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# JB Special Review—Chromatin Structure and Function: Biological Implications in Epigenetics

## Histone variants: essential actors in male genome programming

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**Prior to its transmission to the offspring, the male genome has to be tightly compacted. A genome-scale histone eviction and the subsequent repackaging of DNA by protamines (Prms) direct this essential genome condensation step. The requirement for male germ cells to undergo such a dramatic and unique genome reorganization explains why these cells express the largest number of histone variants, including many testis-specific ones. Indeed, an open chromatin, nucleosome instability and a facilitated process of histone disassembly are direct consequences of the presence of these histone variants in the chromatin of male germ cells. These histone-induced changes in chromatin first control a stage-specific gene expression program and then directly mediate the histone-to-Prm transition process. This review aims at summarizing and discussing a series of recent functional studies of male germ cell histone variants with a focus on their impact on the process of histone eviction and male genome compaction.**

**Keywords:** H2A.L.2; H3.3; protamines; TH2B; transition proteins.

Spermatogenesis is a differentiation process which produces mature spermatozoa, which will deliver the male genome to the oocytes in the female organism. Spermatogenic cells are continuously produced by an asymmetrical division of progenitor cells, named spermatogonia. These cells on the one hand maintain the stem cell population and on the other hand generate meiotic cells, or spermatocytes, which themselves undergo two successive meiotic divisions. The second meiotic division produces haploid cells known as spermatids. The early post-meiotic spermatids are called round spermatids. These cells activate a specific gene expression program that will direct their transformation into spermatozoa, through a series of reorganizations of their various cellular constituents. These reorganizations mostly take place in the subsequent stages of post-meiotic differentiation, in cells known as elongating and

condensing spermatids. Large-scale histone post-translational modifications (PTMs), essentially histone acetylation, signal and regulate the initiation of the process of histone-to-protamine (Prm) replacement in elongating spermatids (1–3).

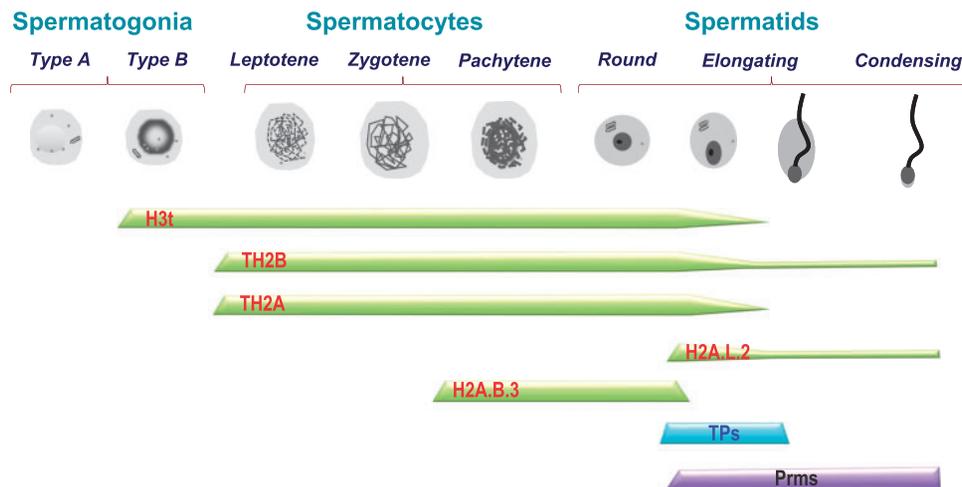
Although still largely unexplored, this genome-wide replacement of histones by Prms is the most dramatic of the known processes involved in chromatin remodelling, genome reorganization and reprogramming (4). In addition to Prms, early investigations also identified other basic non-histone proteins that appear at the time of histone replacement but, in contrast to Prms, totally disappear afterwards (5). These proteins have been named transition proteins (TPs). The early studies of sperm and testis histones in various species also revealed the existence of histones that are only expressed in male germ cells (6). Indeed testis-specific histones were among the first histone variants identified. Additionally, spermatogenic cells not only express a large number of various testis-specific histones, but are also characterized by the presence of some histone variants, which almost entirely replace their canonical counterparts (Fig. 1). Therefore, prior to histone replacement, spermatogenic cell chromatin accumulates unique germline histones in replacement of their canonical counterparts. Histone variants, including the testis-specific ones, will eventually be replaced by Prms.

An open question is whether and how this large number of histone variants, individually and collectively, are involved in directing the spermatogenic gene expression program and in preparing chromatin for the final histone-to-Prm transition. Additionally, it is critical to decipher the individual contribution of each of the specific or generalist histone variants to these unique events. Another related question is the interplay between histones, TPs and Prms in the process of histone eviction. Most of these questions have remained obscure until recently. It is only during the last few years, a long time after the discovery of the first testis-specific histone variants that several pilot studies have started to shed light on the role of histone variants in the stepwise programming of the male genome, in the eviction of histones and in the final genome compaction.

Here, we summarize these recent advances in our knowledge of the role of histone variants, specifically testis-specific histone variants, in spermatogenesis, with a specific focus on the process of genome-wide histone eviction and Prm assembly.

### TH2B

One of the first histone variants discovered belongs to the H2B family and was named TH2B (TSH2B, according to the new nomenclature). Indeed, the protein was



**Fig. 1 Differentiation-dependent expression of testis-specific histone variants during spermatogenesis.** Detailed studies of the indicated histone variants established the precise timing of their expression during the spermatogenic cells differentiation. A fraction of TH2B and H2A.L.2 escape the general histone-to-Prm transition and remains in the mature sperm nuclei.

reported in 1975 (7) as a H2B histone expressed in the testes of several mammalian species.

The first insight into a potential specific function of this H2B variant came from a structural study showing that a histone octamer containing TH2B presents an increased instability compared to a canonical octamer (8). Additional studies showed that TH2B is also present during late spermatogenesis and at the time of histone removal, and that it remains in the transitional structures that are generated during the transformation of nucleosomes into nucleoprotamines (9).

The first functional studies of TH2B in its physiological setting, during spermatogenic cell differentiation, were published only 38 years after its identification, following the generation of different mouse models (10). These studies show that, from the onset of meiosis, TH2B massively replaces H2B, and that most of the genome becomes covered by TH2B-containing nucleosomes. However, surprisingly, in the total absence of TH2B, spermatogenesis seems to occur completely normally, and there is no effect on male fertility.

The investigation of spermatogenesis in TH2B-less mice revealed that, in fact, in the absence of TH2B, a compensatory mechanism leads to the accumulation of H2B, thereby physically compensating the lack of TH2B by an increased production of H2B and allowing the formation of nucleosomes at the genome scale.

This observation raised the question of the functional utility of TH2B during spermatogenesis. Indeed, if H2B can efficiently replace TH2B, then what could be the specific role of TH2B?

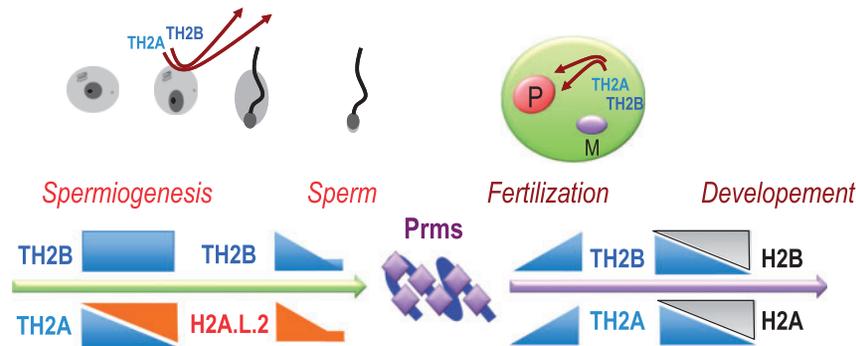
The answer to this question came from the comparative quantitative proteomic analysis of histone PTMs between TH2B-less and wild-type spermatogenic cells. These data show that, in the absence of TH2B, nucleosomes undergo an epigenetic reprogramming to become less stable. Indeed, in TH2B-less nucleosomes, histones gain several chemical modifications that, without any

exception, are all located at structurally strategic positions and present the potential to weaken the overall stability of the nucleosomes. These are H3K122 and H4K77 crotonylation. These two lysines are in contact with DNA and the addition of a bulky crotonyl group with its charge neutralizing capacity is able to affect nucleosome stability. The other PTMs found in TH2B-less nucleosomes are methylations at H4R35, H4R55, H4R67 and H2BR72. Remarkably all these residues are involved in histone–DNA and histone–histone interactions and their methylation has the potential to weaken these intranucleosomal interactions.

Hence, these investigations indicate that the compensatory mechanism that is activated after the loss of TH2B encompasses not only an over-expression of the canonical H2B, but also specific modifications of histones with nucleosomal destabilizing effects. In other words, the primary function of TH2B could be to generate less stable nucleosomes at the whole genome scale, which would lower the energy necessary for the cells to undertake chromosome-wide (sex chromosome inactivation) and genome-wide histone exchanges in later stages.

The nucleosome destabilizing property of TH2B was later confirmed through a series of structural studies. Compared to H2B, the presence of TH2B in a nucleosome leads to the loss of a hydrogen bond with H4 (11). Other structural works considering TH2B and TH2A (an H2A variant co-expressed with TH2B) showed even more dramatic effects on the nucleosomes stability, with significantly reduced histone–DNA contacts and modified histone–histone interactions (12, 13).

TH2B was also found expressed in oocytes and to be assembled again on the paternal genome after fertilization before its total disappearance during later development (Fig. 2) (10, 14, 15). The use of TH2B–TH2A in cell reprogramming showed that their ectopic expression stimulates the reprogramming of somatic cells into



**Fig. 2** The packaging cycles of the male genome with TH2A/TH2B. The histone variants TH2A and TH2B become the major histones in spermatogenic cells, until the replacement of TH2A by H2A.L.2, at the time of histone-to-Prm transition. A fraction of TH2B–H2A.L.2 dimer survives histone replacement. After fertilization, the removal of Prms is associated with the re-assembly of maternal TH2A–TH2B on the male genome. These histones will be entirely replaced by their somatic counterparts during early development. The schemes respectively represent maturing spermatids (left) and a fertilized egg with paternal (P) and maternal (M) genomes (right).

induced pluripotent stem cells, a property that could be related to the ability of these histones to create more accessible chromatin (14).

In contrast to TH2B-less situation, the knock-out of both TH2B and TH2A leads to an arrest of spermatogenesis during meiosis (16). However in this model, although the lack of TH2B induced a compensatory accumulation of H2B, as reported in the TH2B-less model (10), no H2A accumulation was observed (16). Since TH2A is also the main H2A in spermatogenic cells, in *Th2a/Th2b* KO spermatogenic cells, the absence of compensatory gain of H2A expression suggests a significant H2A under-dosage in these cells. The defects observed could therefore be due to an inappropriate chromatin structure and subsequent genome disorganization, rather than to a specific action of TH2A or TH2B (16). In agreement with this hypothesis, two recent reports showed that TH2A could be phosphorylated at the TH2A-specific Thr127 and that this phosphorylation is dispensable for normal spermatogenesis and male fertility (17, 18).

The animal models used to decipher TH2B functions also included mice that expressed a modified endogenous TH2B, bearing three tags at its C-terminal (10). Remarkably, although in these mice all the investigated events at the cellular and molecular levels, i.e. TH2B-tag assembly, transcription, meiosis, sex-chromosome inactivation, H2A.X phosphorylation and histone exchange, etc., occur normally in spermatogenic cells up to the appearance of elongating spermatids, an arrest of spermatogenesis is observed at the time of histone-to-Prm transition. This study demonstrated that TH2B-tag expressed in female animals could also sustain oocyte maturation and embryonic development with no detectable defects and leading to the generation of healthy adult mice (10).

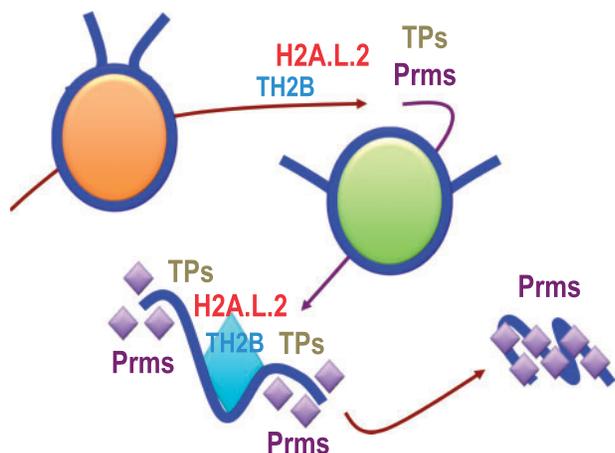
Overall, these data show that the presence of a tag at the C-terminal end of TH2B, interferes very specifically and exclusively with defined and particular events that take place in elongating-condensing spermatids (10). Detailed observations indicated, that in these TH2B-tag expressing male germ cells, although TP and Prms

are produced, Prms are unable to replace histones leading to the accumulation of transitional structures following nucleosome disassembly. Further investigations are required to decipher the exact role of TH2B, and more specifically of its C-terminal region, in the final histone eviction.

## H2A.L.2

In an attempt to identify histones that could facilitate the process of histone-to-Prm transition, surviving histones from spermatids undergoing histone replacement were purified and identified (9). This led to the identification of 5 new histone variants, two H2Bs and three H2As, which were respectively named H2B.L.1, H2B.L.2, H2A.L.1, H2A.L.2 and H2A.L.3 [please see their new nomenclature in (19, 20)]. Among these histones, H2A.L.2 presented all the criteria to be considered as a candidate histone variant involved in histone removal. Indeed, the protein is first detected in spermatids at the same time as TPs (9, 21). Additionally, H2A.L.2 along with TH2B was found in the H3/H4-less transitional structures, which were evidenced in condensing spermatids after extensive micrococcal nuclease (MNase) digestion. Finally, a fraction of H2A.L.2 remains in mature spermatozoa associated with the pericentric regions (9).

The generation of an H2A.L.2-less mouse model proved its critical role in the process of histone-to-Prm replacement. The molecular studies of spermatogenic cells lacking H2A.L.2 revealed its role in the incorporation of TPs onto chromatin. Additionally, a series of *in vitro* nucleosome reconstitution assays demonstrated that the incorporation of H2A.L.2 can drastically modify the structure of the corresponding nucleosome. A more 'open' H2A.L.2-containing nucleosome is able to load TPs, which invade the nucleosome without releasing the DNA. These TPs in turn buffer the incoming Prms and allow their assembly and the final histone eviction. TPs also mediate the processing of Prm2, which is produced as a pre-Prm2 protein (21). In fact the phenotype of H2A.L.2-less spermatogenic cells is very



**Fig. 3** Coordinate actions of histone variants, TPs and Prms in the process of histone-to-Prm replacement. The exchange of TH2B–TH2A by TH2B–H2A.L.2 opens the nucleosomes, allowing the loading of TPs that in turn direct a controlled replacement of histones, Prm assembly and genome compaction.

close to the phenotype of TP-less cells (22–24) suggesting that, in the absence of H2A.L.2, although TPs are produced, they remain non-functional.

Altogether, these observations fully support the idea that the incorporation of H2A.L.2 changes the structure of the nucleosomes allowing chromatin loading of TPs, which in turn buffer and regulate Prm processing and assembly (Fig. 3).

Prms are the true histone displacers. Indeed, they can displace histones even in the absence of TPs (or H2A.L.2). However, although not required for histone removal, TPs are necessary for an efficient Prm-dependent genome compaction.

The major outcome of these investigations is also a new vision of the process of histone-to-Prm transition. Indeed, the general belief was that histones were first replaced by TPs, which were later replaced by Prms to fully compact the genome. The new data described above demonstrate that the full picture of the process requires a histone variant that ‘prepares’ the nucleosomes to undergo disassembly (Fig. 1). In this context, TPs and Prms work together, not successively, to fully transform the nucleosomes into nucleosprotamine structures (Fig. 3).

### H2A.B.3

In an attempt to identify and functionally characterize H2A histone variants that lack the characteristic acidic patch, which is present in most H2As, including canonical H2A, the mouse genome was screened in search for H2A genes with an altered acidic patch (25). The acidic patch is typically composed of a series of six acidic amino acids of H2A, present at the surface of a nucleosome, which mediates the nucleosome–nucleosome interaction and hence chromatin compaction (26, 27). This approach led to the identification of four H2A variants with an altered acidic patch, which were named H2A.Lap1–4, ‘Lap’ standing for Lack of

Acidic Patch. A further analysis showed that some of them had already been identified by Govin and colleagues: H2A.L.2 actually is H2A.Lap3, H2A.L.1 is H2A.Lap2 and H2A.L.3 is H2A.Lap4 (20). H2A.Lap1, now known as H2A.B.3, was uniquely identified in this study and functionally characterized. This H2A variant shows similar structural features as H2A.L.2 but its expression pattern is drastically different (Fig. 1). H2A.B.3 first appears in late pachytene spermatocytes and particularly accumulates in post-meiotic round spermatids but disappears from the elongating spermatids nucleus (25), when H2A.L.2 starts to accumulate (21). The protein was then found enriched at the transcriptional start sites (TSS) of highly active genes in spermatocytes as well as in round spermatids. In these latter cells, H2A.B.3 becomes associated with the X-linked genes that escape sex chromosome transcriptional inactivation (25). Further studies demonstrated that H2A.B.3 has an RNA-binding motif, binds RNA and a series of RNA processing factors and co-localizes with splicing speckles in highly active nuclear subdomains (28). These studies highlight the possibility that H2A.B.3 incorporation at exon–intron boundaries facilitates the recruitment of splicing factors from the splicing speckles.

It is of note that the studies on H2A.B.3 also revealed a specific functional interplay between this histone and the widely-studied H2A.Z in spermatogenic cells. Indeed, several years ago, a microscopic analysis of H2A.Z histone variants revealed a post-meiotic accumulation of H2A.Z on the sex chromosomes (29). A high resolution mapping of H2A.Z and H2A.B.3 revealed an interesting relationship between these two H2A variants. The replacement of H2A.Z by H2A.B.3 at the exon–intron boundaries in spermatogenic cells may play a role in the regulation of splicing at active genes. Additionally, in the surround of genes TSSs, a combination of H2A, H2A.Z and H2A.B.3 could define specific gene categories in terms of expression timing and functional classifications of their products (30). These studies extend the role of histone variants from a specific action on particular chromatin regions to a possibly direct regulation of RNA processing and splicing.

### Testis specific H3

A testis-specific histone H3 was also first reported almost 40 years ago (31). However, similarly to TH2B, it is structural biology that brought hints on the possible functions of this variant, first for the human H3T (32) and then for the mouse H3t variant (33). These studies showed that the alteration of several residues in H3T compared to H3.1, perfectly explains the instability of nucleosomes bearing H3T, observed *in vitro* and *in vivo*. In the case of H3t, only one H3t-specific amino acid, H42, was found to be responsible for creating an ‘open’ nucleosome with flexible DNA at the entry–exit of the nucleosomes (33). The H3t-encoding gene becomes active in differentiating spermatogonia, and its product gradually replaces most of the somatic-type H3s.

In the absence of H3t, a significant reduction of the number of differentiating c-Kit positive spermatogonia was observed, leading to complete male infertility. Further investigations showed a drastic decrease in the number of post-meiotic cells and suggested a possible role for this histone in the entry of cells into meiosis. However, since there was no compensatory increase of H3 gene expression to maintain cellular H3 levels, the observed defects in the absence of H3t could also be explained by histone underdosage.

### H3.3

Besides the mouse models harbouring the inactivation of genes encoding testis-specific histone variants, the targeted inactivation of H3.3-encoding genes in the mouse testis was also performed. H3.3 is a replication-independent H3 that is required for chromatin assembly whenever the replication-dependent pathway is not operational, such as post-fertilization chromatin assembly on the paternal genome (34–36).

In the mouse two independent H3.3 encoding genes, *H3f3a* and *H3f3b*, generate identical H3.3. However individual targeted KO of these genes have different impact on spermatogenesis. The knock-out of *H3f3a* leads to the generation of abnormal spermatozoa (37), while the KO of *H3f3b* has a much more dramatic phenotype including a spermatogenesis arrest at the stage of round spermatid (37, 38).

These functional analyses of H3.3 had been awaited since the interesting observation by de Boer's laboratory of the massive replacement of H3 by H3.3 on the sex chromosomes in meiotic cells (39). During meiosis the unsynapsed X and Y chromosomes create a specific domain in the nucleus of spermatocytes known as the sex body, which undergoes a total transcriptional inactivation during meiosis known as meiotic sex chromosome inactivation (MSCI).

A consequence of this selective incorporation of H3.3 on the sex chromosomes is the creation of interesting combinations of histone variants. In spermatocytes, the macroH2A histone variant was known to accumulate on the sex body (40), while in post-meiotic cells it is H2A.Z that becomes enriched on these chromosomes (29). The presence of both H3.3 and macroH2A in spermatocytes and of H3.3 and H2A.Z in round spermatids increases the probability for these combinations to be present within the same nucleosome. While macroH2A is associated with less active and more stable chromatin domains (41), the H3.3–H2A.Z combination is known to create a particularly unstable nucleosome (42, 43), which could be important for the post-meiotic reactivation of the sex-linked genes.

The generation of these H3.3 KO models therefore provided the possibility to investigate more specifically the importance of H3.3 on the sex chromosome biology during spermatogenesis. However, at least in spermatocytes, no global defects were observed after a reduction in H3.3 expression (37), and the question of the function of H3.3 in MSCI and in the post-meiotic partial reactivation of sex chromosomes at the molecular levels remains open.

### Concluding Remarks

In addition to almost all the histone variants present in somatic cells, spermatogenic cells express a number of unique histone variants of the H2A, H2B and H3 types (Fig. 1).

The structural studies of all these testis-specific variants revealed their capacity to induce nucleosome instability. Remarkably, short H2As, H2A.L.2 and H2A.B.3 as well as H3t are able to generate flexible DNA ends at the entry and exit of nucleosomes. Although these variants induce open chromatin features, these histones are expressed at quite different timings during spermatogenesis. H3t is expressed early in differentiating spermatogonia, while H2A.B.3 is expressed in pachytene spermatocytes and to a greater extent in round spermatids and is eventually replaced by H2A.L.2 in elongating spermatids. Therefore, the configuration of chromatin seems to gradually open from the early stage of commitment of cells into meiotic divisions to the late post-meiotic stage of histone-to-Prm transition. Additionally, spermatocytes express TH2B and TH2A that also form unstable and programmable chromatin. TH2B–TH2A remains in spermatogenic cells until the time of histone replacement, when H2A.L.2 accumulates and becomes the privileged partner of TH2B (Fig. 2).

Therefore, due to the action of these histone variants, the chromatin of spermatogenic cells becomes potentially 'prepared' to undergo histone-to-Prm replacement. Consequently, it is possible to speculate that it is the timing of TPs and Prms expression that decides histones replacement. However, the generation of an H2A.L.2-less mouse model demonstrated that the incorporation of this variant is indispensable for TPs to be efficiently loaded onto chromatin and that TPs incorporation controls the efficiency of the Prm-dependent replacement of histones (21).

Additionally, a genome-wide occurrence of histone hyperacetylation and of other histone acylations also seems necessary for histone replacement (1–3). It can therefore be concluded that a change in the structure of nucleosomes due to the incorporation of testis-specific histone variants is only one facet of the mechanisms involved in general histone eviction and that other mechanisms, mostly histone PTM-based events, are equally important.

Therefore, the future challenge will be to decipher the molecular links between histone variants and histone PTMs in the process of coupled histone eviction and Prm assembly.

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### Conflict of Interest

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