Spermatocyte/spermatid-specific thioredoxin-3, a novel Golgi apparatus-associated thioredoxin, is a specific marker of aberrant spermatogenesis

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Summary

Mammalian germ cells are endowed with a complete set of thioredoxins (Trx), a class of redox proteins located in specific structures of the spermatid and sperm tail. We report here the characterization, under normal and pathological conditions, of a novel thioredoxin with a germ line restricted expression pattern, named spermatocyte/spermatid-specific thioredoxin-3 (SPTRX-3). The human SPTRX-3 gene maps at 9q32, only 50 kb downstream from the TRX-1 gene from which it probably originated as genomic duplication. Consequently, human SPTRX-3 protein comprises a unique thioredoxin domain displaying high homology with the ubiquitously expressed TRX-1. Among the tissues investigated, Sptrx-3 mRNA is found exclusively in the male germ cells at pachytene spermatocyte and round spermatid stages. Light and electron microscopy show SPTRX-3 protein to be predominately located in the Golgi apparatus of pachytene spermatocytes, and round and elongated spermatids, with a transient localization in the developing acrosome of round spermatids. Additionally, increased levels of SPTRX-3, possibly due to overexpression, are observed in morphologically abnormal human spermatozoa from infertile men. Also, SPTRX-3 is identified as a novel post-obstruction autoantigen. Herein we propose that SPTRX-3 can be used as a specific marker for diverse sperm and testis pathologies. SPTRX-3 is the first thioredoxin specific to the Golgi apparatus and its function within this organelle might be related to the post-translational modification of proteins required for germ cell specific functions such as acrosomal biogenesis.
Introduction

The thioredoxin (Trx) family comprises a group of proteins that share a highly conserved active site with the Cys-Gly-Pro-Cys (CGPC) sequence and catalyze redox reactions through the reversible oxidation of the cysteine residues from a dithiol to a disulphide form. Thioredoxins are maintained in their reduced active form by the selenoprotein thioredoxin reductase (TrxR) which transfers electrons from NADPH. Together, NADPH, thioredoxins and their reductases constitute the so-called thioredoxin system (1). All organisms from lower prokaryotes to humans are equipped with distinct thioredoxin systems that participate in diverse cellular processes, including among others modulation of transcription factor-DNA binding activity, DNA synthesis, antioxidant defense, regulation of apoptosis and immune response (1). Most of the functions assigned to thioredoxins are dependent on their ability to behave as general protein disulfide reductases. Moreover, abnormal expression of thioredoxin and/or thioredoxin reductase genes has been correlated with a number of pathologies such as cancer, Alzheimer’s and Parkinson’s diseases, AIDS, etc. (2-5). Eukaryotic organisms have two ubiquitously expressed thioredoxin systems, one in cytoplasm composed of the Trx-1 and TrxR-1, and the other in mitochondria formed by Trx-2 and TrxR-2 (3). Furthermore, a large number of different thioredoxins with novel properties such as organelle specific localization in endoplasmic reticulum (6,7), tissue-specific distribution (8) and microtubule-binding properties (9), have recently been reported in mammals. This complexity is paralleled by the increasing number of splicing variants of both thioredoxin reductase genes. Thus, TrxR-1 gene has been reported to produce at least 21 different mRNAs whose conceptual translation would result in proteins targeting the cytoplasm, mitochondria and nucleus (10). Similarly, TrxR-2
gene could code potential mitochondrial and cytosolic variants depending whether the N-terminal exon coding for the mitochondrial targeting sequence is present or not (11,12).

Based on protein expression pattern, thioredoxins can be separated into two different groups: Group I encompasses all the ubiquitously expressed thioredoxins (TRX-1 (13), TRX-2 (14), TXL-1 (15,16), ERDJ5 (6,7)), found in different subcellular compartments. Group II includes tissue specific thioredoxins such as SPTRX-1 (17,18), SPTRX-2 (19,20) and TXL-2 (9), the first two specifically expressed in male germ cells and the third one in tissues harboring cilia and flagella such as spermatozoa or lung airway epithelia. In addition to mammals, germ-line specific thioredoxins have been reported in lower eukaryotic gonads, such as sea urchin spermatozoa (21) or *Drosophila melanogaster* testis and ovary (22).

The abundance of tissue-specific thioredoxins in male germ cells reflects a key role of this family of proteins in spermatogenesis, which is further supported by the recent finding of a novel testis-specific splicing variant of cytosolic thioredoxin reductase (10) and the Trx and GSSG reductase (TGR), a fusion protein of glutaredoxin and thioredoxin reductase domains predominantly expressed in testis (23). Moreover, TrxR-1 and TrxR-2 mRNA levels in mouse testis are relatively high compared to other tissues such as brain, heart or lung (24).

The major role of the fertilizing spermatozoon is to contribute the male pronucleus to the zygote. For this purpose, the round spermatid undergoes a dramatic morphological and biochemical transformation that gives rise to the highly polarized spermatozoon, through a process known as spermiogenesis (25). Mature spermatozoa are equipped with unique structures that are necessary for the spermatozoon to fulfill its function. For example, the nuclear DNA is linked to
protamines instead of histones to promote a high degree of chromatin condensation and stability (26). Additionally, the sperm head contains the acrosome, an enzyme-filled membrane-enclosed vesicle that is required for the sperm-egg binding and the penetration of egg vestments during fertilization (26). The acrosome is formed from vesicles and saccules derived from the Golgi apparatus during the initial steps of spermiogenesis (27). Another important sperm-specific structure is the tail which is responsible of energy production and mobility of the spermatozoa, and these essential tasks are achieved by the acquisition of additional cytoskeletal structures, namely the outer dense fibers and fibrous sheath, surrounding the sperm axoneme (26,28).

We report here the characterization of a novel thioredoxin, named SPTRX-3, exclusively expressed in testis and localized to the Golgi apparatus of mammalian spermatocytes and spermatids. Interestingly, SPTRX-3 is more abundant in defective spermatozoa from infertile men; in addition, it constitutes a novel sperm post-obstruction autoantigen. Hence, SPTRX-3 arises as a promising marker for several pathologies associated with male reproductive physiology.
Experimental Procedures

cDNA cloning of human, mouse and rat Sptrx-3 genes: The Basic Local Alignment Search Tool (BLAST) (29) was used to perform a survey of various databases at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) to identify new entries encoding potential novel members of the thioredoxin family. Using the sequence of human TRX-1 as bait, we found the expressed sequence tag (EST) AI188241 to encode a novel human putative thioredoxin-like protein. Based on this sequence, the nested forward primers F1 (5´-GAGGCCTGGTGTAATCATGGTACAG-3´) and F2 (5´-CAACAGGGGATTTTCATCAGCAGTACAG-3´) were used for 3´-rapid amplification of cDNA ends (RACE) in a human testis cDNA library (Clontech). Based on the sequence obtained, the nested forward primers R1 (5´-CGAGTTTTGTGTCGGCAGCTGTC-3´) and R2 (5´-CTGTACCATGATTACACCAGGCCTC-3´) were used for 5´-RACE in the same library. The resulting sequences were used to amplify by PCR all the different variants of human SPTRX-3 from the same library. The amplification products were cloned in the pGEM-Teasy vector (Promega) and sequenced in both directions.

For the mouse and rat orthologues, the same strategy was followed using human SPTRX-3 β1 sequence as bait, blasting it against the mouse and rat EST databases (http://www.ncbi.nlm.nih.gov/). By these means one rat and several mouse sequences were obtained (Rat EST AI764117.1 and Mouse EST BY714796, BF149520.1 and AV045293.2) and used to design specific primers at the putative translation initiation and stop codons, to amplify by PCR the mouse and rat Sptrx-3 β1 open reading frames (ORFs) from a mouse or rat testis cDNA libraries (Clontech) respectively.
**Northern Blot Analysis:** Human Multiple Tissue Northern (MTN) blots and human Multiple Tissue Expression (MTE) arrays with poly(A)⁺ RNA from different tissues were purchased from Clontech. The human SPTRX-3 β1 ORF was labeled with [α-³²P] dCTP (Rediprime random primer labeling kit; Amersham Pharmacia-Biotech) and hybridized at 65°C overnight in ExpressHyb Solution following the protocol provided by Clontech. The blots were also hybridized with human β-actin ORF as control. The blots were scanned and quantified with the Gel Pro Analyzer program (Media Cybernetics).

**In Situ Hybridization:** For in situ hybridization, NMRI mice (age 2, 3 and 4 weeks and 3 months) were decapitated and the testes excised. The samples were frozen on dry ice, sectioned with a Microm HM 500 cryostat at 14 μm, and thaw-mounted onto poly-L-lysine-coated glass slides. The sections were stored at -20 °C until use. Three oligonucleotide probes (CATCTTGCTCTTCGGTCCACTTCTGAGGCAC, AGATGAGTCCACATCCACCTGAGCAAACGTGAC and CCACCACAAGCTTGTTTCCAGCATCGCTGAAC) were used. All probes produced similar results when used separately and were 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 (30).

**Antibody production:** Peptide sequences for immunization were derived from the human SPTRX-3 β1 sequence. With the added aim of inducing antigenicity in murids, two different peptides named exon I: (NH₂-) MVQIITDTNEFKTFC (COOH) and exon
V: (Ac-) VTLFSRIKRIIC (-COOH) were synthesized and used to immunize rabbits (Agrisera). After four immunizations, serum was collected and polyclonal antibodies were purified by affinity chromatography using 4mg of an exon I/exon V peptide mix conjugated to UltraLink matrix from Pierce. The specificity of the antibodies was confirmed by ELISA against the peptides (data not shown) and by western-blot analysis.

**Immunocytochemistry:** Paraffin sections containing multiple human tissues (T1065; lot 9994A) were purchased from Dako. In addition, paraffin sections of human testis were used. Immunohistochemistry was performed as described previously (31) either by the ABC method or by the indirect immunofluorescence method using goat anti-rabbit fluorescein isothiocyanate (1:100, 30 min; Roche Molecular Biochemicals) as a secondary antibody. The processed tissue sections were embedded in PBS-glycerol mixture containing 0.1% p-phenylenediamine. The sections were examined with a Nikon Microphot-FXA microscope equipped with proper fluorescent filters.

Processing of Bouin’s fixed and paraffin-embedded testes and subsequent immunoperoxidase staining were described previously (8,32), with some modifications. After deparaffinization and hydration, testicular sections were either subjected or not to an antigen retrieval microwave technique ((33); the antigen retrieval was found to have no advantage). The slides were equilibrated in 300 mm glycine before immunolabeling was conducted using an avidin-biotin complex (ABC) kit (Vector Labs, Burlingame,CA). Non-specific sites were sequentially blocked with avidin and biotin blocking serum, followed by 10% normal goat serum. Primary antibody incubations were conducted overnight at 4°C. After washes in 25 mM Tris-buffered saline with 0.1% Tween, sections were incubated with biotinylated goat anti-
rabbit IgG secondary antibodies (1/200) followed by incubation with ABC using standard procedures (Vector Labs). Peroxidase reactivity was visualized by incubation in 0.05% DAB, pH 7.5 and sections were counterstained with either periodic acid Schiff (PAS) and 0.1% methylene blue or methylene blue alone. For the control, anti-SPTRX-3 antibodies were preincubated for 1 h with SPTRX-3 peptide immunogens at 5x the protein concentration of the antibody dilution, prior to immunolabeling.

Procedures for electron microscopy immunocytochemistry were described previously (8). Affinity-purified anti-SPTRX-3 antibodies were used at a 1:20 dilution, and colloidal gold-conjugated goat anti-rabbit IgG at a 1:20 dilution was the secondary antibody.

**Human Sperm Samples:** Infertile, teratospermic samples originated from patients diagnosed with male factor infertility at the infertility clinic of CEGyR, Buenos Aires Argentina. All 19 patients were previously diagnosed cases of severe male infertility/teratospermia with abnormally low sperm counts and poor sperm motility by WHO criteria as well as abnormally low percentage of normal sperm morphology by Krueger’s strict criteria. All 19 patients and their spouses underwent IVF or ICSI treatment, with only two couples achieving pregnancies, neither of which was carried to term. Control samples from 5 fertile donors with excellent sperm count, motility and morphology by WHO criteria were purchased from Fairfax Cryobank. All sperm samples were collected from informed, consenting donors and handled in accordance with the protocols approved by Internal Review Boards of the University of Missouri-Columbia and CEGyR, Buenos Aires, Argentina.

**Flow cytometry and epifluorescence microscopy:** Procedures were described in detail in (34). Briefly, sperm samples were thawed, washed by centrifugation
through TL-Hepes medium and fixed for 40 minutes in 2% formaldehyde. Sperm suspensions were permeabilized with 0.1% Triton X-100 and blocked in 5% normal goat serum (NGS), collected by centrifugation and split into two equal sperm pellets. One pellet from each donor was incubated for 40 min with the anti-SPTRX3 serum (described above; dil. 1/100), followed by goat anti-rabbit-FITC (Zymed Labs; dil. 1/80). The other half was incubated with monoclonal antibody KM 691 (Kamiya Biomedical Company; dil. 1/100) against recombinant human ubiquitin, an established sperm abnormality marker (34-36), and detected with FITC-conjugated goat anti-mouse IgM (Zymed; dil. 1/80). Blank, negative control samples were prepared by omitting the primary antibody.

Flow cytometry was performed using FACS Scan Analyzer (Becton Dickinson), set at 488 nm wavelength. Relative levels of SPTRX3- or ubiquitin-induced fluorescence in 10,000 individual cells/sample were recorded. Scatter diagrams of visible light and histograms of antibody-induced fluorescence were generated for each sample. The median values of antibody-induced fluorescence (SPTRX-3-medians or Ubi-medians) were compared between infertility patients and fertile donors using statistical tools (ANOVA; Person’s correlation) in MS Excel and SAS version 8.2. Each sample was also screened by epifluorescence microscopy. Ten microliters of each sperm pellet processed for flow cytometry were mounted under a coverslip on a conventional microscopy slide and photographed using Nikon Eclipse 800 microscope with high numerical aperture objectives, and a Cool Snap HQ CCD camera (Roper Scientific), operated by MetaMorph imaging software (Universal Imaging Corp.). Images were archived on recordable CDs and printed on an Epson Stylus Photo 1280 printer using Adobe Photoshop 6.0 software (Adobe Systems).
Western blot analysis:

Elutriated rat germ cells were prepared according to Higgy et al. (37). Elutriated fractions, of approximately 90% purity, were obtained for elongated spermatids, round spermatids and spermatocytes. Rat spermatozoa were obtained from the epididymis and their heads and tails separated and isolated by sonication and gradient centrifugation, respectively, as described by Oko (32). The different cell fractions, spermatozoa and sperm components were boiled for 10 min in sample buffer containing 2% SDS without reducing agent and centrifuged at 5000 x g for 5 min. 5% β-mercapto-ethanol was then added to the isolated supernatant before boiling again and running the supernatant on a 12% SDS-PAGE. Proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose membrane (Schleicher & Schuell. BioScience, Keene, NH) and the immunoreactivity was detected with peroxidase labeled goat anti-rabbit IgG (H+L) (Vector Laboratories, Inc., Berlingame, CA) diluted 1:10,000 (v/v) using enhanced chemiluminescent substrate (Pierce, Rockford, IL) with exposure to x-ray films.

Most mammalian species, including rats, make anti-sperm autoantibodies after obstruction of the male reproductive tract by vasectomy (38). Therefore, we used post-vasectomy rat sera in western blotting experiments to test whether Sptrx-3 was recognized by post-obstruction autoantibodies and thus could be identified as a post-obstruction autoantigen. Adult male Lewis rats weighing 225-275g (Charles River Laboratories) received a bilateral vasectomy under general anesthesia as previously described (39). Sera were collected immediately before the vasectomy and 3 months after the operation. Procedures were conducted with the approval of the Animal Care and Use Committee of the University of Virginia School of Medicine and in accordance with the Guide for the Care and Use of Laboratory Animals and other
relevant publications. Cauda epididymal spermatozoa were obtained by back-flushing fluid from the vas deferens (39). Two-dimensional (2-D) gel electrophoresis and western blotting were performed as previously described (19).

**Transient transfection of human SPTRX-3 in eukaryotic cells:** The pBI-EGFP vector (Clontech) was used to express human SPTRX-3 from a bidirectional promoter where simultaneous GFP expression gives an accurate indication of the transfection efficiency (approximately 60%). The mutagenic primers: 5’-GAGGACGCGTGCCACCATGGTACAGATTAAAG-3’ as forward primer and 5’-CTTGCGCTAGCTATTAATTCTTGAG-3’ as reverse primer were used to amplify human SPTRX-3 from pGEM-Te/SPTRX-3. The forward primer introduced an *Mlu*I site followed by a Kozac sequence (40) and the reverse primer introduced an *Nhe*I site. The amplified DNA was cloned into the *Mlu*I-*Nhe*I sites of pBI-EGFP expression vector and *E. coli* TOP-10 F’ strain was transformed with the recombinant plasmid pBI-EGFP/SPTRX-3. The plasmid was purified using the midi-prep kit (Qiagen) and sequenced. Identical strategy was used with TRX-1 as a control in all experiments.

Transfection experiments were performed with 1 µg of DNA diluted in 10 µl of H2O and 0.5 µl of 0.1M PEI (polyethyleneimine, Sigma-Aldrich). The preparation was mixed thoroughly, incubated at room temperature for 10 min and subsequently added to the medium and applied onto HEK 293 cells. The GFP images were acquired with a Leica laser scanning confocal microscope using the 488 nm line of an ArKr laser, and emitted light was collected at the 500-540 nm wavelength range.
Enzymatic Activity Assays: Enzymatic activity of SPTRX-3 was determined by its capability to reduce insulin disulfide bonds using NADPH as electron donor in the presence of human thioredoxin reductase. The activity assay was performed essentially as described elsewhere (14). In both cases, human TRX-1 was used as control.
Results

cDNA cloning, sequence analysis, genomic organization and chromosomal localization of human, mouse and rat Sptrx-3 genes.

By sequence comparison with human TRX-1 gene, we found that GenBank EST AI188241 (from human testis) encoded a putative novel thioredoxin sequence containing the conserved CGPC active site motif. Based on this sequence we designed primers to perform 5' and 3'-RACE analysis using a human testis cDNA library to clone the full-length cDNA of this novel protein. The complete cDNA sequence consists of a 384 bp ORF, a 97 bp 5'-UTR and a 376 bp 3'-UTR with one canonical poly-(A)+ sequence (Supplemental Data Fig. 1). We also cloned the rat and mouse orthologues of human SPTRX-3 cDNA aided by homology searches. Both mouse and rat Sptrx-3 ORFs encode a protein highly similar to that of human SPTRX-3 (Fig. 1).

Human 3'-RACE analysis rendered four additional products, which were also cloned. Two of them corresponded to different splicing variants of the full-length cDNA described above while the other two arose from alternative splicing of the last exon, translation of which would result in a shorter protein differing at its C-terminus (Fig. 2).

A sequence comparison in the Human Genome Sequence Data Base (http://www.ncbi.nlm.nih.gov/genome/guide/human/) mapped the SPTRX-3 genomic region at human chromosome 9q32 (entry NT_008470) just downstream to TRX-1 gene, between the markers D9S1828 and D9S1835 (based on the deCODE high resolution recombination map of human genome; (41)) (Supplemental Data Fig. 2). Using the Genomatix Software (http://www.genomatix.de/), we have determined that human SPTRX-3 gene spans 35 kb and is organized into six exons and five introns,
all according to the GT/AG rule except for the first exon/intron junction (Supplemental Data Table I). Likewise, we identified the mouse and rat genomic regions at chromosomes 4B3 and 5q22, respectively, both syntenic to that of the human gene and also organized into 6 exons and five introns (Supplemental Data Fig. 2).

Interestingly, SPTRX-3 genomic organization is identical to that of TRX-1, including exon-intron splicing sites, except for SPTRX-3 exon V, which is absent from TRX-1. This conserved organization strongly suggests a genomic duplication event as the explanation for SPTRX-3 origination, which is confirmed by a phylogenetic analysis of all human thioredoxin proteins that places TRX-1 and SPTRX-3 in the same, independent branch (Supplemental Data Fig. 3). As previously mentioned, two different forms of the last exon can be found within SPTRX-3 sequence, designated VI$$\alpha$$ for the shorter and VI$$\beta$$ for the longer exon, respectively (Fig. 2). Notably, exon VI$$\alpha$$ is located immediately upstream of exon VI$$\beta$$ within the genomic sequence as described for glutaredoxin-1 (GRX-1), a related member of the thioredoxin family (42). The remaining variants of SPTRX-3 originate from splicing of exon III alone or together with exon V (see Fig. 2 for nomenclature of these variants). We have chosen the SPTRX-3 $$\beta$$1 form for further work because the vast majority of EST entries in the human, mouse and rat databases correspond to this isoform (to which we will refer as SPTRX-3 onwards), and also because of its similarity with TRX-1.

Human SPTRX-3 ORF encodes for a protein of 127 amino acids (Fig. 1), which corresponds to a unique thioredoxin domain, with an estimated molecular weight of 14.6 kDa. A search for predicted sorting signals to specific subcellular locations at the PSORT II server (http://psort.nibb.ac.jp/) failed to identify any localization or retention signal within SPTRX-3 sequence.
Human, mouse and rat SPTRX-3 proteins show a high degree of homology (Fig. 1). Most of the amino acid residues known to be essential for catalysis, maintenance of three-dimensional structure or protein-protein interactions in previously characterized thioredoxins (43) are conserved in the three orthologues or substituted conservatively. Interestingly, human SPTRX-3, but not rodent Sptrx-3, is the first thioredoxin whose CGPC active site is preceded by an arginine residue instead of a tryptophan. The significance of this substitution remains unclear although it suggests specific properties for the human protein. With regard to structural cysteine residues (those not located at the active site), SPTRX-3 lacks Cys-62 and Cys-73, present in human TRX-1 while Cys-69 (all numbers referred to human TRX-1 residues) is conserved. Two additional cysteines at positions 97 and 98 are present in the three SPTRX-3 orthologues and one more cysteine exists at position 112 in human SPTRX-3. Intriguingly, these three additional cysteines are located in exon V, the only one missing in human TRX-1 (Fig. 1).

**SPTRX-3 mRNA expression is restricted to the male germ-line.**

Multiple Tissue Northern (MTN; Clontech) blots were used to determine the size and tissue distribution of human SPTRX-3 mRNAs using the SPTRX-3 β1 ORF as the probe. Human SPTRX-3 mRNA was detected after 48 hours of exposure only in human testis as a single band of ~0.9 kb in good agreement with the size of the cloned cDNA (Fig. 3A). No signal was obtained in any other tissues after longer exposure. To evaluate the possibility that SPTRX-3 mRNA could be expressed in other tissues not present in these blots, we also screened Multiple Tissue Expression (MTE; Clontech) Array containing poly(A)⁺ RNAs from 72 different human tissues (Supplemental Data Table II). Among the tissues examined, hybridization signal was
observed only in testis mRNA (data not shown). Regarding the expression of Sptrx-3 mRNA during mouse testis development, our in situ hybridization results show that Sptrx-3 mRNA appears during the third week post-partum and increases in the fourth week and up to adult age (Fig. 3C-F). Additionally, in situ hybridization on mouse testis demonstrated that Sptrx-3 mRNA expression was restricted to late primary spermatocytes and round spermatids with no signal in the other testicular cell types (Fig. 3G-I).

**Cellular and subcellular localization of SPTRX-3 protein in testis.**

To examine the expression pattern of SPTRX-3, antibodies were raised and affinity purified against a mix of two different peptides from human SPTRX-3 exon I and exon V (see Fig. 1). Immunohistochemistry of human testicular sections revealed SPTRX-3 expression in spermatocytes and round spermatids (Fig. 4). Close inspection revealed a focal concentration of SPTRX-3 reactivity in the cytoplasm at the apical pole of both pachytene spermatocyte and round spermatid nuclei (Fig. 4A and B). In elongated spermatids, immunostaining shifted to the distal cytoplasm (Fig. 4C). SPTRX-3 reactivity appeared to be in the Golgi apparatus, which is located in a juxta-nuclear position within these cells. To confirm this observation and to clarify the temporal sequence of SPTRX-3 expression, we used the murid model in which the stages of the cycle of the seminiferous epithelium are more clearly delineated. In rat spermatogenesis, SPTRX-3 immunoreactivity was already apparent in early pachytene spermatocytes in stage II of the cycle (Fig. 5A, block arrows) and reached peak intensity in mid-pachytene spermatocytes by stage VII (Fig. 5A). The staining pattern was similar to that in humans, but it was more clearly defined and appeared to correspond to the location of the Golgi apparatus in these cells. This juxta-nuclear
Golgi labeling was retained post-meiotically in round spermatids, in close association with the developing acrosomal granule (Fig. 5A step 2 and 7 spermatids). At the end of acrosome formation, the Golgi apparatus migrated to the distal cytoplasmic lobe of the elongating spermatid where it remained immunoreactive (Fig. 5B) until it disintegrated in the final steps of spermiogenesis. Following Golgi disintegration, immunoreactivity was seen in residual bodies that were phagocytosed the Sertoli cells (Fig. 5A, stage VII) and drawn deep into their cytoplasm (Fig. 5B, stage X).

A similar distribution pattern of Sptrx-3 labeling was found in mouse testis (Figs. 5C and D). Isolated mouse secondary spermatocytes also showed labeling of the juxta-nuclear Golgi apparatus (Fig. 5E). In addition, immunoreactivity was also found in the sperm cytoplasmic droplets (see Fig. 9I) whose main membranous components are saccular derivatives of the Golgi apparatus (44). No signal was detected in the presence of preimmune serum or an antibody preparation pre-adsorbed using a mix of both peptides, thus confirming the specificity of the antibodies and their cross-reactivity in human or rodent samples (Supplemental data Fig. 4).

Immunogold electron microscopy confirmed SPTRX-3 localization to the Golgi apparatus of both spermatocytes and spermatids and also localized SPTRX-3 within the forming acrosome (Fig. 6). The acrosome labeling was transient, lasting only to the end of the cap phase of round spermatid development. Immunolabeling within the contents of the acrosome indicates that SPTRX-3 most likely originates within the Golgi saccules.

To corroborate further the cellular types in which Sptrx-3 is expressed, we performed western blot analysis on extracts of elutriated germ cells from rat testis. A SPTRX-3 immunoreactive 24 kDa band was found in spermatozoa, sperm tails (not in sperm heads), isolated elongated and round spermatids and in spermatocytes, while
a 28 kDa immunoreactive band was only present in spermatozoa and elongated spermatids (Fig. 7). In addition, spermatids and spermatocytes displayed higher co-migrating bands that were cross-reactive and not found in spermatozoa (not shown). Although α-tubulin labeling was used as a control for load equivalence it should be considered only as a rough indicator as the different phases or cell types of spermatogenesis have varying tubulin requirements. Signal was also obtained in mature spermatozoa, which is the result of Sptrx-3 accumulation in the cytoplasmic droplet (see Fig. 9I). Furthermore, isolated sperm heads did not have detectable Sptrx-3, thus demonstrating that its association with the acrosome is transient and restricted to acrosome biogenesis. Interestingly, the Sptrx-3 antibodies recognized a protein of 24 kDa in elutriated rat germ cells, a size that is higher than the 15 kDa calculated on the basis of its amino acid composition. Western blots on bovine testis extracts also showed a major band around 24 kDa (data not shown). Sptrx-3 localization within the Golgi apparatus suggested that posttranslational modifications such as glycosylation might be responsible for the size shift. However, pretreatment of testis and sperm extracts with both N- and O-glycosidases did not modify the banding pattern (data not shown). Nevertheless, it cannot be ruled out that Sptrx-3 has an anomalous migration pattern in SDS gels, as has also been described for Sptrx-1 (18).

**Expression of recombinant SPTRX-3 and enzymatic activity assays.**

To evaluate whether human SPTRX-3 is capable to act as a reducing agent in vitro, we attempted to express recombinant SPTRX-3 in bacteria, coupling it to three different tags: Histidine, GST (Glutathione S-transferase) and MBP (maltose binding protein). Of these three approaches, only MBP fusion with SPTRX-3 rendered enough
soluble protein to carry out enzymatic assays but no activity was detected (data not shown). We then expressed human SPTRX-3 in HEK 293 cells using a bidirectional promoter that simultaneously produces green fluorescent protein as a transfection efficiency control. As shown in Supplemental data Fig 5, cell extracts of SPTRX-3 overproducing cells did not result in increased activity over the corresponding control extracts transfected with the empty vector (only expresses the green fluorescent protein) or untransfected cells, respectively. However, a positive control overexpressing human TRX-1 resulted in a two-fold increase in enzymatic activity. The lack of activity of recombinant SPTRX-3 parallels that of SPTRX-2 and TXL-2 (9,20), two other proteins located in spermatozoa, which suggests that other testis-specific factors might be required in the assay mix.

**Increased levels of SPTRX-3 in spermatozoa of infertile, teratospermic men.**

Once we had characterized SPTRX-3 expression and localization under physiological conditions throughout spermatogenesis, we set out to determine whether SPTRX-3 expression might be altered under pathological conditions. We first investigated SPTRX-3 expression in sperm of infertile men in a flow cytometric assay.

Relative levels of SPTRX-3 in semen of 19 infertile, teratospermic men (further ‘patients’) and 5 fertile donors (further ‘donors’) were compared by flow cytometry in the same trial with the detection of ubiquitin, an established marker of sperm abnormalities (34,35). The average median value of SPTRX-3-induced fluorescence (Fig. 8A), a relative (no dimension) measure of positive immune reaction in samples, was 55.3±8.7 for fertile donors, while it was three times higher (168.7±20.0) in infertility patients. Such high SPTRX-3 levels in teratospermic samples were paralleled by increased sperm ubiquitin-immunoreactivity: average ubiquitin median
values were 64.1±13.1 in fertile donors and 139.2±11.7 in patients. As a result of co-expression of ubiquitin and SPTRX-3, there was a strong positive correlation between their respective flow cytometric medians in all screened samples (r=0.85; p<0.0001). The differences in the sperm content of SPTRX-3 (Fig. 8B, C) and ubiquitin (Fig. 8D, E) between patients and fertile donors were readily identified when the samples of some donors (Fig. 8B, D) and patients (Fig. 8C, E), processed for flow cytometric analysis, were pre-screened using epifluorescence microscopy.

Sub-cellular localization of the SPTRX-3 was determined by immunofluorescence in the ejaculated human spermatozoa and in the isolated mouse spermatids. In human semen samples, the SPTRX-3 was invariably found in the nuclear vacuoles (Fig. 9A) and in the superfluous cytoplasm (Fig. 9B-E) of the morphologically abnormal spermatozoa. Similarly, mouse round spermatids (Fig 9F) contained Sptrx-3 mainly in the form of distinct cytoplasmic foci, comparable to the size and localization of the spermatid Golgi complex. An increased cytoplasmic accumulation of the Sptrx-3 was observed in the apoptotic spermatids (Fig. 9G, H), expressing the pro-apoptotic cell surface protein Fas. Sptrx3 expression in mature mouse spermatozoa (Fig. 9I) was restricted to cytoplasmic droplets. Increased expression has not been observed in defective mouse spermatozoa, since most mouse sperm defects were not due to cytoplasm retention, as seen in humans (data not shown).

**Sptrx-3 is a post-obstruction sperm autoantigen.**

Since Sptrx-2 was previously shown to be an autoantigen (19), we investigated whether Sptrx-3 might also be recognized by post-vasectomy sera, which are known to contain antisperm autoantibodies (39). The reaction of anti-SPTRX-3 serum was compared with that of a set of post vasectomy rat sera on two dimensional blots of
sperm proteins. Post vasectomy sera vary in their patterns of response to different sperm antigens (39,45), and likewise there were variations in the extent to which post vasectomy sera stained spots that co-migrated with those stained by anti-SPTRX-3. Figure 10, panel C shows an example of a serum that stained sperm proteins in a pattern closely resembling that of anti-SPTRX-3 (panel A). A different post vasectomy serum (panel E) bound a smaller number of spots that comigrated with spots stained by anti-SPTRX-3. These results indicate that Sptrx-3 is a post-obstruction sperm autoantigen, at least in some animals. Because vasectomy mimics immunization of rats with spermatozoa (46), the post vasectomy sera reacted with multiple other protein spots (39,45). Study of a limited set of post vasectomy sera showed that two sera stained spots that comigrated with those bound by anti-SPTRX-3 serum (Fig. 10), while two did not, suggesting that up to 50% of vasectomized rats may recognize Sptrx-3 as an autoantigen. While the similarity of staining patterns with anti-SPTRX-3 and a post vasectomy serum (e.g., Fig. 10, C) strongly suggests that Sptrx-3 is an autoantigen, further studies of reactivity of post vasectomy sera with recombinant Sptrx-3 protein will be necessary to determine the incidence of response of vasectomized rats to Sptrx-3 more reliably.
Discussion

Redox regulation has been shown to be important in both the physiology of normal spermatogenesis and the aetiology of some spermatogenic abnormalities (47). We and others have recently identified several novel thioredoxins and thioredoxin reductases, most of them expressed in the male germ cell line (8,10,23). Our discovery of SPTRX-3 brings further complexity to this field and particularly to the testis-specific thioredoxin system since at least five different SPTRX-3 alternative splicing variants exist. It is then reasonable and exciting to speculate about the importance of thioredoxins in regulating spermatogenesis through maintenance of the appropriate redox environment for germ cell differentiation. Human SPTRX-3 and TRX-1 genes appear to have almost identical genomic organizations, with the only difference being an extra exon (exon V) in SPTRX-3. Furthermore, both genes map to a narrow chromosomal region at 9q31-q32 and their protein sequences show approximately 50% identity. All these features strongly support the probable origin of SPTRX-3 as a genomic duplication of a TRX-1 gene ancestor. Through evolution, SPTRX-3 might have acquired the additional sequence corresponding to exon V (testis-specific) providing a novel function in spermatogenesis with the ancestor retaining its original function in a process called neofunctionalization (48). This duplication event can be traced back to at least before the hominid/rodent radiation as both genes are present in mouse and rat.

Similarly to the case with SPTRX-2 and TXL-2 (9,20), we failed to detect any thioredoxin activity when using crude extracts of cells overexpressing recombinant SPTRX-3. One possible explanation is that SPTRX-3 enzymatic activity is dependent on testis-specific thioredoxin reductases. If so, two different TrxRs might fulfill this
function: TGR, a fusion protein of glutaredoxin-like and thioredoxin reductase domains highly expressed in testis (23) or a novel testis-specific splicing variant of TrxR1 with an extra N-terminal glutaredoxin domain (10).

The most striking feature of SPTRX-3 is its localization within the Golgi apparatus of pachytene spermatocytes and, to a lesser extent, spermatids. The Golgi complex is the subcellular compartment where proteins and lipids are modified and sorted, representing the central organelle in the secretory pathway (49). Proteins destined to follow this pathway usually have a hydrophobic stretch of residues at the N-terminus which can act as a sorting motif (50). For instance, hTLP19, a member of the thioredoxin superfamily, contains a potential signal peptide for ER/Golgi localization and secretion (51). Despite SPTRX-3 expression pattern being coincidental with other Golgi-specific proteins, it lacks a clear signal sequence to follow the ER/Golgi pathway. A leaderless pathway independent of ER/Golgi has been proposed for the secretion of some other proteins including TRX-1 (52,53). Regardless of the clear sequence conservation, SPTRX-3 is not expected to follow the ER/Golgi pathway because it localizes exclusively within the Golgi apparatus and there is no evidence for ER localization or secretion of SPTRX-3. Interestingly, it has recently been shown that the acylation with fatty acids at cysteine residues is responsible for the Golgi localization of some proteins, such as GCP16, protein serine kinase PSKH1 or even GFP (54-56). As mentioned above, additional cysteines are present in the testis-specific exon V of SPTRX-3 allowing us to speculate on the possibility of Cys-acylation as the signal for SPTRX-3 translocation into the Golgi apparatus.

In somatic cells, Golgi is an upstream element of a pathway for protein sorting and exocytosis. In male germ cells, the Golgi apparatus is not directly involved in protein exocytosis/secretion. Rather, it participates in the acrosome biogenesis and the
exocytosis function is activated only in a mature spermatozoon reaching the oocyte vestments at fertilization. The assembly of the spermatid/sperm acrosome from Golgi is a complex process involving membrane vesicle docking and fusion. It requires a number of structural and signaling proteins in addition to the nascent enzymes of the acrosomal matrix, which become active only after acrosomal exocytosis. Undoubtedly, such orchestrated incorporation of a great variety of proteins into acrosomal cap requires precisely regulated posttranslational modifications including proteolytic cleavage, glycosylation, phosphorylation, and disulfide bond formation or termination. The transient labeling of SPTRX-3 in the acrosomal granule of secondary spermatocytes and acrosomal cap of round spermatids suggests a possible role of SPTRX-3 in these posttranslational modifications. Importantly, another redox protein, Peroxiredoxin 4 (PRX-4) has been proposed to be involved in acrosome biogenesis and displays an expression pattern resembling that of SPTRX-3 (57). In general, peroxiredoxins are dependent on thioredoxins as electron donors to fulfill their function and therefore, given the colocalization of both proteins, it is likely that SPTRX-3 could be the physiological reductant for PRX-4 in the acrosome.

As the SPTRX-3 labeling reaches peak activity in the Golgi apparatus of pachytene spermatocytes, long before there are any signs of proacrosomic formation, we can not exclude other functions of SPTRX-3 during pachytene spermatocyte or elongated spermatid development. Indeed, some resident Golgi proteins may be accidentally entrapped into secretory vesicles and therefore be present in the acrosome (58) without being specifically required in the structure. Thus, SPTRX-3 is probably not directly involved in acrosome formation, but might fulfill a different acrosome-related function as the post-translational modification of acrosomal enzymes in the Golgi apparatus. Alternatively, during meiosis some proteins are specifically phosphorylated.
to exert their functions and testis-specific MAK kinases governing the progression of pachytene spermatocytes through meiosis have been described (59,60). In this context, previous studies reported on the role of TRX-1 in regulating the activity of the protein kinase ASK-1 through a redox mechanism (61). Therefore, SPTRX-3 might function in a similar manner with testis-specific protein kinases.

In addition to SPTRX-3 expression in normal spermatogenesis, we have also identified unique expression patterns of SPTRX-3 in the pathogenesis of the male reproductive tract. Two-dimensional western-blot analysis showed partial co-migration of protein spots reactive to both anti-SPTRX-3 and post-vasectomy rat sera, suggesting that SPTRX-3 is a novel autoantigen. As previously described, spermatid-specific thioredoxin-2 (SPTRX-2) is found within the sperm tail fibrous sheath and is also an autoantigen (19). Despite their differential localizations, both SPTRX-2 and SPTRX-3 are not expressed until puberty in rodents, when the immune system is completely developed and, therefore, unable to recognize them as autologous-proteins.

More importantly, in mature epididymal spermatozoa, SPTRX-3 is found in the cytoplasmic droplet, where the last remnants of the cytoplasm and organelles are discarded from the sperm cell after differentiation (62). However, in some teratospermic patients SPTRX-3 accumulates and can be detected in the nuclear vacuoles and in the residual cytoplasm of morphologically abnormal spermatozoa. Thus, over-expression or retention of SPTRX-3 in mature spermatozoa might be considered as a novel phenotype marker of male infertility as well as a potential target for developing diagnostic assays and drugs.
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References

1. Arner, E. S., and Holmgren, A. (2000) *Eur J Biochem* **267**, 6102-6109.
2. Gromer, S., Urig, S., and Becker, K. (2004) *Med Res Rev* **24**, 40-89.
3. Hirota, K., Nakamura, H., Masutani, H., and Yodoi, J. (2002) *Ann N Y Acad Sci* **957**, 189-199.
4. Holmgren, A. (2000) *Antioxid Redox Signal* **2**, 811-820.
5. Powis, G., and Montfort, W. R. (2001) *Annu. Rev. Pharmacol. Toxicol.* **41**, 261-295.
6. Cunnea, P. M., Miranda-Vizuete, A., Bertoli, G., Simmen, T., Damdimopoulos, A. E., Hermann, S., Leinonen, S., Huikko, M. P., Gustafsson, J. Å., Sitia, R., and Spyrou, G. (2003) *J Biol Chem* **278**, 1059-1066.
7. Hosoda, A., Kimata, Y., Tsuru, A., and Kohno, K. (2003) *J Biol Chem* **278**, 2669-2676.
8. Miranda-Vizuete, A., Sadek, C. M., Jimenez, A., Krause, W. J., Sutovsky, P., and Oko, R. (2004) *Antioxid Redox Signal* **6**, 25-40.
9. Sadek, C. M., Jimenez, A., Damdimopoulos, A. E., Kieselbach, T., Nord, M., Gustafsson, J. Å., Spyrou, G., Davis, E. C., Oko, R., Van Der Hoorn, F. A., and Miranda-Vizuete, A. (2003) *J Biol Chem*.
10. Rundlöf, A. K., Jarnard, M., Miranda-Vizuete, A., and Arnér, E. S. (2004) *Free Radiac Biol Med* **36**, 641-656.
11. Miranda-Vizuete, A., and Spyrou, G. (2002) *Mol Cells* **13**, 488-492.
12. Rundlöf, A. K., and Arnér, E. S. (2004) *Antioxid Redox Signal* **6**, 41-52.
13. Wollman, E., d'Auriol, L., Rimsky, L., Shaw, A., Jacquot, J., Wingfield, P., Graber, P., Dessarpes, F., Robin, P., Galibert, F., Bertoglio, J., and Fradelizi, P. (1988) *J. Biol. Chem.* **263**, 15506-15512.
14. Spyrou, G., Enmark, E., Miranda-Vizuete, A., and Gustafsson, J.-Å. (1997) *J. Biol. Chem. Biophys. Res. Commun* **243**, 284-288.
15. Miranda-Vizuete, A., Gustafsson, J.-Å., and Spyrou, G. (1998) *Biochem. Biophys. Res. Commun* **211**, 1895-1907.
16. Lee, K.-K., Murakawa, M., Takahashi, S., Tsubuki, S., Kawashima, S., Sakamaki, K., and Yonehara, S. (1998) *J. Biol. Chem.* **273**, 19160-19166.
17. Jimenez, A., Oko, R., Gustafsson, J. Å., Spyrou, G., Pelto-Huikko, M., and Miranda-Vizuete, A. (2002) *Mol Hum Reprod* **8**, 710-718.
18. Miranda-Vizuete, A., Ljung, J., Damdimopoulos, A. E., Gustafsson, J. Å., Oko, R., Pelto-Huikko, M., and Spyrou, G. (2001) *J Biol Chem* **276**, 31567-31574.
19. Miranda-Vizuete, A., Tsang, K., Yu, Y., Jimenez, A., Pelto-Huikko, M., Flickinger, C. J., Sutovsky, P., and Oko, R. (2003) *J Biol Chem* **278**, 44874-44885.
20. Sadek, C. M., Damdimopoulos, A. E., Pelto-Huikko, M., Gustafsson, J. Å., Spyrou, G., and Miranda-Vizuete, A. (2001) *Genes Cells* **6**, 1077-1090.
21. Ogawa, K., Takai, H., Ogiwara, A., Yokota, E., Shimizu, T., Inaba, K., and Mohri, H. (1996) *Mol. Biol. Cell* **7**, 1895-1907.
22. Svensson, M. J., Chen, J. D., Pirrotta, V., and Larsson, J. (2003) *Chromosoma* **112**, 133-143.
23. Sun, Q. A., Kirnarsky, L., Sherman, S., and Gladyshev, V. N. (2001) *Proc Natl Acad Sci U S A* **98**, 3673-3678.
24. Jurado, J., Prieto-Alamo, M. J., Madrid-Risquez, J., and Pueyo, C. (2003) *J Biol Chem* **278**, 45546-45554
25. Oko, R., and Clermont, Y. (1998) in *Encyclopedia of Reproduction* (Neill, D., ed) Vol. 4, pp. 602-609, Academic Press, San Diego
26. Eddy, E. M., and O’Brien, D. A. (1994) in *The physiology of reproduction* (Neill, J. D., ed), pp. 29-77., Raven press, New York
27. Abou-Haila, A., and Tulsiani, D. R. (2000) *Arch Biochem Biophys* **379**, 173-182
28. Curry, M. R., and Watson, P. F. (1995) in *Gametes. The spermatozoon*. (Yovich, J. L., ed), pp. 45-69., Press Syndicate of the Univeristy of Cambridge, Cambridge
29. Altschul, S. F., and Koonin, E. V. (1998) *Trends Biochem Sci* **23**, 444-447.
30. Schultz, R., Suominen, J., Varre, T., Hakovirta, H., Parvinen, M., Toppari, J., and Pelto-Huikko, M. (2003) *Endocrinology* **144**, 767-776
31. Rybnikova, E., Damdimopoulos, A. E., Gustafsson, J.-Å., Spyrou, G., and Pelto-Huikko, M. (2000) *Eur. J. Neurosc.* **12**, 1669-1678
32. Oko, R. (1998) *Andrologia* **30**, 193-206.
33. Shi, S. R., Cote, R. J., and Taylor, C. R. (2001) *J Histochern Cytochem* **49**, 931-937
34. Sutovsky, P., Terada, Y., and Schatten, G. (2001) *Hum Reprod* **16**, 250-258.
35. Rawe, V. Y., Olmedo, S. B., Benmusa, A., Shiigi, S. M., Chemes, H. E., and Sutovsky, P. (2002) *Hum Reprod* **17**, 2119-2127
36. Rechsteiner, M., and Rogers, S. W. (1996) *TIBS* **21**, 267-271.
37. Higgy, N. A., Zackson, S. L., and van der Hoorn, F. A. (1995) *Dev Genet* **16**, 190-200
38. Alexander, N. J., and Anderson, D. J. (1979) *Fertil Steril* **32**, 253-260
39. Flickinger, C. J., Bush, L. A., Williams, M. V., Naaby-Hansen, S., Howards, S. S., and Herr, J. C. (1999) *J Reprod Immunol* **43**, 35-53
40. Kozak, M. (1996) *Mamm. Genome* **7**, 563-574
41. Kong, A., Gudbjartsson, D. F., Sainz, J., Jonsdottir, G. M., Gudjonsson, S. A., Richardsson, B., Sigurdardottir, S., Barnard, J., Hallbeck, B., Masson, G., Shlien, A., Palsson, S. T., Frigge, M. L., Thorgeirsson, T. E., Gulcher, J. R., and Stefansson, K. (2002) *Nat Genet* **31**, 241-247
42. Spyrou, G., Wilson, W., Padilla, C. A., Holmgren, A., and Miranda-Vizuete, A. (2001) *Hum. Genet.* **109**, 429-439
43. Eklund, H., Gleason, F. K., and Holmgren, A. (1991) *Proteins: Structure, function and genetics* **11**, 13-28
44. Oko, R., Hermo, L., Chan, P. T., Fazel, A., and Bergeron, J. J. (1993) *J Cell Biol* **123**, 809-821
45. Flickinger, C. J., Rao, J., Bush, L. A., Sherman, N. E., Oko, R. J., Jayes, F. C., and Herr, J. C. (2001) *Biol Reprod* **64**, 1451-1459
46. Handley, H. H., Flickinger, C. J., and Herr, J. C. (1988) *Biol Reprod* **39**, 1239-1250
47. Aitken, R. J., Ryan, A. L., Curry, B. J., and Baker, M. A. (2003) *Mol Hum Reprod* **9**, 645-661
48. Lynch, M., and Conery, J. S. (2000) *Science* **290**, 1151-1155
49. Donaldson, J. G., and Lippincott-Schwartz, J. (2000) *Cell* **101**, 693-696
50. Blobel, G. (2000) *Chembiochem* **1**, 86-102
51. Liu, F., Rong, Y. P., Zeng, L. C., Zhang, X., and Han, Z. G. (2003) *Gene* **315**, 71-78
52. Rubartelli, A., Bajetto, A., Allavena, G., Wollman, E., and Sitia, R. (1992) *J Biol Chem* **267**, 24161-24164
53. Nickel, W. (2003) *Eur J Biochem* **270**, 2109-2119
54. Ohta, E., Misumi, Y., Sohda, M., Fujiwara, T., Yano, A., and Ikehara, Y. (2003) *J Biol Chem*
55. Brede, G., Solheim, J., Stang, E., and Prydz, H. (2003) *Exp Cell Res* **291**, 299-312
56. McCabe, J. B., and Berthiaume, L. G. (1999) *Mol Biol Cell* **10**, 3771-3786
57. Sasagawa, I., Matsuki, S., Suzuki, Y., Iuchi, Y., Tohya, K., Kimura, M., Nakada, T., and Fujii, J. (2001) *Eur J Biochem* **268**, 3053-3061
58. Moreno, R. D., Ramalho-Santos, J., Sutovsky, P., Chan, E. K., and Schatten, G. (2000) *Biol Reprod* **63**, 89-98
59. Jinno, A., Tanaka, K., Matsushima, H., Haneji, T., and Shibuya, M. (1993) *Mol Cell Biol* **13**, 4146-4156
60. Roeder, G. S., and Bailis, J. M. (2000) *Trends Genet* **16**, 395-403
61. Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., and Ichijo, H. (1998) *EMBO J.* **17**, 2596-2606
62. Ramalho-Santos, J., Moreno, R. D., Wessel, G. M., Chan, E. K., and Schatten, G. (2001) *Exp Cell Res* **267**, 45-60
63. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res* **22**, 4673-4680
64. Hermo, L., Oko, R., and Hecht, N. B. (1991) *Anat Rec* **229**, 31-50
Figure legends

**Fig. 1. Alignment of the predicted amino acid sequences of human TRX-1 and human, mouse and rat SPTRX-3 proteins.** The alignment was performed using the W-CLUSTAL program included in the DNASTar package (63). Identical residues are shadowed and the thioredoxin-active site is boxed. Cysteine residues are marked with an asterisk and the arrow indicates the arginine residue at the human SPTRX-3 thioredoxin active site. Conserved amino acids (referred to Trx-1, (43)) essential for correct folding or function, apart from those of the active site, are marked with a circle. The sequences of the peptides used for rabbit immunizations are indicated by black lines.

**Fig. 2. Human SPTRX-3 splicing variants.** A) Comparative exon organization between human TRX-1 and the five different human SPTRX-3 splicing variants identified in this study. The exon organization is identical for the first four exons preceding SPTRX-3 exon V, which is not present in TRX-1 sequence. Alternative usage of two forms of exon VI defines α and β variants of SPTRX-3. B) PCR amplification of the different human SPTRX-3 ORF splicing variants.

**Fig. 3. Expression pattern of Sptrx-3 mRNA.** A) Human Multiple Tissue Northern blot was hybridized with human SPTRX-3 β1 ORF probe resulting in one labeled mRNA species at 0.9 kb only in testis. β-actin was used as control. B) In situ hybridization of mouse liver, used as negative control tissue, shows no signal. C) At two weeks no staining is present in mouse testis. D) At three weeks several seminiferous tubules (arrowheads) are clearly labeled. E) At four weeks all tubules are...
strongly stained. F) In adult mouse, all seminiferous tubules exhibit intense signal while interstitial tissue (arrows) is negative. G) In a dipped section of three weeks old testis, late primary spermatocytes (arrowheads) show strong signal while Leydig cells (arrows) are not labeled. H) In adult testis strong signal is seen in round spermatids (rs) but elongating spermatids (es) are devoid of labeling. I) High magnification showing grains over round spermatids (rs) and late primary spermatocytes (arrowheads) while basally situated spermatogonia and Sertoli cells (arrows) are not labeled. Bar in the figure represents 0.4mm (B,C), 0.6mm (D, E, F), 40μm (G,H) and 15μm (I). P.B.L., peripheral blood leukocytes.

**Fig. 4. Expression pattern of SPTRX-3 protein in human testis.** Human testicular sections immunoperoxidase stained with anti-Sptrx 3 antibody and counterstained with periodic acid-schiff (PAS) and methylene blue. A) A section through seminiferous tubules in various stages of the sixth stage cycle of the seminiferous epithelium. Immunoperoxidase staining is restricted to pachytene spermatocytes and both round and elongated spermatids. In both pachytene and round spermatids the immunoreactivity appears peripheral to the nucleus with a predominately polar orientation, suggestive of the Golgi apparatus. R, round spermatids; P, pachytene spermatocytes. E, elongated spermatids. B) A higher magnification section through a seminiferous tubule whose lower portion is in stage II of the cycle. The arrows point to the immunoperoxidase reactions at polar ends of round spermatid nuclei. C) Section through a seminiferous tubule in stage V. Immunoperoxidase reactivity is associated with the periphery of the pachytene spermatocyte nuclei (asterisks) and also within the cytoplasmic lobe of elongated spermatids (arrows). The nuclei of cone shaped,
elongated spermatids (E) absorb the methylene blue and therefore appear dark blue. Bars, 10 μm.

**Fig. 5. Expression pattern of Sptrx-3 in mouse and rat testis.** Roman numerals refer to the stages of the cycle of the seminiferous epithelium A) Rat seminiferous tubules in stages II and VII of the cycle immunoperoxidase-stained with anti-Sptrx3 antibody and counterstained with methylene blue. In Stage II the Golgi apparatus (GA) of step 2 round spermatids (RS) are immuno-reactive and are in a juxta-nuclear (N) position associated with the formation of the acrosome. Immunostaining of the Golgi apparatus (block arrows) of early pachytene spermatocytes is also apparent. In stage VII the immuno-reactive Golgi apparatus of the step 7 round spermatid begins to dissociate from the newly assembled acrosome (see GA-RS in white font). Lower in the epithelium, larger Golgi Apparati (GA) of mid-pachytene spermatocytes (P) are immunostained intensely. Higher in the epithelium, darkly immunostained residual bodies (RB) are evident. Bar, 10 μm. B) Section through a rat seminiferous tubule in stage X of the cycle, immunoperoxidase stained with anti-Sptrx-3 antibody and counterstained with PAS and methylene blue. Bolded arrows point to the immuno-reactive Golgi apparatus next to the nuclei of pachytene spermatocytes while lined arrows point to the immuno-reactive Golgi apparatus of step 10 spermatids that are no longer associated with acrosome development but have migrated to the distally located, cytoplasmic lobe. The reactivity in the latter is slowly diminishing. Note that the PAS counterstained acrosomes of step 10 spermatids are not immuno-reactive. Residual bodies deep in the Sertoli cytoplasm are still intensely immuno-reactive. E, elongating spermatids; P, pachytene spermatocytes. Bar, 10 μm. C,D) Mouse testicular sections, immunoperoxidase stained with anti-Sptrx-3 and counterstained
with PAS and methylene blue. Arrows point to immunostained Golgi apparatus in its supra-nuclear position adjacent to pachytene spermatocyte nuclei (C & D) or round spermatid nuclei (C). E, elongated spermatids; P, pachytene spermatocytes; R, round spermatids. Bars, 10 μm. E) Mouse isolated pachytene spermatocytes express Sptrx-3 (red) in association with the Golgi apparatus or the acrosomic granule.

**Fig. 6.** Electron micrograph of a step 3 and 6 rat spermatid immunogold labeled with anti-SPTRX3 antibody. Labeling is specific to the Golgi apparatus (GA) and forming acrosome of the round spermatid. AG, acrosomic granule; AV, acrosomic vesicle; AC, acrosomic cap; N, nucleus of spermatid. Bar, 0.2 μm.

**Fig. 7.** Immunoblot developmental analysis of Sptrx-3. Western blot lanes loaded with SDS extracts of whole sperm (WS), sperm tails, and isolated elongated (ES) and round (RS) spermatids and spermatocytes (SP), immunoreacted with anti-alpha-tubulin (64) and anti-SPTRX-3 antibodies. The western blot was stripped and re-probed to accommodate immunostaining with both antibodies.

**Fig. 8.** SPTRX-3 is over-expressed in the spermatozoa of infertile, teratospermic men. A) Dual flow cytometric analysis of SPTRX-3 and sperm quality marker ubiquitin in a semen sample from fertile donor (left column), and an infertile, teratospermic patient (center). Blank, negative control sample (right column) was generated using semen sample from the same fertile donor as shown in left column. Top row shows histograms and median values of relative fluorescence in samples processed with anti-SPTRX-3 serum and an appropriate fluorescently conjugated secondary antibody; middle row shows histograms and medians of the same subjects’ sperm
samples processed with anti-ubiquitin antibodies. Empty curves in the ‘patient’ and ‘blank’ histograms represent the histogram of the fertile donor. SPTRX-3 medians shown are the actual values for these particular samples and should not be confused with average median values described in Results. Bottom row shows scatter diagrams of visible light, illustrating the prevailing cell size in normal and infertile sample. The increased number of spots (each dot represents one cell) in the upper right corner of the histogram of the patient’s sample (arrows, center) is indicative of a large number of morphologically abnormal, large spermatozoa, including those with superfluous cytoplasm. B-E) Epifluorescence microscopy of SPTRX-3 (B, C) and ubiquitin (D,E) in sperm samples of a fertile donor (B,D) and a teratospermic patient (C,E; same subjects as shown in A). Corresponding differential interference contrast (DIC) images are shown in panels B’-E’.

**Fig. 9. Expression of SPTRX-3 in human spermatozoa and in mouse spermatids and spermatozoa.** In human sperm samples, SPTRX-3 (red) is invariably found in the nuclear vacuoles (A) and in the superfluous cytoplasm (B-E) of morphologically abnormal spermatozoa. Similarly, mouse round spermatids (F) express Sptrx-3 mainly in distinct cytoplasmic foci (probably Golgi), with increased expression in presumably apoptotic spermatids (G, bottom H), expressing the pro-apoptotic cell surface protein Fas (green). Sptrx-3 expression in mature mouse spermatozoa (I) is restricted to cytoplasmic droplets. Increased expression has not been observed in defective mouse spermatozoa.

**Fig. 10. Two dimensional (2-D) western blots of rat sperm proteins stained with anti-SPTRX-3 serum or post vasectomy rat sera.** The arrows and arrowheads
denote comparable points in each of the three blots. A) Antiserum raised against SPTRX-3 peptides bound two main constellations of proteins, at 24-29 kDa, pI 4.7-7.2, and 17-22 kDa, pI 5.8-7.5. The post vasectomy serum shown in panel C bound trains of protein spots at 24-29 and 17-22 kDa that migrated in a very similar pattern to that for SPTRX-3 (A). The second post vasectomy serum (E) showed a different pattern; although it did not react with the entire constellation of protein spots, it did stain spots that co-migrated with those at the acidic end of the 20-25 kDa train (short arrow) and at the basic end of the 17-22 kDa train (arrowhead). Blots stained with the corresponding pre-immune or pre-vasectomy sera (B, D, and F) showed no reaction or very faint staining; although two spots at 17-18 kDa, pI ~7.0-7.3, were lightly stained in panel F, their staining with the corresponding post vasectomy serum was much increased (E).
Supplemental Data Fig. 1. Nucleotide and amino acid sequence of human SPTRX-3. The nucleotide numbers are indicated on the right and the amino acid numbers are shown on the left. The two stop codons in frame with the ATG are underlined. The thioredoxin active site is boxed. The canonical poly-(A)^+ sequence is displayed in bold.

Supplemental Data Fig. 2. Chromosomal localization of the human, mouse and rat Sptrx-3 orthologues. The human SPTRX-3 gene maps to 9q32 region, is flanked by the genes TRX-1 and MUSK1 and is organized into six exons with two different alternative forms for the sixth exon (VIα and VIβ). Similarly, mouse Sptrx-3 gene is located between the genes Trx-1 and a polydomain-pending gene at the 4B3 region. Finally, rat Sptrx-3 maps at 5p24 region flanked by Trx-1 and a polydomain-pending gene as well as the mouse orthologue.

Supplemental Data Fig. 3. Unrooted phylogenetic tree of the deduced amino acid sequences of all human thioredoxins. The alignment of the sequences was performed using the ClustalX program and the tree was generated with 10000 bootstrap trials. The bootstrap values are indicated at the nodes.

Supplemental Data Fig. 4. Immunostaining of human seminiferous tubules with anti-SPTRX-3 antibody. A) Immunoreactivity, as described above, appears in pachytene spermatocytes, round and elongated spermatids with the labelling peak reaching the pachytene spermatocyte step. B) Anti-SPTRX-3 antibodies pre-absorbed with the peptides against they were raised show no immunoreactivity. Bars, 10 μm.
Supplemental Data Fig. 5. Mammalian expression of recombinant human SPTRX-3 and thioredoxin activity analysis. Left panel shows the bidirectional expression of human TRX-1 and SPTRX-3 with GFP in HEK 293 cells. Note that transfection efficiency is comparable with the three different constructs. Right upper panel shows the western-blot analysis of TRX-1 and SPTRX-3 expression. Anti-TRX-1 antibody detects the endogenous protein in pBI-EGFP and non-transfected lanes as well as the TRX-1 overexpression in pBI-EGFP/TRX-1 lane. Anti-SPTRX-3 detects SPTRX-3 expression only when HEK 293 cells are transfected with the pBI-EGFP/SPTRX-3 construct. Right lower panel shows the thioredoxin activity assay in the crude protein extracts of transient transfections with the empty vector, with TRX-1 or SPTRX-3. TRX-1 has been used as a positive control and protein extracts from HEK 293 cells and transfections with the empty vector were used as negative controls.
| Gene   | 5'-UTR  | ORF    | 3'-UTR  |
|--------|---------|--------|---------|
| TRX-1  | ATG     | WCGPC  | TAG     |
|        | I       | II     | III     |
|        | IV      | V      | V       |
|        | SPTRX-3 | I      | II      |
|        | SPTX-3  | III    | IV      |
|        | SPTX-3  | V      | VIβ     |
| SPTX-3 | I       | II     | III     |
|        | IV      | V      | VIβ     |
| SPTX-3 | I       | II     | III     |
|        | IV      | V      | VIβ     |

Jiménez et al., Figure 2
Jiménez et al., Figure 3A
Jiménez et al., Figure 4
Jiménez et al., 2004. Figure 5
Jiménez et al., Figure 6
Jiménez et al., Figure 7
Jiménez et al., Figure 8
Jiménez et al., Figure 9
Jiménez et al., Figure 10
TGATTTCCAGGCAGGATACTGTAATAAATAGGAGACAGCTACAGTGATCCAACTAAACCA
1
MVQIIKDT

ACAGGGGATTTTCATCAGCACTTCCCTGTTGTAATCATGGTACAGATTATTAAGACACG
120

AATGAATTAAAAACATTTTTGACAGTGCCCCAGACAAACTCCGACATGTTTCAATTTCT
9NEFKTFLTAAHGKLAVQFS

TCGAACGGTGTTGGCTCTGCAAAAGGATGTTTCTGTTTTCCATGCTATGCTGTGAAA
240

MVICITE

TACCAAAATGATATTATTGCTAATGTGATGTAACAATTTTCCGAGCTGGCTGAACCT
300

NFKEFTFAQLSKPMHFSV

TCGAAACGGTGTTGGCTCTGCAAAAGGATGTTTCTGTTTTCCATGCTATGCTGTGAAA
240

SKRCPPMKRMFPVFHAMS

MVICITE

TTCTCAAGAATCAAAGAAATAATTGCTGTTTATAGAAGTGCTGCTGACACTGATT
420

FSRIKRIICCYRSFGMSNL

TTTGAGTTTTTGTGAGGCCATGTCTAAAAATTGGAACAGCTAAAGATTAAGGAA
480

FEFCGADAKKLEAKTQELM.

GCTGATCTCCAAGGCCAATACATTGTGACATTGAAAAGGCAAGAGCAATAATGTGTT
540

TGCTTTTACATTCCAGCAATACTGTAGTGGCTACTGCTCTCTCTCTTAATGCTTTTGCT
600

GTTGAAATCCATAATGCAATCTCACTGCAAAATTTGCTAAAACACTGTTGGAATATGAGAGA
660

TTGCATCTATTCCATTTCCAACTTGGGCTTCTCTACTGCTCAGCACACTCCTTGGGC
720

AGTGGTTTTATGACCTTCACAAGAGAGGTCTCTGAGCAGCTAATGATTACAGGTCATCAA
780

GATTTCAGAGATTAGCAAGAGTCACATTCTCTCCAGTCAGGTAACAAATAATTGT
840

CTTAGTGCCTCGTG

856
Jiménez et al., Supplemental Data Figure 2
Jiménez et al. Supplemental Data Figure 4
Jiménez et al., Supplemental Data Figure 5
**Supplemental Data Table I.** Intron-exon organization of human SPTRX-3.

| Exon | Exon size (bp) | Intron | Intron size (kb) | Sequence at exon/intron junction | AA | Position | Type |
|------|----------------|--------|------------------|----------------------------------|-----|----------|------|
| 1    | 121            | 1      | 3.4              | ACACGgcaag ttcagAATGA            | T/N | 8/9      | 0    |
| 2    | 105            | 1      | 4.9              | TCCATgtgag tgaagGCTAT            | H/A | 43/44    | 0    |
| 3    | 60             | 2      | 2.9              | CTCCGgtgag cccagGAGCT           | P/E | 63/64    | 0    |
| 4    | 66             | 3      | 21.6             | AGAAGgtgag ctagGTAAC            | K/V | 85/86    | 0    |
| 5    | 66             | 4      | 0.9              | GGTAAggtgaa aacagATTTT          | F   | 109      | 1    |
| 6    | 439            | 5      | 0.9              | GGTAAgtgaa aacagATTTT          | F   | 109      | 1    |
**Supplemental Data Table II.** List of tissues contained in the MTE Array 2 (Clontech).

| Tissue                                                                 |
|------------------------------------------------------------------------|
| whole brain                                                            |
| cerebral cortex                                                        |
| frontal lobe                                                           |
| parietal lobe                                                          |
| occipital lobe                                                         |
| temporal lobe                                                          |
| paracentral gyrus of cerebral cortex                                   |
| pons                                                                   |
| cerebellum left                                                        |
| cerebellum right                                                       |
| corpus callosum                                                        |
| amygdala                                                               |
| caudate nucleus                                                        |
| hippocampus                                                            |
| medulla oblongata                                                      |
| putamen                                                                |
| accumbens nucleus                                                      |
| thalamus                                                               |
| heart                                                                  |
| aorta                                                                  |
| atrium, left                                                           |
| atrium, right                                                          |
| ventricle, left                                                        |
| ventricle, right                                                       |
| interventricular septum                                                |
| apex of the heart                                                      |
| esophagus                                                              |
| stomach                                                                |
| duodenum                                                               |
| jejunum                                                                |
| ileum                                                                  |
| ileocecum                                                              |
| appendix                                                               |
| colon, ascending                                                       |
| colon, transverse                                                      |
| colon, descending                                                      |
| rectum                                                                 |
| kidney                                                                 |
| skeletal muscle                                                        |
| spleen                                                                 |
| thymus                                                                 |
| peripheral blood leukocyte                                             |
| lymph node                                                             |
| bone marrow                                                            |
| trachea                                                                |
| lung                                                                   |
| placenta                                                               |
| bladder                                                                |
| uterus                                                                 |
| prostate                                                               |
| testis                                                                 |
| ovary                                                                  |
| liver                                                                  |
| pancreas                                                               |
| adrenal gland                                                          |
| thyroid gland                                                          |
| salivary gland                                                         |
| promyelocytic leukemia, HL-60                                           |
| Hela S3                                                                |
| chronic myelogenous leukemia, K562                                     |
| lymphoblastic leukemia, MOLT-4                                         |
| Burkitt's lymphoma, Raji                                               |
| Burkitt's lymphoma, Daudi                                              |
| colorectal adenocarcinoma, SW480                                       |
| lung carcinoma, A549                                                   |
| fetal brain                                                            |
| fetal heart                                                            |
| fetal kidney                                                           |
| fetal liver                                                            |
| fetal spleen                                                           |
| fetal thymus                                                           |
| fetal lung                                                             |
Spermatocyte/spermatid-specific thioredoxin-3, a novel golgi apparatus-associated thioredoxin, is a specific marker of aberrant spermatogeneration

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