REPRODUCTION
REVIEW

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

Jianqiang Bao and Mark T Bedford

Department of Epigenetics and Molecular Carcinogenesis, The University of Texas MD Anderson Cancer Center, Smithville, Texas, USA

Correspondence should be addressed to Jianqiang Bao; Email: JBao2@mdanderson.org

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 by 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. Although 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 discuss recent advances in our understanding of how epigenetic players, such as histone variants and histone writers/readers/erasers, rewire the haploid spermatid genome to facilitate histone substitution by protamines in mammals.

Reproduction (2016) **151** R55–R70

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, the primordial germ cell (PGC) lineage is committed at embryonic day 6.5 (E6.5) in mice. They keep proliferating and migrating to the genital ridge around E10.5—E11.5, when 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 10–11 translocation proteins TET1/2 and DNMT3A/B, which induce the active DNA demethylation and methylation, respectively, in both male and female primordial germ cell population (Vincent *et al.* 2013, Zhao & Chen 2013).

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 the seminiferous tubules, undergo multiple rounds of self-renewal and sequentially differentiate into progenitor spermatogonia, 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 substages: 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 reorganization, 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 reorganized and packaged into highly condensed nuclei of the spermatozoa. One of the dramatic changes that occur 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). Although the morphological changes

ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/REP-15-0562
Online version via www.reproduction-online.org

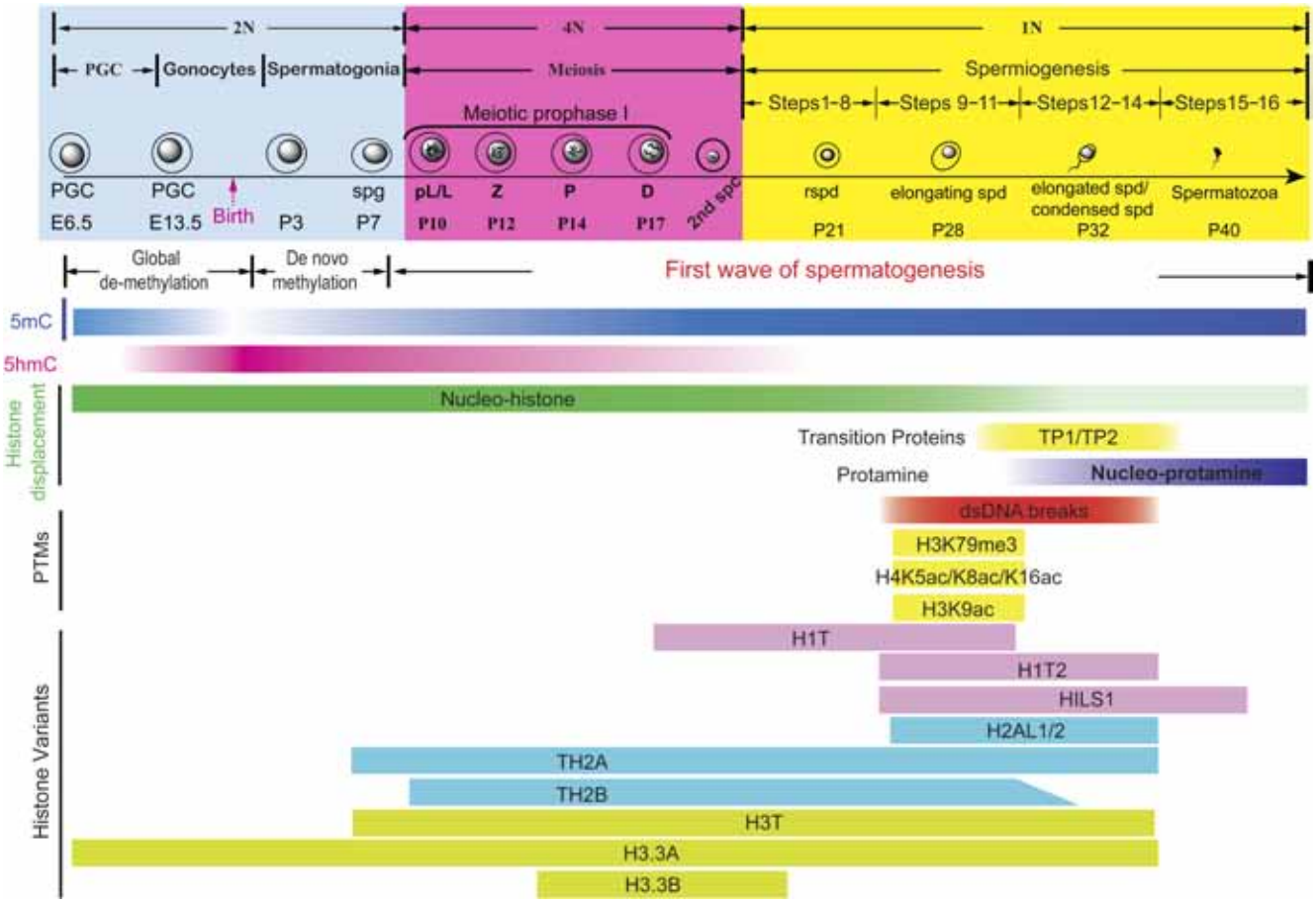

Figure 1 Summary of critical time points and epigenetic events through germ line development in mice. Germ line development in mice undergoes diploid stage (primordial germ cells and spermatogonia), tetraploid stage (spermatocytes), and haploid stage (spermatids) maturation, as highlighted with different colors on the basis of chromosome contents. In addition to the alteration of genetic DNA methylation (5mC and 5hmC), the nucleohistone-based chromatin structure is gradually substituted by the nucleoprotamine structure during late stages of spermiogenesis. Potential histone variants and PTMs involved in histone-to-protamine transition are highlighted in different colors.

throughout all steps of spermatid development are well characterized, the molecular basis underlying the highly orchestrated chromatin reorganization, 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, germ line-specific genes are activated in a well-defined sequence through different stages of spermatogenesis. The difficulty in 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 has been identified to be successfully used for FACS purification of specific types of differentiated cell population, 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 focus on how different histone variants and selective histone posttranslational modifications are critical for histone displacement, transition protein association and displacement, and protamine deposition during nuclear elongation of haploid spermatids. We also address the potential roles of the emerging posttranslational 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.

Histone variants

Distinct from somatic cells, germ cells express many, spatiotemporally regulated sets of core histone variants, although the majority of histones and these variants are evicted from nucleosomes during the histone-to-protamine transition 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: (1) 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 germ line undergoes a directional differentiation following the commitment of SSCs to advanced spermatogonial fate. (2) Histone variants tend to comprise 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 macro-H2A 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 germ line-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. (3) 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.

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 and 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 the spermatids of mammalian testis (Yan *et al.* 2003).

Table 1 A summarized table showing validated and proposed candidate factors involved in histone-to-protamine transition.

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, and 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, and defects in TNP1 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, and reduced protamine incorporation (mouse)	Bush *et al.* (2013), Yuen *et al.* (2014)
H3t	spc, rspd, espd	Nucleus	?	Tachiwana *et al.* (2008, 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,b)
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, sub-fertile, 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, and defects in H4K5/K8/K12ac (mouse)	Bell *et al.* (2014)
Camk4	espd	Nucleus	Male sterility, impaired spermatids, retention of TNP2, and loss of PRM2 (mouse)	Wu *et al.* (2000)
PARP1/2	espd	Nucleus	Morphologically abnormal sperm, sub-fertility, and retention of core histones (mouse)	Meyer-Ficca *et al.* (2005, 2009)
Chromatin readers/remodelers				
PYGO2	espd (step 8–12)	Nucleus	Abnormal nuclear condensation, disrupted H3K9ac, and 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 (steps 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, and retention of TH2B (mouse)	Shang *et al.* (2007), Berkovits & Wolgemuth (2011)

spg, spermatogonia; spc, spermatocytes; rspd, round spermatids; espd, elongating/elongated spermatids, NA, not applicable.

Amino acid sequence analysis revealed that it is the least conserved H1 variant and has evolved rapidly in mammals. *Hils1* is an intron-less gene located in intron 8 of the α-sarcoglycan protein-coding gene and accounts for ~10% of total chromatin protein in the mouse spermatids (Yan *et al.* 2003, Iguchi *et al.* 2004). Most

importantly, HILS1 protein was specifically detected in spermatids between steps 9 and 15, a critical time window when haploid spermatids undergo histone-to-protamine transition. Intriguingly, a recent LC/MS analysis has 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 is likely a critical player during histone replacement, although a *Hils1* KO mouse model has yet to be established.

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 and 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 and 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 rendered 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, using *in vitro* isotopic labeling 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, both *Th2a* and *Th2b* genes are juxtaposed on chromosome 17 in mouse genome and share a common transcriptional promoter localized between the two genes, suggesting that 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 that both TH2A and TH2B induce chromatin instability. In mouse 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 and 16 were observed in the double-null testes, and they exhibited much lower amounts of transition protein 1 (TNP1 (TP1)) and protamine 2 (PRM2) in the nuclear chromatin fraction, compared with those of the WT spermatids. Given that the mRNA levels of *Tnp1* 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).

H3 variants

In addition to two canonical histones H3.1 and H3.2 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 and Table 1). H3.3 differs from canonical H3.1 variant by five amino acids and is encoded by two gene paralogs in the mammalian 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). Although the difference in 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 mouse 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 that *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 TNP1 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 compared with canonical H3.1-assembled nucleosomes. Thus, it is considered 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, 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.

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. However, 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.

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 cross talk 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 considered 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 have 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 has 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 on 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.

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 and 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 independent 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 it is not replaced by protamine 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 acetyl lysine binding domain-containing protein, BRDT, is found to be 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 subfamily members conserved in humans and mice (Dhar *et al.* 2012). Structural studies demonstrated that the first bromodomain is sufficient to recognize one or more acetyl lysines (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 onward (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-shaped head morphology and highly condensed, small ball-like structures; however, 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, which regulates 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, although there was no difference in the expression levels of transition proteins and protamines between WT and KO, TNP2 (TP2) protein did not co-localize with DNA in the nucleus, compared with the overlapped localization of TNP2 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 which 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). Although it remains 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 removes acetyl moiety from substrates. This suggests that a dynamic balance between acetylation and deacetylation of histones is absolutely essential for successful spermiogenesis.

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 conjugation of ubiquitin 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 transfer of ubiquitin moiety 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); however, 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 compared with WT sperm, where only basic PRM1 and PRM2 proteins were detected, thus suggesting that 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 imply 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).

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, AdoMet, 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 germ line development, it is well known that members from the protein lysine methyltransferases (PKMTs) display highly dynamic expression patterns. However, their physiological roles during spermatogenesis, particularly 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 has demonstrated that histone H3K79 methylation is exclusively detected in the elongating spermatids preceding the histone-to-protamine transition, and also correlates with histone H4 hyperacetylation (Fig. 1). This methyl mark is highly conserved in the testes in drosophila, mouse, and human, suggesting that it plays an important role during histone displacement (Dottermusch-Heidel *et al.* 2014a,b). Similar to 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 in both histone and non-histone proteins. There are nine members in the PRMT family, which can be categorized into three types: type I enzymes catalyze the formation of asymmetrical dimethylarginine (PRMT1, PRMT3, CARM1 (PRMT4), PRMT6, and PRMT8), while type II enzymes catalyze the synthesis of symmetrical dimethylarginine (PRMT5 and PRMT9) (Yang *et al.* 2015). Type III enzymes merely catalyze the mono-methylation of the arginine residues in the substrates (PRMT7). Although PRMT8 is only expressed in neuron, other members of the PRMT family 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 has been recently discovered that a substantial proportion of histones is hyper-methylated (on lysine and arginine residues) when purified from human 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).

Poly-ADP-ribosylation

Poly-ADP-ribosylation, known 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 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 affect 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 super-coiled DNA relaxation during nucleoprotein exchange. The 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 and 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, 2015). More strikingly, markedly abnormal retention of core histone H3, testis-specific TH2B, histone linker HIST1H1T (H1T), and HILS1 was 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,b).

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; however, 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 (TNP1 and TNP2) 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).

Transition proteins and their PTMs

Transition proteins 1 and 2 (TNP1 and TNP2) are the basic chromosomal proteins present in a specific time window (condensing spermatids) preceding the protamine deposition in the germ line in mammals (Heidaran & Kistler 1987, Heidaran *et al.* 1988). The TNP1 protein is only 55 amino acids (~6.2 kD) in size, and it is highly conserved across the mammalian species. In contrast, TNP2 exhibits a high degree of sequence divergence across mammals, with only about 50% amino acid homology between mice and humans (Kremling *et al.* 1989, Keime *et al.* 1992). At the protein level, TNP1 is more abundantly expressed, accounting for ~60% of the basic proteins in elongating spermatids of the mice, compared with TNP2. *In vitro* biochemical studies demonstrated that TNP1 tends to relax the DNA in the nucleosomal core particles by reducing the melting temperature of DNA, whereas TNP2 is prone to compact the nucleosomal DNA by increasing the melting temperature, suggesting that TNP2 is a DNA-condensing protein, while TNP1 can promote the eviction of the nucleosomal histones (Singh & Rao 1987, Kundu & Rao 1995, 1996, Kolthur-Seetharam *et al.* 2009). Moreover, these functional differences might also reflect their unique roles during mammalian 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 testis 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 wild-type 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 TNP2 protein in the *Tnp1* KO mice (Yu *et al.* 2000). The testicular phenotype for *Tnp2* KO mice was even more mild with normal testis weights, sperm morphology, histone displacement, and fertility albeit with smaller litter size (Zhao *et al.* 2001, 2004b). Increased levels of TNP1 protein were also observed in *Tnp2*-null spermatids, suggesting that both TNP1 and TNP2 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.

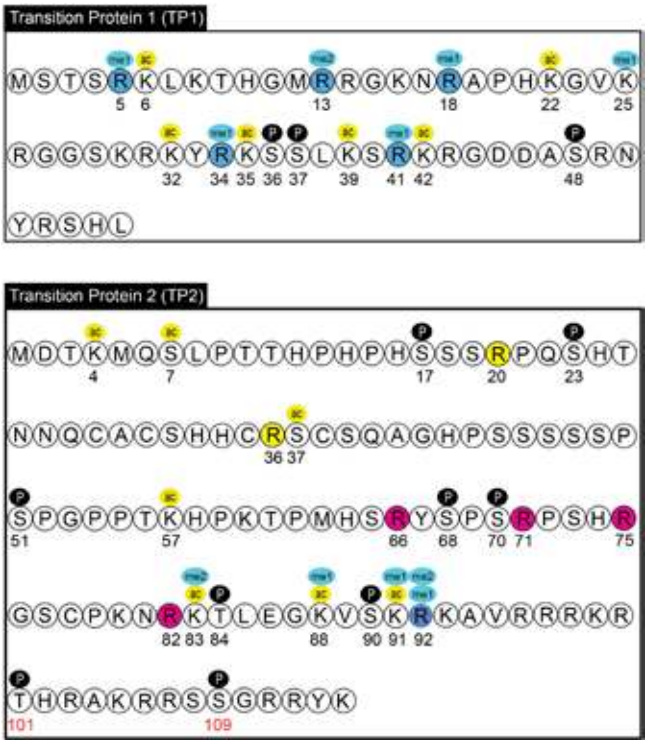

Figure 2 Summary of posttranslational modification profiles in transition proteins identified in the mouse testes. A combination of varied PTMs observed in the TPs in mice testes. The following PTMs as highlighted in different colors: lysine methylation, arginine methylation, lysine acetylation, and serine phosphorylation. Most modified residues were identified by the proteomic LC/MS strategy, while threonine 101 (T101) and serine 109 (S109) in TNP2 were validated through *in vitro* kinase assay (Meetei *et al.* 2002, Nikhil *et al.* 2015). Among them, monomethylation of lysine 88 in TNP2 (TP2K88me1) and monomethylation of arginine 92 in TNP2 (TP2R92me1) were validated as *bona fide* methyl marks that specifically tag TNP2 in elongating/elongated spermatids in the rat testis (Nikhil *et al.* 2015). p, phosphorylation; ac, acetylation; me, methylation.

Importantly, there was a severe retention of histones found in the double-mutant mice, suggesting that 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, which 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 mammalian species, especially those from TNP1. Biochemical analyses suggested that PRMT1 and PRMT4 are the predominant arginine methyltransferases involved in the methylation of endogenous TNP1 and TNP2 proteins. In vitro methylation assay confirmed that PRMT4 can methylate multiple arginine sites in TNP2, 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 TNP2 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 that both methyl marks play vital roles during histone-to-protamine transition (Nikhil *et al.* 2015).

Protamines and their PTMs

Similar to transition proteins, protamines are also basic proteins that are enriched for lysine 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 humans (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 TNP2 and loss of PRM2 association (Wu *et al.* 2000). Although 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. However, the loss of merely a single posttranslational phosphorylation event in the PRM2, which caused the severe defects in the basic protein exchange in the *Camk4* KO mice, implies that regulated cross talk takes place between specific PTMs on the protamines and certain effector proteins (such as 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 be discovered due to the long stretches of arginines in the PRM1 protein that make mass spectrometry difficult.

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), 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 ~110 aa motifs found in many genes of all eukaryotes, and they were the first protein module identified that specifically recognized acetylated lysine substrates before 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, 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 although TP and protamine 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, these data support 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 and 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 cross talk between H3K4me3 and H3K9ac, because PYGO2 "reads" the H3K4me3 mark through its PHD domain and recruits histone acetyltransferase (HAT) activity (Nair *et al.* 2008).

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 humans, will be first 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, although our knowledge of the details of the stepwise 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, posttranslational histone modifications, and chromatin remodelers that are found in somatic cells, but also many testis-specific factors, such as 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 that 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 facilitates 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, such as 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), has been 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 germ line, 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 comprised 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". Although these "players" have been intensively studied 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 germ line development. Multiple germ line-specific *Cre* lines are available, such as fetal PGC cell-specific *Tnap-Cre* and *Ddx4-Cre* lines, the prospermatogonia-specific *Stra8-Cre* line, spermatocyte-specific *Hspa2-Cre* and *Pgk2-Cre* lines, and haploid spermatid-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 germ line 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, as 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 trans-generational 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.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by NIH grant DK062248.

Acknowledgment

The authors apologize to researchers whose papers are not cited in this study due to space constraints. The authors thank Rebecca Deen for text editing.

References

Akhtar A & Becker PB 2000 Activation of transcription through histone H4 acetylation by MOF, an acetyltransferase essential for dosage compensation in Drosophila. *Molecular Cell* **5** 367–375.

Aldrich JC & Maggert KA 2015 Transgenerational inheritance of diet-induced genome rearrangements in Drosophila. *PLoS Genetics* **11** e1005148. (doi:10.1371/journal.pgen.1005148)

Awe S & Renkawitz-Pohl R 2010 Histone H4 acetylation is essential to proceed from a histone- to a protamine-based chromatin structure in spermatid nuclei of Drosophila melanogaster. *Systems Biology in Reproductive Medicine* **56** 44–61.

Balhorn R 2007 The protamine family of sperm nuclear proteins. *Genome Biology* **8** 227.

Bao J, Wu J, Schuster AS, Hennig GW & Yan W 2013 Expression profiling reveals developmentally regulated lncRNA repertoire in the mouse male germline. *Biology of Reproduction* **89** 107. (doi:10.1095/biolreprod.113.113308)

Barchi M, Geremia R, Magliozzi R & Bianchi E 2009 Isolation and analyses of enriched populations of male mouse germ cells by sedimentation velocity: the centrifugal elutriation. *Methods in Molecular Biology* **558** 299–321.

Bedford MT & Richard S 2005 Arginine methylation an emerging regulator of protein function. *Molecular Cell* **18** 263–272.

Bell EL, Nagamori I, Williams EO, Del Rosario AM, Bryson BD, Watson N, White FM, Sassone-Corsi P & Guarente L 2014 SirT1 is required in the male germ cell for differentiation and fecundity in mice. *Development* **141** 3495–3504.

Berkovits BD & Wolgemuth DJ 2011 The first bromodomain of the testis-specific double bromodomain protein Brdt is required for chromocenter organization that is modulated by genetic background. *Developmental Biology* **360** 358–368.

Bramlage B, Kosciessa U & Doenecke D 1997 Differential expression of the murine histone genes H3.3A and H3.3B. *Differentiation* **62** 13–20. (doi:10.1046/j.1432-0436.1997.6210013.x)

Brunner AM, Nanni P & Mansuy IM 2014 Epigenetic marking of sperm by post-translational modification of histones and protamines. *Epigenetics Chromatin* 7 2**.**

Bush KM, Yuen BT, Barrilleaux BL, Riggs JW, O'Geen H, Cotterman RF & Knoepfler PS 2013 Endogenous mammalian histone H3.3 exhibits chromatin-related functions during development. *Epigenetics Chromatin* **6** 7. (doi:10.1186/1756-8935-6-7)

Cai L, Rothbart SB, Lu R, Xu B, Chen WY, Tripathy A, Rockowitz S, Zheng D, Patel DJ, Allis CD *et al.* 2013 An H3K36 methylation-engaging Tudor motif of polycomb-like proteins mediates PRC2 complex targeting. *Molecular Cell* **49** 571–582.

Castillo J, Amaral A & Oliva R 2014 Sperm nuclear proteome and its epigenetic potential. *Andrology* **2** 326–338. (doi:10.1093/molehr/gau079)

Catena R, Ronfani L, Sassone-Corsi P & Davidson I 2006 Changes in intranuclear chromatin architecture induce bipolar nuclear localization of histone variant H1T2 in male haploid spermatids. *Developmental Biology* **296** 231–238. (doi:10.1016/j.ydbio.2006.04.458)

Chang YF, Lee-Chang JS, Panneerdoss S, MacLean JA 2nd & Rao MK 2011 Isolation of Sertoli, Leydig, and spermatogenic cells from the mouse testis. *Biotechniques* **51** 341–342, 344.

Chen P, Zhao J, Wang Y, Wang M, Long H, Liang D, Huang L, Wen Z, Li W, Li X *et al.* 2013 H3.3 actively marks enhancers and primes gene transcription via opening higher-ordered chromatin. *Genes & Development* **27** 2109–2124.

Chen P, Wang Y & Li G 2014 Dynamics of histone variant H3.3 and its coregulation with H2A.Z at enhancers and promoters. *Nucleus* **5** 21–27.

Cho C, Willis WD, Goulding EH, Jung-Ha H, Choi YC, Hecht NB & Eddy EM 2001 Haploinsufficiency of protamine-1 or -2 causes infertility in mice. *Nature Genetics* **28** 82–86.

Couppez M, Martin-Ponthieu A & Sautiere P 1987 Histone H4 from cuttlefish testis is sequentially acetylated. Comparison with acetylation of calf thymus histone H4. *Journal of Biological Chemistry* **262** 2854–2860.

Dantzer F & Santoro R 2013 The expanding role of PARPs in the establishment and maintenance of heterochromatin. *FEBS Journal* **280** 3508–3518.

De Lucia F, Faraone-Mennella MR, D'Erme M, Quesada P, Caiafa P & Farina B 1994 Histone-induced condensation of rat testis chromatin: testis-specific H1t versus somatic H1 variants. *Biochemical and Biophysical Research Communications* **198** 32–39. (doi:10.1006/bbrc.1994.1005)

Dhar S, Thota A & Rao MR 2012 Insights into role of bromodomain, testis-specific (Brdt) in acetylated histone H4-dependent chromatin remodeling in mammalian spermiogenesis. *Journal of Biological Chemistry* **287** 6387–6405. (doi:10.1074/jbc.M111.288167)

Dottermusch-Heidel C, Gartner SM, Tegeder I, Rathke C, Barckmann B, Bartkuhn M, Bhushan S, Steger K, Meinhardt A & Renkawitz-Pohl R 2014a H3K79 methylation: a new conserved mark that accompanies H4 hyperacetylation prior to histone-to-protamine transition in Drosophila and rat. *Biology Open* **3** 444–452.

Dottermusch-Heidel C, Klaus ES, Gonzalez NH, Bhushan S, Meinhardt A, Bergmann M, Renkawitz-Pohl R, Rathke C & Steger K 2014b H3K79 methylation directly precedes the histone-to-protamine transition in mammalian spermatids and is sensitive to bacterial infections. *Andrology* **2** 655–665.

Drabent B, Bode C, Miosge N, Herken R & Doenecke D 1998 Expression of the mouse histone gene H1t begins at premeiotic stages of spermatogenesis. *Cell and Tissue Research* **291** 127–132.

Drabent B, Benavente R & Hoyer-Fender S 2003 Histone H1t is not replaced by H1.1 or H1.2 in pachytene spermatocytes or spermatids of H1t-deficient mice. *Cytogenetic and Genome Research* **103** 307–313. (doi:10.1159/000076818)

Dyson MH, Rose S & Mahadevan LC 2001 Acetyllysine-binding and function of bromodomain-containing proteins in chromatin. *Frontiers in Bioscience* **6** D853–D865. (doi:10.2741/Dyson)

Eitoku M, Sato L, Senda T & Horikoshi M 2008 Histone chaperones: 30 years from isolation to elucidation of the mechanisms of nucleosome assembly and disassembly. *Cellular and Molecular Life Sciences* **65** 414–444.

Escalier D 2006 Knockout mouse models of sperm flagellum anomalies. *Human Reproduction Update* **12** 449–461.

Fantz DA, Hatfield WR, Horvath G, Kistler MK & Kistler WS 2001 Mice with a targeted disruption of the H1t gene are fertile and undergo normal changes in structural chromosomal proteins during spermiogenesis. *Biology of Reproduction* **64** 425–431. (doi:10.1095/biolreprod64.2.425)

Feng CW, Bowles J & Koopman P 2014 Control of mammalian germ cell entry into meiosis. *Molecular and Cellular Endocrinology* **382** 488–497. (doi:10.1038/ncomms10845)

Filipescu D, Szenker E & Almouzni G 2013 Developmental roles of histone H3 variants and their chaperones. *Trends in Genetics* **29** 630–640. (doi:10.1016/j.tig.2013.06.002)

Gaucher J, Boussouar F, Montellier E, Curtet S, Buchou T, Bertrand S, Hery P, Jounier S, Depaux A, Vitte AL *et al.* 2012 Bromodomain-dependent stage-specific male genome programming by Brdt. *EMBO Journal* **31** 3809–3820.

Gibson BA & Kraus WL 2012 New insights into the molecular and cellular functions of poly(ADP-ribose) and PARPs. *Nature Reviews Molecular Cell Biology* **13** 411–424.

Godmann M, Lambrot R & Kimmins S 2009 The dynamic epigenetic program in male germ cells: its role in spermatogenesis, testis cancer, and its response to the environment. *Microscopy Research and Technique* **72** 603–619. (doi:10.1126/science.aab2006)

Govin J, Caron C, Lestrat C, Rousseaux S & Khochbin S 2004 The role of histones in chromatin remodelling during mammalian spermiogenesis. *European Journal of Biochemistry* **271** 3459–3469. (doi:10.1016/j.gyobfe.2006.07.012)

Govin J, Caron C, Rousseaux S & Khochbin S 2005 Testis-specific histone H3 expression in somatic cells. *Trends in Biochemical Sciences* **30** 357–359. (doi:10.1083/jcb.200604141)

Govin J, Escoffier E, Rousseaux S, Kuhn L, Ferro M, Thevenon J, Catena R, Davidson I, Garin J, Khochbin S *et al.* 2007 Pericentric heterochromatin reprogramming by new histone variants during mouse spermiogenesis. *Journal of Cell Biology* **176** 283–294.

Govin J, Dorsey J, Gaucher J, Rousseaux S, Khochbin S & Berger SL 2010 Systematic screen reveals new functional dynamics of histones H3 and H4 during gametogenesis. *Genes & Development* **24** 1772–1786. (doi:10.1101/gad.1954910)

Grimes SR Jr & Henderson N 1984 Hyperacetylation of histone H4 in rat testis spermatids. *Experimental Cell Research* **152** 91–97. (doi:10.1023/A:1006807716339)

Gu B, Watanabe K & Dai X 2012 Pygo2 regulates histone gene expression and H3 K56 acetylation in human mammary epithelial cells. *Cell Cycle* **11** 79–87. (doi:10.1016/j.stem.2013.04.012)

Guerrero-Bosagna C & Skinner MK 2014 Environmentally induced epigenetic transgenerational inheritance of male infertility. *Current Opinion in Genetics and Development* **26** 79–88. (doi:10.1038/srep20837)

Hammoud SS, Low DH, Yi C, Carrell DT, Guccione E & Cairns BR 2014 Chromatin and transcription transitions of mammalian adult germline stem cells and spermatogenesis. *Cell Stem Cell* **15** 239–253.

Hassa PO & Hottiger MO 2008 The diverse biological roles of mammalian PARPS, a small but powerful family of poly-ADP-ribose polymerases. *Frontiers in Bioscience* **13** 3046–3082. (doi:10.1186/s12943-015-0474-2)

Hayashi K, Yoshida K & Matsui Y 2005 A histone H3 methyltransferase controls epigenetic events required for meiotic prophase. *Nature* **438** 374–378. (doi:10.1161/STROKEAHA.115.010819)

Hazzouri M, Pivot-Pajot C, Faure AK, Usson Y, Pelletier R, Sele B, Khochbin S & Rousseaux S 2000 Regulated hyperacetylation of core histones during mouse spermatogenesis: involvement of histone deacetylases. *European Journal of Cell Biology* **79** 950–960. (doi:10.1078/0171-9335-00123)

Heidaran MA & Kistler WS 1987 Transcriptional and translational control of the message for transition protein 1, a major chromosomal protein of mammalian spermatids. *Journal of Biological Chemistry* **262** 13309–13315.

Heidaran MA, Showman RM & Kistler WS 1988 A cytochemical study of the transcriptional and translational regulation of nuclear transition protein 1 (TP1), a major chromosomal protein of mammalian spermatids. *Journal of Cell Biology* **106** 1427–1433.

Henikoff S & Smith MM 2015 Histone variants and epigenetics. *Cold Spring Harbor Perspectives in Biology* **7** a019364.

Hong E, Lim Y, Lee E, Oh M & Kwon D 2012 Tissue-specific and age-dependent expression of protein arginine methyltransferases (PRMTs) in male rat tissues. *Biogerontology* **13** 329–336. (doi:10.3346/jkms.2014.29.9.1188)

Huh NE, Hwang IW, Lim K, You KH & Chae CB 1991 Presence of a bi-directional S phase-specific transcription regulatory element in the promoter shared by testis-specific TH2A and TH2B histone genes. *Nucleic Acids Research* **19** 93–98.

Iguchi N, Tanaka H, Yamada S, Nishimura H & Nishimune Y 2004 Control of mouse hils1 gene expression during spermatogenesis: identification of regulatory element by transgenic mouse. *Biology of Reproduction* **70** 1239–1245. (doi:10.1095/biolreprod.103.024760)

Izadyar F, Spierenberg GT, Creemers LB, den Ouden K & de Rooij DG 2002 Isolation and purification of type A spermatogonia from the bovine testis. *Reproduction* **124** 85–94.

Jason LJ, Moore SC, Lewis JD, Lindsey G & Ausio J 2002 Histone ubiquitination: a tagging tail unfolds? *Bioessays* **24** 166–174. (doi:10.1002/bies.v24:2)

Keime S, Kumm S, Luerssen H & Engel W 1992 The nucleotide sequence of boar transition protein 2 (TNP2) cDNA and haploid expression of the gene during spermatogenesis. *Animal Genetics* **23** 373–378.

Kennedy BP & Davies PL 1980 Acid-soluble nuclear proteins of the testis during spermatogenesis in the winter flounder. Loss of the high mobility group proteins. *Journal of Biological Chemistry* **255** 2533–2539. (doi:10.1002/ece3.2015.5.issue-16)

Khadake JR & Rao MR 1995 DNA- and chromatin-condensing properties of rat testes H1a and H1t compared to those of rat liver H1bdec; H1t is a poor condenser of chromatin. *Biochemistry* **34** 15792–15801.

Kolthur-Seetharam U, Pradeepa MM, Gupta N, Narayanaswamy R & Rao MR 2009 Spatiotemporal organization of AT- and GC-rich DNA and their association with transition proteins TP1 and TP2 in rat condensing spermatids. *Journal of Histochemistry & Cytochemistry* **57** 951–962. (doi:10.1369/jhc.2009.953414)

Kouzarides T 2007 Chromatin modifications and their function. *Cell* **128** 693–705. (doi:10.1016/j.cell.2007.02.005)

Kremling H, Luerssen H, Adham IM, Klemm U, Tsaousidou S & Engel W 1989 Nucleotide sequences and expression of cDNA clones for boar and bull transition protein 1 and its evolutionary conservation in mammals. *Differentiation* **40** 184–190. (doi:10.1111/j.1432-0436.1989.tb00597.x)

Krishnamoorthy T, Chen X, Govin J, Cheung WL, Dorsey J, Schindler K, Winter E, Allis CD, Guacci V, Khochbin S *et al.* 2006 Phosphorylation of histone H4 Ser1 regulates sporulation in yeast and is conserved in fly and mouse spermatogenesis. *Genes & Development* **20** 2580–2592.

Kundu TK & Rao MR 1995 DNA condensation by the rat spermatidal protein TP2 shows GC-rich sequence preference and is zinc dependent. *Biochemistry* **34** 5143–5150.

Kundu TK & Rao MR 1996 Zinc dependent recognition of a human CpG island sequence by the mammalian spermatidal protein TP2. *Biochemistry* **35** 15626–15632.

Kycia I, Kudithipudi S, Tamas R, Kungulovski G, Dhayalan A & Jeltsch A 2014 The Tudor domain of the PHD finger protein 1 is a dual reader of lysine trimethylation at lysine 36 of histone H3 and lysine 27 of histone variant H3t. *Journal of Molecular Biology* **426** 1651–1660.

Lahn BT, Tang ZL, Zhou J, Barndt RJ, Parvinen M, Allis CD & Page DC 2002 Previously uncharacterized histone acetyltransferases implicated in mammalian spermatogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **99** 8707–8712.

Leitch HG, Tang WW & Surani MA 2013 Primordial germ-cell development and epigenetic reprogramming in mammals. *Current Topics in Developmental Biology* **104** 149–187. (doi:10.1016/j.cell.2015.04.053)

Levesque D, Veilleux S, Caron N & Boissonneault G 1998 Architectural DNA-binding properties of the spermatidal transition proteins 1 and 2. *Biochemical and Biophysical Research Communications* **252** 602–609. (doi:10.1006/bbrc.1998.9687)

Li W & Mills AA 2014 Packing for the journey: CHD5 remodels the genome. *Cell Cycle* **13** 1833–1834. (doi:10.1371/journal.pone.0150294)

Li F, Mao G, Tong D, Huang J, Gu L, Yang W & Li GM 2013 The histone mark H3K36me3 regulates human DNA mismatch repair through its interaction with MutSalpha. *Cell* **153** 590–600. (doi:10.1016/j.cell.2013.03.025)

Li W, Wu J, Kim SY, Zhao M, Hearn SA, Zhang MQ, Meistrich ML & Mills AA 2014a Chd5 orchestrates chromatin remodelling during sperm development. *Nature Communications* **5** 3812.

Li Y, Wen H, Xi Y, Tanaka K, Wang H, Peng D, Ren Y, Jin Q, Dent SY, Li W *et al.* 2014b AF9 YEATS domain links histone acetylation to DOT1L-mediated H3K79 methylation. *Cell* **159** 558–571.

Lin R, Leone JW, Cook RG & Allis CD 1989 Antibodies specific to acetylated histones document the existence of deposition- and transcription-related histone acetylation in Tetrahymena. *Journal of Cell Biology* **108** 1577–1588. (doi:10.1083/jcb.108.5.1577)

Lin Q, Sirotkin A & Skoultchi AI 2000 Normal spermatogenesis in mice lacking the testis-specific linker histone H1t. *Molecular and Cellular Biology* **20** 2122–2128. (doi:10.1128/MCB.20.6.2122-2128.2000)

Liu Z, Zhou S, Liao L, Chen X, Meistrich M & Xu J 2010 Jmjd1a demethylase-regulated histone modification is essential for cAMP-response element modulator-regulated gene expression and spermatogenesis. *Journal of Biological Chemistry* **285** 2758–2770. (doi:10.1074/jbc.M109.066845)

Lu LY, Wu J, Ye L, Gavrilina GB, Saunders TL & Yu X 2010 RNF8-dependent histone modifications regulate nucleosome removal during spermatogenesis. *Developmental Cell* **18** 371–384. (doi:10.1016/j.devcel.2010.01.010)

Ma T, Keller JA & Yu X 2011 RNF8-dependent histone ubiquitination during DNA damage response and spermatogenesis. *Acta Biochimica et Biophysica Sinica (Shanghai)* **43** 339–345. (doi:10.1093/abbs/gmr016)

Marfella CG & Imbalzano AN 2007 The Chd family of chromatin remodelers. *Mutation Research* **618** 30–40. (doi:10.1002/jcp.22308)

Martianov I, Brancorsini S, Catena R, Gansmuller A, Kotaja N, Parvinen M, Sassone-Corsi P & Davidson I 2005 Polar nuclear localization of H1T2, a histone H1 variant, required for spermatid elongation and DNA condensation during spermiogenesis. *Proceedings of the National*

Academy of Sciences of the United States of America **102** 2808–2813. (doi:10.1073/pnas.0406060102)

Matzuk MM, McKeown MR, Filippakopoulos P, Li Q, Ma L, Agno JE, Lemieux ME, Picaud S, Yu RN, Qi J *et al.* 2012 Small-molecule inhibition of BRDT for male contraception. *Cell* **150** 673–684.

McCarrey JR, Geyer CB & Yoshioka H 2005 Epigenetic regulation of testis-specific gene expression. *Annals of the New York Academy of Sciences* **1061** 226–242. (doi:10.1095/biolreprod.114.125757)

Meetei AR, Ullas KS, Vasupradha V & Rao MR 2002 Involvement of protein kinase A in the phosphorylation of spermatidal protein TP2 and its effect on DNA condensation. *Biochemistry* **41** 185–195. (doi:10.1021/bi0117652)

Meistrich ML & Hess RA 2013 Assessment of spermatogenesis through staging of seminiferous tubules. *Methods in Molecular Biology* **927** 299–307. (doi:10.1016/j.celrep.2015.07.057)

Meistrich ML, Trostle-Weige PK, Lin R, Bhatnagar YM & Allis CD 1992 Highly acetylated H4 is associated with histone displacement in rat spermatids. *Molecular Reproduction and Development* **31** 170–181. (doi:10.1002/(ISSN)1098-2795)

Meistrich ML, Mohapatra B, Shirley CR & Zhao M 2003 Roles of transition nuclear proteins in spermiogenesis. *Chromosoma* **111** 483–488. (doi:10.1002/(ISSN)1526-968X)

Meyer-Ficca ML, Scherthan H, Burkle A & Meyer RG 2005 Poly(ADP-ribosyl)ation during chromatin remodeling steps in rat spermiogenesis. *Chromosoma* **114** 67–74. (doi:10.1007/s00412-005-0344-6)

Meyer-Ficca ML, Lonchar J, Credidio C, Ihara M, Li Y, Wang ZQ & Meyer RG 2009 Disruption of poly(ADP-ribose) homeostasis affects spermiogenesis and sperm chromatin integrity in mice. *Biology of Reproduction* **81** 46–55. (doi:10.1095/biolreprod.108.075390)

Meyer-Ficca ML, Ihara M, Lonchar JD, Meistrich ML, Austin CA, Min W, Wang ZQ & Meyer RG 2011a Poly(ADP-ribose) metabolism is essential for proper nucleoprotein exchange during mouse spermiogenesis. *Biology of Reproduction* **84** 218–228. (doi:10.1095/biolreprod.110.087361)

Meyer-Ficca ML, Lonchar JD, Ihara M, Meistrich ML, Austin CA & Meyer RG 2011b Poly(ADP-ribose) polymerases PARP1 and PARP2 modulate topoisomerase II beta (TOP2B) function during chromatin condensation in mouse spermiogenesis. *Biology of Reproduction* **84** 900–909. (doi:10.1095/biolreprod.110.090035)

Meyer-Ficca ML, Ihara M, Bader JJ, Leu NA, Beneke S & Meyer RG 2015 Spermatid head elongation with normal nuclear shaping requires ADP-ribosyltransferase PARP11 (ARTD11) in mice. *Biology of Reproduction* **92** 80.

Miller D, Brinkworth M & Iles D 2010 Paternal DNA packaging in spermatozoa: more than the sum of its parts? DNA, histones, protamines and epigenetics. *Reproduction* **139** 287–301. (doi:10.1530/REP-10-0536)

Mishra LN, Gupta N & Rao SM 2015 Mapping of post-translational modifications of spermatid-specific linker histone H1-like protein, HILS1. *Journal of Proteomics* **128** 218–230. (doi:10.1016/j.jprot.2015.08.001)

Montellier E, Rousseaux S, Zhao Y & Khochbin S 2012 Histone crotonylation specifically marks the haploid male germ cell gene expression program: post-meiotic male-specific gene expression. *Bioessays* **34** 187–193.

Montellier E, Boussouar F, Rousseaux S, Zhang K, Buchou T, Fenaille F, Shiota H, Debernardi A, Hery P, Curtet S *et al.* 2013 Chromatin-to-nucleoprotamine transition is controlled by the histone H2B variant TH2B. *Genes & Development* **27** 1680–1692.

Moriniere J, Rousseaux S, Steuerwald U, Soler-Lopez M, Curtet S, Vitte AL, Govin J, Gaucher J, Sadoul K, Hart DJ *et al.* 2009 Cooperative binding of two acetylation marks on a histone tail by a single bromodomain. *Nature* **461** 664–668.

Musselman CA, Avvakumov N, Watanabe R, Abraham CG, Lalonde ME, Hong Z, Allen C, Roy S, Nunez JK, Nickoloff J *et al.* 2012 Molecular basis for H3K36me3 recognition by the Tudor domain of PHF1. *Nature Structural & Molecular Biology* **19** 1266–1272.

Nair M, Nagamori I, Sun P, Mishra DP, Rheaume C, Li B, Sassone-Corsi P & Dai X 2008 Nuclear regulator Pygo2 controls spermiogenesis and histone H3 acetylation. *Developmental Biology* **320** 446–455. (doi:10.1016/j.ydbio.2008.05.553)

Nikhil G, Pradeepa MM, Anayat BU & Satyanarayana RM 2015 Mapping of post-translational modifications of transition proteins, TP1 and TP2 and identification of protein arginine methyltransferase 4 and lysine methyltransferase 7 as methyltransferase for TP2. *Journal of Biological Chemistry* **290** 12101–12122.

Oliva R & Dixon GH 1991 Vertebrate protamine genes and the histone-to-protamine replacement reaction. *Progress in Nucleic Acid Research and Molecular Biology* **40** 25–94.

Oliva R & Mezquita C 1982 Histone H4 hyperacetylation and rapid turnover of its acetyl groups in transcriptionally inactive rooster testis spermatids. *Nucleic Acids Research* **10** 8049–8059.

Orsi GA, Couble P & Loppin B 2009 Epigenetic and replacement roles of histone variant H3.3 in reproduction and development. *International Journal of Developmental Biology* **53** 231–243. (doi:10.1016/j.cub.2014.08.010)

Pai CC, Deegan RS, Subramanian L, Gal C, Sarkar S, Blaikley EJ, Walker C, Hulme L, Bernhard E, Codlin S *et al.* 2014 A histone H3K36 chromatin switch coordinates DNA double-strand break repair pathway choice. *Nature Communications* **5** 4091.

Patel DJ & Wang Z 2013 Readout of epigenetic modifications. *Annual Review of Biochemistry* **82** 81–118.

Pentakota SK, Sandhya S, A PS, Chandra N & Satyanarayana Rao MR 2014 Mapping post-translational modifications of mammalian testicular specific histone variant TH2B in tetraploid and haploid germ cells and their implications on the dynamics of nucleosome structure. *Journal of Proteome Researc* **13** 5603–5617. (doi:10.1021/pr500597a)

Pivot-Pajot C, Caron C, Govin J, Vion A, Rousseaux S & Khochbin S 2003 Acetylation-dependent chromatin reorganization by BRDT, a testis-specific bromodomain-containing protein. *Molecular and Cellular Biology* **23** 5354–5365.

Pradeepa MM & Rao MR 2007 Chromatin remodeling during mammalian spermatogenesis: role of testis specific histone variants and transition proteins. *Society of Reproduction and Fertility supplement* **63** 1–10.

Pruslin FH, Imesch E, Winston R & Rodman TC 1987 Phosphorylation state of protamines 1 and 2 in human spermatids and spermatozoa. *Gamete Research* **18** 179–190. (doi:10.1002/(ISSN)1554-3919)

Qin S, Guo Y, Xu C, Bian C, Fu M, Gong S & Min J 2013 Tudor domains of the PRC2 components PHF1 and PHF19 selectively bind to histone H3K36me3. *Biochemical and Biophysical Research Communications* **430** 547–553.

Rao BJ & Rao MR 1987 DNase I site mapping and micrococcal nuclease digestion of pachytene chromatin reveal novel structural features. *Journal of Biological Chemistry* **262** 4472–4476. (doi:10.1038/srep21575)

Rathke C, Baarends WM, Awe S & Renkawitz-Pohl R 2014 Chromatin dynamics during spermiogenesis. *Biochimica et Biophysica Acta* **1839** 155–168.

Rivera C, Gurard-Levin ZA, Almouzni G & Loyola A 2014 Histone lysine methylation and chromatin replication. *Biochimica et Biophysica Acta* **1839** 1433–1439. (doi:10.1093/nar/gkv929)

Rose KL, Li A, Zalenskaya I, Zhang Y, Unni E, Hodgson KC, Yu Y, Shabanowitz J, Meistrich ML, Hunt DF *et al.* 2008 C-terminal phosphorylation of murine testis-specific histone H1t in elongating spermatids. *Journal of Proteome Research* **7** 4070–4078.

Salva A, Klinefelter GR & Hardy MP 2001 Purification of rat leydig cells: increased yields after unit-gravity sedimentation of collagenase-dispersed interstitial cells. *Journal of Andrology* **22** 665–671.

Samson M, Jow MM, Wong CC, Fitzpatrick C, Aslanian A, Saucedo I, Estrada R, Ito T, Park SK, Yates JR 3rd *et al.* 2014 The specification and global reprogramming of histone epigenetic marks during gamete formation and early embryo development in *C. elegans*. *PLoS Genetics* **10** e1004588.

Sarg B, Chwatal S, Talasz H & Lindner HH 2009 Testis-specific linker histone H1t is multiply phosphorylated during spermatogenesis. Identification of phosphorylation sites. *Journal of Biological Chemistry* **284** 3610–3618.

Shang E, Salazar G, Crowley TE, Wang X, Lopez RA & Wolgemuth DJ 2004 Identification of unique, differentiation stage-specific patterns of expression of the bromodomain-containing genes Brd2, Brd3, Brd4, and Brdt in the mouse testis. *Gene Expression Patterns* **4** 513–519.

Shang E, Nickerson HD, Wen D, Wang X & Wolgemuth DJ 2007 The first bromodomain of Brdt, a testis-specific member of the BET sub-family of double-bromodomain-containing proteins, is essential for male germ cell differentiation. *Development* **134** 3507–3515. (doi:10.1242/dev.004481)

Shinagawa T, Huynh LM, Takagi T, Tsukamoto D, Tomaru C, Kwak HG, Dohmae N, Noguchi J & Ishii S 2015 Disruption of Th2a and Th2b genes causes defects in spermatogenesis. *Development* **142** 1287–1292.

Shinohara T, Orwig KE, Avarbock MR & Brinster RL 2000 Spermatogonial stem cell enrichment by multiparameter selection of mouse testis cells.

Proceedings of the National Academy of Sciences of the United States of America **97** 8346–8351. (doi:10.1095/biolreprod.102.009977)

Shires A, Carpenter MP & Chalkley R 1975 New histones found in mature mammalian testes. *Proceedings of the National Academy of Sciences of the United States of America* **72** 2714–2718.

Shirley CR, Hayashi S, Mounsey S, Yanagimachi R & Meistrich ML 2004 Abnormalities and reduced reproductive potential of sperm from Tnp1- and Tnp2-null double mutant mice. *Biology of Reproduction* **71** 1220–1229.

Sin HS, Barski A, Zhang F, Kartashov AV, Nussenzweig A, Chen J, Andreassen PR & Namekawa SH 2012 RNF8 regulates active epigenetic modifications and escape gene activation from inactive sex chromosomes in post-meiotic spermatids. *Genes & Development* **26** 2737–2748.

Singh J & Rao MR 1987 Interaction of rat testis protein, TP, with nucleic acids *in vitro*. Fluorescence quenching, UV absorption, and thermal denaturation studies. *Journal of Biological Chemistry* **262** 734–740.

Small CL, Shima JE, Uzumcu M, Skinner MK & Griswold MD 2005 Profiling gene expression during the differentiation and development of the murine embryonic gonad. *Biology of Reproduction* **72** 492–501.

Smith L 2011 Good planning and serendipity: exploiting the Cre/Lox system in the testis. *Reproduction* **141** 151–161.

Song N, Liu J, An S, Nishino T, Hishikawa Y & Koji T 2011 Immunohistochemical analysis of histone H3 modifications in germ cells during mouse spermatogenesis. *Acta Histochemica et Cytochemica* **44** 183–190.

Stanley FK, Moore S & Goodarzi AA 2013 CHD chromatin remodelling enzymes and the DNA damage response. *Mutation Research* **750** 31–44.

Steilmann C, Paradowska A, Bartkuhn M, Vieweg M, Schuppe HC, Bergmann M, Kliesch S, Weidner W & Steger K 2011 Presence of histone H3 acetylated at lysine 9 in male germ cells and its distribution pattern in the genome of human spermatozoa. *Reproduction, Fertility and Development* **23** 997–1011.

Szenker E, Ray-Gallet D & Almouzni G 2011 The double face of the histone variant H3.3. *Cell Research* **21** 421–434. (doi:10.1038/cr.2011.14)

Tachiwana H, Osakabe A, Kimura H & Kurumizaka H 2008 Nucleosome formation with the testis-specific histone H3 variant, H3t, by human nucleosome assembly proteins in vitro. *Nucleic Acids Research* **36** 2208–2218.

Tachiwana H, Kagawa W, Osakabe A, Kawaguchi K, Shiga T, Hayashi-Takanaka Y, Kimura H & Kurumizaka H 2010 Structural basis of instability of the nucleosome containing a testis-specific histone variant, human H3T. *Proceedings of the National Academy of Sciences of the United States of America* **107** 10454–10459. (doi:10.1073/pnas.1003064107)

Tan M, Luo H, Lee S, Jin F, Yang JS, Montellier E, Buchou T, Cheng Z, Rousseaux S, Rajagopal N *et al.* 2011 Identification of 67 histone marks and histone lysine crotonylation as a new type of histone modification. *Cell* **146** 1016–1028.

Tanaka H, Iguchi N, Isotani A, Kitamura K, Toyama Y, Matsuoka Y, Onishi M, Masai K, Maekawa M, Toshimori K *et al.* 2005 HANP1/H1T2, a novel histone H1-like protein involved in nuclear formation and sperm fertility. *Molecular and Cellular Biology* **25** 7107–7119.

Tang WW, Dietmann S, Irie N, Leitch HG, Floros VI, Bradshaw CR, Hackett JA, Chinnery PF & Surani MA 2015 A Unique gene regulatory network resets the human germline epigenome for development. *Cell* **161** 1453–1467.

Thakar A, Gupta P, Ishibashi T, Finn R, Silva-Moreno B, Uchiyama S, Fukui K, Tomschik M, Ausio J & Zlatanova J 2009 H2A.Z and H3.3 histone variants affect nucleosome structure: biochemical and biophysical studies. *Biochemistry* **48** 10852–10857.

Trostle-Weige PK, Meistrich ML, Brock WA, Nishioka K & Bremer JW 1982 Isolation and characterization of TH2A, a germ cell-specific variant of histone 2A in rat testis. *Journal of Biological Chemistry* **257** 5560–5567.

Trostle-Weige PK, Meistrich ML, Brock WA & Nishioka K 1984 Isolation and characterization of TH3, a germ cell-specific variant of histone 3 in rat testis. *Journal of Biological Chemistry* **259** 8769–8776. (doi:10.1016/0012-1606(85)90137-X)

Ullas KS & Rao MR 2003 Phosphorylation of rat spermatidal protein TP2 by sperm-specific protein kinase A and modulation of its transport into the haploid nucleus. *Journal of Biological Chemistry* **278** 52673–52680.

Venkatesh S & Workman JL 2015 Histone exchange, chromatin structure and the regulation of transcription. *Nature Reviews Molecular Cell Biology* **16** 178–189.

Vincent JJ, Huang Y, Chen PY, Feng S, Calvopina JH, Nee K, Lee SA, Le T, Yoon AJ, Faull K *et al.* 2013 Stage-specific roles for tet1 and tet2 in DNA demethylation in primordial germ cells. *Cell Stem Cell* **12** 470–478.

Weake VM & Workman JL 2008 Histone ubiquitination: triggering gene activity. *Molecular Cell* **29** 653–663. (doi:10.1074/jbc.M114.597948)

Welchman RL, Gordon C & Mayer RJ 2005 Ubiquitin and ubiquitin-like proteins as multifunctional signals. *Nature Reviews Molecular Cell Biology* **6** 599–609. (doi:10.1038/nrm1700)

Wendt KD & Shilatifard A 2006 Packing for the germy: the role of histone H4 Ser1 phosphorylation in chromatin compaction and germ cell development. *Genes & Development* **20** 2487–2491.

Wu JY, Ribar TJ, Cummings DE, Burton KA, McKnight GS & Means AR 2000 Spermiogenesis and exchange of basic nuclear proteins are impaired in male germ cells lacking Camk4. *Nature Genetics* **25** 448–452. (doi:10.1038/78153)

Yan W, Ma L, Burns KH & Matzuk MM 2003 HILS1 is a spermatid-specific linker histone H1-like protein implicated in chromatin remodeling during mammalian spermiogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **100** 10546–10551.

Yan W, Si Y, Slaymaker S, Li J, Zheng H, Young DL, Aslanian A, Saunders L, Verdin E & Charo IF 2010 Zmynd15 encodes a histone deacetylase-dependent transcriptional repressor essential for spermiogenesis and male fertility. *Journal of Biological Chemistry* **285** 31418–31426.

Yang Y, Hadjikyriacou A, Xia Z, Gayatri S, Kim D, Zurita-Lopez C, Kelly R, Guo A, Li W, Clarke SG *et al.* 2015 PRMT9 is a type II methyltransferase that methylates the splicing factor SAP145. *Nature Communications* **6** 6428.

Yu YE, Zhang Y, Unni E, Shirley CR, Deng JM, Russell LD, Weil MM, Behringer RR & Meistrich ML 2000 Abnormal spermatogenesis and reduced fertility in transition nuclear protein 1-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America* **97** 4683–4688.

Yuen BT, Bush KM, Barrilleaux BL, Cotterman R & Knoepfler PS 2014 Histone H3.3 regulates dynamic chromatin states during spermatogenesis. *Development* **141** 3483–3494.

Zarnescu O 2007 Immunohistochemical distribution of hyperacetylated histone H4 in testis of paddlefish *Polyodon spathula*: ultrastructural correlation with chromatin condensation. *Cell and Tissue Research* **328** 401–410.

Zhang L, Wang J, Pan Y, Jin J, Sang J, Huang P & Shao G 2014 Expression of histone H3 lysine 4 methylation and its demethylases in the developing mouse testis. *Cell and Tissue Research* **358** 875–883.

Zhao H & Chen T 2013 Tet family of 5-methylcytosine dioxygenases in mammalian development. *Journal of Human Genetics* **58** 421–427. (doi:10.1002/prca.v10.3)

Zhao M, Shirley CR, Yu YE, Mohapatra B, Zhang Y, Unni E, Deng JM, Arango NA, Terry NH, Weil MM *et al.* 2001 Targeted disruption of the transition protein 2 gene affects sperm chromatin structure and reduces fertility in mice. *Molecular and Cellular Biology* **21** 7243–7255.

Zhao M, Shirley CR, Hayashi S, Marcon L, Mohapatra B, Suganuma R, Behringer RR, Boissonneault G, Yanagimachi R & Meistrich ML 2004a Transition nuclear proteins are required for normal chromatin condensation and functional sperm development. *Genesis* **38** 200–213.

Zhao M, Shirley CR, Mounsey S & Meistrich ML 2004b Nucleoprotein transitions during spermiogenesis in mice with transition nuclear protein Tnp1 and Tnp2 mutations. *Biology of Reproduction* **71** 1016–1025.

Zhuang T, Hess RA, Kolla V, Higashi M, Raabe TD & Brodeur GM 2014 CHD5 is required for spermiogenesis and chromatin condensation. *Mechanisms of Development* **131** 35–46.

Received 25 November 2015
First decision 4 January 2016
Revised manuscript received 19 January 2016
Accepted 5 February 2016