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Epigenetic regulation of the histone-to-protamine transition during spermiogenesis

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Abstract

In mammals, male germ cells differentiate from haploid round spermatids to flagella-containing motile sperm, in a process called spermiogenesis. This process is distinct from somatic cell differentiation in that the majority of the core histones are replaced sequentially, first by transition proteins and then protamines, facilitating chromatin hyper-compaction. This histone-to-protamine transition process represents an excellent model for the investigation of how epigenetic regulators interact with each other to remodel chromatin architecture. While early work in the field highlighted the critical roles of testis-specific transcription factors in controlling the haploid-specific developmental program, recent studies underscore the essential functions of epigenetic players involved in the dramatic genome remodeling that takes place during wholesale histone replacement. In this review, we will discuss recent advances in our understanding of how epigenetic players, like histone variants and histone writers/readers/erasers, rewire the haploid spermatid genome to facilitate histone substitution by protamines in mammals.

Keywords

Testis; Spermatogenesis; Chromatin Remodeling; Epigenetics; Spermiogenesis; Histone Variants; Transition Protein; Protamine; Histone Methylation; Histone Acetylation

1. Introduction

Epigenetics, referring to the phenotypic inheritance of traits in the progeny without altering the genetic DNA code, is involved in a wide range of biological processes, including germ cell development. During embryonic development, primordial germ cells (PGCs) lineage is committed at embryonic day 6.5 (E6.5) in the mice. They keep proliferating and migrating to the genitor ridge around E10.5~11.5, when the sex identity is determined in the gonad (Fig. 1) (Leitch *et al.* 2013, Feng *et al.* 2014). Meanwhile, they undergo global genome-wide *de novo* reprogramming mainly orchestrated by the DNA demethylases and methyltransferases, such as ten-eleven translocation proteins (TET1/2) and DNMT3A/B, which induce the active DNA de-methylation and methylation, respectively, in both the male and female primordial germ cell populations (Vincent *et al.* 2013, Zhao & Chen 2013).

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After birth, the production of sperm is initiated during male puberty. Spermatogonial stem cells (SSC), localized in a niche close to the basal compartment of seminiferous tubules, undergo multiple rounds of self-renewal and sequentially differentiate into progenitor spermatogonia, and A type and B type spermatogonia (Fig. 1). After postnatal day 8 (P8), a population of committed spermatogonia progressively develops into spermatocytes, which are characterized by a prolonged meiotic prophase I (with four sub-stages − leptotene, zygotene, pachytene and diplotene). Following meiosis, a single spermatocyte gives rise to four haploid spermatids that are interconnected through the cytoplasmic bridges, which subsequently undergo a dramatic morphological change and nuclear chromatin re-organization, through a process known as spermiogenesis (Fig. 1) (Govin *et al.* 2004). In mice, spermatid development is divided into a total of 16 steps on the basis of nuclear elongation and acrosome morphology (the acrosome is the cap-like structure at the anterior of the spermatids). Between Steps 1 and 8, round spermatids maintain highly active transcriptional output, followed by Steps 9–11 (elongating spermatids), in which the nucleus starts to elongate, and transcriptional machinery starts to shut down, and finally by Steps 12–14 (condensed spermatids). Spermatids at Steps 15–16 (spermatozoa) exhibit typical hook-type head morphology ready to be released into the lumen in the seminiferous tubules (Fig. 1) (Meistrich & Hess 2013). During spermatid development, the paternal genome is re-organized and packaged into highly condensed nuclei of the spermatozoa. One of the dramatic changes that occurs lies in the transition from nucleosome-based chromatin to protamine-based chromatin arrays, which facilitates the condensation of sperm heads and protects the paternal DNA from damage and mutagenesis (Fig. 1) (Rathke *et al.* 2014). While the morphological changes throughout all steps of spermatid development are well characterized, the molecular basis underlying the highly orchestrated chromatin re-organization, in particular the transition from histone-to-protamine replacement, remains largely unknown (Rathke *et al.* 2014). This could be ascribed to a lack of experimental approaches that can recapitulate germ cell development *in vitro*. For example, one cannot carry out the loss of function (LOF) or gain of function (GOF) assays in the germ cells in culture, thus rendering traditional approaches, such as RNAi utilized to explore gene functions, impossible. Furthermore, germ cells are extremely heterogeneous as demonstrated by their highly dynamic morphology and distinct gene expression profiles at each stage of development (Small *et al.* 2005, Bao *et al.* 2013). In general, germline-specific genes are activated in a very defined sequence through the different stages of spermatogenesis. The difficulty of studying spermiogenesis is further compounded by the fact that there are also other somatic cell types present together with the germ cells, such as Sertoli cells, Leydig cells and other interstitial cells. Unlike somatic organs, in which a combination of surface protein markers have been identified to be successfully used for FACS purification of specific types of differentiated cells populations, there are currently no surface marker-based methods that can effectively purify the different stages of spermatocytes or spermatids from the testis, although a few protein markers have been applied to isolate SSC-enriched population of germ cells (Shinohara *et al.* 2000, Izadyar *et al.* 2002). Moreover, there remain shortcomings to the currently available methods used for crude purification of different types of germ cells. For instance, there are considerable variations in the widely adopted sedimentation velocity-based purification system, such as 2%-4% BSA sedimentation and centrifugal elutriation, not only between different labs, but also among different operators

(Salva *et al.* 2001, Barchi *et al.* 2009, Chang *et al.* 2011). Therefore, a gene knockout (KO) strategy is the only efficient approach currently being utilized to decipher the gene functions *in vivo* during spermatogenesis. In this review, we will evaluate the current advances in our understanding of epigenetic mechanisms underlying the late stage of spermiogenesis that can be gleaned from mouse KO studies. Specifically, we will focus on how different histone variants and selective histone post-translational modifications are critical for histone displacement, transition protein association and displacement, and protamines deposition during nuclear elongation of haploid spermatids. We will also address the potential roles of the emerging post-translational modifications (PTMs) of transition proteins and protamines *per se*. Epigenetic defects during histone-to-protamine transition not only lead to reduced fertility, but are also potentially transmitted to the next generation. Thus, a better understanding of these epigenetic mechanisms will help us identify more therapeutic targets for male infertility treatment.

2. Histone variants

Distinct from somatic cells, germ cells express many, spatiotemporally-regulated sets of core histones variants, although the majority of histones and these variants are evicted from nucleosomes during the histone-to-protamine stage (Fig. 1) (McCarrey *et al.* 2005, Govin *et al.* 2007, Pradeepa & Rao 2007). Intriguingly, some of the histone variants are exclusively detected in testes and are not present in somatic cells. This could be ascribed to the three intrinsic features that histone variants have: i) Canonical histones are only synthesized during the S-phase of the cell cycle and incorporated into the nucleosomes in a replication-dependent manner, while histone variants are expressed throughout the whole cell cycle (Henikoff & Smith 2015, Venkatesh & Workman 2015). These are especially crucial for germ cells, because the germline undergoes a directional differentiation following the commitment of SSCs to advanced spermatogonial fate. ii) Histone variants tend to consist of varied composition of hydrophobic or hydrophilic amino acids, which are prone to be associated with either “open” or “repressive” chromatin states by stabilizing or destabilizing the mono-nucleosomes. For example, H2A.Z and H3.3 usually coincide with active gene expression, while macroH2A is generally found in transcriptionally inert chromatin (Filipescu *et al.* 2013, Chen *et al.* 2014, Venkatesh & Workman 2015). Interestingly, haploid spermatid development requires activation of a distinct set of germline-specific genes, which are required for acrosome formation (*Acrosin*), and nuclear elongation and flagella assembly (*Spata16*) (Escalier 2006), as well as the repression of the somatic gene expression program. iii) Histone variants comprise special PTM marks (histone code) that can serve as docking sites for effector proteins to confer downstream signaling pathways (Rose *et al.* 2008). In general, some histone variants are present from early meiotic spermatocytes through the late elongated spermatids, while some are exclusively present in the haploid spermatids right before histone-to-protamine replacement (Orsi *et al.* 2009). This suggests that these variants most likely assist in packaging the paternal genome during the later stage of spermatid development.

2.1. H1 variants

In contrast to other types of somatic H1 variants, testis-specific H1 subtype variant (*H1t*) gene transcript was exclusively detectable as early as mid- to late pachytene spermatocytes (Drabent *et al.* 2003), and maintained high expression levels until the elongating spermatid stage (Fig. 1 & Table 1). H1T protein sequence is highly divergent from other subtypes of H1 variant, sharing only 50% homology with its closest homolog within the H1 family. However, its protein amount has been estimated to account for up to 55% of the total linker H1 protein in the histones of germ cells (Drabent *et al.* 1998). *In vitro* studies showed that H1T binds much less tightly to H1-depleted oligonucleosomes than to other somatic H1 subtypes, serving to maintain a relatively decondensed, open chromatin configuration, which is required for meiotic recombination and histone replacement (De Lucia *et al.* 1994, Khadake & Rao 1995). Both the expression pattern of H1T and its biochemical properties support its roles in the transition stage of histone replacement. Unexpectedly, *in vivo* knockout mouse models demonstrated that *H1t*–deficient testes did not exhibit any detectable anomalies in spermatogenesis (Lin *et al.* 2000). *H1t* KO mice were viable, fertile and grew normally in appearance. However, it is impossible to exclude an essential role of *H1t* in spermatogenesis, because other H1 subtypes increased proportionally to compensate for the loss of *H1t* in the KO testes as corroborated by reverse-phase HPLC (Lin *et al.* 2000, Fantz *et al.* 2001).

Another testis-specific H1 variant, H1T2, is exclusively expressed in the haploid spermatids with weak expression from Steps 2 to 4, with its protein levels increased from Steps 5 to 12 (Catena *et al.* 2006). Interestingly, taking advantage of a specific monoclonal antibody generated against the C-terminal sequence, which distinguishes H1T2 from other subtypes of H1, H1T2 was specifically detected at the apical pole region of polarized spermatid nuclei. Unlike *H1t*, constitutive ablation of *H1t2* led to greatly reduced fertility. Histological examination unveiled morphological anomalies observed in >80% sperm recovered from cauda epididymis, abnormal spermatid elongation and defective DNA condensation in the elongating spermatids (Martianov *et al.* 2005, Tanaka *et al.* 2005), suggesting a critical role of H1T2 in the replacement of histones by protamines.

Similar to H1T2, the third member of linker H1 variants, HILS1 (spermatid-specific linker histone H1-like protein), was found to be only expressed in spermatids of mammal testis (Yan *et al.* 2003). Amino acid sequence analysis revealed that it is the least conserved H1 variant and it has evolved rapidly in mammals. *Hils1* is an intron-less gene located in intron 8 of the alpha-sarcoglycan protein-coding gene and accounts for ~10% of total chromatin protein in the mice spermatids (Yan *et al.* 2003, Iguchi *et al.* 2004). Most importantly, HILS1 protein was specifically detected in spermatids between Steps 9–15, a critical time window when haploid spermatids undergo histone-to-protamine transition. Intriguingly, a recent LC/MS analysis uncovered 15 novel PTMs in HILS1 protein sequence, among which a few were further confirmed to be present in elongating and elongated spermatids (Mishra *et al.* 2015). Based on these features, it is speculated that HILS1 will be a critical player during histone replacement, although a *Hils1* KO mouse model has yet to be established.

2.2. H2A and H2B variants

In addition to canonical H2A histone, there are multiple H2A variants present in mammals, including H2A.X, which is involved in double strand breaks (DSBs), H2A.Z that is often enriched in the transcriptional start sites (TSS) of active genes, and the testis-specific H2A variant, TH2A (Fig. 1 & Table 1). The testis-specific histone variant H2B (TH2B), which was initially discovered in mammalian testicular histone extracts in 1975 (Shires *et al.* 1975), is the major variant form of somatic H2B in testis. Using TH2B–specific antibody, both Western blot analysis and immunohistochemistry demonstrated that TH2B accumulates in the leptotene spermatocytes starting at P10 and maintains high expression levels thereafter (Montellier *et al.* 2013, Shinagawa *et al.* 2015). In contrast, the expression levels of somatic H2B dramatically declined by P16 (Fig. 1 & Table 1) (Rao & Rao 1987). This inverse correlation of expression levels between H2B and TH2B in the germ cells implies that TH2B, as opposed to H2B, might play a major role in meiotic and post-meiotic germ cells. An *in vivo* mouse model, which has three consecutive affinity tags at the C-terminus of TH2B protein, generated a dominant-negative effect and renders the male mice infertile (Montellier *et al.* 2013). Interestingly, the C-terminal tag did not cause any obvious fine-tuned effects on meiosis, such as meiotic recombination, meiotic sex chromosome inactivation (MSCI), phosphorylation of H2AX, nor the chromosome-wide H3 displacement by H3.3. In contrast, the TH2B-tag led to severe abnormalities in the elongating spermatids and a dramatically declined number of elongated spermatids (Montellier *et al.* 2013). Nonetheless, it is worthwhile noting that constitutive *Th2b* KO mice are viable and fully fertile without any observed phenotypes, suggesting a compensatory mechanism that rescued TH2B deficiency in testis. Detailed examination shows that in *Th2b*-null testis, somatic H2B was significantly upregulated. Most importantly, by using *in vitro* isotopic labelling in conjugation with the HPLC/MS/MS strategy, they found enhanced arginine methylation occurred at H4R35, H4R55, H4R67, and H2BR72 in spermatids from *Th2b* KO testis (Montellier *et al.* 2013). These data suggest that somatic histones might substitute for the functions of testis-specific histone variants by implementing compensatory PTMs.

Interestingly, the *Th2a* gene and its paralog *Th2b* are juxtaposed on chromosome 17 in mice genome and share a common transcriptional promoter localized between the two genes, suggesting TH2A and TH2B act together in a coordinated fashion in germ cells (Trostle-Weige *et al.* 1982, Huh *et al.* 1991). In line with this observation, biochemical analysis demonstrated both TH2A and TH2B induce chromatin instability. In mice testis, the protein levels of both TH2A and TH2B increase rapidly during first wave of spermatogenesis, starting from the spermatogonia, whereas H2A and H2B exhibit opposite tendencies, displaying decreased expression trends. Simultaneous inactivation of TH2A and TH2B caused a number of defects during meiosis of spermatocytes and chromatin condensation in the spermatids. Specifically, many morphologically abnormal, degenerated spermatids between Steps 10–16 were observed in the double null testes, and they exhibited much lower amounts of transition protein 1 (TP1) and protamine 2 (PRM2) in the nuclear chromatin fraction, as compared to those of the WT spermatids. Given that the mRNA levels of TP1 were comparable between WT and *Th2a*/ *Th2b*-null spermatids, it is conceivable that TH2A/TH2B are required to guide the deposition of transition proteins and protamines (Shinagawa *et al.* 2015).

2.3. H3 variants

In addition to two canonical H3.1 and H3.2 histones that differ by only one amino acid, there are also three additional H3 variants found in mammals: H3.3, H3T and CENP-A (Fig. 1 & Table 1). H3.3 differs from canonical H3.1 variant by five amino acids and is encoded by two gene paralogs in the mammal genomes, *H3f3a* and *H3f3b*. Both encode the same H3.3 protein sequence although they have divergent regulatory elements and untranslated regions at 5' and 3' ends (Szenker *et al.* 2011). While the difference of amino acid sequences between H3.1 and H3.3 appears to be subtle, biochemical studies demonstrated that H3.3 incorporation gives rise to a more open chromatin configuration and facilitates transcription by disrupting the higher-order chromatin structure in spite of its minute effect on the mono-nucleosomal stability. In agreement with this, CHIP analysis showed that H3.3 is generally linked to transcriptionally active regions that are marked by H3K4me3, while H3.1 is typically associated with repressive transcription (Thakar *et al.* 2009, Chen *et al.* 2013). In mice testes, *H3f3a* transcripts are detected at low levels in all types of germ cells, such as spermatogonia, spermatocytes and spermatids. By comparison, *H3f3b* is largely expressed in the meiotic prophase of spermatocytes (Bramlage *et al.* 1997). Targeted ablation of *H3f3b* caused male sterility as a result of apparently reduced levels of H3.3 protein in the meiotic spermatocytes and round/elongating spermatids, suggesting *H3f3a* transcription is subject to a distinct regulatory mechanism that cannot compensate for *H3f3b*'s function. *H3f3b*-null germ cells exhibit reduced H3.3 incorporation, increased apoptosis, elevated H3k9me3 methylation, and disrupted expression of a cohort of spermatogenesis-related genes. Importantly, the TP1 protein was abnormally deposited in asynchronous spermatids while PRM1 protein was not detectable in late (>Step 11) elongated spermatids and in mature sperm, suggesting that *H3f3b* is essential for histone-to-protamine replacement (Yuen *et al.* 2014).

H3T, also known as H3.4, was initially found exclusively in mammalian testis, although more recent studies show that H3T protein is expressed at low levels in other somatic tissues as well (Trostle-Weige *et al.* 1984, Govin *et al.* 2005). Amino acid sequence alignment demonstrates a difference of only five residues between H3.1 and H3T. However, biochemical studies clearly indicate that H3T-incorporated nucleosomes are significantly unstable as compared to canonical H3.1-assembled nucleosomes. Thus, it is thought that H3T plays a critical role during meiotic chromosome re-configuration in spermatocytes and in the nucleochromatin repackaging process in spermatids (Tachiwana *et al.* 2008, Tachiwana *et al.* 2010). However, *H3t* mouse knockout studies have not yet been performed, so the precise *in vivo* role of this histone variant has yet to be established.

3. Histone modifications

Histones are subject to dynamic PTMs that constitute one of the key mechanisms by which the gene expression is tightly controlled in a spatiotemporally specific manner (Kouzarides 2007). Histone modifications can also serve as epigenetic marks that can be faithfully passed on to the offspring (Guerrero-Bosagna & Skinner 2014). Covalent conjugation of different PTMs has a significant impact on the local chromatin conformation by affecting the stability of histone octamer and the interaction between DNA and histones. For example, lysine

acetylation reduces the positive charge of the histones, resulting in their weaker interaction with the negatively charged DNA molecules wrapped around it, thereby increasing the nucleosomal fluidity. On the other hand, diverse PTMs or their combinations (the histone code) can act as "docking sites" for the recruitment of effector molecules (readers), which promote further signaling from the chromatin scaffold (Patel & Wang 2013, Venkatesh & Workman 2015). Thus, distinct histone PTMs, in conjunction with spermiogenesis specific effector modules, provides an attractive means to facilitate the chromatin remodeling and histone-to-protamine replacement.

3.1. Phosphorylation

Histone phosphorylation catalyzed by kinases is involved in a broad range of cellular processes related to diseases and development. Phosphorylated PTMs are commonly observed on serine, threonine and tyrosine residues present on all four core histones. These modifications exert a profound impact on gene expression, either by serving as "docking sites" for effector proteins or by regulatory crosstalk with other PTMs such as methylation and ubiquitination. Throughout the process of spermatogenesis, it has long been observed that core histones display dynamic phosphorylation modifications (Govin *et al.* 2010, Song *et al.* 2011). Among them, H4S1 phosphorylation is highly conserved from Drosophila to mammals. In Drosophila, H4S1ph was detected in meiotic spermatocytes, with an abundance of this mark in spermatids undergoing chromatin compaction, and was thus thought to be a prerequisite for successive histone replacement by basic proteins in late spermatids (Krishnamoorthy *et al.* 2006, Wendt & Shilatifard 2006). More recently, global LC/MS studies identified many phosphorylated residues that exist on the different histone variants expressed in testis (Sarg *et al.* 2009, Pentakota *et al.* 2014, Mishra *et al.* 2015), with some phosphorylation events exclusively present in elongating spermatids. For instance, the Rao group recently identified nine serine phosphorylation sites and one threonine phosphorylation site on HILS1 protein. Immunofluorescent staining showed that antibodies specifically generated against HILS1 and HILS1-Y78p recognized antigens present in elongating and condensing spermatids (Mishra *et al.* 2015). The same group also uncovered novel serine phosphorylated residues in TH2B (Pentakota *et al.* 2014). Despite the discovery of many phosphorylation modifications in the core histones and histone variants of the germ cells, their physiological roles remain largely unstudied.

3.2. Acetylation

Increased acetylation of histone H4 is the earliest, physiological event long known to precede the histone-to-protamine transition and is conserved across multiple species, including mammals and flies (Fig. 1 & Table 1) (Oliva & Mezquita 1982, Grimes & Henderson 1984, Meistrich *et al.* 1992, Zarnescu 2007). It has been hypothesized that histone H4 hyper-acetylation *per se* can unpack the higher-order chromatin structure, so as to help in the eviction of histones, as well as the incorporation of basic proteins. H2A, H2B and H4 were detected to be acetylated in spermatogonia and pre-leptotene spermatocytes, but under-acetylated in meiotic spermatocytes and in round spermatids. However, in the elongating spermatids, three lysine residues (H4K5, H4K8 and H4K16) at the N-terminal tail of H4 become hyper-acetylated independently of DNA replication (Oliva & Dixon 1991, Lahn *et al.* 2002, Govin *et al.* 2004, Eitoku *et al.* 2008, Awe & Renkawitz-Pohl 2010). In

addition, acetylated H4 disappears progressively in an anterior-caudal pattern similar to that of chromatin condensation in spermatids, reinforcing the theory that there is a direct link between H4 hyper-acetylation, histone replacement and chromatin condensation (Hazzouri *et al.* 2000). In support of this notion, histone H4 remains under-acetylated in species, such as carp and winter flounder, where they are not replaced by protamines during spermiogenesis (Kennedy & Davies 1980).

Based on the "histone code" hypothesis, the acetyl moiety groups on lysine residues might be read by an effector module to confer downstream signaling. Indeed, an acetyllysine binding domain-containing protein, BRDT, is found specifically expressed in the spermatocytes and the haploid spermatids (Shang *et al.* 2004). BRDT has two bromodomains, which specifically recognized the acetylated lysine module, belonging to one of the four BET sub-family members conserved in humans and mice (Dhar *et al.* 2012). Structural studies demonstrated that the first bromodomain is sufficient to recognize one or more acetyllysines (Moriniere *et al.* 2009). Genetic deletion of the first bromodomain resulted in male sterility due to the morphologically abnormal development of the spermatids from Step 9 onwards (Table 1) (Shang *et al.* 2007, Dhar *et al.* 2012, Gaucher *et al.* 2012). Specifically, in these mice, starting at Step 9, elongating spermatids were observed with mis-shapened head morphology and highly condensed, small ball-like structures, but surprisingly meiosis progresses normally (Shang *et al.* 2007, Gaucher *et al.* 2012). In support of the functional role of BRDT *in vivo*, a bromodomain-specific small-molecule inhibitor, JQ1, which specifically binds to the acetyl-lysine binding pocket, induced spermatogenic deficiency when applied to mice (Matzuk *et al.* 2012).

Interestingly, there seems to be a well-balanced mechanism that fine tunes the acetylation levels in germ cells. SIRT1 is a member of the sirtuin family of NAD^+-dependent deacetylase, that regulate diverse biological processes. Germ cell-specific *Sirt1* KO mice displayed reduced fecundity and an increased proportion of abnormal spermatozoa (Table 1) (Bell *et al.* 2014). In the elongating and elongated spermatids, while there was no difference in the expression levels of transition proteins and protamines between WT and KO, TP2 protein did not co-localize with DNA in the nucleus, as compared to the overlapped localization of TP2 and DNA in control mice, leading to the condensation defect in the KO testis (Bell *et al.* 2014). However, SIRT1 is likely functional through a H4 acetylation-independent mechanism in that the acetylation levels of H4K5, H4K8 and H4K12 declined in the KO testes.

Another example supporting the notion that histone acetylation levels in the spermatids must be precisely regulated is the ZMYND15 and its protein complex (Table 1). ZMYND15 is a testis-specific protein that is specifically present in the nuclei of spermatocytes and haploid spermatids. *Zmynd15* KO mice exhibited severe developmental defects in the elongating and elongated spermatids causing male sterility without any obvious meiotic arrest (Yan *et al.* 2010). Whereas it remains currently unclear whether there is a defect in the histone replacement in those apoptotic and sloughing spermatids in the *Zmynd15* KO testis, biochemical examination demonstrated that ZMYND15 interacts with histone deacetylases (HDAC)(Yan *et al.* 2010), a class of enzymes that remove acetyl moiety from substrates.

This suggests that a dynamic balance between acetylation and deacetylation of histones is absolutely essential for successful spermiogenesis.

3.3. Ubiquitination

Ubiquitin is a small, 76-residue eukaryotic protein that was originally found to be covalently attached to target proteins to signal their degradation by the 26S proteasome, known as the ubiquitin-proteasome system (Jason *et al.* 2002, Welchman *et al.* 2005). Growing evidence demonstrates that ubiquitin conjugation to the substrates can be interpreted into other diverse signal pathways involved in DNA damage response, cell cycle regulation, and metabolism (Weake & Workman 2008). Ubiquitination is generally a successive process catalyzed by three enzymes. Ubiquitin-activating enzyme (E1) activates ubiquitin, which is subsequently transferred to ubiquitin-conjugating enzyme (E2). Both ubiquitin-activated E2 and protein substrates are specifically recognized by the ubiquitin-protein ligase (E3), which catalyzes the ubiquitin moiety transfer to the substrate proteins. In some cases, multiple rounds of ubiquitination generate polyubiquitin chains on target proteins (Jason *et al.* 2002, Welchman *et al.* 2005), but substrates can also be mono-ubiquitinated. One example is the histone mono-ubiquitination, which occurs on H2A or H2B (Weake & Workman 2008). RNF8 is an ubiquitin E3 ligase that contains a RING domain at the C-terminal. It promotes recruitment of downstream DNA damage response factors at the damage sites by ubiquitinating H2A and H2B. In addition, ubiquitinated H2A and H2B are highly enriched in the XY body (Sex body) in the pachytene spermatocytes and in elongating spermatids. Intriguingly, genetic inactivation of *Rnf8* led to the disappearance of ubiquitinated H2A from the XY body, where MSCI occurred, but had no effect on meiosis of spermatocytes (Table 1) (Sin *et al.* 2012). Instead, loss of *Rnf8* caused significant developmental defects in the late steps of spermatids (Lu *et al.* 2010). Histologically, fewer numbers of elongated spermatids were produced in the *Rnf8*-null testes. The DNA condensation was compromised and there was a wide range of morphological anomalies in the sperm heads and tails due to the defects in the displacement of histones by protamines. The majority of canonical histones were abnormally retained in *Rnf8*-null mature sperm as compared to WT sperm, where only basic PRM1 & 2 proteins were detected, thus suggesting RNF8-mediated ubiquitination of H2A/H2B is necessary for the transition from histones to nucleoprotamines in sperm chromatin (Lu *et al.* 2010). Moreover, there was a dramatic decrease in the acetylation of H4K16, a speculated hallmark for histone-to-protamine replacement, in the *Rnf8*-null germ cells. Preliminary data implies that ubiquitinated H2A/H2B can serve as a “tag” to target the MOF acetyltransferase complex, which is responsible for H4K16 acetylation, and is also highly expressed in the elongating/elongated spermatids (Akhtar & Becker 2000). However, the mechanistic details of RNF8 function in spermatogenesis remain largely unknown (Ma *et al.* 2011).

3.4. Methylation

Protein methylation is observed on both lysine and arginine residues and is catalyzed by methyltransferases, which biochemically transfer the methyl moiety from the principal methyl donor, Ado-Met, to protein substrate residues (Bedford & Richard 2005, Rivera *et al.* 2014). Histone methylation represents one of the most widely studied PTMs that confer profound impact on chromatin dynamics during cell proliferation, differentiation and

transformation. Throughout germline development, it is well-known that members from the protein lysine methyltransferases (PKMTs) display highly dynamic expression patterns. However, their physiological roles during spermatogenesis, in particular during post-meiotic haploid spermatid development, remain largely untapped (Hayashi *et al.* 2005, Godmann *et al.* 2009, Hammoud *et al.* 2014, Samson *et al.* 2014, Zhang *et al.* 2014). An interesting recent study demonstrated that histone H3K79 methylation is exclusively detected in the elongating spermatids preceding the histone-to-protamine transition, and also correlates with histone H4 hyper-acetylation (Fig. 1). This methyl mark is highly conserved in the testes in drosophila, mouse, and human, suggesting it plays an important role during histone displacement (Dottermusch-Heidel *et al.* 2014a, Dottermusch-Heidel *et al.* 2014b). Like lysine methylation, arginine methylation, which is catalyzed by a family of proteins called protein arginine methyltransferases (PRMTs), is also a very common PTM that has been demonstrated to be involved in a diverse range of cellular processes (Bedford & Richard 2005). Arginine methylation is commonly detected on both histone and non-histone proteins. There are 9 members in the PRMT family, which can be categorized into three types: Type I enzymes catalyzes the formation of asymmetrical di-methylarginine (PRMT1, PRMT3, PRMT4, PRMT6 and PRMT8), while Type II enzymes catalyzes the synthesis of symmetrical di-methylarginine (PRMT5 and PRMT9) (Yang *et al.* 2015). Type III enzymes merely catalyzes the mono-methylation of the arginine residues in the substrates (PRMT7). Whereas PRMT8 is only expressed in neuron, other members of the PRMT family all exhibit broad tissue expression distribution, including in the testes (Hong *et al.* 2012). Interestingly, three members (*Prmt1, Prmt4 and Prmt5*) display elevated mRNA expression levels during the first wave of spermatogenesis, with the highest expression levels detected in haploid spermatids, indicating that these enzymes might be critical for ordered histone-to-protamine transition during spermiogenesis (Nikhil *et al.* 2015).

Using advanced mass spectrometry technology, it was recently discovered that a substantial proportion of histones are hypermethylated (on lysine and arginine residues) when purified from humans sperm (Brunner *et al.* 2014). Of those histone methyl marks identified in H3, a few were well-explored in previous studies; e.g., H3K9me3 is a hallmark of repressive heterochromatin, whereas H3K27me3 is especially enriched in developmental gene promoter regions poised for early embryonic gene expression. H3K36 methylation is related to DNA repair and transcriptional elongation, while the function for H3R83 methylation is unknown (Li *et al.* 2013, Pai *et al.* 2014). Also of great interest, is the role of the reported methylation of H4R19 and H4R23 (Brunner *et al.* 2014), which are possibly indicative of new histone marks for histone eviction, and their functional roles awaiting future studies. Furthermore, the methylation of H2BK117 and H2BK121 might reflect that they serve as marks for TH2B replacement, instead of marks for histone displacement, as the majority of the H2B protein is replaced by TH2B during haploid spermatid development (Montellier *et al.* 2013, Shinagawa *et al.* 2015).

3.5. Poly-ADP-ribosylation

Poly-ADP-ribosylation, knowns as PARylation, is also a common PTM observed in higher eukaryotes. This modification is catalyzed by poly(ADP-ribose) (PAR) polymerases (mainly PARP1 and PARP2) and reversed by PAR glycohydrolase (PARG). In response to genotoxic

insults or naturally occurring DNA strand breaks (DSBs), the DNA-binding domains of PARP1 and PARP2 enzymes can efficiently recognize the DSBs. As a result of this DNA engagement, their enzymatic activity is activated, and an ADP-ribose polymer is generated on their target protein. PARylation marks are quite short-lived owing to the rapid and specific removal of PAR by PAR glycohydrolase, which is present ubiquitously in most tissues. PARylation has been functionally linked to a broad array of signaling pathways that impact gene expression, RNA and protein localization, and the maintenance of heterochromatin (Hassa & Hottiger 2008, Gibson & Kraus 2012, Dantzer & Santoro 2013). Naturally occurring physiological DSBs are present in elongating spermatids of Steps 9–13 during the histone-to protamine-based chromatin transition stage (Fig. 1), which involves a more open chromatin structure associated with nuclear supercoiled DNA relaxation during nucleoprotein exchange. Highest protein levels of PARP1 and PARP2 were specifically detected in the nuclei of rat spermatids at Steps 12–14 (Table 1). Consistent with this, immunofluorescent staining using antibodies against ADP-ribose polymer uncovered prominent formation of this PTM in elongating rat spermatids between Steps 11–14, a critical developmental window preceding the nucleoprotein exchange during spermiogenesis (Meyer-Ficca *et al.* 2005). Genetic deletion of either *Parp1* gene ($Parp1^{-/-}$) or one isoform encoded by the *Parg* gene ($Parg110^{-/-}$) led to reduced fertility or sterility, caused by the sperm with nuclear abnormalities, including aberrant nuclear shape, defective nuclear condensation and increased amounts of double strand breaks in the cauda sperm (Meyer-Ficca *et al.* 2009, Meyer-Ficca *et al.* 2015). More strikingly, markedly abnormal retention of core histone H3, testis-specific TH2B, histone linker HIST1H1T (H1T) and HILS1 were found in the KO sperm. These results clearly showed that poly-ADP-ribosylation is an integral component of chromatin remodeling machinery in the elongating spermatids during histone removal (Meyer-Ficca *et al.* 2011a, Meyer-Ficca *et al.* 2011b).

4. Chromatin remodelers

Chromatin remodelers exert dynamic modulation of chromatin architecture to allow access to highly condensed DNA by the transcriptional machinery, as well as to provide access for histone replacement. The Chromodomain-helicase-DNA binding (CHD) proteins belong to a family of chromatin remodelers consisting of nine members, which are characterized by tandem Chromodomains, N-terminal to the ATPase/Helicase domain (Marfella & Imbalzano 2007, Stanley *et al.* 2013). CHD5 is categorized into Class II CHD proteins that are defined by a C-terminal coiled-coil region, as well as a tandem PHD domain, instead of a typical DNA-binding domain. Although present ubiquitously in multiple somatic tissues, CHD5 exhibits preferential expression in the mouse brain. In the testes, CHD5 was specifically detected in the nuclei, resembling the staining pattern of H3K27me3 that is specifically enriched in the heterochromatic chromocenter in both, the haploid round spermatids and elongating spermatids (Table 1) (Li *et al.* 2014a). *Chd5* KO mice were viable and grossly normal in appearance, but KO males had a significantly decreased sperm output and motility, as well as a high proportion of sperm with abnormal head morphology. Histological examination revealed a decreased number of elongated spermatids (Table 1). Strikingly, in the purified elongating and elongated spermatids, aberrant retention of nucleosomal histones and elevated levels of transition proteins (TP1 & 2) and protamines were observed in the KO

testes. Moreover, immunofluorescence also indicated that H4 acetylation was compromised in the elongating/elongated spermatids of KO testes. These data suggest an indispensable role of CHD5 in histone-to-protamine replacement in the developing spermatids in the mouse testes (Li & Mills 2014, Li *et al.* 2014a).

5. Transition proteins and their PTMs

Transition protein 1 and 2 (TP1 & 2) are the basic chromosomal proteins present in a specific time window (condensing spermatids) preceding the protamine deposition in the germline in mammals (Heidaran & Kistler 1987, Heidaran *et al.* 1988). The TP1 protein is only 55 amino acids (~6.2kD) in size, and it is highly conserved across the mammal species. In contrast, TP2 exhibits a high degree of sequence divergence across mammals with only about 50% amino acids homology between mouse and human (Kremling *et al.* 1989, Keime *et al.* 1992). At the protein level, TP1 is more abundantly expressed, accounting for ~ 60% of the basic proteins in elongating spermatids of the mouse, as compared with TP2. *In vitro* biochemical studies demonstrated that TP1 tends to relax the DNA in the nucleosomal core particles by reducing the melting temperature of DNA, whereas TP2 is prone to compact the nucleosomal DNA by increasing the melting temperature, suggesting that TP2 is a DNA-condensing protein while TP1 can promote the eviction of the nucleosomal histones (Singh & Rao 1987, Kundu & Rao 1995, Kundu & Rao 1996, Kolthur-Seetharam *et al.* 2009). Moreover, these functional differences might also reflect their unique roles during mammal spermiogenesis (Levesque *et al.* 1998). Indeed, single targeted deletion of either *Tnp1* or *Tnp2* led to subtle morphological alteration in mouse models (Yu *et al.* 2000, Zhao *et al.* 2001, Shirley *et al.* 2004). For example, *Tnp1* KO mice had no changes in the testes weights or sperm production. Only electron microscopy revealed a rod-like chromatin shape in the step 13 spermatids of *Tnp1* KO mice versus fine chromatin fibrils observed in the wildtype counterpart (Yu *et al.* 2000, Zhao *et al.* 2004a). In addition, the motility of *Tnp1* KO sperm was dramatically reduced, and only ~60% of KO males were fertile. Unexpectedly, the histone displacement appeared to be normal in the *Tnp1*-null mice, presumably because of the feedback upregulation of TP2 protein in the *Tnp1* KO mice (Yu *et al.* 2000). The testicular phenotype for *Tnp2* KO mice was even more mild with normal testes weights, sperm morphology, histone displacement and fertility albeit with smaller litter size (Zhao *et al.* 2001, Zhao *et al.* 2004b). Increased levels of TP1 protein were also observed in *Tnp2*-null spermatids, suggesting both TP1 and TP2 can compensate for each other *in vivo*. In support of this hypothesis, mice with double KO of *Tnp1* and *Tnp2* had a general decrease in sperm morphology, motility, and chromatin condensation. Importantly, there was a severe retention of histones found in the double mutant mice, suggesting both TPs function redundantly yet have their unique roles during spermiogenesis (Meistrich *et al.* 2003, Shirley *et al.* 2004, Zhao *et al.* 2004b).

Unlike histones, TPs are highly enriched for arginine and lysine residues, where trypsin prefers to cut, thus posting a significant challenge to identify the landscape of PTMs of residues in the TPs by mass spectrometry. Early biochemical studies identified that few amino acids of TPs can be modified by kinases *in vitro*, that might play an important role in spermiogenesis (Meetei *et al.* 2002, Ullas & Rao 2003). The global PTMs of TPs have been recently established by Rao's laboratory (Nikhil *et al.* 2015). As summarized in Fig. 2, both

TPs possess a considerable number of PTMs observed on serine, lysine and arginine residues, including acetylation, phosphorylation and methylation. Notably, many residues with PTMs are highly conserved across mammal species, especially those from TP1. Biochemical analyses suggested that PRMT1 and PRMT4 are the predominant arginine methyltransferases involved in the methylation of endogenous TP1 and TP2 protein. *In vitro* methylation assay confirmed PRMT4 can methylate multiple arginine sites in TP2, including R71, R75 and R92 (Nikhil *et al.* 2015). Most importantly, by utilizing two well-validated antibodies against lysine 88 (TP2K88me1) and arginine 92 (TP2R92me) in the TP2 protein, it was found that these two methyl marks displayed similar temporal enrichment in the elongating spermatids in the rats, with a specific enrichment window during nuclear condensation of spermatid development, suggesting both methyl marks play vital roles during histone-to-protamine transition (Nikhil *et al.* 2015).

6. Protamines and their PTMs

Similar to transition proteins, protamines are also basic proteins that are enriched for lysines and cysteine residues. Most mammals have one protamine gene. However, in both humans and mice, there are two genes (protamine 1 - *Prm1* and protamine 2 - *Prm2*) clustered on the same chromosome (Balhorn 2007). Interestingly, distinct from transition protein genes, genetic ablation of one allele of either *Prm1* or *Prm2* results in the sterility of male mice, a phenomenon called “haploinsufficiency” (Cho *et al.* 2001). It has been long proposed that both PRM1 and PRM2 are phosphorylated *in vivo* in human (Pruslin *et al.* 1987). Recently, utilizing a newly developed peptide-based bottom-up MS/MS strategy, a group identified a total of 11 PTMs, including acetylation, phosphorylation and methylation, on the protamines of mice sperm (Brunner *et al.* 2014). Importantly, the S55 of PRM2 was reported to be a candidate phosphorylated substrate residue for CAMK4, a multifunctional serine/threonine protein kinase that was specifically detected in the late stage of spermatids (Wu *et al.* 2000). In the targeted *Camk4* KO male mice, there was a profound impairment in late elongated spermatids. Specifically, transition protein displacement by PRM2 was disrupted as evidenced by the prolonged retention of TP2 and loss of PRM2 association (Wu *et al.* 2000). While direct genetic evidence showing the vital role of S55 phosphorylation in regulating the late spermiogenesis is still missing, *in vitro* kinase assays validated that PRM2 is a *bona fide* substrate for CAMK4 kinase. On the other hand, the loss of merely a single post-translational phosphorylation event in the PRM2, which caused the severe defects in the basic protein exchange in the *Camk4* KO mice, implies that regulated crosstalk takes place between specific PTMs on the protamines and certain effector proteins (like readers and chromatin remodelers), which are essential for the histone-to-protamine transition. Furthermore, we must point out that there are likely additional PTMs that have yet to been discovered due to the long stretches of arginines in the PRM1 protein that make mass spectrometry difficult.

7. Epigenetic readers

As stated above, epigenetic marks on histones are usually interpreted by effector proteins (readers), which transmit the signal further downstream. Simultaneous acetylation of multiple N-terminal lysine residues of H4 (H4K5, H4K8 and H4K12, etc), also known as H4

hyperacetylation, is a well-documented event occurring right before histone displacement that is seen in all species that feature histone-to-protamine replacement (Couppez *et al.* 1987, Lin *et al.* 1989, Meistrich *et al.* 1992). It is plausible that histone acetylation *per se* attenuates the interplay between histone and DNA to facilitate histone removal. More importantly, there is evidence supporting a role for "acetylated H4" and its "reader" BRDT in regulating the removal of histones during late spermiogenesis (Table 1) (Pivot-Pajot *et al.* 2003, Dhar *et al.* 2012). Bromodomains are highly conserved ~110aa motifs found in many genes of all eukaryotes, and they were the first protein module identified that specifically recognized acetylated lysine substrates prior to the recently identified "YEATS" domain, the second conserved motif specifically binding acetylated lysines (Dyson *et al.* 2001, Moriniere *et al.* 2009, Li *et al.* 2014b). Among four mammalian genes (*Brd2*, *Brd3*, *Brd4* and *Brdt*) of BET (bromodomain and extra terminal) family of bromodomain-containing proteins, *Brdt* is unique in that it is exclusively detected in testes, with high expression levels in spermatocytes and haploid spermatids (Shang *et al.* 2004, Shang *et al.* 2007, Gaucher *et al.* 2012). Consistent with its restricted expression pattern, *Brdt* KO mice are viable and exhibit sterility only in the males. Specifically, round spermatids deficient for *Brdt* display a fragmented chromocenter, a heterochromatin structure that forms just after completion of meiosis in round spermatids (Shang *et al.* 2007, Berkovits & Wolgemuth 2011, Gaucher *et al.* 2012). Morphological anomalies can be discerned in the haploid spermatids starting at Step 9, a time point when histone hyperacetylation occurs. Importantly, it has been reported that the BRDT protein co-localized with acetylated H4 chromatin in elongating spermatids (Shang *et al.* 2007), although other studies found that BRDT protein is either not detected in elongating spermatids or only partially overlap with acetylated histone (Shang *et al.* 2007, Berkovits & Wolgemuth 2011, Dhar *et al.* 2012). Interestingly, immunofluorescent studies demonstrated that whereas TPs and protamines proteins are both synthesized, their localization remains in the cytoplasm of the spermatids, leading to the accumulation of both nuclear TH2B and cytoplasmic protamines in the spermatids (Gaucher *et al.* 2012). Collectively, this data supports BRDT having a specific role during the histone-to-protamine transition stage by recognizing acetyl histone marks in the mammalian testes. However, the stepwise regulation of acetylated histones by BRDT, and how BRDT interacts with epigenetic remodeling complexes to facilitate this replacement, remains to be explored.

PYGO2 is another example representative of the crucial roles of epigenetic readers in chromatin remodeling in elongating spermatids (Table 1) (Nair *et al.* 2008, Gu *et al.* 2012). PYGO2 comprises a C-terminal plant homeodomain finger (PHD), which is a highly conserved motif that can recognize the H3K4me3 motif. PYGO2 is specifically detected in the nuclei of elongating spermatids between Steps 8–12 by immunostaining. Genetic abrogation of *Pygo2* resulted in male sterility caused by aberrant post-meiotic gene expression and abnormal nuclear condensation (Nair *et al.* 2008). In addition, the acetylation of H3K9, which normally coexisted with acetylated H4 in elongating spermatids, was disrupted in *Pygo2* KO testis, although the H3K4me3 mark is maintained. Mechanistically, there might be a crosstalk between H3K4me3 and H3K9ac, because PYGO2 "reads" the H3K4me3 mark through its PHD domain and recruits histone acetyltransferase (HAT) activity (Nair *et al.* 2008).

8. Perspective

In somatic cells, DNA is wound around histone octamers twice to form the nucleosomes, the basic unit of chromatin structure that is further coiled into solenoids, giving rise to the higher-degree chromatin packaging inside the nucleus. Distinct from somatic cells, the majority of histone proteins, ~99% of histones in mice and ~90% in human, will be firstly replaced by transition proteins, and finally by the protamines during the late stage of haploid spermatid development (Rathke *et al.* 2014, Venkatesh & Workman 2015). The DNA-protamine structure, known as nucleoprotamines, is super-coiled into toroids in the sperm, leading to the super-higher compaction of DNA in the sperm head. The nucleoprotamine-based chromatin structure inside the sperm nucleus is 6–20 times more condensed than nucleosome-based chromatin structure, and is believed to be essential for paternal procreation: 1) Super-coiled chromatin enables sperm to shed the majority of the cytoplasm, endowing sperm with rapid “swimming capability”, thus allowing them to transit effectively in the female reproductive tract in order to fertilize eggs in the oviduct; 2) Hyper-compaction of the chromatin can effectively protect the genetic DNA materials from physical stress and DNA damage; 3) Emerging studies demonstrate that PTMs found on protamines constitute a unique “protamine code” critical for cell reprogramming during early embryonic development (Miller *et al.* 2010, Castillo *et al.* 2014). Thus, any defects formed during histone-to-protamine transition would not only lead to male sterility, but also might elicit developmental anomalies in the next generation.

Currently, while our knowledge of the details of the step-wise nucleoprotamine replacement during late spermiogenesis is still in its infancy, it is quite clear that nucleohistone displacement by nucleoprotamine is an intricate (germ cell-autonomous) process, involving not only those common histone variants, post-translational histone modifications and chromatin remodelers that are found in somatic cells, but also many testis-specific factors, like testis-specific histone variants (TH2B, H1T , etc) and BRDT (Rathke *et al.* 2014, Venkatesh & Workman 2015). It is known that some epigenetic regulators, such as TH2A/B and H3.3A/B, appear very earlier during meiosis before the histone-to-protamine transition in the elongating spermatids (Fig. 1), suggesting germ cells have a long temporal window to prepare all the histone replacement machinery. After meiosis, the specific PTMs present in canonical histones, TPs and Protamines catalyzed by various “writers” could be recognized by respective epigenetic “readers” in the haploid spermatids. This interlacing network of epigenetic regulator interactions ultimately facilitate the subsequent histone replacement by TPs and finally by Protamines. Obviously, hyper-acetylation of H4 is not the sole histone PTM marking histones for removal in the elongating spermatids. Therefore, it is paramount to decipher the genome-wide histone PTMs that accumulate right before the initiation of histone eviction in the elongating haploid spermatids. Indeed, some new histone modifications, like H3R83me1, H3K117me3 and H2BK121me3 were discovered recently in the residual histones in the mature sperm (Brunner *et al.* 2014). Also supporting this hypothesis, a novel histone lysine PTM, crotonylation (Kcr), was recently discovered specifically marking the genes that are active in post-meiotic spermatids. This might constitute an integral component of the testicular “histone code” for histone removal in the elongating spermatids (Tan *et al.* 2011, Montellier *et al.* 2012). With the recent advance of

more sensitive LC/MS in conjugation with the purification of specific stage of elongating spermatids by centrifugal elutriation, it is conceivable that additional histone PTMs that occur just at the onset of histone displacement in the elongating spermatids will be unveiled in the near future (Castillo *et al.* 2014, Samson *et al.* 2014). Nonetheless, we must bear in mind that, owing to a lack of *in vitro* cell culture systems, it remains difficult to rapidly elucidate the *in vivo* roles of individual PTM as we rely heavily on transgenic mouse models to infer the functional significance of the different histone marks.

Considering the abundance of histone variants and potential for novel histone PTMs present in the germline, our current understanding of how epigenetic signals are transmitted to the downstream signaling by epigenetic "readers" is clearly understudied. For example, the PHF1 protein, which is comprised of an N-terminal Tudor domain and two C-terminal PHD fingers, has been underscored to play important roles in Polycomb repressive complex 2 (PRC2)-mediated transcriptional repression through stimulating H3K27me3 activity by binding to H3K36me3 (Musselman *et al.* 2012, Cai *et al.* 2013, Qin *et al.* 2013). Interestingly, a recent investigation showed that PHF1 also binds to, the H3TK27me3 mark, a testis-specific H3 variant that is mostly expressed in the testis (Kycia *et al.* 2014), suggesting that some well-studied somatic epigenetic "readers" might play distinct but yet-to-be-identified roles specifically in germ cells.

Epigenetic marks are typically deposited by "writers", recognized by "readers" and eliminated by "erasers". while these "players" have been intensively studying in somatic cells, their physiological functions during germ cell development have been largely overlooked. The availability of the "Cre-LoxP" system provides a powerful approach to explore those epigenetic regulators throughout the whole process of germline development. There are multiple germline-specific *Cre* lines available, such as fetal PGC cell-specific *Tnap-Cre* and *Ddx4-Cre* lines, the prospermatogonia-specific *Stra8-Cre* line, spermatocytes-specific *Hspa2-Cre* and *Pgk2-Cre* lines, and haploid spermatids-specific *Aqp2-Cre* and *Tspy-Cre* lines (Smith 2011). These invaluable *Cre* lines, combined with many available floxed mouse models, provide a great opportunity for us to probe the physiological roles of epigenetic players in germline development by generating germ cell stage-specific KO mice.

A deep understanding of epigenetic reprogramming during the late stage of spermiogenesis allows us not only to develop novel approaches for the diagnosis and treatment of fertility in the clinics, but also to discover novel biomarkers for male contraception. In addition, since the haploid male and female gamete genome will be reprogrammed to form the diploid zygotic genome, it is likely that any epigenetic defect is going to be transmitted to the offspring, which might elicit severe birth defects and developmental disorders. Recent studies also strengthen the multigenerational and transgenerational inheritance of parental epigenetic markers across multiple species (Guerrero-Bosagna & Skinner 2014, Aldrich & Maggert 2015, Tang *et al.* 2015), highlighting the extreme importance of understanding the epigenetic mechanisms underlying histone-to-protamine transition during male gamete production in mammals.

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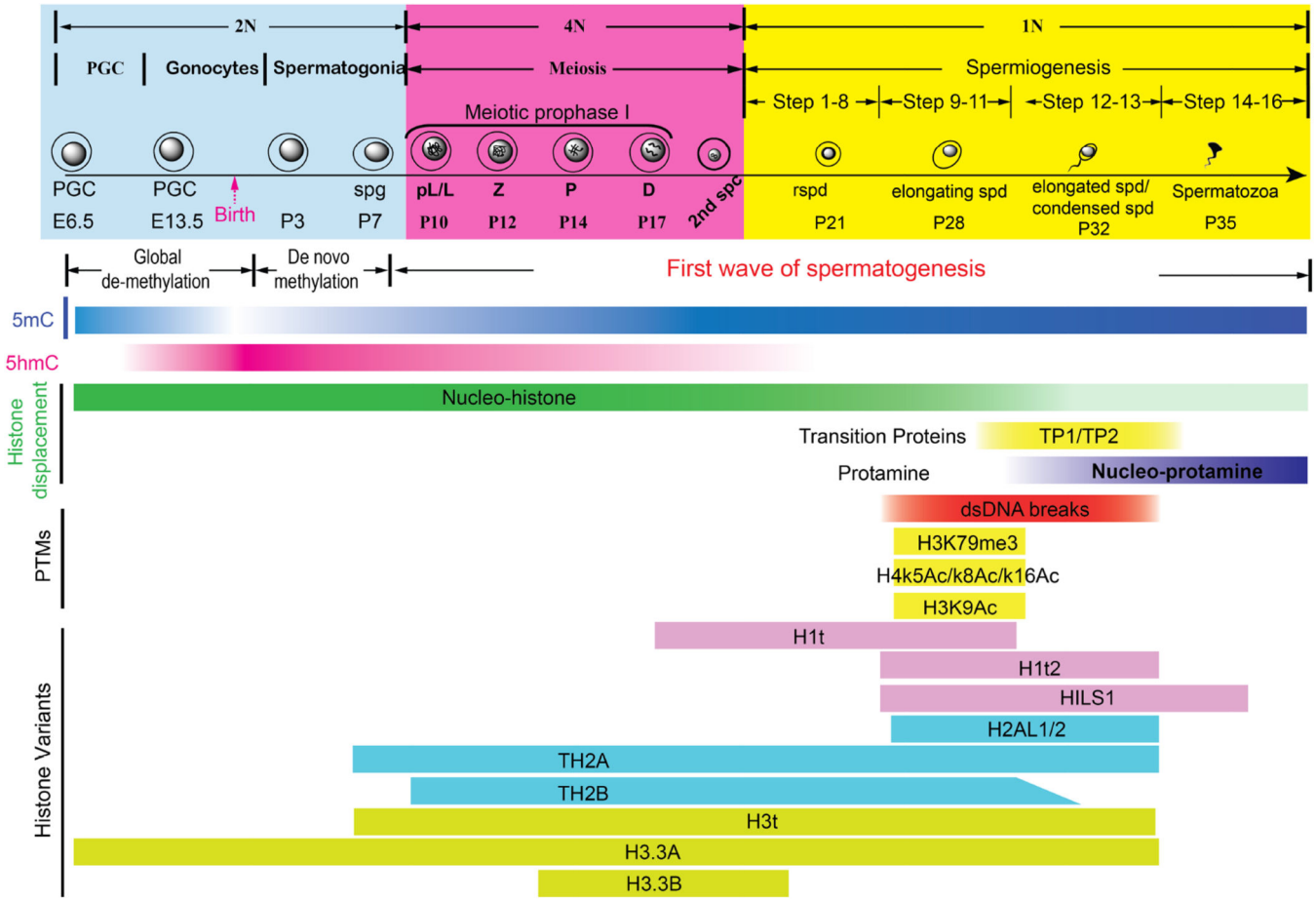

Figure 1.

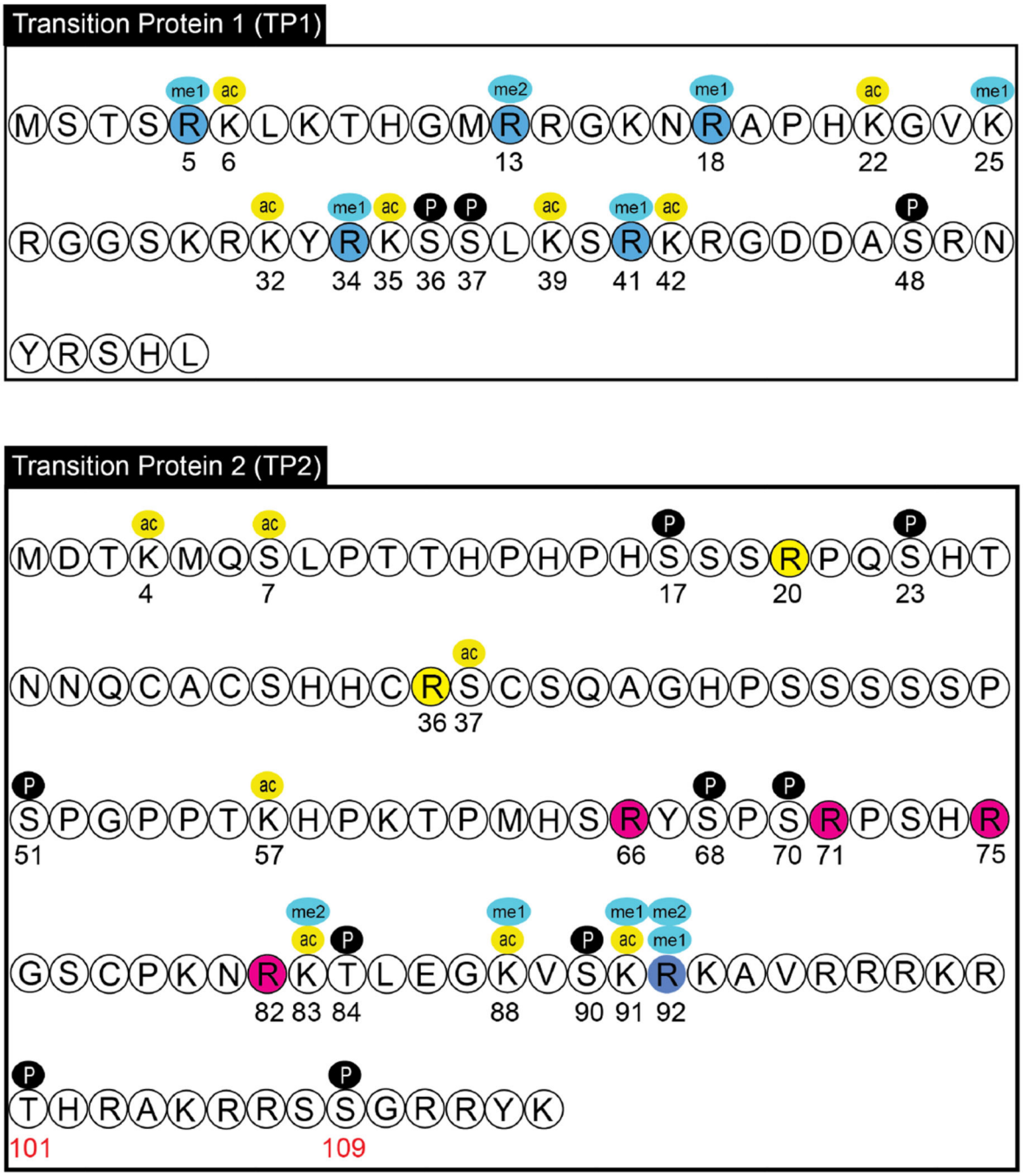

Figure 2.

Table 1

A summarized table showing validated and proposed candidate factors involved in histone-to-protamine transition: spg, spermatogonia; spc, spermatocytes; rspd, round spermatids; espd, elongating/elongated spermatids, NA, not applicable.

	Name	Expression	Localization	KO phenotype	References
Histone variants	H1t	spc, rspd	nucleus	No detectable phenotype (mouse)	(Lin *et al.* 2000)
	H1T2	rspd, espd	nucleus	Reduced fertility, morphological anomalies of sperm, defective DNA condensation (mouse)	(Martianov *et al.* 2005, Tanaka *et al.* 2005)
	TH2A	spg, spc, rspd, espd	nucleus	Double KO of TH2A/TH2B led to infertility, degenerated spermatids, defects in TP1 and PRM2 transportation to nucleus (mouse)	(Trostle-Weige *et al.* 1982, Shinagawa *et al.* 2015)
	TH2B	spc, rspd, espd	nucleus	TH2B KO mice are normal with compensatory enhancement of PTMs. C-terminal tagged TH2B Tg males were sterile. (mouse)	(Montellier *et al.* 2013, Shinagawa *et al.* 2015)
	H2AL1/2	espd	Pericentric regions	?	(Govin *et al.* 2007)
	H3.3	Spg, spc, rspd, espd	nucleus	Testicular atrophy; reduced and abnormal sperm; male infertility; reduced protamine incorporation; (mouse)	(Bush *et al.* 2013, Yuen *et al.* 2014)
	H3t	spc, rspd, espd	nucleus	?	(Tachiwana *et al.* 2008, Tachiwana *et al.* 2010)
Histone modifications	H3K9me1/2	spc, rspd, espd	nucleus	NA	(Liu *et al.* 2010)
	H3K79me3	espd (Step 9~11)	nucleus	NA	(Dottermusch-Heidel *et al.* 2014a, Dottermusch-Heidel *et al.* 2014b)

	Name	Expression	Localization	KO phenotype	References
	H3K9ac	Espd	nucleus	NA	(Nair *et al.* 2008, Steilmann *et al.* 2011)
	H4K5/K8/K12ac	espd (Step 9~)	nucleus	NA	(Oliva & Mezquita 1982, Awe & Renkawitz-Pohl 2010)
	RNF8	spc, spd	nucleus	Normal MSCI, subfertile, defective nucleosome removal (mouse)	(Lu *et al.* 2010, Ma *et al.* 2011)
	SIRT1	spg, spc (Step 1~9)	nucleus	Decreased sperm output, reduced fertility, abnormal sperm head morphology, defects in H4K5/K8/K12ac (mouse)	(Bell *et al.* 2014)
	Camk4	espd	nucleus	Male sterility, Impaired spermatids, retention of TP2, loss of PRM2 (mouse)	(Wu *et al.* 2000)
	PARP1/2	espd	nucleus	Morphologically abnormal sperm; subfertility; retention of core histones (mouse)	(Meyer-Ficca *et al.* 2005, Meyer-Ficca *et al.* 2009)
Chromatin readers/remodelers	PYGO2	espd (Step 8~12)	nucleus	Abnormal nuclear condensation; disrupted H3K9ac; infertility (mouse)	(Nair *et al.* 2008, Gu *et al.* 2012)
	ZMYND15	rspd, espd	nucleus	Male infertility; sloughing of late spermatids (mouse)	(Yan *et al.* 2010)
	CHD5	rspd (Step 4~10)	nucleus	reduced and abnormal sperm; male sub-fertility; failed histone-to-protamine transition (mouse)	(Li *et al.* 2014a, Zhuang *et al.* 2014)
	BRDT	Spc, rspd, espd (?)	nucleus	Abnormal sperm morphology; infertility; retention of TH2B (mouse)	(Shang *et al.* 2007, Berkovits & Wolgemuth 2011)