Aberrant Expression of \textit{Dynein light chain 1} (\textit{DYNLT1}) is Associated with Human Male Factor Infertility* \textsuperscript{S}

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\textit{DYNLT1} is a member of a gene family identified within the t-complex of the mouse, which has been linked with male germ cell development and function in the mouse and the fly. Though defects in the expression of this gene are associated with male sterility in both these models, there has been no study examining its association with spermatogenic defects in human males. In this study, we evaluated the levels of \textit{DYNLT1} and its expression product in the germ cells of fertile human males and males suffering from spermatogenic defects. We screened fertile (\(n = 14\)), asthenozoospermic (\(n = 15\)), oligozoospermic (\(n = 20\)) and teratozoospermic (\(n = 23\)) males using PCR and Western blot analysis. Semiquantitative PCR indicated either undetectable or significantly lower levels of expression of \textit{DYNLT1} in the germ cells from several patients from across the three infertility syndrome groups, when compared with that of fertile controls. \textit{DYNLT1} was localized on head, mid-piece, and tail segments of spermatozoa from fertile males. Spermatozoa from infertile males presented either a total absence of \textit{DYNLT1} or its absence in the tail region. Majority of the infertile individuals showed negligible levels of localization of \textit{DYNLT1} on the spermatozoa. Overexpression of \textit{DYNLT1} in GC1-spg cell line resulted in the up-regulation of several cytoskeletal proteins and molecular chaperones involved in cell cycle regulation. Defective expression of \textit{DYNLT1} was associated with male factor infertility syndromes in our study population. Proteome level changes in GC1-spg cells overexpressing \textit{DYNLT1} were suggestive of its possible function in germ cell development. We have discussed the implications of these observations in the light of the known functions of \textit{DYNLT1}, which included protein trafficking, membrane vesiculation, cell cycle regulation, and stem cell differentiation. \textit{Molecular & Cellular Proteomics} 14: 10.1074/mcp.M115.050005, 3185–3195, 2015.

The t-complex of the mouse occupies the proximal half of chromosome 17 and contains genes which have profound effects on spermatogenesis. Multiple mutations in several loci in the t-complex appear to interact to cause complete male sterility (1, 2). \textit{Tctex-1} (t-complex testis expressed-1), lately renamed as dynein light chain 1 (\textit{Dynlt1})\textsuperscript{1}, is identified as a candidate gene involved in male sterility in mice (1) and maps to the t-complex in mice (3). \textit{Dynlt1} is a member of a multigene family which is virtually germ cell-specific and is eightfold over expressed in t-homozygotes and 200-fold higher in testis than in other adult tissues (1). The human homologue of the mouse \textit{Dynlt1} is located on chromosome 6q25.2–25.3. The amino acid sequence shows a high degree of similarity to the predicted product of the \textit{Dynlt1} gene of the mouse t complex (4).

\textit{DYNLT1} gene encodes a 14 kDa protein constituting the inner arm L1 of cytoplasmic and flagellar dynein complexes (5, 6). \textit{DYNLT1} is localized to Golgi complexes as well (7). \textit{DYNLT1} protein is present in sperm tails and oocytes (8, 9). A wide range of cellular events are brought about by cytoplasmic dynein and its association with the accessory intermediate, light intermediate, and light chain subunits. These subunits define the interaction of cytoplasmic dynein motor complex with other molecules (10). \textit{DYNLT1} is involved in cargo binding (11), lymphocyte division (8), vesicle transport (12–14), and human embryo implantation (15). \textit{DYNLT1} is known to undergo phosphorylation during apical delivery of rhodopsin (16) and during its interaction with the bone morphogenetic receptor type II (BMPRII) (17). \textit{DYNLT1} can function in dynein-independent fashion as a cell fate regulator by its interaction with G-protein \(\beta\) \(\gamma\) subunit regulating initial neurite sprouting (18), axonal specification, and elongation of hippocampal neurons in culture (11, 19). \textit{GEF-H1} is bound to microtubules by \textit{DYNLT1} and its release without microtubule depolymerization is mediated through the interaction of \textit{DYNLT1} with G proteins (20). \textit{DYNLT1} is a novel marker for neural progenitors in adult brain (21). \textit{DYNLT1} regulatory element was identified which selectively marked nestin* / \textit{GFAP}*/\textit{Sox2}* neural stem-like cells in developing and adult

\* The abbreviations used are: \textit{Dynlt1}, dynein light chain 1; \textit{DMEM}, Dulbecco’s Modified Eagles Medium; BAC, bacterial artificial chromosome.
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The genetic knockdown of **DYNLT1** in radial precursors promoted neurogenesis (23). The use of GFP placed under the control of **DYNLT1** promoter to mark adult neural stem cells and thus allowing the insertion of any nucleotide sequence selectively into neural progenitors has been patented (24).

**DYNLT1** is reported to have functional roles in non-murine germ cells as well. **DYNLT1** was found to be essential during spermatid differentiation in Drosophila (10) and a mouse **DYNLT1** homolog was identified in the dynein light chain of sea urchin sperm flagella (25, 26). However, the expression of **DYNLT1** in human testicular germ cells and its association, if any, with human male factor subfertility are not yet evaluated. This study evaluates the association between **DYNLT1** expression and spermatogenesis in infertile human males and the possible function of **DYNLT1** in spermatogonial cell division and differentiation.

**MATERIALS AND METHODS**

**Reagents**—Ready-To-Go T-primed First-Strand Kit and Hybond™-P PVDF membrane (GE Healthcare, NA, UK), antibodies to **DYNLT1** and β-**ACTIN**, goat anti-rabbit IgG-HRP, goat anti-rabbit IgG-FITC (Santa Cruz Biotechnology, Santa Cruz, CA), Reverse Transcriptase PCR primers (Sigma Genoys, Bangalore, India), Big Dye Terminator v3.1 Cycle Sequencing Kit, Champion™ pET 100 Directional TOPO® Expression kit, Lipofectamine 2000 (Invitrogen, Carlsbad, CA), QIA quick gel extraction kit (Qiagen Gmbh, Hilden, Germany), GC1-spg cell line (GRL:20531) (ATCC, VA, USA), and pEGFPN1 vector (Clonetech, Mountain View, CA) were procured. TRI reagent, agarose, ethidium bromide, Trizma Base, glycine, SDS, CHAPS, Glycerol, PMSF, EGTA, sodium orthovanadate, DAB, Hydrogen peroxide, nickel chloride, poly-L-lysine, parafomaldehyde were purchased from Sigma-Aldrich.

**Semen Collection from Fertile and Infertile Individuals**—This study was approved by the Institutional Review Board and Human Ethics Committee of Rajiv Gandhi Centre for Biotechnology, Thriruvananthapuram, Kerala, India. Volunteering semen donors for the study program were recruited after obtaining a written consent from the subjects. Men who have fathered a child in the preceding 2 years and presenting normal seminogram were considered as fertile males (controls). Male partners of sexually active infertile couple visiting Samad Hospital and the possible function of **DYNLT1** in spermatogonial cell division and differentiation.

**Reverse Transcription PCR Analysis**—Each sperm pellet (10⁷ cells) was homogenized in 1 ml of TRI reagent at 1600 × g for 30 s with 30 s interval using a PT-100 homogenizer probe (Kinematica AG, Luzernstrasse, Lucerne). Total RNA was extracted following protocol recommended by the reagent manufacturer. Briefly, 200 µl of chloroform was added to the homogenate, shaken vigorously for 15 s, incubated for 15 min and centrifuged at 12,000 × g for 15 min at 4 °C. The upper transparent layer was transferred into a new eppendorf tube and RNA precipitated using 0.5 ml isopropanol. The RNA pellet was washed in 70% ethyl alcohol, air dried and suspended in 35 µl sterile DEPC water. 20 µg of the total RNA was heated at 65 °C for 5 min and reverse transcribed to cDNA at 37 °C for 90 min using the Ready To Go T-Prime first strand synthesis kit. The kit utilizes the Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase and an oligo (dT) 18 primer to generate the first strand cDNA. The prepared cDNA was stored at −20 °C.

**PCR primers specific for** **DYNLT1** (DYNLT1_5F, DYNLT1_324R), and ACTB (ACTB -71F, ACTB -490R) were designed using Primer3 software (http://primer3.ut.ee/). The details of the primers used are given in Table I. The polymerase chain reaction was set up as follows: 1 µg of cDNA was mixed with 1X reaction buffer containing 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.5 units thermostable Taq Polymerase and 1 µM each of forward and reverse primers, in a 22 µl reaction mixture. An initial denaturation at 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 30 s and a final extension at 72 °C for 10 min were given to amplify **DYNLT1** from the cDNA in the Gene Amp PCR System 9700 (AB Applied Biosystems, Carlsbad, CA). For the amplification of **ACTB**, an annealing temperature of 58 °C was used, and other conditions remained the same. The PCR products were mixed with 2 µl of gel loading buffer and the products were separated by electrophoresis (100 V, constant voltage) on a 1% agarose gel containing 0.5% of ethidium bromide, using Bio-Rad Gel Electrophoresis SubCell (model 192) and 0.5% TBE running gel buffer. The PCR products were visualized on a UV Transilluminator (GeNei, Bangalore, India) and images captured on Gel Doc image analyzer system using the Quantity One software system (Bio-Rad Laboraties, Hercules, CA). Band intensities of **DYNLT1** were quantitated and were normalized to the corresponding levels of β-**ACTIN** using Phoretix 1D Advanced software, Version 4.01 (Phoretix International, Newcastle upon Tyne, UK).

The **DYNLT1** bands were excised, eluted using QIA quick gel extraction kit according to manufacturer’s instruction, subcloned in pCM4-TOPO vector and recombinant plasmids were isolated. The integration of the gene into the vector was confirmed by DNA sequencing. Automated sequencing reaction was performed using the Big Dye Terminator v3.1 Cycle Sequencing Kit. The sequencing reaction was performed in a Gene Amp PCR System 9700 (Applied Biosystems, Carlsbad, CA), the dye-terminated products were precipitated and were run in an ABI 3730 automated DNA analyzer. The sequences were analyzed by using NCBI nucleotide-nucleotide BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). DNA sequences were aligned using ClustalW (http://www.ebi.ac.uk/clustalw), the alignments were shaded using GENEDOC, version 2.5.0 and a consensus sequence was generated. The conceptual translation of the sequenced data was generated using the JustBio Translater (http://www.justbio.com/index.php?page=translator).

**SDS-PAGE and Western Blotting**—Each sperm pellet was resuspended in 200 µl of the solubilization buffer (187 mM Tris-HCl, pH 6.8, 2% SDS, 0.05% CHAPS, 10% Glycerol, 1 mM PMSF, 1 mM EGTA and 1 mM sodium orthovanadate) and disrupted by sonication (15 MHz, three pulses of 30 s each; Branson Sonifier, Danbury, CT). It was then centrifuged at 7000 × g for 10 min at 4 °C. The supernatant was taken as protein extract and stored at −80 °C.

The protein extracts (20 µg) were mixed with equal volumes of Laemml buffer (1:1) and were heat denatured at 95 °C for 5 min. Extracts were clarified by centrifugation at 18,404 × g for 10 min at
27 °C. Proteins were resolved on a 12% SDS-PAGE at constant voltage (100 V). The proteins were transferred to the PVDF electrophoretic membrane at constant current of 30 mA for 8 h at 4 °C, using a Mini Trans Blot cell (Bio-Rad laboratories). The blotting buffer (pH 8.2) composition was 20% methanol, 25 mM Tris (pH 8.2) and 190 mM Glycine. After blotting, the membranes were pretreated in methanol and incubated in blocker of 5% skimmed milk powder in PBS-T for two hours. Then the membranes were washed three times with PBS-T at 5 mins interval. The membranes were incubated with Dynlt1 antibody at a dilution of 1:1000 in PBS-T for two hours and were washed three times with PBS-T at 5 mins interval. Goat anti rabbit IgG-HRP was added at a dilution of 1:2000 in blocker for one hour. The blots were washed with PBS-T three times at 5 mins interval. The blots were developed by incubating the membranes in 0.05% DAB, 0.1% hydrogen peroxide and 0.04% nickel chloride in PBS-T until the desired contrast was obtained. The blots were photographed on Gel Doc image analyzer system using the Quantity One software system (Bio-Rad Laboratories). Band intensities of Dynlt1 and were quantitated and were normalized to the corresponding levels of beta-ACTIN using Phoretix 1D Advanced software, Version 4.01 (Phoretix International).

**Immunofluorescence assay**—Fifty microliters of the sperm suspension was coated onto Poly-l-Lysine-coated cover slips, fixed using 4% paraformaldehyde for 10 min and permeabilized in 0.25% Triton X-100 for 10 min. These cover slips were incubated for 2 h in PBS containing 2 mg/ml BSA and 100 mM glycine to block the nonspecific binding of cellular proteins to the primary antibodies. The cover slips were washed in three changes of PBS and were subsequently incubated with anti-Dynlt1 antibody at dilution 1:1000 in PBS-T for 2 h at room temperature. The cover slips were again washed three times in PBS and incubated with FITC conjugated goat anti rabbit secondary antibody (dilution 1:2000 in PBS-T) for 1 h in the dark. The cover slips were washed with PBS-T and stored in dark. The cells were imaged under Leica TCS SP2 Confocal Laser Scanning Microscope (Leica TCS SP-II AOB system, Wetzlar, Germany).

**Generation of Dynlt1 Expression Construct**—The mouse homolog of Dynlt1 (Dynlt1b; Accession No. CCDS49936.1) was amplified (342 bp) from mature mouse total testicular cDNA using gene specific forward (5’-ATGGAAGACTTCCAGGCCTCCGA) and reverse (5’-TGGATGGACAGTCCGAAGGTACTGAC) primers with HindIII and SalI forward (5’/H11032/342 bp) from mature mouse total testicular cDNA using gene specific of expression construct (pEGFPN1-X-100 for 10 min. These cover slips were incubated for 2 hi n PBS and incubated for 2 h in PBS containing 2 mg/ml BSA and 100 mM glycine to block the nonspecific binding of cellular proteins to the primary antibodies. The cover slips were washed in three changes of PBS and were subsequently incubated with anti-Dynlt1 antibody at dilution 1:1000 in PBS-T for 2 h at room temperature. The cover slips were again washed three times in PBS and incubated with FITC conjugated goat anti rabbit secondary antibody (dilution 1:2000 in PBS-T) for 1 h in the dark. The cover slips were washed with PBS-T and stored in dark. The cells were imaged under Leica TCS SP2 Confocal Laser Scanning Microscope (Leica TCS SP-II AOB system, Wetzlar, Germany).

**Culturing of Cells and Transfection**—The GC-1 spg cells transfected with pEGFPN1-Dynlt1 construct were harvested and lysed after 48 h post transfection and the total crude protein was extracted using detergent free lysis buffers. GC1-spg cells transfected with empty pEGFPN1 vector served as control. The detergent free hypotonic lysis buffer (10 mM HEPES-pH 7.9, 1.5 mM MgCl₂, 10 mM KCl) and extraction buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl₂; 0.42 mM NaCl; 0.2 mM EDTA; 25% (V/V) Glycerol) yielded crude cytoplasmic and the nuclear fractions respectively and both these fractions were pooled together as crude protein sample. The concentration of the crude protein samples was measured by Bradford assay and the concentration of all the samples was normalized using 50 μM ammonium bicarbonate (ABC) buffer to yield a final concentration of 1 μg/μl.

One hundred micrograms of proteins from each sample was subjected to in-solution trypsin digestion according to the recommendations of the manufacturer. The digested peptide solutions were centrifuged at 18,404 g for 12 min and the supernatant was collected. The supernatant was transferred to autosampler vials (Total Recovery Vial, Waters) for peptide analysis via LC-MS² with ion-mobility. The trypic peptides were separated using a nanoACQUITY UPLC® chromatographic system (Waters, Manchester, UK) employing reversed-phase chromatography technology. Instrument control and data processing were done with MassLynx4.1 SCN781 software. Mass spectral analysis of eluting peptides from the nanoACQUITY UPLC® were carried out on a SYNAPT® G2 High Definition MS™ System [HDMS² System (Waters)]. The acquired ion mobility enhanced MS² spectra was analyzed using ProteinLynx Global SERVER™ v2.5.3 (PLGS, Waters) that uses integrated ProteinLynx™ and MASCOT® database searching (multithreaded) method to maximize number and confidence of protein identifications for protein identification as well as for the label-free relative protein quantification. Data processing included lock mass correction post acquisition. Processing parameters for PLGS were set as follows: noise reduction thresholds for low energy scan ion, 150 counts, high energy scan ion, 50 counts, and peptide intensity, 500 counts (as suggested by manufacturer). The protein identifications were obtained by searching against the mouse database (downloaded from NCBI (ftp://ftp.ncbi.nih.gov/refseq/M_musculus/mRNA_Prot/dated July 22, 2014) containing 77,623 entries. During database search, the parameters for peptide identification were set in such a way that a peptide was required to have at least one fragment ion match, a protein was required to have at least three fragment ion matches and a protein was required to have at least 1 peptide match for identification. Mass tolerance was set to 10 ppm for precursor ions and 20 ppm for fragment ions. Oxidation of methionine was selected as variable modification and cysteine carbamidomethylation was selected as a fixed modification. Tryptsin was chosen as the enzyme used with a specificity of 1 missed cleavage. Proteins identified with at least two distinct peptides with a probability of 0.95 or above were considered as correct identifications. Data sets were normalized using the “auto-normalization” function of PLGS and label-free quantitative analyses was performed by comparing the normalized peak area/intensity of identified peptides between the samples. Furthermore, only a fold change higher than 50% difference (ratio of either <0.50 or >1.5) was considered to be indicative of significantly altered levels of expression.

**Bioinformatics**—The lists of differentially displayed proteins were analyzed using PANTHER classification system (http://www.pantherdb.org/) to categorize the proteins based on their functional relevance. Functional networks of differentially displayed proteins in Dynlt1 overexpressed GC1-spg cells generated using STRING 9.0 (http://string-db.org/newstring cgi/show_network_section.pl). A con-
**RESULTS**

**Western Blot Analysis**—On Western blots, protein extracts from the spermatozoa of all fertile males showed a prominent band at 14 kDa that was recognized by anti-DYNLT1 antibody (Fig. 1A, lanes N5, N3, N6, N7, N8, and N9). This band was very weak in the protein extracts of spermatozoa from many infertile men (Fig. 1A, lanes A32, A209, A329, O253, O238, T228, T233, and T255), though some of the infertile men showed normal levels of expression of DYNLT1 in the germ cells as interpreted from RT-PCR analysis. However, a few infertile men showed normal levels of DYNLT1 expression (Fig. 3A, lanes A204, A34, A37, O75, O124, O207, O35, O244, T234, T235, T236, T245, and T246). ACTB served as the endogenous control. The expression levels of DYNLT1 in infertile men which were significantly lower than those of fertile individuals were expressed as relative band intensities, following quantitation using the phoretix 1D software (Fig. 3C).

**Immunofluorescence Analysis**—The localization of DYNLT1 on spermatozoa was performed using immunocytochemistry. The spermatozoa from fertile males showed DYNLT1 localization on the head, mid-piece and tail regions (Fig. 2, N5). The spermatozoa from infertile males showed a complete absence of DYNLT1 from the tail region, though this protein was present on the head and mid-piece of these cells in asthenozoospermic (Fig. 2, A254), oligozoospermic (Fig. 2, O19) and teratozoospermic (Fig. 2, T244) men.

**RT-PCR Analysis**—PCR using DYNLT1 specific primers from the cDNA prepared from the germ cells obtained from men of proven fertility produced a 350 bp amplicon (Fig. 3A, N5, N3, N6, and N7). This amplicon was eluted and its authenticity was confirmed by direct sequencing (supplemental Fig. S1). The sequence was deposited in the Genbank (Accession No. EU862237). A conceptual translation of this gene yielded a protein with 119 amino acids (supplemental Fig. S1).

Human males clinically diagnosed to have spermatogenic insufficiency showed absence (Fig. 3A, lanes A209, A231, A122, A202, A30, A32, A38, O41, O12, O28, O29, T229, T212, T213, T242, T246, T247, T26, T27, T31, T33, T39, and T34) or low levels (Fig. 3A, lanes O227 and T228) of expression of DYNLT1 in the germ cells as interpreted from RT-PCR analysis. However, a few infertile men showed normal levels of DYNLT1 expression (Fig. 3A, lanes A204, A34, A37, O75, O124, O207, O35, O244, T234, T235, T236, T245, T246, T247, T248, T249, and T250). CD45 was not detected in the cDNA prepared from germ cells of fertile and infertile individuals indicating absence of lymphocyte contamination in the immature germ cells harvested from the semen (Fig. 3B). CD45 amplified from the cDNA prepared from WBC isolated from human blood is shown as positive control.

**Proteome Profiling of GC1-spg Cells Over-expressing DYNLT1**—Single colonies of the E. coli (DH5α) transformed with pEGFPN1-Dynlt1 were screened by colony PCR (Supplemental Fig. S2, A). The amplicons were sequenced and the authenticity of the insert was confirmed by subjecting the sequence obtained to BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi).
GC1-spg cells transfected with pEGFPN1-Dynlt1 and the empty pEGFPN1 vector were incubated for 48 h to achieve the overexpression of DYNLT1 (Fig. 4 and supplemental Fig. S2). The cells were harvested and lysed for protein preparation and subsequent mass spectrometry. The number of distinct peptides and the percentage coverage for each protein assigned for peptide and protein identification are listed in supplemental Tables S2 and S3. The proteomic analysis showed 121 differentially expressed proteins and among them 112 were up-regulated and nine were down-regulated. Among the 121 differentially expressed proteins, only 100 proteins were identified by Uniprot and the respective proteins with Uniprot gene name were listed with their expression level changes (supplemental Table S4). The mass spec predicted proteins which were not identified by Uniprot were tabulated with their fold expression level changes (supplemental Table S5).

The PANTHER classification system was used to categorize the up and down-regulated proteins with respect to DYNLT1 over expression. Those differentially expressed up-regulated proteins were classified into 17 categories according to protein class. The major proportion of the proteins were categorized under chaperones, followed by nucleic acid binding proteins and cytoskeletal proteins (Fig. 5A). Likewise, the down regulated proteins were classified into three categories (Fig. 5B). The STRING prediction tool was used to identify the interactions among the differentially expressed proteins and it showed four clusters of interacting protein sets like ribosomal protein group, cytoskeletal proteins group, chaperones, and metabolic proteins (Fig. 6A). There were no interacting clusters in case of the down regulated proteins (Fig. 6B).

**DISCUSSION**

DYNLT1 gene family was identified as a member of the t-complex of the mouse, which has been linked with male germ cell development and function in mouse (1, 27) and the fly (28, 29). Though defects in DYNLT1 expression were linked to defective spermatogenesis in both mouse and Drosophila, there has been no report evaluating the association between the expression of this gene and defective spermatogenesis in human males. Efforts to assess the role of DYNLT1 by creating mice transgenic for a wild-type bacterial artificial chromosome (BAC) derived from the S1-critical region (containing genes Synj2 and Serac1 which are mutated in t haplotypes) bred onto t haplotype mice revealed that introduction of BAC was sufficient to restore fertility in mice (30). However, the fertility status of the heterozygotes generated in this study and their subsequent progeny was not mentioned. Also, the limited number of progeny containing both BAC ends with the intervening genomic DNA (3.9%) indicates the frailty of the above mentioned study in ruling out DYNLT1 candidature as a fertility determining factor. The data presented in this paper documents for the first time that human males with spermatogenic insufficiency present defective expression of DYNLT1 in germ cells. The mean values of various semen quality parameters of the subjects who participated in this study are given in Table I.

DYNLT1 was expressed aberrantly in infertile individuals compared with fertile individuals upon analysis at the genomic (Fig. 3A and 3C) and proteomic (Fig. 1A and 1B) levels. Our lab reported the abnormal expression of TAR DNA-binding protein (TDP-43) leading to spermatogenic dysfunction recently.
The probability of DYNLT1 being amplified from non-germ cells (like WBC) if any present in the semen was nullified by checking the expression of CD45 in these individuals (Fig. 3B). The localization of DYNLT1 in the tail segments of spermatozoa from fertile human males (Fig. 2), and its striking absence in the spermatozoa from infertile males, strongly
suggests a role for this molecule in flagellar dynamics. DYNLT1 was identified as a component of the microtubular network (5) of the cells and was implicated in functions involving cytoskeletal network and cellular motility (32, 33). Tctex-2, another t-complex protein was also localized in mouse sperm tails (34) and phosphorylated at activation of sperm motility (35).

The genes in t-complex locus of the mouse have important role in male germ cell development and function. The t-complex locus has several genes essential for normal tail length, embryogenesis and spermatogenesis (36). Dynl1 is one of the t complex genes and its chromosomal position and germ cell specific expression make it as a candidate gene for male sterility (37). The presence of DYNLT1 in mouse sperm and its involvement in meiotic drive were reported (38). It is also reported that, DYNLT1 expression is limited to cell cycling progenitors (39) and in young neuronal progenitors (40) in the adult brain. This stem cell/progenitor specific expression in the adult organ raises the possibility of role in maintaining stemness in the mature organs. Stem cells (SCs) are unique cells, possess the capacity to self-renew and as well as have ability to generate differentiated cells (41). The expression of transcription factors like Oct3/4, Sox2, Klf4, c-Myc (42), Nanog and Lin28 (43) were necessary for the maintenance of pluripotency or stemness in both embryo and adult tissues (44). However, the list of proteins which showed significant changes in their levels of expression as a result of Dynlt1 over-expression in GC1 cells did not include any protein associated with stemness.

Spermatogenesis is a complex process during which the transformation of spermatogonial stem cell to spermatozoa occurs via the processes like mitosis, meiosis, and morphological differentiation. The differentiation processes includes nuclear condensation, acrosome and flagellum formation and removal of excess cytoplasm (45). In the present study, majority of the up-regulated proteins categorized by panther classification were molecular chaperones, cytoskeletal proteins, and nucleic acid binding proteins which are involved in cell cycle regulation. In our data, we could see the up-regulation of HSP 70 and HSP 90 classes of chaperones proteins.

Fig. 5. PANTHER classification of proteins up-regulated (A) and down-regulated (B) in GC-1 cells over-expressing DYNLT1.
and chaperonins like T-complex protein (TCP-1) subunits in abundance. The stress induced expression of HSP70 family of chaperones in all population of mouse spermatogenic cells (46) and the abundant expression of TCP-1 during spermatogenesis and its localized distribution over the developing acrosome were reported (47). Also, the targeted disruption of chaperone proteins results in compromised sperm maturation and spermatogenic arrest (48). Therefore the molecular chap-

**Fig. 6.** Functional interactome of up-regulated (A) and down-regulated proteins (B) in GC1-spg cells over-expressing DYNLT1, generated using STRING network prediction algorithm.
Mean sperm quality indicators of the semen samples used in this study. Values indicated are Mean ± S.D. of the observations made.

| Condition | Count | Miotility | Normal forms | Head defect | Neck defect | Tail defect |
|-----------|-------|-----------|--------------|-------------|-------------|-------------|
| N         | 54.43 ± 6.89 | 52.43 ± 4.40 | 67.21 ± 15.02 | 17.86 ± 7.77 | 7.14 ± 2.57 | 8.14 ± 7.73 |
| A         | 71.6 ± 42.92 | 31.27 ± 14.24 | 9.67 ± 4.50 | 47.93 ± 6.08 | 12.27 ± 2.81 | 30.4 ± 4.67 |
| O         | 7.25 ± 6.04 | 35.75 ± 12.51 | 11.33 ± 4.58 | 43.87 ± 7.65 | 13.2 ± 9.56 | 31.73 ± 8.17 |
| T         | 85.96 ± 52.05 | 58.87 ± 7.87 | 10.70 ± 5.10 | 45.65 ± 6.05 | 11.74 ± 2.91 | 31.74 ± 4.40 |

TABLE II

| SL.No: | Primer name | Primer sequence |
|--------|-------------|-----------------|
| 1      | Hdynlt 1-F  | CACCATGAAGAAGACTACAGG |
| 2      | Hdynlt1-R   | TCAAAATAGACAGTCCGAAGG |
| 3      | Hbetaactin -71 F | ACCATGGAATGATGATATCGCC |
| 4      | Hbetaactin -490 R | CACAGCCTGGATAGCAACGTA |

DYNLT1 and Human Male Factor Infertility

Research over the past few decades transformed DYNLT1 from being a sterility locus gene into a gene with multitude of functions. DYNLT1 has recently been used for marking the progenitor cells in a cell population (24). DYNLT1 plays a key role in multiple steps of hippocampal neuron development, including initial neurite sprouting, axon specification, and later dendritic elaboration and these effects are independent from its cargo adaptor role for dynein motor transport (11). DYNLT1 phosphorylated at Thr 94 has a pivotal role in cell cycle progression (26). However, whether male infertility is an outcome of the defective expression of DYNLT1 in the germ line stem cells is yet to be evaluated. In conclusion, this study reports a defect in the expression of DYNLT1 in the germ cells of infertile human males and implicates DYNLT1 in spermatogenic cell division and differentiation.

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** This article contains supplemental Figs. S1 and S2 and Tables S1 to S5.

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REFERENCES

1. Lader, E., Ha, H. S., O’Neill, M., Artzt, K., and Bennett, D. (1989) tctex-1: a candidate gene family for a mouse t complex sterility locus. *Cell* 58, 969–979

2. Ha, H., Howard, C. A., Yeom, Y. I., Abe, K., Uehara, H., Artzt, K., and Bennett, D. (1991) Several testis-expressed genes in the mouse t-complex have expression differences between wild-type and t-mutant mice. *Dev. Genet.* 12, 318–332

3. Lyon, M. F. (2003) Transmission ratio distortion in mice. *Annu. Rev. Genet.* 37, 393–408

4. Roux, A. F., Rommens, J., McDowell, C., Anson-Cartwright, L., Bell, S., Schappert, K., Fishman, G. A., and Musarella, M. (1994) Identification of a gene from Xp21 with similarity to the tctex-1 gene of the murine t complex. *Hum. Mol. Genet.* 3, 257–263

5. Harrison, A., Olds-Clarke, P., and King, S. M. (1998) Identification of the t complex-encoded cytoplasmic dynein light chain tctex1 in inner arm I1 supports the involvement of flagellar dyneins in meiotic drive. *J. Cell Biol.* 140, 1137–1147

Molecular & Cellular Proteomics 14.12
25. Kagami, O., Gotoh, S., Makino, Y., Mohri, H., Kamiya, R., and Ogawa, K. (1996) The mouse t-complex-encoded protein Tctex-1 is a light chain of brain cytoplasmic dynein. J. Biol. Chem. 271, 32281–32287

24. Sung, C. H., and Chuang, J. Z. (2014) Tctex-1 regulatory sequence as stem cell marker. In: Google Patents

23. Campbell, K. S., Cooper, S., Dressing, M., Yates, S., and Buder, A. (1998) Interaction of p56lyn kinase with the dynein light chain, Tctex-1, and colocalization during cytokinesis. J. Immunol. 161, 1728–1737

22. Dedesma, C., Chuang, J. Z., Alfinito, P. D., and Sung, C. H. (2006) Dynein light chain Tctex-1 identifies neural progenitors in adult brain. J. Comp. Neurol. 496:773–786

21. Dedesma, C., Chuang, J. Z., Alfinito, P. D., and Sung, C. H. (2006) Dynein light chain Tctex-1 identifies neural progenitors in adult brain. J. Comp. Neurol. 496, 773–786

20. Liu, X., Huang, J., Chen, T., Wang, Y., Xin, S., Li, J., Pei, G., and Kang, J. (2008) Yamanaka factors critically regulate the developmental signaling network in mouse embryonic stem cells. Cell Res. 18, 1177–1189

19. Chuang, J. Z., Milner, T. A., and Sung, C. H. (2001) Subunit heterogeneity of cytoplasmic dynein: Differential expression of 14 kDa dynein light chain proteins in rat hippocampus. J. Neurosci. 21, 5501–5512

18. Sachdev, P., Menon, S., Kastner, D. B., Chuang, J. Z., Yeh, T. Y., Conde, C., Caceres, A., Sung, C. H., and Sakmar, T. P. (2007) G protein beta gamma subunit interaction with the dynein light-chain component Tctex-1 regulates neurite outgrowth. EMBO J. 26, 2621–2632

17. Meiri, D., Marshall, C. B., Mokady, D., LaRose, J., Mullin, M., Gingras, A., Cott, I., Ikura, M., and Rappol, R. (2014) Mechanistic insight into GPCR-mediated activation of the microtubule-associated RhoA exchange factor Gef-H1. Nat. Commun. 5, 4857. doi: 10.1038/ncomms4857

16. Huw, L. Y., Goldsborough, A. S., Willisson, K., and Artzt, K. (1995) Tctex2: a sperm tail surface protein mapping to the t-complex. Dev. Biol. 170, 183–194

15. Inaba, K., Kagami, O., and Ogawa, K. (1999) Tctex2-related outer arm dynein light chain is phosphorylated at activation of sperm motility. Biochem. Biophys. Res. Commun. 256, 177–183

14. Dang, N., Dang, N., and Dang, N. (2006) Functional analysis of the Drosophila transitory gene Tctex-1. EMBO J. 25, 5085–5098

13. Nagano, F., Orita, S., Sasaki, T., Naito, A., Sakaguchi, G., Maeda, M., Watanabe, T., Kominami, E., Uchiyama, Y., and Takai, Y. (1998) Interaction of Doc2 with tctex-1, a light chain of cytoplasmic dynein. J. Neurosci. 23, 26505–26512

12. Caggese, C., Moschetti, R., Ragone, G., Barsanti, P., and Caizzi, R. (2014) Tctex-1, the Drosophila melanogaster homolog of a putative murine t-complex disruptor encoding a dynein light chain, is required for production of functional sperm. Mol. Genom. Genet. 265, 436–444

11. Li, M. G., Serr, M., Newman, E. A., and Hays, T. S. (2004) The Drosophila tctex-1 light chain is dispensable for essential cytoplasmic dynein functions but is required during spermatid differentiation. Mol. Biol. Cell 15, 3005–3014

10. Chuang, J. Z., Yeh, T. Y., Bollati, F., Conde, C., Canavosio, F., Caceres, A., and Sung, C. H. (2005) The dynein light chain Tctex-1 has a dynein-independent role in actin remodeling during neurite outgrowth. Dev. Cell 9, 75–86

9. Tseng, Y. Y., Gruzdeva, N., Li, A., Chuang, J. Z., and Sung, C. H. (2010) Dynein light chain Tctex-1 regulates neurite outgrowth. EMBO J. 29, 32281–32287

8. Campbell, K. S., Cooper, S., Dressing, M., Yates, S., and Buder, A. (2006) Interaction of p56lyn kinase with the dynein light chain, Tctex-1, and colocalization during cytokinesis. J. Immunol. 161, 1728–1737

7. Tai, A. W., Chuang, J. Z., and Sung, C. H. (1998) Localization of Tctex-1, a cortical precursor cells. J. Cell Biol. 139, 155–166

6. King, S. M., Dillman, J. F., III, Benashski, S. E., Lye, R. J., Patel-King, R. S., and Pfister, K. K. (1996) The mouse t-complex sterility locus. Gene 155, 803–812

5. Reddi, P. P., and Kumar, P. G. (1994) Aberrant expression of TAR DNA binding protein-43 is associated with spermatogenic disorders in men. Reprod. Fertil. Dev. 10

4. Nomoto, A. (2004) Receptor (CD155)-dependent endocytosis of poliovirus receptor CD155 with the dynein light chain Tctex-1 and its implication for poliovirus pathogenesis. J. Biol. Chem. 279, 7897–7904

3. Campbell, K. S., Cooper, S., Dressing, M., Yates, S., and Buder, A. (2006) Interaction of p56lyn kinase with the dynein light chain, Tctex-1, and colocalization during cytokinesis. J. Immunol. 161, 1728–1737

2. Campbell, K. S., Cooper, S., Dressing, M., Yates, S., and Buder, A. (1998) Interaction of p56lyn kinase with the dynein light chain, Tctex-1, and colocalization during cytokinesis. J. Immunol. 161, 1728–1737

1. Campbell, K. S., Cooper, S., Dressing, M., Yates, S., and Buder, A. (1998) Interaction of p56lyn kinase with the dynein light chain, Tctex-1, and colocalization during cytokinesis. J. Immunol. 161, 1728–1737
45. Wang, R., and Sperry, A. O. (2008) Identification of a novel Leucine-rich repeat protein and candidate PP1 regulatory subunit expressed in developing spermatids. *BMC. Cell Biol.* 9, doi: 10.1186/1471–2121-9-9

46. Allen, R. L., O’Brien, D. A., Jones, C. C., Rockett, D. L., and Eddy, E. M. (1988) Expression of heat shock proteins by isolated mouse spermatogenic cells. *Mol. Cell. Biol.* 8, 3260–3266

47. Willison, K., Lewis, V., Zuckerman, K. S., Cordell, J., Dean, C., Miller, K., Lyon, M. F., and Marsh, M. (1989) The t complex polypeptide 1 (TCP-1) is associated with the cytoplasmic aspect of Golgi membranes. *Cell.* 57, 621–632

48. Dun, M. D., Aitken, R. J., and Nixon, B. (2012) The role of molecular chaperones in spermatogenesis and the post-testicular maturation of mammalian spermatozoa. *Hum. Reprod. Update.* 18, 420–435

49. Sperry, A. O. (2012) The dynamic cytoskeleton of the developing male germ cell. *Biol. Cell* 104, 297–305

50. Chateauvieux, S., Ichante, J. L., Delorme, B., Frouin, V., Pietu, G., Langonne, A., Gallay, N., Sensebe, L., Martin, M. T., Moore, K. A., and Charbord, P. (2007) Molecular profile of mouse stromal mesenchymal stem cells. *Physiol Genomics.* 29, 128–138