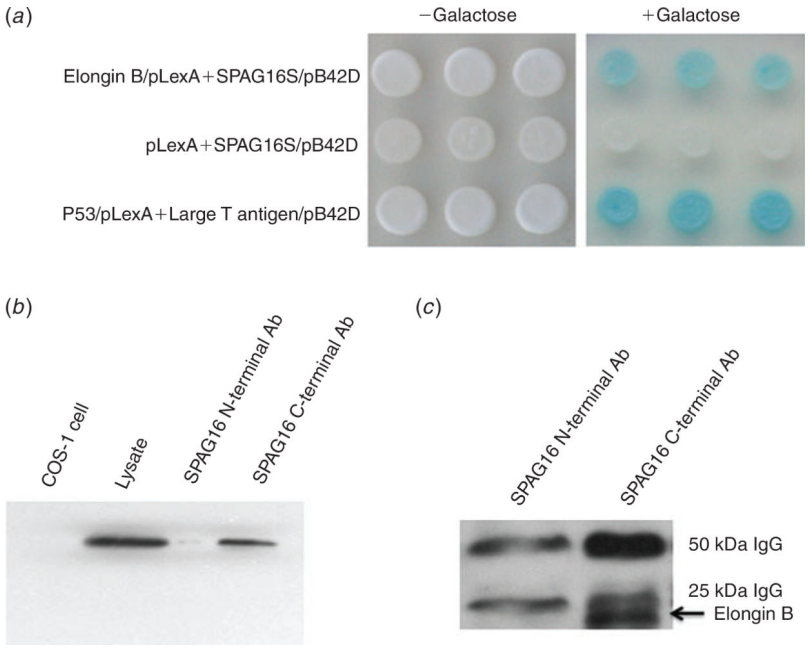

Fig. 1.

Interaction of sperm-associated antigen 16S (SPAG16S) with Elongin B. (*a*) Direct yeast two-hybrid assay to examine the interaction between SPAG16S and Elongin B. Yeast cells (EGY48) were cotransformed with the plasmids indicated and grown in the presence or absence of 2% galactose. Induction of Beta-galactosidose activity indicates a specific interaction between SPAG16S and Elongin B. Yeast cotransformed with p53/pLexA and large T antigen/pB42D were used as a positive control. (*b*) Coimmunoprecipitation of SPAG16S with Elongin B in cultured cells. COS-1 cells were cotransformed with Elongin B/pEGFP-N2 and SPAG16S/pTarget. The cell lysate was immunoprecipitated with antibodies (Abs) against the N- or C-terminus of SPAG16, and then analysed by western blotting with an anti-green fluorescent protein (GFP) Ab. (*c*) Coimmunoprecipitation of SPAG16S with Elongin B in testis extract. Elongin B was pulled down by the anti-SPAG16S Ab.

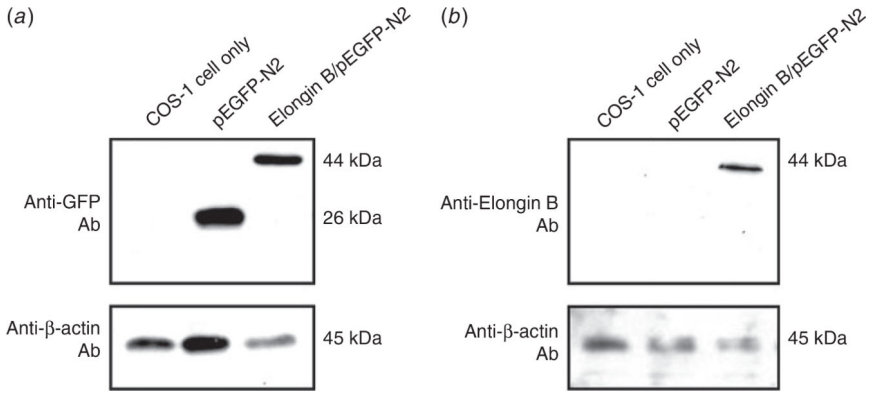

Fig. 2.

Characterisation of an anti-Elongin B polyclonal antibody (Ab). COS-1 cells were transfected with Elongin B/pEGPF-N2 plasmid or an empty pEGPF-N2 plasmid, and cell lysates were used for western blot analysis with (*a*) anti-green fluorescent protein (GFP) Ab or (*b*) anti-Elongin B Ab. (*a*) The anti-GFP Ab cross-reacted with both free GFP protein and GFP-tagged Elongin B fusion protein. (*b*) However, the Elongin B Ab only reacted with the GFP-tagged Elongin B fusion protein, indicating that the Elongin B Ab was specific.

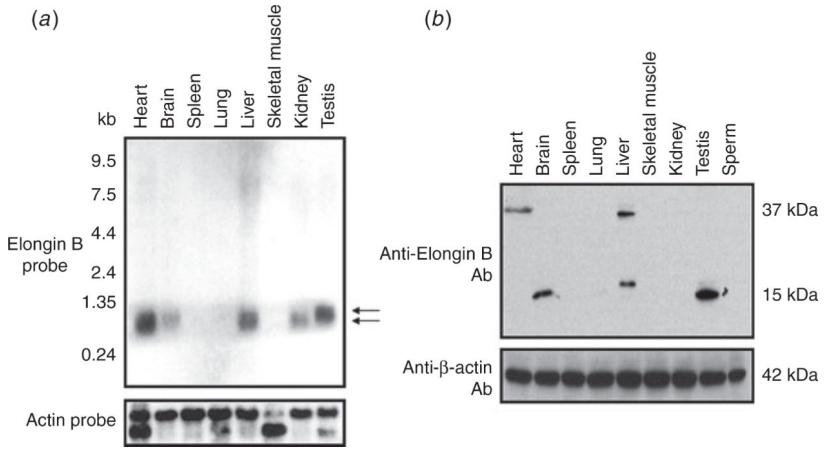

Fig. 3.

Tissue distribution of Elongin B mRNA and protein. (*a*) Northern blot analysis of *Elongin B* mRNA in different mouse tissues, as indicated. *Elongin B* mRNA in the testis is larger than that in other somatic tissues. (*b*) The expression pattern of Elongin B protein was examined in mouse tissues by western blotting. The anti-Elongin B antibody recognised the predicted 18-kDa Elongin B in the brain and testis. In addition, the antibody cross-reacted with proteins of larger molecular weight in the heart and liver. β-Actin was included as a loading control.

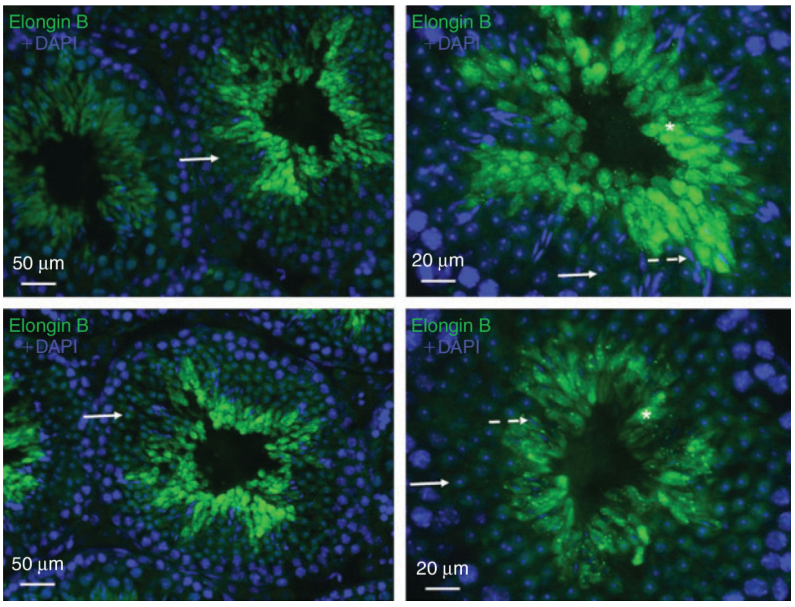

Fig. 4.

Immunofluorescence analysis of Elongin B localisation in the testis. Paraffin sections of mouse testis were immunostained with an anti-Elongin B antibody. No specific signal was detected in spermatogonia and spermatocytes. A weak signal was detected in round spermatids (white arrows), and strong Elongin B staining was present in elongating spermatids (dashed arrows) and residual bodies (asterisks). Nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI; blue).

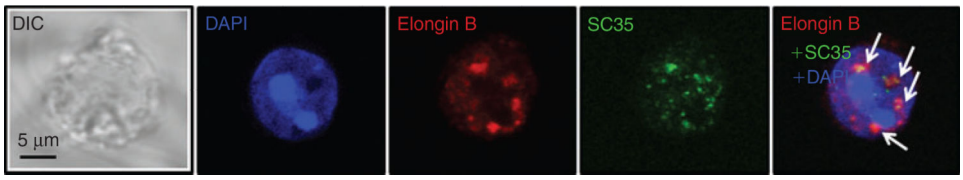

Fig. 5.

Colocalisation of Elongin B and splicing factor SC35 (SC35), a nuclear speckle protein in round spermatids. Isolated testicular cells were double stained with an anti-Elongin B polyclonal antibody (red) and an anti-SC35 monoclonal antibody (green). The two proteins are colocalised in some nuclear speckles in round spermatids (arrows). DAPI, 4′, 6′-diamidino-2-phenylindole.

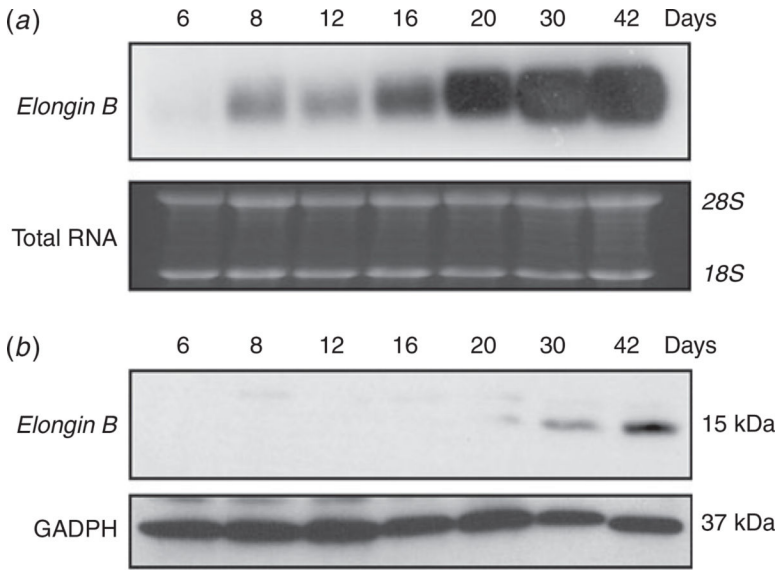

Fig. 6.

Expression profile of Elongin B transcripts and protein during the first wave of spermatogenesis. Testicular expression of Elongin B mRNA and protein was analysed by (*a*) northern blotting and (*b*) western blotting respectively. Total RNA or protein extracts were isolated from mouse testis on different days after birth (6, 8, 12, 16, 20, 30 and 42). Note that protein expression is detected much later than mRNA expression, indicating a translation delay of the gene. *28S* and *18S* rRNA (northern blotting) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; western blotting) were used as loading controls.

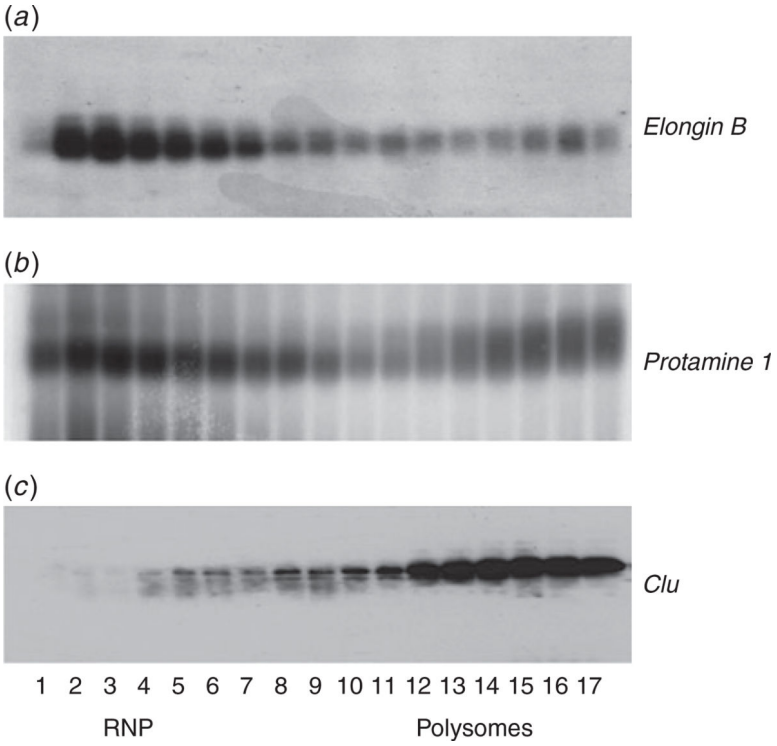

Fig. 7.

(*a*) Sucrose gradient distribution of *Elongin B* transcripts in the mouse testes. Testicular extracts from adult mice were fractionated over a 10–30% sucrose gradient and 17 fractions were collected. RNA was purified from each fraction and hybridised to a radiolabelled *Elongin B* probe in northern blotting. Fraction number 1 is the top of the gradient. (*b*, *c*) Mouse *Protamine 1* (*b*) and *Clu* (*c*) probes were used as controls. RNP, ribonucleoprotein.

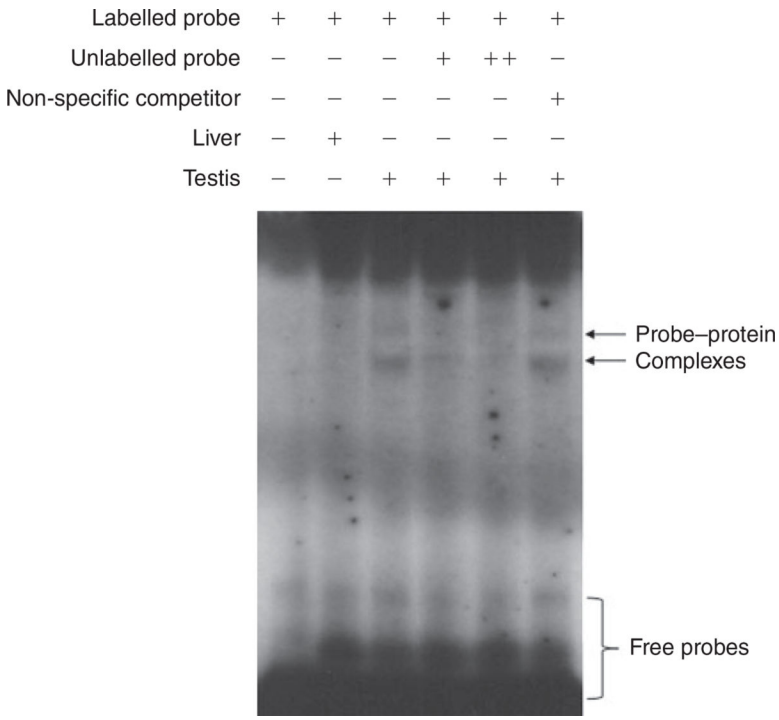

Fig. 8.

Formation of RNA-protein complexes between the 3′-untranslated region (UTR) of *Elongin B* mRNA and mouse testicular extracts. An electrophoretic mobility shift assay was performed using a radiolabelled 3′-UTR of *Elongin B* mRNA and the indicated components. Lane 1, free probe only; Lane 2, probe plus liver extract; Lane 3, probe plus testis extract; Lane 4, probe plus testis extract and 10-fold unlabelled specific probe; Lane 5, probe plus testis extract and 100-fold unlabelled specific probe; Lane 6, probe plus testis extract and non-related unlabelled probe.