



Essential Role of Histone Replacement and Modifications in Male Fertility

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Spermiogenesis is a complex cellular differentiation process that the germ cells undergo a distinct morphological change, and the protamines replace the core histones to facilitate chromatin compaction in the sperm head. Recent studies show the essential roles of epigenetic events during the histone-to-protamine transition. Defects in either the replacement or the modification of histones might cause male infertility with azoospermia, oligospermia or teratozoospermia. Here, we summarize recent advances in our knowledge of how epigenetic regulators, such as histone variants, histone modification and their related chromatin remodelers, facilitate the histone-to-protamine transition during spermiogenesis. Understanding the molecular mechanism underlying the modification and replacement of histones during spermiogenesis will enable the identification of epigenetic biomarkers of male infertility, and shed light on potential therapies for these patients in the future.

Keywords: spermiogenesis, histone-to-protamine transition, histone variants, histone modification, male infertility

INTRODUCTION

Spermatogenesis is the process of male gamete production with successive cellular differentiation, which can be subdivided into spermatogonial mitosis, spermatocytic meiosis and spermiogenesis (Roosen-Runge, 1962; Hess and Renato De Franca, 2008). During spermatogenesis, SSC (spermatogonial stem cells) undergo self-renewal and differentiate into spermatogonia that perform meiosis to generate haploid germ cells and ensure the genetic diversity through meiotic recombination (Rathke et al., 2014; Bao and Bedford, 2016). Then, the haploid germ cells undergo spermiogenesis with a distinct morphological change and chromatin compaction in the sperm nuclei to prevent the paternal genome from mutagenesis and damage (Govin et al., 2004; Bao and Bedford, 2016). During the nuclear chromatin re-organization in spermiogenesis, the majority of the somatic histones are firstly replaced by testis-specific histone variants, and transition proteins (TPs) are subsequently incorporated in the nuclei of spermatids, protamines (PRMs) further replace TPs in the late spermatids to pack the genome into the highly condensed sperm nucleus (Rathke et al., 2014; Bao and Bedford, 2016). During the histone-to-protamine transition, the histone variants and specific histone modifications play essential roles by modulating the chromatin compaction and higher-order chromatin structure (**Table 1**) (Boskovic and Torres-Padilla, 2013; Bao and Bedford, 2016; Hada et al., 2017; Hao et al., 2019). Defects in either the replacement or the modification of histones might result in azoospermia, oligospermia or teratozoospermia, which leads to male infertility (**Table 2**). The focus of this review is on recent advances in our knowledge of how epigenetic regulators, such as histone variants, histone modification and their

TABLE 1 | The main histone variants and modifications during the histone-to-protamine transition.

Type	Histone	Name	Period	Function	Reference
Histone variants	H1	H1T	Spermatocytes to elongating spermatids	Maintain open chromatin configuration and no detectable phenotype in H1t-null testis	Delucia et al., 1994; Khadake and Rao, 1995; Drabent et al., 2000; Fantz et al., 2001
		H1T2	Round spermatids and elongating spermatids	Indispensable for the replacement of histones with protamines and chromatin condensation	Martianov et al., 2005; Tanaka et al., 2005
		HILS1	Elongating and elongated spermatids	Contribute to the open chromatin structure	Yan et al., 2003; Mishra et al., 2018
	H2A	TH2A	Spermatocytes to elongated spermatids	Contribute to the open chromatin structure and cooperate with TH2B to regulate TP2 incorporation	Padavattan et al., 2015; Shinagawa et al., 2015; Padavattan et al., 2017
		H2AL2	Elongating and elongated spermatids	Assemble open nucleosomes and allow TPs incorporation	Govin et al., 2007; Barral et al., 2017
		H2A.B	Spermatocytes to round spermatids	Destabilize chromatin and modulate the dynamics of H2AL2 removal and TP1 incorporation and	Soboleva et al., 2012; Soboleva et al., 2017; Anuar et al., 2019
	H2B	Th2B	Spermatocytes, round spermatids and elongating spermatids	Destabilize chromatin and regulate the TPs and PRMs incorporation	Meistrich et al., 1985; Montellier et al., 2013
	H3	H3.3	All types of germ cell	Contribute to the open chromatin structure, modulate TP1 removal and PRM1 incorporation	Bramlage et al., 1997; Couldrey et al., 1999; Van Der Heijden et al., 2007; Thakar et al., 2009; Chen et al., 2013; Tang et al., 2015
H3T		Spermatocytes, round spermatids and elongating spermatids	Contribute to the open chromatin and required for spermatogonial differentiation and ensures entry into meiosis	Tachiwana et al., 2010; Ueda et al., 2017	
Histone modifications	Acetylation	H4K5/8/12ac	Spermatogonia, spermatocytes and elongating spermatids	Essential for destabilization and remodeling of nucleosomes, TPs incorporation	Hazzouri et al., 2000; Gaucher et al., 2012; Qian et al., 2013; Bell et al., 2014; Dong et al., 2017; Ketchum et al., 2018
		Ubiquitination	H4K16ac	Elongating spermatids	Essential for the recruitment of the MOF acetyltransferase complex to modulate H4K16ac and histone removal
	UbH2A		Spermatocytes and elongating spermatids		
	UbH2B		Spermatocytes and elongating spermatids		
	Methylation	H3K4me3	Spermatogonia, spermatocytes, round spermatids and elongating spermatids	Essential for the recruitment of PYGO2 to recognize HAT to facilitate H3 acetylation; recruit PHF7 to catalyze H2A ubiquitination to facilitate the histone removal	Godmann et al., 2007; Song et al., 2011; Nair et al., 2008; Wang et al., 2019
		H3K9me1/2/3	Spermatogonia, Round spermatids and elongating spermatids	Regulate the <i>Tnps</i> and <i>Prms</i> genes expression	Okada et al., 2007
		H3K36me3	Spermatocytes and round spermatids	Regulate the <i>Tnps</i> and <i>Prms</i> genes expression	Zuo et al., 2018
		H3K79me3	Elongating spermatids	Correlate with histone H4 hyperacetylation to regulate histone-to-protamine transition	Dottermusch-Heidel et al., 2014
	Phosphorylation	γ H2AX	Spermatocytes elongating spermatids	Require for the normal quantities of H3, H4 and PRM2 precursor and intermediate	Li et al., 2005; Spiridonov et al., 2005; Jha et al., 2017
		H4S1	Spermatocyte, round spermatids and elongating spermatids	Essential for chromatin compaction and concomitantly histone accessibility	Krishnamoorthy et al., 2006; Zhang et al., 2016
Other	Crotonylation	Elongating spermatids	Facilitate TP1 and PRM2 incorporation	Liu et al., 2017b	
	PARsylation	Elongating spermatids	Require for histone removal and TP1 incorporation	Meyer-Ficca et al., 2009; Meyer-Ficca et al., 2011; Meyer-Ficca et al., 2015	

TABLE 2 | Mouse models related with the histone-to-protamine transition.

Gene	Phenotype	Function	Reference
<i>H1t</i>	Fertility and no spermatogenesis abnormalities	Dispensable for histone-to-protamine transition	Drabent et al., 2000; Fantz et al., 2001
<i>H1t2</i>	Reduced fertility with delayed nuclear condensation and aberrant elongation of spermatids	Indispensable for the incorporation of PRMs and proper chromatin condensation	Martianov et al., 2005; Tanaka et al., 2005
<i>Th2b</i>	Fertility with normal spermatogenesis in <i>Th2b</i> -null mice Infertility with abnormal spermatozoa in TH2B C-terminus modified mice	Destabilize chromatin and regulate TPs and PRMs incorporation	Montellier et al., 2013
<i>Th2a/Th2b</i>	Infertility with accumulated spermatocytes at interkinesis and abnormal spermatozoa	Indispensable for cohesin release and TP2 incorporation	Shinagawa et al., 2015
<i>H2a2</i>	Infertility and the sperm chromatin show a compaction defects	Assemble open nucleosomes and allow TPs incorporation	Barral et al., 2017
<i>H2a.b</i>	Reduced fertility with abnormal spermatozoa	Destabilize chromatin and modulate the dynamics of H2AL2 removal and TP1 incorporation	Anuar et al., 2019
<i>H3f3a</i>	Reduced fertility with dysmorphic spermatozoa	Require for normal development of some spermatids	Tang et al., 2015
<i>H3f3b</i>	Infertility with abnormal spermatozoa and reduced sperm count	Indispensable for spermatogenesis related genes expression, TP1 removal and PRM1 incorporation	Yuen et al., 2014
<i>H3t</i>	Infertility with azoospermia	Require for spermatogonial differentiation and ensures entry into meiosis	Ueda et al., 2017
<i>Epc1</i>	Infertility with abnormal round spermatids to elongating spermatids transition	Require for round spermatids maturation by regulating histone acetylation and TP2 incorporation	Dong et al., 2017
<i>Tip60</i>	The germ cell is arrested at the RS stage	Contribute to round spermatids maturation by regulating histone acetylation and TP2 incorporation	Dong et al., 2017
<i>Sirt1</i>	Reduced fertility with abnormal spermatozoa and decreased sperm count	Require for acetylation of H4K5, H4K8 and H4K12 histone, and TP2 incorporation	Bell et al., 2014
<i>Brdt</i>	Infertility with complete absence of post-meiotic cells in <i>Brdt</i> -null mice Infertility with abnormal spermatids in <i>Brdt^{ABD1/ABD1}</i> mice	Control the chromatin organization and meiotic sex chromosome inactivation; the first bromodomain of BRDT is essential to link histone removal and TPs, PRMs incorporation	Shang et al., 2007; Dhar et al., 2012; Gaucher et al., 2012; Manterola et al., 2018
<i>Pa200</i>	Reduced fertility with abnormal spermatozoa and decreased sperm count	Recognize acetylated histones and mediate the core histones for acetylation dependent degradation through proteasomes	Khor et al., 2006; Qian et al., 2013
<i>Rnf8</i>	Infertility with abnormal spermatozoa and reduced sperm count	Require for histone ubiquitination and modulate H4K16ac to facilitate histone removal and TPs, PRMs incorporation	Lu et al., 2010
<i>Miw1</i>	Infertility with abnormal spermatozoa and reduced sperm count in <i>Miw1</i> D-box mutations mice	Essential for nuclear translocation of RNF8 and facilitates the histone ubiquitination and further histone removal	Gou et al., 2017
<i>L3mbt2</i>	Reduced fertility with abnormal spermatozoa and decreased sperm count	Require for the RNF8-UbH2A pathway and further PRM1 incorporation	Meng et al., 2019
<i>Phf7</i>	Infertility with abnormal spermatozoa and decreased sperm count	Recognize the H3K4me3/me2 and catalyze H2A ubiquitination to facilitate the histone removal	Wang et al., 2019
<i>Pygo2</i>	Infertile with abnormal spermatozoa and decreased sperm count in <i>Pygo2</i> reduced mice	Recognize H3K4me3 and recruit HAT to facilitate H3 acetylation and expression of <i>Prms</i> , <i>Tnp2</i> , and <i>H1fnt</i> .	Nair et al., 2008
<i>Jhdm2a</i>	Infertile with abnormal spermatozoa and decreased sperm count	Control H3K9 methylation at the promoter of <i>Tnp1</i> and <i>Prm1</i> genes and regulate their expression	Okada et al., 2007
<i>Setd2</i>	Infertility with round spermatid arrest	Catalyze H3K36me3 and facilitate the activation of <i>Tnps</i> and <i>Prms</i> genes	Zuo et al., 2018
<i>Tssk6</i>	Infertility with abnormal spermatozoa	Mediate γ H2AX to possess normal quantities of histone H3, H4 and PRM2 precursor and intermediate	Spiridonov et al., 2005; Jha et al., 2017
<i>Cdyl</i>	Reduced fertility with decreased sperm count and motility	Regulate histone crotonylation to facilitate TP1 and PRM2 incorporation	Liu et al., 2017b
<i>Parp11</i>	Infertility with teratozoospermia	Modulate PARsylation to facilitate chromatin condensation	Meyer-Ficca et al., 2015
<i>Parg110</i>	Reduced fertility with poor sperm chromatin quality	Dispensable for histone removal and TP1 incorporation	Meyer-Ficca et al., 2009; Meyer-Ficca et al., 2011
<i>Tnp1</i>	Reduced fertility with subtle abnormal spermatozoa and decreased spermatozoa	Dispensable for histone displacement as the compensation by TP2 and PRM2 precursor	Yu et al., 2000
<i>Tnp2</i>	Reduced fertility with abnormal spermatozoa	Dispensable for histone displacement but necessary for maintaining the normal processing of PRM2 and the completion of chromatin condensation	Zhao et al., 2001

(Continued)

TABLE 2 | Continued

Gene	Phenotype	Function	Reference
<i>Tnp1/Tnp2</i>	Infertile with abnormal spermatozoa and decreased sperm count	Indispensable for PRM2 incorporation and chromatin condensation	Shirley et al., 2004
<i>Ppm1</i>	Infertile with abnormal spermatozoa and decreased sperm count	Indispensable for spermiogenesis and chromatin condensation	Cho et al., 2001
<i>Ppm2</i>	Infertile with abnormal spermatozoa and decreased sperm count		
<i>Camk4</i>	Infertile with abnormal spermatozoa and decreased sperm count	Mediate the phosphorylation of PRM2 and facilitate basic nuclear proteins removal	Wu et al., 2000

related chromatin remodelers, regulate the highly orchestrated chromatin re-organization and facilitate the histone-to-protamine transition during spermiogenesis.

HISTONE VARIANTS

In eukaryotes, nucleosomes are the packing units of DNA, which contain four types of canonical histones (H2A, H2B, H3, and H4) and the linker histone H1 (Talbert and Henikoff, 2010; Kowalski and Palyga, 2012). While canonical histone expression is typically coupled to DNA replication, some non-canonical histones (histone variants) that are distinct from their canonical paralogues in amino acid sequence, are constitutively expressed and have roles in a wide range of processes (Talbert and Henikoff, 2010). Many histones variants are expressed during spermiogenesis and modulate the chromatin structure to facilitate the histone-to-protamine replacement (Mccarrey et al., 2005; Govin et al., 2007). Here, we summarize the recent advances in our understanding of the role of histone variants during the histone-to-protamine transition.

H1 VARIANTS

Linker histones contribute to form and stabilize the higher-order chromatin structure (Bednar et al., 1998). In mammals, there are about 11 different subtypes of histone H1 (Happel and Doenecke, 2009). Among these, H1T, H1T2, and HILS1 are testis-specific H1 variants (Figure 1) (Happel and Doenecke, 2009).

H1T is exclusively detected as early as mid- to late pachytene spermatocytes, and maintains high expression levels in the elongating spermatids (Figure 1) (Drabent et al., 1996; Drabent et al., 2003). Biochemical and biophysical studies found that, distinct from other somatic H1 variants, H1T binds less tightly to H1-depleted nucleosomes, suggesting it may maintain a relatively open chromatin configuration to facilitate histone replacement during spermiogenesis (Delucia et al., 1994; Khadake and Rao, 1995). Unexpectedly, *H1t*-null mice are fertile and exhibit no spermatogenesis abnormalities, and the histone-to-protamine transition in *H1t*-deficient testis is normal (Drabent et al., 2000; Fantz et al., 2001). Although the expression of some canonical subtypes, including H1.1, H1.2, and H1.4, is enhanced in *H1t*-null mice, elevated levels of H1.1 or H1.2 could not be observed in the *H1t*-deficient spermatids (Drabent et al., 2003), indicating

some other types of H1 variants may play redundant roles in the histone-to-protamine transition.

H1T2 selectively localizes at the apical pole in the nucleus of round and elongating spermatids but not in mature spermatozoa (Figure 1) (Martianov et al., 2005). Distinct from H1T, H1T2 is critical for spermiogenesis, as homozygous *H1t2*-mutant males are infertile due to delayed nuclear condensation and aberrant elongation of spermatids. Further analysis shows the protamine levels are substantially reduced in *H1t2*-null spermatozoa (Martianov et al., 2005; Tanaka et al., 2005), indicating H1T2 is necessary for the incorporation of protamines, and proper chromatin condensation during the histone-to-protamine transition.

HILS1 is strongly expressed in the nuclei of elongating and elongated spermatids (Figure 1) (Yan et al., 2003). HILS1 is the least conserved H1 variant, and a poor condenser of chromatin compared with somatic H1, demonstrating the idea that HILS1 may have a distinct role in the histone-to-protamine transition (Yan et al., 2003; Mishra et al., 2018). In *Drosophila*, *Mst77F* encodes a linker histone-like protein that is similar with the mammalian HILS1 protein and expressed in elongating spermatids (Raja and Renkawitz-Pohl, 2005). The disruption of *Mst77F* cause male sterile as producing spermatozoa with malformed heads. Although the histone-to-protamine transition occurs independently of *Mst77F*, the nuclei of spermatid fail to properly condense after the histone-to-protamine replacement in *Mst77F* mutant male (Kimura and Loppin, 2016). However, the functional roles of HILS1 in mammalian spermiogenesis need further investigation.

H2A VARIANTS

Multiple testis-specific H2A variants have been identified in mammals, including TH2A, H2AL1, H2AL2, H2AL3 and H2A.B (Trostleweige et al., 1982; Govin et al., 2007; Soboleva et al., 2012).

TH2A is present and actively synthesized in early primary spermatocytes and gradually disappears during condensation of spermatid nuclei (Figure 1) (Shires et al., 1976; Trostleweige et al., 1982). TH2A could contribute to the open chromatin structure, as crystal structures of nucleosome core particles (NCPs) with TH2A show the H-bonding interactions between the TH2A/TH2A' L1 loops are lost and the histone dimer-DNA contacts are dramatically decreased (Padavattan et al., 2015; Padavattan et al., 2017). Although a *Th2a*-knockout mouse model has yet

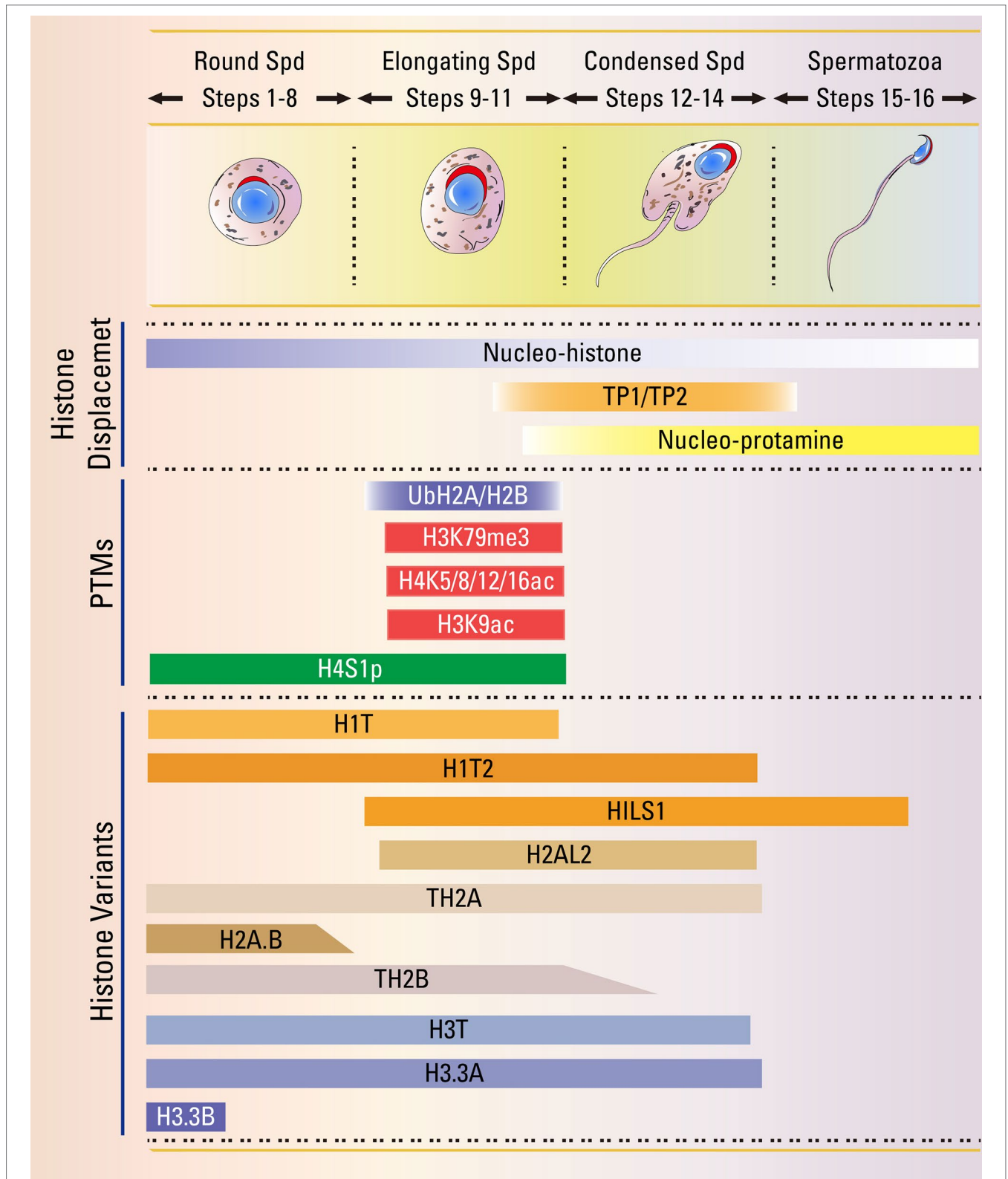


FIGURE 1 | Summary of critical time points and epigenetic events during the histone-to-protamine transition. The haploid germ cells undergo a dramatic morphological change, and nuclear chromatin re-organization starts from round spermatid (Spd) to mature spermatozoa. Detailed studies of the indicated histone variants and histone modification might establish the precise epigenetic events of spermiogenesis.

to be established, mice with knockouts of the testis-specific H2B variants *Th2a* and *Th2b* exhibit male infertility with few sperm in the epididymis (Shinagawa et al., 2015). In this double-knockout mouse, impaired chromatin incorporation of transition protein 2 (TP2) and elevated H2B could be observed in the mutant testis, suggesting the TH2A and TH2B may regulate the function in chromatin dynamics or the total histone levels to facilitate the histone replacement during spermatogenesis (Shinagawa et al., 2015). As the *Th2b*-null male mice show normal spermatogenesis and fertility (Montellier et al., 2013), the histone replacement defect in *Th2a/Th2b* double-knockout male mice is probably caused by the depletion of *Th2a* or their synergistic effect.

In late-developing post-meiotic male germ cells, H2AL2 is specifically expressed in condensing spermatids that correlates with the expression of TPs (Figure 1) (Govin et al., 2007). By comparing *H2al2*-null mice to wild-type mice, H2AL2 was demonstrated to be required to load TPs onto the nucleosome and for efficient PRMs assembly during the histone-to-protamine transition. Additionally, the nucleosome reconstitution assays revealed that the incorporation of H2A.L.2 can drastically modulate the nucleosome structure to facilitate TPs invading the nucleosomes and further transformation (Barral et al., 2017). Thus, H2AL2 could assemble open nucleosomes and allow TPs invading, which further promotes protamine processing and sperm genome compaction.

H2A.B is spatially and temporally regulated during spermatogenesis and detectable from the pachytene stage to the round spermatids (Figure 1) (Soboleva et al., 2012; Soboleva et al., 2017). *In vitro* studies show that H2A.B is able to destabilize chromatin and has unfolding properties to chromatin (Soboleva et al., 2012), indicating H2A.B might promote chromatin reorganization and further histones displacement by TPs. Male *H2a.b*-null male mice are subfertile due to the production of abnormal spermatozoa and clogged seminiferous tubules (Anuar et al., 2019). In *H2a.b*-null elongating spermatids, H2AL2 could not be detected in pericentric heterochromatin, and the replacement of TP1 by protamines appears to be delayed (Anuar et al., 2019). These results indicate H2A.B might modulate the dynamics of H2AL2 and TP1 chromatin incorporation and removal to participate in the histone-to-protamine transition.

H2B VARIANTS

The testis-specific histone variant TH2B is one of the earliest histone variants identified in testis (Shires et al., 1975). TH2B massively replaces somatic H2B during meiosis and remains the main type of H2B in round and elongating spermatids (Meistrich et al., 1985; Montellier et al., 2013), suggesting TH2B might be indispensable for meiotic and post-meiotic germ cells. The crystal structure analysis shows the TH2B could not form the water-mediated hydrogen bonds with H4R78 (Urahama et al., 2014), which may affect the stability of the TH2B nucleosome and facilitate histone replacement during spermiogenesis. In a *Th2b* mutant mouse, which contains modified C-terminus of the TH2B protein and causes a dominant-negative effect, males were infertile and severe abnormalities were seen in the elongating

spermatids, which affected subnucleosomal transitional states during histone replacement (Boskovic and Torres-Padilla, 2013; Montellier et al., 2013). In contrast, *Th2b*-null mice are fertile and show normal spermatogenesis process, indicating a compensatory mechanism that rescues deficiency of TH2B in the histone-to-protamine transition. Indeed, in *Th2b*-null testis, the expression of somatic H2B was significantly increased and elevated methylation of H4R35, H4R55, H4R67, and H2BR72 could be detected in *Th2b*-null spermatids. As H4R35, H4R55, H4R67, and H2BR72 participate in the interactions of histone–DNA and histone–histone, and their methylation may impair these intranucleosomal interactions (Hoghoughi et al., 2018). Thus, the elevated somatic H2B and histone modification in *Th2b*-null spermatids might rescue the *Th2b* deficiency in testis (Montellier et al., 2013; Bao and Bedford, 2016).

In humans, H2BFWT is a testis-specific histone, is synthesized and aggregated in testes, and single nucleotide polymorphisms (SNPs) in this gene is highly associated with male infertility (Churikov et al., 2004; Lee et al., 2009; Ying et al., 2012; Rafatmanesh et al., 2018; Teimouri et al., 2018). And spermatid-specific H2B (ssH2B) and H2BL1 have been identified and are strongly enriched in round or elongating spermatids, similar to that of TPs and protamines (Moss and Orth, 1993; Unni et al., 1995; Govin et al., 2007). However, the functional roles of these H2B variants in the histone-to-protamine transition still need to be further elucidated.

H3 VARIANTS

In addition to the two canonical histones H3.1 and H3.2, three additional H3 variants have been identified and expressed in mammal testes, including H3.3, H3T and H3.5 (Rathke et al., 2014; Bao and Bedford, 2016).

H3.3 differs from canonical H3.1 with five amino acids, is expressed throughout mouse seminiferous tubules, and accumulates in the XY body of spermatocytes (Bramlage et al., 1997; Van Der Heijden et al., 2007). Biochemical and biophysical studies show that H3.3 contributes to an open chromatin configuration and promotes transcription through disrupting the higher-order chromatin structure (Thakar et al., 2009; Chen et al., 2013). H3.3 could be encoded by two gene paralogs in mammal, *H3f3a* and *H3f3b*, and the depletion of either *H3f3a* or *H3f3b* causes male infertility. The disruption of *H3f3a* produces abnormal spermatozoa (Couldrey et al., 1999; Tang et al., 2015), and the loss of *H3f3b* leads to growth defects and death at birth, with surviving *H3f3b*-null males showing complete infertility (Yuen et al., 2014). In *H3f3b*-null germ cells, the TP1 is abnormally deposited in elongating spermatids while PRM1 could not be observed in elongated spermatids and mature spermatozoa, indicating that *H3f3b* is required for chromatin reorganization and the histone-to-protamine transition (Yuen et al., 2014). H3T (H3.4) is exclusively expressed in the spermatocyte and diminishes in the elongating spermatids (Ueda et al., 2017). Biochemical studies clearly indicate that, in the H3T nucleosome, the DNA around the entry-exit regions shows more flexible than that of the H3.1-containing nucleosome, and that

the H3T-containing polynucleosome could form more open configuration than that of H3.1 (Tachiwana et al., 2010). However, the disruption of H3T leads to sterile males with azoospermia, as spermatocyte and spermatids are absent in the *H3t*-null testes (Ueda et al., 2017). Thus, the function of H3T in the later stage of spermatogenesis needs further investigation by using spatially and temporally specific knockout mouse models.

H3.5 is highly expressed in human testis and specifically observed in spermatogonia and spermatocytes (Shiraishi et al., 2017). *In vitro* studies reveal that the H3.5-specific L103 residue, reduces the hydrophobic interaction with histone H4 in the H3.5-containing nucleosome, which corresponds to the H3.3 Phe104 residue (Urahama et al., 2016). H3.5 is significantly reduced in non-obstructive azoospermia (NOA) patients (Shiraishi et al., 2017), whereas the precise roles of H3.5 in spermatogenesis remain largely unknown.

HISTONE MODIFICATION

Covalent conjugation of different post-translational modification of histones has a dramatic effect on the chromatin conformation by affecting the stability of the nucleosome and the histone-DNA interaction (Bao and Bedford, 2016). Many types of histone modifications have been identified to facilitate the histone-to-protamine transition, including acetylation, ubiquitination, methylation, and phosphorylation (Luense et al., 2016).

ACETYLATION

Hyperacetylated histones could facilitate histone eviction, and the acetylation of H2A, H2B, H3, H4 and histone variants have been detected in mammal testis (Grimes and Henderson, 1984a; Grimes and Henderson, 1984b; Oliva and Mezquita, 1986; Oliva et al., 1987). In *Drosophila*, inactivation of histone acetyltransferases by anacardic acid prevents the histones degradation and further a protamine incorporation during spermiogenesis (Awe and Renkawitz-Pohl, 2010), suggesting that histone acetylation is essential for the histone-to-protamine replacement.

H4 acetylation (H4K5ac, H4K8ac, H4K12ac, and H4K16ac) shows a spatial distribution pattern during spermatogenesis and is indispensable for the histone-to-protamine transition (Bao and Bedford, 2016; Ketchum et al., 2018). H4K5ac, H4K8ac and H4K12ac are expressed in spermatogonia and preleptotene spermatocytes, disappear in leptotene to pachytene spermatocytes, reappeared in elongating spermatids, and finally disappeared in condensing spermatids (Figure 1) (Hazzouri et al., 2000; Ketchum et al., 2018). In contrast, H4K16ac could only be detected in elongating spermatids (Figure 1) (Ketchum et al., 2018). *In vitro* analysis shows that H4 acetylation is essential for destabilization and remodeling of nucleosomes, and the incorporation of H4K16ac into nucleosomes prevents the formation of compact chromatin fibers and influence chromatin forming cross-fiber interactions (Tse et al., 1998; Shogren-Knaak et al., 2006; Kan et al., 2009). These findings indicate that H4 acetylation modulates higher order chromatin

structure to facilitate the histone-to-protamine transition. EPC1 (Enhancer Of Polycomb Homolog 1) and TIP60 (Tat-interactive protein, 60 kDa), which are two components for the mammalian NuA4 (nucleosome acetyltransferase of H4) complexes (Figure 2) (Doyon et al., 2004), are co-localized to the nuclear periphery near the acrosomes in both round spermatids and elongating spermatids (Dong et al., 2017). The depletion of either *Epc1* or *Tip60* perturbs histone hyperacetylation, especially H4 acetylation, and affects histone replacement during spermiogenesis (Dong et al., 2017). Another gene that may play a role in acetylation is SIRT1 (Sirtuin 1), a member of the NAD⁺-dependent deacetylase. Germ cell-specific *Sirt1* knockout mice display reduced male fertility due to decreased spermatozoa number and increased proportion of abnormal spermatozoa (Bell et al., 2014; Liu et al., 2017a). In *Sirt1*-null elongating and elongated spermatids, acetylation levels of H4K5, H4K8 and H4K12 are decreased and TP2 could not co-localize in the nucleus, leading to a chromatin condensation defect in *Sirt1*-null spermatozoa (Bell et al., 2014). Thus, SIRT1 may modulate other factors to promote H4 acetylation and the histone-to-protamine transition.

The histone acetylation might be recognized by some chromatin remodelers to confer downstream signaling, and the double bromodomain and extra-terminal domain (BET) proteins have been identified to be critical epigenetic readers binding to acetylated histones and modulating changes in chromatin structure and organization during spermiogenesis (Berkovits and Wolgemuth, 2013). BRDT is a testis-specific BET member protein, which is expressed specifically in spermatocytes and spermatids, and contains two bromodomains that specifically recognize acetylated lysine residues (Shang et al., 2007; Dhar et al., 2012; Manterola et al., 2018). BRDT binds the hyperacetylated histone H4 tail and co-localizes with acetylated H4 in elongating spermatids (Pivot-Pajot et al., 2003; Govin et al., 2006). Remodeling assays have shown BRDT regulated the chromatin reorganization dependent acetylation in round spermatids (Dhar et al., 2012). In mice, the disruption of the first bromodomain in BRDT resulted in male sterility by producing the morphologically abnormal spermatids (Shang et al., 2007). In elongating spermatids with BRDT containing a knockout of bromodomain 1 (BD1), TPs and protamines remained in the cytoplasm and histone replacement did not occur, suggesting BRDT is required for the histone-to-protamine transition by mediating the replacement of acetylated histones (Figure 2) (Gaucher et al., 2012). Furthermore, BRDT was found to bind with the N-terminus of SMARCE1 (SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily E member 1), a member of the SWI/SNF family of ATP-dependent chromatin remodeling complexes (Dhar et al., 2012), indicating BRDT may cooperate with SMARCE1 to facilitate the histone-to-protamine transition during spermiogenesis (Figure 2).

Proteasomes catalyze ATP- and polyubiquitin-dependent protein degradation, and they are made up of a 20S catalytic core particle (CP) and regulatory particle (RP). The 20S CP could be activated by cooperation with various RPs, such as PA700/19S, PA28 α / β , PA28 γ , and PA200 (Stadtmueller and Hill, 2011). PA200 is highly expressed in the testis, and the disruption of PA200

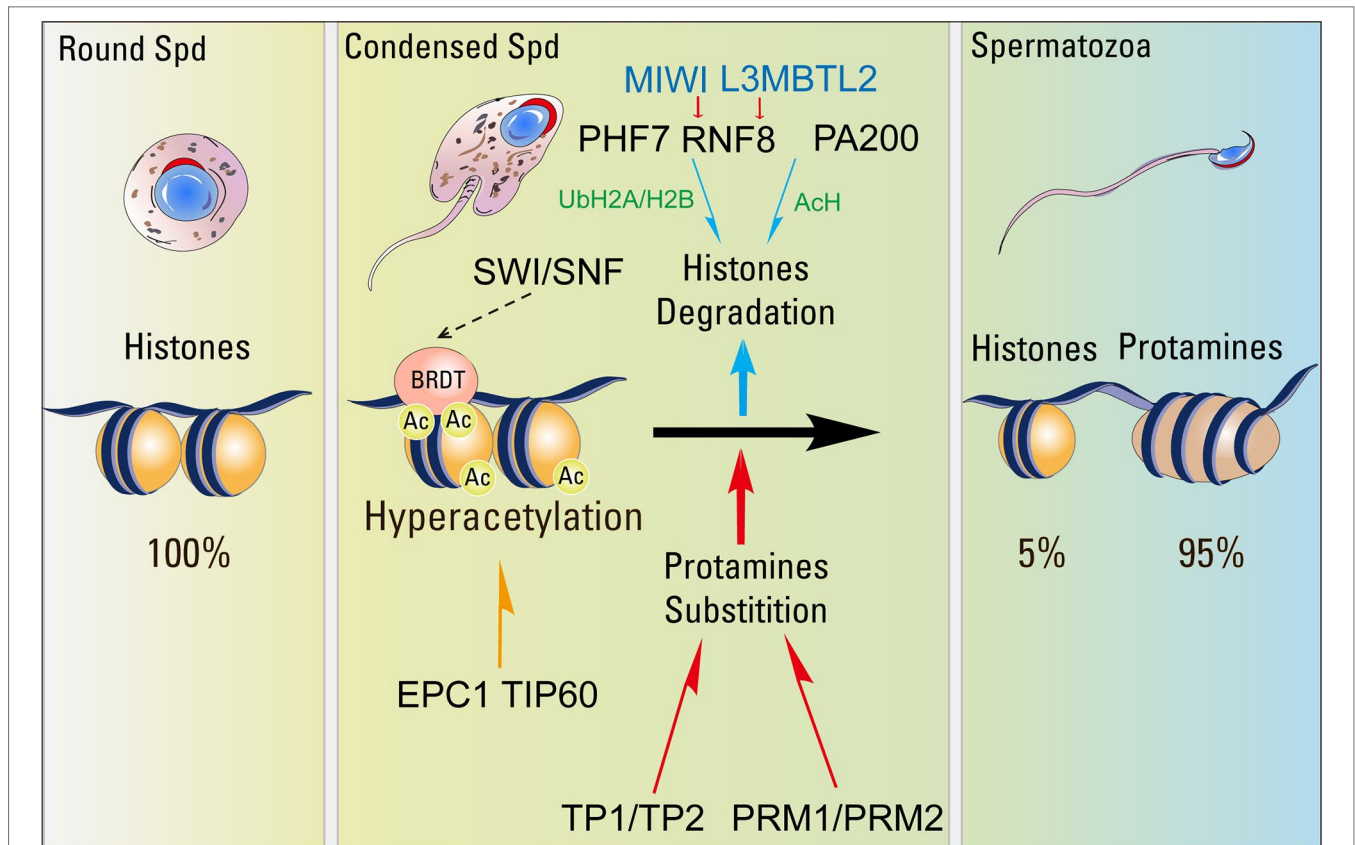


FIGURE 2 | The key factors related to the histone-to-protamine transition. Global incorporation of various H2A, H2B and H3 histone variants creates highly unstable nucleosomes, which then undergo histone hyperacetylation by EPC1/TIP60 or some other nucleosome acetyltransferase complexes. Acetylation at critical lysines further destabilizes the nucleosomes, while tail acetylation generates a platform for the recruitment of BRDT. BRDT interacts with the SWI/SNF family protein then starts the process of histone eviction and replacement by TPs. Evicted acetylated histones would then be recognized by PA200 and degraded by proteasomes during spermatogenesis. RNF8 could catalyze the ubiquitination of H2A. Ubiquitinated H2A and H2B control H4K16ac by regulating the association of MOF to the chromatin and facilitates histone removal in elongating spermatids. MIWI binds to RNF8 in the cytoplasm of early spermatids (Spd) through a Piwi-interacting RNA (piRNA)-independent manner, and promotes the nuclear translocation of RNF8 in late spermatids to catalyze histone ubiquitination and trigger histone removal. L3MBTL2 could interact with RNF8 and facilitate RNF8-dependent histone ubiquitination-related histone removal. PHF7 could recognize the H3K4me3/me2 and catalyze H2A ubiquitination to facilitate histone removal in elongating spermatids.

results in male infertility and severe defects in spermatogenesis (Ustrell et al., 2005; Khor et al., 2006). During spermiogenesis, PA200 regulatory could directly recognize acetylated histones through a bromodomain-like module and promote their ubiquitin-independent degradation. In *Pa200*-null spermatids, results showed that H2B, H3 and elevated H4K16ac could be detected at the end of the elongation stage (Qian et al., 2013). Thus, PA200 specifically recognizes acetylated histones and mediates the core histones for acetylation dependent degradation through proteasomes during spermatogenesis (Figure 2).

UBIQUITINATION

Ubiquitin is a 76 amino acid protein that is attached to target proteins to regulate several cellular processes, such as protein degradation, cell signaling, autophagy, DNA damage responses and so on (Hershko and Ciechanover, 1998; Pickart, 2001; Welchman et al., 2005; Komander and Rape, 2012). Ubiquitinated H2A and

H2B are enriched in spermatocytes and elongating spermatids (Chen et al., 1998; Baarends et al., 1999). RNF8 is an ubiquitin E3 ligase that participates in DDR (DNA damage repair) by catalyzing the ubiquitination of H2A to promote the recruitment of some DNA damage response factors on the damage sites (Ma et al., 2011). The disruption of *Rnf8* causes significant late-stage developmental defects in spermatids due to problematic histone-to-protamine replacement, with the canonical histones being detectable in *Rnf8*-deficient mature spermatozoa (Lu et al., 2010). In *Rnf8*-null mice, both ubiquitinated H2A and H2B are decreased in the testes and H4K16ac is dramatically decreased as well (Lu et al., 2010). Further studies showed that ubiquitinated H2A and H2B were essential for the efficient recruitment of the MOF (males absent on the first) acetyltransferase complex, which is highly expressed in elongating spermatids and responsible for H4K16 acetylation in the chromatin (Akhtar and Becker, 2000; Lu et al., 2010). Thus, RNF8 catalyzed histone ubiquitination could modulate H4K16ac by regulating the localization of MOF on the chromatin and facilitate histone removal in the elongating spermatids.

The RNF8-dependent histone ubiquitination during spermiogenesis could also be modulated by PIWI protein, which is specifically expressed during germline development and enlists piRNAs (Piwi-interacting RNAs) to repress TE (transposable elements) and protect the germ cell genome integrity (Juliano et al., 2011; Siomi et al., 2011; Gou et al., 2017). In mice, *Miwi*, *Mili*, and *Miwi2*, the *Piwi* paralogs, have been identified in the testis and are required for male fertility (Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004; Carmell et al., 2007). During spermiogenesis, MIWI binds to RNF8 in the cytoplasm of early spermatids through a piRNAs-independent manner, and APC/C mediated MIWI degradation in late spermatids is essential for nuclear translocation of RNF8, which catalyzes histone ubiquitination and further facilitates histone removal (Gou et al., 2017). In both humans and mice, mutations in the conserved destruction box (D-box) of HIWI and MIWI proteins, which lead to their stabilization, cause male infertility due to impaired histone ubiquitination and histone-to-protamine transition (Gou et al., 2017). Except MIWI, *L3MBTL2* (Lethal 3 malignant brain tumor like 2), a member of the MBT-domain proteins that is implicated in chromatin compaction, could also interact with RNF8. The depletion of *L3mbtl2* in germ cells affected male fertility by producing abnormal spermatozoa and the decrease of sperm counts. *L3mbtl2* deficiency also caused the reduction of in levels of the RNF8 and histone ubiquitination in elongating spermatids, which further influenced the PRM1 deposition and chromatin condensation during spermiogenesis (Meng et al., 2019).

PHF7 (PHD Finger Protein 7), which contains PHD (plant homeodomain) and RING finger domain, has been identified as a novel H2A ubiquitination E3 ligase in mouse testis (Hou et al., 2012; Wang et al., 2019). PHF7 is specifically located in the elongating spermatid nuclei, and the disruption of *Phf7* led to male mouse infertility as reduction of sperm count and the increased proportion of abnormal spermatozoa (Wang et al., 2019). PHF7 could recognize the H3K4me3/me2 through its PHD domain and catalyze H2A ubiquitination by its RING domain. In *Phf7*-null spermatids, the H2A ubiquitination was dramatically decreased that resulted in the histone retention and protamine replacement defect (Figure 2) (Wang et al., 2019). Therefore, PHF7 has dual roles during the histone-to-protamine transition that works as an epigenetic reader by recognizing H3K4me3/me2 and as an epigenetic writer through catalyzing H2A ubiquitination to promote histone removal.

METHYLATION

Multiple histone methylation have been identified in elongating spermatids, for instance H3K4me2, H3K4me3, H3K9me2, H3K9me3, H3K27me3, H3K79me2, and H3K79me3 (Godmann et al., 2007; Song et al., 2011; De Vries et al., 2012; Dottermusch-Heidel et al., 2014). Among them, the methylation of H3K4 and plus acetylation might help to achieve a more-open chromatin configuration, whereas H3K9 and H3K27 methylation are known to be associated with a more-repressed chromatin configuration (Rathke et al., 2014), indicating a balance of “opened” and “closed” chromatin regions during the histone-to-protamine transition. As

some histone methyltransferases and demethylases are detectable during spermiogenesis (Godmann et al., 2007; Liu et al., 2010; Ushijima et al., 2012), the histone methylation may be dynamically regulated in testis. Although few mouse models exist that allow precise detection of methylation activity that directly regulates histone replacement during spermiogenesis, some studies have revealed that histone methylation may modulate the histone-to-protamine transition through some other ways. PYGO2 (Pygopus homolog 2) comprises a C-terminal PHD finger, which can recognize the H3K4me3 and is specifically located in the elongating spermatid nuclei. In mice, the reduction of *Pygo2* influenced the *Tnp*, *Prm* genes expression and caused the abnormal nuclear condensation, which further led to male sterility (Nair et al., 2008). Furthermore, PYGO2 associates with a histone acetyltransferase (HAT) activity, and the acetylation of H3 is disrupted in *Pygo2* reduced elongating spermatids (Nair et al., 2008), indicating PYGO2 may recognize H3K4me3 through its PHD domain and could recruit HAT to facilitate H3 acetylation and further histone-to-protamine transitions. As described before, PHF7 could recognize the H3K4me3/me2 and catalyze H2A ubiquitination to facilitate the histone-to-protamine transitions (Wang et al., 2019). The predominant histone methyltransferase SETD2 (SET domain-containing 2) catalyzes the H3K36me3, and knocking out *Setd2* in mouse germ cells causes aberrant spermiogenesis, resulting in complete male infertility. Moreover, the disruption of SETD2 causes complete loss of H3K36me3 and impaired activation of *Tnp* and *Prm* genes (Zuo et al., 2018), indicating H3K36me3 may regulate the histone-to-protamine transition by activating *Tnp* and *Prm* genes expression. Contrarily, JHDM2A (JmjC-domain-containing histone demethylase 2A) is an H3K9me2/1-specific demethylase. The loss of *Jhdm2a* in mice exhibits post-meiotic chromatin condensation defects and leads to male infertility. Although global H3K9 methylation has no effect in *Jhdm2a*-null testis, JHDM2A directly binds to and controls H3K9 methylation at the promoter of *Tnp1* and *Prm1* genes, which further regulates the sperm genome packaging and chromatin condensation (Okada et al., 2007).

PHOSPHORYLATION

Histone phosphorylation is involved in various cellular processes (Rossetto et al., 2012; Bao and Bedford, 2016), and dynamic histone phosphorylation have been observed during spermatogenesis (Govin et al., 2010; Bao and Bedford, 2016). The phosphorylation of histone H2AX at residue Ser139 (γ H2AX) plays important roles in many biological processes, such as meiotic recombination and male sex chromosome inactivation in germ cells (Li et al., 2005). γ H2AX is detectable in elongating spermatids, and TSSK6 has been identified to be responsible for the H2AX phosphorylation during spermiogenesis (Jha et al., 2017). In mice, targeted deletion of *Tssk6* leads to male sterility caused by the impairment in morphology and motility of spermatozoa (Spiridonov et al., 2005). In spermatozoa, the loss of TSSK6 blocks γ H2AX formation, resulting in elevated H3, H4 and the precursor and intermediate of PRM2 (Jha et al., 2017). These results indicate that TSSK6 may mediate γ H2AX to participate in the histone-to-protamine transition. H4S1 phosphorylation is highly expressed in mouse spermatocyte,

round and elongating spermatids (Krishnamoorthy et al., 2006; Zhang et al., 2016). H4S1 phosphorylation has been found to be essential for chromatin compaction and concomitantly histone accessibility (Krishnamoorthy et al., 2006; Wendt and Shilatifard, 2006), suggesting that H4S1 phosphorylation is required for histone replacement during spermiogenesis. Outside the canonical histones, many phosphorylated residues have been identified, using mass spectrometry analyses, that exist on different testis-specific histone variants, such as H1T, HILS1, TH2A, TH2B (Sarg et al., 2009; Pentakota et al., 2014; Mishra et al., 2015; Luense et al., 2016; Hada et al., 2017). Although many core histones and histone variants phosphorylation have been identified in germ cells, their physiological roles need further investigation.

OTHER MODIFICATIONS

A variety of histone lysine modifications have been identified, including butyrylation, crotonylation, malonylation, propionylation, and succinylation (Tan et al., 2011; Sabari et al., 2017). Kcr (Lysine crotonylation) is a newly identified histone modification and is detectable in elongating spermatids, which regulated testis-specific genes activation in post-meiotic germ cells (Tan et al., 2011). The CDYL (chromodomain Y-like) protein, which contains a C-terminal CoAP domain that interacts with CoA to achieve its crotonyltransferase activity, may suppress the histone Kcr by converting crotonyl-CoA to β -hydroxybutyryl-CoA. Accordingly, *Cdyl*-deficient male mice show reduced fertility, decreased epididymal sperm count and sperm cell motility, and dysregulated histone Kcr (Liu et al., 2017b). In the *Cdyl*-deficient mouse testes, further analysis showed that the elevated TP1 and PRM2 were localized in a chromatin-free regions (Liu et al., 2017b), suggesting that histone crotonylation is essential for the histone-to-protamine transition during spermiogenesis.

Poly-ADP-ribosylation (PARsylation) is a common protein PTM (post-translational modification) observed in higher eukaryotes and involved in many different fundamental cellular functions. All of core histones and the linker histone H1 can be ADP-ribosylated (Gagne et al., 2006; Messner and Hottiger, 2011), which could be catalyzed by poly(ADP-ribose) polymerases, such as PARP1 and PARP2, and resolved by PARG (PAR glycohydrolase) (Gibson and Kraus, 2012). The PARP1, PARP2 and PARsylation proteins are specifically detected in elongating spermatids (Meyer-Ficca et al., 2005), and the perturbed PARsylation causes reduced male fertility with abnormal retention of core histones, H1T and HILS1 in mature spermatozoa (Meyer-Ficca et al., 2009; Meyer-Ficca et al., 2011; Meyer-Ficca et al., 2015). Thus, PARsylation is essential for the histone-to-protamine replacement, yet the precise PARsylation histone sites need further characterization.

TRANSITION PROTEINS

Between histone eviction and protamine incorporation in the nuclei of spermatids, about ninety percent of the chromatin components consist of TPs, which are arginine- and lysine-rich proteins encoded

by *Tnp1* and *Tnp2* (Meistrich et al., 2003). However, the functional roles of each TP are still controversial (Rathke et al., 2014). TP1 could reduce the melting temperature of DNA and relax the DNA from core particles of nucleosome, whereas TP2 tends to compact the nucleosomal DNA by increasing its melting temperature, indicating TP2 may promote DNA condensation while TP1 facilitates the eviction of the histones (Singh and Rao, 1988; Akama et al., 1998; Kolthur-Seetharam et al., 2009; Rathke et al., 2014). However, a separate study that shown that neither TP1 nor TP2 leads to the conformation changes in supercoiled DNA (Levesque et al., 1998). These differences might reveal their unique roles during mammal spermiogenesis, as single knockout of either *Tnp1* or *Tnp2* leads to little morphological alteration of spermatozoa in mouse models. Elevated TP2 and TP1 proteins could be observed in *Tnp1*-null and *Tnp2*-null spermatids, respectively (Yu et al., 2000; Zhao et al., 2001). Thus, TP1 and TP2 may compensate for each other *in vivo*. Indeed, *Tnp1* and *Tnp2* double-knockout mice show severe abnormal spermiogenesis with a general decrease in sperm motility and abnormal sperm morphology (Shirley et al., 2004). The chromatin condensation is perturbed in the *Tnp1* and *Tnp2* double-knockout mice as severe histones retention is detectable, indicating TPs function redundantly yet have unique roles in the histone-to-protamine transition (Shirley et al., 2004; Zhao et al., 2004; Bao and Bedford, 2016).

PROTAMINES

Protamines are basic proteins that replace TPs in late spermatids (Rathke et al., 2014; Bao and Bedford, 2016). Two protamine genes (*Prm1* and *Prm2*) localize on the same chromosome in both humans and mice (Balhorn, 2007). Protamines tightly interact with DNA *via* a central arginine-rich DNA-binding domain (Balhorn, 2007). Unlike *Tnp* genes, the disruption of either *Prm1* or *Prm2* leads to the male infertility (Cho et al., 2001). Protamines have multiple PTM sites, and a total of 11 PTMs have been identified on the protamines of mouse spermatozoa, including acetylation, phosphorylation and methylation (Brunner et al., 2014). One site of interest is PRM2 S55, which is a candidate phosphorylated substrate residue of CAMK4 (Ca²⁺/calmodulin-dependent protein kinase IV) (Wu et al., 2000). Targeted *Camk4* knockout male mice are infertile, and the transition protein displacement by PRM2 is perturbed as a specific loss of PRM2 and prolonged retention of TP2 in *Camk4*-null spermatids. *In vitro*, PRM2 could be phosphorylated by CAMK4, implicating CAMK4 mediated PRM2 phosphorylation is required for the protamine incorporation during spermiogenesis (Wu et al., 2000). Thus, the specific post-translational modifications on protamines may also be essential for the histone-to-protamine transition.

CONCLUSION AND FUTURE PERSPECTIVES

During the histone-to-protamine transition, many epigenetic regulators work together to facilitate paternal genome re-organization and packaging into the highly condensed nuclei of spermatozoa,

through histone variation, specific histone modification and their related chromatin remodelers. Any defects during the histone-to-protamine transition would lead to male infertility (Bao and Bedford, 2016). While the morphological changes during spermiogenesis are well characterized, the precise molecular mechanisms underlying the chromatin re-organization, in particular the transition from histones to protamines, are still unclear. It's difficult to characterize the dynamic processes that occur during histone eviction, transition protein incorporation and protamine insertion. Moreover, 10% of the spermatozoa population in the epididymis has not yet completed the histone-to-protamine transition (Yoshida et al., 2018). These problems may be ascribed to a lack of experimental methods, which could fully recapitulate germ cell development *in vitro*. Further physiological insights may be gained by developing an *in vitro* germ-cell culture system that more accurately recapitulates the *in vivo* histone-to-protamine transition.

Many histone variants modulate histone replacement by regulating the chromatin structure; therefore, nucleosomes containing these histone variants often maintain a relatively decondensed and open chromatin configuration, facilitating histone replacement during spermiogenesis. The redundant function of histone variants in modulating chromatin configuration ensures that defects in some histone variants have a limited effect on spermatogenesis. Indeed, some mutant histone variants in mouse models are dispensable for male fertility, and mice may show elevated levels of compensatory histones or histone variants. However, the redundant function of histone variants makes it difficult to explore the precise role of each histone variant in histone replacement.

Although many histone modifications have been identified during the histone-to-protamine transition, many studies are descriptive and correlative. The direct manipulation of histone modification sites to reveal function is still urgently needed. With the development of gene editing tools, for example the CRISPR/Cas9 system, mouse models disrupting these histone modifications may be generated and used to elucidate function and *in vivo* relevance in the future. The following open-ended questions still need to be answered to provide in-depth investigation in the field.

- (1) In addition to the histone variants and modifications mentioned above, what other novel histone variants and modifications participate in the histone-to-protamine transition? How can we identify them?
- (2) How and where do histone variants replace canonical histones? What signal is needed to initiate replacement?
- (3) As histone variants and modifications are identified that participate in the histone-to-protamine transition, how do we

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establish an epigenetic modulating network for this process? Which type of histone code is the initiating code?

- (4) Histone hyperacetylation works as a determining event during the histone-to-protamine transition. Is this histone hyperacetylation an initial signal or an indirect consequence of prior events?
- (5) What's the relationship between these histone variants and modifications? What's the mechanism underlying the cross talk between them?
- (6) Chromatin assembly is modulated by histone chaperones or other chromatin remodelers. What's the functional role of histone chaperones during the histone-to-protamine transition? Which histone variants or modifications send a signal to the chaperones?
- (7) How do the transition proteins replace the histone? How do protamines replace the transition proteins? What are the detailed functional roles of transition proteins?
- (8) Are there still post-translational modifications that need to be discovered in order to more accurately describe how histone modification plays a role in spermatozoa maturation?

These questions and their underlying ideas need further investigation and refining to help us more thoroughly understand the complex molecular relationships and exact regulating mechanisms of the histone-to-protamine transition.

AUTHOR CONTRIBUTIONS

WT and HG wrote the manuscript and drew the figures; CL and WL proposed the idea and revised the manuscript. All authors listed have made a substantial, direct and intellectual contribution to the work and approved it for publication.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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