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Identification of cell-specific targets of sumoylation during mouse spermatogenesis

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Abstract

Recent findings suggest diverse and potentially multiple roles of SUMO in testicular function and spermatogenesis. However, SUMO targets remain uncharacterized in the testis due to the complex multicellular nature of testicular tissue, the inability to maintain and manipulate spermatogenesis *in vitro*, and the technical challenges involved in identifying low-abundance endogenous SUMO targets. In this study, we performed cell-specific identification of sumoylated proteins using concentrated cell lysates prepared with de-sumoylation inhibitors from freshly purified spermatocytes and spermatids. One-hundred and twenty proteins were uniquely identified in the spermatocyte and/or spermatid fractions. The identified proteins are involved in the regulation of transcription, stress response, microRNA biogenesis, regulation of major enzymatic pathways, nuclear-cytoplasmic transport, cell cycle control, acrosome biogenesis, and other processes. Several proteins with important roles during spermatogenesis were chosen for further characterization by co-immunoprecipitation, co-localization and *in-vitro* sumoylation studies. GPS-SUMO software was used to identify consensus and non-consensus sumoylation sites within the amino acid sequences of the proteins. The analyses confirmed the cell-specific sumoylation and/or SUMO interaction of several novel, previously uncharacterized SUMO targets such as CDK1, RNAP II, CDC5, MILI, DDX4, TDP-43 and STK31. Furthermore, several proteins that were previously identified as SUMO targets in somatic cells (e.g., KAP1, MDC1) were identified as SUMO targets in germ cells. Many of these proteins have a unique role in spermatogenesis and during meiotic progression. This research opens a novel avenue for further studies of SUMO at the level of individual targets.

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Introduction

Spermatogenesis includes the proliferation of spermatogonia through mitosis, the production of round spermatids from spermatocytes by meiosis, and the post-meiotic maturation of spermatids, termed spermiogenesis. Abnormalities during any of these processes can result in the production of malfunctioning sperm, which may lead to infertility, spontaneous abortion, or birth defects. These facts emphasize the need for a better understanding of spermatogenesis and its regulation, particularly through the characterization of the molecules that have not been well studied in the testis but that regulate important pathways in other tissues.

Various protein post-translational modifications (PTMs) regulate spermatogenesis, one of which is the attachment of small ubiquitin-like modifiers (SUMOs) in a process termed sumoylation. SUMOs are structurally similar to ubiquitin. However, their amino acid sequences differ greatly, with only ~18% similarity (Bayer *et al.* 1998). Four SUMO paralogs have been identified in mammals: SUMO1, 2, 3 and 4. SUMO1 (also named sentrin) shares approximately 50% homology with SUMO 2 and SUMO3, which are usually referred to as SUMO2/3 given that they are 95% identical. During sumoylation, an isopeptide bond forms between a SUMO and the lysine residue of its substrate. This process requires a SUMO-activating enzyme (E1), a SUMO-conjugating enzyme (E2) and a SUMO ligase (E3) (Yeh *et al.* 2000, Wang & Dasso 2009, Yeh 2009, Wilkinson & Henley 2010). Sumoylation often occurs on a target lysine residue within the consensus sequence: ψ -K-X-D/E, where ψ is a hydrophobic amino acid and X can be any amino acid (Rodriguez *et al.* 2001). However, not all consensus sequences are sumoylated, and sumoylation often occurs outside of the consensus sequence (Blomster *et al.* 2010). Notably, SUMO2/3 but not SUMO1 contain the consensus sequence, and mixed SUMO chains with a terminal SUMO1 have been reported (Rodriguez *et al.* 2001). Sumoylation is a dynamic process that can be reversed through the activity of sentrin-specific proteases (SENPs) by the cleavage of the isopeptide bond between the SUMO moiety and the substrate (Mukhopadhyay & Dasso 2007, Yeh 2009, Hannoun *et al.* 2010, Wilkinson & Henley 2010). A diverse set of SUMO target proteins has been identified in somatic cells, including factors that regulate transcription, replication, DNA repair, RNA metabolism, translation, and cellular transport. In addition to the numerous targets of sumoylation that have been identified, there is a growing list of proteins that interact with SUMO non-covalently (Song *et al.* 2004, Chupreta *et al.* 2005, Song *et al.* 2005, Lin *et al.* 2006, Kerscher 2007).

Several developmental processes, including spermatogenesis, have been studied in SUMO1-knockout mice (Zhang *et al.* 2008). In contrast to a previously published study that reported abnormal development of the palate in SUMO1 knockouts (Alkuraya *et al.* 2006), Zhang *et al.* found no abnormalities in mouse development, suggesting that SUMO2 and SUMO3 compensated for the functions of SUMO1. Although these results require further evaluation, they suggest that the SUMO-conjugating machinery, and not an individual SUMO isoform, should be the target of future experiments aiming to inhibit sumoylation. Unfortunately, knockout mice for UBC9, a SUMO-conjugating enzyme, show early embryonic lethality and severe disruptions in mitosis, a finding that supports the indispensable role of sumoylation in mitotic progression (Nacerddine *et al.* 2005). We and other groups have

studied SUMO1 and SUMO2/3 in mouse and human spermatogenesis using advanced cell-imaging techniques and immunodetection analyses. SUMO localized to spermatogonia, the sex chromosomes and the centromeric heterochromatin of spermatocytes, the chromocenters of round spermatids, the centrosome area of elongating spermatids, and the nuclei of testicular somatic cells. These findings are consistent with the diverse and potentially multiple roles of SUMO in testicular function and spermatogenesis, such as spermatogonia proliferation, meiotic sex chromosome inactivation, centromeric heterochromatin organization, and reshaping the spermatid nucleus. (Rogers *et al.* 2004, Vigodner & Morris 2005, Vigodner *et al.* 2006, Brown *et al.* 2008, Metzler-Guillemain *et al.* 2008, Vigodner 2009). In agreement with these results, one study (La Salle *et al.* 2008) showed the dynamic nature of the gene expression levels related to sumoylation during spermatogenesis. Recent studies from our group also revealed changes in global sumoylation following the induction of various stresses in germ cells and sperm (Shrivastava *et al.* 2010, Shrivastava *et al.* 2014). Although these studies provided important initial information about the possible roles of sumoylation in spermatogenesis, little progress has been made in understanding how SUMO regulates the suggested functions. As has been shown in somatic cells, the identification of targets for sumoylation is a critical step toward understanding its cellular functions (Andersen *et al.* 2009, Golebiowski *et al.* 2009, Sarge & Park-Sarge 2009, Tatham *et al.* 2011). TOP2A and synaptonemal complex proteins (SYCP1 and SYCP2) have been co-immunoprecipitated with SUMO from testicular lysates, as shown by our group (Shrivastava *et al.* 2010) and others (Brown *et al.* 2008), respectively. Hundreds of SUMO targets have been identified in somatic cells. However, with the exception of TOP2A and SYCPs, SUMO targets remain uncharacterized in the testis. This knowledge gap is due to the complex, multicellular nature of testicular tissue, the inability to maintain and manipulate spermatogenesis *in vitro*, and the technical challenges involved in identifying low-abundance endogenous SUMO targets. To overcome some of these difficulties, we recently optimized the identification of sumoylated proteins using concentrated cell lysates, isopeptidase inhibitors to prevent de-sumoylation, and a large amount of anti-SUMO antibody crosslinked to agarose beads (Xiao *et al.* 2014). Using this approach, we recently identified a sumoylome of human sperm (Vigodner *et al.* 2013). Several additional sperm targets were identified by another group (Marchiani *et al.* 2014). In this study, numerous sumoylated proteins were uniquely identified in the spermatocyte and/or spermatid fractions using lysates prepared from purified spermatogenic cells. Several proteins with important roles during spermatogenesis were further characterized by co-immunoprecipitation, co-localization and in-vitro sumoylation studies.

Materials and methods

Mice, cell lines, reagents and antibodies

C57BL/6Ncr1 mice were purchased from Charles River (Kingston, NY). The Animal Committee of Albert Einstein College of Medicine, Yeshiva University approved all animal protocols. The mouse Sertoli cell line 15P-1 (ATCC®, CRL-2618) and HEK 293 (ATCC® CRL-1573™) cells were purchased from ATCC (Manassas, VA) and grown in DMEM media with 5% fetal bovine serum (FBS, Life Technologies, 16140-071), 5% bovine growth serum (Fisher Scientific, SH30541.03), 1% penicillin/streptomycin (Life Technologies,

15140-122) and 0.5% Fungizone (Life Technologies, 15290-018) at 32°C with 5% CO₂. The primary human Sertoli cell line (Lonza, MM-HSE-2305) was purchased from Lonza Group Ltd. (Walkersville, MD, USA) and cultured in the Sertoli Cell Growth Medium (SeGM) bullet kit (Lonza, 00191053) at 37°C with 5% CO₂.

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. All reagents for Western blotting and immunofluorescence were purchased from Life Technologies unless otherwise noted. The detergent-removal spin columns (87778), the screw-cap spin columns (69705), and the BCA protein assay kit (23227) were purchased from Thermo Scientific (Rockford, IL, USA). The whole-cell extraction kit (2910) was purchased from Millipore (Temecula, CA, USA). Information regarding the source, vendor and dilution of the antibodies used in this study is summarized in the Supplementary Table 1.

Germ cell separation by velocity sedimentation (STA-PUT)

Mouse testicular germ cells were separated using a STA-PUT sedimentation velocity cell separator (ProScience Inc., Scarborough, Ontario, Canada) and a published procedure (La Salle *et al.* 2009). The details of the procedure are summarized in the Supplementary materials.

Flow cytometry analysis

Chosen STAPUT fractions were centrifuged at 500 g for 7 min at 4°C followed by aspiration of all but 1 ml of supernatant. The pellets were then resuspended with a brief, gentle vortex. From each resulting cell suspension, 150 µl was individually mixed with propidium iodide (PI) staining solution (PBS with 1% (v/v) RNase A, 10 µg/ml PI, and 1% (v/v) Igepal CA-630) and incubated at 37°C for 15 min in the dark. After incubation, the samples were filtered through a nylon mesh and subjected to flow cytometric analysis of PI-stained DNA fractions (Supplementary Figure 1A). The flow cytometry and the subsequent analyses were processed by CytoSoft software (Millipore, Billerica, MA, USA). Fractions containing spermatocytes (tetraploid cells) and spermatids (haploid cells) with a purity above 80% and 90%, respectively, were pooled together for cell slides, protein extraction, or spermatocyte culture (Supplementary materials, Fig.1).

Immunofluorescence (IF)

Pooled fractions were centrifuged at 500 g for 7 min and resuspended in 5 ml of Krebs Ringer Buffer (KRB) media (120 mM NaCl, 4.8 mM KCl, 25.2 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄·7H₂O, 1.3 mM CaCl₂, 1 × Pen/Strep/Glu (Life technologies), 1 × essential amino acid, 1 × non-essential amino acid (Lonza, Walkersville, MD, USA), and 11.1 mM dextrose). Twenty-five microliters of the cell suspension was smeared onto a poly-L-lysine microscope slide (Polysciences Inc., 22247, Warrington, PA, USA), and the rest was subjected to protein extraction. The slides were completely air dried, and the cells were fixed with 1% paraformaldehyde (PFA) in PBS (Polysciences, 18814) for 10 min. The cells were then washed twice with PBS before being permeabilized with 0.3% Igepal CA-630 for 10 min. The cells were then blocked with Image-iT® FX signal enhancer for 30 min and then rinsed with PBS. Subsequently, the cells were incubated with the appropriate antibodies

in PBS containing 1% BSA for 2 h (Supplementary Table 1). Following a PBS wash, the cells were incubated with fluorophore-conjugated secondary antibodies diluted 1:150 in PBS containing 1% BSA for 1 h. The cells were then washed, and the nuclei were stained with 4 $\mu\text{g/ml}$ of 4,6-diamino-2-phenylindole (DAPI) for 5 min. After one wash, the slides were mounted with ProLong Gold antifade reagent. The images were collected with a Nikon inverted fluorescence microscope and 60 \times and 100 \times objective lenses with DAPI, fluorescein isothiocyanate and CY-5 filter sets. At least two slides with at least 50 cells on each slide were analyzed. The images are representative of the pattern obtained for the entire slide analyzed.

Protein extraction

Both denaturing and non-denaturing lysis buffer was used for cell lysis. Many proteins are covalently and/or non-covalently modified by SUMO. It has therefore been suggested that the use of a denaturing lysis buffer containing a high percentage of SDS would have the benefits of immediately denaturing isopeptidases and eliminating non-covalent interactions with SUMO, thereby leaving only covalent SUMO binding in place (Sarge & Park-Sarge 2009, Tatham *et al.* 2009, Barysch *et al.* 2014). The high percentage of SDS, however, would prevent sumoylated proteins from binding to anti-SUMO antibodies during the IP procedure. As a result, SDS is usually either significantly diluted (e.g., 1:10) or removed from the lysis buffer by other means (Sarge & Park-Sarge 2009, Tatham *et al.* 2009, Barysch *et al.* 2014). However, these manipulations can cause certain proteins to re-nature and non-covalent interactions to reform. In our studies of several SUMO targets, no significant difference was found between the results of a co-IP performed using denaturing and non-denaturing lysis buffers (Supplementary Fig. 2). In both cases, the conclusions concerning possible protein sumoylation were based on the presence of high-molecular weight protein conjugate/s (one or numerous) detected with both anti-SUMO and anti-target protein antibodies above the band corresponding to the non-modified form of the protein. The presence of a band corresponding to the molecular weight of the non-modified protein would suggest a non-covalent interaction.

To produce denatured lysates, cell pellets were re-suspended in modified 2 \times Laemmli buffer (150 mM Tris-HCl pH 7.2, 4% sodium dodecyl sulfate (SDS), 20% glycerol and 20 mM N-ethylmaleimide (NEM, an isopeptidase inhibitor that blocks the activities of SENPs (Suzuki *et al.* 1999)) with 5 \times 10⁶ cells for every 300 μl of buffer. The solution was sonicated until the sample become liquid and then boiled at 100°C for 10 min. The lysates were then collected after centrifugation at high speed at room temperature for 15 min. The denaturing lysate was subjected to SDS removal using detergent-removal spin columns (Thermo Scientific) in accordance with the manufacturer's instructions. In brief, the SDS-containing lysate was slowly dropped onto the top of the compact resin of detergent-removal spin columns that had been washed with PBS and equilibrated and then incubated for two minutes at room temperature. The columns were then centrifuged at 1,000 g for two minutes at room temperature, and the flow-through fractions were collected and pooled together as the SDS-removed lysate.

To produce the non-denatured lysate, 5×10^6 cells were re-suspended in 300 μ l of whole cell extraction buffer (produced from the Millipore kit) supplemented with NEM at a final concentration of 20 mM. This cell suspension was then drawn through a 27-gauge needle five times and incubated on ice for 15 min. The lysate was collected after a centrifugation at high speed at 4°C for 20 min. Lysates from 5–6 or 2–3 STA-PUT separations (for spermatocytes and spermatids, respectively) were collected to produce sufficient amounts of proteins for IP (500–1,300 μ g of protein).

Immunoprecipitation (IP)

In all of the experiments, the lysates were pre-cleared by mixing them with control agarose resin (from Thermo Scientific, 26150) at 4°C for 1 h on an orbital rotator. Each 600 μ l of the pre-cleared lysate (0.5–1.3 mg of protein) was mixed with 120 μ l of SUMO1-agarose conjugate in a screw-cap spin column (Thermo Scientific) at 4°C. This was performed overnight on an orbital rotator and followed by centrifugation at 4000 rpm (Eppendorf, 5415C) at 4°C for 1 min. The amount of antibody was determined in preliminary experiments using an increasing antibody concentrations and analyzing its ability to precipitate the maximal amount of sumoylated proteins from lysates containing 1 mg of protein (not shown). The retained sumoylated proteins were washed twice with whole-cell extraction buffer supplemented with 20 mM NEM and then eluted with 50 ml acidic elution buffer (Thermo Scientific, 21004). Usage of the antibody-agarose conjugates prevented the heavy and light immunoglobulin chains from appearing in the eluted fraction, facilitating the subsequent mass spectrometry analysis.

For IP using other antibodies, the lysates were pre-cleared using protein A/G agarose beads and incubated with the antibodies overnight (Supplementary Table 1) Mouse or Rabbit IgG was used as a corresponding negative control. The lysates were washed as described above and incubated with protein A/G agarose beads overnight. This step was followed by additional washes and elution as described above.

Gel electrophoresis and Western Blotting

Gel electrophoresis was performed using NuPAGE 4 – 12% gradient Bis-Tris polyacrylamide gels and MOPS running buffer as previously described (Vigodner *et al.* 2013). The membrane (Novex nitrocellulose membrane, 0.45 μ m pore size, Life Technologies) was first blocked with 2% membrane blocking agent (GE Healthcare UK Limited, RPN2125V, Little Chalfont, Buckinghamshire, UK) in PBS + 0.02% (v/v) Tween 20 (PBS-T) for 1 h at room temperature. The membrane was then incubated with primary antibodies in PBS containing 2% BSA and 0.1% sodium azide for either 2 h at room temperature or overnight at 4°C. Following three washes with PBS-T, the membrane was further incubated with secondary antibodies that were diluted to 1:5000 in PBS-T for 1 h at room temperature. The secondary antibodies used in this study included the following: ECL™ anti-rabbit IgG HRP linked (GE Healthcare UK Limited, NA934V), goat anti-mouse IgG (H + L) HRP (Millipore, AP308P), and goat anti-rat IgG-HRP (Santa Cruz, sc-2032). The image collection and quantitative analyses were performed using the Universal Hood II and Quantity One Software (Bio-Rad Laboratories, Hercules, CA). Depending on the quality of the images after protein separation followed by a Western blot with a particular antibody,

the representative images for the SUMO IP results are presented either for denaturing (CDK1, STK31, and the largest subunit of RNAP II) or non-denaturing (MDC1, KAP1, MILI, DDX4, CDC5, TDP-43) protein lysates.

Gel fixation and staining

The samples to be subjected to mass spectrometry analysis were run on NuPAGE 4 – 12% gradient Bis-Tris polyacrylamide gels at 150 V for 5 min. The gels were fixed using a solution containing 50% (v/v) methanol and 7% (v/v) acetic acid at room temperature for 20 min and washed with distilled water 3 times for 5 min each. The fixed gels were then stained by incubation in GelCode® Blue Stain Reagent (Thermo Scientific, 24590) at room temperature for 1 hour. This step was followed by washing with distilled water overnight to remove excess staining solution. The stained regions were cut into 3 gel bands, digested and analyzed by LC-MS/MS for mass spectrometry.

Mass spectrometry analysis

Mass spectrometry (MS) was performed with the assistance of the Laboratory for Macromolecular Analysis and Proteomics at the Albert Einstein College of Medicine of Yeshiva University and is described below.

In-gel trypsin digestion and nanospray LC-MS/MS was performed as described in (Wang *et al.* 2014). Briefly, Coomassie-stained cut protein gel bands were first reduced with TCEP, alkylated with iodoacetamide and digested with trypsin. Nanospray LC-MS/MS was performed using a Linear Ion Trap (LTQ) mass spectrometer (Thermo Scientific) with the RSLC chromatography system (Thermo Scientific). The ten most intense ions determined from an initial survey scan (300–1600 m/z) were selected for fragmentation (MS/MS). The raw data files were converted to mgf text files (Mascot generic file) with Proteome Discoverer 1.2 and then merged and searched against the human or mouse NCBI database (April, 2014) using the in-house Mascot Protein Search engine (Matrix Science) with an automatic decoy database search. The following search parameters were used: trypsin, 2 missed cleavages; fixed modification of carbamidomethylation (Cys); variable modifications of deamidation (Asn and Gln), pyro-glu (Glu and Gln) and oxidation (Met); monoisotopic masses; peptide mass tolerance of 2 Da; and product ion mass tolerance of 0.6 Da. The Mascot-identified proteins were further validated with Scaffold (version 4, Proteome Software) using 99% and 95% protein and peptide probability, respectively, and a minimum of 2 peptides. The peptide and protein FDR (false discovery rates) were adjusted to 1% or less.

In vitro sumoylation assay

In vitro sumoylation assays were performed with the SUMOylation kit from Active Motif, Inc. (40120, Carlsbad, CA), following the manufacturer's protocol. The mouse GST-CDK1 recombinant protein was purchased from Sino Biological Inc. (Beijing, China).

Bioinformatics analysis

All of the putative sumoylation sites and SUMO-interactive motifs (SIMs) of the proteins were identified with SUMOsp 2.0 (The CUCKOO Workgroup, USTC). The identified proteins were divided into functional groups based on a literature search.

Results

I. Separation of spermatocytes and spermatids

The spermatocytes and spermatids were separated with a STA-PUT procedure that utilizes differential sedimentation velocity at the unit gravity of different cell types (Bellve *et al.* 1977, La Salle *et al.* 2009). The contents of the fractions collected from the separation were examined using microscopy and flow cytometry to identify tetraploid and haploid cells. The details of the procedure and the analysis of fraction purity are described in Supplementary Fig. 1 A and B.

II. Identification of sumoylated targets

For the identification of SUMO targets, proteins were extracted from isolated fractions using denaturing buffer followed by SDS removal and immunoprecipitated with anti-SUMO1-agarose conjugates as described in the Materials and Methods. Agarose resin without cross-linkage to antibodies was used as a negative control. Western blotting confirmed the successful enrichment of sumoylated proteins in the IP fraction compared with the negative controls (Fig. 1A). The precipitated proteins and their corresponding negative controls were then briefly run on gels, and the gels were subsequently fixed and stained (Fig. 1B). The stained regions were cut into three gel bands, digested and analyzed by LC-MS/MS for mass spectrometry. In addition to the enrichment of specific proteins, some non-specific background was observed in the negative controls. After protein digestion, mass spectrometry analyses revealed approximately 120 proteins uniquely in the antibody fractions but none in any of the negative controls (Table 1). These identified proteins were subdivided into eight groups according to their previously published functions (Matunis *et al.* 1996, Moroiianu 1998, Stopka *et al.* 2000, Goldberg *et al.* 2003, Myojin *et al.* 2004, Cramer 2006, Stark & Taylor 2006, Bao *et al.* 2012, Vourekas *et al.* 2012, Wang *et al.* 2012, Lasko 2013) (Fig. 2). The largest group, with 33%–34% of the spermatocyte and spermatid SUMO targets, included proteins involved in transcription, RNA interaction and stability, and splicing. This group included numerous ribosomal and heterogeneous nuclear ribonucleoproteins, important splicing factors, and several novel SUMO targets with important roles in germ cells, such as the largest subunit of RNA polymerase II (RNAP II), DEAD (Asp-Glu-Ala-Asp) box polypeptide DDX4 and DDX42, PIWI-like protein 2 (MILI/PIWIL2), TAR DNA-binding protein -43 (TDP-43), and paraspeckles component 1 (Table 1, Fig. 2).

Glycolytic and mitochondrial enzymes were found to be sumoylated in both spermatocytes (16%) and spermatids (10%), together with other enzymes (10–11%). Several proteins involved in ubiquitination, including ubiquitin-activating and ubiquitin-conjugating enzymes, ubiquitin hydrolases, and proteasome subunits were identified as SUMO targets in spermatocytes (9%) and in spermatids (3%) (Table 1, Fig. 2).

Stress-related and heat shock proteins represented 6–7% of sumoylated targets in spermatocytes and spermatids. Membrane-associated, vesicle trafficking, and ER proteins represented 11% of sumoylated proteins in spermatocytes and 14% in spermatids. This group included Rab 7 and Rab 11, calreticulin and acrosin-binding protein precursors, and the testis-specific ER protein calmeglin (Table 1, Fig. 2).

Cytoskeletal proteins represented only 2% of spermatocyte but 12% of spermatid SUMO targets. Conversely, proteins involved in DNA-break repair and chromatin remodeling were primarily sumoylated in spermatocytes (7%) but not in spermatids (1%). Importantly, this group included several proteins implicated in the regulation of chromatin remodeling during meiosis, such as mediator of DNA damage checkpoint protein 1 (MDC1), SWI/SNF-related regulator of chromatin 4 and 5 (SMARCA4 and 5), and poly [ADP-ribose] polymerase 1 (PARP1) (Table 1, Fig. 2).

Proteins that mediate nuclear-cytoplasmic transport comprised 3% of the spermatocyte and 5% of the spermatid sumoylome. Importantly, the major known sumoylated target in cells, Ran GTPase activating protein 1 (RanGAP1), was identified in both spermatocyte and spermatid fractions. This finding serves as a positive control for the specificity of SUMO target identification. Other proteins in this group included Ran binding protein 5, importin beta, and exportin 2, which are known to interact with RanGAP1 (Roscioli *et al.* 2012).

Cell cycle regulators represented 2% and 4% of sumoylated proteins in spermatocytes and spermatids, respectively. These important regulators of meiosis and mitosis include CDK1 (CDC2), testis-specific serine/threonine kinase 31 (STK31) and cell division cycle 5-like protein (CDC5) (Table 1, Fig. 2).

III. Confirmation of possible sumoylated targets

GPS-SUMO, a tool for the prediction of sumoylation sites and SUMO-interaction motifs (SIMs), was used to identify consensus and non-consensus sequences for possible sumoylation within the amino acid sequences of the proteins (Zhao *et al.* 2014). Because approximately 40% of proteins that are sumoylated are not sumoylated within the consensus sequence, the prediction algorithm of GPS-SUMO is based on the analysis of 983 manually collected consensus and non-consensus sumoylation sites in 545 proteins and 137 known SIMs in 80 proteins (Zhao *et al.* 2014). Several proteins with important roles during spermatogenesis (displaying an infertility phenotype upon inactivation or/and germ cell-specific proteins) were analyzed for the presence of sumoylation sites, and several of those with multiple and/or conserved sites were chosen for further characterization. These proteins included MDC1, KAP1, MILI, DDX4, CDK1, CDC5, STK31, TDP-43 and the largest subunit of RNAP II. Co-IP assays performed with anti-SUMO1 and anti-target protein antibodies alongside their negative controls were followed by Western blot analyses with antibodies against the target proteins or SUMO.

III.1. RNAP II

RNA polymerase II (RNAP II) is a master regulator of transcription in both germ and somatic cells. RNAPII was identified as SUMO-interacting protein specifically in the

spermatocyte fraction mass spectrometry screen (Table 1). In lysates from whole testis lysate, purified spermatocytes, spermatids and human embryonic kidney (HEK) cells, an isoform of RNAP II was specifically identified with an anti-SUMO antibody followed by Western blotting with an antibody against the largest subunit of RNAP II, suggesting that this is a sumoylated isoform (Fig. 3A). IP with an anti-RNAP II antibody did not successfully enrich for sumoylated isoforms of the proteins (not shown), which was likely due to the less efficient recognition by the antibody of the sumoylated isoform of the protein compared with the non-sumoylated form. Bioinformatics analysis revealed the presence of only non-consensus sumoylation sites in the amino acid sequence of RNAP II. However, the two sequences were evolutionary conserved between mouse and human (Supplementary Table 2).

III.2. MDC1

MDC1 is an important regulator of the DNA damage response. Male-specific infertility in *Mdc1*^{-/-} mice is due to meiotic arrest (Lou *et al.* 2006). MDC1 is an essential factor for establishing sex chromosome-wide silencing in the pachytene stage (Kunin *et al.* 2010, Ichijima *et al.* 2011). MDC1 was specifically identified in the spermatocyte fraction in our mass-spectrometry screen (Table 1). Several MDC1 isoforms in the range of 160–260 kDa that may correspond to splice isoforms of the protein were pulled down using co-IP with anti-SUMO antibody followed by MDC1 Western blot from the whole testis, purified spermatocyte but not spermatid fraction lysate (Fig. 3B). Sumoylation of MDC1 in somatic cells was previously reported (Yin *et al.* 2012). Bioinformatics analysis revealed the presence of several consensus sumoylation sites in both the mouse and human amino acid sequences of MDC1 (Supplementary Table 2).

III.3. MILI

MILI, a mammalian member of the *Piwi* gene family, binds to piRNAs. Spermatogenesis in *mili*-null mice is blocked at early prophase of the first meiosis, and the mice are sterile (Kuramochi-Miyagawa *et al.* 2004). MILI was specifically identified in the spermatocyte fraction in the mass-spectrometry screen (Table 1). IP with an anti-SUMO antibody followed by Western blotting with an anti-MILI antibody identified an apparent non-covalent interaction of the protein with SUMO or sumoylated proteins in spermatocytes and whole testis lysate (a band of approximately 110 kDa, Fig. 3C). The signal in spermatid fraction was very weak suggesting either an absence or a low level of SUMO and MILI interaction in these cells. Higher molecular weight (and presumably sumoylated) isoforms of MILI precipitated better with an anti-MILI antibody than with the anti-SUMO antibody followed by Western blotting with either the anti-SUMO or anti-MILI antibody. Overall, these results suggest that there is both covalent and non-covalent modification of MILI by SUMO. MILI is a germ cell specific protein, and therefore somatic data are not shown. Bioinformatics analysis revealed the presence of several consensus sumoylation sites in both the mouse and human amino acid sequences of the protein (Supplementary Table 2).

III.4. DDX4

DDX4 is the mouse VASA homologue (MVH) that is expressed exclusively in germ cells. It interacts with MILI to regulate microRNA-mediated RNA silencing (Kuramochi-Miyagawa *et al.* 2004). DDX4 is required for the development of male germ cells. Male mice that are homozygous for a targeted mutation of *Mvh* produce no sperm in the testes, with spermatogenic arrest at early meiosis in a manner similar to MILI-deficient mice (Tanaka *et al.* 2000). Several isoforms of the protein ranging from 60–80 kDa were detected by Western blotting using an anti-DDX4 antibody in spermatocytes, spermatids and whole testis lysate. These proteins may be the result of alternative splicing (Luo *et al.* 2013), Fig. 3D). The largest isoform (approximately 80 kDa) may be a sumoylated form of the protein, as this isoform was specifically identified by reciprocal co-IP using both SUMO and DDX4 antibodies. Although DDX4 was identified in spermatocyte fraction in the mass-spectrometry screen (Table 1), a more sensitive approach of Western blotting supported sumoylation of DDX4 in both spermatocytes and spermatids. DDX4 is a germ-cell specific protein, and therefore somatic data are not shown. Bioinformatics analyses revealed the presence of several consensus sumoylation sites in both the mouse and human amino acid sequences of DDX4, supporting possible sumoylation (Supplementary Table 2).

III.5. KAP1

KRAB domain-associated protein 1 (KAP1, TIF1 β , TRIM28) is a transcriptional repressor known to play essential roles in chromatin remodeling in early embryonic development and spermatogenesis. During spermatogenesis, KAP1 is preferentially associated with the heterochromatin structures of spermatocytes, spermatids and Sertoli cells. KAP1 was identified in both the spermatocyte and spermatid fractions in the mass-spectrometry screen (Table 1). IP with an anti-SUMO antibody followed by KAP1 Western blot analysis identified a possible non-covalent interaction of the protein with SUMO (a band at approximately 100 kDa, Fig. 4A) in the whole testis, spermatocyte, spermatid, and HEK cell lysate. Higher molecular weight SUMO-conjugates were clearly detected by IP with an anti-KAP1 antibody followed by Western blotting with either an anti-SUMO or anti-KAP1 antibody (Fig. 4A). KAP1 is an important protein in Sertoli cells. Therefore, to further evaluate the possible sumoylation of KAP1 in those cells, we also employed primary human and transformed mouse Sertoli cell lines. In all Sertoli cells, co-IP analyses revealed possible covalent and non-covalent interaction of KAP1 with SUMO (Fig. 4B, human and mouse Sertoli cell lines are shown). These results suggest that Sertoli cell lines can be used to a certain degree to study the regulation of sumoylation in Sertoli cells. Sumoylation of KAP1 in somatic cells was previously reported (Li *et al.* 2007, Campbell & Izumiya 2012). Bioinformatics analysis revealed the presence of several consensus sumoylation sites in both the mouse and human amino acid sequences of the protein (Supplementary Table 2).

III.6. CDK1

CDK1 (CDC2) is a crucial and indispensable regulator of both mitosis and meiosis (Diril *et al.* 2012). CDK1 was identified in both the spermatocyte and spermatid fractions in the mass-spectrometry screen (Table 1). Lysates from the whole testis and purified spermatocytes and spermatids were obtained, and two isoforms of CDK1 were identified in

the SUMO pull-down from the whole testis and spermatocytes, suggesting both the covalent and non-covalent interaction of CDK1 with SUMO (Fig. 5A). Only one isoform was observed at the detectable level in the spermatid and HEK cell lysate. The higher-molecular weight isoform was also highly enriched after IP with an anti-CDK1 antibody followed by Western blotting with an anti-SUMO antibody (Fig. 5A). Some background signal was detected in the negative control when highly concentrated lysates were used for the CDK1 IP analysis (whole testis). However, the background was significantly lower than the specific signal and was not detected upon IP with anti-SUMO antibodies. To confirm the possible sumoylation of CDK1 and given the importance of the protein in both germ and somatic cells, an *in vitro* sumoylation reaction was performed with a commercially available recombinant GST-CDK1 protein, sumoylation enzymes (E1, E2), and either normal SUMO or a mutant SUMO incapable of forming an isopeptide bond (Fig. 5B). Western blot analysis with an anti-CDK1 antibody revealed the presence of a sumoylated CDK1 band above the non-modified GST-CDK1 when using the normal (N) but not the mutant (M) SUMO isoform (Fig. 5B). When detected with an anti-SUMO antibody, multiple bands were observed in the sample with normal SUMO, most likely corresponding to sumoylated E1 and E2 in addition to sumoylated CDK1. However, these bands were not observed in the sample with the mutant SUMO isoform. Bioinformatics analysis revealed the presence of the consensus sumoylation site in the amino acid sequence of the mouse but not the human CDK1 (Supplementary Table 2). However, the alignment of the two sequences revealed a difference in only one amino acid, with a possible target lysine still present at the same position. We examined whether another important cell cycle regulator, CDK2 (not identified by our screen), contains a consensus sequence for sumoylation and detected no such sequence in CDK2 (not shown).

III.7. CDC5

CDC5 is a DNA-binding protein involved in cell cycle control. Using lysates from the whole testis, spermatocytes, spermatids, and HEK cells, reciprocal co-IP using anti-SUMO and anti-CDC5 antibodies supported the mostly non-covalent interactions of CDC5 with SUMO (Fig. 5C, a band approximately 110 kDa) but also some weak bands of higher molecular weight, which can correspond to sumoylated isoforms of the protein (brackets). Although CDC5 was identified in the spermatid fraction in the mass-spectrometry screen, Western blotting supported interaction between CDC5 and SUMO in both spermatocytes and spermatids. Bioinformatics analysis revealed the presence of two consensus sumoylation sites conserved between mouse and human in the amino acid sequences of CDC5 (Supplementary Table 2).

III.8. STK31

StkTK31 is a germ cell specific protein kinase. StkTK31 was identified in both the spermatocyte and spermatid fractions in the mass-spectrometry screen (Table 1). Co-IP analysis with anti-SUMO and anti-STK31 antibodies using the whole testis, spermatocyte and spermatid lysates supported mostly covalent (Fig. 5D, a band at approximately 110 kDa) and some non-covalent (a band just below 80 kDa in some fractions) interactions of STK31 with SUMO (Fig. 5D). Although STK31 was only identified in the spermatocyte fraction in the mass-spectrometry screen, Western blotting supported possible interaction between

CDC5 and SUMO in both spermatocytes and spermatids. STK31 is a germ-cell specific protein, and somatic data are not shown. Bioinformatics analysis revealed the presence of multiple conserved consensus sumoylation sites in the amino acid sequences of STK31 in mouse and human (Supplementary Table 2).

III.9. TDP-43

TDP-43 is an evolutionarily conserved, ubiquitously expressed DNA/RNA-binding protein. In testis, it binds to the promoter of the testis-specific mouse *Acrv1* gene in spermatocytes and spermatids, but ACRV1 is expressed exclusively in spermatids. Mutations in the TDP-43 promoter-binding motifs lead to the premature transcription of *Acrv1* in spermatocytes. TDP-43 may be involved in pausing RNAPII at the *Acrv1* promoter in spermatocytes (Lalmansingh *et al.* 2011). One mechanism regulating the different activity of TDP-43 in spermatocytes and spermatids could be a posttranslational modification. Our mass spectrometry screen identified TDP-43 as SUMO target in spermatocytes but not in the spermatid fraction (Table 1). Notably, IP with an anti-SUMO antibody followed by TDP-43 Western blot analysis of the whole testis and purified germ cell fractions (Fig. 6A) confirmed that SUMO and TDP-43 interact specifically in spermatocytes but not in spermatids (Fig. 6A and B; two membranes with increasing amounts of protein are shown). The purity of the fractions was confirmed using an anti-SYCP3 antibody showing a prominent band specifically in the spermatocyte fraction (Fig. 6B, SYCP3). Interestingly, two bands were identified using IP in HEK cells with an anti-SUMO antibody followed by TDP-43 Western blot analysis, suggesting both covalent and non-covalent interactions between TDP-43 and SUMO. Bioinformatics analysis revealed the presence of a conserved non-consensus sequence at the same residues in the mouse and human proteins (Supplementary Table 2).

IV. Colocalization studies

Partial colocalization of SUMO with its putative targets (orange signal) supported their possible sumoylation. Several targets showed a certain degree of colocalization between SUMO in the large heterochromatic regions of spermatocytes (MDC1 in the XY body (Fig. 7 A₁ and A₂, arrowheads) and KAP1 in the centromeric heterochromatin and partially in the XY body of spermatocytes (arrow and arrowheads, respectively, Fig. 7B₁ and B₂) and chromocenters of round spermatids (Fig. 7C₁ and C₂, arrowheads). KAP1 also colocalized with SUMO in certain areas of human and mouse Sertoli cells as well as in mouse Sertoli cell lines (Fig. 7, D₁, D₂; E₁, E₂, in insert: mouse Sertoli). The degree of colocalization varied between the cells, suggesting either a cell-cycle or stage-dependent behavior. Several targets of SUMO involved in transcriptional regulation and microRNA biogenesis (e.g., CDC5 (F₁, F₂, G₁, G₂), STK31 (H₁, H₂)) showed some overlap with SUMO that resulted in sparse orange signals detectable primarily in the DAPI-poor areas of spermatocytes. These areas may correspond to intrachromosomal territories (Branco & Pombo 2006).

Discussion

In this study, immunoprecipitation and Western blot analysis with an anti-SUMO1 antibody were used to identify SUMO targets in testicular cells. Our previous study showed similar

localization and Western blot patterns of SUMO1 and SUMO2/3 in testicular cells and sperm. Together with an absence of defects in spermatogenesis in SUMO1-knockout mice (Zhang *et al.* 2008), these data suggest that the functions and targets of SUMO1 and SUMO2/3 overlap in testicular cells. In other cell types, certain proteins are preferentially modified by either SUMO1 or 2/3. These data suggest that the presence of specific SUMO1 and SUMO2/3 targets during spermatogenesis cannot be excluded.

Our results suggest the role of sumoylation as a major player in the regulation of transcription, stress responses, the regulation of major enzymatic pathways, nuclear-cytoplasmic transport, cell cycle control, acrosome biogenesis and other functions in spermatogenesis. Interestingly, proteins involved in ubiquitination and sumoylation, DNA repair, and chromatin remodeling are highly sumoylated in spermatocytes, whereas cytoskeleton proteins are highly modified by SUMO in spermatids. It is possible that these proteins are more dynamically regulated specific cell types, as consistent with their functions.

Several proteins identified as SUMO targets in this study were previously found to be sumoylated in somatic cells (studies from other groups) or in human sperm (a study from our group). These proteins included RanGAP1, MDC1, KAP1, heat shock proteins, heteronucleoproteins, and several splicing factors (Mahajan *et al.* 1998, Vassileva & Matunis 2004, Li *et al.* 2007, Matafora *et al.* 2009, Li *et al.* 2010, Yin *et al.* 2012, Vigodner *et al.* 2013). Although previous studies in mammalian cells did not identify RNAP II as a SUMO target, studies in yeast identified the largest subunit of RNAP II as being sumoylated (Chen *et al.* 2009). In a similar manner, CDK1 was identified as a SUMO target in *Drosophila* (Nie *et al.* 2009). These previous data strongly support our findings and the specificity of our identification. Our results are further supported by the fact that all of the studied targets, with the exception of RNAP II and TDP-43, contain one or more consensus sumoylation sequence/s, and many of these sequences are evolutionarily conserved (Supplementary table 2). RNAP II and TDP-43 have non-consensus sequences, but those sequences are evolutionarily conserved.

Immunoprecipitation of endogenous proteins is challenging because sometimes only a small fraction of certain proteins can be sumoylated at a given time. In some experiments, possible sumoylated isoforms were precipitated with and anti-SUMO antibody but not with the antibody against the target protein (e.g., DDX4, Fig. 3D). These results are probably due to a lower affinity of specific antibodies to the sumoylated form of the protein. For other targets (e.g., MILI, CDC5, Fig. 3C and 5C), sumoylated isoforms were only precipitated with the antibody against the target protein and not with the SUMO antibody. These results can likely be explained by a very large number of sumoylated proteins in cells and a limited ability of the anti-SUMO antibody to precipitate 100% of the sumoylated proteins, particularly those with a very low degree of sumoylation.

Localization studies support the mass spectrometry results regarding a potentially diverse role of sumoylation in germ cells (Fig. 7). Several targets show a partial overlap with SUMO (orange signal). For example, KAP1 is preferentially associated with the heterochromatin structures of spermatocytes, spermatids and Sertoli cells. Interestingly, in somatic cells

KAP1 can catalyze its own sumoylation and transcriptional repression in a phosphorylation-dependent manner (Li *et al.* 2007, Li *et al.* 2010). Germ cell expression of KAP1 is required for spermatogenesis in the mouse. However, the shedding of immature germ cells in mutant mice was attributed to impaired paracrine interactions between germ and Sertoli cells (Weber *et al.* 2002, Herzog *et al.* 2011). Therefore, the role and regulation of KAP1 sumoylation in the heterochromatic region of germ and Sertoli cells should be further examined.

MDC1, which colocalizes with SUMO in the XY body, was recently implicated in spreading heterochromatin over the sex chromosome during MSCI (Ichijima *et al.* 2011). In somatic cells, MDC1 is sumoylated in response to the formation of double-stranded DNA breaks (Yin *et al.* 2012). SUMO and several other proteins involved in heterochromatin formation were absent from the sex body of *Mdc1*-null spermatocytes. Based on these results, the authors of this early study concluded that MDC1 is upstream of SUMO during MSCI. Our results complicated this interpretation somewhat because the sumoylated target itself is not present in *Mdc1*-null spermatocytes. MDC1 also plays a role in DNA double-stranded break repair and can be SUMO modified in response to ionizing radiation. Sumoylated MDC1 then recruits the ubiquitin ligase RNF4, which mediates ubiquitination at the DNA damage site (Yin *et al.* 2012). Our previous study showed that SUMO is also localized to the DNA breaks in germ cells (Shrivastava *et al.* 2010). These data, together with the results of this study, suggest that MDC1 can be sumoylated at the meiotic DNA breaks. Whether this modification activates ubiquitination remains to be determined, but our data support a close crosstalk between ubiquitination and sumoylation. Specifically, several ubiquitin-activating and ubiquitin-conjugating enzymes as well as ubiquitin hydrolases were identified as SUMO targets (Table 1). Furthermore, similar to previous results from our group obtained for human sperm, ubiquitin was identified in SUMO pulldowns. These results suggest that some proteins are simultaneously modified by sumoylation and ubiquitination (Vigodner *et al.* 2013).

Several targets implicated in the regulation of transcription and/or microRNA biogenesis were found to interact with SUMO (Fig. 3). Interestingly, MILI was mostly sumoylated in spermatocytes. These data are consistent with a specific role for this protein during meiosis.

TDP-43 is an ubiquitously expressed transcription factor that is highly conserved through evolution (Lalmansingh *et al.* 2011). There has been an increasing interest in this protein since mislocalized TDP-43 was found in the intracellular ubiquitinated inclusions in the brains of patients with frontotemporal lobar degeneration with ubiquitin-positive inclusions, amyotrophic lateral sclerosis, and Alzheimer disease (Neumann *et al.* 2006, Lalmansingh *et al.* 2011). In the testis, TDP-43 regulates the spermatid-specific transcription of *Acrv1*, and mutations in the *Acrv1* promoter-binding motifs of TDP-43 cause premature expression of *Acrv1* in spermatocytes. Surprisingly, TDP-43 is also found at the *Acrv1* promoter in spermatocytes, where it was suggested to regulate RNAP II pausing by an unknown mechanism. Our results revealed a striking difference in the sumoylation or SUMO-interaction of TDP-43 in spermatocytes and spermatids, suggesting that these interactions can contribute to RNAP II pausing or other cell-specific mechanisms in spermatocytes.

Further studies will characterize how sumoylation regulates the functions of TDP-43 in germ cells and other tissues.

An interesting finding of this study was that several kinases (CDK1, CDC5 and STK31) were identified as being sumoylated. CDK1 (CDC2) is a crucial and indispensable regulator of both mitosis and meiosis (Diril *et al.* 2012). Although CDK1 was not reported to be sumoylated in somatic cells, it was identified as a target of sumoylation in the *Drosophila* embryo, supporting our finding (Nie *et al.* 2009). Further studies will need to uncover how the sumoylation of CDK1 affects mitotic and meiotic progression.

STK31 was first identified as a germ cell-specific kinase (Wang *et al.* 2001) and was reported to interact with MIWI, suggesting a role in miRNA biogenesis and spermatogenesis (Bao *et al.* 2012). However, genetic studies utilizing mouse models have shown that STK31 is dispensable for spermatogenesis and oogenesis. Nevertheless, human STK31 was reported to be expressed in gastrointestinal cancers, including esophageal, gastric, colon and colorectal cancers (Yokoe *et al.* 2008, Fok *et al.* 2012). The knockdown of *Stk31* in colon cancer cells promotes cell differentiation and suppresses tumorigenicity (Fok *et al.* 2012). The role and regulation of STK31 by sumoylation in tumorigenesis remain to be characterized.

CDC5 is a DNA-binding protein involved in cell cycle control. Similar to MILI and DDX4, CDC5 is associated with the production of microRNAs through interactions with their gene promoters and RNAP II (Zhang *et al.* 2013). In yeast, CDC5 is also involved in the regulation of the SUMO pathway and modulates the maintenance and dissolution of cohesion at centromeres (Baldwin *et al.* 2009, Attner *et al.* 2013). The functions of CDC5 and its regulation by posttranslational modifications in mammalian spermatogenesis are not yet known.

Although the present data focused on proteins uniquely identified in the antibody fraction and not in the control fraction, some proteins, including those with an important role in spermatogenesis (such as HSP 70–2, phosphorylated H2AX, other histones, topoisomerase 2 alpha, and PIWI-like protein 1), were identified in both fractions (data not shown). The observation that these proteins bind to beads in a non-specific manner does not exclude the possibility that sumoylation or interaction with SUMO occurs. Indeed, TOP2A was identified as a specific target of sumoylation in our previous work (Shrivastava *et al.* 2010). Further studies will need to be conducted to test the possible sumoylation of these proteins. Furthermore, there may be additional proteins in germ cells that are modified by sumoylation but that were below the detection level of the technique used in this study.

Interestingly, most of the sumoylated proteins analyzed in this study also non-covalently interact with SUMO or sumoylated proteins. Bioinformatics analysis revealed that only four proteins (MDC1, RNAP II, STK31, TDP-43) contained a sumo-interacting motif (SIM, not shown). These data suggest that a greater number of proteins can interact with SUMO or sumoylated proteins non-covalently, regardless of the presence of a SIM.

In conclusion, this study identified and confirmed the sumoylation of several novel, previously uncharacterized SUMO targets, such as CDK1, RNAP II, CDC5, MILI, DDX4,

TDP-43 and STK31. Furthermore, several proteins that were previously identified as SUMO targets in somatic cells (e.g., KAP1, MDC1) were identified as SUMO targets in germ cells. Many of these proteins have unique roles in spermatogenesis, particularly during meiotic progression. This research opens a novel avenue for further studies of SUMO at the level of individual targets. If the sumoylation sites of the selected proteins are not identified, site-directed mutagenesis can then be employed to mutate candidate lysine residues to arginine to determine whether this substitution causes the disappearance of the sumoylated isoform(s). New approaches to identify sumoylated sites with mass spectrometry are under development but require further validation (Hsiao *et al.* 2009). After identification of the acceptor lysine residue(s), an attempt can be made to produce specific antibodies against the sumoylated form of the protein that can then be used for localization and interaction studies. Analysis of the functional consequences of the mutations in the sumoylated sites of the identified proteins, as well as other aspects of impaired sumoylation in germ cells, can be addressed both *in vitro* and *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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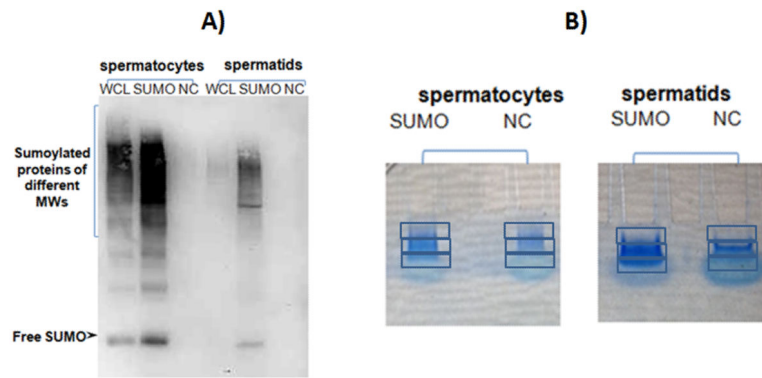


Figure 1.

(A) Western blotting confirmed the successful enrichment of the IP fraction for sumoylated proteins in spermatocytes and spermatids when compared to the negative controls. Whole-cell lysate (WCL), negative control (NC) and immunoprecipitated fraction (SUMO) are shown.

(B) The precipitated proteins and their corresponding negative controls were briefly run on gels, and the gels were subsequently fixed and stained. The stained regions were cut into three gel bands, digested and analyzed by LC-MS/MS for mass spectrometry.

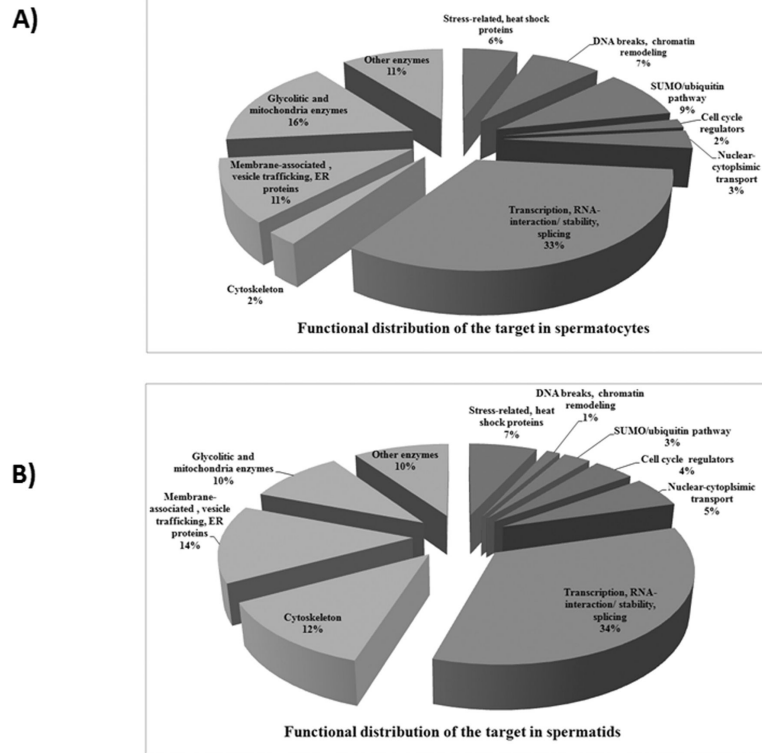


Figure 2. Functional distribution of SUMO targets in mouse spermatocytes (A) and spermatids (B). The percentage of sumoylated proteins from a certain functional group out of 100% of the identified sumoylated proteins is shown.

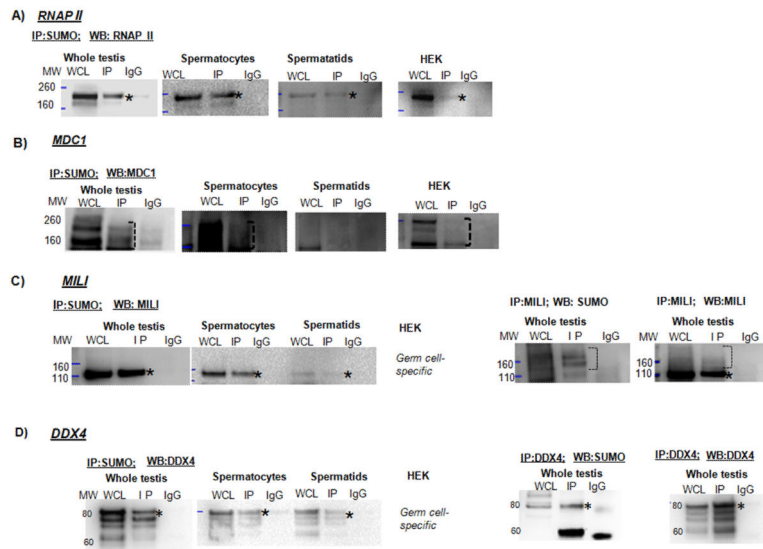


Figure 3.

Co-IP analysis of SUMO and RNAP II (**A**), MDC1 (**B**), MILI (**C**), and DDX4 (**D**). Whole-cell lysate (WCL), negative control (IgG) and IP fractions are shown. The migrating positions of the molecular weight (MW) markers are indicated.

(**A**) Co-IP analysis of SUMO and RNAP II. In lysates prepared using denaturing buffer from whole testis, purified spermatocytes, spermatids, and Human Embryonic Kidney (HEK)-293 cells, two isoforms of RNAP II were identified. The isoform with a higher molecular weight (asterisk) was identified by IP with an anti-SUMO antibody followed by Western blotting with an anti-RNAP II antibody.

(**B**) Using whole testis, purified spermatocytes, and Human Embryonic Kidney (HEK)-293 cells lysate prepared using non-denaturing buffer, IP with an anti-SUMO antibody followed by Western blotting with an MDC1 antibody detected multiple isoforms of MDC1 (bracket). There was no specific signal identified in the spermatid fraction,.

(**C**) IP using anti-SUMO or anti-MILI antibodies followed by Western blotting with either anti-MILI or anti-SUMO antibodies. Using spermatocytes, spermatids and whole testis lysate prepared using non-denaturing buffer, IP with an anti-SUMO antibody followed by Western blotting with an anti-MILI antibody identified a non-covalent interaction of the protein with SUMO or sumoylated proteins (a band approximately 110 kDa; asterisk). The signal in spermatids was at a low level. Sumoylated conjugates of higher molecular weight were precipitated with an anti-MILI antibody followed by Western blotting with either the anti-SUMO or anti-MILI antibody.

(**D**) IP using SUMO or DDX4 antibodies followed by Western blotting with either DDX4 or SUMO antibodies. Several isoforms of the protein could be detected on the Western blot using an anti-DDX4 antibody and non-denaturing lysis buffer. The largest isoform (approximately 80 kDa, asterisk) could be a sumoylated form of the protein, as it was specifically identified by reciprocal co-IP using both SUMO and DDX4 antibodies.

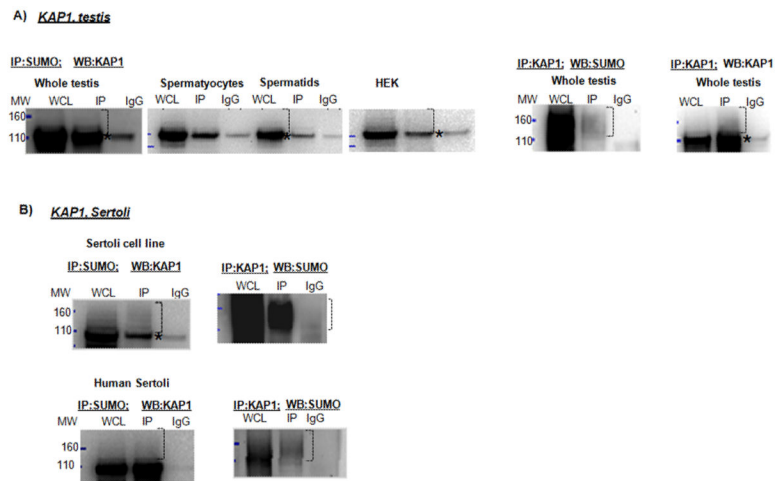


Figure 4.

Co-IP analysis of SUMO and KAP1. IP using non-denaturing lysis buffer with anti-SUMO antibody followed by KAP1 Western blot analysis identified a possible non-covalent interaction of the protein with SUMO (a band approximately 100 kDa, asterisk). Higher molecular weight SUMO-conjugates were clearly detected by IP with an anti-KAP1 antibody followed by Western blotting with either an anti-SUMO or anti-KAP1 antibody (bracket). In a similar manner, co-IP analyses of KAP1 and SUMO in Sertoli cells and cell lines revealed possible covalent and non-covalent interactions of KAP1 with SUMO.

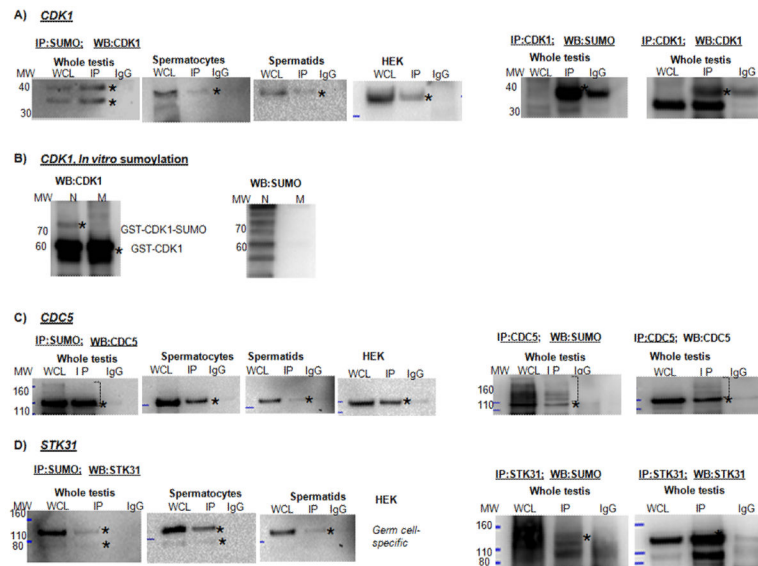


Figure 5.

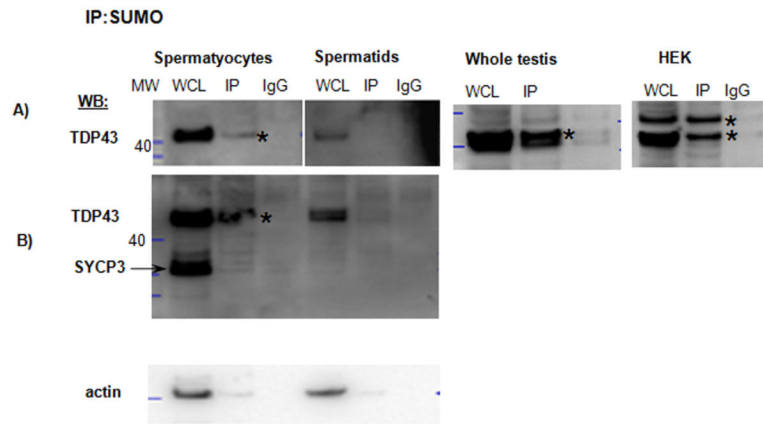
Co-IP analysis of SUMO and CDK1 (A), CDC5 (C), and STK31 (D) and the *in vitro* sumoylation analysis of CDK1 (B).

(A) Co-IP analysis of SUMO and CDK1. Lysates prepared using denaturing lysis buffer from whole testis and purified spermatocytes were obtained, and two isoforms of CDK1 were identified in the SUMO pulldown, suggesting both the covalent and non-covalent interaction of CDK1 with SUMO. Only one isoform was detectable in spermatids and HEK cells. The higher-molecular weight isoform (asterisk) was also highly enriched after IP with an anti-CDK1 antibody followed by Western blotting with an anti-SUMO antibody.

(B) To confirm the possible sumoylation of CDK1, an *in vitro* sumoylation reaction was performed with a recombinant GST-CDK1 protein, sumoylation enzymes (E1, E2), and either normal (N) or a mutant (M) SUMO incapable of forming an isopeptide bond. Western blot analysis with an anti-CDK1 antibody revealed the presence of a sumoylated CDK1 band above the non-modified GST-CDK1 when using the normal (N) but not mutant (M) SUMO isoform. When detecting with an anti-SUMO antibody, multiple bands were observed only in the sample with normal SUMO, most likely corresponding to sumoylated E1 and E2 in addition to sumoylated CDK1.

(C) Reciprocal co-IP using non-denaturing lysis buffer, anti-SUMO and anti-CDC5 antibodies support both covalent (bracket) and non-covalent interactions of CDC5 with SUMO (asterisk).

(D) Co-IP analysis using non-denaturing lysis buffer, anti-SUMO and anti-STK31 antibodies supported possible non-covalent and covalent interactions of StkTK31 with SUMO. The asterisk indicates a possible sumoylated isoform found above the non-sumoylated isoform of STK31.

**Figure 6.**

Co-IP analysis of SUMO and TDP-43. IP using non-denaturing lysis buffer with anti-SUMO antibody followed by TDP-43 Western blot analysis identified a prominent specific signal in the whole testis and spermatocyte but not in the spermatid fractions (**A** and **B** show two different membranes with increasing amount of proteins). The same membrane was re-probed with anti-SYCP3 antibody (SYCP3) to confirm the purity of the spermatocyte and spermatid fractions and with an actin antibody to show a comparable level of the whole cell lysates used for the IP. Two bands were identified in HEK cells, suggesting both covalent and non-covalent interactions between TDP-43 and SUMO.

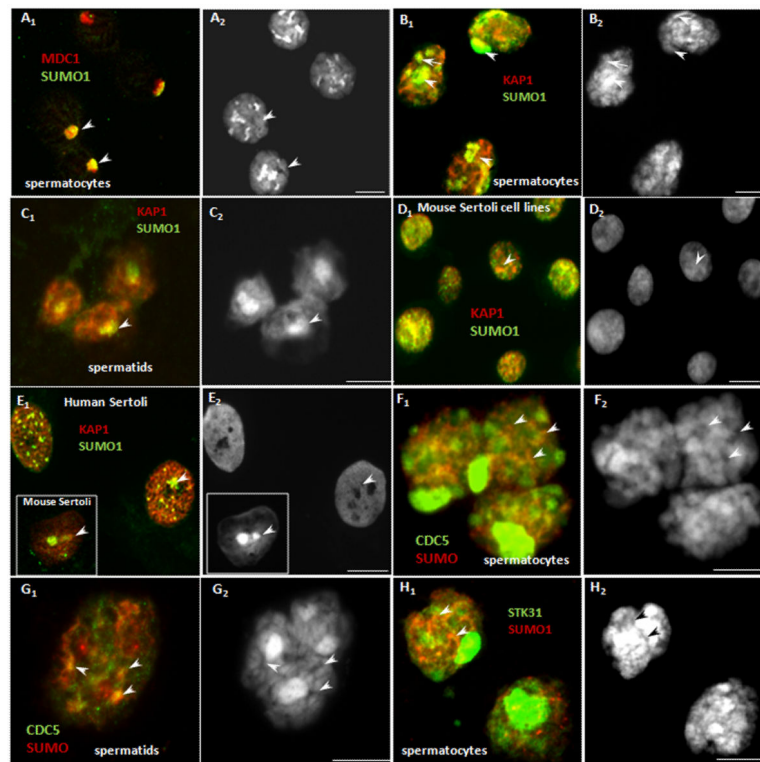


Figure 7.

Immunofluorescent localization of SUMO and its putative targets in testicular cells. Targets, color-coding and cell type are indicated for each image. A mouse monoclonal anti-SUMO1/GMP antibody was used in (**A₁**), and a rabbit monoclonal anti-SUMO1 was used in (**B–H**). Nuclei are stained by DAPI (blue). For all images, immunofluorescent staining is shown alongside the corresponding DAPI images to demonstrate the chromatin structure. Scale bar is 10 μ m. SUMO colocalized with MDC1 in the XY body (Fig. 7 **A₁** and **A₂**, arrowheads) and KAP1 in the centromeric heterochromatin and partially in the XY body of spermatocytes (Fig. 7 **B₁** and **B₂**, arrowheads and arrow, respectively) and chromocenters of round spermatids (Fig. 7 **C₁** and **C₂**, arrowheads). KAP1 also colocalized with SUMO in certain areas of human and mouse Sertoli cells as well as in mouse Sertoli cell lines (**D₁**, **D₂**; **E₁**, **E₂**, an insert: mouse Sertoli). CDC5 (**F₁**, **F₂**, **G₁**, **G₂**), and STK31 (**H₁**, **H₂**) were detectable primarily in the DAPI-poor areas of cells that may correspond to intrachromosomal domains (arrows).

Table 1

Identification of sumoylated proteins unique to the spermatocyte or spermatid IP samples via tandem mass spectrometry. Protein ID, molecular weight, and the number of unique peptides identified for each protein in spermatocytes and/or spermatids is indicated. Proteins of specific interest in the field of spermatogenesis are indicated in bold.

	Accession number *	MW	<i>Number of unique peptides</i>	
			spermatocytes	spermatids
Stress-related, heat shock proteins				
heat shock 70 kDa protein 4	gi 112293266 (+3)	94 kDa	18	11
inducible heat shock protein 70	gi 118490060 (+4)	70 kDa		7
stress-70 protein, mitochondria –	gi 162461907 (+5)	73 kDa	5	4
heat shock 70 kDa protein 1-like	gi 124339838 (+3)	71 kDa		2
heat shock protein 105 kDa	gi 114145505 (+5)	96 kDa		3
glutathione S-transferase P 1	gi 10092608 (+5)	24 kDa	2	
1-Cys peroxiredoxin protein 2	gi 3789944 (+3)	25 kDa	2	
dnaJ homolog subfamily B member 1 (Heat shock 40 kDa protein 1)	gi 9055242 (+2)	38 kDa	2	
protein DJ-1	gi 55741460 (+1)	20 kDa	2	
Ephx1 protein	gi 34784388 (+4)	51 kDa	2	
DNA breaks, chromatin remodeling			spermatocytes	spermatids
mediator of DNA damage checkpoint protein 1 (MDC1)	gi 132626693 (+5)	185 kDa	8	
SWI/SNF related regulator of chromatin SMARCA5	gi 148678936 (+1)	116 kDa	5	
SWI/SNF related regulator of chromatin SMARCA4	gi 148693261 (+8)	185 kDa	2	
matrin-3	gi 25141233 (+4)	95 kDa	3	3
ruvB-like	gi 9790083 (+1)	50 kDa	3	
ruvB-like 2	gi 6755382 (+1)	51 kDa	2	
AT rich interactive domain 2 (ARID, RFX-like)	gi 262231796	196 kDa	3	
damage specific DNA binding protein 1	gi 148709424 (+5)	108 kDa	3	
poly [ADP-ribose] polymerase 1	gi 20806109 (+4)	113 kDa	2	
SUMO/ubiquitin pathway			spermatocytes	spermatids
tripartite motif protein 28 (TRIM28, KAP1) SUMO ligase	gi 148706135 (+3)	89 kDa	7	2
Ubqln1 protein, partial	gi 16307349 (+8)	47 kDa	3	
ubiquitin-associated protein 2-like isoform 3	gi 260166704 (+9)	117 kDa		
ubiquitin-conjugating enzyme E2 N	gi 309262615 (+3)	23 kDa	2	
ubiquitin-activating enzyme E1-like 2, isoform CRA_a	gi 148706006 (+4)	119 kDa	2	
ubiquitin carboxyl-terminal hydrolase isozyme L3	gi 139948802 (+1)	26 kDa	2	
ubiquitin carboxyl-terminal hydrolase LI	gi 148705826 (+2)	26 kDa	2	
proteasome subunit alpha type-4	gi 6755196 (+2)	29 kDa	2	
proteasome subunit beta type-3	gi 6755202	23 kDa	2	
26S protease regulatory subunit 4	gi 6679501	49 kDa	2	
26S proteasome non-ATPase regulatory subunit 1 (Psm1)	gi 116283726 (+5)	93 kDa	2	
26S proteasome non-ATPase regulatory subunit 2 (Psm2)	gi 27692965 (+8)	67 kDa	2	
26S proteasome non-ATPase regulatory subunit 6 (Psm6)	gi 28175479 (+3)	44 kDa		4
Cell cycle regulators			spermatocytes	spermatids

<u>cyclin-dependent kinase 1</u>	gi 31542366 (+3)	34 kDa	4	3
<u>serine/threonine kinase 31</u>	gi 13603843 (+2)	115 kDa	3	
<u>cell division cycle 5-like protein</u>	gi 22779899 (+3)	92 kDa		2
ASR2B	gi 13517493 (+3)	100 kDa		2
Nuclear-cytoplasmic transport			spermatocytes	spermatids
<u>RAN GTPase activating protein 1</u>	gi 148672614 (+5)	73 kDa	13	2
<u>Ran-specific GTPase-activating protein</u>	gi 153792001 (+2)	24 kDa		2
Ran binding protein 5	gi 12057236 (+2)	124 kDa	6	2
Karyopherin (importin) beta 1	gi 30931411 (+4)	97 kDa	4	2
exportin-2	gi 12963737	110 kDa	2	
Transcription, RNA-interaction/ stability, splicing			spermatocytes	spermatids
<u>DEAD (Asp-Glu-Ala-Asp) box polypeptide 4</u>	gi 148686462 (+2)	78 kDa	4	
<u>DEAD (Asp-Glu-Ala-Asp) box polypeptide 42</u>	gi 133777033 (+6)	89 kDa	2	
<u>piwi-like protein 2</u>	gi 10946610	109 kDa	5	
<u>paraspeckle component 1</u>	gi 225543409 (+2)	59 kDa	2	
<u>RNA polymerase II largest subunit</u>	gi 2145091 (+2)	217 kDa	3	
RNA-binding protein EWS	gi 88853581 (+4)	69 kDa	3	3
RNA-binding protein 14	gi 86262142 (+1)	36 kDa	7	4
<u>TAR DNA-binding protein 43 isoform 1</u>	gi 21704096 (+4)	45 kDa	2	
PC4 and SFRS1-interacting protein	gi 19527168	51 kDa	5	6
elongation factor 2	gi 33859482 (+8)	19 kDa	4	4
splicing factor, proline- and glutamine-rich	gi 23956214 (+1)	60 kDa		
splicing factor, arginine/serine-rich 14	gi 148696857 (+4)	120 kDa	2	
splicing factor 3B subunit 1	gi 15214281 (+1)	146 kDa	3	
splicing factor 1 protein	gi 14318588 (+10)	67 kDa	6	2
splicing factor, arginine/serine-rich 15	gi 109150409			2
far upstream element (FUSE)-binding protein 2	gi 163954948 (+2)	75 kDa	7	9
far upstream element (FUSE) binding protein 3	gi 224922832 (+2)	62 kDa		2
fusion, derived from t(12;16) malignant liposarcoma	gi 148685669 (+6)	98 kDa	3	6
interleukin enhancer-binding factor 3 isoform 1	gi 111607430 (+7)	60 kDa		3
T-complex protein 1 subunit epsilon	gi 6671702 (+6)	68 kDa	6	5
nonsense mRNA reducing factor 1 NORF1	gi 12836885 (+4)		7	2
THO complex subunit 4	gi 6755763 (+1)	27 kDa	3	2
HLA-B-associated transcript 3	gi 148694699 (+7)	111 kDa	2	
polypyrimidine tract binding protein 2	gi 148680404 (+4)	68 kDa	4	
prohibitin-2	gi 126723336 (+2)	33 kDa	2	
Abce1 protein, partial [gi 45219736 (+3)	65 kDa	2	
Enhancer of mRNA-decapping protein 4	gi 145566774 (+4)	152 kDa	3	
protein strawberry notch homolog 1	gi 124487087 (+2)	154 kDa	3	
<u>Ribonucleoproteins</u>			spermatocytes	spermatids
heterogeneous nuclear ribonucleoprotein A/B isoform 1	gi 146260280 (+5)	95 kDa	5	3
heterogeneous nuclear ribonucleoprotein A1 isoform b	gi 85060507 (+6)	16 kDa		6
heterogeneous nuclear ribonucleoprotein U	gi 148681230 (+6)	49 kDa	5	

heterogeneous nuclear ribonucleoprotein H	gi 10946928 (+2)	123 kDa	2	5
small nuclear ribonucleoprotein N	gi 3142634 (+2)	25 kDa	3	
heterogeneous nuclear ribonucleoprotein Q isoform 1	gi 114145493 (+9)	70 kDa		4
heterogeneous nuclear ribonucleoprotein L	gi 183980004 (+6)	64 kDa	2	
heterogeneous nuclear ribonucleoprotein A3 isoform a	gi 31559916 (+11)	40 kDa		2
heterogeneous nuclear ribonucleoprotein r protein, 116 kDa U5 small nuclear ribonucleoprotein component isoform b	gi 158508674 (+5)	109 kDa	2	
heterogeneous nuclear ribonucleoprotein D-like	gi 148664250 (+2)	46 kDa		2
<i>Ribosomal proteins</i>			spermatocytes	spermatids
60S ribosomal protein L31-like isoform 1	gi 82898755 (+6)	14 kDa	2	
60S ribosomal protein L21	gi 31560385 (+13)	39 kDa	2	
60S ribosomal protein L14	gi 13385472 (+1)	24 kDa		2
60S ribosomal protein L22	gi 6677775	15 kDa	2	
60S ribosomal protein L23-like	gi 407262287 (+2)	14 kDa	2	
40S ribosomal protein S13	gi 15029927 (+2)	16 kDa	2	
40S ribosomal protein S20 isoform 2	gi 4506697	13 kDa	2	
40S ribosomal protein S19	gi 12963511 (+3)	16 kDa	2	
16S ribosomal protein	gi 200796 (+1)	87 kDa	2	2
ribosomal protein S26	gi 1527176 (+3)	13 kDa	2	2
Ribosomal protein S9	gi 21594169 (+1)	23 kDa	3	2
Ribosomal protein S23	gi 72679974 (+5)	16 kDa	2	
La ribonucleoprotein domain family member 1	gi 147744571 (+3)	121 kDa		2
Cytoskeleton			spermatocytes	spermatids
lamin-B1	gi 188219589 (+2)	67 kDa	3	6
alpha-actinin-4	gi 11230802	105 kDa	2	
filamin-C	gi 124487139 (+2)	291 kDa		10
filamin-B	gi 145966915 (+2)	277 kDa		8
talin 1	gi 148670519 (+4)	270 kDa		3
vinculin	gi 148669535 (+3)	124 kDa		3
coiled-coil domain containing 39	gi 148703084 (+2)	107 kDa	2	
kinesin family member 5B	gi 148691088 (+3)	110 kDa		4
protein syndesmos	gi 13385314 (+1)	23 kDa		3
plectin	gi 122065897 (+24)	534 kDa		2
myosin light chain, regulatory B-like	gi 71037403 (+4)	20 kDa		3
Membrane-associated, vesicle trafficking, ER proteins			spermatocytes	spermatids
acrosin-binding protein isoform 1 precursor	gi 188035922 (+1)	61 kDa	2	
GPI-anchored membrane protein 1	gi 148695758 (+7)	84 kDa	4	4
ras-related protein Rab-11B	gi 6679583 (+3)	24 kDa	5	2
ribophorin I	gi 148666824 (+4)	68 kDa		2
ribophorin	gi 1468961 (+4)	66 kDa	7	2
calreticulin precursor	gi 6680836 (+5)	48 kDa		2
rab7	gi 1050551 (+1)	24 kDa	3	2
Programmed cell death 6 interacting protein	gi 20071292 (+8)	96 kDa	4	2

calmegin, isoform CRA_b	gi 148678956 (+3)	71 kDa	3	2
calnexin precursor	gi 6671664 (+1)	67 kDa	2	
solute carrier family 2, facilitated glucose transporter member 3	gi 261862282 (+2)	53 kDa	3	3
transmembrane emp24-like trafficking protein 10	gi 148670919 (+2)	26 kDa		2
ras-related protein Rab-14	gi 18390323 (+3)	24 kDa	3	
SEC22 vesicle trafficking protein homolog B	gi 14290512 (+1)	25 kDa	2	
ERO1-like beta	gi 109730421 (+3)	54 kDa	2	
p162 protein	gi 1205976 (+7)	162 kDa	2	
zinc finger protein 289	gi 148695611 (+4)	58 kDa	2	
Glycolytic and mitochondria enzymes			spermatocytes	spermatids
cytochrome c oxidase subunit 6C	gi 16716343	8 kDa	2	
cytochrome b-c1 complex subunit 2, mitochondrial precursor	gi 22267442 (+2)	48 kDa	2	
cytochrome b-c1 complex subunit 1, mitochondrial precursor	gi 46593021 (+3)	53 kDa	3	
cytochrome c oxidase subunit IV	gi 1372988 (+2)	20 kDa	3	
dihydrolipoamide S-acetyltransferase precursor	gi 16580128 (+2)	59 kDa	3	
trifunctional enzyme subunit alpha, mitochondrial precursor	gi 33859811 (+1)	83 kDa	6	
trifunctional enzyme subunit beta, mitochondrial precursor	gi 21704100 (+4)	51 kDa	2	
phosphoglycerate mutase 1	gi 114326546 (+1)	29 kDa		2
L-lactate dehydrogenase A chain isoform 2	gi 257743039 (+5)	40 kDa	3	2
L-lactate dehydrogenase B chain	gi 6678674	37 kDa	2	2
phosphate carrier protein, mitochondrial precursor	gi 19526818 (+6)	40 kDa	2	
fructose-bisphosphate aldolase A isoform 1 precursor	gi 293597567 (+2)	45 kDa		5
aldehyde dehydrogenase 2, mitochondria	gi 148687772 (+5)	55 kDa		4
Me1 protein	gi 13096987 (+6)	64 kDa	2	2
ATP synthase subunit O, mitochondrial precursor	gi 20070412 (+2)	23 kDa	3	
inositol-3-phosphate synthase 1	gi 12963757	61 kDa	2	
inner membrane protein, mitochondrial	gi 148666538 (+10)	81 kDa	2	
creatine kinase B-type	gi 10946574 (+3)	43 kDa		3
acyl-CoA hydrolase	gi 14587839 (+6)	38 kDa	2	
N(4)-(beta-N-acetylglucosaminy)-L-asparaginase isoform 1 precursor	gi 54292135	37 kDa	3	
carnitine O-palmitoyltransferase 2, mitochondrial precursor	gi 162138915 (+3)	74 kDa	2	
isocitrate dehydrogenase 3 (NAD+) alpha	gi 148693872 (+5)	40 kDa	2	
enoyl-CoA delta isomerase 1, mitochondrial precursor	gi 31981810 (+1)	32 kDa	2	
coiled-coil-helix-coiled-coil-helix domain containing 3	gi 148681756 (+2)	23 kDa	2	
Other enzymes			spermatocytes	spermatids
GMP synthase [glutamine-hydrolyzing]	gi 85861218 (+4)	77 kDa	2	
tripeptidyl peptidase II	gi 148664483 (+4)	139 kDa	2	
Aspartyl-tRNA synthetase	gi 14250408 (+6)	57 kDa	2	
triosephosphate isomerase, partial	gi 1864018 (+5)	23 kDa		4
retinol dehydrogenase 11 precursor	gi 19482172 (+3)	35 kDa		2
hydroxysteroid (17-beta) dehydrogenase 4	gi 148677986 (+3)	33 kDa	3	
Immunoglobulins			spermatocytes	spermatids
Ig heavy chain V region TE32	gi 110285 (+14)	13 kDa	2	2

kappa-Ig light chain (111 AA)	gi 930228	12 kDa	9	
Unknown functions in testis			spermatocytes	spermatids
ataxin 2-like	gi 148685438	113 kDa	10	4
testis specific 10	gi 148682582 (+2)	80 kDa	4	
neuroleukin	gi 200065 (+8)	63 kDa	2	2
myelin expression factor 2 isoform 1	gi 244790087 (+4)	63 kDa	2	
platelet-activating factor acetylhydrolase IB subunit gamma	gi 6679201	26 kDa	2	
interleukin enhancer-binding factor 2	gi 13385872 (+2)	43 kDa	2	
tetratricopeptide repeat protein 21B	gi 114158711 (+1)	151 kDa	2	
annexin A3	gi 148688409 (+6)	36 kDa		2
high density lipoprotein (HDL) binding protein	gi 148708002 (+5)	144 kDa		2

* the number showing in the bracket following “+” means how many more accession number also refer to this target