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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.

Keywords

PTIP; DNA damage; DNA repair; Spermatogenesis

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.

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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.

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 hours after hCG injection. Sera were harvested after overnight incubation at 4°C, centrifuged at 1000rpm for 10min, 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, Rhox8 - AGGAACTGGAGCGCATTTT, TCAGGCATTTCCTGTATTTGG, Ptip - GCCAGGGGAATAGATGTTCA, CAGCTACCCTTGTGCTCCA, Ppia - CGAGCTCTGAGCACTGGAG, GATGCCAGGACCTGTATGCT.

Results

PTIP expression in the male germ cell lineage

In order to address whether PTIP functions in the male germline, we first examined when and where PTIP is localized during different stages of spermatogenesis by staining germ cell spreads from wild-type testes with specific antibodies against γH2AX, a marker of DNA damage, PLZF, a marker for Type-A spermatogonia (Payne and Braun, 2006), and PTIP. PTIP was detected in primary spermatocytes, identified by PLZF+ nuclear localization in Type A spermatogonia of the early male germ cell lineage (Fig. 1A, top panel). Additionally, punctate foci of PTIP and PLZF were observed, but the proteins did not show co-localization. Next, PTIP immunostaining was performed with γH2AX, a marker of dsDNA breaks, to distinguish cells undergoing meiosis I, such as pachytene spermatocytes, from other spermatocytes (Fig. 1A, bottom panel). Other spermatogonial cells, identified by their small, rounded γH2AX-negative nuclei, stained strongly for PTIP. Primary spermatocytes within the pachytene stage are readily identified by the intense γH2AX stained XY body. These cells possess high levels of PTIP within the decondensed regions of the nucleus, as marked by decreased DAPI intensity. Note that PTIP is not recruited to the XY body, which is transcriptionally inactive and undergoes restricted synapsis between the pseudo-autosomal regions of the X and Y chromosomes during this stage (Fernandez-Capetillo et al., 2003). The PTIP protein can be identified in the nucleus of round spermatids except within the intense DAPI-stained nucleoli. Additionally, a strong PTIP+ focus near the periphery of the nuclei can be identified in most round spermatids. Finally, PTIP localization in the elongating spermatid is low or undetectable except for a small region of staining at the periphery of the DAPI-positive region of the developing acrosome.

During meiosis prophase I, sister chromatids align through the formation of the synaptonemal complex and progress through four stages: leptotene, zygotene, pachytene and

diplotene. To further characterize PTIP localization during synapsis, germ cell spreads were co-stained using SCP3 to identify the lateral elements of the synaptonemal complex (Fig. 1B). The distribution of PTIP in the zygotene phase shows PTIP localization within decondensed areas of the nucleus (Fig. 1B). Nuclear PTIP staining persists in the primary spermatocyte through the early pachytene stage as the strands near completion of synapsis between homologous chromosomes (Fig. 1B). Notice that PTIP and SCP3 co-localization is only identified at a few regions in the nucleus during these stages suggesting that PTIP function is primarily within the decondensed chromatin during meiosis I. During the pachytene stage, the SCP3-stained synaptonemal complexes are separate, thick filaments within a large nucleus (Fig 1B). During this stage, nuclear PTIP staining is dramatically reduced to background levels with only a few foci present (Fig. 1B). The dynamic localization of PTIP in the nucleus through meiosis I suggests multiple possible molecular functions, such as roles in genomic stability, DNA repair, synapsis formation, or post-synaptic transcriptional activation.

Generation of conditional PTIP knockout mice

To better understand the functions of PTIP in adult tissues, the ROSA-CreER transgene was bred onto the *Paxip*$^{fl/fl}$ strain allowing the conditional deletion of PTIP upon the administration of 4OH-Tamoxifen. At 6 or 7 weeks of age, male *Paxip*$^{fl/fl}$ mice (labeled PTIP+) or ROSA-Cre-ER; *Paxip*$^{fl/fl}$ mice (labeled PTIP−) were injected with 4OH-TAM for five days. As an additional control, male Rosa-Cre-ER; *Paxip*$^{fl/fl}$ mice (labeled PTIP− Control) that were not injected with 4OH-Tamoxifen were analyzed in parallel. At 6 weeks post-treatment, PTIP+ mice remained healthy while PTIP− mice were displaying multiple abnormalities including reduced weight/growth, cloudy eyes, and dermatitis. Testes were harvested at 6 weeks after treatment for analysis. PTIP− testes were significantly smaller and atrophied compared the PTIP+ controls (Fig. 2A). Testis weight, normalized to body weight, showed that the PTIP− testes were on average 25% of PTIP+ controls (Fig. 2B). To verify the deletion of PTIP in the testis, both Western analysis (Fig. 2C) and qPCR analysis of PTIP cDNA (Fig. 2D) was performed and showed significant reduction of the protein and mRNA, respectively. Since testis weight was reduced in PTIP conditional knock down animals, studies were performed to evaluate testis endocrine function and response to hCG challenge. At 6 weeks post tamoxifen injection both PTIP+ and PTIP− males had serum testosterone below levels of assay detection (0.05 ng/ml). Following hCG challenge both PTIP+ and PTIP− males had elevated serum testosterone, 17.2±2.2 and 4.5±1.7 ng/ml (mean ±SE) respectively. This indicating that Leydig cell steroidogenesis was preserved in PTIP+ and PTIP− males although serum levels were significantly lower in PTIP− males compared to PTIP+ ($P<0.01$).

At 2 weeks post-tamoxifen injection, histology revealed little difference in the morphology between PTIP+ and PTIP− testis (Fig. 2E). However, at 6 weeks post-tamoxifen treatment, histological analysis of PTIP− testis reveals a clear defect in sperm maturation, with decellularization and fibrosis within the tubules compared to PTIP+ littermates (Fig. 2F). Noticeably, PTIP− testes were nearly devoid of elongating spermatids and spermatozoa. Additionally, spermatogenesis was halted within multiple stages of the spermatogenic cycle within the PTIP− testis as indicated by different remaining cell types within a single tubule. A few rare PTIP− tubules consisting entirely of round spermatids were identified suggesting a secondary block in spermatogenesis later in development. Sertoli cells, with their characteristic prominent nucleoli, were identified along the basement membrane within the PTIP− tubules.

Gene expression analysis of PTIP− testis

In order to confirm the arrested spermatogenesis and to analyze the effects of the PTIP mutation on gene expression, RNA from whole testis was isolated, cDNA prepared, and qPCR was performed for genes expressed at specific stages of germ cell development (Fig. 3). Genetic markers for spermatogonia, spermatocytes, spermatids and sertoli cells were examined in PTIP+ and PTIP− samples. PTIP is needed to maintain the pluripotency of embryonic stem cells in vitro, as PTIP deletion results in loss of pluripotency markers and eventual differentiation to the trophobast lineage (Kim et al., 2009). Interestingly, the spermatogonium markers tested were similar in both the PTIP+ and PTIP− testis (Fig. 3). Oct4 mRNA was marginally decreased in the PTIP− testis compared to the PTIP+, although Oct4 protein was not significantly affected in protein lysates (see Fig. 4C). PLZF and Stra8 mRNA levels were not significantly different in all samples suggesting the spermatogium population persists. However, gene expression changes in Kit, a spermatogonia marker, were significantly increased in PTIP− samples. Although Kit expression may be increased within the spermatogonium population, Kit and other gene expression changes could represent immune cell infiltration due to the multiple pathologies found in the PTIP− mice.

Expression of genes known to function in spermatocyte development was also analyzed (Fig. 3). The Bmp8 and Ccna1 mRNA levels were downregulated within the PTIP− treated population, while the mRNA of Scp3 and Clgn were not affected significantly. These changes suggest PTIP is required to activate or maintain spermatocyte gene expression during Meiosis I. All the marker genes tested of the spermatid stage of development were significantly different. PTIP− testis showed marked decreases of Crem, Camk4, Odf1, Tnp1 and Prm1 expression, corroborating the loss of elongating spermatids and spermatozoa identified by histology and immunostaining. Additionally, Sertoli cell markers, Rhox1 and Rhox8, were significantly increased within the PTIP− testis which may reflect the decreased numbers of germ cells compared to sertoli cells. Overall, the expression analysis shows a significant block in spermiogenesis in the PTIP− testis compared to the control. Additionally, the analysis of earlier spermatocyte markers suggests disrupted gene expression within meiosis I of the PTIP− spermatocyte. However, these early changes may also be due to changes in the distribution of germ cell populations in the PTIP− testis.

Histone and Protein Analyses

During embryogenesis, loss of PTIP significantly reduced the global levels of H3K4me3 in both mice and flies (Fang et al., 2009; Patel et al., 2007). In order to examine histone methylation more directly, immunofluorescence was performed on sections of PTIP+ and PTIP− testis to identify potential differences in PTIP dependent histone methylation in stem cells during spermatogenesis. Immunostaining of PLZF, a marker of Type A spermatogonia, was used to identify the stem cell population in both PTIP+ and PTIP− testes (Payne and Braun, 2006). These stem cells were localized in a characteristic position next to the basal lamina (Fig. 4A). We then utilized antibodies specific for H3K4me2, H3K4me3 and H3K27me3, as these histone modifications show characteristic staining patterns within the nucleus, depending on the stage of spermatogenesis (Hayashi et al., 2005; Payne and Braun, 2006). These histone methylation staining patterns of pachytene spermatocytes identified in germ cell spreads were not noticeably different (Fig 4B). Distinguishable, punctate regions of staining are found in the pachytene spermatocytes when using the H3K27me3 antibody, while H3K4me2 and H3K4me3 antibodies identified a general staining pattern throughout the nucleus (Fig 4B). Similar staining profiles can still be identified in the PTIP− primary spermatocytes compared to the PTIP+ cells for these histone marks. Interestingly, PTIP− spermatocytes have significantly increased nuclear γH2AX staining compared to PTIP+ spermatocytes in which γH2AX staining is restricted to the XY body. By Western analysis of H3K4me3 levels from whole testicular lysates only a slight decrease of H3K4me3 levels

were observed within the PTIP− samples compared to the PTIP+ controls (Fig. 4D). Although changes of histone methylation status at specific loci cannot be ruled out, this data suggests the loss of PTIP does not have a gross effect upon global H3K4me2, H3K4me3 or H3K27me3 patterns.

As spermatogenesis progresses, protamines replace much of the histones within the spermatid nucleus. Levels of Prm1 are much lower in PTIP− testes (Fig. 4D) and likely reflect the fact that few spermatids reach the stage of histone replacement. We also examined proteins known to sense and regulate the repair of ds DNA breaks. Notably, levels of DNAPKcs, phopho-ATM, p53, and phospho-p53 were all reduced in the PTIP− testicular lysates (Fig. 4E). The overall levels of γH2AX were not measurably different, although as discussed below, the pattern of staining was qualitatively different.

Immunostaining for DNA Damage and Repair Proteins

The differences in p-ATM, p-p53, and DNAPKcs levels suggest a potential defect in the repair of double stranded breaks that mediate crossing over in meiosis. Phosphorylation of H2AX (also known as γH2AX) is the earliest indicator of dsDNA breaks and can be identified at varying levels throughout spermatogenesis. In prophase of meiosis I, γH2AX coats the XY chromosomes, termed the XY body, so that a γH2AX positive aggregate is easily discernible. A second protein, 53BP1 is also localized to nuclear foci after ds DNA breaks and is required for a successful DNA damage response (Ward et al., 2003). Thus we co-stained sections of PTIP+ and PTIP− testes for γH2AX and 53BP1 localization (Fig 5A). In cross-sections of tubules, strong γH2AX/53BP1 staining of XY bodies identified the pachytene stage cells that are close to the basement membrane in both the PTIP+ and PTIP− testis (Fig 5A). However, in the PTIP− testis, yH2AX/53BP1 positive pachytene cells within most tubules are also located in the lumen of the tubules that are devoid of advanced spermatocytes (Fig 5A). Additionally, many primary spermatocytes lining the basement membrane and within the lumen possess high γH2AX staining in the entire nucleus, not just the XY body, compared to cells within the PTIP+ tubules. Pre-pachytene cells were also identified in sections by their smaller size, location next to the basement membrane, and diffuse staining for γH2AX and 53BP1 (Fig 5A). Again, PTIP− cells had marginally increased levels and identifiable foci of staining for γH2AX compared to PTIP+. Similar results were also observed for Rad51, a DNA-binding damage repair protein that co-localizes with γH2AX (Fig. 5B). Again, strong immunostaining of both Rad51 and γH2AX proteins was observed in spermatocytes near the basement membrane of the PTIP+ tubule, but also in cells within the lumen of PTIP− tubules. In the pre-pachytene cells near basement membranes of the PTIP− tubule, high levels of Rad51 protein marked small nuclear foci that were rarely seen wild type testes (Fig 5B).

Immunostaining of SCP3, a lateral component of the synaptonemal complex, was used to verify the integrity of the synapse between sister chromatids in the pachytene stage (Fig. 6). Co-immunostaining of γH2AX and SCP3 testis sections revealed the presence of the SCP3 staining filaments in both PTIP+ and PTIP− tubules suggesting the synapses are not affected in the PTIP− cells (Fig 6A). Closer inspection of primary spermatocytes in the diplotene stage of meiosis I revealed the disintegrating synaptonemal complex appearing as SCP3+ foci present in both PTIP+ and PTIP-. However, intense γH2AX immunostaining decorated the condensed chromatids in the PTIP− tubule (Fig 6B). Unfortunately, we could not localize PTIP on sections of normal testes, most likely due to high background and low antibody affinity. Nevertheless, consistent with previous immunostaining for other DNA damage markers, this data suggests PTIP− spermatocytes are unable to progress to the secondary spermatocyte stage due to a block in meiosis I. This developmental arrest is likely due to genomic instability and the inability to repair dsDNA breaks.

Discussion

The production of male gametes requires the maintenance of the spermatogonial lineage, the successful recombination of sister chromatids during meiosis I, and the complex differentiation of primary spermatocytes to mature spermatozoa. The deletion of PTIP results in a dramatic developmental arrest of spermatogenesis, resulting in testicular atrophy and a near complete lack of spermatozoa. Based on the analysis of markers for DNA double stranded breaks and DNA repair, we propose that PTIP is required to maintain genomic integrity and potentially assist in DNA repair during meiotic prophase. Failure to repair these breaks, leads to arrested development and infertility.

Several lines of evidence point to two potential functions for PTIP in the epigenetic regulation of gene expression and in the DNA damage response and repair pathways. In both mice and flies (Fang et al., 2009; Patel et al., 2007), germline null PTIP mutant embryos show a global reduction in H3K4me3, consistent with its role as an adaptor protein that links MLL3/4 to chromatin. In more differentiated cells, the loss of PTIP result in fewer and more locus specific changes in the H3K4me3 status (Daniel et al., 2010; Lefevre et al., 2010; Stein et al., 2011). In response to ionizing radiation induced dsDNA breaks, PTIP is known to interact with a second complex that includes 53BP1 and the MRN complex (Cho et al., 2007; Wu et al., 2009). Loss of PTIP can sensitize cells to radiation and reduce the efficiency of double stranded break repair (Cho et al., 2003; Daniel et al., 2010; Wang et al., 2010). Of course, these two functions may be related, as changes in histone methylation at sites of DNA repair may also be required (Faucher and Wellinger, 2010). In addition, a recent study links histone methylation to dsDNA break introduction and repair (Buard et al., 2009), as H3K4me2 and H3K4me3 preceded dsDNA breaks at recombination hotspots suggesting that histone methylation is involved in targeting dsDNA break and repair machinery during homologous recombination. However, given the persistence of H3K4me3-staining within the PTIP− primary spermatocyte nuclei, PTIP is unlikely to be needed for global maintenance of the H3K4me3 status. Rather, PTIP appears essential to maintain genomic integrity during exchange of genetic material between sister chromatids.

The cessation of spermatogenesis at the primary spermatocyte stage as well as the increased intensity and number of cells staining positive for γH2AX, 53BP1 and RAD51 within the PTIP− testis suggests a dramatic effect on the sensing or repair of DNA damage. Interestingly, γH2AX and 53BP1 staining of the XY body during pachytene stage of spermatogenesis in the PTIP− testis is maintained, consistent with previous work that demonstrates unique recruitment mechanism for these proteins to the XY body (Mahadevaiah et al., 2001; Romanienko and Camerini-Otero, 2000).

Although systemic deletion of PTIP in adult males led to general deterioration of the health of the animals, the endocrine response to hCG was intact, albeit reduced. This reduction in testosterone might also reflect the smaller size of the PTIP− testis rather than a direct defect in the hormonal response pathways. We favor the interpretation that testicular atrophy is due to arrested spermatogenesis and the failure to repair double-stranded DNA breaks. Deletion of other DNA sensing or repair proteins in the mouse has revealed a similar phenotype to PTIP, in which spermatogenesis is halted within meiosis I and specifically before synapse is completed. The deletion of H2AX, in which the phosphorylated form marks double-strand DNA breaks, results with the cessation of spermatogenesis during the pachytene-stage (Celeste et al., 2002). Also, loss of the damage-sensor kinase, ATM, results in a significant meiotic disruption in the early leptonema stage due to the mislocalization of ATR, DMC1, and RAD51 to chromatin (Barlow et al., 1998). The conditional deletion of ATR in the adult mouse using the Cre-ER inducible system also resulted in testicular atrophy, decellularization and loss of secondary spermatocytes similar to the PTIP− phenotype

(Ruzankina et al., 2007). Additionally, deletion of the DNA repair protein BRCA1 on a p53 heterozygous background results in the failure of spermocytes to progress beyond the diplotene-stage of meiosis I (Xu et al., 2003). All of these mutant phenotypes are consistent with the interpretation that PTIP also functions in the DNA repair pathways that are required for the completion of meiosis.

In conclusion, PTIP plays critical functions in genomic stability and may act in DNA damage repair during meiosis I. The inactivation of PTIP results in a dramatic decellularization of the seminiferous tubules and loss of spermatid and spermatozoa populations. PTIP− primary spermatocytes possess significantly increased levels of H2AX-phosphorylation along with changes in 53BP1 and Rad51 localization indicative of unrepaired dsDNA breaks supporting this hypothesis. These results demonstrate the importance of PTIP-mediated genomic stability during homologous recombination of meiosis I.

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References

Barlow C, Liyanage M, Moens PB, Tarsounas M, Nagashima K, Brown K, Rottinghaus S, Jackson SP, Tagle D, Ried T, Wynshaw-Boris A. Atm deficiency results in severe meiotic disruption as early as leptonema of prophase I. Development. 1998; 125:4007–17. [PubMed: 9735362]

Buard J, Barthes P, Grey C, de Massy B. Distinct histone modifications define initiation and repair of meiotic recombination in the mouse. Embo J. 2009; 28:2616–24. [PubMed: 19644444]

Celeste A, Petersen S, Romanienko PJ, Fernandez-Capetillo O, Chen HT, Sedelnikova OA, Reina-San-Martin B, Coppola V, Meffre E, Difilippantonio MJ, Redon C, Pilch DR, Olaru A, Eckhaus M, Camerini-Otero RD, Tessarollo L, Livak F, Manova K, Bonner WM, Nussenzweig MC, Nussenzweig A. Genomic instability in mice lacking histone H2AX. Science. 2002; 296:922–7. [PubMed: 11934988]

Cho EA, Prindle MJ, Dressler GR. BRCT domain-containing protein PTIP is essential for progression through mitosis. Mol Cell Biol. 2003; 23:1666–73. [PubMed: 12588986]

Cho YW, Hong T, Hong S, Guo H, Yu H, Kim D, Guszczynski T, Dressler GR, Copeland TD, Kalkum M, Ge K. PTIP associates with MLL3- and MLL4-containing histone H3 lysine 4 methyltransferase complex. J Biol Chem. 2007; 282:20395–406. [PubMed: 17500065]

Daniel JA, Santos MA, Wang Z, Zang C, Schwab KR, Jankovic M, Filsuf D, Chen HT, Gazumyan A, Yamane A, Cho YW, Sun HW, Ge K, Peng W, Nussenzweig MC, Casellas R, Dressler GR, Zhao K, Nussenzweig A. PTIP Promotes Chromatin Changes Critical for Immunoglobulin Class Switch Recombination. Science. 2010; 329:917–923. [PubMed: 20671152]

Fang M, Ren H, Liu J, Cadigan KM, Patel SR, Dressler GR. Drosophila ptip is essential for anterior/posterior patterning in development and interacts with the PcG and trxG pathways. Development. 2009; 136:1929–38. [PubMed: 19429789]

Faucher D, Wellinger RJ. Methylated H3K4, a transcription-associated histone modification, is involved in the DNA damage response pathway. PLoS Genet. 2010:6.

Fernandez-Capetillo O, Mahadevaiah SK, Celeste A, Romanienko PJ, Camerini-Otero RD, Bonner WM, Manova K, Burgoyne P, Nussenzweig A. H2AX is required for chromatin remodeling and inactivation of sex chromosomes in male mouse meiosis. Dev Cell. 2003; 4:497–508. [PubMed: 12689589]

Glaser S, Lubitz S, Loveland KL, Ohbo K, Robb L, Schwenk F, Seibler J, Roellig D, Kranz A, Anastassiadis K, Stewart AF. The histone 3 lysine 4 methyltransferase, Mll2, is only required briefly in development and spermatogenesis. Epigenetics Chromatin. 2009; 2:5. [PubMed: 19348672]

Gohler T, Munoz IM, Rouse J, Blow JJ. PTIP/Swift is required for efficient PCNA ubiquitination in response to DNA damage. DNA Repair (Amst). 2008; 7:775–87. [PubMed: 18353733]

Gong Z, Cho YW, Kim JE, Ge K, Chen J. Accumulation of Pax2 transactivation domain interaction protein (PTIP) at sites of DNA breaks via RNF8-dependent pathway is required for cell survival after DNA damage. J Biol Chem. 2009; 284:7284–93. [PubMed: 19124460]

Hayashi K, Yoshida K, Matsui Y. A histone H3 methyltransferase controls epigenetic events required for meiotic prophase. Nature. 2005; 438:374–8. [PubMed: 16292313]

Jean-Faucher C, Berger M, de Turckheim M, Veyssiere G, Jean C. Testosterone and dihydrotestosterone levels in epididymis, vas deferens, seminal vesicle and preputial gland of mice after hCG injection. J Steroid Biochem. 1985; 23:201–5. [PubMed: 4033119]

Jowsey PA, Doherty AJ, Rouse J. Human PTIP facilitates ATM-mediated activation of p53 and promotes cellular resistance to ionizing radiation. J Biol Chem. 2004; 279:55562–9. [PubMed: 15456759]

Kim D, Patel SR, Xiao H, Dressler GR. The role of PTIP in maintaining embryonic stem cell pluripotency. Stem Cells. 2009; 27:1516–23. [PubMed: 19544464]

Lefevre GM, Patel SR, Kim D, Tessarollo L, Dressler GR. Altering a histone H3K4 methylation pathway in glomerular podocytes promotes a chronic disease phenotype. PLoS Genet. 2010; 6:e1001142. [PubMed: 21060806]

Lu LY, Wu J, Ye L, Gavrilina GB, Saunders TL, Yu X. RNF8-dependent histone modifications regulate nucleosome removal during spermatogenesis. Dev Cell. 2010; 18:371–84. [PubMed: 20153262]

Mahadevaiah SK, Turner JM, Baudat F, Rogakou EP, de Boer P, Blanco-Rodriguez J, Jasin M, Keeney S, Bonner WM, Burgoyne PS. Recombinational DNA double-strand breaks in mice precede synapsis. Nat Genet. 2001; 27:271–6. [PubMed: 11242108]

Munoz IM, Jowsey PA, Toth R, Rouse J. Phospho-epitope binding by the BRCT domains of hPTIP controls multiple aspects of the cellular response to DNA damage. Nucleic Acids Res. 2007; 35:5312–22. [PubMed: 17690115]

Munoz IM, Rouse J. Control of histone methylation and genome stability by PTIP. EMBO Rep. 2009; 10:239–45. [PubMed: 19229280]

Patel SR, Kim D, Levitan I, Dressler GR. The BRCT-domain containing protein PTIP links PAX2 to a histone H3, lysine 4 methyltransferase complex. Dev Cell. 2007; 13:580–92. [PubMed: 17925232]

Payne C, Braun RE. Histone lysine trimethylation exhibits a distinct perinuclear distribution in Plzf-expressing spermatogonia. Dev Biol. 2006; 293:461–72. [PubMed: 16549060]

Romanienko PJ, Camerini-Otero RD. The mouse Spo11 gene is required for meiotic chromosome synapsis. Mol Cell. 2000; 6:975–87. [PubMed: 11106738]

Ruzankina Y, Pinzon-Guzman C, Asare A, Ong T, Pontano L, Cotsarelis G, Zediak VP, Velez M, Bhandoola A, Brown EJ. Deletion of the developmentally essential gene ATR in adult mice leads to age-related phenotypes and stem cell loss. Cell Stem Cell. 2007; 1:113–26. [PubMed: 18371340]

Sasaki H, Matsui Y. Epigenetic events in mammalian germ-cell development: reprogramming and beyond. Nat Rev Genet. 2008; 9:129–40. [PubMed: 18197165]

Schwab KR, Patel SR, Dressler GR. Role of PTIP in class switch recombination and long-range chromatin interactions at the immunoglobulin heavy chain locus. Mol Cell Biol. 2011; 31:1503–11. [PubMed: 21282469]

Stein AB, Jones TA, Herron TJ, Patel SR, Day SM, Noujaim SF, Milstein ML, Klos M, Furspan PB, Jalife J, Dressler GR. Loss of H3K4 methylation destabilizes gene expression patterns and physiological functions in adult murine cardiomyocytes. J Clin Invest. 2011; 121:2641–50. [PubMed: 21646717]

Ventura A, Kirsch DG, McLaughlin ME, Tuveson DA, Grimm J, Lintault L, Newman J, Reczek EE, Weissleder R, Jacks T. Restoration of p53 function leads to tumour regression in vivo. Nature. 2007; 445:661–5. [PubMed: 17251932]

Wang X, Takenaka K, Takeda S. PTIP promotes DNA double-strand break repair through homologous recombination. Genes Cells. 2010

Ward IM, Minn K, van Deursen J, Chen J. p53 Binding protein 53BP1 is required for DNA damage responses and tumor suppression in mice. Mol Cell Biol. 2003; 23:2556–63. [PubMed: 12640136]

Wu J, Prindle MJ, Dressler GR, Yu X. PTIP regulates 53BP1 and SMC1 at the DNA damage sites. J Biol Chem. 2009

Wykes SM, Krawetz SA. The structural organization of sperm chromatin. J Biol Chem. 2003; 278:29471–7. [PubMed: 12775710]

Xu X, Aprelikova O, Moens P, Deng CX, Furth PA. Impaired meiotic DNA-damage repair and lack of crossing-over during spermatogenesis in BRCA1 full-length isoform deficient mice. Development. 2003; 130:2001–12. [PubMed: 12642502]

Yan W, Shao Z, Li F, Niu L, Shi Y, Teng M, Li X. Structural basis of gammaH2AX recognition by human PTIP BRCT5-BRCT6 domains in the DNA damage response pathway. FEBS Lett. 2011

- The PTIP protein is implicated in histone methylation and DNA repair.
- We use an inducible Cre drive to delete PTIP in adult male mice.
- Loss of PTIP results in arrested spermatogenesis and infertility.
- Germ cells are unaffected but spermatids cannot progress due to DNA damage.
- The results point to an essential role for PTIP in sister chromatid exchange.

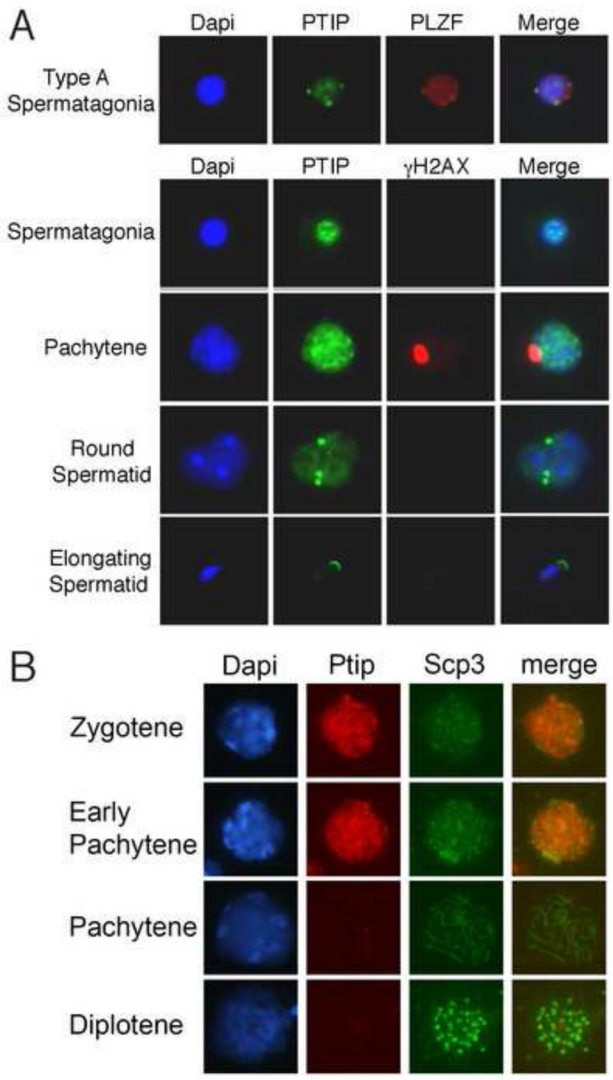

Figure 1. Nuclear PTIP Protein is Expressed in the Male Germline

A) Germ cell spreads from wildtype adult mice stained for PLZF (red, top panel), γ-H2AX (red, bottom panel), and PTIP (green). Low levels of nuclear PTIP can be found within the nucleus of PLZF+ Type A spermatogonia. Higher levels of PTIP are seen in γ-H2AX- spermatogonia cells and the zygotene/pachytene primary spermatocyte. Notice PTIP staining is excluded from the γ-H2AX-stained XY body of the zygotene/pachytene spermatocyte. Later, PTIP staining is reduced within the nuclei of round spermatids. Notice PTIP staining does not co-localize within the nucleolus and a single, intense foci is found at the periphery of the nucleus in each cell. In the elongating spermatid, PTIP staining is absent from the condensed chromatin, but is localized to periphery of the acrosome. B) Dynamic localization of PTIP during meiotic prophase. Immunostaining of PTIP (red) and the lateral synaptonemal complex protein, SCP3 (green) revealed general nuclear staining of PTIP with few areas of co-localization between the PTIP and SCP3 during the zygotene and early pachytene stages. PTIP is decreased in the nucleus except for a few small foci during the pachytene and diplotene stage.

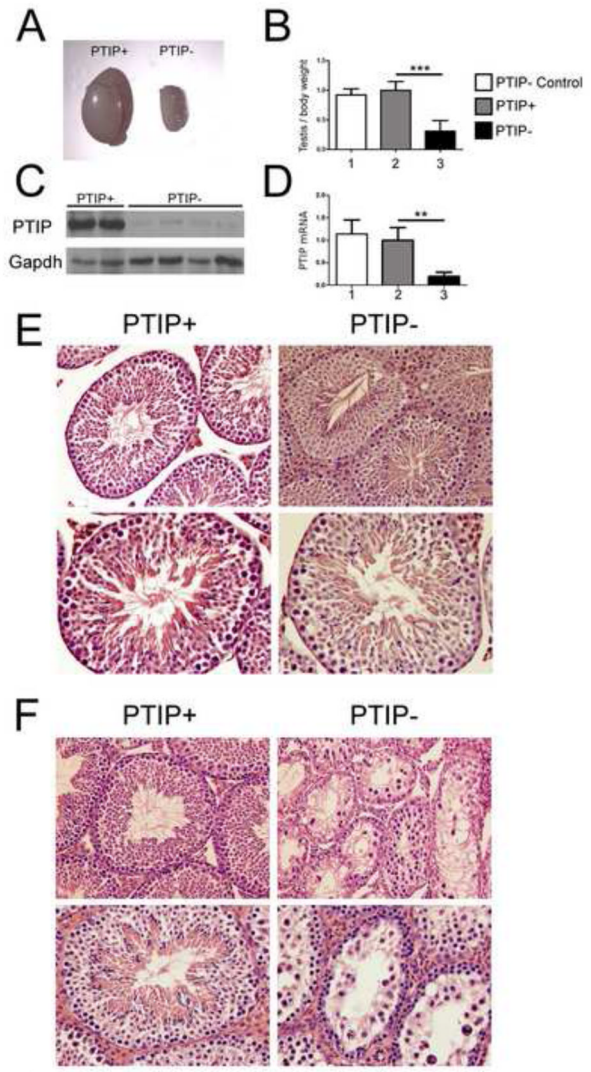

Figure 2. Testicular Atrophy and Decellularization of the Seminiferous Tubules in PTIP− Testes

A) Six week after tamoxifen treatment the PTIP− testes are significantly smaller in size than the PTIP+ control. B) Testicular weight of uninjected PTIP− controls (n=2), injected PTIP+ (n=4), and injected PTIP− (n=4) mice normalized to body weight. C) Western blot of testicular cell lysates demonstrating reduction of PTIP protein in the PTIP− testes compared to injected PTIP+ testes. D) Q-PCR of testicular cDNA showing significant reduction of PTIP mRNA in PTIP− animals. E and F) Representative hematoxcylin and eosin (HE) staining of testis sections from PTIP+ and PTIP− mice. E) Histology of the PTIP+ and PTIP − testis harvested 2 weeks after Tamoxifen treatment reveals normal morphology including spermatocytes, round spermatids, and elongating spermatids. F) PTIP+ and PTIP− testis harvested 6 weeks after Tamoxifen treatment. Notice the significant decrease of tubular cell density, loss of round spermatids and elongating spermatids in the PTIP− testis. Error bars represent 1 S.D. **, $p<0.01$; ***, $p<0.001$, Student's T test for independent variables.

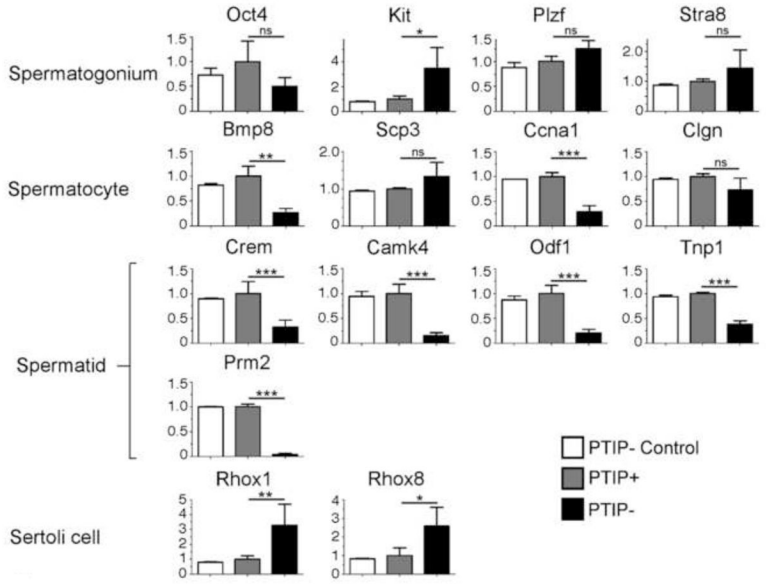

Figure 3. RT-qPCR Analysis of Testicular Marker Genes

Total RNAs isolated from control PTIP− (n=2), PTIP+ (n=4), and PTIP− (n=4) testes were assayed with gene specific primers as indicated. The spermatogonium markers, Oct4, Plzf, and Stra8, persist and are not significantly changed in the PTIP− testis suggesting the progenitor cell compartment is not disturbed. However, Kit expression is upregulated. Expression patterns of spermatocyte markers, Bmp8 and Ccna1 in PTIP− testis are significantly decreased, whereas Scp3 or Clgn are not significantly affected. By the spermatid stage, gene expression is significantly decreased in the PTIP− testis correlating with the phenotypic loss of round and elongating spermatids. Sertoli cell markers, Rhox1 and Rhox8, in the PTIP− testis were significantly increased over controls indicating the persistence of these cells. Error bars represent 1 S.D. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, Student's T test for independent variables.

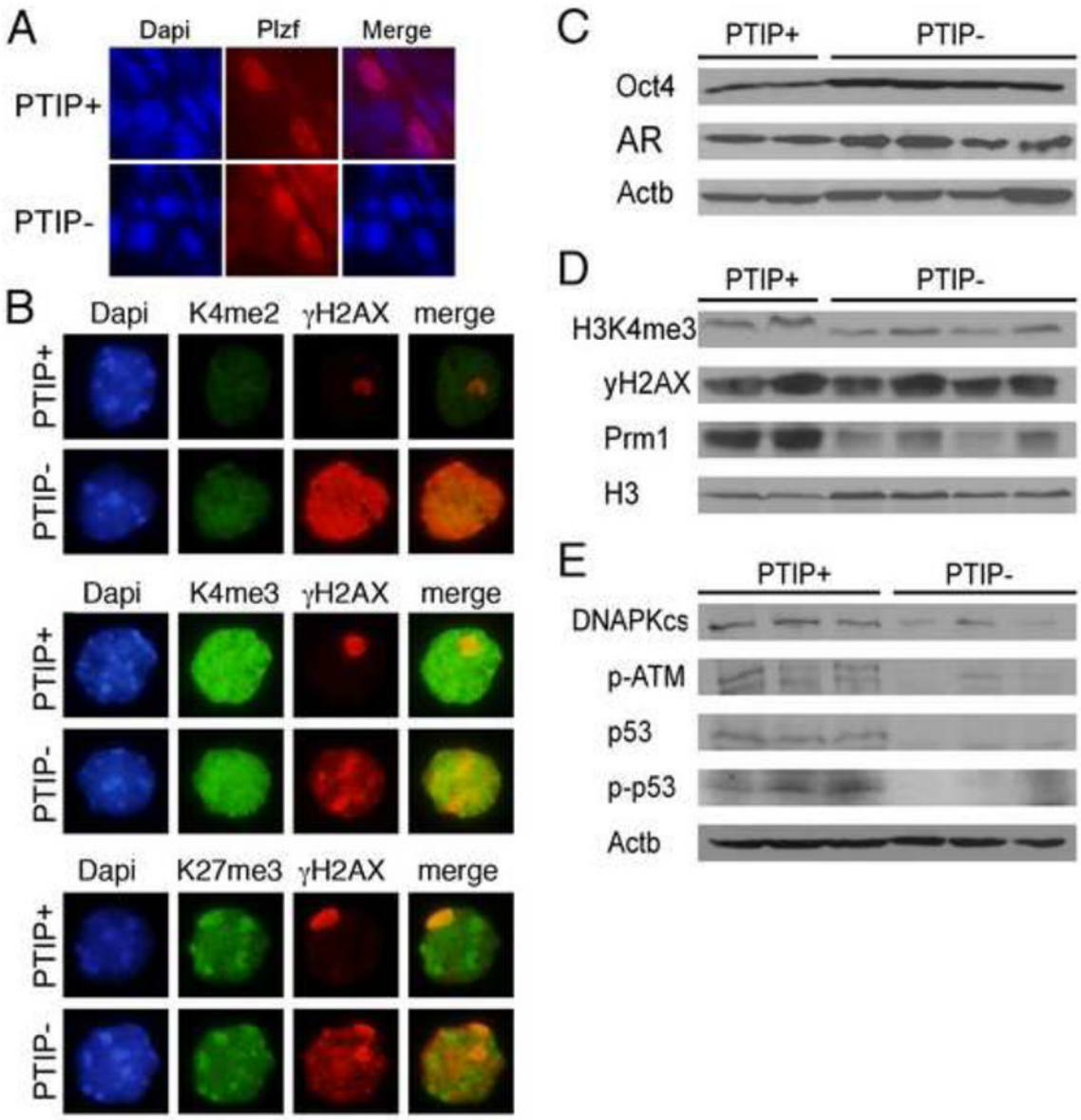

Figure 4. Analysis of Global Histone Methylation in Spermatogonial and Primary Spermatocyte cells

A) Immunostaining of PTIP+ and PTIP− testis sections using antibodies for PLZF (red) reveals PLZF+ spermatogonium in the PTIP− tubules. B) Immunostaining of primary spermatocytes in germ cell spreads with antibodies against γH2AX+ (red) or histone H3 methylation (green) as indicated. H3K4me2, H3K4me3 and H3K27me3 immunostaining patterns are not qualitatively or quantitatively different between PTIP+ and PTIP− primary spermatocyte cells. Note that γH2AX staining in the PTIP− spermatocytes is not limited to the XY body and extends diffusely throughout the nucleus. C–E) Western blotting of whole testis lysates from PTIP+ and PTIP− animals probed with the antibodies indicated. C) Westerns for the androgen receptor, AR, and Oct4 demonstrating the endocrine nuclear receptor and germ stem cell marker, respectively, are present in the PTIP− testis. D) Global H3K4me3 and γH2AX levels are not measurably changed between samples, but Prm1, a protamine, is reduced in the PTIP− testis correlating with loss of spermiogenesis. E) DNA

repair complex protein expression analyses shows decreased DNA-PKcs, phospho-ATM, p53, and phospho-p53 in the PTIP− testis.

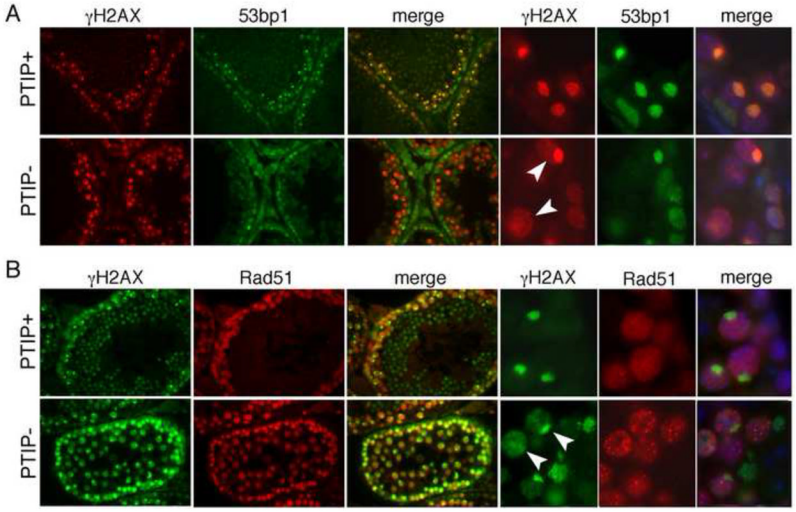

Figure 5. Immunostaining for DNA Damage and Repair Proteins

PTIP+ and PTIP− testis sections were stained for γ-H2AX, Rad51, and 53BP1 as indicated. A) Both 53BP1 (green) and γH2AX (red) co-stain the XY body in PTIP+ and PTIP− tubules and allow identification of primary spermatocytes in the pachytene stage (arrows). Strong staining of 53BP1 and γH2AX is present in the primary spermatocytes near the basil lamina of the PTIP+ tubule undergoing meiotic recombination. The γH2AX+ and 53BP1+ primary spermatocytes comprise almost the entire cell population present within the PTIP− tubule. Notice no spermatocytes have advanced beyond meiosis I in the PTIP− tubule. B) Similar results were observed when staining for γH2AX (green) and Rad51 (red), a DNA damage sensor, in the PTIP+ and PTIP− tubule.

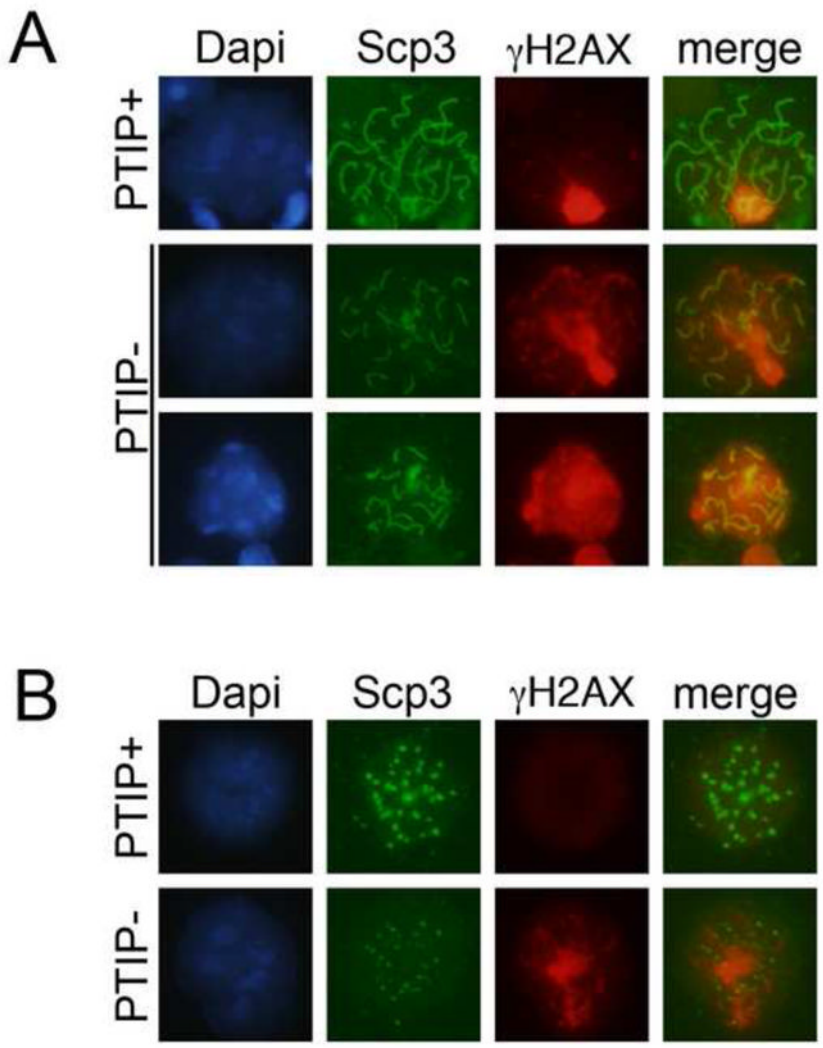

Figure 6. Germ Cell spread Immunostaining for the Synaptonemal Complex and DNA damage

A) Formation of the synaptonemal complex appears similar in the PTIP− and PTIP+ testis, as SCP3-stained filaments (green) can be identified within pachytene cells of both PTIP+ and PTIP− tubules. Notice γH2AX staining, which is restricted to the XY body in PTIP+ cells, persists within the nucleus in the PTIP− pachytene cells, suggesting that dsDNA breaks remain unrepaired as synapse is completing. Additionally, PTIP− cells can also be identified in which γH2AX staining has expanded throughout the nucleus. B) PTIP+ and PTIP− tubules possessing primary spermatocytes in the diplotene stage of meiosis I can be distinguished by SCP3+ nuclear foci and densely stained DAPI+ condensing chromatids. However, γH2AX staining of the PTIP− chromatids persists when compared PTIP+ chromatids. The persistence γH2AX staining in the PTIP− testis suggests incomplete DNA repair and cessation of spermatogenesis during meiosis I.