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Arrested spermatogenesis and evidence for DNA damage in PTIP mutant testes

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ABSTRACT

The differentiation of mature sperm from male germ cells requires both chromatin remodeling and compaction as well as DNA double stranded break repair of sister chromatids. We examined the function of PTIP, a protein implicated in both DNA repair and histone methylation, during spermatogenesis by using a conditional, inducible mutation in adult male mice. Loss of PTIP led to the developmental arrest of spermatocytes, testicular atrophy, and infertility. By immunostaining with specific markers for different stages of spermatogenesis and for proteins involved in DNA damage and repair mechanisms, we conclude that the lack of PTIP results in genomic instability and DNA damage resulting in the cessation of spermatogenesis in meiosis I. These data underscore the importance of PTIP in the DNA repair process associated with the development of mature spermatozoa.

Introduction

In mammals, the generation of spermatozoa is a continuous process that encompasses multiple molecular mechanisms for generating mature haploid sperm. Such mechanisms include stem cell maintenance, cellular differentiation, DNA damage and repair during recombination, epigenetic remodeling of DNA and histone modification, and chromatin compaction (Sasaki and Matsui, 2008). This complex process requires two rounds of meiosis to produce the haploid gametes from a diploid germ cell. Before a germ cell completes meiosis I, chromosome pairs undergo homologous recombination involving the formation of synaptonemal complexes in which double strand (ds) DNA breaks occur followed by repair such that genetic material is exchanged between maternal and paternal chromosomes. Following meiosis I, the haploid secondary spermatocytes undergo another cell division resulting in the separation of individual chromatid strands to generate two spermatid cells. Spermatids then undergo a differentiation program, involving extensive chromatin compaction and replacement of histones with protamine, as the mature spermatozoa are formed.

The nuclear protein PTIP is implicated in both the DNA damage response and in the epigenetic modification of gene expression states (Munoz and Rouse, 2009). After ionizing irradiation, PTIP localizes to characteristic nuclear foci that also contain γH2AX, 53BP1, and the MRN complex, all of which are associated with the repair of DNA double stranded breaks (Gong et al., 2009; Jowsey et al., 2004; Munoz et al., 2007; Wu et al., 2009). Loss of PTIP renders cells hypersensitive to ionizing radiation and prevents 53BP1 localization to nuclear foci (Wu et al., 2009). Furthermore, PTIP may function as an adaptor protein for ATM and 53BP1, to link phospho-ATM to the site of a double stranded break (Gohler et al., 2008; Jowsey et al., 2004; Yan et al., 2011).

In addition to its association with DNA repair proteins, PTIP is part of the MLL3/4 histone H3K4 methylation complex that modifies chromatin during development and in response to specific inputs (Cho et al., 2007; Patel et al., 2007). PTIP links the MLL3/4 complex to tissue specific DNA binding proteins such as Pax2 and Pax5 to promote H3K4 trimethylation and gene activation (Patel et al., 2007; Schwab et al., 2011). Loss of PTIP reduces the global level of H3K4me3 in mammalian post-gastrulation embryos (Patel et al., 2007), in embryonic stem cells (Kim et al., 2009), and in *Drosophila* embryogenesis (Fang et al., 2009), suggesting that it can interact with a large number of loci. In more differentiated cells, PTIP is needed to maintain the epigenetic status, the stability of the transcriptome, and cellular phenotypes (Lefevre et al., 2010; Stein et al., 2011). This dual role as an adaptor protein in DNA repair and histone methylation is best illustrated at the immunoglobulin heavy chain locus in B cells where PTIP is needed for histone methylation at promoter regions upon isotype switching and for the efficient repair of the switch region after a double stranded break occurs (Daniel et al., 2010). In fact, a specific point mutation in PTIP can rescue the histone methylation function but not the DNA repair function, suggesting that these functions are separable.

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In spermatogenesis, the importance of double stranded breaks and DNA repair required for mediating crossing over is clear. Histone methylation changes are also implicated as conditional deletion of MLL4/KMT2D in the mouse results in arrest of spermatogenic development at the pachytene stage of meiosis I (Glaser et al., 2009). Further epigenetic modifications culminate in the replacement of most, but not all, histones with protamines (Wykes and Krawetz, 2003). Thus, the study of spermatogenesis offers a comprehensive model system to better understand the function of specific proteins in both DNA repair and chromatin remodeling. Using a tamoxifen-inducible Cre system, we successfully deleted *Paxip1*, the gene that encodes PTIP protein, in the adult mouse and studied the effects on spermatogenesis. In the testis of PTIP deleted mice, global levels of H3K4me3 were not significantly affected, stem cell populations were present, but the advancement through spermatogenesis was halted during meiosis I prophase in PTIP mutant testes. This phenotype suggests that PTIP plays a critical function in the maintenance of genomic stability during meiosis I in the germ cell lineage.

Materials and methods

Animals

The *Paxip1* conditional floxed (fl) allele containing the 5′ regulatory sequences and exon 1 flanked with loxP sites has been previously described (Kim et al., 2009). The ROSA-CreER allele was bred on to the *Paxip1*$^{fl/fl}$ strain which allows for tamoxifen-induced nuclear translocation of CreER (Ventura et al., 2007). At 6 to 7 weeks of age, male mice were injected with 20 mg/kg of 4-OH tamoxifen (H7904, Sigma) dissolved in peanut oil. Injections were given 5 times, once per day for 5 days. Tissues were then harvested at 2 or 6 weeks post-injection for analysis.

Histology and immunofluorescence

Testes were dissected and fixed overnight in 4% PFA in 1X DPBS, washed, and then processed for paraffin embedding. 5-micron sections were cut, dewaxed, rehydrated and processed for hematoxylin and eosin staining or processed for standard indirect immunofluorescence using epitope unmasking as described previously (Lefevre et al., 2010; Patel et al., 2007). Micrographs were taken using a Nikon ES800 fluorescent microscope and digital spot camera. All exposure times were set manually to insure equivalence among sections.

hCG challenge and serum testosterone analysis

Six week post-tamoxifen injected PTIP+ and PTIP- males were injected with 10 IU human Chorionic Gonadotropin (hCG) to induce Leydig cell testosterone production (Jean-Faucher et al., 1985). The remaining PTIP+ and PTIP− males were untreated and used as a control group. Whole blood was harvested 2 h after hCG injection. Sera were harvested after overnight incubation at 4 °C, centrifuged at 1000 rpm for 10 min, snapped frozen and stored at −80 °C until testosterone assay. Serum testosterone levels were measured using the competitive chemiluminescence immunoassay run on an ADVIA Centaur following manufacturer's instructions (Siemens HealthCare Diagnostics, Tarrytown, NY). The limits of detection of testosterone were 0.05 ng/ml. The inter-assay coefficients of variations of testosterone were less than 11.3%; and the intra-assay coefficients of variations of testosterone were less than 11.8%.

Germ cell spreads

Germ cell spreads were performed as previously described (Lu et al., 2010). Briefly, germ cells were dissociated from testes by mincing, resuspended in a hypotonic buffer, washed, resuspended in 0.1 M sucrose, spread on a slide dipped in 1% PFA–0.15% Triton X-100, pH 9.2, and dried. Standard immunofluorescence was performed as above.

Antibodies

Rabbit anti-PTIP has been described previously (Patel et al., 2007). Rabbit anti-H3K4me3 (#39159) and anti-H3K27me3 (#39155) were obtained from Active Motif. Mouse monoclonal Anti-γH2AX (Ser 139) (#05–636) and mouse anti-H3 (#05–499) were obtained from Upstate. Anti-PLZF (ab104854) and rabbit anti-H3K4me2 (Ab7766) were purchased from Abcam. Goat anti-SCP3 (M-14), goat anti-Actb (C-11), goat anti-Gapdh (V-18), rabbit anti-Rad51 (H-92), rabbit anti-Androgen Receptor (N-20), rabbit anti-Oct4 (H-134), rabbit anti-PRM1 (A-17), and rabbit anti-53BP1 (H-300) were obtained from Santa Cruz Biotechnology. Anti-DNA-PKcs (MC-365) was purchased from Kamiya Biotech. Rabbit anti-phospho-p53 (Ser 15) (#9284) was obtained from Cell Signaling. Mouse anti-p53 (Ab-6) was purchased from Calbiochem. Mouse phospho-ATM (Ser 1981) (#200-301-500) was purchased from Rockland. Secondary Antibodies were obtained from Abcam. Secondary antibody only controls performed ensured nonspecific staining was at background levels (data not shown).

Western analysis

Testes were minced, lysed in 2X SDS sample buffer, and boiled. Lysates were separated using SDS-PAGE and transferred to PVDF membranes. Standard western analysis was performed, with Beta-actin, Gapdh, and histone H3 used to normalized protein concentration.

Gene expression analysis

Total RNA was isolated from whole testis using Trizol reagent (Invitrogen). 5 micrograms of total RNA was reverse transcribed generating cDNA using oligo(dT) primers (Superscript II cDNA kit, Invitrogen). cDNAs were amplified with the iTaq Sybr green master mix (Bio-Rad) in a Prism 7500 (Applied Biosystem). Peptidylprolyl isomerase A (PPIA) was used as an endogenous control to normalize targets. Primers were designed to span introns to help control for genomic DNA contamination. The gene specific primers used were as follows:

Oct4—AGACCACCATCTGTCGCTTC, GGTCTCCAGACTCCACCTCA,
Plzf—GAGCACACTCAAGAGCCACA, GTGGCAGAGTTTGCACTCAA,
Kit—CCCTTGAAAAGGCCAACAT, GAGTTGACCCTCACGGAATG,
Stra8—GCTTTTGACGTGGCAAGTTT, AACACAGCCAAGGCTTTTGA,
Bmp8—GTGTGCTTTCCCACTGGACT, GAGGTGGCACTCAGTTTGGT,
Sycp3—CAACAACAAAAGATTTTTCAGCA,
TTTGCAACATAGCCATTTCTTTT,
Ccna1—TTCTGGAAGCTGACCCATTC, GGCAAGGCACAATCTCATTT,
Clgn—AGAATGGGAGGCACCACATA, TCTGGGTTGGGAATCTTCTG,
Crem—GCGACAACCGCATCAGAG, TCCTTCCCTGTTTTCCTTATTT,
Camk4—CCCATGGGTCACAGGTAAAG,
TGTGGTTCTCTTGGATGCTG,
Odf1—AGATCCTCTGGGCGATTTCT, TGATGTTCGGGTGTGAGAGA,
Tnp1—ACAAGGGCGTCAAGAGAGGT, CATCACAAGTGGGATCGGTA,
Prm2—GAAGGCGGAGGAGACACTC, GGGAGGCTTAGTGATGGTG,
Rhox1—AATGTGGCCTCAGCAACAG, TCTGCACTTTGGCTTCACAC,