# Cloning, expression and characterization of mouse sperm specific thioredoxin-1 (Sptrx-1) gene and protein

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**Abbreviations:** DAPI, 4',6-diamidino-2-phenylindole, dihydrochloride; DTNB, 5,5´-dithiobis-(2-nitrobenzoic acid); FISH, fluorescent *in situ* hybridization; FS, fibrous sheath; GST, glutathione S-transferase; ODF, outer denser fibers; ORF, open reading frame; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; RACE, rapid amplification of cDNA ends; Trx, thioredoxin; UTR, untranslated region

Mouse Sptrx-1 GenBank accession number: AF196282

## SUMMARY

Thioredoxins (Trx) are proteins that participate in different cellular processes via redox-mediated reactions. We have recently described two novel members of this family in humans that display a sperm specific expression pattern, named Sptrx-1 and Sptrx-2 respectively. We report here the cloning and characterization of the mouse Sptrx-1 gene and protein which are similar to those described for the human orthologue. Mouse Sptrx-1 open reading frame encodes for a protein of 462 amino acids composed of an N-terminal repetitive domain of 15 residues motif followed by a C-terminal domain typical of thioredoxins. Mouse Sptrx-1 gene sequence is interrupted by only one intron of 525 bp located in the 5´-UTR, and using FISH analysis we have mapped its chromosomal location at 17E1.2-1.3. Northern blot analysis identifies testis as the only tissue expressing mouse Sptrx-1 mRNA, and by *in situ* hybridization we find a strong labeling in the testis seminiferous tubules mostly in the round spermatids. Affinity purified antibodies against human Sptrx-1 crossreact well with the mouse protein which confirms expression in seminiferous tubules at the late steps of spermiogenesis. By indirect immunofluorescence microscopy in mouse epididymal sperm, we identify Sptrx-1 labeling from the postacrosomal region to the end of the principal piece. Recombinant mouse Sptrx-1 displays protein disulfide reducing activity in the enzymatic assay coupled to NADPH and thioredoxin reductase. The availability of the mouse Sptrx-1 gene sequence is the first step aiming to the generation of knock-out mice, whose characterization will provide significant information regarding the *in vivo* function of Sptrx-1 and its possible implication in several sperm anomalies.

# INTRODUCTION

Thioredoxins (Trx) are a family of proteins that are conserved in all organisms through evolution and are characterized by the sequence of their highly conserved active site Cys-Gly-Pro-Cys (CGPC). Thioredoxins participate in different cellular mechanisms, mainly redox reactions, by the reversible oxidation of their active site from the dithiol form to disulfide (Arner and Holmgren, 2000; Powis and Montfort, 2001). To be active, thioredoxins must be in their reduced form, and this state is maintained by the flavoenzyme thioredoxin reductase (TrxR) at expense of the reducing power of NADPH, thus forming the so-called thioredoxin system (Holmgren and Björnstedt, 1995). The functions ascribed to thioredoxins are continuously increasing since they were initially discovered as electron donors for ribonucleotide reductase, an essential enzyme in DNA synthesis (Laurent *et al*., 1964). In addition, thioredoxins reduce other metabolic enzymes like PAPS reductase or methionine sulfoxide reductase, regulate transcription factor DNA binding activity, act as antioxidant molecules, modulate apoptosis and have also been implicated in many pathological situations (Powis and Montfort, 2001).

All organisms so far investigated, from bacteria to mammals, contain several thioredoxin systems (see introduction of Spyrou *et al*., 2001). During the recent years, the thioredoxin field has experienced an important expansion as new members of the family have been described and new functions of known members of this family have been reported. The most recent breakthroughs in the field have been the characterization of a complete thioredoxin system in mitochondria of eukaryotic organisms (Laloi *et al*., 2001; Miranda-Vizuete *et al*., 2000); the discovery that, in *Drosophila,* thioredoxin reductase is able to reduce glutathione as this organism lacks a functional glutathione reductase (Kanzok *et al*., 2001); the characterization of a novel form of thioredoxin reductase with a N-terminal extension displaying high homology to glutaredoxins (another redox enzyme

closely related, both structurally and functionally, to thioredoxins) (Sun *et al*., 2001) and the identification of the first two members of the family with a tissue specific expression pattern, exclusively located in the tail of human spermatozoa, named Sptrx-1 and Sptrx-2 (Miranda-Vizuete *et al*., 2001; Sadek *et al*., 2001).

The mammalian sperm tail is divided into three main regions running in a proximal to distal direction away from the sperm head: the mid-piece (that is attached to the sperm head by the neck or connecting piece), the principal piece and the end piece (for a scheme on sperm tail organization see (Eddy and O´Brien, 1994) and (Curry and Watson, 1995)). The flagellum of the mammalian spermatozoa is organized around the central axoneme which consists of 9+2 microtubule doublets, similar to that seen in the cilia and flagella of all eukaryotic cells and extends through the full length of the flagellum (Eddy and O´Brien, 1994). In addition, mammalian spermatozoa contain characteristic cytoskeletal elements associated with the axoneme, namely outer dense fibers (ODF) and fibrous sheath (FS), that do not have any counterparts in any other cell type (Oko, 1998). The function of ODF and FS is not fully elucidated but it seems to be related to the control of flagellar motion (Curry and Watson, 1995) and protection against shearing forces during epididymal transit (Baltz *et al*., 1990). However, evidence for a more active than merely structural role of the ODF and FS in sperm function is increasing, supported by the fact that among their constituent proteins there are several displaying either enzymatic or regulatory functions. For example in the FS, GAPDS produces ATP via the glycolytic pathway. This is critical for the transition to hyperactivated motility as well as capacitation (Williams and Ford, 2001). Moreover, the initiation and maintenance of sperm motility is regulated by a cascade of phosphorylation / dephosphoryaltion events (reviewed by (Tash and Bracho, 1994)). In this respect AKAP, also located in the FS, tethers cAMP-dependent protein kinase A, directing and specifying the actions of the

kinase in close proximity to the sperm´s axonemal machinery (reviewed by (Feliciello *et al*., 2001)).

Sptrx-1 transiently associates to the longitudinal columns of the FS during sperm tail elongation, but it is not a structural FS component as it is discharged to the residual body and cytoplasmic droplet after tail assembly is completed (Y. Yu, R. Oko and A. Miranda-Vizuete, in preparation). Sptrx-2 is also located in the sperm FS, but its expression pattern differs to that of Sptrx-1 in that it remains as a structural component of the FS in the mature spermatozoa (R. Oko and A. Miranda-Vizuete, unpublished results). We report here the cloning, chromosomal localization and characterization of the mouse Sptrx-1 gene as a first step in the attainment of the Sptrx-1 knock-out strategy.

## MATERIALS AND METHODS

**cDNA / genomic cloning and chromosomal localization of mouse Sptrx-1 gene:** The Basic Local Alignment Search Tool (BLAST) (Altschul and Koonin, 1998) was used to perform a survey of different databases at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) to identify entries encoding the putative mouse orthologue of human Sptrx-1. Using this approach we found the mouse EST entries AU021712 and AU021742, that displayed high homology to the thioredoxin domain of human Sptrx-1. Based on these sequences, specific nested primers (available upon request) were used for 5´- and 3´-RACE in a mouse testis cDNA library (Clontech). The resulting sequences were used to amplify by PCR the full-length cDNA of mouse Sptrx-1 cDNA from the same library. The amplification product was cloned in the pGEM-Teasy vector (Promega) and sequenced in both directions. The mouse Sptrx-1 ORF was used to screen a BAC mouse genomic library (genetic background ES129/SvJ) (Genome Systems, Inc.). Positive clones were confirmed to contain the mouse Sptrx-1 by PCR amplification and subsequent sequencing using primers designed at the flanking positions of mouse Sptrx-1 cDNA. For Sptrx-1 gene chromosomal localization one of these positive clones was labeled with digoxigenin dUTP by nick translation. This labeled probe was combined with sheared mouse DNA and hybridized to normal metaphase chromosomes derived from mouse embryo fibroblasts cells in a solution containing 50% formamide, 10% dextran sulfate and 2X SSC. Specific hybridization signals were detected by incubating the hybridized slides in fluoresceinated antidigoxigenin antibodies followed by counterstaining with DAPI.

**Northern blot analysis and *in situ* hybridization**: A mouse multiple tissue Northern blot and mouse RNA Master blots with poly(A)$^+$ RNA from different tissues were purchased from Clontech. The mouse Sptrx-1 ORF was labelled with [α-$^{32}$P]dCTP (Rediprime random primer labeling kit, Amersham) and hybridized at 60°C overnight in Ultrahyb™ Solution following the protocol provided by Ambion. The blot was also hybridized with mouse β-actin as control. The blots were scanned and quantified with the Gel Pro Analyzer program (Media Cybernetics). For *in situ* hybridization, mouse testes were frozen on dry ice, sectioned with Microm HM 500 cryostat at 14 μm and thaw-mounted onto Polysine glass slides (Menzel). The sections were stored at –20ºC until use. Four different oligonucleotide probes (based on mouse Sptrx-1 cDNA sequence) were used, all producing similar results when used separately and were usually used simultaneously to intensify the signal. Several control probes with the same length and similar GC content and specific activity were used to determine the specificity of the hybridization. *In situ* hybridization was carried out as described previously (Kononen and Pelto-Hiukko, 1997).

**Expression and purification of mouse recombinant Sptrx-1.** The ORF encoding mouse Sptrx-1 was cloned into the *BamHI-EcoRI* sites of the pGEX-4T-1 expression vector (Pharmacia) and used to transform *E. coli* BL21(DE3). A single positive colony was inoculated in 1 liter of LB medium plus ampicillin and grown at 37ºC until $A_{600}$= 0.5. The production of the fusion protein was induced by addition of 0.5 mM IPTG and growth was continued for another 3.5 h. Overexpressing cells were harvested by centrifugation and frozen until use. The cell pellet was resuspended in 40 ml 20 mM Tris-HCl, 1 mM EDTA and 150 mM NaCl plus protease inhibitor cocktail at the concentration recommended by

manufacturer (SIGMA). Lysozyme was added to a final concentration of 0.5 mg / ml with stirring for 30 min on ice. Sarkosyl (1%) was added and the cells were disrupted by 10 min sonication The supernatant was cleared by centrifugation at 15,000 x *g* for 30 min and loaded onto a glutathione sepharose 4B column (Pharmacia Biotech). Binding to the matrix was allowed to occur for 2 h at room temperature. Thrombin (5U per mg fusion protein) was used to remove GST by incubation overnight at 4°C. The resulting protein preparation was then subjected to ion exchange chromatography using a HiTrap Q column (Pharmacia Biotech) and mouse Sptrx-1 was eluted using a gradient of NaCl. Protein concentration was determined from the absorbance at 280 nm using a molar extinction coefficient of 11.290 $M^{-1}$ $cm^{-1}$. A western blot on mouse recombinant Sptrx-1 was performed as previously described for human Sptrx-1 using the same affinity purified antibodies (Miranda-Vizuete *et al*., 2001).

**Enzymatic activity assays.** Thioredoxin reductase and NADPH were used as electron donors to determine the enzymatic activity of mouse Sptrx-1 and the assay was performed essentially as described previously (Spyrou *et al*., 1997). Briefly, aliquots of mouse Sptrx-1 were added to 40 µl of a reaction mixture composed of 200 µl of Hepes (1 M), pH 7.6, 40 µl of EDTA (0.2 M), 40 µl of NADPH (40 mg / ml), and 500 µl of insulin (10 mg / ml). The reaction was initiated by the addition of 10 µl of thioredoxin reductase from calf thymus (3.0 A412 unit), and incubation was continued for 20 min at 37 °C. The reaction was stopped by the addition of 0.5 ml of 6 M guanidine-HCl, 1 mM DTNB, and the absorbance at 412 nm was measured. Human Trx-1 and Sptrx-1 were used as controls.

**Immunohistochemistry and immunofluorescence analysis**. CD mice were anesthetized, and the testis and epididymides were fixed by perfusion through the abdominal aorta and heart, respectively, in Bouin's fixative. Fixed tissues were washed extensively in 75% alcohol before being completely dehydrated in ethanol and embedded in paraffin. For light microscopy immunocytochemistry, 5 µm paraffin sections were deparaffinized and hydrated through a graded series of ethanol concentrations before immunoperoxidase localization with anti-Sptrx-1 antibody by standard procedures (see (Oko, 1998)). Staging of the cycle of the seminiferous epithelium and determining the steps of spermiogenesis was done according to the classifications of Leblond and Clermont (Leblond and Clermont, 1952).

## RESULTS

**cDNA cloning, sequence analysis, genomic organization and chromosomal localization of mouse Sptrx-1 gene.** By sequence comparison we found that Genbank EST entries AU021712 and AU021742 partially encoded a putative mouse protein with very high homology with human Sptrx-1 sequence. Therefore, we designed specific primers based on these sequences and performed 5'- and 3'-RACE PCR analysis in a mouse testis cDNA library to clone the full-length cDNA sequence of this putative protein. The complete sequence of the cDNA consists of an ORF of 1386 bp, a 5'-UTR of 315 bp including one stop codon in frame and a very short 3'-UTR of 17 bp before the poly(A)$^+$ tail (Fig. 1). Analysis of the mouse Sptrx-1 ORF identified 5 different potential start methionine residues (Fig. 1). We propose methionine 4 as the putative start site based on the similarity to human Sptrx-1 sequence. A potential polyadenylation signal is present within the ORF in close proximity to the stop codon, explaining the short 3´-UTR. Mouse Sptrx-1 ORF encodes a protein of 462 amino acids with an estimated molecular mass of 52 kDa and a pI of 5.24. It has an identical domain organization to that of the human protein: a N-terminal domain characterized by a unique arrangement organized as repeats of a 15-residue motif shortly after the start methionine and a C-terminal thioredoxin domain. As shown in Figure 1, the repeated motif has a general pattern (PKSSEDIIQ(S/P)KK(E/G)DR) which is highly conserved and rich in both basic (Lys) and acidic residues (Glu, Asp) which are responsible for the low pI and net charge at pH 7.0 of -17.28. Other features previously described for the human Sptrx-1 protein (Miranda-Vizuete *et al.*, 2001) also pertain to mouse Sptrx-1, including the presence of two additional cysteines (residues 443 and 450, respectively) in the thioredoxin domain at identical positions to that of mouse Trx-1 protein (Matsui *et al.*, 1995) (which serves as module for the rest of the members of the thioredoxin family of proteins) and human

Sptrx-1 (Fig. 2). Also similar to human Sptrx-1, mSptrx-1 contains several potential phosphorylation sites for different protein kinases and two highly scored PEST sequences for proteasome-dependent degradation centered at positions 12 and 315, respectively (Fig. 1). However, while we could not predict any coiled-coil domain within human Sptrx-1 protein, we find that mouse Sptrx-1 is predicted to organize as coiled-coil between residues 324 to 381 (we used the Lupa´s algorithm at http://psort.ims.u-tokyo.ac.jp/form2.html). A coiled-coil is a bundle of α-helices that are wound into a superhelix, which might be important for the maintenance of the oligomeric structure (Lupas, 1997). Interestingly, mouse Sptrx-1 lacks one of the repetitive domains (Figure 2), close to the C-terminal thioredoxin domain and this could be an explanation for the difference between the human and mouse protein structures.

Next, we used the mouse Sptrx-1 ORF to screen a mouse genomic library (genetic background ES129/SvJ) and identified seven BAC clones containing the mouse Sptrx-1 gene. We sequenced three of these clones in both directions and found that, similarly to humans, mouse Sptrx-1 gene contains only one intron of 525 bp located in the 5´-UTR (Figure 3A).

To determine the chromosomal localization of mouse Sptrx-1 we performed FISH analysis in mouse metaphase chromosomes using one of the above clones as probe. A initial experiment resulted in specific labeling of the middle region of a small chromosome which was believed to be chromosome 17 on the basis of DAPI staining. To confirm this point we conducted a second experiment with a probe specific for the telomeric region of mouse chromosome 17 which was cohybridized with the mouse Sptrx-1 genomic clone. This experiment resulted in the specific labeling of the telomere and the middle portion of chromosome 17 (Figure 3B). Measurement of 10 specifically labeled chromosomes 17 demonstrated that the mouse genomic clone is located at a position which is 71% of the distance from the heterochromatic-euchromatic boundary to

the telomere of chromosome 17, an area that corresponds to band 17E1.2-1.3 (Figure 3C). A total of 80 metaphase cells were analysed with 75 exhibiting specific labeling. We have confirmed this position in Celera Mouse Database (http://www.celera.com) where mouse Sptrx-1 is flanked by Vapa and Rip1 genes, both located in the middle portion of chromosome 17 (data not shown). Remarkably, Vapa protein contains a major sperm protein (MSP) domain, which is found in proteins involved in sperm motility and are capable to oligomerise to form filaments (Skehel *et al*., 2000). Moreover, several genes in the close proximity to mouse Sptrx-1 also display a testis specific expression pattern such as Ssrzf1 (spermatid-specific RING zing finger protein 1, also termed sperizin) or fert1 (fer testis tyrosine kinase) (Fischman *et al*., 1990; Fujii *et al*., 1999).

**Tissue expression of mouse Sptrx-1 mRNA.** First, we used multiple-tissue Northern blots to determine the size and tissue distribution of mouse Sptrx-1 mRNAs (using the ORF as the probe), which was only detected in mouse testis as a single band of approximately 1.4 kb in good agreement with the size of the cloned cDNA (Fig. 4A). To evaluate the possibility that mouse Sptrx-1 mRNA could be expressed in other tissues not present in these blots, we also screened an RNA dot blot containing poly(A)$^+$ RNAs from 22 different mouse tissues. Among the tissues examined, a hybridization signal was observed only in testis mRNA (Figure 4B).

To further investigate the expression pattern of mouse Sptrx-1 mRNA, *in situ* hybridization was performed in mouse testis sections. Sptrx-1 mRNA is identified in a vast majority of the seminiferous tubules (Figure 4C) and analysis of mouse testis sections at higher magnification clearly shows a strong labeling in round spermatids with no signal in the remainder of the testicular cells (Fig. 4D). This expression pattern is consistent with the *in situ* data reported for human Sptrx-1 mRNA (Miranda-Vizuete *et al*., 2001).

**Expression and enzymatic activity of mouse Sptrx-1 protein.** Recombinant human Sptrx-1 migrated in SDS-PAGE at 90 kDa size although its theoretical size is 53 kDa (Miranda-Vizuete *et al*., 2001). This apparent discrepancy can be explained by the potential α-helical structure of the N-terminal repetitive domain. Using specific polyclonal antibodies directed to the repetitive N-terminal domain of human Sptrx-1 we were able to demonstrate the presence of a similar protein in extracts of murine testis (Miranda-Vizuete *et al*., 2001). To confirm this point we produced recombinant mouse Sptrx-1 and showed that it migrates in SDS gels at a similar size to its human orthologue, and it is readily recognized by the antibodies raised against the human protein (Figure 5A, inset). We evaluated the reducing activity of mouse Sptrx-1 using NADPH and calf thymus thioredoxin reductase and found that the mouse protein is able to reduce the disulfide bonds of insulin at a similar rate to that of the human protein (Fig. 5A).

**Tissue expression and cellular localization of mouse Sptrx-1 protein.** To address whether mouse Sptrx-1 protein distribution resembles that of the human counterpart (Miranda-Vizuete *et al*., 2001), we performed immunohistochemical analysis in mouse testis sections. As shown in Figure 5B, mouse seminiferous tubules immunostain mostly in the tail region of elongating spermatids and this staining is abolished when using the antibody previously preadsorbed with the mouse recombinant protein. A more details analysis shows that mouse Sptrx-1 is expressed at late steps of spermiogenesis, coincidental with the assembly of the tail structures such as the FS or ODF (Figure 5C). This result is consistent with that obtained in rat testis sections where Sptrx-1 expression peaks at steps 14-16 (stages XIV-III) and transiently associates to the longitudinal columns of the FS (Y. Yu, R. Oko and A. Miranda-Vizuete, in preparation). Other cellular

types such as spermatogonia, spermatocytes, Leydig and Sertoli cells are devoid of signal (data not shown).

## DISCUSSION

The progress in the sequencing of mammalian genomes, including the human, has provided an invaluable tool for the discovery of new genes (Baltimore, 2001). Practically all protein families have expanded in numbers during the recent years, and this is also the case for the thioredoxin family. Thioredoxins are a class of redox proteins that function as general protein disulfide reductases by the reversible oxidation of their conserved active site (Cys-Gly-Pro-Cys) (Holmgren and Björnstedt, 1995). In mammals, five proteins with the active site CGPC have been described to date: Trx-1 which is mostly cytosolic but can translocate into the nucleus upon certain stimuli and is also secreted (Powis and Montfort, 2001); Trx-2 a mitochondrial enzyme (Spyrou *et al*., 1997); Txl-1 an ubiquitous protein of unknown function (Miranda-Vizuete *et al*., 1998) and Sptrx-1 and Sptrx-2, the first two members of the family with a tissue specific distribution in the flagellum of the human spermatozoa (Miranda-Vizuete *et al*., 2001; Sadek *et al*., 2001). In this context, we report here the cloning and characterization of the mouse Sptrx-1 gene and protein.

Mouse Sptrx-1 gene is identical in structure to its human orthologue (indicating a common ancestor originated before the rodent / primate radiation), with only one intron of about 0.5 kb that interrupts the 5´-UTR of the mRNA. Furthermore, both mouse and human Sptrx-1 mRNAs have an extremely short 3´-UTR with a potential polyadenylation signal within the ORF. However, while human mRNA contains two potential start methionine residues in frame, the mouse gene has five. Taken together, all these features point to the direction of an exquisite regulation of the Sptrx-1 mRNA expression. Indeed, the synthesis of many sperm tail polypeptides is regulated at the translational level as their transcripts are synthesized and accumulate during the haploid phase of spermatogenesis while the spermatid nucleus is still transcriptionally active. Translation

then occurs after the condensation of the spermatid nucleus (Catalano *et al.*, 2001; Oko and Clermont, 1989). Major translational regulatory mechanisms are, for instance, the presence of upstream AUG codons in the 5´-UTR prior to the main ORF (Gray and Wickens, 1998), introns harboring regulatory sequences (mostly the first intron and located within the 5´-UTRs) that modulate the expression of their mRNA (Gray and Wickens, 1998) or the control for the choice of the polyadenylation site (Zhao *et al*., 1999). All these mechanisms might account for Sptrx-1 regulation as its mRNA is mostly found in round spermatids while the protein expression starts later (at step 9) and peaks at step 15 of rat spermiogenesis cycle (Y. Yu, R. Oko and A. Miranda-Vizuete, in preparation).

Mouse and human Sptrx-1 proteins are quite similar in their overall domain structure with a N-terminal repetitive domain consisting of a 15 residue motif highly conserved among repetitions and a C-terminal domain typical of thioredoxins (Miranda-Vizuete *et al.*, 2001). However, a detailed homology analysis between the two orthologues reveals a much higher amino acid identity at the thioredoxin domain (63%) than at the N-terminal repetitive domain (42%). This difference might reflect a more strict requirement for the Sptrx-1 dependency of thioredoxin activity while the higher divergence at the N-terminus might be a consequence of specific interactions with other FS components with also a high divergence between human and mouse spermatozoa. This is further supported by the presence of a coiled-coil domain in the mouse protein, which might be important for the regulation of oligomeric structure of the native mouse protein, whereas the human protein does not require this domain. The presence of coiled-coil proteins in other sperm proteins has just been reported for ODF2 and ODF3, thus suggesting that this coiled-coil organization might be required for some fibrillar proteins that conform the sperm tail (Petersen *et al.*, 2002)

Mouse Sptrx-1 gene maps at chromosome 17, which has been shown to play a pivotal role in male fertility, as it contains a naturally occurring variant of several genes,

termed the *t haplotypes,* shown to influence male but not female fertility ((Olds-Clarke, 1997) and references therein). The variant alleles of genes at the *t haplotypes* are linked together in four inversions at the proximal part of chromosome 17 and males carrying two different *t* haplotypes are sterile because their spermatozoa exhibit severe motility defects and also are unable to penetrate zona pelucida-free oocytes. This phenotype is reduced in spermatozoa from mice carrying only one *t* haplotype, as they exhibit only mild motility and delay in penetration of zona pellucida-free oocytes (Olds-Clarke, 1997). Several gene candidates for sterility factors have been identified within the inversions of the *t* haplotypes, although their specific role in the infertility phenotype is still unknown (reviewed in (Olds-Clarke, 1997)). Mouse Sptrx-1, a novel sperm specific gene, maps at a more distal part of chromosome 17 and, therefore, outside the *t* haplotype interval. However, the Sptrx-1 locus at 17E1.2-1.3 also contains other genes with a testis specific expression such as Vapa, Ssrzf1 and fert1 (Fischman *et al*., 1990; Fujii *et al*., 1999; Skehel *et al*., 2000). Whether chance or selection has resulted in the accumulation of so many genes involved in sperm function in chromosome 17, the end result is an excellent model system for understanding the genetic basis of mammalian fertilization.

We have recently found that Sptrx-1 transiently associates to the longitudinal columns of the fibrous sheath during tail elongation in rat spermiogenesis (Y. Yu, R. Oko and A. Miranda-Vizuete, in preparation). We show here that the mouse protein is also expressed during sperm tail assembly and therefore a conserved expression pattern in mammals pertains to Sptrx-1. Several pathologies, resulting in male sterility and affecting the correct assembly of the sperm tail have been described ((Chemes, 2000) and references therein), among those, dysplasia of the fibrous sheath (DFS) is the best characterized. The main features of DFS are a marked hypertrophy and hyperplasia of random fibrous sheath components that form thick rings or broad meshes without the orderly disposition in longitudinal columns and transversal ribs that characterize the

normal FS (Chemes *et al*., 1998; Rawe *et al*., 2001). Although a genetic basis has been proposed to underlie this pathology, no genes have been thus far reported to be involved in this disease. Because of the transient association to the longitudinal columns of the FS, Sptrx-1 can be considered as candidate gene for DFS as a defect in its expression or activity might result in abnormal sperm motility due to severe abnormalities of the FS. One way to explore this possibility is the generation of knock-out mice in Sptrx-1 and the data here reported are thus the first step towards this approach.

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## LEGENDS FOR THE FIGURES

**Figure 1. Nucleotide and amino acid sequence of mouse Sptrx-1.** Three 5´-UTR ATG in frame are in boldface and boxed. The upstream stop codon in frame is in boldface and underlined. The down arrow indicates the position of the intron within the 5´-UTR. The two PEST sequences are double underlined. The thioredoxin domain is shadowed and the WCGPC active site within the domain is boxed. The putative polyadenylation signal is in boldface and underlined.

**Figure 2. Alignment of the predicted amino acid sequence of human and mouse Sptrx-1.** Identical residues are boxed. The arrowheads show the common cysteine residues in human and mouse Sptrx-1 proteins while the asterisks indicate the cysteine residues in the human protein not conserved in the mouse orthologue.

**Figure 3. Genomic organization and chromosomal localization of mouse Sptrx-1 gene.** A) The sequence of the 5´-UTR intron is shown with the conserved GT/AG dinucleotides at the intron junction in boldface. B) Fine mapping of the mouse Sptrx-1 gene shown by FISH analysis. A telomeric probe for mouse chromosome 17 is pointed out by a white arrowhead while the doublet signal for mouse Sptrx-1 is indicated by a red arrowhead, on a blue-painted full set of mouse chromosomes. C) Ideogram of G-banded mouse chromosome 17 with the possible locus for mouse Sptrx-1 gene depicted by a red arrowhead.

**Figure 4. Expression pattern of mouse Sptrx-1 mRNA.** A) Mouse multiple tissue Northern blot. The mouse Sptrx-1 probe hybridized with one mRNA species at 1.7 kilobases only in testis. β-actin was used as control. B) Mouse RNA master blot where

Sptrx-1 probe hybridized only with testis mRNA (arrow). For a complete list of mouse tissues see CLONTECH homepage (http://www.clontech.com/archive/OCT97UPD/MasterBlot.shtml). C) Strong signal for Sptrx-1 mRNA can be seen in large number of seminiferous tubules in mouse testis section. Bar, 200 μm. D) In dipped section signal for Sptrx-1 mRNA is seen in round spermatids (indicated by triangles), while pachytene spermatocytes (ps), elongating spermatids (es), Leydig cells (Lc) and other testicular cells are devoid of signal. Bar, 30 μm.

**Figure 5. Enzymatic activity, western blot and immunohistochemical analysis of mouse Sptrx-1 expression in mouse testis** A) Purified mouse Sptrx-1 was assayed for its ability to reduce insulin disulfide bonds in the presence of NADPH and calf thymus thioredoxin reductase. □, human Trx-1 reduced; O, mouse Sptrx-1 oxidized; Δ, human Sptrx-1 oxidized. The reaction was initiated by adding 5 μl of calf thymus thioredoxin reductase (50 $A_{412}$ units) and stopped after 20 min by the addition of 6M guanidine HCl, 1 mM DTNB. The experiments were repeated three times and a representative experiment is shown. The inset shows the titration of mouse recombinant Sptrx-1 protein using the antibodies raised against the human orthologue. B) Light microscopy micrograph of portions of para-formaldehyde fixed mouse seminiferous tubules either incubated with anti Sptrx-1 antibody (left panel) or antibody preadsorbed with recombinant mouse Sptrx-1 (right panel). Only the former is immunoperoxidase reactive (black precipitate), indicating that the tail immunoreactivity in the seminiferous tubular lumen is specific. Bar 10 μm. C) Stage identifiable sections through mouse seminiferous tubules immunoperoxidase-stained with affinity purified anti Sptrx-1 serum diluted 1/20. Staining (black precipitate) of the elongating sperm tails is specially evident in stages X (spermatid step 10), XI (step 11) and II (step 16) of the cycle while faint immunostaining

is first detected in stage IX (step 9). By stage V-VI (steps 17-18) immunostaining is practically absent from luminal tails. Bar 40 μm.

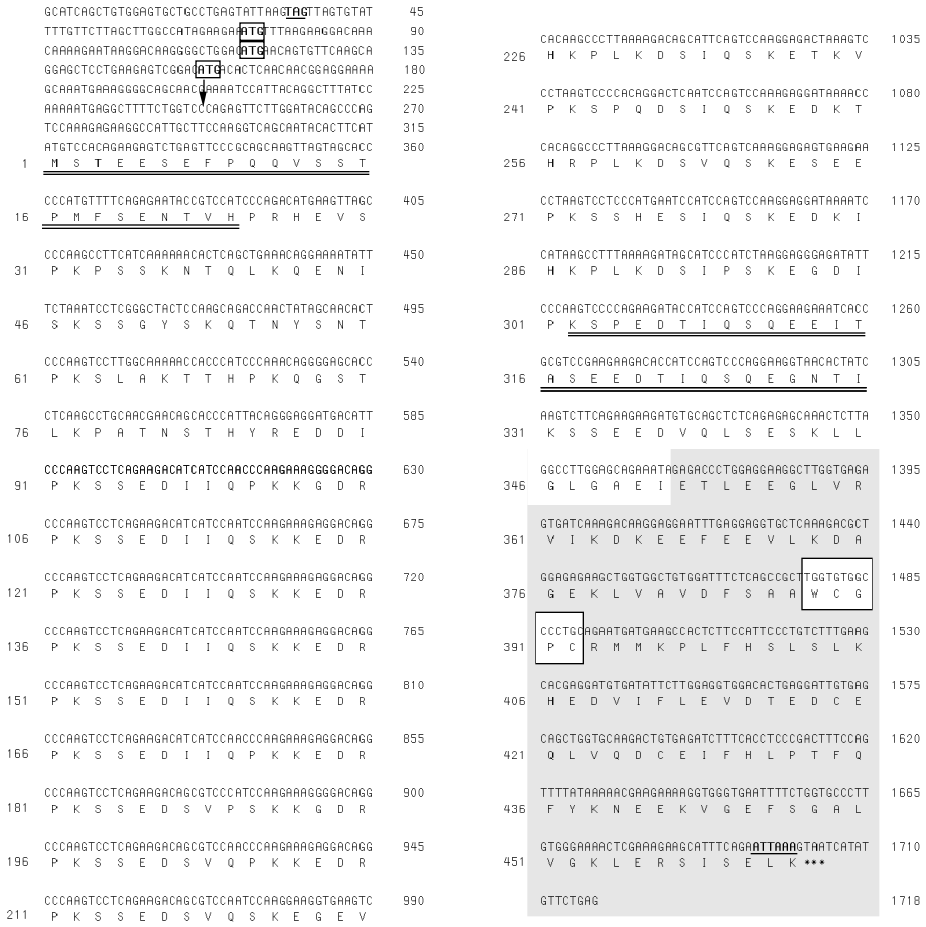

Figure 1

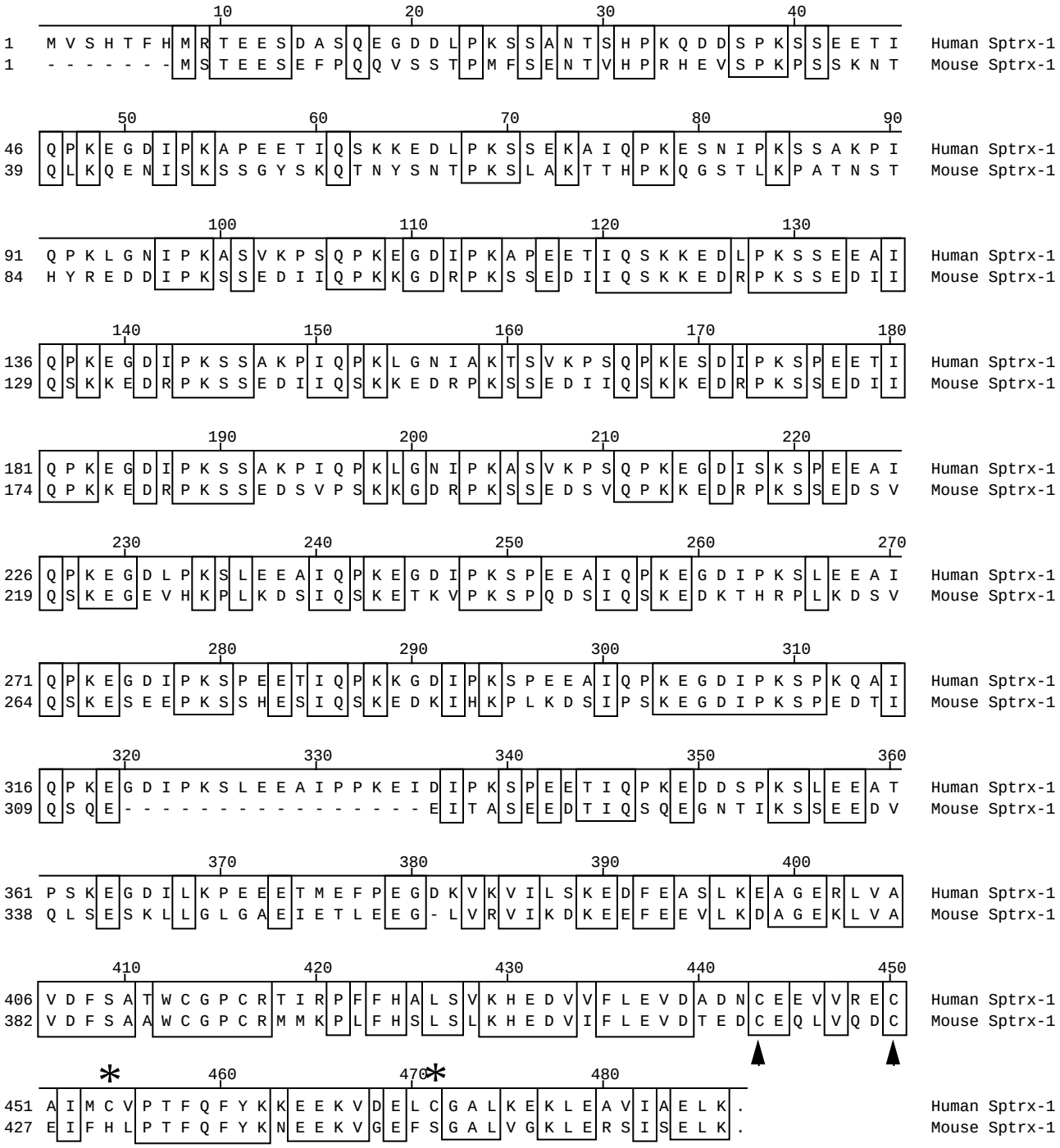
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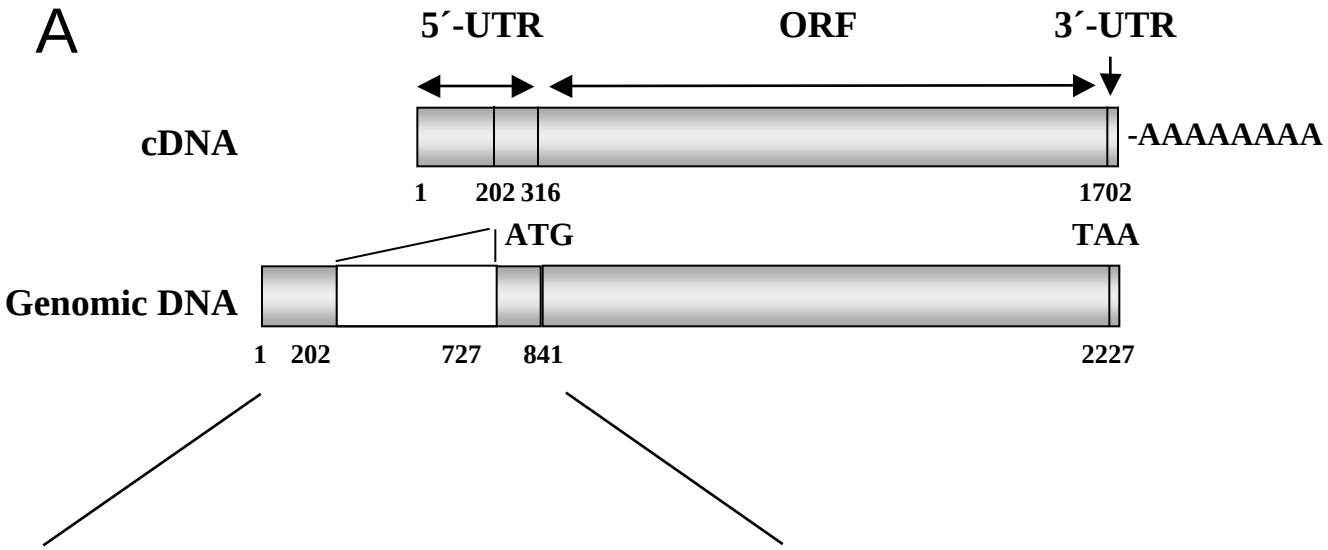
Figure 2

A

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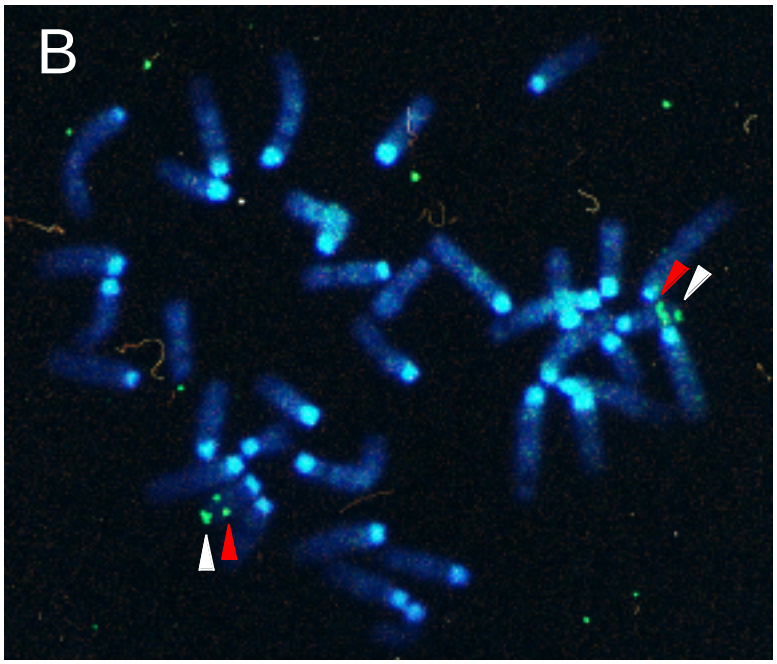
B



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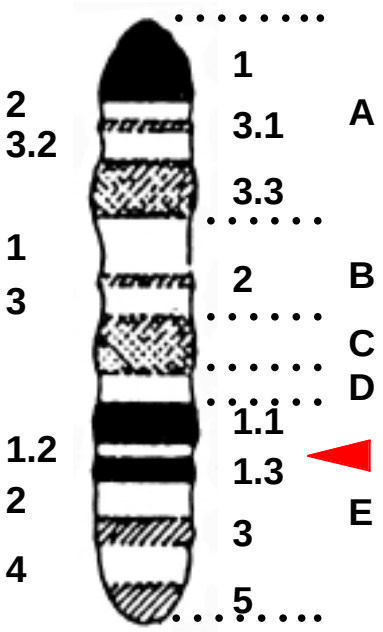


Chr. 17

Figure 3

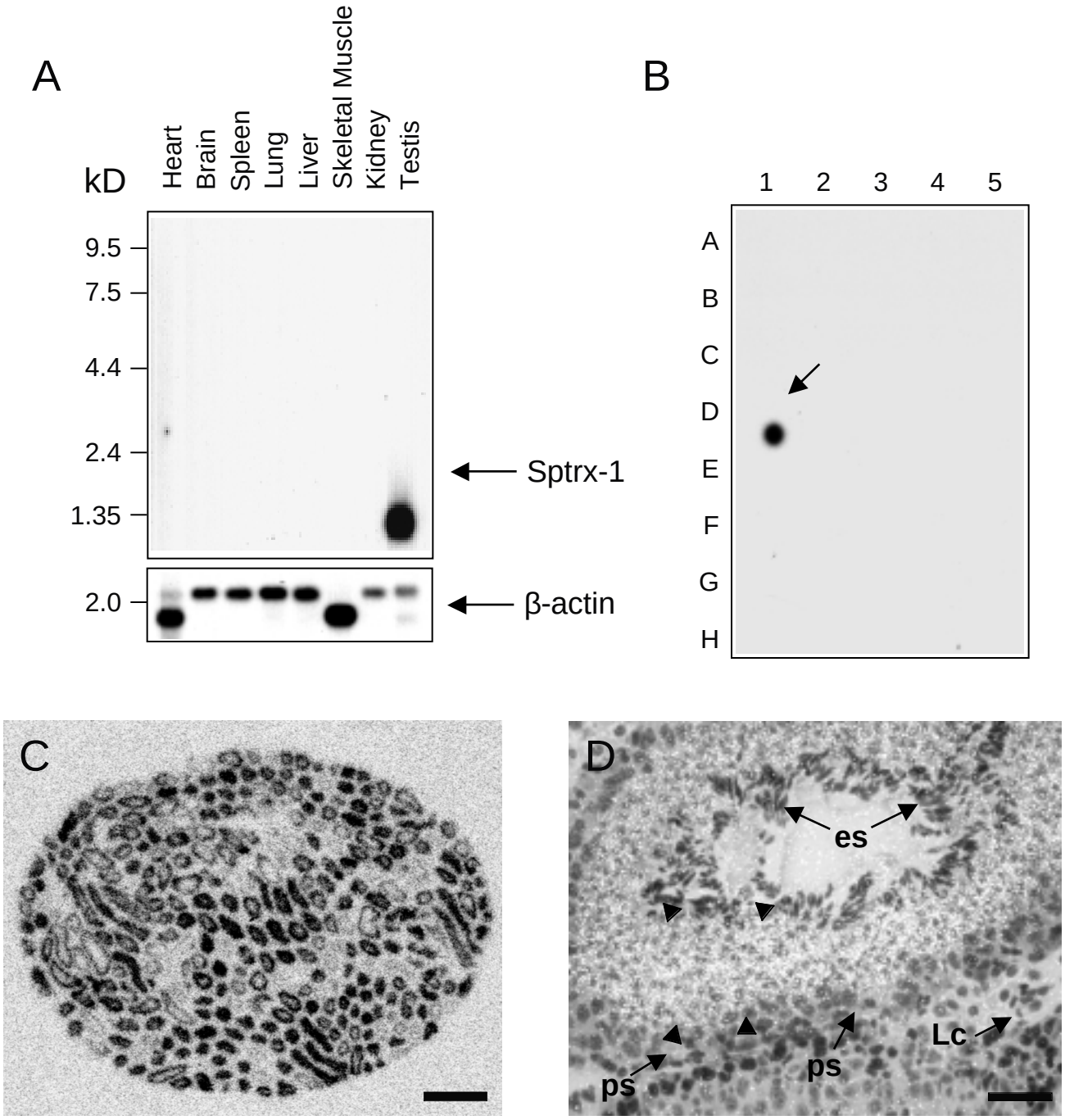
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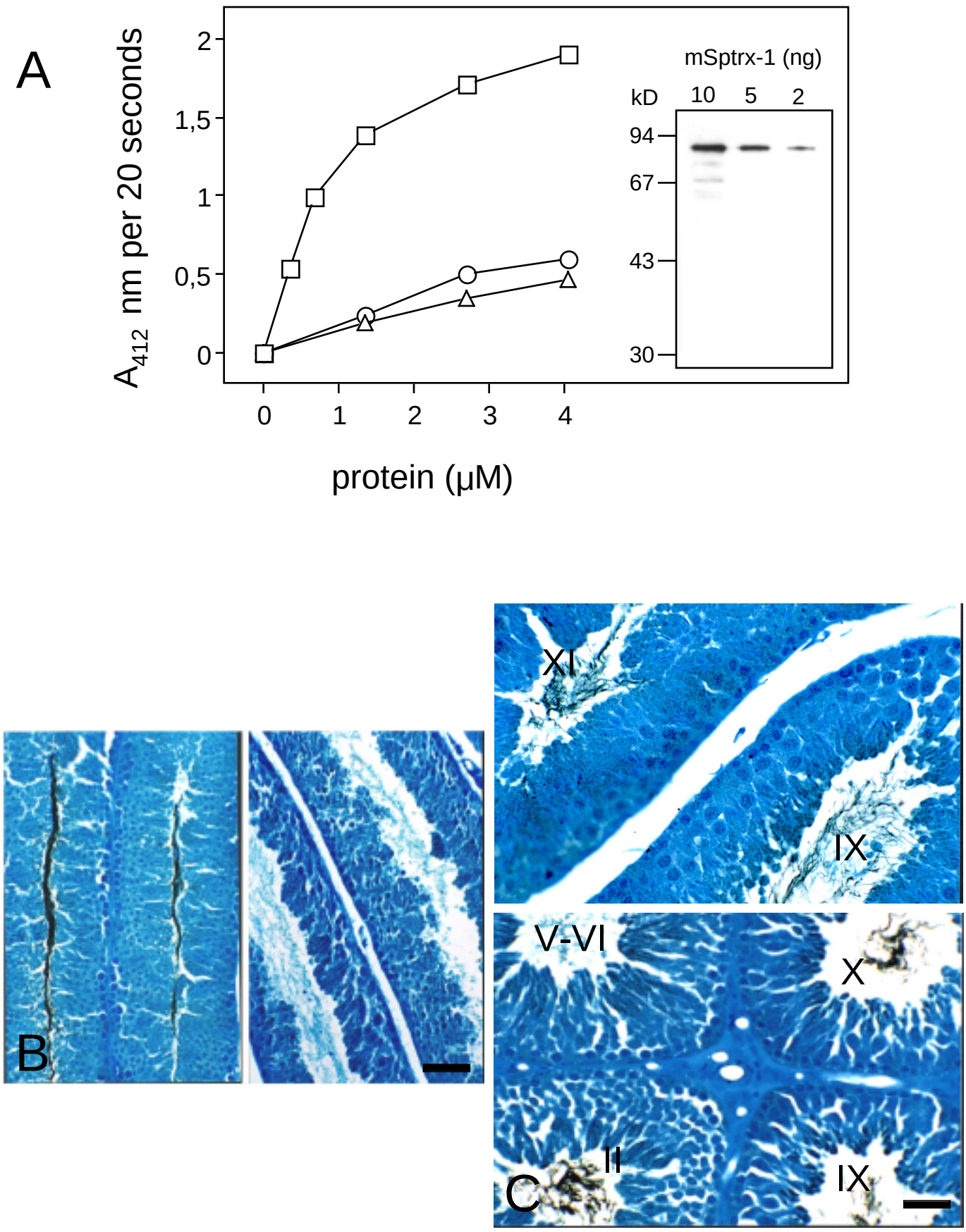
Figure 4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B
XI
IX
V-VI
X
II
C
IX

Figure 5