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TSGA10 is a Centrosomal Protein, Interacts with *ODF2* and Localizes to Basal Body

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Abstract

TSGA10 is overexpressed in some cancers, during neural development, in embryogenesis, and in several tissues with actively dividing cells. *TSGA10* protein localization to the sperm tail has been previously described. The protein is cleaved into two parts, which appear to play different functions in the sperm tail: the 27-KDa N-terminal is localized to the fibrous sheath in the principal piece, whereas its 55-KDa C-terminal of the *TSGA10* forms filaments, decreases transcriptional activity of hypoxia-inducible factor (HIF)-1-alpha and accumulates in the midpiece of mature spermatozoa. Using colocalization, and coimmunoprecipitation assays, we show that *TSGA10* interacts with 'Outer Dense Fiber 2' (*ODF2*), a centrosome scaffold component associated with mother centrioles. Also, our yeast two-hybrid assay shows that the full-length *TSGA10* protein and its 55-KDa C-terminus portion predominantly interact with *ODF2*. However, the truncated N-terminus 27-KDa fibrous sheath component of *TSGA10* fails to bind *ODF2*. Our experiments examining the localization of *TSGA10*, demonstrated that the full length *TSGA10* protein localizes to perinuclear structures, colocalizes with γ-tubulin, and associates with the centrosome and basal body. The *TSGA10* 55-KDa C-terminus, but not its 27-KDa N-terminus also localizes to the centrosome and basal body. Our Real-time PCR data indicated that the levels of *TSGA10* and *ODF2* genes expressions correlate, in mice testes. Finally, we propose that *TSGA10* is a ciliary-centrosomal protein and therefore is a good candidate for further investigation in ciliopathies, as well as, cancer biology.

Keywords: *TSGA10*; *ODF2*; Centrosome; Basal body; Sperm tail

Abbreviations: AD: Activation Domain; BD: Binding Domain; CBB: Centriole and Basal Body; CT_1: C- Terminal 1; CT_2: C-Terminal 2; FS: Fibrous Sheath; HIF1-alpha: Hypoxia-Inducible Factor; NT: N-Terminal; *ODF2*: Outer Dense Fiber 2; *TSGA10*: Testis-Specific Gene 10 Protein

Introduction

The fibrous sheath (FS) and outer dense fiber (ODF) proteins are the main structural components in the sperm tail. The fibrous sheath is a cytoskeletal structure (two longitudinal columns) surrounding the axoneme in the principal piece of the sperm's flagellum and links to the axonemal doublets 3 and 8 [1]. The axoneme is a conserved cytoskeletal and motor structure composed of a 9-microtubule doublet ring, surrounding a central pair of microtubules which is nucleated from a distal area of the basal body called the transition zone [2]. Microtubules are essential for chromosome segregation in mitosis and for organelle movement and positioning in interphase cells [3]. FS and ODF proteins may also play a role in chromatin division during spindle formation in mitosis, as well as, serving an obvious purpose in sperm motility [4,5].

These ciliary proteins may also coordinate multiple intracellular signaling events. About 80% of known FS proteins have anchoring sites for cAMP-dependent protein kinase A including "A Kinase Anchoring Protein 4" (AKAP4), AKAP3 and TAKAP-80. Some AKAPs (e.g. AKAP350) are centrosomal-specific proteins [6] and in many cases AKAPs scaffold multiple enzymes that are typically protein kinases, phosphatases, or other second messenger- dependent mediators [7,8].

TSGA10 is a 82-KDa protein with major expression in sperm tail. Post-translational cleavage gives 2 proteins: a 27- KDa fibrous sheath protein (*TSGA10*-N) localizing to the principal piece, and a 55-KDa component (*TSGA10*-C) that localizes to the midpiece of mature spermatozoa [9,10]. This *TSGA10*-C isoform decreases transcriptional activity of HIF1-alpha [11]. We have also reported a 65-KDa component of *TSGA10*, which could result from transcription of the first 16 exons [9].

In vertebrates, it has been recently shown that *TSGA10* is a paralog of BLD10/CEP135, sharing a high degree of similarity (65%). BLD10/CEP135 as a coiled-coiled centrosomal protein is one of the proteins required for centriole and basal body (CBB) biogenesis and in assembly and maintenance of both microtubule and centrosome functionalities (12-13). Also *Drosophila* BLD10/CEP135 represents an ancestor of the 'BLD10/CEP135 and *TSGA10*' family, localizing in a more distal region of the basal body and functions in both centriole and flagella biogenesis [12].

Several reports have shown that *TSGA10* is expressed in many cancer cells, as a cancer testis gene [13-18]. More recently, some advanced stages of esophageal squamous cell carcinomas (ESCCs) have been correlated with down- regulation of *TSGA10* [19]. Thus, the role of *TSGA10* in different cancers is still elusive.

ODF2 is a major outer dense fibre protein and is a sperm

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axonemal and centrosomal self-interacting coiled-coil scaffold protein with affinity for microtubules [20], involved in the recruitment of γ-tubulin into centrosomes [21,22]. Also, *ODF2* plays an important role in nucleolar function in embryogenesis [23] and during preimplantation development in mice [24]. *ODF2* localizes to centrosomes, basal bodies and the photoreceptor primary cilium in adult tissues [25,26], and is indispensable for the generation of primary cilia [27]. Both *TSGA10* and *ODF2* proteins share a significant alignment with the coiled-coil proteins, including myosins, lamins, tropomyosins.

In this study we investigate possible functions of the *TSGA10* protein by its localization with the basal body and centrosome in somatic cells, and its direct interaction with *ODF2* in testis.

Material and Methods

Plasmid construction

In order to create bait vectors for the two-hybrid study, the cDNAs encoding mouse *TSGA10* were amplified by PCR using the vector EGFP-C2-*TSGA10* [9] as the template. The forward and reverse primers were 5'–CACTCTCCCATGGGAAGCTTAATGATGAGAAAT-3' (*NcoI* site underlined) and 5'- TCGAATTCTCAAATCTCACTGTGAACATG-3' (*EcoRI* site underlined), respectively. The PCR product (~2070 bp) was digested with the restriction enzymes *NcoI* and *EcoRI* and fused in frame with the Gal4-DNA binding domain of the vector pGBKT7-T (CLONTECH) using the same restriction sites. Also the 5' and two different 3' truncated *TSGA10* cDNA -coding for NT (N-terminal), CT_1 and CT_2 (C-terminal) constructs respectively- were cloned in bait vectors separately using the above-mentioned primers as well as: a reverse primer for amplification of the *TSGA10-NT* (5'-TGGAATTCTCTCTCCCTGGCAATCTGAC-3') (restriction site *EcoRI* underlined), a forward primer for amplification of the *TSGA10*-CT_1 (5'-GATTCCATGGGCAG TTGGACGAGACAAAT-3') (restriction site NcoI underlined), and a forward primer for amplification of *TSGA10-CT*$_2$ (5'-AAAAACCATGGGCTCTGATACTCAGCGACATCT-3'). The resulting bait constructs, pGBKT7-full-length- *TSGA10 (TSGA10-FL), TSGA10-NT, TSGA10-CT*$_1$*, and TSGA10-CT*$_2$ were used in the two-hybrid screening. A rat-testis cDNA library (a gift from Dr. Frans van der Hoorn, University of Calgary, Alberta, Canada) constructed on the basis of the isolated 'prey' plasmid was used. It was made using 2 µg of the primer (5'- AAGCGGCCGCGTCGACAT-3'), which harbours NotI and SalI restriction sites, and 6 µg of poly$(A)^+$ RNA, then EcoRI adapters were added, and cDNA fragments greater than 400 base pairs were isolated and inserted into the *EcoRI* and *SalI* site of pGAD424 (CLONTECH).

Yeast two-hybrid analysis

Two-hybrid screening (covering ~ 2 x 10^6 independent clones for each bait construct) was performed by transforming the yeast strain AH109 first with the bait plasmid (pGBKT7-*TSGA10*), followed by a second transformation with the rat testis library plasmids described above. Primary positive clones were obtained on selective synthetic dropout medium (–Leu, -Trp, -His, -Ade) as well as (-His, -Leu, -Trp) to check transformation efficiency. *TSGA10* and prey insert gene expression were confirmed by PCR. The pGAD424 plasmids encoding putative mouse *TSGA10*-interacting proteins were isolated, tested for self-activity and re-tested for interaction. Inserts of confirmed interacting sequences were sequenced to ensure in-frame coding sequence with the AD (activating domain) of Gal4. Positive clones were further assayed for galactosidase activity to characterise the strength of interaction. Also, these candidate prey vectors were transformed into AH109 yeast strains carrying either the full-length, N, or C-terminal portions of *TSGA10* bait fragments, on a small scale. For yeast mating, extracted prey vector was transformed into Y187 yeast strain and mated with AH109 containing the bait constructs described above. To accurately establish the sequence of interacting proteins, cDNA inserts from pGAD/cDNA plasmids were sequenced twice on an automated DNA sequencer (Applied Biosystems).

Generation of *TSGA10* antibodies

Antibodies against *TSGA10* N-terminus and *TSGA10* C-terminus were generated based on the procedure described in our previous report [9].

Cell culture, immunocytochemistry, and direct fluorescent staining

MDCK, and COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, high glucose) and incubated at 37°C, 5% CO2. To confirm the co-localization of *TSGA10* and *ODF2*, MDCK cells were seeded on sterilized glass cover slips treated with poly-L-lysine (Sigma) in 6-well plates. One day after seeding, cells were fixed with methanol in -20°C for 10 minutes, and incubated with primary antibody diluted 1:50 (v/v) in blocking solution for 1 h at 37°C and washed in PBS. After washing, they were incubated for 1 h at 37°C with the relevant secondary antibody (FITC-conjugated goat anti-rabbit and rabbit anti-goat IgG; Jackson Immunoresearch Laboratory, Inc., West Grove, PA) in blocking solution and washed in PBS before staining with DAPI. Cover slips were mounted on glass slides with Permount SP15-100 (Fisher Scientific, Edmonton, Alberta, Canada), mounted and analyzed by fluorescence microscopy (Zeiss Vision, Mannheim, Germany). To verify and compare subcellular localizations of the full-length and 55-KDa isoform of *TSGA10*, the constructs of *TSGA10* fused to GFP were used and the COS-7 cell line was transfected with these constructs using "Fugene 6" (Roche, USA). Then the *TSGA10* protein was detected using antisera against its C-terminus and by immunocytochemistry.

Immunofluorescence analysis

Respiratory epithelial cells were obtained by nasal brush biopsy (cytobrush plus, Medscand Malmö, Sweden) and suspended in cell culture medium. Samples were spread onto glass slides, air dried and stored at -80°C until use. Cells were treated with 4% paraformaldehyde, 0.2% Triton-X 100 and 1% skim milk prior to incubation with primary (at least 2 hours) and secondary (30 minutes) antibodies at room temperature. Appropriate controls were performed omitting the primary antibodies. Rabbit polyclonal anti-a/b-tubulin obtained via Cell Signaling Technology (USA). Highly cross adsorbed secondary antibodies (Alexa Fluor 488, Alexa Fluor 546) were obtained from Molecular Probes (Invitrogen). DNA was stained with Hoechst 33342 (Sigma). Confocal images were taken on a Zeiss LSM 510 i-UV.

In Vitro Translation and Co-Immunoprecipitation

In vitro translations were performed using the bait (pGBKT7) and prey (pGADT7) plasmids described above and the TNT^- system (Promega) in the presence of $[^{35}S]$Cys and legends as recommended by the manufacturer. Inserts in above-mentioned vectors can be efficiently translated in vitro using T7 RNA polymerase (Pharmacia Biotech Inc.) to produce RNA.

Fifteen µl of in vitro translation reactions and 0.5 µl of T7 Tag monoclonal antibody (Novagen) were added to 25 µl of protein A-Sepharose beads (Pharmacia), which had been preincubated in immunoprecipitation (IP) buffer (10% glycerol, 50 mM Hepes-KOH, pH 8.0, 100 mM glutamate, 6 mM MgOAc, 0.5 mM dithiothreitol, 1 mM EGTA, 0.1% Nonidet P-40, 0.5 mg/ml bovine serum albumin) and mixed with 250 µl of fresh IP buffer. The reactions were incubated on a shaker for 6 h at 4°C, spun in a microcentrifuge, and washed 3 times with 1.5 ml of IP buffer. To confirm the proper translation, samples were denatured and analyzed by electrophoresis on SDS-polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue, destained, and dried, and proteins were detected by autoradiography using Kodak XAR film.

Similarly, to confirm *TSGA10* and *ODF2* interaction, the in vitro translation reactions were preincubated separately with 1 µl of (rabbit) anti-*TSGA10* (2) and (goat) anti-*ODF2* (Santa Cruz Biotechnology, CA) antibodies and protein

G beads in immunoprecipitation (IP) buffer (10% glycerol, 50 mM Hepes-KOH, pH 8.0, 100 mM glutamate, 6 mM MgOAc, 0.5 mM dithiothreitol, 1 mM EGTA, 0.1% Nonidet P-40, 0.5 mg/ml bovine serum albumin) and mixed with 250 µl of fresh IP buffer, at 4°C on a shaker overnight. The precipitates were then used for immunoblotting after washing 3 times with IP buffer-Titon X-100 (1%) including protease inhibitor cocktail (Sigma). SDS-PAGE, was performed according to standard procedures using testis tissue, yeast colonies including the interacting (*ODF2*) proteins, and mammalian cell protein. For detection of *TSGA10* and *ODF2* on the blot, the purified anti-*TSGA10* and anti-*ODF2* IgG fractions were used at a dilution of 1:5000. Visualization was with secondary anti-rabbit and anti-goat antibodies conjugated to horseradish peroxidase using the ECL Plus detection system (Amersham Pharmacia Biotech).

Determination of the *TSGA10* and *ODF2* genes expression levels in mouse testes by Real-time PCR

Mice Tetes were washed with PBS and total RNA was isolated using TRIzol reagent (Invitrogen). Total RNA (3 µg) was isolated from the smashed testes, treated with DNase I (Promega, Madison, WI), and reverse transcribed using random hexamer primers (Roche Diagnostics, Indianapolis IN) and MMLV reverse transcriptase (Promega) (45). Real-Time PCR was performed using iQTM SYBR Green supermix (Bio-Rad, Hercules, CA) on a sequence detection system. All samples were run in triplicate, and *ODF2* and *TSGA10* expression levels were normalized to 18s and determined $2^{-\Delta Ct}$. The sequences of the forward and reverse primers used for *ODF2* and *TSGA10* are shown in Table 1. The primers generated a 152-bp, and 165bp amplicons for the *ODF2* and *TSGA10*, respectively.

Results

ODF2 was selected by a two-hybrid screen using *TSGA10* full-length as bait

To identify novel binding partners of the *TSGA10* protein, we used yeast two-hybrid assay to screen a rat testis cDNA library (fused

qRT-PCR Primers	Sequences
ODF2 (Forward)	5' CTGCCTTGTTAAGGTGTTGATGTC 3'
ODF2 (Reverse)	5' TCATGGCCTTGAAGGATACCA 3'
TSGA10 (Forward)	5' AAGGCTCACTTGGAACAGCGGATA 3'
TSGA10 (Reverse)	5' ACTCTCGGTGTCCATTGCCTTTCT 3'

Table 1A: Sequences of the *TSGA10* and *ODF2* primers used for qRT-PCR.

Prey Bait	Cotransformation	Mating	X-Gal
TSGA10-NT (aa# 1-347)	—	—	—
TSGA10- CT_1 (aa# 215-689)	+	+	+
TSGA10- CT_2 (aa# 331-689)	—	—	—
Full *TSGA10* (aa# 1-689)	+	+	+
pGBKT7-p53	—	—	—

Table 1B: Summarized results of the yeast two-hybrid assay described in the table of Figure 1.

to Gal4- AD and pre-transformed in pGAD424; Clontech) and full-length *TSGA10* fused to Gal4-BD (subcloned in pGBKT7; Clontech). When the AD (activation domain) and the BD (binding domain) are brought into close proximity, they initiate transcription of four yeast reporter genes: HIS3, ADE2, lacZ and MEL1, via the GAL promoter. Yeast was co-transformed with the *TSGA10* bait and the testis cDNA fusion library (the prey library), and plated at medium stringency (dropout medium minus His, Leu, Trp), in order to identify low affinity interactors. One hundred and seventy nine positive colonies were identified from the testis library. Owing to the large number of positives, colonies were replated in high stringency medium (minus Ade, His, Leu, Trp) and tested by galactosidase assay. In total, 4 positive strong interactions were identified in this screen, comprising 3 independent prey clones. One prey clone contained the cDNA for *ODF2* (accession no. BC078857). This prey clone encodes a 3'-end of *ODF2* (encoding C-terminal protein portion) and interaction was further verified by multiple assays, including galactosidase, cotransformation and yeast mating. For yeast matings, different yeast strains, transformed with interactor-AD and with *TSGA10*-BD, were mated, plated on selective medium and subsequently, positive colonies were counted. pGAD424-*ODF2* could not activate the lacZ reporter gene by itself, nor in combination with pGBKT7 or p53/GAL4

DBD (encodes a p53/GAL4 DBD protein (Figure 1). Based on the data, it seems that the interaction domain of *TSGA10* with *ODF2* is present in the C-terminal end of the 55-KDa isoform (CT_1), since there is no interaction between CT_2 and *ODF2*. No interaction was detected between NT and *ODF2*.

ODF2 isoform interacts with full length and 55-KDa fragment of *TSGA10* C-terminus

To confirm the association of *TSGA10* and *ODF2* fragments, and to determine physiological relevance of the protein interactions identified in yeast, an in vitro translation of the transcripts followed by co-immunoprecipitation experiments of the translates were performed in mammalian cells. To verify the interaction of *TSGA10* and *ODF2* by Co-IP, in vitro translation was recruited utilizing the constructs plasmids of *TSGA10* bait and *ODF2* prey and the TNT^{-} system (Promega) in the presence of [^{35}S]Cys. The translation reactions (200 ul each) were used for co- immunoprecipitation to ensure that full length *TSGA10* and *ODF2* protein isoform interact and associate with each

other. In the immunoprecipitation of translation products, using *ODF2* antibody, probed with the antibody against *TSGA10* N-terminal head, only the 82-KD component of full-length *TSGA10* (but not the 27-KD fibrous sheath component) was precipitated. This suggests that only the 82-KD component of full-length *TSGA10* is associated with *ODF2*. However, in IP of the translation reactions, using *ODF2* antibody, probed with the antibody against *TSGA10* 55- KDa fragment, both the 82-KD full-length band, and its 55-KD fragment were associated with *ODF2* (Figure 2A). A 74-KD band appeared in both blots, which

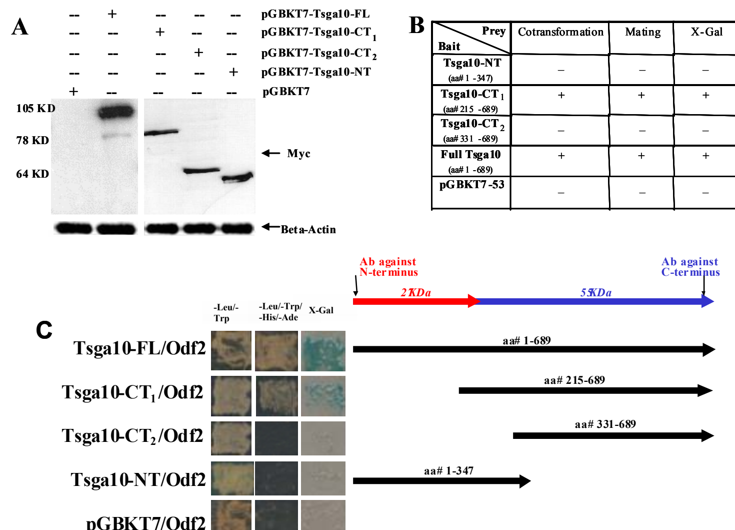

Figure 1: Yeast two-hybrid interaction between *ODF2* and various regions of *TSGA10*. The yeast two-hybrid system was used to test physical association between *TSGA10* and *ODF2*. The full-length *TSGA10*-bait construct was used for screening of the testis library; its N,C-termini half bait constructs were used to test any possible interaction with the interacting *ODF2* prey.

A) Biochemical confirmation of *TSGA10* constructs expression by using total lysates (60 µg of each) of the yeast cells transformed by *TSGA10* constructs (Full-length (FL), the carboxyl terminus 1 (CT1), carboxyl terminus 2 (CT2) or amino terminus (NT)), as well as, control empty vector (pGBKT7) run in 10% SDS-PAGE and visualized with anti-myc antibody (because pGBKT7 contains a c-Myc epitope tag). B) The table shows the exact number of *TSGA10* aminoacids in its various constructs (Full –FL-, CT1, CT2 and NT). It also summarises the results of interaction of *TSGA10* amino and carboxyl termini constructs with *ODF2* using yeast transformation and mating and its confirmation by X-Gal assay. C) Representative yeast colonies in the selective medium (-Leu/-His and –Leu/-His/-Trp/-Ade) after transformation of *TSGA10* and *ODF2* constructs as well as schematic figure of the *TSGA10* bait constructs subcloned in pGBKT7 (Clontech), sequence domains of both the carboxyl terminus (C) and amino terminus (N) regions of mouse *TSGA10* (see marked starting and ending amino acid residues). Colony lift assay shows positive interaction between *ODF2* and both full-length (FL) or the carboxyl terminus 1 (CT1) of *TSGA10*, whereas incubation with the carboxyl terminus 2 (CT2), or the amino terminus (NT) of *TSGA10* did not result in any interaction. The interactions in the colony lift assay were examined after a 24-h incubation of filter paper in 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) solution. This suggests that only the full-length *TSGA10* and the CT1 terminus (55 KD) of *TSGA10*, but not the CT2 or N-termini interact with *ODF2*.

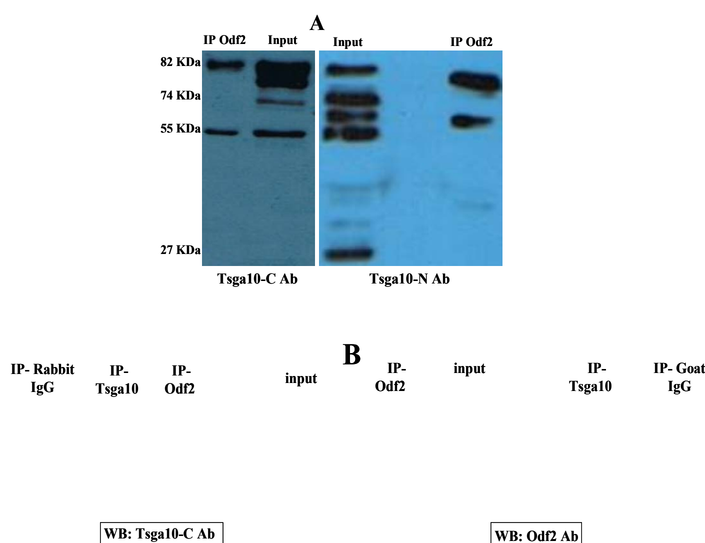

Figure 2: Identification of *ODF2* interaction with full length and 55-KDa fragment of *TSGA10* by co-immunoprecipitation. A) Total protein from rat sperm was extracted (in the buffer containing 1% SDS, and 2% DTT), then ODF component was separated and collected via sucrose gradient subjected to immunoprecipitation (IP) followed by Western blot analysis (WB) using antibodies against *TSGA10* N- and C-termini. Immunoblots from left to right: panel 1, IP: performed using the anti-*ODF2* antibody, WB: anti-*TSGA10*-C antibody; panel 2, IP: goat anti-*ODF2* antibody, WB: rabbit anti-*TSGA10*-N antibody. Apart from full-length 82-KDa *TSGA10* and its spliced form (74 KDa) protein components, different sized proteins (27 KDa and 55KDa) are recognised by antibodies against the N and C-termini, respectively. *ODF2* could only bind to full-length as well as its C-terminal 55-KDa component; B) Translated products from *in vitro* translation experiment (according to the protocol mentioned in the "Materials and Methods"), were used to examine *TSGA10* and *ODF2* interaction. Two panels of direct and reverse IP followed by WB, showed association of full-length *TSGA10* (82 KDa) and *ODF2* (84 KDa) proteins. In each IP, relevant IgG was also used as negative control to rule out any non-specific binding of protein to IgG. Left panel, IP: performed using the goat anti-*ODF2* antibody, WB: anti-*TSGA10*-C antibody; Right panel, IP: anti-*TSGA10*, WB: anti-*ODF2* antibody.

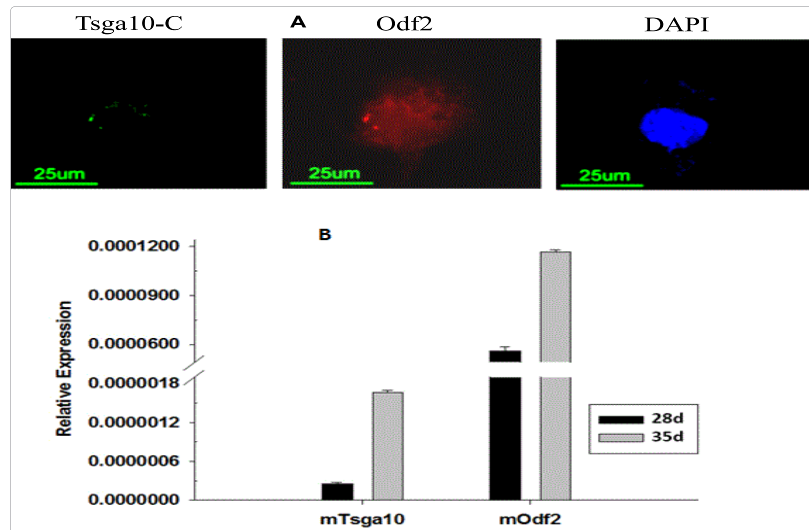

Figure 3: Colocalization and correlation of *TSGA10* and *ODF2* expression in MDCK mammalian cell line. A) MDCK cells were fixed on glass slides and stained with rabbit anti-*TSGA10* (left panel) and goat anti-*ODF2* antisera (middle panel). The *TSGA10* antiserum was affinity purified against the C-terminal peptide aa# 676-689, followed by incubation with anti-rabbit and anti-goat FITC-coupled secondary antibodies. Controls were blocked with the relevant peptide and showed no signal (not shown). In the right panel, the nuclei are stained with DAPI. B) The expression levels of both genes have been detected using Real-time PCR in 28 and 35 days after birth. The results showed a correlation in mice mature testis. Values presented are means + SEM from triplicate cultures.

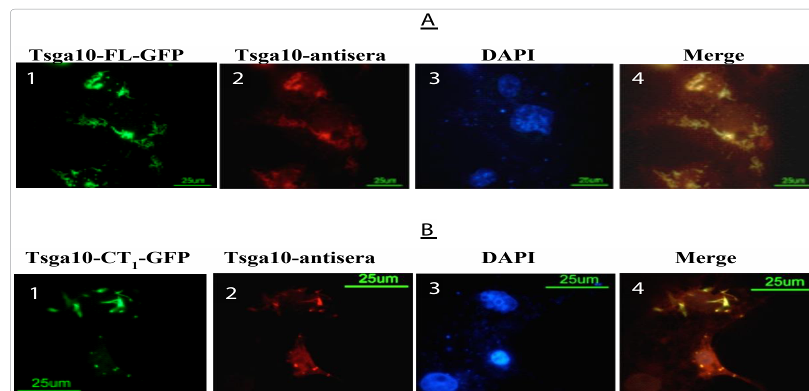

Figure 4: Immunolocalization of the *TSGA10* protein by transfection the COS-7 cells using full-length *TSGA10*-GFP (A) and *TSGA10*-CT1-GFP (B) constructs. By immunofluorescence, *TSGA10* antibody detects specifically the *TSGA10* protein (red, panels 2 in A&B) fused to GFP (green, panels 1 in A&B) and then merged (panels 4 in A&B). DAPI (blue, panels 3 in A&B) used for the nuclei staining. In each of A and B figures, several examples of overexpressed full-length and Carboxyl terminus (CT1) *TSGA10* are presented.

probably represents the splicing variant of the *TSGA10* protein where exon 16 is spliced out without frame shift [9]. Endogenous *TSGA10* also specifically interacted with *ODF2*. Endogenous *ODF2* interacted with *TSGA10*, as well. However, no interaction was detectable with the control antibody (relevant IgG) (Figure 2B).

Colocalization and co-expression of *TSGA10* and *ODF2*

Double immunofluorescence experiments for endogenous *TSGA10* and *ODF2* provided further evidence for interaction between *TSGA10* and *ODF2*. The colocalization experiment was performed using antibody against the *TSGA10*-C-terminus (Figure 3A). Using the N-terminus-specific antibody, the *TSGA10* protein appears to be localized within the nucleus (not shown), while using antibody against *TSGA10*-C-terminus it seems to be localized in a single structure close to the nucleus (Figure 3A), suggesting that the protein is associated with an organelle such as the centrosome.

Since the expression of interacting *TSGA10* and *ODF2* has been shown in sperm tail and in mouse embryo (during embryogenesis), it was of interest to study the correlation of their expression levels. As the result, a correlated expression level has been detected for *ODF2* and *TSGA10* in mouse testes under basal conditions, with expression of both genes increasing in 28 and 35 days after birth (Figure 3B). Collectively, the findings suggest that *TSGA10* and *ODF2* expression levels in mice testes correlate with each other, and these two genes most likely function together in male germ cell.

Over-expressed *TSGA10* shows perinuclear localization

Exogenously over-expressed full-length and the 55 KDa isoform of *TSGA10* occupy a perinuclear localization in COS-7 cells, transfected with EGFP-C2-*TSGA10* (full- length, FL) and EGFP-C2-*TSGA10*-CT1 (consisting of aa# 215-689), which expresses *TSGA10* with a N-terminal GFP tag (Figure 4). The same pattern of perinuclear localization was

observed by overexpression of the 55-KDa component, although the full-length form seems to be more abundant. We have demonstrated that the *TSGA10*-C is sufficient for *TSGA10* interaction with *ODF2*, and its perinuclear localization targets *TSGA10* efficiently to the centrosome and co-localizes with it.

TSGA10 associates with the centrosome and localizes at the ciliary base

Since *TSGA10* interacts with the centrosomal protein *ODF2*, we examined whether *TSGA10* might also be a centrosome-associated protein. To test this, COS-7 cells were examined for colocalization of *TSGA10* and the centrosome marker γ-tubulin by double immunofluorescence cytochemistry utilizing fluorescent microscopy. Figure 5 shows the *TSGA10* colocalized with γ-tubulin, although this was not as distinct when using a specific antibody against *TSGA10*-N-terminus (not shown). Furthermore, it was hypothesized that *TSGA10* protein is present wherever there is a conserved ciliary structure [9]. Therefore, in a further study using a specific antibody against *TSGA10*-C-terminus and high-resolution immunofluorescence confocal microscopy of respiratory epithelial cells carrying motile cilia, *TSGA10* has been localized to the ciliary base which is consistent with localization at basal bodies and transition zone (Figure 5C).

The 55-KDa *TSGA10* isoform interacts with *ODF2* and localizes to centrosomes

Upon confirmation that the full-length *TSGA10* protein interacts with *ODF2*, we tried to determine which part of the *TSGA10* protein was responsible for the observed interaction. So the interactions between isolated cDNA inserts from pGAD/*ODF2* plasmid and some gments of *TSGA10* were studied. These partial fragments include *TSGA10*-NT (aa #1-347), *TSGA10*-CT_1 (aa #215-689), and *TSGA10*-CT_2 (aa #331-689). In the yeast two-hybrid study, the C-terminus 55 KDa-component of *TSGA10* (CT_1), as well as, the full-length protein were shown to interact with *ODF2* protein; neither NT nor CT_2 truncated fragments of the *TSGA10* showed any interaction with *ODF2* in yeast. After transformation of the yeast, the expression of these inserts and the expression of the fusion proteins were verified by PCR and Western blotting respectively using polyclonal antibodies (Figure 1). None of the bait *TSGA10* constructs alone was able to confer yeast growth after transformation together with pGAD424 containing no insert.

Discussion

In this study we showed that *TSGA10* sperm tail protein is a ciliary-centrosomal protein, similar in location to *ODF2*, which is also a major sperm tail protein. Expression of these two proteins is not restricted to male germ cells, but also found in somatic cells, being components of (primary) cilium and centrosome. *TSGA10* has been shown to be expressed in apical cytoplasmic membrane of ciliated cell [28]. Additionally, *TSGA10* and *ODF2* interact with each other, and the C-terminal fragment of each protein is responsible for this interaction.

Recent data have highlighted the role of centrosomal proteins in integrated "cell brain" complexes that coordinate all cellular processes [29]. Our data suggest that after processing, the C-terminal (55 KDa) and N-teminal (27 KDa) components of *TSGA10* may have different functions. *TSGA10* is therefore, a multifunctional protein: its expression has been previously shown during spermatogenesis, embryogenesis, and neural development, as well as, in some cancers (as a cancer-testis antigen) [15]. On the other hand, the prey clone of *ODF2* that interacted with *TSGA10* includes the C-terminal end of *ODF2*, where one of the two leucine zipper motifs is located [30]. *TSGA10* has also at least a conserved region of the leucine zipper motif starting from N-terminal end of the 55-KDa (CT_1) isoform. *TSGA10* and *ODF2* may, therefore, interact via their leucine zippers; and this may speculate the fact that there is no interaction between CT_2 (but CT_1) and

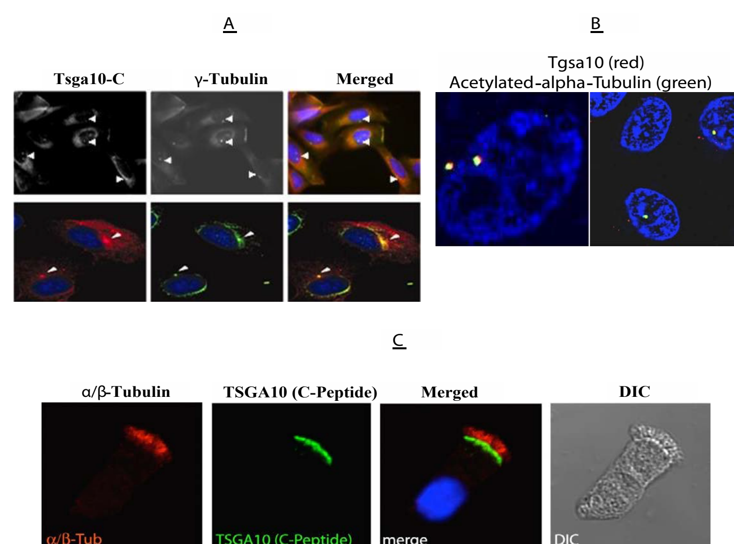

Figure 5: Association of *TSGA10* with the centrosome and basal body. A) Double immunofluorescence stain using antisera against *TSGA10*-C (left panel), anti-γ-tubulin (middle panel), and merged images (right panel) shows co-localization of these two proteins at a distinct cellular spot that seems to be associated with centrosomes. The experiments were carried out in COS-7 (top, x400; bottom, x1000) cells cultured on coverslips. DAPI (blue) was used to visualise nuclei. Immunostained COS-7 cells are filtered for a better presentation (top panel), while *TSGA10* and γ-tubulin are stained in red and green (bottom panel), respectively. B) Merged images of double immunofluorescence stain using antisera against *TSGA10* and anti-γ-tubulin show co-localization (yellow) of these two proteins at two cellular spots that seems to be centrosomes. C) Sublocalization of *TSGA10* in respiratory epithelial cells. Co-staining with antibodies against rabbit anti-a/b tubulin (red) and mouse-anti-*TSGA10* antibodies (green). a/b tubulin localizes to the entire length of the ciliary axonemes. In respiratory cells *TSGA10* localizes to the ciliary base, which is consistent with localization at the basal bodies and the transition zone.

ODF2, while there is also no interaction between NT and *ODF2*. The importance of leucine zipper motif in interactions of sperm tail proteins has already been highlighted [30,31]. In further studies, it would be interesting to investigate whether these two proteins (*TSGA10* and *ODF2*) are interacting by dimerization (making a dimerized HLH) via their leucine zipper motifs, which may result in a synergistic regulatory effect on spindle formation and centrosome amplification. Interaction of some centrosomal proteins such as *ODF2*, CEP110, CEP135, Ninein and CEP170 with each other (via their conserved coiled-coil domains and leucine zipper motifs) has already been reported to be crucial for proper function and maturation of the centrosome [4,32].

Considering the result of this study, *TSGA10* may have a role in ciliogenesis, as it belongs to the BLD10/CEP135 family of evolutionary conserved proteins, which are required for centriole and basal body biogenesis. On the other hand, BLD10 has a crucial role in central microtubule pair and axonemes, and similar to *TSGA10*, shows a similar localization in a more distal region of the basal body [12]. Considering all above facts and its association with *ODF2* and CEP135, at least *TSGA10* C-terminal fragment is a CBB and ciliary-centrosomal protein that probably plays a major role in central pair of motile cilia. Although crucial role of *TSGA10* and its interaction with *ODF2* in centrioles/basal bodies -which are essential mainly for the generation of cilia [26] - should be confirmed in further experimental studies, *TSGA10* function in ciliogenesis has been already proposed [9] and recently highlighted [12]. Given *TSGA10* role in ciliogenesis and its contribution to ciliary structure, it is a good candidate in the study of ciliopathies including polycystic kidney disease, primary ciliary dyskinesia, nephronophthisis, Senior-Loken syndrome, Joubert syndrome, Meckel syndrome, oral-facial-digital syndrome, Alström syndrome, Bardet–Biedl syndrome (BBS) and hydrocephalus [33].

Based on our study here, both *TSGA10* and *ODF2* genes are obviously expressed in ES cells, but certainly are consistent with the notion that germ cell genes are part of the ES cell repertoire. *TSGA10* may also play a role in centrosome-related functions via its "ATPase chromosome segregation" and "structural maintenance of chromosome protein 1" (SMC1) domains. Here we have shown that during anaphase and telophase, *TSGA10* transfers from centrosome to central spindle where formation of cleavage furrow happens. Although SMC1 is localized to centrosomes throughout the cell cycle in microtubule-independent manner [34,35], and many centrosomal proteins have SMC domain, *TSGA10* role in spacing or even dimerization within the molecule should be confirmed in more substancial experiments. The dynamic localization change from chromosomes to central spindle, may propose *TSGA10* cytoskeletal protein as a 'chromosome passenger', which regulate chromosomal functions including segregation. Considering so many conserved potential sites for serine/threonine phosphorylation, *TSGA10* may function as either an Aurora-B protein or a substrate and modulator for Aurora-B. Such a role of *TSGA10* in chromosome segregation during mitosis could result, consistent with its over-expression in some cancers – thus explaining *TSGA10* as a cancer/testis antigen [15-17]. Centrosome amplification itself can also initiate tumorigenesis [36]. Therefore *TSGA10* overexpression in reported cancers can be speculated via misamplification of centrosome. On the other hand, consistent with a possible role of *TSGA10* in ciliogenesis, it is specifically expressed in astrocyte and over-expressed in astrocytoma and brain tumors [37] –in which primary cilia is expressed and primary ciliogenesis defects have been reported, respectively [38]. Meanwhile, an altered interaction between centrosomal proteins *TSGA10* and *ODF2* may result in centrosome instability and/or ciliogenesis defect as a primary biologic event in cancers which both proteins play a role in.

Considering all above, *TSGA10* may stimulate centrosome amplification -such as an oncogene-, and be regulated by HIF-1. Therefore, further studies to investigate *TSGA10* specific and detailed role in mitosis via centrosome amplification and/or regulation by Aurora-B is suggested. Both *TSGA10* and *ODF2* have also been reported as autoantigens in some autoimmune conditions [39,40]. The antigenic expressions of centrosomal proteins in some autoimmune diseases, and presence of autoantibodies reacting with a group of centrosomal proteins have been previously studied [41]. Therefore, *TSGA10* and *ODF2* interaction may modulate autoimmunity and be also good candidate proteins for treatment of some autoimmune disorders (e.g. SLE) [42] which is subject for the further studies.

An association of activated ERM family members with scaffolding proteins is reported [43]. The facts that *TSGA10* has an ERM domain and *ODF2* is a scaffolding protein may imply a strong association of *TSGA10* with membrane proteins. This may be via tubulin glutamylation, which is involved in the regulation of intracellular traffic, including chromosomes, and in the regulation of ciliary and flagellar motility [44,45].

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