Mapping of Post-translational Modifications of Transition Proteins, TP1 and TP2, and Identification of Protein Arginine Methyltransferase 4 and Lysine Methyltransferase 7 as Methyltransferase for TP2

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Background: Transition proteins replace 90% of the nucleosomal histones during nucleo-histone to nucleo-protamine chromatin reconfiguration in mammalian spermiogenesis.

Results: Major transition proteins, TP1 and TP2, harbor several post-translational modifications. TP2 is methylated by PRMT4 and KMT7 methyltransferase.

Conclusion: Endogenous transition proteins, TP1 and TP2, exhibit extensive post-translational modifications.

Significance: This work provides insight into the chromatin remodeling events during spermiogenesis and establishment of the sperm epigenome.

In a unique global chromatin remodeling process during mammalian spermiogenesis, 90% of the nucleosomal histones are replaced by testis-specific transition proteins, TP1, TP2, and TP4. These proteins are further substituted by sperm-specific protamines, P1 and P2, to form a highly condensed sperm chromatin. In spermatozoa, a small proportion of chromatin, which ranges from 1 to 10% in mammals, retains the nucleosomal architecture and is implicated to play a role in transgenerational inheritance. However, there is still no mechanistic understanding of the interaction of chromatin machinery with histones and transition proteins, which facilitate this selective histone replacement from chromatin. Here, we report the identification of 16 and 19 novel post-translational modifications on rat endogenous transition proteins, TP1 and TP2, respectively, by mass spectrometry. By in vitro assays and mutational analysis, we demonstrate that protein arginine methyltransferase PRMT4 (CARM1) methylates TP2 at Arg71, Arg75, and Arg92 residues, and lysine methyltransferase KMT7 (Set9) methylates TP2 at Lys88 and Lys91 residues. Further studies with modification-specific antibodies that recognize TP2K88me1 and TP2R92me1 modifications showed that they appear in elongating to condensing spermatids and predominantly associated with the chromatin-bound TP2. This work establishes the repertoire of post-translational modifications that occur on TP1 and TP2, which may play a significant role in various chromatin-templated events during spermiogenesis and in the establishment of the sperm epigenome.

Eukaryotic genomic DNA is packaged with canonical histones and its variants constituting the nucleosomal architecture of chromatin. Chromatin proteins, including histones, harbor several post-translational modifications, and this epigenetic landscape of chromatin proteins along with effector proteins determines the chromatin structure and mediates several chromatin-templated processes (1, 2). This chromatin architecture is continuously reconfigured during male germ cell differentiation. Mammalian spermatogenesis is a developmental process in which diploid spermatogonial stem cells differentiate to haploid spermatooza. Spermiogenesis is the last phase of spermatogenesis, during which haploid round spermatids undergo change in their cellular and nuclear morphology to form mature sperm, accompanied by a remarkable change in chromatin structure and composition (3–7). The nucleosomal architecture of haploid round spermatids is transformed into toroidal nucleoprotamine fiber in the mature spermatozoa in a two-step process. In the first step, intermediate basic proteins, transition proteins replace 90% of the histones. In the second step, highly basic proteins, protamines replace transition proteins to occupy 90–99% of the sperm chromatin in different mammals (3–9).

In rats, spermiogenesis comprises of 19 steps. During the first eight steps, haploid round spermatids are transcriptionally active, which ceases in step 9 or 10 as the chromatin reconfiguration process is initiated (4). Histone eviction begins with histone H4 hyperacetylation during steps 9–12, initiating the deposition of the transition proteins. Brdt, a double bromodomain protein, is suggested to facilitate eviction of histones by recognizing acetylated histone H4 and through its interaction with Smarca1 (BAF57), a key member of ATP-dependent remodel-
ing complex SWI/SNF (10, 11). Transition proteins (TP1, TP2, and TP4) replace most of the histones and briefly organize the chromatin during spermiogenesis steps 12–15. Protamines (P1 and P2) are deposited with a concomitant complete replacement of the transition proteins during steps 16–19 to decorate the sperm chromatin (3, 4).

A small fraction of the sperm chromatin, which is about 10% in humans and 1% in rodents, remains nucleosomal at specific genomic loci (8, 9, 12). Sperm nucleosomes are largely present in intergenic and intrinsic regions and associate with retrotransposable elements and centromeric repeats. A small subset of sperm nucleosomes are retained in the promoter regions of development-regulatory proteins, which become activated in preimplantation embryos (13–15). Retained nucleosomes also include non-canonical histone variants H2A-Bbd, H2AL1/L2, H2BL1, TH2B, and H3.3 (3). Many of the sperm nucleosomal loci exhibit characteristic active and repressive histone modifications, reflecting their expression status in embryogenesis (9, 16). Recently, mouse sperm protamines also have been demonstrated to harbor post-translational modifications (17). Thus, sperm chromatin has a defined epigenetic signature and is suggested to play a fundamental role in transgenerational inheritance (17, 18). However, the mechanism of selective histone modification, the post-translational modifications of transition proteins may also play a critical role in the chromatin restructuring process during mammalian spermiogenesis, we embarked to comprehensively identify PTMs of the two major transition proteins, TP1 and TP2. Through mass spectrometric analysis of purified endogenous TP1 and TP2, we demonstrate 16 novel modifications for TP1 and 19 novel modifications for TP2, which include arginine mono- and/or dimethylation, lysine acetylation, lysine mono- and/or di-methylation, serine acetylation, and threonine and serine phosphorylation. Furthermore, we demonstrate PRMT4 (also known as CARM1) and KMT7 (also known as Set7/9 or Set9) as the enzymes responsible for the catalysis of arginine and lysine methylation of TP2, respectively. The results described here establish the PTM repertoire of TP1 and TP2, which, in combination with the diverse roles of chromatin modifiers and readers, should provide insights into the molecular mechanisms underlying the global chromatin remodeling event during the late steps of mammalian male germ cell differentiation and consequently establishment of the sperm epigenome.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections—**PRMT4 mammalian expression constructs with N-terminal FLAG tag were transfected in HEK293T cells (ATCC) by Lipofectamine 2000 reagent (Invitrogen). Transfection was carried out in 9-mm dishes according to the manufacturer’s instructions. The ratio of reagent to plasmid DNA used was 2:1, and 1 μg/ml plasmid was transfected. M2-agarose beads were used to immunoprecipitate PRMT4 variants, and beads were used as an enzyme source for *in vitro* methylation assays as described previously (33).

**Animals—**Male Wistar Rats (*Rattus norvegicus*), female rabbits (New Zealand White) and female mice (BALB/c) were obtained from the institute animal facility. All procedures for handling animals were approved by the animal ethics committee of the Jawaharlal Nehru Centre for Advanced Scientific Research (Bangalore, India).

**Plasmid Constructs—**RNA was isolated from rat round spermatids using TRIzol reagent (Invitrogen), and the cDNAs were synthesized using oligo(dT) primers by ThermoScript reverse transcriptase (Invitrogen) following the manufacturer’s instructions. Complementary cDNAs of PRMT4 variants were cloned in p3XFLAG-CMV-10 vector between HindIII and BglII sites. Recombinant TP2 bacterial expression plasmid, which is codon-optimized, was used for TP2 protein expression as described previously (32, 34, 35). This plasmid construct was used as template for introducing mutations. Plasmids expressing

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4 The abbreviations used are: PTM, post-translational modification; WB, Western blot; SRS, sonication-resistant spermatids; PCA, perchloric acid; AUT, acid-urea-Triton; TP, transition protein; rTP2, recombinant TP2; PRMT, protein arginine methyltransferase; K25me1, K39me1, K88me1, K91me1, and R92me1; lysine 25, 39, 88, and 91 and arginine 92 monomethylation, respectively; K33me2 and R92me2, lysine 83 and arginine 92 dimethylation, respectively; H3R17me2a, histone H3 arginine 17 asymmetric dimethylation.
ing mutant PRMT4 or rTP2 were generated by site-directed mutagenesis (Agilent) following the manufacturer’s protocol. Primer sequences used for cloning and mutagenesis are listed in Table 1.

Expression and Purification of TP2 Protein—TP2 plasmid constructs were transformed into *Escherichia coli* Rosetta cells (Novagen). Protein expression was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h at 37 °C shaking. Bacterial cells from 1 liter of culture were pelleted at 3,300 × *g* for 10 min at 4 °C. Bacterial cell pellet was resuspended in 25–30 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 0.57 M NaCl, 10 mM β-mercaptoethanol, 0.2 mM PMSF) and 1 mg/ml chicken egg white lysozyme. After a 30 min incubation on ice with intermittent vortexing, cells were lysed by sonication at a pulse of 5 s on/7 s off, 40% amplitude for 10 min, and centrifuged at 13,400 × *g* at 4 °C for 60 min. Supernatant was centrifuged again for 40 min, followed by another spin of 30 min until the supernatant was clarified. Heparinagarose beads (Sigma) were equilibrated in lysis buffer, and 500 μl of beads were added to the supernatant and kept for 4 h (to overnight) binding in an end-to-end rotor at 4 °C. The supernatant was passed three times through the column at a slow flow rate, allowing beads to settle, followed by a wash with 5 volumes of lysis buffer. Bound proteins were eluted by a step gradient of increasing salt concentration of 0.6, 1, and 1.4 M NaCl in lysis buffer, and 500-μl fractions were collected. The fractions were checked on 15% SDS-polyacrylamide gel for the presence of TP2 protein, and the selected fractions were dialyzed against 1% acetic acid with 10 μM ZnSO4. Diafiltered samples were lyophilized and reconstituted in water.

Antibodies—The following antibodies were used: anti-PRMT4 (P4995, Sigma; WB, 1:500; immunofluorescence, 1:50), anti-KMT7 (ab119400, Abcam; WB, 1:500; immunofluorescence, 1:50), anti-H3 (39163, Active Motif; WB, 1:5,000), anti-H3R17me2a (07-214, Millipore; WB, 1:500), anti-Rme1 (8015, Cell Signaling; WB, 1:1,000), anti-Rme2a (39231, Active Motif; WB, 1:500), anti-Rme2s (07-413, Millipore; WB, 1:500), anti-Kme1/2 (ab23366, Abcam; WB, 1:500), and anti-H4 (05–858, Millipore; WB, 1:10,000). Anti-TP1 and anti-TP2 antibodies were obtained as described previously (36).

Immunization and Purification of Antibodies—TP2 polyclonal antibodies were raised by immunizing female New Zealand White rabbits and BALB/c mice with recombinant TP2 expressed and purified from *E. coli*. Rat TP2/K88me1 (KLH-CRKTLGKme1VSKRKA) and R92me1 (KLH-CEGK-VSKRme1KAVRRR) polyclonal antibodies were raised by injecting synthetic peptides in female BALB/c mice and female New Zealand White rabbits, respectively. Antigens were conjugated to keyhole limpet hemocyanin via the cystine residue. After sufficient boosters, blood was collected from the marginal ear vein of rabbit. The blood was collected from mouse by retro-orbital blood collection method. Blood was kept at room temperature to allow it to clot. Separated serum was transferred to fresh tubes. Antibodies were purified by sequential precipitation by caprylic acid and ammonium sulfate and resuspended in PBS (37).

Affinity Purification of Antibody with Immobilized Peptide—SulfoLink immobilization kit for peptides (Thermo Scientific) was used to affinity-purify the antibody. Briefly, peptide was coupled to the SulfoLink column as per the manufacturer’s protocol. Nonspecific sites were blocked by l-cysteine. The column was washed extensively with PBS. IgG was bound to the peptide-coupled column for 3 h at 4 °C. The column was washed with PBS, and antibody was eluted with 0.1 M glycine pH 2.5 and immediately neutralized by adding one-tenth volume of Tris-HCl, pH 10, and dialyzed against PBS. Affinity-purified antibody was stored in small aliquots at −20 °C.

Affinity Purification of Antibody by Antigen/Protein Immobilization on Membrane—The purified rTP2 protein was resolved by SDS-PAGE and transferred onto nitrocellulose membrane using a semidry transfer apparatus (GE Healthcare). After transfer, membrane was stained with Ponceau S staining solution (0.1% (w/v) Ponceau, 1% (v/v) acetic acid), the protein band was marked, and additional membrane was cut and discarded. Membrane was washed extensively with PBS, and IgG was bound to the membrane for 3 h at 4 °C. Nitrocellulose membrane was washed with PBS, followed by washes with water, and antibody was eluted with 0.1 M glycine, pH 2.5, and immediately neutralized by adding one-tenth volume of Tris-HCl, pH 10, and dialyzed against PBS. Affinity-purified antibody was stored in small aliquots at −20 °C.

Purification of Endogenous TP1 and TP2—Rat testes (6 animals, 60–80 days old) were decapsulated and homogenized in 5 volumes of cell lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl2, 0.1% Nonidet P-40, 1 mM PMSF, 10 mM NaHSO4, 10 mM sodium butyrate, 1 μM TSA, protease inhibitor mixture, phosphatase inhibitor mixture). The homogenate was filtered through four layers of bandage cloth and centrifuged at 1,500 × *g* for 10 min at 4 °C. The pellet of crude nuclei was resuspended in 5 volumes of 0.4 N HCl, kept on ice with occasional vortexing for 30 min, and centrifuged at 10,000 × *g* for 10 min at 4 °C. Acid-soluble proteins were precipitated with a step gradient of 0–3% and 3–30% trichloroacetic acid (TCA) and sequentially washed with ice-cold acetone containing 0.05% HCl and three times with ice-cold acetone. The pellet was dissolved in water. Proteins from the 3–30% TCA fraction were fractionated in two rounds of chromatography on a C18 column (Waters’ XBridge BEH300 Prep C18, 5-μm OBD, 19 × 150 mm) using AKTA purifier 10 (General Electric) by employing a gradient of solvent A (5% acetonitrile, 0.1% TFA) and solvent B (90% acetonitrile, 0.1% TFA). The gradient conditions employed during the first run were as follows: 0–19% solvent B in 15 min, 19–23% solvent B in 30 min, and 23–100% solvent B in 10 min at a flow rate of 2 ml/min. Fractions containing the transition proteins, obtained during 19–23% solvent B separation, were pooled and separated further using the following gradient conditions: 0–18% solvent B in 15 min, 19–21% solvent B in 30 min, and 21–100% solvent B in 10 min at a flow rate of 2 ml/min. The peak corresponding to the transition proteins, TP1 and TP2, appeared at 19.5% solvent B. Fractions were col-
lected and dried to completion in a SpeedVac (Thermo Fisher) and resuspended in water. An aliquot of the protein fractions was checked for its composition in 15% SDS-PAGE and confirmed by Western blotting with anti-TP1 and TP2 antibodies. Fractions containing transition proteins were lyophilized and stored at −20 °C.

**Mass Spectrometry, Data Analysis, and Validation**—Two approaches were employed for the mass spectrometric characterization of the post-translational modifications of endogenous transition proteins, TP1 and TP2. In the first approach, TPs were chemically derivatized with propionic anhydride, before and after proteolytic cleavage by trypsin (38). The lyophilized protein sample obtained after RP-HPLC separation comprising endogenous TP1 and TP2 was reconstituted in 100 mM ammonium bicarbonate, pH 8.0. About 20 μg of protein was propionylated in vitro twice, before and after trypsin digestion, as described previously (38). The tryptic peptides were analyzed by LC-MS/MS (Easy-nLC 1000-Q-Exactive, Thermo) at PTM Biolabs, Co., Ltd. (Chicago, IL). Peptides were loaded on a trapping column (Acclaim PepMap 100, 2 cm long, 75-μm inner diameter, 3-μm resin, Thermo Fisher) and eluted over an analytical column (Acclaim PepMap, RSLC, 15 cm long, 50-μm inner diameter, 2-μm resin, Thermo Fisher) at 300 nl/min. The mass spectrometer was operated in data-dependent mode, with MS and MS/MS performed in the Orbitrap at 70,000 and 17,500 full width at half-maximal resolution, respectively.

In the second approach, endogenous TPs were cleaved by trypsin, chymotrypsin, and elastase independently to generate suitable peptides from the entire length of protein. Endogenous TP1 and TP2 proteins were resolved in 15% SDS-PAGE, and gel bands containing 2 μg of protein each were digested with trypsin, chymotrypsin, and elastase independently. The TP1 and TP2 gel bands were excised and washed with 25 mM ammonium bicarbonate. Reduction and alkylation were performed sequentially with 10 mM dithiothreitol and incubated at 60 °C, followed by treatment with 50 mM iodoacetamide at room temperature. Digestion was performed either with trypsin (sequencing grade, Promega) at 37 °C for 4 h or with chymotrypsin (sequencing grade, Promega) at 37 °C for 12 h or with elastase (Promega) at 37 °C for 12 h, and the reaction was terminated by adding formic acid to a final concentration of 0.1%. The supernatant was used directly for LC-MS/MS analysis. The lyophilized protein sample obtained after RP-HPLC separation comprising endogenous TP1 and TP2 was reconstituted in 100 mM ammonium bicarbonate, pH 8.0. About 20 μg of protein was propionylated in vitro twice, before and after trypsin digestion, as described previously (38). The tryptic peptides were analyzed by LC-MS/MS (Easy-nLC 1000-Q-Exactive, Thermo) at PTM Biolabs, Co., Ltd. (Chicago, IL). Peptides were loaded on a trapping column (Acclaim PepMap 100, 2 cm long, 75-μm inner diameter, 3-μm resin, Thermo Fisher) and eluted over an analytical column (Acclaim PepMap, RSLC, 15 cm long, 50-μm inner diameter, 2-μm resin, Thermo Fisher) at 300 nl/min. The mass spectrometer was operated in data-dependent mode, with MS and MS/MS performed in the Orbitrap at 70,000 and 17,500 full width at half-maximal resolution, respectively.

Mass spectra and fragmentation tables for all of the identified peptides were manually validated to be correct. Manual validation for all the modified peptides was performed by considering the mass error on the parent ion and also by b and y ions of the modified peptides. For modification like phosphorylation, characteristic neutral loss was also considered in addition. The search parameters employed were as follows. Mass error was set as ±10 ppm for peptide mass tolerance and 0.02 Da for fragment mass tolerance. Enzyme was specified as trypsin or none (for chymotrypsin and elastase), allowing up to three missed cleavages for the direct digestion approach and up to six missed cleavages for propionylated samples for tryptic peptides. Carbamidomethyl on Cys was specified as a fixed modification, whereas acetylation on Lys/Ser, phosphorylation on Ser/Thr/Tyr, methylation of Lys (mono-, di-, or trimethylation)/Arg (mono- or dimethylation), crotonylation on Lys, and succinylation on Lys were specified as variable modifications. Propionylation on Lys and peptide N termini were also included when analyzing samples derivatized with propionic anhydride. Mascot DAT files were parsed into the Scaffold software for validation and filtering and to create a non-redundant list per sample. Data were filtered using a minimum protein value of 99.9%, a minimum peptide value of 80% (Prophet scores), and a requirement of at least two unique peptides per protein. Scaffold results were exported as mzIdentML and imported into Scaffold PTM in order to assign site localization probabilities using Ascore (39). All of the spectra generated with acceptable Ascore and localization probability for modified peptides were manually validated to be correct. Mass spectra and fragmentation tables for all of the identified post-translational modifications are given in supplemental Data S1 and S2 for TP1 and TP2, respectively.

**Centrifugal Elution and Flow Cytometric Analysis**—Rat testes were decapsulated and minced, and seminiferous tubules were digested with 10 mg of collagenase in 25 ml of DMEM at 30 °C for 1 h. Cells were filtered through four layers of bandage cloth, and cells were pelleted at 500 × g for 10 min at 4 °C and resuspended in 8–10 ml of running buffer DPBS (PBS, 0.1% glucose, 0.2% BSA). Cells were injected into the elutriation rotor and were separated as described previously (27). The fractions collected were washed with Dulbecco’s PBS and microscopically analyzed after DAPI staining, and an aliquot was fixed in 70% ethanol overnight at −20 °C. The rest of the cells were immediately frozen in liquid nitrogen and stored at −80 °C. The cell pellet of fixed cells was obtained by centrifugation at 500 × g for 10 min at 4 °C. Cells were washed three times with Dulbecco’s PBS and suspended in staining buffer (0.3% Nonidet P-40, 50 μg/ml RNase A, 25 μg/ml ethidium bromide). Cells were incubated at 37 °C for 1 h, staining was allowed to continue at 4 °C for at least 2–3 h, and then purity was monitored by flow cytometry.
RNA Purification and Quantitative Real-time PCR Assays—Total RNA for cDNA synthesis was obtained from 10-day-old rat testes for gametic diploid cells and from elutriated tetraploid (pachytene spermatocytes) and haploid cells (round spermatids) by the TR1zol method (Invitrogen) according to the manufacturer’s instructions. RNA was subjected to DNase I treatment (New England Biolabs) to remove any contaminating genomic DNA. Complementary DNAs were synthesized by SuperScript reverse transcriptase (Invitrogen). Real-time PCR analysis was performed with three biological replicates using EvaGreen dye (Qarta) in a Corbett Rotor-gene 6000 machine. Melt curve analysis was done to ensure specific amplification. Difference in values of Ct (threshold cycle) were used to calculate the fold difference in expression of mRNA in gametic diploid, tetraploid, and haploid cells by the ΔΔCt method (2−ΔΔCt) of analysis. Nascent polypeptide-associated complex (pachytene spermatocytes) and haploid cells (round spermatids) by the TRIzol method (Invitrogen) according to the manufacturer’s instructions. RNA was subjected to DNase I treatment (New England Biolabs) to remove any contaminating genomic DNA. Complementary DNAs were synthesized by SuperScript reverse transcriptase (Invitrogen). Real-time PCR analysis was performed with three biological replicates using EvaGreen dye (Qarta) in a Corbett Rotor-gene 6000 machine. Melt curve analysis was done to ensure specific amplification. Difference in values of Ct (threshold cycle) were used to calculate the fold difference in expression of mRNA in gametic diploid, tetraploid, and haploid cells by the ΔΔCt method (2−ΔΔCt) of analysis. Nascent polypeptide-associated complex α was used as an internal normalization control, because its expression level across different spermatogenic cells was found to be unchanged in microarray experiments (27). Primer sequences used for quantitative and semiquantitative PCR are listed in Table 1.

Isolation of Sonication-resistant Spermatid (SRS) Nuclei—Rat testes were homogenized in 6–8 volumes of buffer A (10 mM Tris–HCl, pH 7.4, 10 mM sodium meta-bisulfit, 0.1 mM PMSF, 0.34 M sucrose, 0.1% Triton X-100). The homogenate was filtered through four layers of bandage cloth and centrifuged at 4,000 × g for 10 min at 4 °C. The pellet of crude nuclei was suspended in 3–5 volumes of buffer B (10 mM Tris–HCl, pH 7.4, 10 mM sodium meta-bisulfit, 0.1 mM PMSF) and subjected to sonication at a pulse of 10 s on/10 s off, 40% amplitude for 15 min. Sonicate was centrifuged at 10,000 × g for 10 min. The pellet was resuspended in 3–5 volumes of buffer B, and the suspension was layered over a 10 ml cushion of 1.5 M sucrose in 10 mM DTT, 0.2% Triton X-100, and protease inhibitor mixture) and centrifuged at 16,000 × g for 10 min at 4 °C to obtain chromatin proteins. The extracted proteins were precipitated by 30% TCA at 4 °C. After they were kept on ice for 30 min, the proteins were recovered by centrifugation at 12,000 × g for 10 min. The pellet of proteins thus obtained was sequentially washed with ice-cold acetone containing 0.05% HCl and three times with ice-cold acetone. The pellet recovered was dried, dissolved in water, and stored in aliquots at −20 °C.

In Vitro Methylation Reaction—Proteins were incubated in 1X HMT buffer (as recommended by the manufacturer), 1 μl (1.1 μCi) of S-adenosyl-L-[methyl-3H]methionine, and methyltransferase enzyme (PRMT1, M0234, New England Biolabs; PRMT4, 14-575, Millipore; PRMT4 and PRMT4-E267Q, 31347, and 31348, Active Motif; PRMT5, SRP0145, Sigma; KMT7, 14-469, Millipore) in a 30-μl reaction volume and incubated at 30 °C for 30 min. To visualize the radiolabeled proteins, the reaction products were precipitated with 25% trichloroacetic acid, sequentially washed with ice-cold acetone containing 0.05% HCl and three times with ice-cold acetone, resuspended in water, resolved electrophoretically on 15% SDS-PAGE, and subjected to fluorography using a solution containing 22.5% 2,5-diphenyloxazole in DMSO. Gels were dried, and autoradiography was performed at −80 °C for 2–3 days.

V8 Protease Digestion of TP2 and Autoradiography—In vitro methylated TP2 (3 μg) was digested with V8 protease (from Staphylococcus aureus; Sigma) in 50 mM ammonium bicarbonate, pH 7.8, at 37 °C for 8 h at an enzyme/protein ratio of 1:50, and the products were separated on 15% SDS-polyacrylamide gel. The gel was exposed for autoradiography as described above.

Western Blotting—The protein samples were resolved by SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes using standard procedures. After transfer, the membrane was blocked using 5% skimmed milk powder in wash buffer PBST (1× PBS with 0.1% Tween 20) for 1 h at room temperature. The membrane was then incubated with primary antibody for 2 h or overnight at 4 °C on a rocking platform. The unbound antibody was removed by washing the membrane with wash buffer and probed with an appropriate HRP-conjugated secondary antibody in PBST with 1% skimmed milk powder and incubated for 1 h. The membrane was then washed extensively and developed using the chemiluminescence kit (Thermo Scientific).

Dot Blot Analysis—Peptides, 2 μg each (backbone peptide, TP2K88 (CPKNRKTLEGVSRKAVRRR) and TP2R92 (CEGKVSRRKVARR)) and methylated peptide, TP2K88me1 (CPKNRKTLEGKme1VSRKAVRRR) and TP2R92me1 (CEGKVSRRKme1KAVRRR), were spotted onto nitrocellulose membrane, and once the membrane was dry, it was blocked with PBST containing 5% skimmed milk powder for 1 h. After
blocking, the membrane was incubated with primary antibody (prepared in blocking buffer) for 1 h. The blot was washed three times with PBST, and it was incubated with secondary antibody (prepared in blocking buffer) for 1 h. After incubation, the blot was washed three times with PBST and once with PBS, and then ECL substrate was added, and autoradiography was

### TABLE 1

| Gene     | Accession ID | Primer sequence (5’-3’) | Length (bp) | Purpose          |
|----------|--------------|--------------------------|-------------|------------------|
| PRMT1    | NM_024362.1  | FP ACACGTGCTGCAAGCTCG    | 199         | Real time PCR    |
|          |              | RP TCCACCAGTCCACGGGG     |             |                  |
| PRMT3    | NM_053557.1  | FP TGGCCGAGTGAGTCGCTGAC  | 253         | Real time PCR    |
|          |              | RP GCAACTGCGGTGCACATGGC  |             |                  |
| PRMT4    | V1-AB201114.1| FP GCCAGCACCATGCTGACCA   | 211         | Real time PCR    |
| V2-AB201115.1|             | RP TGTTGCGCTGGCTAGGG     |             |                  |
| V3-AB201116.1|             |                           |             |                  |
| V4-AB201117.1|             |                           |             |                  |
| V5-KJ672502|             |                           |             |                  |
| PRMT4 v2 | AB201115.1   | FP TGGCAACGCTCAAGCTCAC   | 193         | Real time PCR    |
|          |              | RP ATGGACATGGGAGGCAATAGA |             |                  |
| PRMT4 v3 | AB201116.1   | FP AGCAGTGTATCTGCTGGCCTC | 126         | Real time PCR    |
|          |              | RP CACTGTGTTAGCTGGCCACTGG|             |                  |
| PRMT5    | NM_00108867.2| FP AGTCGAGAGTCTGCTGAGCA  | 132         | Real time PCR    |
|          |              | RP CCGCGTGAGGAGACAGGACAT|             |                  |
| PRMT6    | NM_00106466.1| FP GGTGAGACTGCGGAGAGAAG  | 161         | Real time PCR    |
|          |              | RP TGGCGGAGGAGAGGACATCA  |             |                  |
| PRMT7    | NM_00104153.1| FP CTCGGACGCTGCCATAGCA   | 173         | Real time PCR    |
|          |              | RP GATTCAGTTGCTCCGCAAC   |             |                  |
| PRMT10   | NM_001191599.1| FP AAGGCGATGCGCTGGCTG   | 152         | Real time PCR    |
|          |              | RP GCAGCCACGACGCTACAGC   |             |                  |
| FBXO10   | XM_00107167.4| FP TTGCCGCCGCAAGATGGGAAG| 153         | Real time PCR    |
|          |              | RP CGATCAAGCTCCCGGCTC    |             |                  |
| FBXO11   | NM_181631.2  | FP TGACGACACTGGAAGAGTGGC | 155         | Real time PCR    |
|          |              | RP GGTGCAGCTTGTGTAGTGG   |             |                  |
| NACA     | NM_001198562.1| FP ATGGTCAACATCGGTTCCTC | 352         | Real time PCR    |
|          |              | RP GGACAGCTTTGCTGTCAG    |             |                  |
| PRMT4    | V1-AB201114.1| FP GCGGGCTCCGAGGCTGAC    | 211         | Semi-quantitative PCR |
| V2-AB201115.1|             | RP V1, 2, 4 & 5          |             |                  |
| V3-AB201116.1|             | GGTCCGGGATGGACATGGCG     |             |                  |
| V4-AB201117.1|             | V1-224 V2-355 V3-139 V4-155 V5-245 | |                  |
| V5-KJ672502|             |                           |             |                  |
| KMT7 Set I | NM_001109558.1| FP GACGGATTACACACGAGGTT | 208         | Semi-quantitative PCR |
|          |              | RP CCACATAGGCTTGCAGA     |             |                  |
| KMT7 Set II | NM_001109558.1| FP CTGCAAGGCACCTATGGGA  | 227         | Semi-quantitative PCR |
|          |              | RP CCATAGAGGCGAGTCTCAG   |             |                  |
| KMT7 Set III | RM_001109558.1 | FP | CAGAGGACTGCAGCTCTATGG | 223 | Semi-quantitative PCR |
| PRMT4 | V1-AB201114.1 | FP | GATGACAAGCTTTAAGCCAGCGGCAGCCG | | |
| | V3-AB201116.1 | FP | GAATTCCAGATTCACAATCCACTCATA | | Cloning |
| | V5-AB20117.1 | FP | GATGACAGATTCACATGACAGCCAGCCG | | |
| | V5-KJ672502 | FP | GATGACAAGCTTTAAGCCAGCGGCAGCCG | | |
| PRMT4 (1-490) | V1-AB201114.1 | RP | GAATTCAAGATCTCTTGTATGAGAAGAACCAGGC | 1470 | Cloning |
| PRMT4-E267Q | V1-AB201114.1 | RP | GGTGACGGTCTTACAGGTCTTGTGTT | Not applicable | |
| rTP2 R20K | NM_017057.2* | FP | CAGAGGACTGCAGCTCTATGG | | |
| rTP2 R36K | NM_017057.2* | FP | GCCTGAGCCCAACCTGCAAACAG | Not applicable | |
| rTP2 R66K | NM_017057.2* | FP | CCCC AACACCCATCGACTCTAAA | | |
| | | RP | GCTGAGGAGTGGAAGGAGTATTT | | |
| rTP2 R71K | NM_017057.2* | FP | GCCTGAGCCCAACCTGCAAACAG | Not applicable | |
| | | RP | GCTGAGGAGTGGAAGGAGTATTT | | |
| rTP2 R75K | NM_017057.2* | FP | GGCTGAGCCCAACCTGCAAACAG | Not applicable | |
| | | RP | GCCTGAGGAGTGGAAGGAGTATTT | | |
| rTP2 R82K | NM_017057.2* | FP | CAGAGGACTGCAGCTCTATGG | | |
| | | RP | GCTGAGGAGTGGAAGGAGTATTT | | |
| rTP2 R92K | NM_017057.2* | FP | GCCTGAGGAGTGGAAGGAGTATTT | | |
| rTP2 R71K, R75K | NM_017057.2* | FP | GCCTGAGGAGTGGAAGGAGTATTT | | |
| rTP2 K88R | NM_017057.2* | FP | GAGCTGAGGAGTGGAAGGAGTATTT | | |
| rTP2 K91R | NM_017057.2* | FP | GAGCTGAGGAGTGGAAGGAGTATTT | | |
| | | RP | GCCTGAGGAGTGGAAGGAGTATTT | | |
performed. All of the synthetic peptides were purchased from Storkbio Ltd.

Peptide Competition Assay—A peptide competition assay was performed to block the antibody epitopes by preincubating with ~50-fold molar excess of peptide used for immunization. TP2K88me1 and R92me1 methylation-specific antibody was blocked independently with methylated peptide TP2K88me1 (CPKNRTLEGKme1VSKRKAVR) and TP2R92me1 (CEGKVSKRme1KAVRRR), respectively, for 3 h. After blocking the blot with 5% skimmed milk powder, peptide-blocked antibodies were allowed to bind with the blotted protein for 1 h at room temperature. The rest of the steps are the same as for the Western blot protocol described above.

AUT-PAGE—Endogenous TP1 and TP2 protein (10 μg each) were resolved in AUT-PAGE and transferred to nitrocellulose membrane, as described previously (41), and subsequently probed with anti-TP1 and anti-TP2 antibodies.

Decondensation and Immunofluorescence of Testicular Cells—Total testicular cells were decondensed by resuspending in decondensation buffer (PBS, 10 mM dithiothreitol) and incubated at 4 °C for 1 h in an end to end rotator (36). Cells were obtained after centrifuging at 1,500 × g for 10 min at 4 °C and were washed twice with ice-cold PBS. Total testicular cell smears were fixed with 4% paraformaldehyde (in PBS) and permeabilized with 0.1% Triton X-100 (in PBS) for 15 min. 1% BSA in PBS was used for blocking nonspecific sites. Smears were incubated with primary antibodies (anti-PRMRT4, anti-KMT7, anti-TP2, anti-TP2K88me1, and anti-TP2R92me1), counterstained with corresponding secondary antibodies conjugated with Alexa Fluor 488 and/or Alexa Fluor 568. Nuclei were stained with DAPI (Sigma). Colocalization experiments were carried out by simultaneous incubation of smears with two different primary antibodies raised in different organisms. Images were acquired by a Zeiss confocal laser scanning microscope (LSM 510 META, Carl Zeiss). Analysis for colocalization was done using LSM browser software, which creates a scatter plot and gives the number of colocalized and individual pixels. A cut-mask image shows only the region containing colocalized pixels.

Phylogenetic Analysis—To determine the sequence divergence of transition proteins TP1 and TP2 among mammals, sequences of eight mammals were retrieved from UniProt (TP1: rat (P02317), mouse (P10856), human (P09430), chimpanzee (F7VJM6), rhesus macaque (F7VL8), cow (P17305), pig (P17306); TP2: rat (P11101), mouse (P11378), human (Q05952), chimpanzee (B3LF34), rhesus macaque (Q9N1A3), dog (O77645), cow (P26377), pig (P29258)). Sequence alignment was carried out using MUSCLE software, and the phylogenetic tree was constructed by the neighbor-joining method using MEGA software version 6. Bootstrap resampling and reconstruction was done 1,000 times to confirm reliability of the phylogenetic tree analysis. The evolutionary distances were computed using the JTT matrix-based method and are in units of the number of amino acid substitutions/site. All positions with less than 95% site coverage were eliminated (i.e. fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position) (42).

RESULTS

Purification of Endogenous TP1 and TP2 from Rat Testis—Rat testicular nuclei were extracted with 0.4 N HCl, and acid-soluble proteins were precipitated sequentially using a step gradient of 0–3% and 3–30% TCA. Transition proteins, TP1 and TP2, were precipitated in the 3–30% TCA fraction (Fig. 1A). Proteins present in the 3–30% TCA fraction were loaded on a C18 column and separated by RP-HPLC. In the first chromatographic run, transition proteins were partially separated from the bulk of the other basic proteins (Fig. 1B). Fractions containing TPs as monitored by Western blot analysis were pooled (Fig. 1C) and then fractioned using a shallower gradient of solvent B in the second run (Fig. 1D). Fractions containing transition proteins, TP1 and TP2, were pooled and checked for purity in SDS-PAGE, and their identity was confirmed by Western blotting using anti-TP1 and anti-TP2 antibodies (Fig. 1E). In order to examine the presence of differentially modified forms, endogenous purified transition proteins were resolved in AUT-PAGE, because it can separate variants and post-translationally modified forms of basic proteins (41). Multiple bands were observed for TP2 (13 kDa), which also reacted with anti-TP2 antibodies, indicating the presence of multiple covalently modified forms of endogenous TP2. However, TP1, due to its small size (6.4 kDa), migrated along with the dye front as a sharp band even in the AUT-PAGE (Fig. 1F).

Mass Spectrometry Analysis of Endogenous TP1 and TP2—Transition proteins are highly basic proteins, with lysine and arginine constituting about 38 and 24% of the rat TP1 and TP2 protein, respectively. This means that regions with frequent lysine and arginine residues after tryptic digestion will result in very short peptides, which are technically unsuitable for characterization by mass spectrometry. To circumvent this problem, two independent approaches were employed. In the first approach, TPs were chemically derivatized with propionyl anhydride before and after proteolytic cleavage by trypsin. Propionic anhydride derivatization adds propionyl group at the peptide N termini, unmodified and monomethylated lysine thus neutralizing their charge, making peptides less hydrophilic and suitable for separation by RP-HPLC (38). In the second approach, endogenous TPs were cleaved by trypsin, chymotrypsin, and elastase independently, to generate suitable peptides from the entire length of protein.

Sequence coverage of 91% was obtained for TP1, and 16 novel modifications on 15 different amino acid residues were identified. These include serine phosphorylation at Ser36, Ser37, and Ser48; lysine methylation at Lys25 and Lys39; lysine acetylation at Lys6, Lys22, Lys35, Lys37, Lys39, and Lys42; and arginine methylation at Arg3, Arg13, Arg18, Arg43, and Arg44. In addition, K32ac was observed only in association with R34me (Fig. 2A). Spectral information of identified post-translational modifications of TP1 is listed in Table 2 (see supplemental Data S1 for MS plots of TP1 PTMs and the corresponding fragmentation table).

For TP2, a sequence coverage of 84% was achieved, covering most of the N-terminal two-thirds of the protein. A small stretch of C-terminal sequence of TP2 (residues 97–114) was
not covered in the mass spectrometry analysis due to the presence of many arginine residues within this sequence. For endogenous TP2, 19 novel modifications on 15 different amino acid residues were identified. These include serine phosphorylation at Ser17, Ser23, Ser51, Ser68, Ser70, and Ser90; serine acetylation at Ser7 and Ser37; threonine phosphorylation at Thr84; lysine methylation at Lys83, Lys88, and Lys91; lysine acetylation at Lys4, Lys57, Lys83, Lys88, and Lys91; and arginine methylation at Arg92 (Fig. 2B). Earlier in vitro studies have shown that acetylation and phosphorylation of TP2 occur in its C terminus, which reduces its DNA condensation property (25, 30, 32). In the C terminus (residues 87–114) of endogenous TP2, seven PTMs on 4 residues were identified, including the sites for acetylation and phosphorylation. This provides further support for the earlier in vitro studies (30–32). However, the region harboring PKA phosphorylation sites, Thr101 and Ser109, identified by in vitro biochemical and mutational analysis (30), was not covered in the present mass spectrometry analysis. Spectral information of identified post-translational modifications of TP2 is listed in Table 3 (see supplemental Data S2 for MS plots of TP2 PTMs and the corresponding fragmentation table).

Sequence Divergence of TP1 and TP2 and Conservation of PTM Sites in Mammals—To determine the extent of sequence divergence of the two major transition proteins, TP1 and TP2, during evolution, phylogenetic analysis was carried out using TP1 and TP2 sequences of eight mammals belonging to four
animal orders. This includes rat and mouse from rodentia; human, rhesus macaque, and chimpanzee from primates; pig and cow from cetartiodactyla; and dog from carnivora (43). Among the two major transition proteins, TP2 sequence has diverged more than TP1 across mammalian species, as indicated by the differences in their branch length (Fig. 3, A and B). This is indicative of different evolutionary constraints on TP1 and TP2, and possibly the two major transition proteins fulfill distinct roles in mammalian spermiogenesis. For better appreciation of the post-translational modifications of transition proteins, TP1 and TP2, sequence conservation of the modified residues across selected mammals was analyzed. Most of the post-translationally modified amino acid residues of TP1 are conserved across the analyzed mammals except for the phosphorylation sites Ser36, Ser37, and Ser48 (Fig. 3 C). Among the 15
Post-translationally modified amino acid residues of TP2, only 4 residues are conserved. Many of the phosphorylation and arginine methylation sites are present only in rodents (Fig. 3D). This presumably indicates that some of these modifications present on the non-conserved amino acid residues are lineage- and/or organism-specific. In the subsequent work, we focused on the novel arginine and lysine methylation of the two major transition proteins.

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**Post-translational Modification Repertoire of TP1 and TP2**

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**A**

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**B**

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**C**

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**D**

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Arginine Methylation of Transition Proteins TP1 and TP2—

Arginine methylation is mediated by protein arginine methyltransferases (PRMTs). Among these, type I PRMTs (PRMT1, -2, -3, -4, -6, and -8) and type II PRMTs (PRMT5 and -7) catalyze the reaction of the formation of asymmetric dimethylarginines and symmetric dimethylarginines, respectively, through a monomethylarginine intermediate on the terminal nitrogen (ω) of the guanidino group of arginine. PRMT7 generates only monomethylarginine for some substrates, referred to as type III activity. In yeast, type IV activity has been described where internal nitrogen (δ) of the guanidino group is methylated. Besides these, there are additional putative PRMTs, like PRMT9 (FBXO11), PRMT10, and FBXO10, that are not well characterized (44–46). The role of arginine methylation of nuclear basic proteins in mammalian spermatogenesis has not been studied in detail except that PRMT5 has been shown to methylate PIW1 proteins during spermiogenesis (47).

To confirm the observed arginine methylation of the transition proteins TP1 and TP2 in mass spectrometry analysis, endogenously purified proteins were probed with anti-methylarginine antibodies. Both of the transition proteins TP1 and TP2 showed the presence of monomethylarginine and asymmetric dimethylarginine. However, no signal was observed for symmetric dimethylarginine (Fig. 4A). In order to identify PRMT(s) responsible for the methylation of transition proteins during spermiogenesis, the expression pattern of rat PRMTs was checked by quantitative PCR in the haploid spermatid (round spermatid) stage, where transcripts for transition proteins are made. PRMT1, PRMT4, and PRMT5 showed higher transcript levels compared with other PRMTs (Fig. 4B). The expression of these selected PRMTs was further checked in gametic diploid spermatogonial cells, tetraploid pachytene spermatocytes, and haploid round spermatids, and they all showed elevation in expression at progressive stages of differentiation. PRMT4 showed a much higher level of expression uniquely in the round spermatids (Fig. 4C). Four alternative splice variants of PRMT4, v1, v2, v3, and v4, have been reported in rats. They share a common N terminus, which encompasses the methyltransferase domain, and differences among the variants are limited to the C terminus (33). Expression levels of PRMT4 v2 and v3 were analyzed by quantitative PCR using primers for unique stretches of amino acid sequences present in their C termini. The results indicate that v2 and v3 transcripts do not account for the observed up-regulation of PRMT4 in round spermatids; therefore, these PRMT4 transcripts correspond to other splice variants (Fig. 4C).

To investigate whether PRMT1, PRMT4, and PRMT5 can methylate the transition proteins TP1 and TP2 and/or other spermatid protein(s), an in vitro methylation reaction of acid-soluble spermatid proteins was performed. Acid-soluble proteins were extracted using 0.4 N HCl and 10% PCA (perchloric acid) from the SRS nuclei, which comprise elongating and condensing spermatids. All three tested PRMTs methylated several basic proteins from SRS nuclei, including the proteins at the position of transition proteins and histones (Fig. 4D). This is indicative of an important role of arginine methylation in mammalian spermiogenesis. We observed differences in the methylation pattern between the in vitro methylation reaction of acid-soluble proteins extracted by 0.4 N HCl and 10% PCA at the position of TP1 and TP2 protein, and this could be due to inaccessibility of sites that may be masked by association with other proteins present in the extract (Fig. 4D). To confirm the methylation of TP1, an in vitro methylation assay was performed using endogenous TP1 and TP2 from rat testis as substrates. TP1 and TP2 appeared to be potential substrate for PRMT1 and PRMT4, respectively, whereas a faint signal was observed at the position of TP1 with PRMT4 and at the position of TP2 with PRMT5 (Fig. 4E). Because we had a bacterial expression construct of codon-optimized recombinant TP2 (34, 35), an in vitro methylation reaction of rTP2 was performed. PRMT4 methylated rTP2, whereas PRMT1 and PRMT5 showed no activity for rTP2 in the tested buffer conditions (Fig. 4F). To further establish that TP2 is a substrate for PRMT4, an in vitro methylation reaction was performed using an active site mutant of PRMT4 harboring the E267Q mutation, which abolishes its methyltransferase activity (48). As expected, PRMT4-E267Q could not methylate both histone H3 (positive control) and rTP2 (Fig. 4G). In our subsequent study, we focused on TP2 methylation by PRMT4 because its detailed characterization and identification of the modification site(s) can be better appreciated in the context of the known functional domains of TP2.

PRMT4 Splice Variants with the Methyltransferase Domain Can Methylate TP2—The appearance and localization pattern of PRMT4 protein was examined at different steps of spermiogenesis by immunofluorescence. PRMT4 is abundantly present in transcriptionally active round spermatids, reflecting its presumptive role in the transcription process. PRMT4 is present at reduced levels in elongating spermatids and is present in all of the steps where TP2 is expressed (Fig. 5A). The presence of PRMT4 in sonication-resistant spermatids was confirmed by Western blot analysis using SRS nuclear lysate. Multiple bands were observed in Western blot, which could be either due to the presence of alternatively spliced variants or due to post-translational modifications of PRMT4 (Fig. 5B). Notably, PRMT4 is an established transcriptional co-activator and catalyzes H3R17me2a modification (49). H3R17me2a modification was indeed observed in histone H3 extracted from both round spermatids and TP2.
matids and SRS nuclei, demonstrating that PRMT4 is enzymatically active in spermatids (Fig. 5C). H3R17me2a observed in elongating spermatids is puzzling, and it probably serves some transcription-independent function, because step 11 onward spermatids are transcriptionally inert. Notably, H3R17me2a has not been identified on the retained nucleosomes in the sperm (17).

Because multiple bands were observed for PRMT4 in the Western blot analysis of SRS nuclear lysate (Fig. 5B), it was pertinent to check all PRMT4 variants for their ability to methylate TP2 in vitro. All of the four known splice variants of PRMT4 were cloned using round spermatid cDNA of rats, and in addition, a novel PRMT4 v5 was identified, and its sequence variation is depicted in Fig. 5D. A graphical representation of the exon-intron structure of the C terminus of rat PRMT4 and its isoforms is depicted in Fig. 5E. All of the PRMT4 variants (v1–v5) have an intact methyltransferase domain in their N termini, with arrows indicating differences among the variants. Earlier, it was observed that PRMT4 transcripts in round spermatids belong to a variant other than v2 and v3 (Fig. 4C). To identify the most abundant variant, the primer sets indicated in Fig. 5E were used to amplify PRMT4 isoforms by semi-quantitative PCR. A blue-green primer set generates different size amplicons for v1, v2, v4, and v5, whereas a blue-yellow primer set amplifies only v3. Expected variant amplicon sizes were demonstrated by using cloned PRMT4 variants as template. PRMT4 v1 was found to be the most abundant transcript in round spermatids, followed by much reduced levels of v3, whereas v2, v4, and v5 were barely detectable (Fig. 5F).

In the present study, commercially available recombinant mouse PRMT4 enzyme (catalog no. 14-575, Millipore), which has the same sequence as the v1 of rat PRMT4, was used for in vitro methylation of TP2 (Fig. 4F). In order to test the potential of other PRMT4 variants to methylate TP2, their mammalian expression constructs with N-terminal FLAG tag were transfected in HEK293T cells. The expressed PRMT4 variants were captured on M2-agarose beads and used as an enzyme source.
FIGURE 5. All PRMT4 variants can methylate TP2. A, localization of TP2 and PRMT4 in different steps of spermiogenesis (early spermatids to late spermatids represented from top to bottom). Scale bar, 5 μm. B, Western blot analysis showing the presence of PRMT4 in SRS nuclear lysate. C, Western blot analysis of H3R17me2a (catalyzed by PRMT4) in acid-soluble proteins extracted by 0.4N HCl from round spermatids (RS) and SRS nuclei. Acid-soluble proteins from round spermatids have a small quantity of TP2, which begins to express in step 9, whereas TP1 is present only in SRS nuclei acid-soluble proteins and begins to express in step 10 of rat spermiogenesis. Western blot for histone H3 and H4 was used for assessing protein loading. D, small stretch of rat chromosome 8 in the rn5 rat genome database showing genomic sequence from the C terminus of the PRMT4 gene, highlighting the difference between variants 1 and 5 (intron is depicted in black, and exon is depicted in blue). Additional exonic sequence in PRMT4 variant 5 compared with variant 1 located in the rat sequence is underlined with black. E, graphical representation of the rat PRMT4 gene organization, highlighting the C terminus of PRMT4 isoforms generated by alternative splicing. Blue bars, exons; gray bars, introns. Black vertical bars, possible splice site; orange vertical bars, stop codon. Black arrowheads, positions of splice sites, indicating the differences in PRMT4 variants. A common set of primers (blue and green arrows) can amplify variants 1, 2, 4, and 5, generating different amplicon sizes. Another set (blue and yellow arrows) is for amplifying variant 3. The PRMT4 N terminus (residues 1–490) has an intact methyltransferase domain; the position of residue 490 is indicated. F, semiquantitative PCR to determine the presence of PRMT4 alternative splice variants in round spermatids. Cloned PRMT4 variants were amplified by primer sets mentioned above to demonstrate the expected amplicons. The amplicon size for different variants is indicated. PRMT4 v1 (more abundant) and v3 are present in round spermatids. G, in vitro methylation of histone H3 and TP2 using PRMT4 variants v2, v3, v4, and v5 and PRMT4-E267Q expressed in HEK293T cells. H, in vitro methylation of histone H3 and TP2 using the PRMT4(1–490) fragment comprising residues 1–490 with an intact methyltransferase domain.
All PRMT4 variants methylated histone H3 and TP2. The active site mutant of PRMT4-E267Q (negative control) did not methylate histone H3 and TP2 (Fig. 5G). The first 490 residues of PRMT4 comprising precore, core, and C-extension sequence from the postcore region account for the intact methyltransferase domain and are essential for its catalytic activity (50). PRMT4(1–490) methylated both histone H3 and TP2 (Fig. 5H).

The results clearly show that all PRMT4 variants containing the intact methyltransferase domain could methylate both histone H3 and TP2, despite the differences in their C-terminal sequences.

PRMT4 Methylates TP2 at Arg71, Arg75, and Arg92—Mass spectrometry analysis of endogenous protein in mass spectrometry analysis. B. V8 protease digestion of in vitro methylated TP2 by PRMT4, which cleaves TP2 to generate amino acids 1–86 (N terminus) and amino acids 87–114 (C terminus). The N terminus of TP2 is predominantly methylated, whereas the C terminus also showed the presence of a modification site. C. in vitro methylation of TP2 R92K mutant, indicating that Arg92 is the site for PRMT4 modification in the C terminus of TP2. D. in vitro methylation of single site arginine to lysine mutant proteins, Arg71 and Arg75. They are the major sites for PRMT4 methylation in vitro. G. in vitro methylation of TP2 R71K and R75K mutant proteins by PRMT4(1–490), consisting of a catalytically efficient methyltransferase domain.

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ylation levels by more than 90% (Fig. 6E). TP2 Arg71 and Arg75 were reconfirmed as the in vitro sites of PRMT4 methylation in the N terminus of TP2 (Fig. 6F). In Fig. 5, G and H, it was observed that any variant with an intact methyltransferase domain can methylate TP2. We used PRMT4(1–490) with an intact methyltransferase domain to methylate TP2 with the R71K/R75K mutation. These mutations in the TP2 sequence abolished methylation, further confirming them as the major sites for methylation in vitro (Fig. 6G). The N-terminal region of TP2 was covered in the mass spectrometry analysis but did not reveal the presence of methylation at Arg71 and Arg75. This suggests that it could be of low abundance or may not have been detected in mass spectrometry due to some technical reasons. Alternatively, PRMT4 preferentially methylates at Arg71 and Arg75 in vivo, although it can methylate Arg71 and Arg75 in vitro, suggesting that additional factors might regulate site accessibility for methylation in vivo.

Lysine Methylation of TP2 by KMT7—Lysine methylation is catalyzed by lysine methyltransferases, which are classified into eight distinct subfamilies based on the presence of protein domains like the SET domain or sharing of homologous sequences. They transfer one, two, or three methyl groups from S-adenosyl-L-methionine to the e-amino group of lysine to generate mono-, di-, or trimethyllysine in histones and various non-histone proteins (51, 52). Mass spectrometry analysis of endogenous transition proteins revealed TP1 to be monomethylated at Lys25 and Lys39 and TP2 to be dimethylated at Lys83 and monomethylated at Lys88 and Lys91 (Fig. 7A). Lysine methylation of endogenous transition proteins was reconfirmed by probing purified endogenous protein with anti-mono- and dimethyllysine antibodies (Fig. 7B). Among lysine methyltransferases, KMT7 is known to methylate many non-histone proteins (52). Based on the site of methylation in known substrates and peptide array, KMT7 appears to have a broad recognition sequence motif (53, 54). We tested the possibility of KMT7-mediated methylation of spermatid proteins. As observed previously with arginine methyltransferases, KMT7 also methylated many basic proteins extracted by 0.4 N HCl and 10% PCA from sonication-resistant spermatid nuclei, suggesting the importance of protein methylation in mammalian spermiogenesis (Fig. 7C). Further, both endogenous TP1 and TP2 are methylated by KMT7 in vitro, with TP2 being a better substrate (Fig. 7D). Surprisingly, a double band was observed at the position of TP2; the upper band could be a modified form of TP2, which could be a better substrate for KMT7, or some minor impurity migrating at that position being methylated by KMT7. KMT7-

FIGURE 7. Lysine methyltransferase KMT7 methylates TP2. A, MS plots depicting lysine methylation sites on endogenous TP1 and TP2 identified by mass spectrometry analysis (viz. K25me1 and K39me1 for TP1 and K83me2, K88me1, and K91me1 for TP2). B, Western blot analysis of endogenous transition proteins, TP1 and TP2, with antibodies against mono- and dimethyllysine. C, in vitro methylation of 0.4 n HCl (lane 1) and 10% PCA (lane 2) extract of rat SRS nuclear proteins by KMT7. KMT7 methylated several acid-soluble proteins, including at the position of TP2, as indicated. D, in vitro methylation of purified endogenous transition proteins, TP1 and TP2, and histone H3 (positive control) by KMT7. E, in vitro methylation of recombinant TP2 and histone H3 by KMT7.
mediated methylation of TP2 was further confirmed by performing an in vitro methylation reaction using recombinant TP2 as the substrate (Fig. 7E). Histone H3, which is a known substrate for KMT7, served as a positive control.

KMT7 Methylates TP2 at Lys88 and Lys91—Further experiments were directed to establish the presence of KMT7 in the haploid spermatids. Primers were designed that amplified different regions of KMT7, and this confirmed the presence of KMT7 transcripts in round spermatids (Fig. 8A). Indirect immunofluorescence was also performed to look for protein expression in elongating/condensing spermatids. KMT7 was found to be present in all of the steps where TP2 is expressed, with most of the KMT7 being localized in the cytoplasm of the spermatid nuclei, and relatively fewer KMT7 pixels were observed within the spermatid nuclei (Fig. 8B). To confirm that KMT7 is indeed present in the elongating and condensing spermatid nuclei, Western blot analysis was performed, which established the presence of KMT7 in the SRS nuclear lysate (Fig. 8C).

Mass spectrometry data of endogenous TP2 revealed lysine methylation at the Lys83, Lys88, and Lys91 amino acid residues. In vitro methylation of rTP2 by KMT7 was performed and then subjected to V8 protease digestion. An autoradiogram showed that only the C terminus of TP2 is methylated by KMT7 (Fig. 8D). This indicates that Lys88 and Lys91 could be the possible sites for KMT7 because Lys83 resides in the N-terminal fragment generated after V8 protease cleavage. The Lys88 and Lys91 residues present in the C terminus of rat TP2 were mutated to arginine and subjected to in vitro methylation by KMT7. An in vitro methylation assay revealed Lys88 as the major site of methylation because it resulted in more than 80% loss of modification, with Lys91 being a minor site (Fig. 8E). It is quite possible that K83me2 could be a potential substrate for some other lysine methyltransferase; however, this has not been addressed in the present study.

TP2K88me1 and TP2R92me1 Are Present in Elongating to Condensing Spermatids—To obtain insights into the role of TP2 methylation, modification-specific antibodies were raised against K88me1 and R92me1 peptides. TP2K88me1 and TP2R92me1 were identified in the mass spectrometry analysis of endogenous protein and established as the methylation site for KMT7 and PRMT4, respectively, by in vitro mutational analysis. Dot blot analysis was performed to check the specificity of the anti-TP2K88me1 and anti-TP2R92me1 affinity-purified antibodies. Both antibodies reacted only with the respective methylated peptides and not with the corresponding backbone sequence (Fig. 9A). To validate further the monospecificity of the anti-TP2K88me1 and anti-TP2R92me1 antibodies, Western blot analysis was performed with acid-soluble proteins extracted from 35- and 70-day-old rat testis nuclei. 70-day-old adult rat testis contains all of the germ cells, including mature spermatozoa, whereas 35-day-old rats are devoid of spermatids and therefore lack spermatid-specific transition proteins. Both methylation-specific antibodies and anti-TP2 antibodies lit a single band in the lane with proteins extracted from the adult rat (70 day old) testicular nuclei. This signal was completely abolished when methylation-specific antibodies were preincubated with a ~50-fold molar excess (peptide to IgG) of the respective methylated peptides, as indicated (Fig. 9B). In order to establish whether TP2K88me1 and TP2R92me1 are present in the nucleoplasm or bound to chromatin, total testicular nuclei was fractionated into soluble and chromatin fractions.
Western blot analysis revealed that both TP2K88me1 and TP2R92me1 predominantly associated with chromatin (Fig. 9C).

Next, we were curious to examine at what steps of spermiogenesis the methylation of TP2 took place. Polyclonal anti-TP2 antibodies used in earlier experiments were raised against rat endogenous TP2 and may recognize epitope(s) comprising post-translational modifications present on the endogenous protein. To avoid any complications that can arise from such overlapping specificity, rTP2 was expressed in *E. coli* and was used to raise TP2 polyclonal antibodies by immunizing female New Zealand White rabbits and BALB/c mice as described under “Experimental Procedures.” The specificity of these antibodies was confirmed by Western blot analysis with rat testicular lysate (Fig. 9D). An immunofluorescence experiment with
Post-translational Modification Repertoire of TP1 and TP2

The essential steps involved in chromatin reorganization during mammalian spermiogenesis have been known for more than 3 decades, but the molecular mechanisms that transform the nucleo-histone architecture to nucleo-protamine fiber via the deposition of intermediate basic proteins, transition proteins, still remains elusive. Besides the changes in chromatin constitution, multiple events take place during spermiogenesis, namely (a) global transcriptional shutdown, (b) DNA repair, (c) territorial movement of chromosomes, (d) shaping of spermatid nuclei, (e) establishment of specialized chromatin domains, and (f) proteasomal machinery to remove evicted chromatin proteins during spermiogenesis (3). This is indicative of the necessity for the involvement of chromatin remodeling machinery, chaperones, chromatin readers, and repair machinery and their interaction with transition proteins and protamines. We now report for the first time a comprehensive study of the post-translational modification repertoire of the transition proteins, TP1 and TP2, which constitute the majority of the chromatin basic protein in spermatids.

These PTMs may play an active role in several aspects of spermiogenesis processes. The presence of post-translational modifications can alter transition protein interaction with DNA and ensuing chromatin condensation. In the case of TP1, basic amino acids flanking Tyr$^{33}$ and Tyr$^{51}$ have been proposed to be involved in the destabilization of chromatin, as determined by the quenching of tyrosine fluorescence upon binding of TP1 with DNA (21). Notably, TP1 does not have a defined condensation domain because none of the TP1 peptides could mimic the condensation achieved by full-length protein, suggesting the involvement of basic amino acids across the length of the protein (55). It is possible that post-translational modifications identified on TP1 can modulate its destabilization property (Fig. 10A). Three functional domains have been elucidated for TP2, including two zinc fingers in its N terminus (zinc finger 1: His$^{12}$, His$^{13}$, His$^{16}$, and His$^{24}$; zinc finger 2: Cys$^{29}$, Cys$^{31}$, Cys$^{35}$, and Cys$^{38}$), nuclear localization signal (NLS; residues 87–95), and DNA condensing domain (residues 87–114), as depicted. In vitro phosphorylation sites for the PKA Ca$^{2+}$, Thr$^{501}$ and Ser$^{109}$, which were not covered in mass spectrometry analysis of endogenous TP2, are also depicted.

FIGURE 10. PTMs in the context of functional properties of TP1 and TP2. A, PTM sites identified on TP1. The Tyr$^{33}$ and Tyr$^{51}$ residues are also indicated. B, PTM sites in the context of functional domains of TP2 (viz. zinc finger domains (zinc finger 1: His$^{12}$, His$^{13}$, His$^{16}$, and His$^{24}$; zinc finger 2: Cys$^{29}$, Cys$^{31}$, Cys$^{35}$, and Cys$^{38}$), nuclear localization signal (NLS; residues 87–95), and DNA condensing domain (residues 87–114), as depicted). In vitro phosphorylation sites for the PKA Ca$^{2+}$, Thr$^{501}$ and Ser$^{109}$, which were not covered in mass spectrometry analysis of endogenous TP2, are also depicted.

These antibodies revealed the same localization pattern of TP2 as with the previous anti-TP2 antibodies raised against the endogenous TP2 in different steps of spermiogenesis. An immunofluorescence experiment using anti-rTP2 antibody as a reference showed that TP2K88me1 and TP2R92me1 showed a very similar progression across the steps of spermiogenesis. TP2K88me1 and TP2R92me1 appear in the elongating spermatids and later show elevated levels in elongated spermatids. During mid-spermiogenesis, TP2K88me1 and TP2R92me1 staining is present throughout the nucleus. TP2K88me1 and TP2R92me1 signal progressively disappeared from the head to the tail region of the differentiating spermatids. This was accompanied by the condensation of nuclei moving from the head to the tail region. A cut-mask image representing the colocalized pixels and pixel counts for TP2, TP2K88me1, and TP2R92me1 are depicted for each of the presented spermatid (Fig. 9, E and F).

DISCUSSION

The essential steps involved in chromatin reorganization during mammalian spermiogenesis have been known for more than 3 decades, but the molecular mechanisms that transform the nucleo-histone architecture to nucleo-protamine fiber via the deposition of intermediate basic proteins, transition proteins, still remains elusive. Besides the changes in chromatin constitution, multiple events take place during spermiogenesis, namely (a) global transcriptional shutdown, (b) DNA repair, (c) territorial movement of chromosomes, (d) shaping of spermatid nuclei, (e) establishment of specialized chromatin domains, and (f) proteasomal machinery to remove evicted chromatin proteins during spermiogenesis (3). This is indicative of the necessity for the involvement of chromatin remodeling machinery, chaperones, chromatin readers, and repair machinery and their interaction with transition proteins and protamines. We now report for the first time a comprehensive study of the post-translational modification repertoire of the transition proteins, TP1 and TP2, which constitute the majority of the chromatin basic protein in spermatids.
spermiogenesis and requires an active process to enter the nucleus, whereas TP1 makes a passive entry into the nucleus. Many of the amino acid residues that undergo post-translational modification on endogenous transition proteins, TP1 and TP2, are present on non-conserved amino acids. In some cases, homologous amino acids are present in other organisms that may be post-translationally modified, conferring functional conservation, but many others lack any such homologous amino acid residues and thus appear to be lineage- and/or organism-specific. Interestingly, most of the PTMs identified on mouse protamines are also present on non-conserved amino acid residues (17). This indicates that sequence divergence of transition proteins and protamines and the presence of post-translational modifications on non-conserved residues might contribute to the remodeling of sperm chromatin in a unique manner characteristic to each of the different mammalian species.

It is intuitive to believe that post-translational modifications of transition proteins, in addition to modulating their DNA/chromatin interaction properties, may also mediate interaction with the chromatin machinery to bring about histone replacement and other spermiogenic events. TP2 protein is already known to interact with two chaperones, HSPA2 and NPM3 (32, 61). Interaction of NPM3 with TP2 is abolished in the presence of TP2 C-terminal acetylation catalyzed by KAT3B (p300) (32). Many of the histone modification reader proteins are expressed during spermiogenesis like Brdt, Cdyl, and NRDC (10, 11, 62–65), and it is quite likely that some of these readers may recognize PTMs of TPs and protamines and confer structural/functional changes to spermatid chromatin architecture. Acetylation-mediated proteasomal degradation has been shown to be necessary for the removal of evicted acetylated histone H4 during spermiogenesis (66), and a similar mechanism might also operate for the transition proteins for their degradation in late spermiogenic steps. In this context, notably, both endogenous major transition proteins, TP1 and TP2, are acetylated, as demonstrated in the present study. Chromosomes occupy specific positions in the spermatozoal nucleus, and this territorial reorganization begins in spermatids with simultaneous shaping of nuclei during spermiogenesis associated with change in chromatin composition (67, 68). Phosphorylated protamine P1 interacts with lamin B receptor, a component of the inner nuclear membrane (69). Transition proteins TP1 and TP2 show a distinct staining pattern with AT- and GC-rich DNA as they are spatio-temporally organized during spermiogenesis (36). It is possible that TPs, by virtue of their modification(s), may interact with the components of nuclear lamina (similar to phosphorylated protamine P1) and manchette and help in the reorganization of chromatin and shaping of nuclei (68). Among all of the PTMs associated with TP2, the presence of TP2K88me1 and TP2R92me1 has been demonstrated in elongating and condensing spermatids by modification-specific antibodies (Fig. 9). The availability of these two modification-specific antibodies would facilitate efforts in future to identify interacting proteins and to address some of the proposed functions mentioned above.

Protein-modifying enzymes have been extensively characterized with respect to histones; however, not much is known about their presence and role in chromatin remodeling events during spermiogenesis and more specifically with respect to transition proteins and protamines (30, 31, 70). The present study has demonstrated the presence of various PRMTs and KMT7 in spermatids and also the existence of several characteristic potential substrates (Figs. 4D and 7C). For a comprehensive understanding of the mechanisms involved in chromatin remodeling events during spermiogenesis, it becomes necessary to identify and characterize the role of chromatin-modifying enzymes that carry out covalent modification of basic proteins expressed in the spermiogenesis process.

For a very long time, sperm was believed to contribute just the genetic material to the offspring because histones are replaced by protamines in sperms to achieve compact packaging, hydrodynamic shape, and resistance to genotoxic insults (71, 72). Studies in the last decade have shown that sperm also contributes to the epigenetic landscape of the zygote (73, 74) and carries the epigenetic information in four forms: non-random genomic occupancy of canonical histones, histone variants, and protamines (8, 9, 12); the presence of post-translational modifications on histones and protamines (9, 15, 17); the DNA methylation pattern of spermatozoa (75–78); and spermatozoal RNA (79, 80). Environmental insults are known to alter the sperm epigenome, which results in offspring with clinical manifestations (81). Therefore, it is very important to understand the mechanisms and the various steps involved in the sperm epigenome establishment, its transmission to the zygote, and influence of the environment. Selective histone replacement by transition proteins during mid-spermiogenesis can create an imprint, which we propose as spermatid epigenome, that may directly or indirectly influence the sperm epigenome landscape. Some of the questions to be addressed to understand this transformation process are the following. (a) Is the genomic distribution of histones and protamines entirely decided during the replacement of histones by transition proteins, or do protamines also displace some of the histones directly, besides occupying the transition protein bound chromatin loci? (b) Do chromatin regions that escape histone eviction bear characteristic epigenetic marks? (c) Are PTMs of testicular sperm histones and protamines identical to or different from what has been identified in the mature sperm? (d) Besides the possible role of PTMs of TPs and protamines in chromatin-templated processes, do these PTMs mark specific chromatin domains and also influence the DNA methylation pattern? It would be worthwhile to map the epigenomic landscape of a few mammalian model organisms, from condensing spermatids, and compare them with mature epididymal sperm, particularly with respect to positioning and the PTM repertoire of nucleosomal histones, transition proteins, and protamines, to understand how spermatid epigenome finally shapes the sperm epigenome.

Acknowledgments—We thank B. S. Suma and Anitha G. for help with confocal microscopy and Sanger sequencing, respectively. We thank PTM Biolabs, Co., Ltd. (Chicago, IL) and MS Bioworks, LLC (Ann Arbor, MI) for mass spectrometry analysis.
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