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TCTEX1D4 Interactome in Human Testis: Unraveling the Function of Dynein Light Chain in Spermatozoa

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Abstract

Studies were designed to identify the TCTEX1D4 interactome in human testis, with the purpose of unraveling putative protein complexes essential to male reproduction and thus novel TCTEX1D4 functions. TCTEX1D4 is a dynein light chain that belongs to the DYNT1/TCTEX1 family. In spermatozoa, it appears to be important to sperm motility, intraflagellar transport, and acrosome reaction. To contribute to the knowledge on TCTEX1D4 function in testis and spermatozoa, a yeast two-hybrid assay was performed in testis, which allowed the identification of 40 novel TCTEX1D4 interactors. Curiously, another dynein light chain, TCTEX1D2, was identified and its existence demonstrated for the first time in human spermatozoa. Immunofluorescence studies proved that TCTEX1D2 is an intra-acrosomal protein also present in the midpiece, suggesting a role in cargo movement in human spermatozoa. Further, an *in silico* profile of TCTEX1D4 revealed that most TCTEX1D4 interacting proteins were not previously characterized and the ones described present a very broad nature. This reinforces TCTEX1D4 as a dynein light chain that is capable of interacting with a variety of functionally different proteins. These observations collectively contribute to a deeper molecular understanding of the human spermatozoa function.

Introduction

Spermatozoa are highly specialized cells with the purpose of crossing the female reproductive tract and ultimately fertilizing the oocyte (Sutovsky and Mnandhar, 2006). Human spermatozoa, when released from testis, are morphologically mature, although functionally incapable of movement (Reid et al., 2011). In epididymis, spermatozoa acquire functional attributes, with motility being the most important one. Curiously, alterations in gene expression cannot account for the functional modifications since transcription is impaired in spermatozoa, mainly because of DNA packing by protamines (Reid et al., 2011). Intracellular control mechanisms are mainly responsible for the functional modifications that occur in spermatozoa. Reversible phosphorylation appears to be essential in motility acquisition in the epididymis, since lower phosphorylation levels of structural and functional proteins are associated with immotile spermatozoa in the caput of the epididymis, while higher levels of phosphorylation in the cauda of the epididymis are associated with motile spermatozoa (Fardilha et al., 2011b; Publicover and Barratt, 2011).

Phosphoprotein phosphatase 1 catalytic subunit gamma, isoform 2 (PPP1CC2), a testis-specific and sperm-enriched phosphatase, is involved in spermatozoa motility acquisition (Chakrabarti et al., 2007; Huang et al., 2002; Smith et al., 1996; Vijayaraghavan et al., 1996). A yeast two-hybrid screen performed in a human testis cDNA library identified the protein t-complex testis expressed protein 1 domain containing 4 (TCTEX1D4) as a PPP1CC2 binding partner (Fardilha et al., 2011b).

TCTEX1D4 is a dynein light chain protein of the DYNT1/TCTEX1 family (Korrodi-Gregório et al., 2013). Dyneins are responsible for moving cargo, organelles, and macromolecular complexes, from the periphery towards the center of the cell along microtubules. In spermatozoa flagellum, dyneins are essential for flagellar whipping since they power the slide between the doublets of microtubules. On the other hand, dyneins are involved in intraflagellar transport along the axoneme of spermatozoa. Due to the cargo diversity, dyneins are involved in a wide range of different processes such as mitosis and embryonic development (Hook and Vallee, 2006; Karcher et al., 2002; King, 2000; Sakakibara and Oiwa,

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2011). TCTEX1D4 was first described as a binding partner of endoglin in various tissues such as vascular endothelial and smooth muscle cells, placenta, and testis (Meng et al., 2006). In human spermatozoa, TCTEX1D4 is present in the flagellum, and to a lesser extend, in the head. Such distribution may hint a role in sperm motility, intraflagellar transport, and in acrosome reaction (Korrodi-Gregório, 2013).

The work described here was designed to identify the TCTEX1D4 interactome in human testis with the purpose of unraveling putative protein complexes essential to male reproduction and thus novel TCTEX1D4 functions. A yeast two-hybrid approach allowed the identification of 40 novel interactors for TCTEX1D4 among which was TCTEX1D2, another dynein light chain.

Materials and Methods

Yeast two-hybrid screen

The Matchmaker Gal4 Two-Hybrid System 2 (Clontech, Saint Germain-en-Laye, France) was used to perfom the yeast two-hybrid screen. The *TCTEX1D4* cDNA (clone IMAGE 30531412) was inserted between *NcoI* and *SalI* of pAS2-1 vector (Esteves et al., 2013), after which pAS2-1-TCTEX1D4 was transformed into a Y187 yeast strain. Yeast transformations were perfomed using a standard lithium acetate method (Gietz et al., 1995). This expression vector was first used to confirm the expression of the resulting fusion protein (GAL4-TCTEX1D4) and also to confirm that this protein did not activate *per se* the reporter genes. For library screening, the yeast strain Y187 transformed with pAS2-1-TCTEX1D4 was mated with yeast strain AH109 expressing a human testis cDNA library in the pACT-2 vector, a GAL activation domain expression vector (Clontech). Half the mating mixture was plated on high stringency medium (quadruple dropout: SD/-Ade/-His/-Leu/-Trp) and the other half in low stringency medium (triple dropout: SD/-His/Leu/-Trp) and incubated at 30°C. The colonies obtained in the low strigency medium were replica-plated in high strigency medium and all colonies that survied the high stringency medium were, finally, plated onto selective medium containing X-α-Gal at 30°C. MEL-1 expression was checked by the appearance of a blue color.

Sperm extracts

All donors were informed about the use of sperm samples for scientific purposes and a written informed consent was obtained. Also the local Ethics Committee approved the study and the procedures were in compliance with the current national laws. Human semen samples were collected from adult healthy donors by masturbation into an appropriate sterile container. All samples were analyzed according to the World Health Organization (WHO) criteria by experienced technicians and only samples considered normal were used (WHO, 2012). For all methods sperm cells were washed three times with 1X PBS.

Western blot analysis

After washing, sperm cells were dissolved in 1% SDS, and further sonicated and boiled. Extracts were mass normalized using BCA assay, and increasing quantities of protein were separated by SDS-PAGE (14%) and electrotransferred to a nitrocellulose membrane. Afterwards, the membrane was incubated with anti-TCTEX1D2 in a 1:1000, overnight at 4°C with shaking (SAB1103419- Sigma-Aldrich Química, S.A., Sintra, Portugal). Finally the membrane was incubated for 1 h at room temperature with anti-rabbit infrared secondary antibodies (1:5000, Li-Cor Biosciences UK Ltd, Cambridge, UK). Beta-tubulin was used as loading control (1:5000, Zymed Laboratories Inc., Cambridge, UK). The images were obtained using Odyssey Infrared Imaging System (Li-Cor Biosciences UK Ltd).

Blot overlay

TCTEX1D4 cDNA was PCR-amplified from pAS2-1-TCTEX1D4 using the primers: forward, 5′-GCGAATTCA TGGCCAGCAGGCCTC-3′ and reverse 5′-CCGCTCGGT CACTCGCAGTAGAGC-3′. The obtained cDNA was inserted into pET-28a vector (Novagen, Madison, WI) and transformed in bacteria *E. coli* Rosetta (DE3) strain for protein expression. After bacterial lysis, proteins were boiled, sonicated, separated by SDS-PAGE (14%), and transferred to a nitrocellulose membrane. A TCTEX1D2 indirect-immunoprecipitation was performed from sperm extracts. After washing, sperm cells were lysed with RIPA and proteases inhibitors were added (1 mM PMSF; 10 mM benzamidine; 5 μM pepstatin A; 2 μM leupeptin; 1.5 μM aprotinin; 1 mM EGTA, all from Sigma-Aldrich Química, S.A., Sintra, Portugal) and sonicated three times in 5 sec cycles. The sperm lysate was centrifuged (1600 *g*, 15 min, 4°C), the supernatant collected carefully and incubated with dynabeads protein G (Invitrogen, Life Technologies S.A., Madrid, Spain) for 1 h at 4°C, for pre-clearance. Afterwards, the dynabeads were removed and the sperm lysate was incubated with 5 μg of anti-TCTEX1D2 antibody overnight at 4°C. The lysate was then incubated with dynabeads for 1 h and 30 min at 4°C. The dynabeads were washed two times with 1X PBS and finally with a nondenaturing solution (glycine 0.1 M pH 2.5–3.0). The membrane containing pET-TCTEX1D4 was incubated with the TCTEX1D2 immunoprecipitated fraction overnight at 4°C with shaking. After washing with 1X PBS, the membranes were incubated with anti-TCTEX1D2 antibody and anti-rabbit infrared secondary antibodies. The images were obtained using Odyssey Infrared Imaging System (Li-Cor Biosciences).

Immunocytochemistry of TCTEX1D2 and TCTEX1D4

After washing, sperm cells were applied to a coverslip and left to dry. Fixation was performed with 4% paraformaldehyde (10 min) and permeabilization with ice-cooled methanol (2 min). Blocking was achieved by incubation with 3% BSA in 1X PBS at room temperature for 30 min. After washing, the samples were incubated either with anti-TCTEX1D2 antibody (1:200 SAB1103419, Sigma-Aldrich Química) or with anti-TCTEX1D4 (CBC8C) (1:150) for 2 h, followed by incubation with anti-rabbit Texas Red (1:300, T6391, MolecularProbes, Eugene, OR) for 1 h and mounted in Mowiol 4-88 (Sigma-Aldrich Química). Nuclei were stained with Hoechst 33258 (1:2000, Polysciences Europe GmbH, Eppelheim, Germany). To perform the co-localization studies with acrosome marker and to mimic biologic context, sperm cells were subject to a swim-up test as described previously (De Jonge and Barratt, 2013) to recover the highly motile sperm cells.

After, $1\ldots10^6$ motile spermatozoa were incubated in capacitating medium (37°C, 5%CO_2) for 2 h and noncapacitating medium (37°C) for 1 h (De Jonge and Barratt, 2013). To induce the acrosome reaction, capacitated spermatozoa were incubated with progesterone (3 μM- Sigma-Aldrich Química) for 30 min at 37°C. Then, the sperm samples were applied to the coverslip and left to dry. Fixation and permeabilization was performed with 95% ethanol for 30 min and washed twice with 1X PBS. The following procedure was identical as previously described. All images were obtained using an Olympus IX-81 inverted epifluorescence microscope (Olympus Portugal–Opto-Digital Tecnologias, S.A., Lisboa, Portugal).

In silico analysis

To characterize TCTEX1D2, several search engines were used. Amino acidic TCTEX1D2 sequence or Uniprot ID was used for *in silico* analysis. For secondary structure Eukaryotic Linear Motif (ELM) (Dinkel et al., 2012) and Psipred v3.3 were (http://bioinf.cs.ucl.ac.uk/psipred/) used. For putative phosphorylation sites and kinases were used: NetPhos 2.0 (Blom et al., 1999), Disphos 1.3 (http://www.dabi.temple.edu/disphos/) NetPhosK 1.0 (Blom et al., 2004), KinasePhos2 (Wong et al., 2007), Scansite (Obenauer et al., 2003), and ELM. For other putative post-translational modifications Prosite (de Castro et al., 2006); ELM; NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/); SignalP 1.4 (Petersen et al., 2011); Myristoylator (Bologna et al., 2004), and NMT (http://mendel.imp.ac.at/myristate/SUPLpredictor.htm) were used. Only results with scores above 0.8 and/or with high conservation scores were considered. To reinforce the data acquired in the previously stated databases, NetSurfP 1 (Petersen et al., 2009) was used to determine the absolute surface accessibility of the amino acids (April 27, 2013). To determine TCTEX1D2 homology between placental mammals species and to evaluate the evolutionary conservation of possible post-translational modifications and motifs, Ensembl and NCBI sequence of TCTEX1D2 orthologs were retrieved (April 16, 2013) and visualized in BioEdit software. ClustalW2 alignment was used to determine TCTEX1D2 homology between orthologs.

With the purpose of complement TCTEX1D4 interactome obtained by yeast-two hybrid, interactors already described were retrieved using Psicquic View (April 23, 2013) (Del-Toro et al., 2013). A total of 44 interactors were used for further characterization (genomic contigs and ORFs were left out). Full-length codifying sequences of all identified interacting proteins was retrieved from NCBI with the purpose of performing a PPP1 binding motif search using ScanProsite (de Castro, 2006). All PPP1 binding motif (PPP1BM) sequences searched are described in Supplementary data Table S1 (Supplementary data are available online at www.liebertpub.com). Expression (transcripts per million) of all interactors was obtained from UNIGENE (April 26, 2013), as well as protamine 2 (testis specific/enriched protein, used as control). Gene expression is inferred from ESTs (expressed sequence tags) counts from cDNA libraries. The testis expression level of all TCTEX1D4 interacting proteins was converted to percentage of transcripts in testis.

Mouse Genome Informatics (MGI) (http://www.informatics.jax.org/) and Phenopedia (Yu et al., 2010) databases were used to search male infertility phenotypes associated with the interactors retrieved. The search was performed using the Uniprot ID of all TCTEX1D4 interactors (April 26, 2013) for MGI and for Phenopedia the keyword used was ''male infertility'' (May 22, 2013). Database for annotation, visualization, and integrated discovery (DAVID) was used to retrieve functional data of TCTEX1D4 interacting proteins. DAVID is an analytic tool with the goal of extracting biological annotations and statistically highlights the most enriched from a list of genes (does not recognized isoforms) (Huang da et al., 2009). Biological process, molecular function, and cellular component of each interactor were obtained (GeneOntology), as well as associated pathways (KEGG). In all databases and tools used, whenever possible, *Homo sapiens* was chosen as the predefined species.

Results

Identification of TCTEX1D4 interacting proteins

A yeast two-hybrid screen, using TCTEX1D4 as bait, was performed against a human testis cDNA library. The number of clones screened was $2.59\ldots10^6$, which produced 494 positive clones. From these, 86 clones were sequenced corresponding to 40 different proteins, 1 open reading frame, and 3 genomic contigs.

All TCTEX1D4 interactions identified are novel, since in databases, only TGFBR3, ACVR2A, TGFBR2 and ENG are described as TCTEX1D4 binding proteins (Table 1). Since

Table 1. TCTEX1D4 Interactome Described in Databases

Database results			
Proteins	*Uniprot ID*	*Gene locus*	*PPP1BM*
ACVR2A (Activin receptor type-2A)	P27037	2q22.3	–
ENG (Endoglin)	P17813	9q34.11	–
PPP1CA (Phosphoprotein phosphatase catalytic subunit alpha)	P62136	11q13	RGVSF
PPP1CC1 (Phosphoprotein phosphatase catalytic subunit gamma 1)	P36873	12q24.1-q24.2	RGVSF
PPP1CC2 (Phosphoprotein phosphatase catalytic subunit gamma 2)	P36873	12q24.1-q24.2	RGVSF
TGFBR2 (TGF-beta receptor type-2)	P37173	3p22	FGSKVR
TGFBR3 (TFG-beta receptor type 3)	Q03167	1p33-p32	KSVVF KSVNW KRVHF

Psicquic View was used to obtain TCTEX1D4 interacting proteins already described and only interactions proven by one experimental result were considered.

PPP1CC2 co-localizes with TCTEX1D4 and is involved in spermatozoa physiology, more specifically in spermatozoa motility, the presence of the PPP1BM in TCTEX1D4 interacting proteins may indicate a putative interaction with PPP1. Among the TCTEX1D4 interacting proteins, we found that 53% have a PPP1BM. However, PPP1BM are not specific to PPP1 isoforms (Table 2).

The clone identified most frequently was an inhibitor of CDK2, Cyclin A1 interacting protein (INCA1). Differences in the number of positive clones between INCA1 (represents around 22% of the identified proteins) and other TCTEX1D4 interacting proteins, can hint the level of expression of such protein in human testis. Isoform specificity shows us if the clone identified corresponds to a unique isoform or may correspond to several isoforms. The fact that it corresponds to a unique isoform narrows the interaction to testis.

Validation of TCTEX1D4/TCTEX1D2 interaction

One of TCTEX1D4 interacting proteins identified was TCTEX1D2, another dynein light chain. This protein is only described once in the literature, in *Chlamydomonas* (DiBella et al., 2004). The work here presented confirms the presence of TCTEX1D2 in human spermatozoa for the first time. Increasing quantity of total protein was used and TCTEX1D2 was detected using a polyclonal antibody for TCTEX1D2 (Fig. 1A). A single prominent band was detected at 16 kDa, which is in agreement with the predicted molecular weight for TCTEX1D2 (16,122 Da). The amount of TCTEX1D2 in the extract seems to be low; however, the sensitivity of the antibody for Western blot might also account for this outcome.

To validate the interaction between TCTEX1D2 and TCTEX1D4, TCTEX1D2 was immunoprecipitated from human sperm cells to obtain an enriched TCTEX1D2 extract. Figure 1B shows that when a TCTEX1D4 enriched bacterial extract is incubated with TCTEX1D2 immunoprecipitated, TCTEX1D2 is detected at 27 kDa, the TCTEX1D4 molecular weight.

Subcellular localization of TCTEX1D2 and TCTEX1D4 in human spermatozoa

Localization studies were performed showing that TCTEX1D2 is primarily located in the head, particularly in the acrosome (Fig. 2A), and TCTEX1D4 in midpiece (Korrodi-Gregório et al., 2013). In immunofluorescence, the TCTEX1D2 antibody is more sensitive than for Western blot. Co-localization studies were not performed since both antibodies for the above proteins were produced in rabbit.

To understand the biologic meaning of TCTEX1D2 and TCTEX1D4 in fertilization, spermatozoa were capacitated, the acrosome reaction was induced, and an acrosome marker was used. *Pisum sativum* agglutinin associated with FITC (PSA-FITC) is a negative staining of acrosome, which means that acrosome reaction is assessed by the disappearance of the labeling. Before acrosome reaction, the co-localization pattern between PSA-FITC and TCTEX1D2 supports the evidence that TCTEX1D2 is an intra-acrosomal protein (Fig. 2B, arrowhead). After acrosome reaction, both TCTEX1D2 and PSA staining disappeared, further confirming that TCTEX1D2 is an acrosomal protein (Fig. 2B). Also, to a lesser extent, TCTEX1D2 is present in the midpiece (Fig. 2B, arrow). In spermatozoa that did not suffer acrosome reaction, 37% ($n = 100$) of spermatozoa present an acrosome plus midpiece pattern, and 63% ($n = 100$) an acrosomal localization for TCTEX1D2.

The most common localization pattern for TCTEX1D4 is head and midpiece, which is in accordance with results shown by Korrodi-Gregório et al. (2013) (Fig. 2C, cross). Nevertheless, the amount of TCTEX1D4 present in the head (Fig. 2C, stars) appears to be less than in the midpiece (Fig. 2C, cross), and in some spermatozoa the location is not altered by the induction of the acrosome reaction. Again, in spermatozoa that did not suffer acrosome reaction, 60% ($n = 100$) of spermatozoa present on the head plus midpiece pattern for TCTEX1D4 and 40% ($n = 100$) present TCTEX1D4 in the midpiece.

In silico *characterization of TCTEX1D2*

In order to identify physiological relevant motifs and post-translational modifications, a bioinformatics analysis of TCTEX1D2 was performed. According to ELM and Psipred, TCTEX1D2 amino acid sequence is divided in two distinct domains: a small disordered domain (1–21) and a larger globular domain (22–142). The globular domain comprises one alpha helix, followed by four beta strands. Figure 3 shows that TCTEX1D2 contains four putative phosphorylated serines (8, 10, 42, 62), two of them in the disordered domain. According to NetSurfP, serine 42 is more likely to suffer phosphorylation since it has an absolute surface accessibility of 0.70, while serine 8 and 10 have a score below 0.2 and serine a score of 0.49. Four kinases were found to putatively phosphorylate TCTEX1D2: ataxia telangiectasia mutated (ATM, S8, S10, S42, S62), protein kinase B (PKB, S10), polo-like kinase 1 (PLK1, S62), and casein kinase 2 (CK2, S62). No potential threonines or tyrosines phosphorylation sites were found. No other post-translational modifications were found in TCTEX1D2. However, a putative binding site for MAPK1 was identified. A PPP1BM (35–40) was also identified in TCTEX1D2, corresponding to the F-X-X-[RK]-X-[RK] motif present in apoptotic proteins.

Homology studies showed that serine 42 is highly conserved among placental mammals (Fig. 4, arrowhead), unlike serines 8, 10, and 62. Also in Figure 4 it is possible to observe that the PPP1BM (Fig, 4, box 1) and the MAPK1 binding motif (Fig. 4, box 2) are highly conserved and are present in all mammalian species analyzed.

Profiling TCTEX1D4 interactome—Analysis in silico

Testis expression of TCTEX1D4 interacting proteins. Figure 5 represents the testis expression in % of the TCTEX1D4 interacting proteins and protamine 2 (PRM2), which were retrieved from UNIGENE (http://www.ncbi.nlm.nih.gov/unigene). Protamine 2 is a well-described testis-specific protein responsible for DNA packing in spermatozoa (Reid et al., 2011). Since the purpose of the expression profile was to identify testis-specific proteins, PRM2 was used as a control. Two proteins, CRISP2 and TSC21, present the same level of expression in testis as PRM2, which indicates that they are testis specific. Data mining confirmed that both proteins are described as testis specific (Busso et al., 2005; Cohen et al., 2008; Yu et al., 2007). Also, FNDC8 presents a

Table 2. Partial Human Testis TCTEX1D4 Interactome

Testis yeast two-hybrid results					
Proteins	*No. clones*	*Uniprot ID*	*Gene locus*	*Isoform specificity*	*PPP1BM*
ACTN1 (Alpha-actinin-1)	1	P12814	14q22-q24	All isoforms (3)	RVGW
ATXN7L1 (Ataxin-7-like protein 1)	1	Q9ULK2	7q22.3	Isoform 1 and 3	–
CCDC89 (Coiled-coil domain-containing protein 89)	5	Q8N998	11q14.1	–	–
CRISP2 (Cysteine-rich secretory protein 2)	4	P16562	6p12.3	All isoforms (5)	–
CTSB (Cathepsin B)	1	P07858	8p22	–	RVMF
CTSL1 (Cathepsin L1)	1	P07711	9q21.33	Isoform 1	RSVDW
FBLN1 (Fibulin-1)	1	P23142	22q13.31	Isoform 3	–
FLOT1 (Flotillin-1)	1	O75955	6p21.3	–	RARA
FNDC8 (Fibronectin type III domain-containing protein 8)	3	Q8TC99	17q12	–	–
GALNTL2 (Polypeptide N-acetylgalactosaminyltransferase 15)	1	Q8N3T1	3p25.1	–	KEIHF
GARS (Glycine–tRNA ligase)	1	P41250	7p15	–	–
GEMIN4 (Gem-associated protein 4)	1	P57678	17p13	–	LSVLF
GNB2L1 (Guanine nucleotide-binding protein subunit beta-2-like 1)	1	P63244	5q35.3	–	LSVAF
IDH3B (Isocitrate dehydrogenase [NAD] subunit beta, mitochondrial)	1	O43837	20p13	All isoforms (3)	KIKF
HNRNPF (Heterogeneous nuclear ribonucleoprotein F)	1	P52597	10q11.21	–	FVVKLR
IFT88 (Intraflagellar transport protein 88 homolog)	2	Q13099	13q12.1	All isoforms (2)	KAIKF GILK
INCA1 (Inhibitor of CDK interacting with cyclin A1)	19	Q0VD86	17p13.2	All isoforms (2)	–
LRRC71 (Leucine-rich repeat-containing protein 71)	1	Q8N4P6	1q23.1	–	KYVFF
MAN2B1 (Lysosomal alpha-mannosidase)	2	O00754	19cen-q13.1	Isoform 1	RKVNW RVAW
OSGEP (Probable tRNA threonylcarbamoyladenosine biosynthesis protein)	4	Q9NPF4	14q11.2	–	–
PDXP (Pyridoxal phosphate phosphatase)	2	Q96GD0	22cen-q12.3	–	–
QRICH1 (Glutamine-rich protein 1)	1	Q2TAL8	3p21.31	All isoforms (2)	–
RANBP9 (Ran-binding protein 9)	1	Q96S59	6p23	–	RMIHF
RBM4B (RNA-binding protein 4B)	1	Q9BQ04	11q13	–	–
SECISBP2 (Selenocysteine insertion sequence-binding protein 2)	2	Q96T21	9q22.2	–	LNVAW
SMURF1 (E3 ubiquitin-protein ligase SMURF1)	1	Q9HCE7	7q22.1	All isoforms (3)	RIYF LMVKF
STAM2 (Signal transducing adapter molecule 2)	1	O75886	2q23.3	–	LMVEW
TCTEX1D2 (Tctex1 domain-containing protein 2)	1	Q8WW35	3q29	–	FQQRFR
TECR (Very-long-chain enoyl-CoA reductase)	1	Q9NZ01	19p13.12	–	–
TSC21 (Testis-expressed sequence 37 protein)	1	Q96LM6	2p11.2	–	–
TIMM50 (Mitochondrial import inner membrane translocase subunit TIM50)	1	Q330K1	19q13.2	–	–
TTC28 (Tetratricopeptide repeat protein 28	2	Q96AY4	22q12.1	All isoforms (2)	FVEKVR
UBE2D2 (Ubiquitin-conjugating enzyme E2 D2)	4	P62837	5q31.2	Isoform 2	KVAF LTIHF
UQCRC1 (Cytochrome b-c1 complex subunit 1, mitochondrial)	1	P31930	3p21.3	–	RDVVF
USP33 (Ubiquitin carboxyl-terminal hydrolase 33)	1	Q8TEY7	1p31.1	All isoforms (3)	–
WDR73 (WD repeat-containing protein 73)	2	Q6P4I2	15q25.2	–	LRVTW
ZIM2 (Zinc finger imprinted 2)	1	Q9NZV7	19q13.4	–	–
ZNF335 (Zinc finger protein 335)	2	Q9H4Z2	20q13.12	–	–
ZNF562 (Zinc finger protein 562)	1	Q6V9R5	19p13.2	All isoforms (3)	FEEKTK
ZNF638 (Zinc finger protein 562)	2	Q14966	2p13.1	All isoforms (2)	FMAKQR FISRYR
Chromosome 11 genomic contig	1	_	11	_	–
Chromosome 11 genomic contig, GRCh37.p5 Primary Assembly	2	_	11	_	–
Chromosome 19 genomic contig, GRCh37.p5	1	_	19	–	_
Chromosome 12 open reading frame 63	1	_	12		KEVHF

Gene locus was retrieved from NCBI. PPP1BM were obtained by full sequence scan using ScanProsite tool.

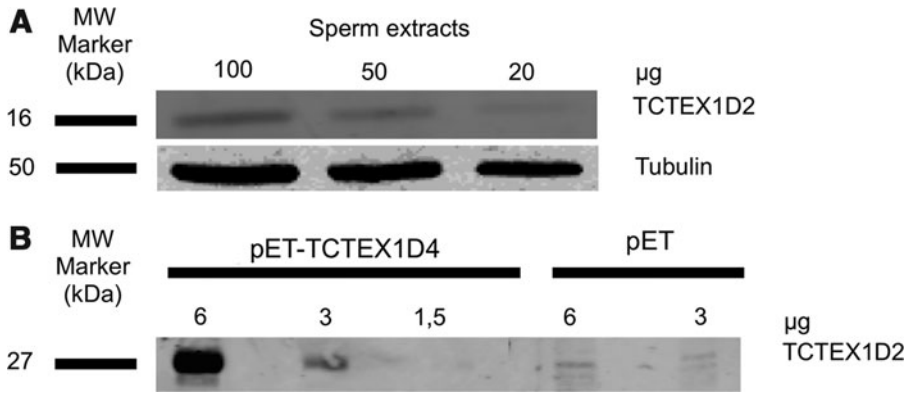

FIG. 1. TCTEX1D2 is present in sperm cells and interacts with TCTEX1D4. **(A)** TCTEX1D2 detection in human sperm extracts. Increasing quantity of total protein was used; TCTEX1D2 was detected at 16 kDa. Beta-tubulin was used as a loading control. **(B)** An enriched bacterial extract of TCTEX1D4 was incubated with an enriched TCTEX1D2 extract, which resulted in detection of TCTEX1D2 at 27 kDa (TCTEX1D4 molecular weight). pET was used as a control.

high level of expression in testis but there are no references to support it as being a testis-specific protein. LRRC71, although not presenting the levels of expression in testis as high as PRM2, has more than 50% of its ESTs expressed in testis. INCA1, CCDC89, and IFT88 present more than 10% of expression in testis. Other TCTEX1D4 interacting proteins have less than 10% expression in testis.

Associated phenotype. With the purpose of identifying TCTEX1D4 interacting proteins with male infertility phenotypes, a search in MGI and Phenopedia databases was performed. RANBP9, PPP1CC, ACVR2A, TGFBR3, and CTLS1 knockouts in mice present phenotypes that interfere with the several stages of spermatogenesis, sperm cells physiology, histology, and morphology of male reproductive system. In Phenopedia, the only hit with male infertility phenotype was CRISP2. Other TCTEX1D4 interacting proteins either present a phenotype with no relation with male infertility or do not have an associated phenotype.

Gene ontology profile. Molecular function, biologic process, and cellular component characterization of TCTEX1D4 interactome were retrieved from DAVID. Notice that each protein can be classified in more than one category. In Figure 6A, the molecular functions of TCTEX1D4 interacting proteins are represented. The most prevalent molecular function of TCTEX1D4 interacting proteins is cation binding. In this category are included proteins that interact non-covalently with charged atoms or groups of atoms with a positive charge, for example, calcium and magnesium. 37.8% of the TCTEX1D4 interacting proteins have an unknown molecular function. TCTEX1D4 interacting proteins tend to bind to a diversity of molecules, specifically RNA (11.1%), sugars (8.9%), and proteins. Particularly in protein binding, receptor binding and protein complex binding are the most common molecular functions, representing 11.1%. It is noteworthy to mention that all categories of protein binding (SMAD binding, cytokine binding, growth factor binding, enzyme binding, protein complex binding, and receptor binding) are heavily influenced by the fact that TCTEX1D4 interacting proteins retrieved from databases are involved in the TGF-beta pathway. However, most of the proteins with cation binding function were identified in the testis yeast two-hybrid system (Supplementary data Table S2).

TCTEX1D4 interacting proteins are categorized in a myriad of biologic processes (Fig. 6B). Whereas 35.6% of the TCTEX1D4 interacting proteins are not classified in a biologic process, the phosphate metabolic process represents 17.8% of the TCTEX1D4 interacting proteins. Also, a representative category is proteolysis (17.8%). Curiously, TCTEX1D4 interacting proteins appear to be involved in *de novo* synthesis of proteins since the RNA metabolic processes, transcription, and translation, represent, respectively, 17.8%, 11.1%, and 4.4% of the TCTEX1D4 interacting proteins (Supplementary data Table S3).

Finally, concerning the cellular component of TCTEX1D4 interacting proteins, most of the TCTEX1D4 interacting proteins do not have a defined subcellular localization (Fig. 7A). However, 20% of the TCTEX1D4 interacting proteins are integral to membranes, which means that the proteins penetrate at least one layer of any bilayer membrane. Mitochondrial membranes (4.4%) and plasma membranes (4.4%) are the most representative bilayer membranes in which TCTEX1D4 interacting proteins are localized. Also, several proteins are associated with specific cellular organelles, such as mitochondrion (13.3%), lytic vacuole (6.7%), Golgi apparatus (4.4%), and endoplasmic reticulum (4.4%).

TCTEX1D4 interacting proteins in the cellular component are shown in Supplementary data (Supplementary data Table S4).

KEGG pathway profile. According to Figure 7A, 77.8% of the TCTEX1D4 interacting proteins are not associated with a specific signaling pathway. However, 6.7% of the proteins are associated with endocytosis and the TGF-beta pathway, which is strongly influenced by the TCTEX1D4 interacting proteins of TGF-beta family. Adherent's junction, lysosome, and insulin signaling pathways represent each 6.7% of the TCTEX1D4 interacting proteins (Supplementary data Table S5).

Discussion

TCTEX1D4 has been identified as an endoglin interactor in placenta (Meng et al., 2006) and as a PPP1CC2 interactor

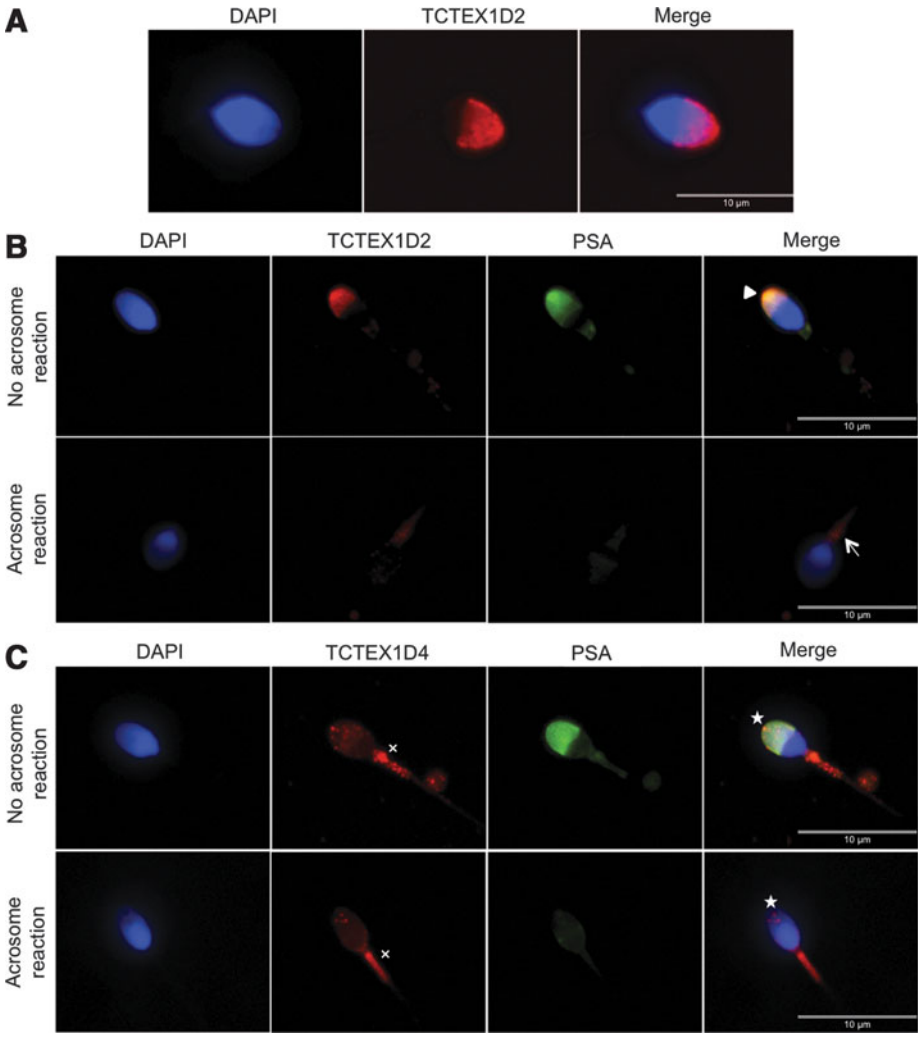

FIG. 2. Cellular localization of TCTEX1D2 and TCTEX1D4 in human spermatozoa. Human spermatozoa stained with anti-TCTEX1D2 (**A**, **B**, and **C**) anti-TCTEX1D4 (CBC8C) were detected using a Texas Red secondary antibody, PSA-FITC, and Hoechst 33258. All images were obtained with a 100X magnification in a Olympus IX-81 inverted epifluorescence microscope. **(A)** Human spermatozoa stained with anti-TCTEX1D2. **(B)** Human spermatozoa stained with anti-TCTEX1D2 and PSA-FITC, with and without acrosome reaction. *Arrowhead* represents the co-localization between TCTEX1D2 and PSA. *Arrow* indicates the staining of TCTEX1D2 in the midpiece. **(C)** Human spermatozoa stained with anti-TCTEX1D4 (CBC8C,) and PSA-FITC, with and without acrosome reaction. *Crosses* show TCTEX1D4 localization in midpiece. *Stars* represent the acrosomal localization of TCTEX1D4 before and after acrosome reaction.

in testis (Fardilha et al., 2011b), and appears to be essential in MTOC dynamics and sperm motility (Korrodi-Gregório et al., 2013). Nevertheless, information regarding the exact function of TCTEX1D4 is still sparse. Thus, a testis TCTEX1D4 yeast two-hybrid was performed to identify novel TCTEX1D4 interactions and consequently to have a glance at TCTEX1D4 functions in testis. Forty novel interactors were identified being INCA1, the most common. INCA1 was first described as an interactor and substrate of cyclin A1-CDK2 complex. Interestingly, cyclin A1 and INCA1 have highest expression levels in human normal testis. INCA1 was described as a regulator of the cell cycle, and is associated with testis maturation having a role in spermatogenesis (Baumer et al., 2011; Diederichs et al., 2004). The fact that INCA1 is highly present in testis, and together with TCTEX1D4 are located in the nucleus can indicate the involvement of TCTEX1D4 in meiosis and mitosis, particularly in spermatogenesis (Baumer et al., 2011; Korrodi-Gregório et al., 2013).

Another TCTEX1D4 interacting protein identified was also a dynein light chain, TCTEX1D2. This protein was only described in *Chlamydomonas* flagellum I1 inner arm and although not essential for inner arm assembling, seems to be crucial to flagellum stabilization (DiBella et al., 2004). Our results show for the first time the presence of TCTEX1D2 in human sperm cells. The interaction between both proteins was proven by yeast two-hybrid and blot overlay. However, none of the above methods take into account subcellular

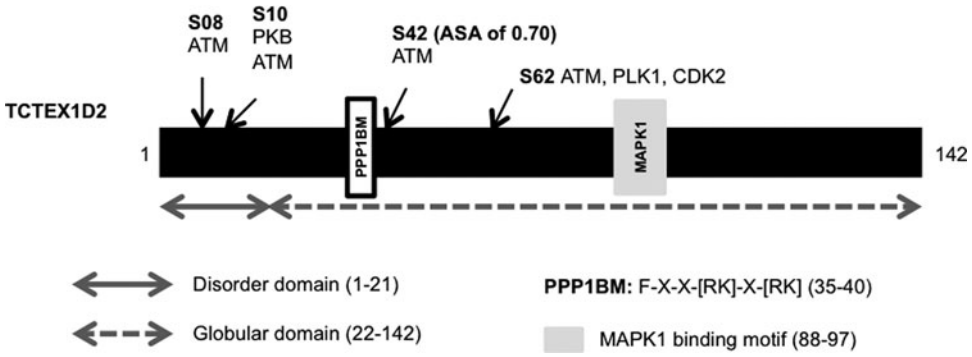

FIG. 3. Schematic representation of human TCTEX1D2. Both TCTEX1D2 domains are represented in the putative motifs and phosphorylation sites for kinases. ASA, absolute surface acessibility; ATM, ataxia telangiectasia mutated; CDK2, cyclin-dependent kinase 2; MAPK1, mitogen-activated protein kinase 1; PKB, protein kinase; and PLK1, polo–like kinase 1.

localization and therefore we cannot state that both proteins interact *in vivo*. A first immunofluorescence study showed that TCTEX1D2 was mainly in the head, particularly in the acrosome region. With the goal of mimicking as accurately as possible the physiological context of human sperm cells, spermatozoa were capacitated and acrosome reaction was induced. The results showed that TCTEX1D2 is mainly an intra-acrosomal protein and to a lesser extent present in the midpiece. Interestingly, these results are not in accordance with the work in *Chlamydomonas* and may hint at a unique role for TCTEX1D2 in mammals, notably in humans. It was already proved that DLC8, a dynein light chain highly

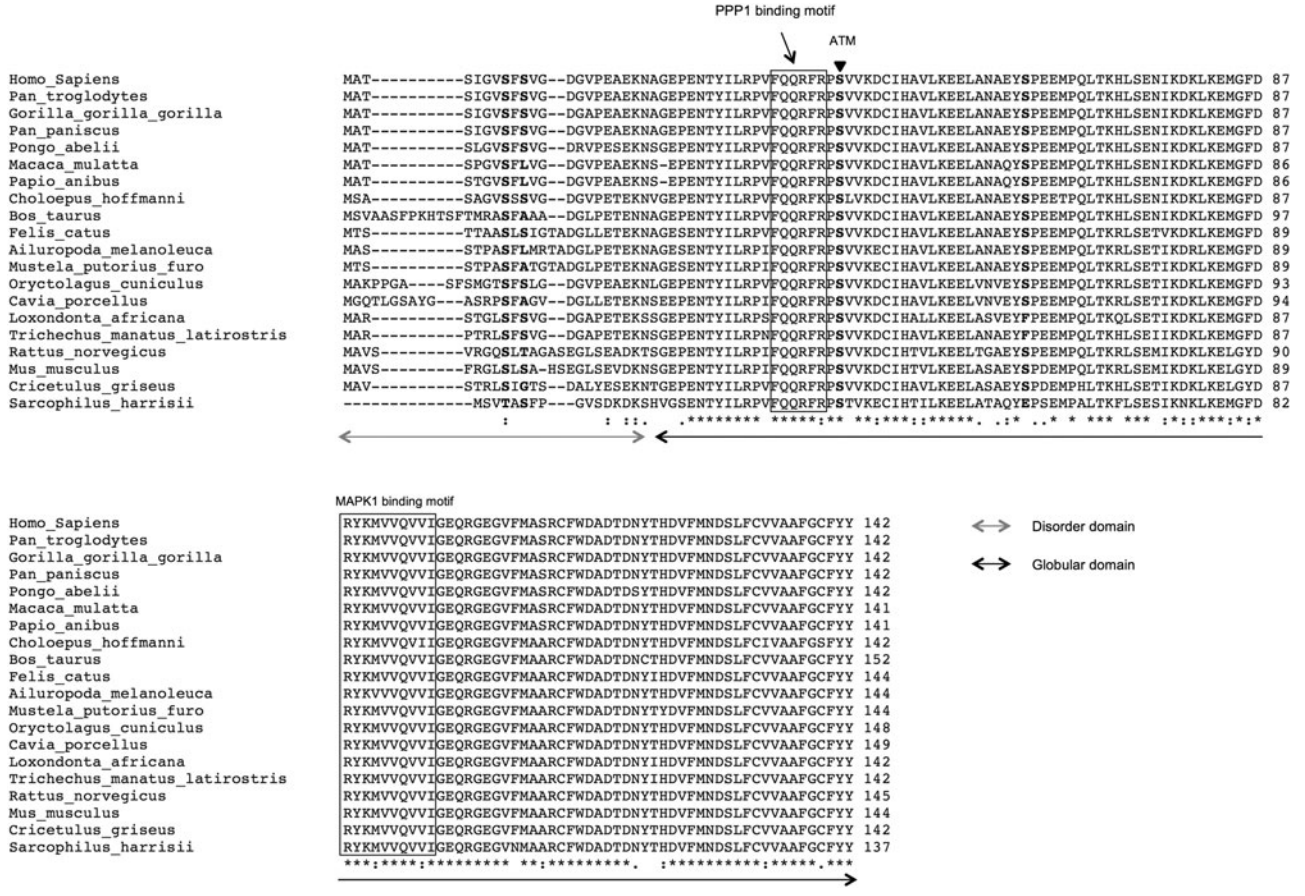

FIG. 4. Alignment of TCTEX1D2 orthologs in placental mammals. Sequences were obtained in Ensembl and NCBI. ClutalW2 was used to align all sequences. ATM, ataxia telangiectasia mutated; MAPK1, mitogen-activated protein kinase 1; PPP1, phosphoprotein phosphatase 1. *(asterisk) indicates positions which have a single, fully conserved residue; : (colon) indicates conservation between groups of strongly similar properties; and . (period) indicates conservation between groups of weakly similar properties.

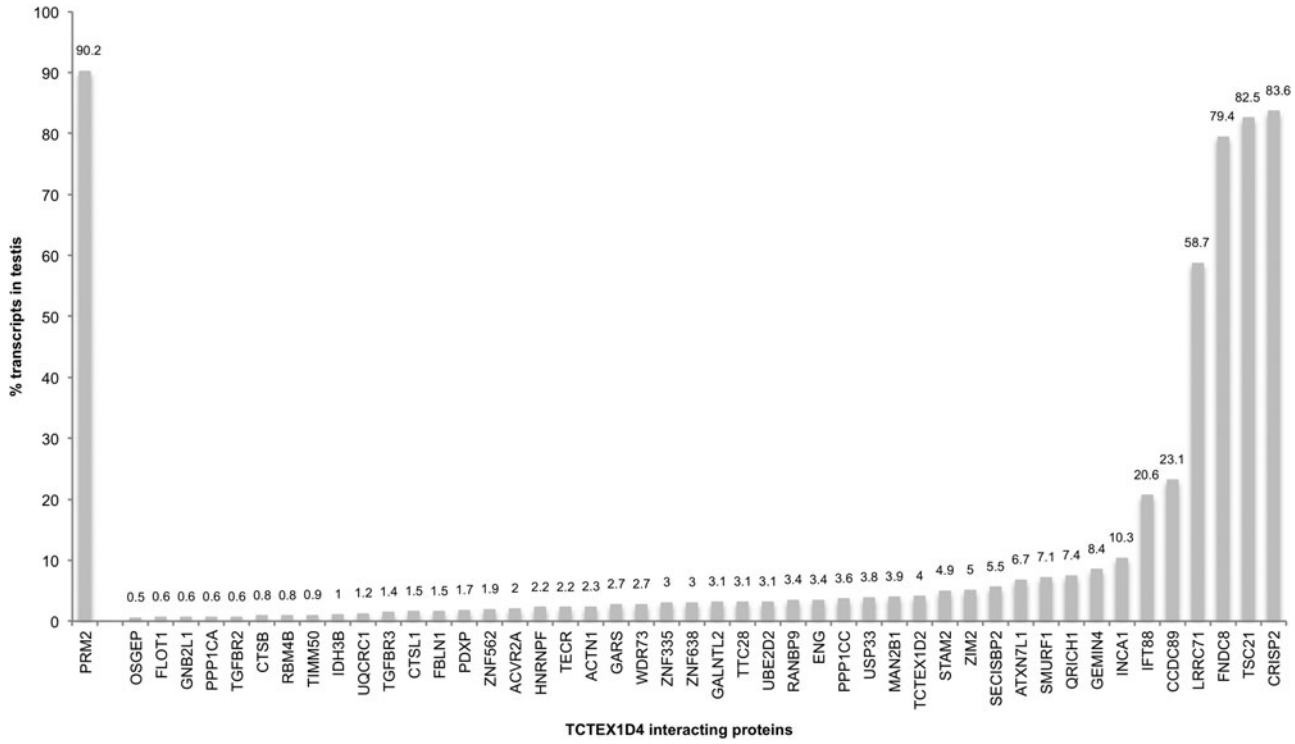

FIG. 5. Testis expression of the TCTEX1D4 interacting proteins. Transcripts per million of all TCTEX1D4 interacting proteins was retrieved form UNIGENE and converted to percentage of transcripts in testis.

conserved, displays nonrelated transport functions. This protein plays a role in apoptosis, cell cycle, protein inhibition, and promotion of protein dimerization (Mohan and Hosur, 2009). TCTEX1D2 localization in the acrosome may hint dynein nonrelated functions for this protein in mammalian spermatozoa. On the other hand, TCTEX1D4 is mainly localized in the midpiece and in the acrosome to a lesser extent, which agrees with the results previously obtained (Korrodi-Gregório et al., 2013). These results suggest that in human spermatozoa the interaction between TCTEX1D4 and TCTEX1D2 will occur in the acrosome and in the midpiece. Acrosome is formed at early stage of spermiogenesis and consists in one single large acrosomic granule. During sperm epididymal passage, some intra-acrosomal modifications occur, mostly biochemical changes and distribution of several antigens (Abou-Haila and Tulsiani, 2000; Toshimori, 1998). TCTEX1D2/TCTEX1D4 complex may be responsible for such changes in the distribution, working as dynein light chains and transporting proteins along the acrosome. TCTEX1D2/TCTEX1D4 complex or TCTEX1D2

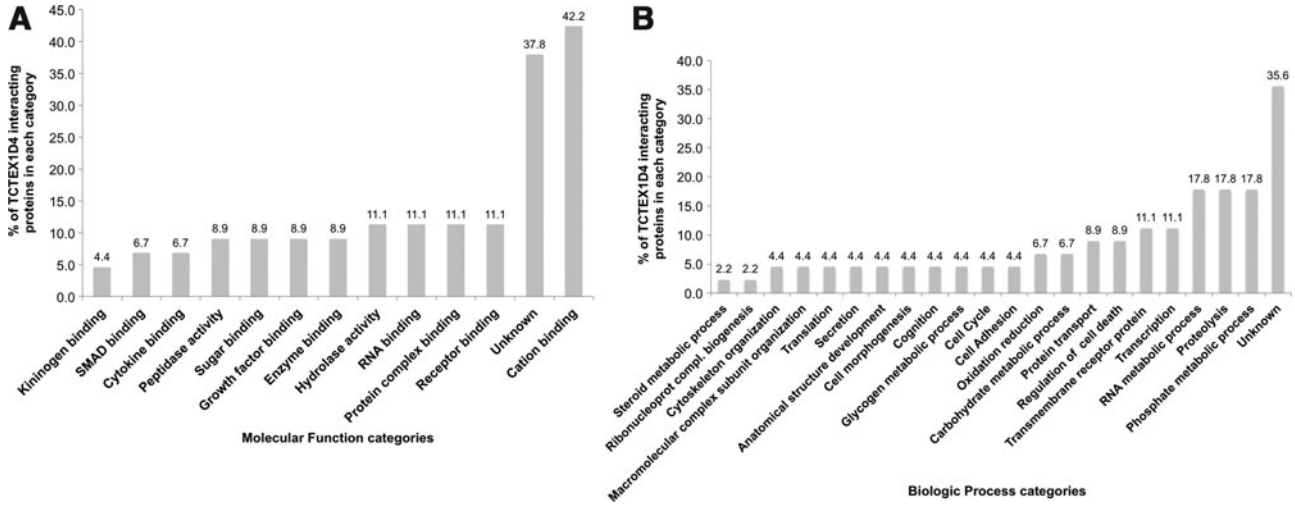

FIG. 6. Molecular function, biologic process, cellular component, and signaling pathways of the TCTEX1D4 interacting proteins. **(A)** Molecular functions; **(B)** biologic process were retrieved using DAVID. Percentage of TCTEX1D4 interacting proteins in each category was calculated and plotted.

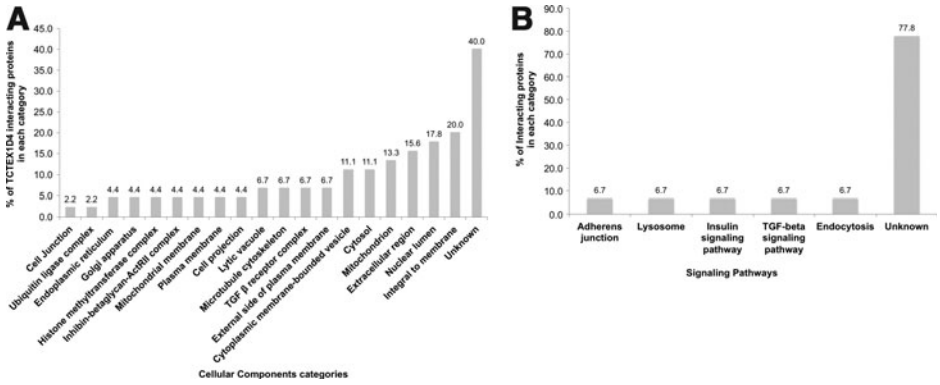

FIG. 7. Cellular component and signaling pathways of the TCTEX1D4 interacting proteins. **(A)** Cellular component and **(B)** signaling pathways were retrieved using DAVID; percentage of TCTEX1D4 interacting proteins in each category was calculated and plotted.

throughout its interacting proteins can control calcium and kinase receptors localization, which are essential for acrosome reaction. Since the acrosome reaction depends on protein phosphorylation and PPP1CC2 is present in the acrosome, an interplay between the two dynein lights chains and the phosphatase could also happen.

Regarding the function of TCTEX1D2/TCTEX1D4 complex in the midpiece, this complex may have a key role in energy production, since the midpiece is highly enriched in mitochondria. As translation is impaired in spermatozoa, intraflagellar transport has a vital role, allowing the re-location of existing proteins, vesicles, and organelles. Being TCTEX1D2 and TCTEX1D4 dynein light chains, they can be responsible for this transport in the midpiece (Silverman and Leroux, 2009).

Since TCTEX1D4 was identified as a PPP1CC2 interactor, a search for PPP1BM in all TCTEX1D4 interacting proteins was performed to unravel possible tri-complexes. More than half the interactors present a PPP1BM, which may indicate a putative formation of trimers. In fact, a PPP1BM was also identified in TCTEX1D2, which is usually present in apoptotic proteins, such Bcl-2 and Bad, and confers an apoptotic signature (Godet et al., 2010).

To better understand the biological function of human TCTEX1D2, an *in silico* analysis was performed. Phosphorylation appears to be the only post-translational modification of TCTEX1D2. Four putative phosphorylation serines were identified, with serine 42 having the highest probability of phosphorylation by ATM. Interestingly, all kinases that putatively phosphorylate TCTEX1D2 (ATM; PKB; PLK1; CD2) are involved in the regulation of spermiogenesis and spermatogenesis (Anai et al., 2005; Barlow et al., 1998; Mannowetz et al., 2010; Matsubara et al., 1995).

TCTEX1D4 partial testis interactome was also analyzed with a goal of profiling TCTEX1D4 interactome and ultimately to unveil more information regarding the role of TCTEX1D4 in testis and spermatozoa. The analysis of the testis expression level of the interactors revealed that CRISP2 and TSC21 are testis-specific proteins with well supported data (Busso, 2005; Yu, 2007). CRISP2, in testis, acts as a bridging molecule between spermatogenic and Sertoli cells (Maeda et al., 1998; 1999). In human spermatozoa, CRISP2 remains in sperm acrosome even after acrosome reaction, in the equatorial segment (Busso, 2005), and in the flagellum is present in the outer dense fiber (Busso et al., 2007; Cohen, 2008; O'Bryan et al., 1998). Identification of TCTEX1D4/CRISP2 interaction reinforces the presence of TCTEX1D4 in the acrosome and its possible involvement in the acrosome reaction and fertilization. In testis, TCTEX1D4 is present in the cytoplasm of spermatogenic and Sertoli cells, particularly near the cell–cell junction, as well as CRISP2 (Korrodi-Gregório et al., 2013; Maeda, 1998; 1999). These subcellular localizations support the hypothesis that TCTEX1D4 can be responsible for the retrograde transport of CRISP2, functioning as a dynein light chain. A male infertility phenotype search was performed allowing the identification of key TCTEX1D4 interacting proteins. PPP1CC, CRISP2, CTSL1, RANBP9, and ACVR2A knockouts lead to phenotypes of male infertility. This was not surprising due to functions already described for these proteins (Fardilha et al., 2011a; Kumar et al., 2001; Maeda, 1998; Puverel et al., 2011). However, there is no bibliographic support that points to the role of CTSL1 in male infertility. The fact that TCTEX1D4 interacts with proteins essential for male fertility supports the involvement of this protein in functions specific to testis and/or spermatozoa.

To determine whether the TCTEX1D4 interactions were biologically relevant, functions, processes, and subcellular distributions of the TCTEX1D4 interactome were obtained and analyzed. The results revealed that most of the interactors are not characterized. However, the variety of functions, processes, and subcellular distributions found is expected since TCTEX1D4 is a dynein light chain. Cation binding proteins are the most predominant category and this is relevant because sperm motility and acrosome reaction are processes that depend heavily on calcium concentration (Gupta and Bhandari, 2011; Publicover and Barratt, 2011). The possibility of TCTEX1D4 being involved in calcium regulation is promising. In biologic processes, the TCTEX1D4 interacting proteins that are involved in phosphatase metabolism are also involved in the TGF-beta pathway, and were not identified in the testis yeast two-hybrid system. On the other hand, proteins involved in *de novo* synthesis of proteins can indicate a relevant role of TCTEX1D4. Concerning the subcellular localization, most TCTEX1D4 interacting proteins are associated with membranes, which is not surprising, since dyneins are responsible for transport and organization of the cytosol.

Conclusion

A TCTEX1D4 yeast two-hybrid screen in testis was performed with the goal of identifying the TCTEX1D4 partial interactome and revealing putative functions of this protein. TCTEX1D2 was identified as a TCTEX1D4 partner and for the first time was shown to be present in human spermatozoa. Also, immunofluorescence studies showed that TCTEX1D2 and TCTEX1D4 are intra-acrosomal proteins and are present in the midpiece of human spermatozoa. The TCTEX1D4/TCTEX1D2 subcellular localizations in the cytoskeleton, in the acrosome, and in the mitochondria hint a function in intraflagellar transport, acrosome reaction, and energy control, respectively. An *in silico* analysis of TCTEX1D4 partial interactome inferred that TCTEX1D4 may have key roles in male reproduction since it interacts with two testis-specific proteins and six proteins that, when knockout, lead to male infertility. Interestingly, most TCTEX1D4 interacting proteins are not characterized and the ones that are have a variety of molecular functions, since they are involved in a myriad of biologic process and are distributed through the cell. Nevertheless, since almost half of the TCTEX1D4 interacting proteins bind to cations, it reinforces the involvement of TCTEX1D4 in the acrosome reaction.

Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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