Sptrx-2, a fusion protein composed of one thioredoxin and three tandemly repeated NDP–kinase domains is expressed in human testis germ cells

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Abbreviations: 7-AAD, 7-aminoactinomycin D; DAD, dynein arm deficiency; EST, expressed sequence tag; GFP, green fluorescent protein; GST, glutathione S-transferase; IC1, intermediate chain-1; ORF, open reading frame; PCD, primary ciliary dyskinesia; PEI, polyethylenimine; RACE, rapid amplification cDNA ends; Trx, thioredoxin; TrxR, thioredoxin reductase; UTR, untranslated region.

Running title: Sptrx-2, a novel sperm specific thioredoxin

Human Sptrx-2 GenBank accession number: AF305596

ABSTRACT

Background: Thioredoxins (Trx) are small redox proteins that function as general protein disulfide reductases and regulate several cellular processes such as transcription factor DNA binding activity, apoptosis and DNA synthesis. In mammalian organisms, thioredoxins are generally ubiquitously expressed in all tissues with the exception of Sptrx which is specifically expressed in sperm cells (Miranda-Vizuete *et al.* 2001).

Results: We report here the identification and characterization of a novel member of the thioredoxin family, the second with a tissue-specific distribution in human sperm, termed Sptrx-2. The Sptrx-2 ORF encodes for a protein of 588 amino acids with two different domains: an N-terminal thioredoxin domain encompassing the first 105 residues and a C-terminal domain composed of three repeats of a NDP kinase domain. The Sptrx-2 gene spans about 51 kb organized in 17 exons and maps at locus 7p13-14. Sptrx-2 mRNA is exclusively expressed in human testis, mainly in primary spermatocytes while Sptrx-2 protein expression is detected from the pachytene spermatocytes stage onwards, peaking at round spermatids stage. Recombinant full-length Sptrx–2 expressed in bacteria displayed neither thioredoxin nor NDP kinase enzymatic activity.

Conclusions: The sperm specific expression of Sptrx-2, together with its chromosomal assignment to a position reported as a potential locus for flagellar anomalies and male infertility phenotypes such as primary ciliary dyskinesia suggests that it might be a novel component of the human sperm axonemal organization.

INTRODUCTION

Thioredoxins (Trx) are small ubiquitous proteins that are conserved in all organisms through evolution. They are characterized by the sequence of their highly conserved active site Cys-Gly-Pro-Cys (CGPC) and participate in different cellular mechanisms, mainly redox reactions by the reversible oxidation of their active site from the dithiol form to disulfide (Arner & Holmgren 2000; Powis & Montfort 2001). To be active, thioredoxins must be reduced 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 & Björnstedt 1995). All organisms so far investigated contain several thioredoxins. Thus, *E. coli* has two thioredoxins, both cytosolic, and the lower eukaryote *Saccharomyces cerevisiae* has three, two cytosolic and one mitochondrial (Grant 2001; Laurent *et al.* 1964; Miranda-Vizuete *et al.* 1997a; Pedrajas *et al.* 1999). The increasing complexity of higher eukaryotes is also reflected in the thioredoxin family. For instance, a homology search in *Caernohabditis elegans* and *Drosophila melanogaster* genomes identify up to eight proteins containing the active site WCGPC. The number of thioredoxins in mammalian organisms is lower although it is expected to increase once the human genome sequence has been fully searched. Currently, in humans there are three ubiquitous forms, Trx-1, Trx-2 and Txl-1 and only one tissue specific Sptrx expressed in spermatozoa (Holmgren & Björnstedt 1995; Miranda-Vizuete *et al.* 2000; Miranda-Vizuete *et al.* 1998; Miranda-Vizuete *et al.* 2001).

Initially discovered as an electron donor for ribonucleotide reductase, an essential enzyme in DNA synthesis, many other functions have since then been ascribed to thioredoxins. They 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 & Montfort 2001).

Based on protein sequence organization two distinct groups can be identified within the thioredoxin family. Group I includes those proteins that exclusively encode a thioredoxin domain while group II is composed of fusion proteins of thioredoxin domains plus additional domains. Among those belonging to group I are *E. coli* Trx-1, the three yeast thioredoxins or mammalian Trx-1 and Trx-2 (Grant 2001; Holmgren & Björnstedt 1995; Laurent *et al.* 1964; Spyrou *et al.* 1997). Examples of group II thioredoxins are *E. coli* Trx-2, that has an N-terminal extension that resembles the structure of a zinc finger domain (Miranda-Vizuete *et al.* 1997a) or the DLC14 and DLC16 of *Chlamydomonas reinhardtii* flagellar outer dynein arm that possess a thioredoxin domain followed by a C-terminal extension of unknown function (Patel-King *et al.* 1996). Also belonging to group II are mammalian Txl-1 and Sptrx which have additional domains displaying no homology with any other protein in the databases (Miranda-Vizuete *et al.* 1998; Miranda-Vizuete *et al.* 2001). Another member of group II is sea urchin IC1 protein, a sperm outer dynein arm intermediate chain which is composed of an N-terminal thioredoxin domain followed by three nucleoside diphosphate (NDP) kinase domains (Ogawa *et al.* 1996).

NDP-kinase represents a large family of structurally and functionally conserved proteins from bacteria to humans also known as nm23 that, in general, catalyzes the transfer of γ-phosphates between nucleosides and deoxynucleoside di- and tri-phosphates, therefore playing a pivotal role in maintaining a balanced pool of nucleotides (Postel *et al.* 2000). In humans, eight different members of the nm23 family have been reported to date (nm23-H1 to H8), and as with the thioredoxin family, nm23 proteins can also be classified into two groups based on sequence alignment and phylogenetic analysis (Lacombe *et al.* 2000). Group I is composed of

nm23-H1 to H4, and is characterized by a similar genomic organization, the formation of hexamers of identical subunits and the classical enzymatic activity of NDP kinases. Group II encompasses nm23-H5 to H8 genes which are defined by a more divergent sequence, have an NDP kinase active site sequence that is not strictly conserved and have considerable variation in the lengths of their N- and C-terminal domains (Lacombe *et al.* 2000). Besides the kinase function, human nm23 proteins have been implicated in cell growth, tumor suppression metastasis and development (Lombardi *et al.* 2000). In addition, nm23-H2 has been shown to be the transcription factor Puf for the proto-oncogene c-*myc* (Postel *et al.* 1993). Most human nm23 proteins are found in cytosol, although nm23-H4 is shown to be a mitochondrial enzyme (Lacombe *et al.* 2000; Milon *et al.* 2000). Furthermore, nm23-H5, H6 and H8 have been shown to have a tissue-specific distribution, mostly expressed in human testis (Lacombe *et al.* 2000; Munier *et al.* 1998). We have described a similar situation in the thioredoxin family and have also identified mitochondrial and sperm specific members (Miranda-Vizuete *et al.* 2001; Spyrou *et al.* 1997).

We report here the characterization of a novel member of the thioredoxin family, the second with a tissue specific distribution in human testis, termed Sptrx-2 based on its expression pattern in spermatozoa. The Sptrx-2 sequence has been independently deposited in public databases (GenBank entry NM_016616) as a member of the nm23 family (nm23-H8) due to the presence of three NDP kinase domains located at the C-terminus of the thioredoxin domain. Thus, Sptrx-2 must be classified as a member of the group II of thioredoxins. Sptrx-2 displays high homology with sea urchin IC1 (intermediate chain-1) protein, a component of the sperm axonemal outer dynein arm complex (Ogawa *et al.* 1996). Taken together, Sptrx-2 appears to be a new component of the human sperm axoneme architecture and its possible role in human sperm motility and fertility is discussed.

RESULTS

cDNA cloning, sequence analysis, genomic organization and chromosomal localization of human Sptrx-2 gene. By sequence comparison we found that Genbank entry AF202051 encoded a putative novel human thioredoxin-like sequence. As no information regarding tissue expression was stated in this entry we performed a BLAST search in Genbank EST database to determine which cDNA library we should use to confirm AF202051 sequence. The search resulted in the identification of three matches from testis, total fetus and male germ cell tumors, respectively (GenBank entries: AL043096, AI077399 and AW590130). Therefore, we designed specific primers based on AF202051 sequence and performed 5'- and 3'-RACE PCR analysis in a human testis cDNA library to clone the full-length cDNA sequence of this novel protein. The complete sequence of the cDNA obtained consists of an ORF of 1767 bp, a 5'-UTR of 70 bp including two stop codons in frame and a 3'-UTR of 166 bp before the poly(A)$^+$ tail (Fig. 1). Human Sptrx-2 ORF encodes a protein of 588 amino acids with an estimated molecular mass of 67.3 kDa and a pI of 4.82. It is interesting that Sptrx-1 pI (Miranda-Vizuete *et al.* 2001) is identical to that of Sptrx-2. Analysis of the human Sptrx-2 sequence identified two distinct domains: an N-terminal domain (comprising the first 105 residues) similar to thioredoxins and a C-terminal domain composed of three tandemly repeated NDP kinase domains (Fig. 2A). Interestingly, the Sptrx-2 protein domain organization resembles the one of IC1, an outer dynein arm of sea urchin axoneme (Ogawa *et al.* 1996). Regarding the N-terminal thioredoxin domain, some of the structural amino acids that are conserved in previously characterized mammalian thioredoxins like Asp-26, Trp-31, Pro-40 or Gly-91 (numbers referred to those of human Trx-1) are also conserved in Sptrx-2 (Fig. 2B). However, other important residues shown to be essential for catalysis,

maintenance of three-dimensional structure or protein-protein interactions are changed, for instance Phe-11, Ala-29, Asp-58 or Lys-81 (Eklund *et al.* 1991). The alignment of all known human thioredoxins was used to perform a phylogenetic analysis including a novel thioredoxin-like protein, named Txl-2[1]. As shown in Figure 3A and 3B, mitochondrial thioredoxin (Trx-2) separates from the rest of the human thioredoxins quite early in evolution and the remaining members are clustered into three different groups: Trx-1 and Sptrx-1, Sptrx-2 and Txl-2, and finally Txl-1. Sptrx-1 is a retrogene originating from human Trx-1 mRNA; whereas, Sptrx-2 and Txl-2 have identical intron/exon organization[1] suggesting they have originated from a genomic duplication event. Txl-1 is placed alone in an independent branch.

Additionally, the Sptrx-2 C-terminal domain consists of three repeats of an NDP kinase domain. The first domain (NDPk-A) is truncated while the second and third (NDPk-B and NDPk-C) are complete (Fig. 2A). As noted previously, Sptrx-2 has also been reported as a novel member of the NDP kinase family of proteins, termed nm23-H8. However, no published report on this protein has been done except a brief mention in a review (Lacombe *et al.* 2000). In this review, a protein alignment and phylogenetic analysis of all human NDP kinase domains is done, showing that the Sptrx-2 NDPk-B and NDPk-C domains belong to NDP kinase group II.

In X-ray crystallography and site-directed mutagenesis studies, nine residues essential for catalysis and stability of nm23 proteins have been identified (Lacombe *et al.* 2000; Lombardi *et al.* 2000). Surprisingly, the Sptrx-2 NDPk-B domain only has two out of nine conserved residues while the NDPk-C domain has seven conserved residues regarded as crucial for enzymatic activity. Furthermore, the sequence of the active site in the NDPk-B domain (NXXY) is different from the remaining nm23 proteins (NXXH) where X can be any residue (Fig. 2A).

[1] Antonio Miranda-Vizuete *et al.*, manuscript in preparation.

Comparison of the protein sequence with PROSITE database (Bairoch *et al.* 1997) identified, along with the above mentioned thioredoxin and NDP kinase domains, several potential phosphorylation sites for protein kinases CKI, CKII, GSK3 and PKC with only one potential site for PKA. In addition, a highly scored PEST sequence for proteasome-dependent degradation centered at position 242 (Fig. 1).

A homology search in GenBank identified Sptrx-2 genomic region in the genomic BAC clone AC018634. Sptrx-2 gene spans about 51 kb and is organized into 17 exons and 16 introns all of them conforming to the GT/AG rule (Table 1). Finally, the BAC clone AC018634 has been mapped to human chromosome 7p13-14, between the markers D7S485 and D7S528, by PCR screening of a human-rodent radiation panel.

Tissue and cellular expression of human Sptrx-2 mRNA. Multiple-tissue Northern blots were used to determine the size and tissue distribution of human Sptrx-2 mRNAs using the ORF as the probe. Human Sptrx-2 mRNA was only detected in human testis as a single band of approximately 2.4 kb in good agreement with the size of the cloned cDNA (Fig. 4). It should be noted that a very long exposure of the blots was required to identify the hybridization signal which suggests that Sptrx-2 mRNA might not be expressed at high levels, consistent with the low number (three) of matching EST sequences in the human database. To evaluate the possibility that Sptrx-2 mRNA could be expressed in other tissues not present in these blots, we also screened an RNA dot blot containing poly(A)$^+$ RNAs from 50 different human tissues. Among the tissues examined, hybridization signal was observed only in testis mRNA (data not shown). The testis specific mRNA expression obtained by Northern blot analysis is in agreement with that of Mehus and Lambeth obtained by RT-PCR (Lacombe *et al.* 2000).

To further investigate the expression pattern of Sptrx-2 mRNA, *in situ* hybridization was performed in human testis sections showing that it is expressed in primary spermatocytes and round spermatids with no signal in the remainder of the testicular cells (Fig. 5). Similar results have been obtained using mouse Sptrx-2 cDNA in mouse testis sections (M. Pelto-Huikko and A Miranda-Vizuete, unpublished results). This expression pattern slightly differs from that of Sptrx-1 mRNA which is mainly found in round and elongating spermatids (Miranda-Vizuete *et al.* 2001) and is also consistent with the three Sptrx-2 matches in GenBank EST database in testis, male germ cell tumor and total fetus. Hybridization of human Sptrx-2 cDNA probe in human tissues other than testis resulted in no signal (data not shown).

Expression and enzymatic activity of human Sptrx-2 protein. Recombinant human Sptrx-2 migrated in SDS-PAGE at 67 kDa size in good agreement with its theoretical size while a truncated form of Sptrx-2 expressing only the N-terminal thioredoxin domain migrated approximately at 12 kDa (Fig. 6 inset). Members of the NDP kinase family have been described to be hexamers in their native conformations (Lacombe *et al.* 2000). To evaluate whether this was also the case for Sptrx-2 we performed a gel filtration chromatography and found that the protein migrates as a monomer (Fig. 6).

Enzymatic activity of thioredoxins is usually assayed by their capacity to reduce the disulfide bonds of insulin using either DTT as artificial reductant or NADPH and thioredoxin reductase as a more physiological reducing system (Arner & Holmgren 2000). We were unable to detect any Sptrx-2 enzymatic activity using calf thymus thioredoxin reductase and NADPH with either the full-length protein or the truncated form (data not shown). However, when using DTT as reductant we were able to detect activity with the truncated form expressing only the thioredoxin domain. When compared with human Trx-1 used as control, truncated Sptrx-2

displayed a similar enzymatic activity profile although at a 1:5 molar ratio and with a marked latency phase (Fig. 7). Taken together, these data suggest that Sptrx-2 is not an efficient protein disulfide reductase *in vitro*.

NDP kinases catalyze the transfer of a terminal phosphate residue from NTPs to NDPs according to a ping-pong mechanism. The first step of this reaction consists of the autophosphorylation of the enzyme at a conserved histidine of the active site (Biondi *et al.* 1996; Lecroisey *et al.* 1995). As noted above, the NDPk-C domain of Sptrx-2 has the typical active site sequence NXXH while the NDPk-B domain has a substitution of the histidine residue by a tyrosine, which might also be phosphorylated (Fig. 2A). However, Sptrx-2 is unable to undergo autophosphorylation under the same experimental conditions that allowed positive control yeast NDP kinase autophosphorylation (data not shown) (Milon *et al.* 2000).

To assess the possibility that Sptrx-2 could display enzymatic activity in an *in vivo* system we overexpressed human Sptrx-2 in HEK293 cells by transient transfections experiments. Cells overexpressing full-length Sptrx-2 did not show significant differences on enzymatic activity over the control values, due to the high levels of endogenous thioredoxin activity (data not shown).

Tissue expression and cellular and subcellular localization of human Sptrx-2 protein. Affinity-purified antibodies were used to study the expression pattern of human Sptrx-2 protein in different human tissues and cell lines. As shown in Figure 8A, only human testis and sperm extracts expressed Sptrx-2 as a band of approximately 67 kDa in good agreement with the migration of the recombinant protein in SDS gels. No signal was detected in any other tissue or cell line used as control, including those harbouring flagella tissues like lung and trachea. The antibodies also recognized a band of similar size in rat, mouse and bull testis extracts (data not shown). To address the question of whether Sptrx-2 protein distribution

paralleled the mRNA expression, we performed immunohistochemical analysis in human testicular sections that revealed that Sptrx-2 expression was restricted to spermiogenesis, starting at the pachytene spermatocytes level and peaking at the round and elongating spermatid stage (Figure 8B). Other testicular cell types like spermatogonia, Sertoli and Leydig cells were devoid of signal. Immunofluorescence analysis of human ejaculated spermatozoa identifies Sptrx-2 signal localized from the caudal region of the head to the end of the principal piece of sperm tail (Figure 8C).

To determine whether Sptrx-2 protein might be targeted to any subcellular compartment during spermiogenesis, we constructed plasmid vectors to express it as a fusion protein carrying GFP at its C-terminus. The plasmid was transiently transfected into HEK293 cells and the fluorescent fusion protein detected by confocal microscopy. Cells transfected with the control plasmid expressing only the GFP protein showed fluorescence in both cytosol and nucleus, as its small size (27 kDa) allows it to translocate into the nucleus passively (data not shown). The fusion protein Sptrx-2/GFP showed a clear cytosolic distribution although a weaker punctuated pattern could also be found in the nucleus (Fig. 9A). When this image was merged with DNA staining (Fig. 9B) a yellow colour indicating colocalization was obtained in the perinuclear region and also scattered through the nucleus (Fig. 9C). There was also a dotted yellow pattern in the cytosol, which might correspond to mitochondria as they also contain DNA. The presence of Sptrx-2 in nucleus is not likely to be an artefact as a control with Sptrx-1 or Txl-2 GFP fusion constructs had a clear cytosolic localization with no nuclear fluorescent signal (A. Miranda-Vizuete, unpublished results) and the subcellular localization of the Sptrx-2/GFP fusion protein *in vivo* was similar to that of fixed cells (data not shown). In addition, it is important to point out that Sptrx-2 sequence analysis on PSORT II program (http://psort.ims.u-tokyo.ac.jp/form2.html) identified two putative pat7 nuclear targeting sequences centred at residues 50 (PLFRKLK) and 308 (PDFKKMK),

respectively (Hicks & Raikhel 1995), and a putative mitochondrial pre-sequence cleavage site at residue 16 (REVQLQTVINN↓QS), although the overall prediction is cytosolic (Gavel & von Heijne 1990). These data might explain the pattern obtained with the Sptrx-2/GFP fusion protein.

DISCUSSION

The first form of thioredoxin (now termed Trx-1) was described in *E. coli* as early as 1964 (Laurent *et al.* 1964) whereas the identification of the mammalian mouse and human homologues had to wait for 25 years (Tagaya *et al.* 1989; Wollman *et al.* 1988). Since 1996 our group has been engaged in the search of novel members of the thioredoxin family of proteins, which resulted in the identification of a mitochondrial thioredoxin system (Trx-2 and TrxR2), a ubiquitous thioredoxin-like protein of unknown function (Txl-1) and the first tissue-specific member of the family in human spermatozoa (Sptrx-1) (Miranda-Vizuete *et al.* 2000; Miranda-Vizuete *et al.* 1998; Miranda-Vizuete *et al.* 2001). All known thioredoxins fall into two groups depending on whether they are composed of only a thioredoxin domain, (Trx-1 and Trx-2, Group I) or have additional domains (Txl-1 and Sptrx-1, Group II). We report here the identification of the second tissue-specific member of the family, named Sptrx-2, also in male germ cells.

Sptrx-2 is organized into one N-terminal thioredoxin domain followed by three NDP kinase domains, the first of them incomplete. A similar protein arrangement has been described for IC1, a sea urchin protein which has been classified as one of the three intermediate chains of axonemal outer arm dynein sperm axoneme (Ogawa *et al.* 1996). Thus IC1 is also composed of a thioredoxin domain, three tandemly repeated NDP kinase domains and only differs from Sptrx-2 in a C-terminal part rich in glutamic acid residues. Sptrx-2 has been proposed to be the human homologue of IC1 based on sequence similarity (Lacombe *et al.* 2000). The occurrence of thioredoxin domains in other flagellar proteins have also been reported in outer arm dynein light chains DLC14 and DLC16 from *Chlamydomonas* flagella (Patel-King *et al.* 1996). However, to our knowledge, thioredoxin activity has not been properly assayed in

these flagellar proteins. In our study, we have failed to detect any enzymatic thioredoxin activity when assaying recombinant full-length Sptrx-2 (expressed in bacteria) with NADPH and calf thymus thioredoxin reductase or DTT. Only when expressing the thioredoxin domain alone we were able to detect a significant activity in the DTT assay. The acquisition of a thioredoxin domain in flagellar proteins occurs early in evolution and is most likely a consequence of molecular or enzymatic requirements for a specific function in flagellum movement (Patel-King *et al.* 1996). The lack of thioredoxin activity in Sptrx-2 most probably depends on its three-dimensional structure, not only of the full-length protein but also of the thioredoxin domain itself that impedes the access of the natural catalyst thioredoxin reductase to its active site. This implies that another mechanism might operate to maintain Sptrx-2 in an active reduced state. One possible explanation is the newly described TRG, a fusion protein of a glutaredoxin and a thioredoxin reductase module which is highly expressed in testis and might function as a reducing system for Sptrx-2 (Sun *et al.* 2001). Alternatively, phosphorylation is one of the most well–established cellular mechanisms by which a conformational change in a protein is achieved (Cohen 2000) and the major regulatory pathway in testis and sperm physiology (Eddy & O´Brien 1994). Sptrx-2 has multiple phosphorylation sites and one might speculate that phosphorylation of the protein could induce a conformational change that would make the thioredoxin active site accessible to thioredoxin reductase.

The Sptrx-2 thioredoxin domain is followed by one incomplete (lacking the active site) and two complete NDP kinase domains at the C-terminus of the protein (domains A, B and C respectively). NDP kinases catalyze the transfer of γ-phosphates between tri- and diphosphonucleosides and are autophosphorylated as an intermediate in the reaction (Biondi *et al.* 1996). This autophosphorylation has been shown to occur at the Nδ position of the conserved histidine of the active site N*XX*H.

While the NDPk-C domain has this conserved active site sequence, the B domain lying in the middle of the tandem has the histidine substituted by a tyrosine residue, which is also phosphorytable thus suggesting possible novel features in the catalytic mechanism. As with the thioredoxin activity, we have not been able to identify any autophosphorylation activity in Sptrx-2, which is also the case of the other NDP kinase specifically expressed in testis, nm23-H5 (Munier *et al.* 1998). Taken together, the lack of both thioredoxin and kinase activities in Sptrx-2 while the structures of the respective domains are so conserved might suggest that interaction with other proteins or cofactors is needed for Sptrx-2 to function.

Sptrx-2 protein expression is restricted to spermiogenesis, being detected in pachytene spermatocytes, round and elongating spermatids within human seminiferous tubules. In mature human sperm Sptrx-2 is mostly found in the tail, further supporting the hypothesis of being the human homologue of sea urchin IC1 protein. The synthesis of the sperm cytoskeletal polypeptides, has been shown to occur in the cytoplasm of spermatids, which is consistent with the expression pattern obtained for Sptrx-2 and corresponds to the growth of the structures that organize the sperm tail, including the axoneme (Oko 1998). Taken together, it is reasonable to assume that Sptrx-2 might be a component of the human sperm axonemal machinery and therefore be a candidate gene to several male infertility phenotypes.

With this aim we first screened the Mendelian Cytogenetics Network Database (http://mcndb.imbg.ku.dk/) to determine whether any breakpoint in the region where Sptrx-2 maps has been reported to be associated with an infertility phenotype. We found four reported breakpoints mapping at Sptrx-2 locus and, interestingly, three of them had an associated trait of infertility (accession ID number: 702748, 702844 and 702852). Primary ciliary dyskinesia (PCD) is an autosomal recessive disorder (OMIM 242650) characterized by a failure of proper ciliary and flagellar movement whose clinical manifestations are chronic respiratory infections, male

infertility and situs inversus (Blouin *et al.* 2000; Cowan *et al.* 2001). The motility of cilia and flagella is generated in the axoneme which has been estimated to be composed of more than 250 polypeptides (Dutcher 1995). The axoneme consists of a core of 9 peripheral + 2 central microtubule doublets (composed of proteins named tubulins) connected by outer and inner dynein arms (composed of heavy, intermediate and light chains) plus other accessory proteins. Electron microscopy studies of sperm of patients affected by PCD reveal anomalies in both microtubule and dynein arm organization and as a consequence PCD is a genetically highly heterogeneous disease (Blouin *et al.* 2000). Our Northern blot analysis has included, in addition to testis, other cilia harbouring tissues like trachea, brain or lung; however, we have failed to detect Sptrx-2 mRNA hybridization in any other tissue than testis. As all cilia and flagella share the same organization the question arises whether Sptrx-2 gene might code for a sperm-specific axoneme dynein protein. So far, dynein proteins are classified into two major groups, cytoplasmic and axonemal with no further tissue specific distinction (Milisav 1998). However, there is considerable precedent that similar dynein genes could be expressed either in somatic tissue or male germ cells as cases describing patients with normal somatic cilia while defective spermatozoa due to lack of dynein arms or viceversa have been reported (Phillips *et al.* 1995 and references therein). Differential gene expression in somatic or germ tissue axoneme is by no means limited to dynein arms (Neesen *et al.* 1997; Milisav *et al.* 1996) as a testis specific α-tubulin has been identified in mice (Distel *et al.* 1984). Dynein arm deficiency (DAD) is a subgroup of PCD and a recent linkage analysis has identified 7p, the region where Sptrx-2 maps, as a potential genomic region harbouring gene(s) involved in DAD (Blouin *et al.* 2000). Thus, similarity with sea urchin IC1, male germ cell specific expression and chromosomal mapping in candidate regions for male infertility phenotypes suggest that Sptrx-2 is a

gene coding for a sperm axonemal specific form of dynein arm and therefore a good candidate for male infertility diseases associated with primary ciliary dyskinesia. Further work is in progress to decipher the functional role of Sptrx-2 in human spermatogenesis which will shed more light on the newly discovered subset of thioredoxin proteins with a testis specific expression.

EXPERIMENTAL PROCEDURES

cDNA cloning of human Sptrx-2 gene: The Basic Local Alignment Search Tool (BLAST) (Altschul & 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 new entries encoding potential novel members of the thioredoxin family. Using human Trx-1 and Trx-2 ORF as bait (Miranda-Vizuete *et al.* 1997b; Wollman *et al.* 1988) we found entry AF202051 to encode a putative thioredoxin-like sequence. This entry was named nm23-H8 by virtue of its additional sequence homology to members of the NDP kinase family. Based on this sequence, the primers F1 (5´-CCTGTTTTGTTAGATAAATGGCAAGC-3´) and R1 (5´-GTTTTCACAGTATATACTTTAGTTTTCC-3´) were used to amplify by PCR the Sptrx-2 ORF from a human testis cDNA library (Clontech). The amplification product was cloned in the pGEM-Teasy vector (Promega) and sequenced in both directions. The nested forward primers F2 (5´-GAAGCAAAAGCGGTTGTTAATAGAC-3´) and F3 (5´-GGAAAACTAAAGTATATACTGTG) and the nested reverse primers R2 (5´-CTTTGATTATTGATGACTGTCTG-3´) and R3 (5´-GCTTGCCATTTATCTAACAAAACAGG-3´) were used for 3´- and 5´-RACE, respectively, in the same library to determine the sequence of the Sptrx-2 UTR.

Northern blot analysis: Human multiple tissue Northern blots with poly(A)$^+$ RNA from different tissues were purchased from Clontech. The human Sptrx-2 ORF was labelled with [α-^{32}P]dCTP (Rediprime random primer labeling kit, Amersham) and hybridized at 42°C overnight in Ultrahyb™ Solution following the protocol

provided by Ambion. The blots were also hybridized with human β-actin as a control.

Expression and purification of human Sptrx-2 recombinant protein. The ORF encoding human Sptrx-2 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. 1% sarkosyl was added and cells disrupted by 10 min sonication and 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 human Sptrx was eluted using a gradient of NaCl. For gel filtration chromatography Sptrx-2 preparation from ion exchange chromatography was applied to a Superdex G-75 prep. grade column (Amersham Pharmacia Biotech) under non-denaturing conditions, equilibrated with the same buffer as the protein preparation. Protein concentration was determined from the absorbance at 280 nm using a molar extinction coefficient of 47,730 M^{-1} cm^{-1}. The cloning, overexpression and purification of the truncated form of human Sptrx-2 (hΔSptrx-2) was identical to

that described for the full-length protein except that the ion exchange purification step was not required as the protein eluted in a pure form following thrombin cleavage.

Enzymatic activity assays. Two different assays were used to determine the enzymatic activity of human Sptrx-2, both based on the ability of the protein to reduce insulin disulfide bonds *in vitro.* In the DTT assay, DTT is used as reductant and the assay was carried out as previously described (Wollman *et al.* 1988). Briefly, 25 µl of reaction mixture composed of 40 µl of 1M Tris-HCl pH7.4, 10 µl 0.2 M EDTA pH 8.0 and 200 µl insulin (10 mg/ml) were added to the different enzyme preparations in a final volume of 200 µl. The reaction was initiated by adding 1 µl of 0.1 M DTT and increase of 600 nm absorbance at 30ºC was recorded for 30 min. The second assay used thioredoxin reductase and NADPH as electron donors for thioredoxin and was performed essentially as described elsewhere (Spyrou *et al.* 1997). Briefly, aliquots of Sptrx-2 and ΔSptrx-2 were preincubated at 37 °C for 20 min with 2 µl of: 50 mM Hepes, pH 7.6, 100 µg/ml bovine serum albumin, and 2 mM DTT in a total volume of 70 µl. This step allows total reduction of the protein. Then, 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) were added. 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. In both assays human Trx-1 was used as control.

Human Sptrx-2 *in situ* hybridization analysis: Riboprobes (sense and antisense) were generated from Sptrx-2 ORF template using MEGAscript-II transcription kit (Ambion, Austin, TX). Probes were labelled with digoxigenin-UTP (Boehringer Mannheim, Mannheim, Germany). Paraffin sections of human testis and human multi tissue slides (T1065; Dako, Copenhagen, Denmark) were deparaffinized with xylene and rehydrated with ethanol and air dried. Sections were hybridized for 18 h at 55˚C with the labelled probes diluted in hybridization buffer (4 x SSC, 50% formamide, 1x Denhardt´s solution, 1% sarcosyl, 10% dextran sulphate and 250µg/ml yeast RNA). Sections were subsequently washed twice in 2 x SSC at room temperature, 0.5 x SSC at 60˚C and in 0.1 x SSC at 60˚C for 15 min, each. Then the tissues were incubated with alkaline phosphatase conjugated anti-digoxigenin antibody (dil. 1:750; Boehringer Mannheim) for 2 h. The signal was visualized using Vector Alkaline Phosphatase kit-II (Vector Laboratories, Burlingame, CA) and the sections were mounted. Images were taken with Nikon FXA microscope equipped with PCO Sensicam digital camera (PCO, Kelheim, Germany) and the images were processed with CoralDraw9 software (Corel Corporation Ltd., Ontario, Canada).

Preparation of spermatozoa and extraction of sperm proteins. Semen samples from healthy donors were allowed to liquefy at room temperature and separated from seminal plasma by centrifugation (1000 x *g*) for 10 min at room temperature. After two washes in PBS the pelleted spermatozoa were frozen at -20°C until use. The sperm pellet was solubilized in a lysis buffer containing Tris-HCl 0.1 M pH 8.0, NaCl 0.15 M, protease inhibitor cocktail (Boehringer Mannheim) and phosphatase inhibitor cocktail (SIGMA) at the concentration recommended by the manufacturers. Samples were then subjected to three cycles of freezing/thawing in dry ice-ethanol, incubated for 30 min on ice and centrifuged at 14000 rpm for 30 min. The soluble fraction was used for further analysis.

Antibody production, immunoblotting analysis and immunocytochemistry. Purified GST-hSptrx-2 was used to immunize rabbits (Zeneca Research Biochemicals). After six immunizations, serum from rabbits was purified by ammonium sulfate precipitation. Affinity purified antibodies were prepared using a cyanogen bromide-activated Sepharose 4B column, onto which 0.5 mg recombinant Sptrx-2 fragment had been coupled using the procedure recommended by the manufacturer (Pharmacia). Specificity of the antibodies was tested by western blotting using recombinant Sptrx–2 and total cell extracts. Immunodetection was performed with horseradish peroxidase-conjugated donkey anti-rabbit IgG diluted 1:5000 following the ECL protocol (Amersham Corp.). For immunocytochemistry, paraffin sections containing multiple human tissues (T1065, Lot: 9994A) were obtained from Dako (Copenhagen). In addition routine paraffin sections of human testis were used. For immunofluorescence analysis human sperm samples were obtained from healthy volunteers. Immunocytochemistry was performed as described previously (Rybnikova *et al.* 2000) either by the ABC-method or by indirect immunofluorescence method using goat anti-rabbit-FITC (1:100, 30 min, Boehringer-Mannhein) as a secondary antibody. The fluorescence samples were embedded in PBS-glycerol mixture containing 0.1% *p*-phenylenediamine. The sections were examined with Nikon Microphot-FXA microscope equipped with proper fluorescent filters.

Green fluorescent protein analysis. We used the pEGFP-N3 vector (Clontech) to express the GFP at the C-terminus of hSptrx-2. For that purpose we used the mutagenic primers GFP-F1 (5'-GTTGAATTCGCCACCATGGCAAGCAAAAAAC-3´) as forward primer and GFP-R1 (5'-GTTTTCCTCAGGATCCCTCAAAGAGTCTATT-3') as reverse primer to amplify human Sptrx-2 from pGEM-T/hSptrx-2. The forward

primer introduces an *Eco*RI site followed by a Kozac sequence (Kozak 1996) and the reverse primer introduces a *Bam*HI site. The amplified DNA was cloned into the *Eco*RI-*Bam*HI sites of pEGFP-N3 expression vector and *E. coli* XL1-Blue strain was transformed with the recombinant plasmid, pGFP-hSptrx-2. The plasmid was purified using the midi-prep kit (Qiagen, Chatsworth, CA) and sequenced.

Transfection was performed with 1 μg of DNA diluted in 10 μl of H_2O and 0.5μl of 0.1 M PEI. The mixture was mixed thoroughly, incubated at room temperature for 10 min and subsequently added to the medium and applied onto HEK293 cells grown in coverslips. 48 hours after transfection cells were fixed with 3.7% paraformaldehyde for 20 min at room temperature. The nucleus was stained with 1 μM 7-aminoactinomycin D (7-AAD) (Molecular Probes) for 30 min.

The GFP pictures were acquired with a Leica laser scanning confocal microscope. For GFP excitation we used the 488 line of an ArKr laser and emitted light was collected between 500 and 540 nm. 7-AAD was excited with the 568 line and emitted light was collected between 640 and 680 nm.

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LEGENDS TO THE FIGURES

Fig. 1. Nucleotide and amino acid sequence of human Sptrx-2. The nucleotide numbers are displayed on the right and the amino acid numbers on the left. The two stop codons in frame within the 5´-UTR are underlined. The internal PEST sequence and the polyadenylation signal AATAAA are double underlined. The conserved thioredoxin active site and the two putative NDP kinase active site motifs are boxed.

Fig. 2. A) Organization of human Sptrx-2 protein. Numbers refer to those of the amino acid sequence shown in Figure 1. **B) Alignment of the predicted amino acid sequence of Sptrx-2 thioredoxin domain with all the known human members of the thioredoxin family.** Alignment was performed using the W-Clustal program included in the DNASTART package (Thompson *et al.* 1994). Identical residues are shadowed and thioredoxin active site sequence CGPC is shown in bold and boxed. Numbers are referred to those of human Trx-1 (Wollman *et al.* 1988).

Fig. 3. A) Phylogenetic analysis of all the members of the human thioredoxin family. The phylogenetic analysis was produced by applying the neighbour-joining method of Saitou and Nei to the alignment data (Saitou & Nei 1987). The scale indicates the number of amino acid substitutions per hundred residues. **B) Percentage of similarity of human thioredoxins.** The data are derived from the protein alignment performed in Figure 2 using the same software package.

Fig. 4. Expression pattern of human Sptrx-2 mRNA. Human multiple tissue poly(A)$^+$ mRNA blots (Clontech) were hybridized with the Sptrx-2 ORF probe identifying only one mRNA species at 2.4 kb in testis. A long-term exposure of the

blots was necessary to have enough signal. β-actin was used as control to determine the relative amount of mRNA from each tissue. P.B.L., peripheral blood leukocytes.

Fig. 5. *In situ* hybridization analysis of human Sptrx-2 mRNA distribution. Human testis section hybridized with digoxigenin labelled Sptrx-2 antisense probe. Signal can be seen in late primary spermatocytes (arrowheads) and in round spermatids (arrows). The rest of the testicular cells are devoid of signal. Bar represents 30μm (A), 15μm (B) and 10μm (C, D).

Fig. 6. Expression and purification of human recombinant Sptrx-2 protein. Full length human Sptrx-2 and a truncated form expressing only the N-terminal thioredoxin domain, ΔSptrx-2, were purified as GST fusion protein and thrombin cleaved with 5 U/mg protein. On inset, both proteins (4 μg) were separated by SDS-PAGE 12%, Lane 1 Sptrx-2, Lane 2 ΔSptrx-2. On chromatogram, full length Sptrx-2 was run on a gel filtration chromatography column with the following markers: a) Albumin, b) Ovalbumin, c) Chymotrypsinogen A and d) Ribonuclease A.

Fig. 7. Enzymatic activity of human Sptrx-2. Purified Sptrx-2 and ΔSptrx-2 were assayed for its ability to reduce insulin disulfide bonds using DTT as electron donor: (O) Trx-1 at 5 μM, (❑) ΔSptrx-2 at 25 μM, (Δ) ΔSptrx-2 at 5 μM, (◇), Sptrx-2 at 25 μM. The reaction was initiated by adding 1 μl of 100 mM DTT and followed for 30 min at 30ºC.

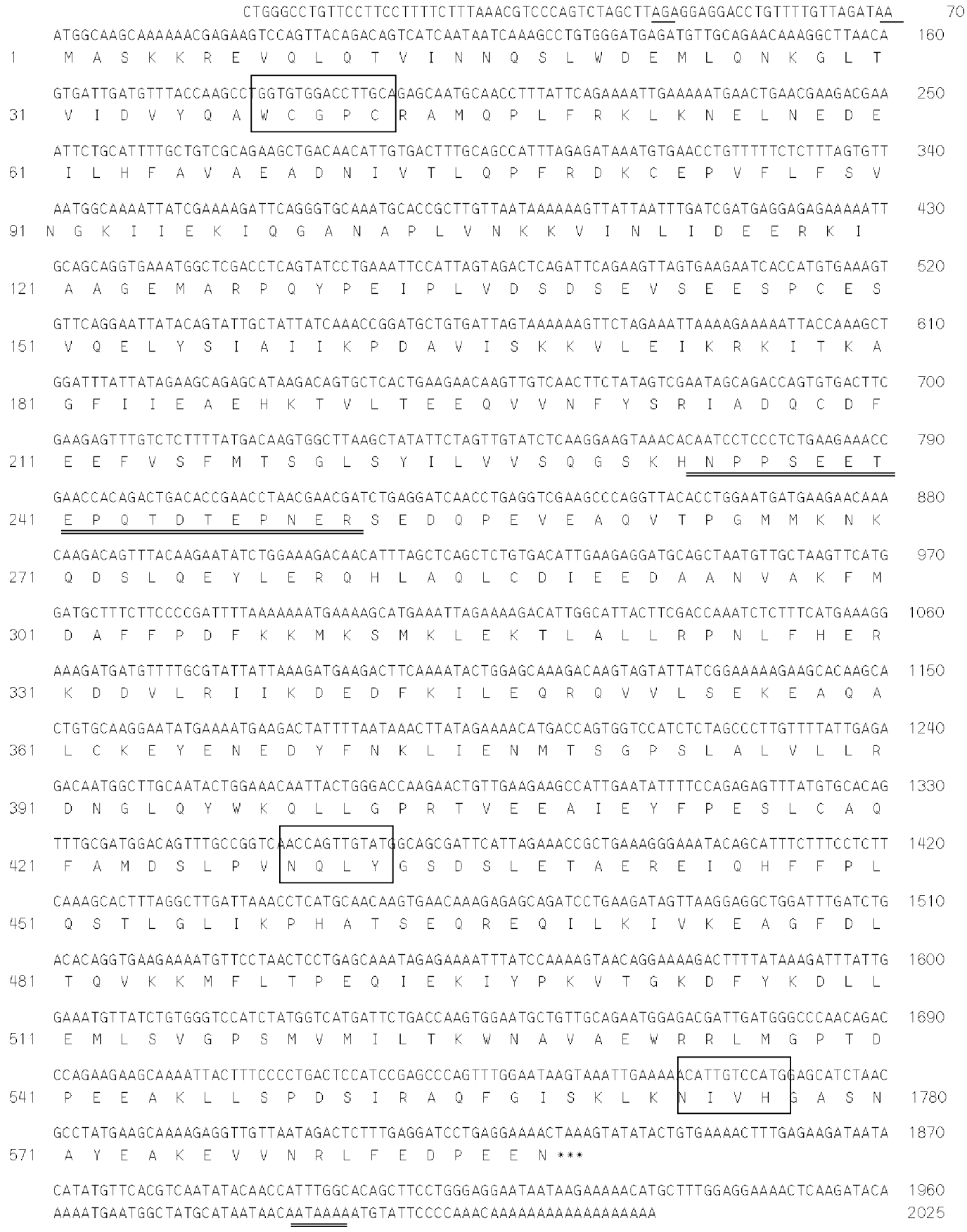
Fig. 8. Tissue and cellular distribution of human Sptrx-2 protein. (A) Sptrx-2 expression in different human tissues and cell lines. All extracts were at 10 μg except sperm at 1 μg. (B) Immunocytochemistry of human testis showing strong Sptrx-2

labeling in the apically localized spermatids (black arrowheads) and spermatozoa tails (white arrowheads). Spermatogenic cells at earlier stages of development and Leydig cells are devoid of staining. Image is at 20x amplification. (C) Immunofluorescent demonstration of Sptrx-2 in human ejaculated spermatozoa. Labeling is present from the postacrosomal region and the neck through the middle and principal piece. The anterior part of the head is unlabeled. Bar represents 4 μm.

Fig. 9. Subcellular distribution of human Sptrx-2 protein. (A) HEK 293 cells transfected with Sptrx-2/GFP construct show green fluorescence mostly in the cytosol although some diffuse signal is also found scattered through the nucleus. (B) The same cells were stained further with the DNA-selective dye 7-AAD, resulting in a red staining of nuclear and mitochondrial DNA. (C) Overlapping of both images demonstrates co-localization of both signals (yellow color) only in some spots in the cytosol and in the perinuclear region, indicating mitochondrial localization. In addition, there is a diffuse yellow staining within the nucleus. Micrographs were obtained from a single focal section.

TABLE I. Genomic organization of human Trx7 gene.

Exon size		*Intron size*		*Sequence at exon/intron junction*		*Residue at junction*			*Coding information*
Exon	*(bp)*	*Intron*	*(bp)*	*5´splice donor*	*3´splice acceptor*	*AA*	*Position*	*Type*	
1	>63								5´-UTR
2	40	1	1.203	TTTGTgtaag	gatagTAGAT				5´-UTR / Trx
3	58	2	78	TACAGgtggg	tgcagACAGT	Q/T	11/12	0	Trx
4	107	3	193	AACAGgtata	tctagTGATT	V	31	1	Trx
5	72	4	6.538	CTGTCgtaag	tccagGCAGA	V/A	66/67	0	Trx
6	117	5	4.682	GTGTTgtaag	tatagAATGG	V/N	90/91	0	Trx
7	67	6	1.251	CTCAGgtaat	catagTATCC	Q/Y	129/130	0	Interface
8	74	7	885	TGTTCgtaag	tccagAGGAA	Q	152	1	Interface
9	93	8	1.103	GAAAAgtaag	tgaagATTAC	K/I	176/177	0	NDPk-A
10	196	9	2.084	ACCAGgtatg	tgtagTGTGA	Q/C	207/208	0	NDPk-A
11	176	10	8.933	GACAGgtata	gacagTTTAC	S	273	2	Interface
12	145	11	7.295	GAAAGgtagg	tttagATGAT	D	332	1	NDPk-B
13	108	12	697	ACCAGgtaga	aatagTGGTC	S	380	2	NDPk-B
14	152	13	3.024	GAGAGgtagg	caaagTTTAT	S	416	2	NDPk-B
15	145	14	6.037	AAGAGgtaaa	cttagAGCAG	E	467	1	NDPk-C
16	238	15	2.295	TCTGTgtaag	tgcagGGGTC	V	515	2	NDPk-C / 3´-UTR
17	>156	16	3.136	GTGAAgtagc	cctagAACTT				3´-UTR


CTGGGCCTGTTCCTTCCTTTTCTTTAAACGTCCCAGTCTAGCTTAGAGGAGGACCTGTTTTGTTAGATAA 70
ATGGCAAGCAAAAAACGAGAAGTCCAGTTACAGACAGTCATCAATAATCAAAGCCTGTGGGATGAGATGTTGCAGAACAAAGGCTTAACA 160
1 M A S K K R E V Q L Q T V I N N Q S L W D E M L Q N K G L T
GTGATTGATGTTTACCAAGCCTGGTGTGGACCTTGCAGAGCAATGCAACCTTTATTCAGAAAATTGAAAAATGAACTGAACGAAGACGAA 250
31 V I D V Y Q A W C G P C R A M Q P L F R K L K N E L N E D E
ATTCTGCATTTTGCTGTCGCAGAAGCTGACAACATTGTGACTTTGCAGCCATTTAGAGATAAATGTGAACCTGTTTTTCTCTTTAGTGTT 340
61 I L H F A V A E A D N I V T L Q P F R D K C E P V F L F S V
AATGGCAAAATTATCGAAAAGATTCAGGGTGCAAATGCACCGCTTGTTAATAAAAAAGTTATTAATTTGATCGATGAGGAGAGAAAAATT 430
91 N G K I I E K I Q G A N A P L V N K K V I N L I D E E R K I
GCAGCAGGTGAAATGGCTCGACCTCAGTATCCTGAAATTCCATTAGTAGACTCAGATTCAGAAGTTAGTGAAGAATCACCATGTGAAAGT 520
121 A A G E M A R P Q Y P E I P L V D S D S E V S E E S P C E S
GTTCAGGAATTATACAGTATTGCTATTATCAAACCGGATGCTGTGATTAGTAAAAAAGTTCTAGAAATTAAAAGAAAAATTACCAAAGCT 610
151 V Q E L Y S I A I I K P D A V I S K K V L E I K R K I T K A
GGATTTATTATAGAAGCAGAGCATAAGACAGTGCTCACTGAAGAACAAGTTGTCAACTTCTATAGTCGAATAGCAGACCAGTGTGACTTC 700
181 G F I I E A E H K T V L T E E Q V V N F Y S R I A D Q C D F
GAAGAGTTTGTCTCTTTTATGACAAGTGGCTTAAGCTATATTCTAGTTGTATCTCAAGGAAGTAAACACAATCCTCCCTCTGAAGAAACC 790
211 E E F V S F M T S G L S Y I L V V S Q G S K H N P P S E E T
GAACCACAGACTGACACCGAACCTAACGAACGATCTGAGGATCAACCTGAGGTCGAAGCCCAGGTTACACCTGGAATGATGAAGAACAAA 880
241 E P Q T D T E P N E R S E D Q P E V E A Q V T P G M M K N K
CAAGACAGTTTACAAGAATATCTGGAAAGACAACATTTAGCTCAGCTCTGTGACATTGAAGAGGATGCAGCTAATGTTGCTAAGTTCATG 970
271 Q D S L Q E Y L E R Q H L A Q L C D I E E D A A N V A K F M
GATGCTTTCTTCCCCGATTTTAAAAAAATGAAAAGCATGAAATTAGAAAAGACATTGGCATTACTTCGACCAAATCTCTTTCATGAAAGG 1060
301 D A F F P D F K K M K S M K L E K T L A L L R P N L F H E R
AAAGATGATGTTTTGCGTATTATTAAAGATGAAGACTTCAAAATACTGGAGCAAAGACAAGTAGTATTATCGGAAAAAGAAGCACAAGCA 1150
331 K D D V L R I I K D E D F K I L E Q R Q V V L S E K E A Q A
CTGTGCAAGGAATATGAAAATGAAGACTATTTTAATAAACTTATAGAAAACATGACCAGTGGTCCATCTCTAGCCCTTGTTTTATTGAGA 1240
361 L C K E Y E N E D Y F N K L I E N M T S G P S L A L V L L R
GACAATGGCTTGCAATACTGGAAACAATTACTGGGACCAAGAACTGTTGAAGAAGCCATTGAATATTTTCCAGAGAGTTTATGTGCACAG 1330
391 D N G L Q Y W K Q L L G P R T V E E A I E Y F P E S L C A Q
TTTGCGATGGACAGTTTGCCGGTCAACCAGTTGTATGGCAGCGATTCATTAGAAACCGCTGAAAGGGAAATACAGCATTTCTTTCCTCTT 1420
421 F A M D S L P V N Q L Y G S D S L E T A E R E I Q H F F P L
CAAAGCACTTTAGGCTTGATTAAACCTCATGCAACAAGTGAACAAAGAGAGCAGATCCTGAAGATAGTTAAGGAGGCTGGATTTGATCTG 1510
451 Q S T L G L I K P H A T S E Q R E Q I L K I V K E A G F D L
ACACAGGTGAAGAAAATGTTCCTAACTCCTGAGCAAATAGAGAAAATTTATCCAAAAGTAACAGGAAAAGACTTTTATAAAGATTTATTG 1600
481 T Q V K K M F L T P E Q I E K I Y P K V T G K D F Y K D L L
GAAATGTTATCTGTGGGTCCATCTATGGTCATGATTCTGACCAAGTGGAATGCTGTTGCAGAATGGAGACGATTGATGGGCCCAACAGAC 1690
511 E M L S V G P S M V M I L T K W N A V A E W R R L M G P T D
CCAGAAGAAGCAAAATTACTTTCCCCTGACTCCATCCGAGCCCAGTTTGGAATAAGTAAATTGAAAAACATTGTCCATGGAGCATCTAAC
541 P E E A K L L S P D S I R A Q F G I S K L K N I V H G A S N 1780
GCCTATGAAGCAAAAGAGGTTGTTAATAGACTCTTTGAGGATCCTGAGGAAAACTAAAGTATATACTGTGAAAACTTTGAGAAGATAATA 1870
571 A Y E A K E V V N R L F E D P E E N ***
CATATGTTCACGTCAATATACAACCATTTGGCACAGCTTCCTGGGAGGAATAATAAGAAAAACATGCTTTGGAGGAAAACTCAAGATACA 1960
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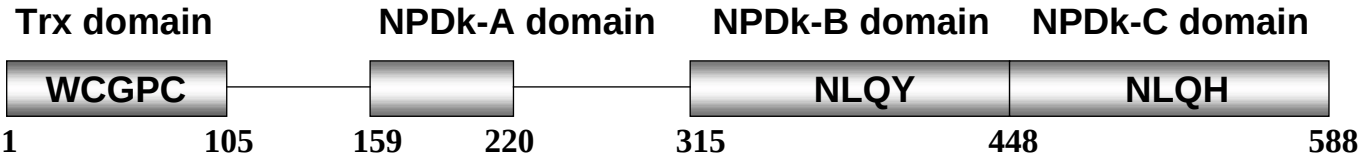
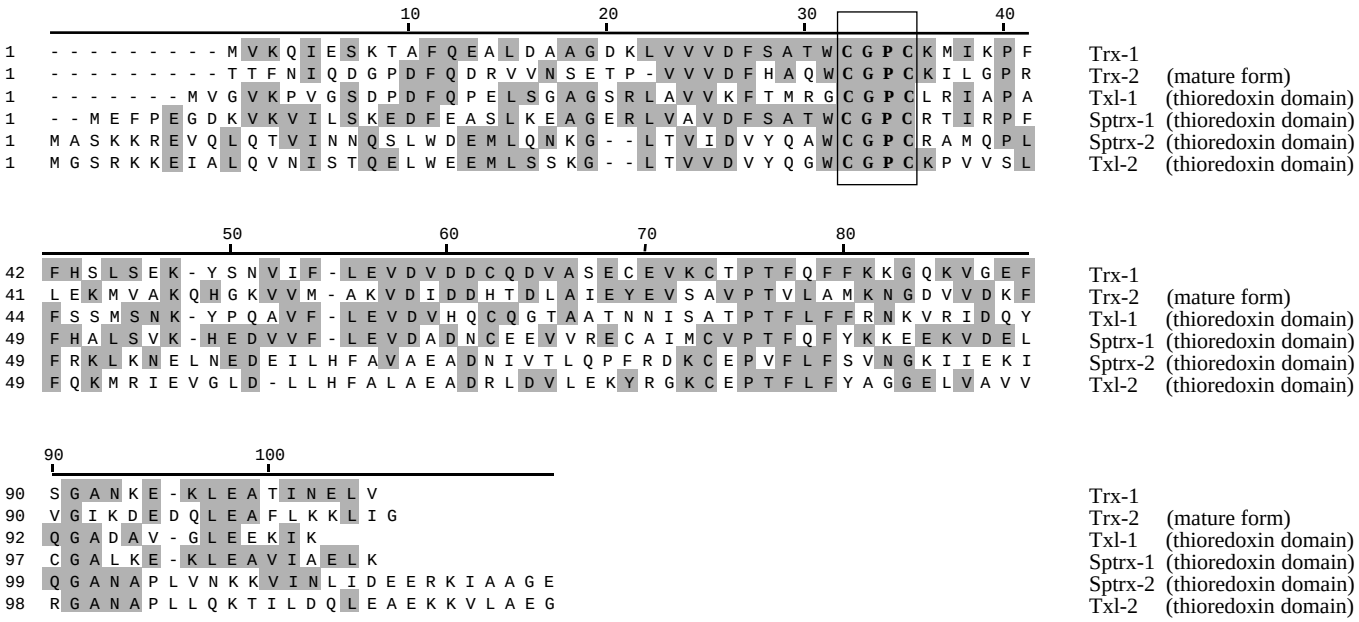
A

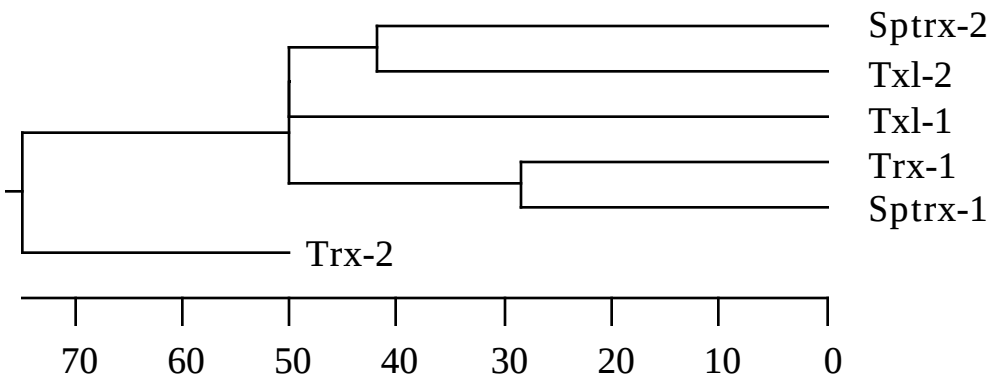


B



Sadek *et al.* (2001) Figure 2

A

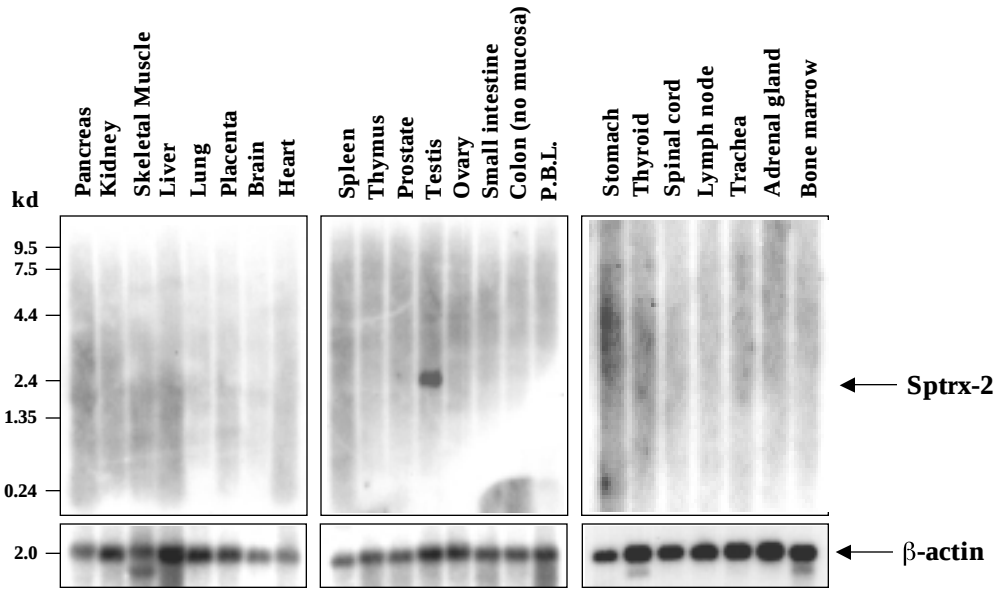


B

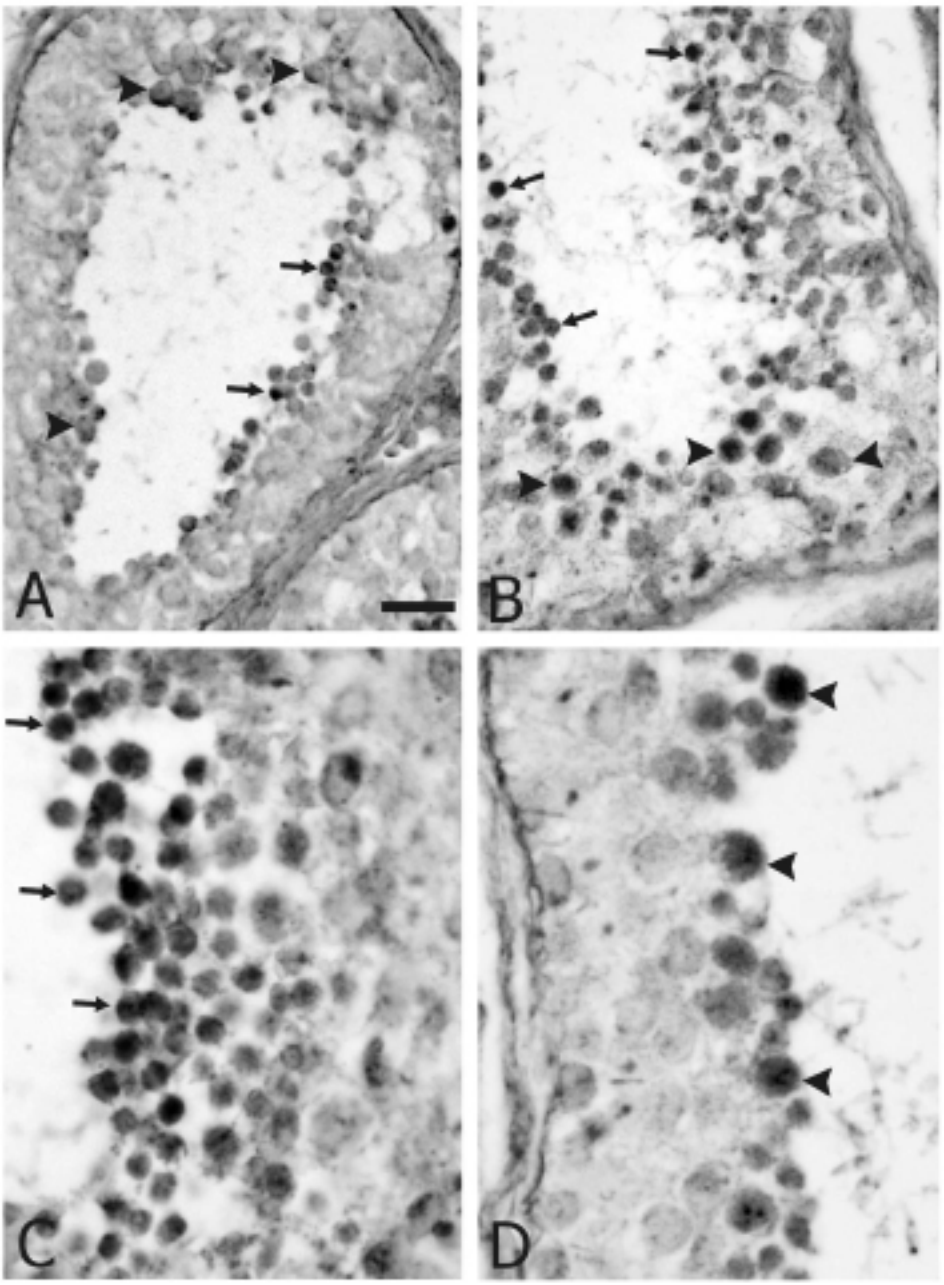
	Trx-1	Trx-2	Txl-1	Sptrx-1	Sptrx-2	Txl-2
Trx-1	■	41.9	46.5	58.1	24.8	25.6
Trx-2		■	32.5	31.6	16.0	20.2
Txl-1			■	38.6	21.1	17.5
Sptrx-1				■	23.1	23.9
Sptrx-2					■	49.2
Txl-2						■

Percent Similarity

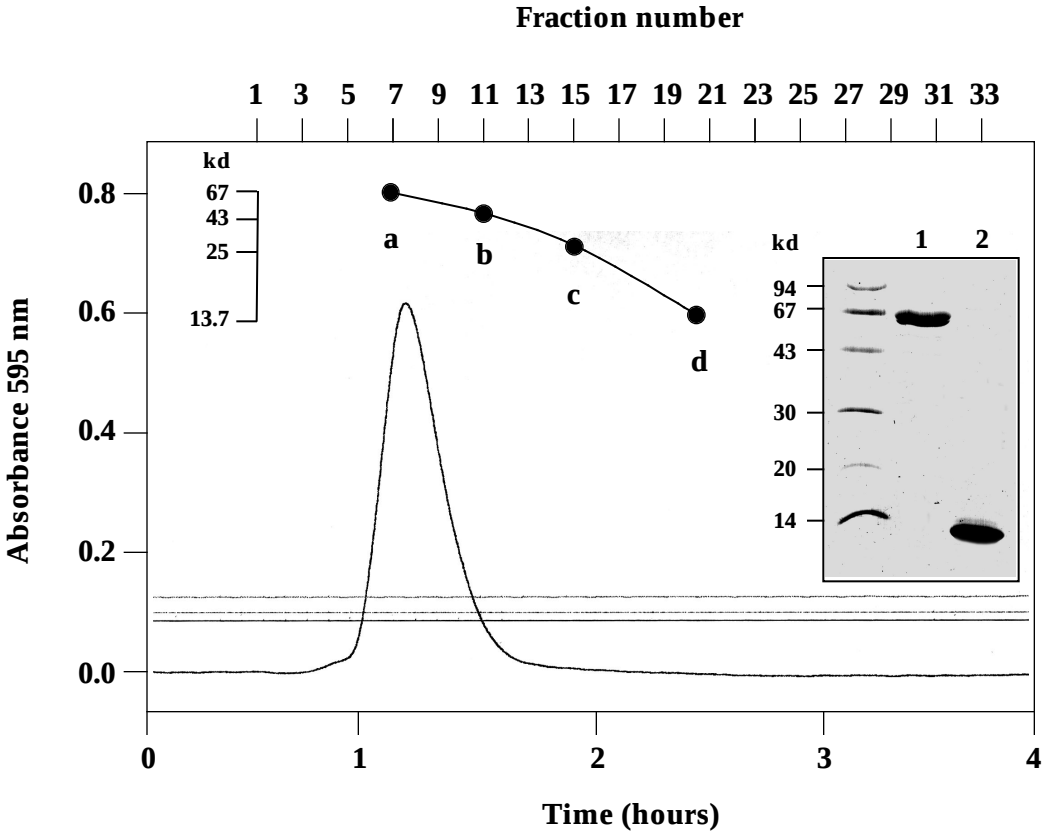
Sadek *et al.* (2001) Figure 3



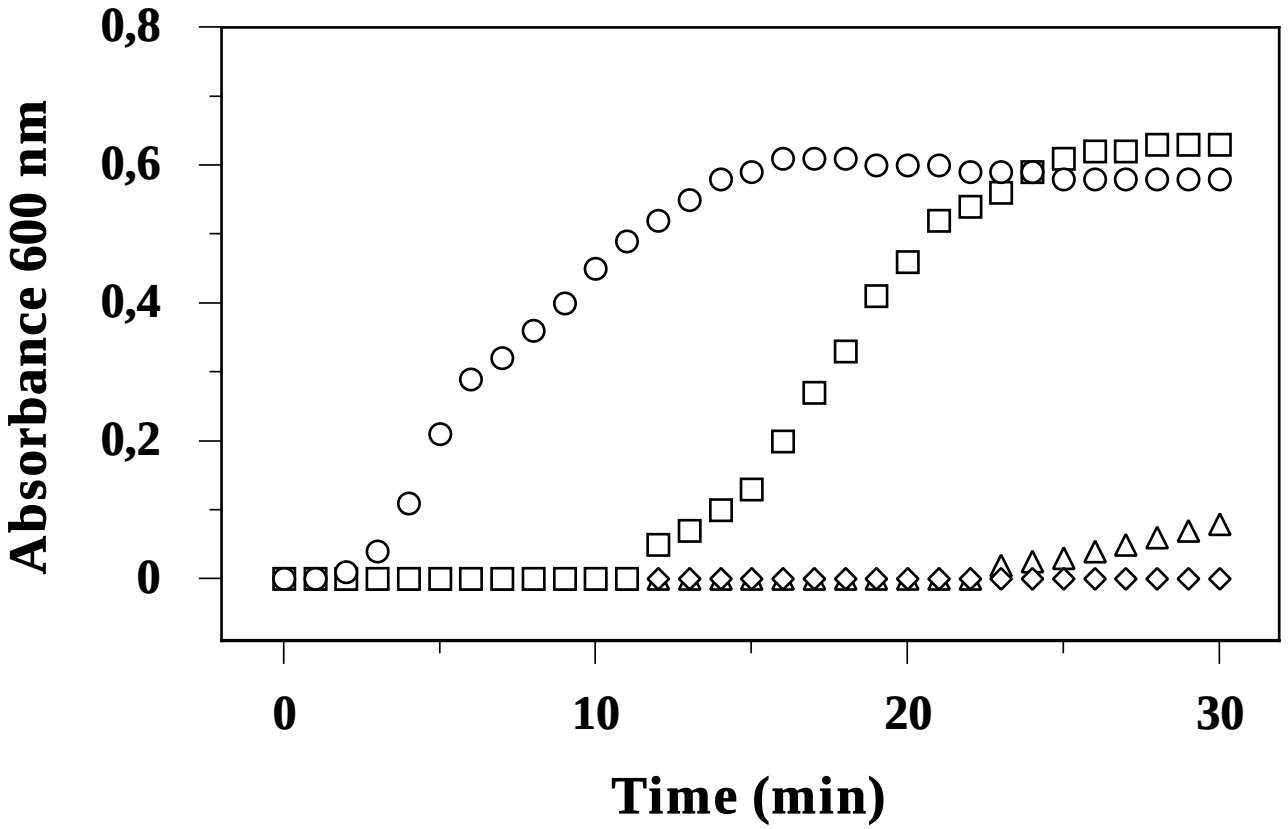
Sadek *et al.* (2001) Figure 4



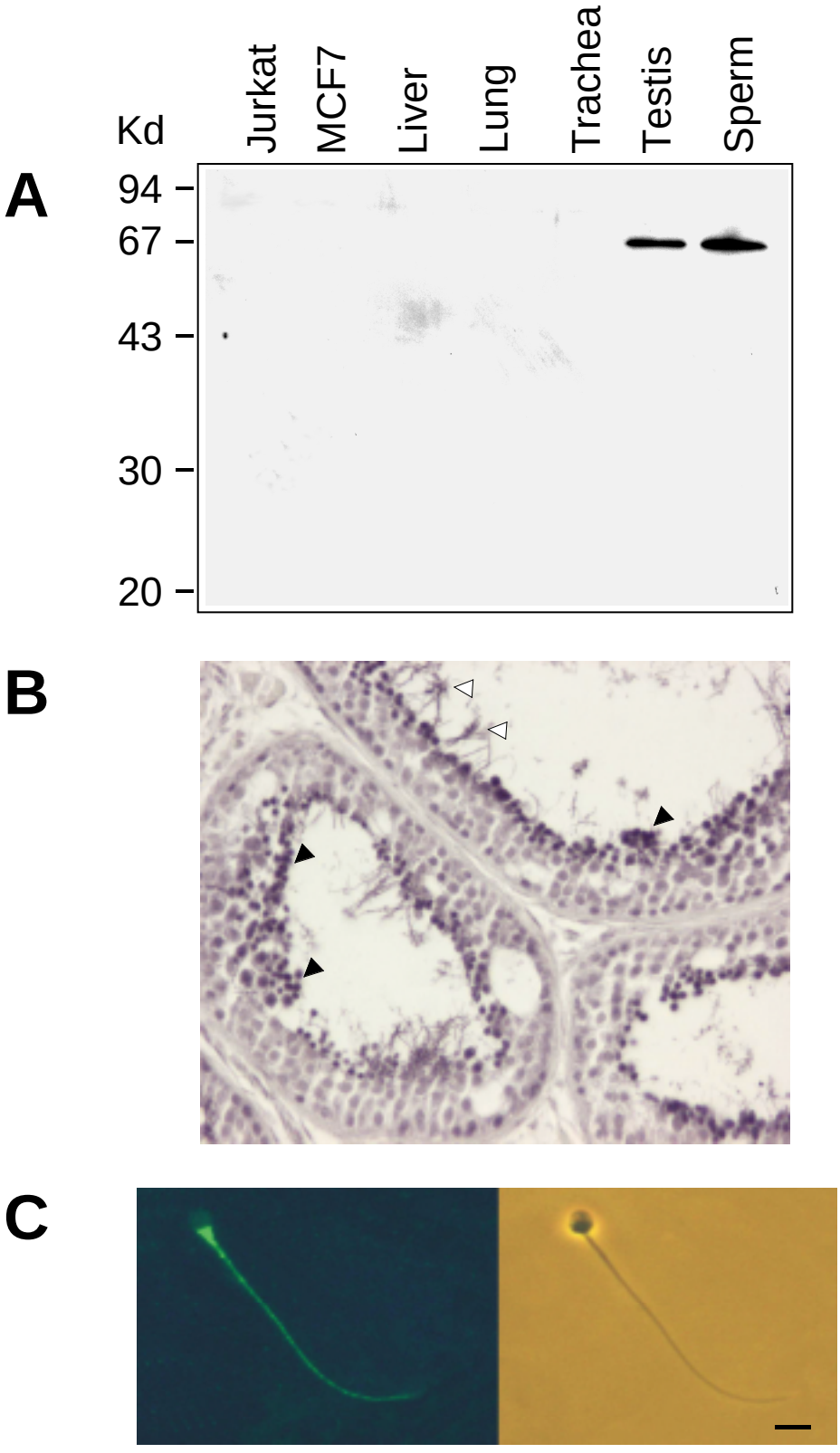
Sadek *et al.* (2001) Figure 5



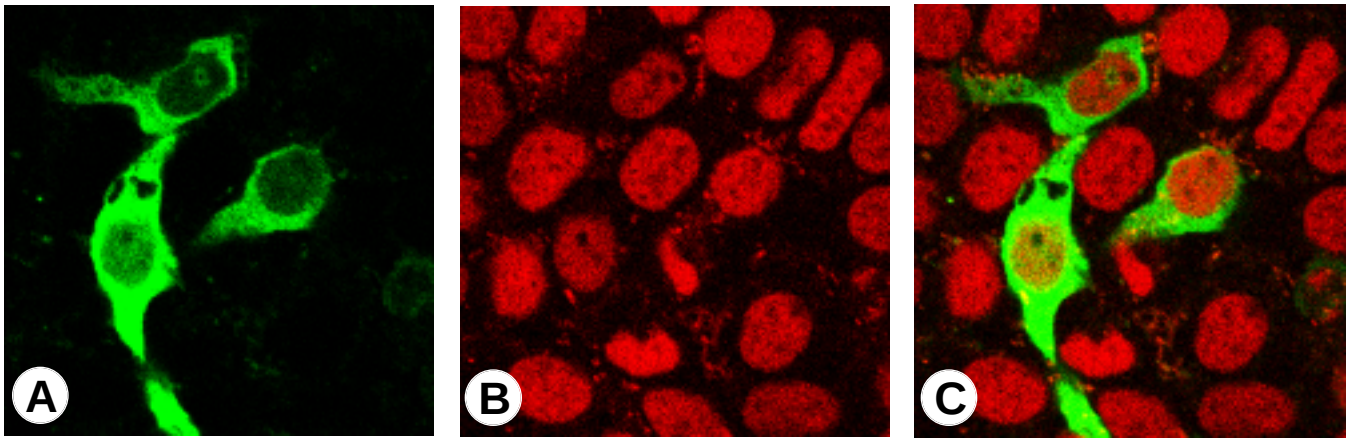
Sadek *et al.* (2001) Figure 6



Sadek *et al.* (2001) Figure 7



Sadek *et al.* (2001) Figure 8



Sadek *et al.* (2001) Figure 9