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Review

Chromatin dynamics during spermiogenesis ☆☆

Christina Rathke [a], Willy M. Baarends [b], Stephan Awe [c,1], Renate Renkawitz-Pohl [a,*]

[a] Philipps-Universität Marburg, Fachbereich Biologie, Entwicklungsbiologie, 35043 Marburg, Germany
[b] University Medical Center Rotterdam, Department of Reproduction and Development, Erasmus MC, 3000 DR Rotterdam, Netherlands
[c] Institut für Molekularbiologie und Tumorforschung, Philipps-Universität Marburg, Emil-Mannkopff-Str. 2, 35037 Marburg, Germany

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ABSTRACT

The function of sperm is to safely transport the haploid paternal genome to the egg containing the maternal genome. The subsequent fertilization leads to transmission of a new unique diploid genome to the next generation. Before the sperm can set out on its adventurous journey, remarkable arrangements need to be made during the post-meiotic stages of spermatogenesis. Haploid spermatids undergo extensive morphological changes, including a striking reorganization and compaction of their chromatin. Thereby, the nucleosomal, histone-based structure is nearly completely substituted by a protamine-based structure. This replacement is likely facilitated by incorporation of histone variants, post-translational histone modifications, chromatin-remodeling complexes, as well as transient DNA strand breaks. The consequences of mutations have revealed that a protamine-based chromatin is essential for fertility in mice but not in *Drosophila*. Nevertheless, loss of protamines in *Drosophila* increases the sensitivity to X-rays and thus supports the hypothesis that protamines are necessary to protect the paternal genome. Pharmaceutical approaches have provided the first mechanistic insights and have shown that hyperacetylation of histones just before their displacement is vital for progress in chromatin reorganization but is clearly not the sole inducer. In this review, we highlight the current knowledge on post-meiotic chromatin reorganization and reveal for the first time intriguing parallels in this process in *Drosophila* and mammals. We conclude with a model that illustrates the possible mechanisms that lead from a histone-based chromatin to a mainly protamine-based structure during spermatid differentiation. This article is part of a Special Issue entitled: Chromatin and epigenetic regulation of animal development.

1. Introduction

Germ cells mediate the transfer of genetic information from generation to generation and are thus pivotal for maintenance of life. Spermatogenesis is a continuous and precisely controlled process that leads to the formation of haploid sperm capable of fertilization.

The process of spermatogenesis is highly conserved among many organisms and can be subdivided into three crucial phases: a mitotic amplification phase, a meiotic phase, and a post-meiotic phase also known as spermiogenesis (Fig. 1A). The meiotic phase ensures haploidization of the genome as well as an independent assortment of genetic information within individual germ cells. Germ cells in the post-meiotic phase can be subdivided into early spermatids with round nuclei, intermediate spermatids with elongating nuclei, and spermatids with condensed nuclei [1]. In many organisms (e.g., humans, mice, *Drosophila*), male germ cells undergo a series of morphological transformations during spermiogenesis to build a sperm with its typical species-specific shape from an initially round cell [2–6]. A common feature of spermatogenesis is that transcription stops at a defined point during germ cell differentiation. Thus, translational repression and storage of mRNAs, such as those encoding protamines, are crucial for completion of spermiogenesis [7,8] (Fig. 1A). Within the scope of this review, we will mainly focus on chromatin reconstruction during spermiogenesis.

Already in the last third of the 19th century, Miescher and Kossel described salt-like linkages of nucleic acid with two different complexing substances: protamines and histones [9–12]. In addition, they identified histones as the major chromatin proteins in somatic cells, and either histones (e.g., in carp) or protamines (e.g., in salmon) as the chromatin proteins of sperm cells [9–12]. We now know that in many organisms spermiogenesis is accompanied by a dramatic reorganization of chromatin from a nucleosomal histone-based structure to a structure largely based on protamines [1,7,13–15]. The replacement of histones by protamines is gradual [16]. First, some of the canonical histones are replaced by testis-specific histone variants. Subsequently, so-called transition proteins are incorporated as nucleosomes are removed, and finally,

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* Corresponding author. Tel.: +49 6421 2821502; fax: +49 6421 2821538.
E-mail address: renkawit@biologie.uni-marburg.de (R. Renkawitz-Pohl).
[1] Present address.

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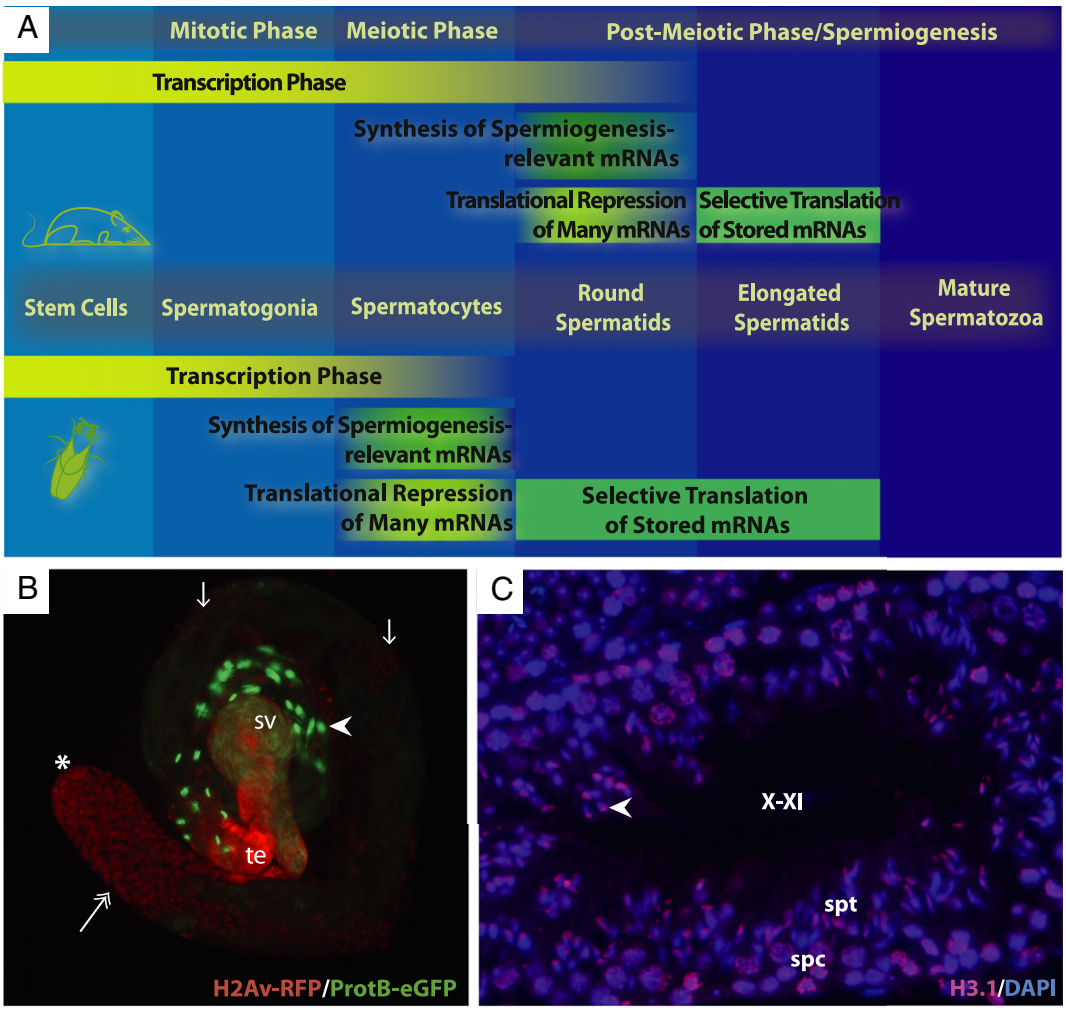

Fig. 1. Comparison of spermatogenesis in mice and flies. (A) Schematic drawing of the different stages of spermatogenesis. Spermatogenesis is characterized by a mitotic proliferation phase, a meiotic phase, and a post-meiotic phase known as spermiogenesis. In mice, global transcription in male germ cells is repressed by the time round spermatids start to elongate. In *Drosophila*, hardly any gene activity is detectable during spermiogenesis. Thus, post-meiotic spermatid differentiation is programmed by translationally repressed mRNAs synthesized in spermatocytes (in flies) or in round spermatids (in mice). During spermiogenesis, translationally repressed mRNAs are gradually released and spermatid differentiation-relevant proteins are synthesized. (B) Testis of a double transgenic fly that expresses the fusion proteins H2Av-RFP and ProtB-eGFP. The asterisk marks the tip of the testis tube, where stem cells localize. Expression of H2Av-RFP is visible in mitotic spermatogonia and meiotic spermatocytes (double arrow) up to early post-meiotic spermatids (arrows). During spermiogenesis, H2Av-RFP vanishes and ProtB-eGFP becomes detectable (arrowhead). Somatic cells of the terminal epithelia (te) and the seminal vesicle (sv) also express H2Av-RFP. (C) Cross-section of a mouse seminiferous tubule at stages X–XI, stained with DAPI (blue) and with an antibody that recognizes histone H3.1 (red). All cells except condensed spermatids express H3.1. Spermatids in the process of removing the histones are partially positive for H3.1 (arrowhead). The areas containing spermatocytes (spc) and elongating and condensing spermatids (spt) are indicated.

protamines generate the tightly packaged sperm nucleus [17,18]. In both flies and mammals, specific histone modifications and transient formation of DNA breaks precede or accompany protamine deposition [19–24]. Over the past two decades, many chromatin components that specifically function during spermiogenesis have been described. However, the exact regulatory cascade of events of chromatin reorganization in developing sperm as well as the reason why sperm possess such an unusual chromatin composition are still poorly understood.

Here, we highlight the current knowledge on mammalian and *Drosophila* spermiogenesis. In Section 2 we provide an overview of the histone-to-protamine transition. In Section 3, we briefly discuss the potential function of protamines. Sections 4–6 discuss the different chromatin components of spermatids during post-meiotic chromatin remodeling in more detail. We then present current mechanistic insights into this process in Sections 7 and 8. In Sections 9 and 10, we briefly discuss the possible relevance of residual histones within sperm nuclei as well as protamine removal after fertilization. We conclude with a model that integrates the current knowledge and highlight the questions to be addressed in future research.

2. From histones to protamines — an overview

The process of spermatogenesis in mammals and in *Drosophila* species is similar (Fig. 1A), although the size and shape of mature sperm as well as the time span for spermatogenesis differ considerably (Fig. 2) [25]. The testes of *Drosophila* and all mammalian species contain all stages of spermatogenesis, from stem cells to mature sperm, and spermatogenesis occurs within a tubular structure (Fig. 1B, C). Germ cells develop in close contact with the surrounding somatic cells, known as cyst cells in *Drosophila* and Sertoli cells in mammals. In *Drosophila*, germ cells move from the apical tip to the basal end of the tubule during differentiation, while in mammals, germ cells move from the periphery to the lumen, followed by transport to the epididymis, a long tubule that stores the mature sperm [3,26]. Thus, in *Drosophila*, the process of spermatogenesis can be followed from proximal to distal along a single testis tubule (Fig. 1B), whereas in mammalian testis, many tubules are present and specific associations of cell types can be found together in a single cross-section (Fig. 1C). In mammals, the number of stages that can be observed varies among species [26]. For a general overview on fly

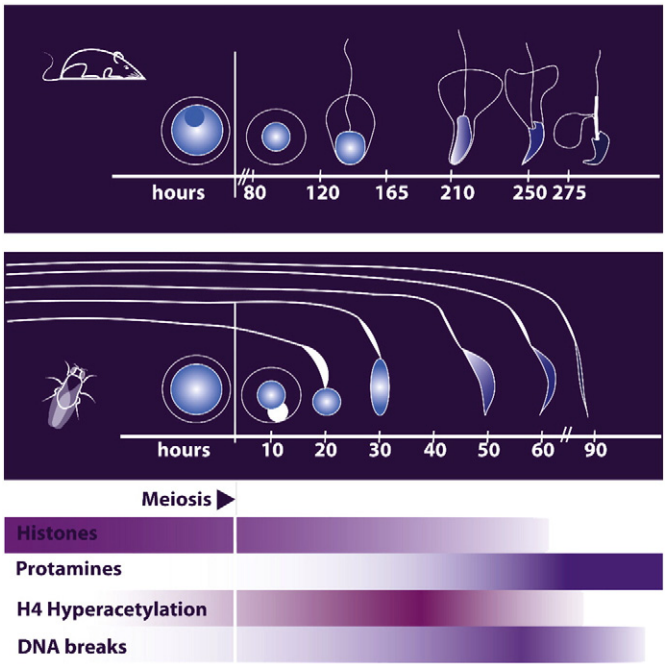

Fig. 2. Key chromatin remodeling events during spermiogenesis in mice and flies. Shortly after meiosis, spermatids are characterized by a round nucleus that elongates and reshapes during spermiogenesis. In parallel, the nucleosomal histone-based chromatin configuration is replaced by a mainly protamine-based tightly compacted structure. This chromatin reorganization is accompanied by hyperacetylation of histone H4 as well as the transient appearance of DNA breaks.

and mammalian spermatogenesis, we refer the reader to the *Drosophila* Testis Gene Expression Database FlyTED (http://flyted.zoo.ox.ac.uk) [27] and SpermatogenesisOnline (http://mcg.ustc.edu.cn/sdap1/spermgenes) [28].

As cells enter the meiotic prophase, homologous chromosomes need to pair in preparation for the first meiotic division. In mammals, chromosome pairing is preceded by and depends on the induction of meiotic DNA double-strand breaks [29,30]. This then leads to genetic recombination and crossover formation. A striking difference between *Drosophila* and mammalian spermatogenesis is the lack of a synaptonemal complex in flies and thus lack of recombination in male germ cells [31,32]. Following meiosis and during spermiogenesis in both flies and mammals, round spermatids differentiate into mature sperm. During this process, the nuclear volume dramatically reduces, and histones are gradually replaced by protamines (Fig. 2). This exchange is associated with both hyperacetylation of histone H4 and DNA breaks (Fig. 2) [19,23,33]. The histone-to-protamine transition can be easily visualized in testes of double-transgenic flies that express the red-fluorescent-protein-labeled histone H2Av-RFP and the enhanced-green-fluorescent-protein-labeled protamine ProtB-eGFP (Fig. 1B). Live imaging in *Drosophila* has revealed that substitution of histones by protamines takes place 50–60 h after the meiotic divisions and lasts about 5–6 h [34]; live imaging of the histone-to-protamine transition in mice has not yet been performed. However, based upon immunolocalization analyses of (acetylated) canonical histones [35], transition proteins [36–39], and protamines [40], the known overall duration of spermatogenesis, and the relative duration of each stage [41], the histone-to-protamine transition in mice is thought to start approximately 156 h after completion of meiosis. This corresponds to a stage when spermatids have reached step 10 of spermiogenesis and are elongating. Histones are no longer detected in condensing spermatids in step 12, which form 30–36 h later. At step 15, the transition proteins have disappeared. Taken together, the duration of the complete histone-to-protamine transition in mice can be estimated to encompass maximally 120–126 h.

3. What is the function of protamination?

In many species, protamines constitute the most abundant chromatin components of mature sperm [42]. It is believed that protamines stabilize sperm chromatin through their assembly in the minor groove of DNA [43]. The high level of arginines within protamines mediates strong DNA binding, while the cysteines facilitate formation of inter- and intra-protamine disulfide bonds essential for the formation of highly compacted chromatin [16]. In addition, it has been proposed that zinc ions contribute to DNA–protamine packaging by linking protamines with zinc bridges [44]. Various hypotheses explain why sperm exhibit such a specialized chromatin structure. First, condensation of sperm chromatin may help to generate a compact hydrodynamic shape. Second, the compaction of chromatin may protect the paternal genome from physical and chemical damage. And third, protamines could be involved in epigenetic regulation [13,16,42]. For example, swimming tests with deer sperm have shown that sperm with more elongated heads swim faster [45]. In mice, disruption of already one allele of either *Prm1* or *Prm2* leads to male sterility [46]. Nuclei of sperm with reduced amounts of protamines are less resistant to chemical disruption than nuclei of wild-type sperm [46]. In addition, protamine reduction causes distinct changes in chromatin packing, in the shape and organization of the acrosome, and in the overall architecture of the sperm [43]. Swimming tests for these protamine-deficient sperm cells have not been performed, but since flagellar morphology is also aberrant, the effect of aberrant head shape alone could not have been tested in this model. Following intracytoplasmic sperm injection (ICSI), *Prm2*-deficient epididymal sperm are able to activate most metaphase-II-arrested mouse eggs, but only few develop to the blastocyst stage [43]. Also in humans, reduced protamine levels correlate with infertility, and sperm with damaged DNA negatively affect development of human embryos after ICSI [47]. In flies, the two identified protamines are not essential for male fertility. However, it is very likely that sperm of protamine-deficient flies still contain proteins that perform functions similar to that of protamines. Nevertheless, protamine-deficient *Drosophila* sperm have an increased sensitivity to X-rays [48]. Together, these data indicate that protamination protects the genome against damage. More detailed analyses of the cause of developmental arrest of ICSI-derived embryos injected with protamine-deficient sperm should reveal whether defective protamination also affects the early zygotic gene expression program, or whether DNA damage of the paternal genome interferes with further development of the embryos.

4. Histone variants in male germ cells

In most eukaryotic cells, nucleosomes are the packing units of DNA. Nucleosomes are octamers formed by two molecules of each of the canonical core histones H2A, H2B, H3, and H4 [49]. The linker histone H1 binds to the nucleosomal and linker DNA and contributes to a higher-order chromatin structure [50]. The major functions of canonical histones are genome packaging and gene regulation. The non-canonical histones (histone variants) play a role in a wide range of processes, such as transcription initiation and DNA repair by establishing a distinct chromosomal domain to carry out a specialized function [51]. Currently, variants of all four canonical histones and the linker histone H1 are known. Histone H4 belongs to the most slowly evolving proteins in eukaryotes, and variants have been described only in a few species [52,53]. In mammals, no histone H4 variant is known. In flies, the histone H4 replacement gene *H4r* has been described, and the encoded protein has been found in the sperm proteome [54,55]. Nevertheless, to date there is no evidence that H4r plays an essential role in *Drosophila* spermiogenesis. While some non-canonical histones are ubiquitously expressed, others are restricted to few tissues or cells, e.g., male germ cells. Here, we will focus on H1, H2A, H2B, and H3 variants that are expressed in post-meiotic germ cells and might be involved

in loosening the chromatin structure in preparation for the histone-to-protamine transition.

4.1. H1 variants

In mammals, 11 different subtypes of histone H1 are known. Among them, H1t, H1T2, and HILS1 (Fig. 3) are expressed specifically in the testis [56]. The linker histone variant H1t becomes detectable first in pachytene spermatocytes and persists until chromatin reorganization in post-meiotic stages [57,58]. H1t-deficient mice are fertile and exhibit no spermatogenesis abnormalities, but enhanced gene expression of the canonical subtypes H1.1, H1.2, and H1.4 has been observed in H1t-deficient spermatids, compared to controls [59]. The testis-specific H1 variant H1T2 is expressed in haploid male germ cells until the histone-to-protamine transition. Loss of H1T2 causes defects in post-meiotic nuclear condensation and greatly reduces male fertility [60,61]. In spermatid nuclei, H1T2 localizes in a cap-like structure at the apical pole under the acrosome. This specific localization possibly confers polarity to the spermatid nucleus, which in turn could be essential for proper DNA condensation [62]. The spermatid-specific linker histone H1-like protein (HILS1) is strongly expressed in nuclei of elongating and elongated spermatids [63–65]. As HILS1 remains detectable in spermatids that do not contain core histones, it may fulfill other and/or additional functions compared to linker histones, at least at this late stage of spermiogenesis [64].

In *Drosophila*, three proteins specifically expressed in the testis with similarity to linker histone proteins have been described. Synthesis of all three proteins, named Don Juan (DJ), Don Juan Like (DJL), and Mst77F (Fig. 4), is restricted to post-meiotic male germ cells [66–69]. Of these three proteins, only Mst77F shows a significant similarity to mammalian HILS1 and is essential for male fertility. Like HILS1, Mst77F can still be detected when core histones have been removed [17]. In contrast to HILS1, Mst77F can even be detected in distinct regions in the nuclei of mature sperm [17,48]. Furthermore, Mst77F is associated with microtubules during nuclear shaping. Hence, a dual function for Mst77F in nuclear shaping and chromatin reorganization has been proposed [48]. In mammals, an H2B variant has been reported to reside outside the spermatid nucleus, in the subacrosomal compartment, and might be involved in homing the acrosome on top of the nucleus [70,71]. However, this H2B variant appears to function exclusively outside the nucleus. And a dual function-inside as well as outside the sperm nucleus-have so far not been reported for any mammalian histone variant, including HILS1.

4.2. H2A and H2B variants

In *Drosophila*, only a single H2A variant (H2Av) has been described to date. Histone H2Av shows similarity to both mammalian H2A variants (H2A.X and H2A.Z) [72]. Within the testis, H2Av in flies and H2A.X and H2A.Z in mammals can be detected in male germ cells until spermiogenesis and vanish at the time of chromatin reorganization (Figs. 3 and 4) [17,23,73,74]. In mammals, several testis-specific or testis-enriched H2A and H2B variants during spermatogenesis have been reported (Fig. 3). The histone variants TH2A and TH2B (testis-specific variants of histones H2A and H2B) are specifically expressed in the testis, and both have been detected in post-meiotic spermatids [75,76]. During condensation of spermatid nuclei, both TH2A and TH2B gradually disappear [77–80]. In humans, TH2B can still be detected in mature sperm [76]. The spermatid-specific H2B (ssH2B) variant is specifically synthesized and expressed in round spermatids

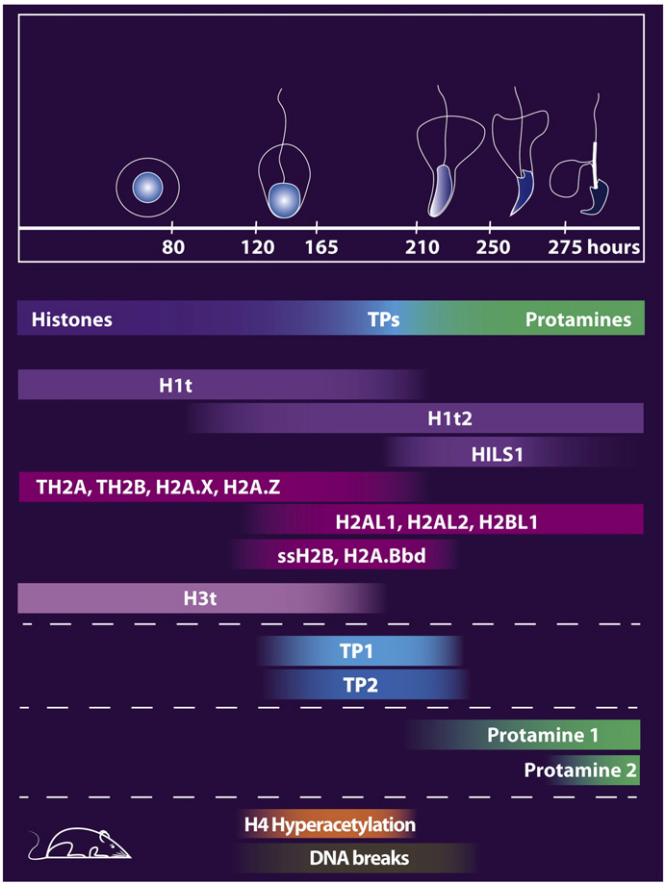

Fig. 3. Scheme displaying the major chromatin components expressed during spermatid differentiation in mice. Chromatin reorganization initiates with incorporation of histone variants into chromatin. In round and early spermatids, many histone variants can be detected. Some variants vanish as chromatin compacts, while others remain. During spermatid elongation, histones are first replaced by transition proteins and subsequently by protamines.

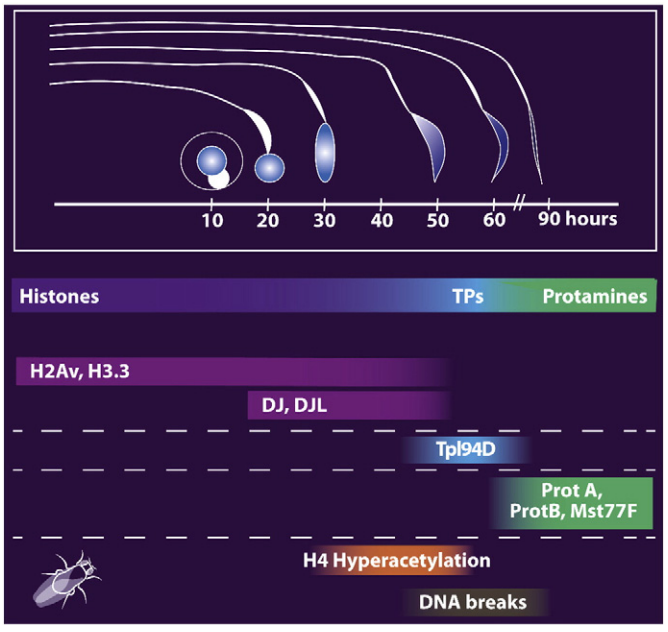

Fig. 4. Scheme displaying the major chromatin components expressed during spermatid differentiation in flies. In round and early spermatids, histone variants and the histone-like proteins DJ and DJL can be detected. These proteins vanish together with the bulk of histones. Post-meiotic chromatin reorganization involves a virtually complete exchange of histones by protamines and Mst77F. At the time of chromatin reorganization, transient expression of Tpl94D can be observed. Note that only the nuclear expression of Mst77F is depicted here.

and starts to decrease before the bulk of histones becomes degraded during chromatin compaction [81,82]. As mammalian round spermatids are transcriptionally active, it is possible that ssH2B might be involved in transcriptional regulation. The non-canonical H2A.B.bd is enriched in human and mouse testes [83,84]. H2A.B.bd has been found in protein fractions of mammalian testes at the time when histones are highly acetylated (see Section 7). In addition, H2A.B.bd has been observed in the nucleosomal chromatin fraction of mature human sperm [85]. As H2A.B.bd is able to destabilize and unfold chromatin in vitro, this variant might assist in chromatin reorganization and displacement of histones by transition proteins [85–87]. In 2007, Govin et al. discovered five histone variants expressed mainly in testes: H2AL1, H2AL2, H2AL3, H2BL1, and H2BL2 [22]. H2AL1, H2AL2, H2AL3, and H2BL1 mRNAs are strongly enriched in round and elongating spermatids compared to pachytene spermatocytes, while H2BL2 mRNA is present in very low levels in meiotic and post-meiotic stages. Immunostainings using antibodies that recognize H2AL1 and H2AL2 (H2AL1/L2) or H2BL1 show an accumulation of H2AL1/L2 and H2BL1 in condensing spermatids similar to that of transition proteins and protamines (Fig. 3). In addition, H2AL1 and H2AL2 localize to the pericentric regions in condensing spermatids and may participate in specific reprogramming of pericentric heterochromatin. Additionally, H2AL1/L2 and H2BL1 remain associated with chromatin of mature sperm isolated from epididymis [22].

4.3. H3 variants

In addition to the two canonical histones H3.1 and H3.2, three non-canonical H3 variants have been identified in mammals: H3.3, H3t, and CENP-A [88]. Also two primate-specific H3 variants (H3.X and H3.Y) have been characterized [89], and more recently, H3.5 has been discovered in humans [90]. H3.5 is specifically expressed in seminiferous tubules of human testes [90]. The histone variant H3t (Fig. 3) is highly enriched in male germ cells, but small amounts have also been detected in somatic cells [91,92]. H3t is synthesized in spermatogonia, and remains detectable in spermatocytes and early spermatids [92]. The non-canonical histone H3.3 is encoded by two different genes (H3.3A and H3.3B) that encode the same amino acid sequence. In mammals, H3.3A mRNAs can be detected in male germ cells until post-meiotic stages, while H3.3B mRNAs are restricted to meiotic prophase [93].

In *Drosophila*, H3.3A is strongly transcribed in testes [94]. H3.3 is expressed in mitotic, meiotic, and post-meiotic male germ cells and disappears with the bulk of histones (Fig. 4) [23,94]. H3.3-deficient flies are male sterile and exhibit chromatin defects in male germ cells [95]. CENP-A (CID in *Drosophila*) is known to replace H3 in centromere-specific nucleosomes [96]. Also in male germ cells, CENP-A is associated with centromeres in mammals and flies and appears to persist in mature sperm [97,98].

5. Transition proteins: transiently expressed non-histone chromatin components in spermatids

Between histone removal and protamine deposition in mammalian spermatids, about 90% of the basic chromatin components consists of transition proteins (Fig. 3) [99]. Mouse transition protein 1 (TP1) and 2 (TP2), encoded by *Tnp1* and *Tnp2*, are arginine- and lysine-rich proteins that bind strongly to DNA [100–106]. The functional activities of each transition protein are still under debate. It has been reported that TP1 decreases the melting temperature of DNA, relaxes the DNA in nucleosomal core particles, and stimulates the DNA-relaxing activity of topoisomerase I [107–110], which indicates that TPs could help chromatin remodeling by making the DNA more flexible. However, others have reported that neither TP1 nor TP2 is able to cause topological changes in supercoiled DNA [111]. In addition, it has been proposed that the TPs can mediate DNA and chromatin condensation [101,112]. Finally, TP1 has been found to stimulate repair of single-strand DNA breaks [113]. *Tnp1* knockout in mice leads to severely reduced sperm motility and to elevated levels of TP2 and of some protamine 2 precursors in spermatid nuclei that might partially compensate for the lack of TP1. About 60% of *Tnp1*-deficient males is sterile [114]. In contrast, *Tnp2*-deficient mice are fertile and display only mild sperm abnormalities [115]. In mice lacking both TPs, histone displacement and protamine deposition proceed relatively normally, while chromatin condensation is irregular in all spermatids. Furthermore, many late spermatids show DNA breaks, the number of epididymal sperm is drastically reduced, and mice are sterile. Surprisingly, ICSI of mature spermatids of *Tnp2*-deficient mice produces offspring, while epididymal sperm are incapable of fertilizing [39,116]. Together, these data indicate that there is some functional redundancy between TP1 and TP2, and that they are not required for histone removal and protamine loading, yet are important for the proper regulation of chromatin structure. In *Drosophila*, true homologs of transition proteins have not been identified. Nevertheless, the arginine-rich HMG-box containing protein transition protein-like 94D (Tpl94D) is specifically expressed in spermatid nuclei at the time of the histone-to-protamine transition (Fig. 4) and might be a functional homolog of mammalian transition proteins [23].

6. Protamines and chromatin components of mature sperm chromatin

Transition proteins are subsequently replaced by the highly basic protamines in late spermatids (Figs. 3 and 4). Mammalian protamines are arginine- and cysteine-rich proteins with a low molecular mass and are associated with DNA in late spermatids and mature sperm [117,118]. Most mammalian species have only one type of protamine present in sperm, while the genomes of mice and humans encode two protamine types (Fig. 3): Protamine 1 (PRM1 or P1) and Protamine 2 (PRM2 or P2) [13,119,120]. Whereas protamines of the P2 family (P2, P3, and P4), which differ only in the N-terminal extension of 1–4 residues, are generated by proteolysis from a precursor encoded by a single gene, protamine 1 is synthesized as a mature protein [42]. Just as in mice and humans, also the genome of *Drosophila* encodes two different protamines [17]. These protamines, ProtA and ProtB (encoded by *Mst35Ba* and *Mst35Bb*, respectively) (Fig. 4), are cysteine-rich but less arginine-rich than mammalian P1 and P2 [17]. In mice, PRM1 and PRM2 are essential for male fertility, and even deletion of a single copy of either *Prm1* or *Prm2* leads to infertility [46]. In humans, nuclei of normal sperm cells contain similar amounts of protamine 1 and 2 (P1/P2 ratio of 1) [121,122]; deviations from this ratio correlate with male infertility [16,123,124]. Surprisingly, in *Drosophila*, ProtA and ProtB are not essential for fertility [48]; when both protamines are missing, only about 20% of the sperm nuclei displays abnormal morphology [48]. However, one has to consider that these two protamines are not the only chromatin components of sperm in flies (Fig. 4). The HILS1-like protein Mst77F (see Section 4.1) marks distinct chromatin regions within sperm nuclei, and its deposition is independent of the incorporation of protamines [48]. It needs to be clarified whether further chromatin components exist in mature sperm that might be functionally redundant with protamines.

In mammals and flies, protamination of the paternal genome is a late event during spermiogenesis, occurring when transcription has already ceased. It has long been known that mammalian protamine expression is regulated by distinct transcriptional and translational control mechanisms [125]. In *Drosophila*, mRNAs relevant for spermiogenesis (e.g., protamines) are synthesized in primary spermatocytes, and these mRNAs are then translationally repressed until the appropriate phase during spermiogenesis has been reached when the proteins are needed (Fig. 1A) [8,17,25]. In post-meiotic stages, transcription of only a few genes has been reported [126,127]. In mammals, early round spermatids are highly transcriptionally active [128]. During spermatid maturation, transcriptional activity gradually decreases and becomes undetectable in late spermatids (Fig. 1A) [128]. The mRNAs for protamines and transition proteins are synthesized in

round spermatids, and like *Drosophila* protamine mRNAs, stored in a translationally repressed state [7]. In flies, transcription of genes required for spermiogenesis depends on testis-specific TATA-box-binding-protein-associated factors (tTAFs) [129–132]. It has previously been proposed that the bromodomain protein tBRD-1, which is expressed specifically in the testis, plays a role in regulation of transcription of translationally repressed mRNAs in flies [133]. Furthermore, some functionally identified mRNA regions regulate protein synthesis in late spermatids. The 5′-UTR regulates translational repression of *protamine* mRNA, while translational activation depends on sequences present in the open reading frame [8]. Only recently have *protamine* and *Mst77F* genes importantly been shown to be direct target genes of tTAFs [8]. Testis-enriched TAF variants, e.g., TAF7L and TAF4b, have been identified also in mammalian male germ cells [134,135]. Thus, in both mammals and flies, proper spatiotemporal expression of major spermatid and sperm chromatin components is mediated by intricate cell-type-specific mechanisms of transcriptional and translational control. A detailed comparison of transcriptional events in male germ cells of mammals and flies has recently been reviewed by White-Cooper and Davidson [136].

7. Histone modifications that precede histone removal during spermiogenesis

During spermiogenesis, most histones are replaced by transition proteins and subsequently by protamines. This drastic alteration in chromatin configuration is expected to require mechanisms to promote eviction of nucleosomes in favor of incorporation of transition proteins, followed by a subsequent exchange of transition proteins for protamines. In addition to the incorporation of histone variants, specific histone modifications also affect the higher-order chromatin structure and play important roles in gene regulation and maintenance of genome integrity [137]. Histones are commonly post-translationally modified at the amino-terminal tails. The most-studied histone modifications include acetylation, methylation, phosphorylation, ubiquitination, and SUMOylation. These modifications can be recognized by proteins and facilitate downstream events on chromatin, resulting in a more open or closed chromatin configuration [138–140]. In both flies and mammals, hyperacetylation of histone H4 has been observed in elongating spermatids just prior to histone removal (Figs. 3 and 4) [23,141,142]. It has been hypothesized that H4 hyperacetylation in mammalian spermatids leads to an open chromatin structure that facilitates and induces histone displacement [13,143]. In mice and humans, reduced levels of histone H4 hyperacetylation in sperm correlates with impaired fertility [141,144]. Previously established testis organ cultures have enabled us to address the functional importance of histone hyperacetylation in *Drosophila*. Indeed, inhibition of histone acetyltransferases (HATs) in in vitro cultures of *Drosophila* testes leads to spermiogenesis arrest, and the chromatin remains in a histone-based configuration [34]. Thus, hyperacetylation appears to be a prerequisite for proper chromatin reorganization. However, premature acetylation does not induce premature chromatin reorganization in *Drosophila*, which suggests that H4 hyperacetylation is not the sole inducer of chromatin remodeling [34]. Also, in mouse, premature expression of either PRM1 or TP2 in early round spermatids causes abnormalities in chromatin structure and infertility [145,146]. This indicates that these proteins can be incorporated in chromatin that is not yet hyperacetylated, although the timing of histone hyperacetylation has not been analyzed in these mouse models. Within the amino-terminal tail of histone H4, four lysines can be acetylated: lysine 5, lysine 8, lysine 12, and lysine 16 (H4K5, H4K8, H4K12, H4K16). In elongating spermatids of mice, acetylation of H4K5, H4K8, and H4K12 but not H4K16 has been observed [22,35]. In contrast, others describe acetylation of all four lysines in elongating spermatids [147,148]. In humans, H4K8 and H4K16 acetylations occur in elongating spermatids [149]. In addition, acetylation of histone H3 (H3K9) can be detected in elongating spermatids of humans and mice [24,150]. In mice, the plant homeodomain (PHD) finger protein PYGO2 is expressed in elongating spermatids at stages when chromatin remodeling occurs and might recruit the histone acetyltransferase that is responsible for H3, but not H4 hyperacetylation [151]. In bovine testes, the histone acetyltransferase (HAT) MYST4 that acetylates histone H3 and H4 can be specifically detected in nuclei of elongating spermatids [152]. While many histone modifications disappear in late elongating spermatids in parallel with the replacement of histones by transition proteins, others can still be detected in the fraction of chromatin that still has a nucleosome-based structure in mature sperm, e.g., H3K9 acetylation in humans or H4K8 and H4K12 in mice [148,150]. In mice, almost all retained histones in sperm are thought to localize to the (peri)centromeric heterochromatin [148]. Beside acetylation, strong histone methylation signals have been observed in elongating spermatids, such as strong H3K4 mono-, di-, and tri-methylation in mice and flies [23,153]. These modifications together with acetylations might assist in achieving a more-open chromatin configuration. Nevertheless, H3K9 mono-, di-, and tri-methylation as well as H3K27 di- and tri-methylation known to be associated with a repressed chromatin also occur in elongating spermatids in mammals and flies [23,149]. Expression of different histone methyltransferases and demethylases has been observed during spermatid elongation in flies and mammals [153–156]. This coexistence of both types of enzymes might be crucial to balance regions of “opened” and “closed” chromatin.

Another modification that may play a role in chromatin remodeling during spermiogenesis is histone ubiquitination. Ubiquitinated histones H2A and H2B are enriched during meiosis and in elongating spermatids in mice [157,158]. Likewise, in *Drosophila*, mono-ubiquitination of histone H2A precedes hyperacetylation of histone H4 [23]. The RING domain protein RNF8 in mice exhibits ubiquitin E3 ligase activity, and it has been postulated that it regulates ubiquitination of histone H2A and H2B as a prerequisite for histone H4K16 acetylation in spermatids [159]. However, more recently, Sin et al. reported that H4K8 acetylation and H4K16 acetylation are not affected in elongating spermatids of *RNF8*-deficient mice [160], leaving this issue unresolved. The identification of 67 novel histone modifications [161] indicates that we have just scratched the surface in trying to unravel the histone code, and in understanding how histone modifications could regulate the histone-to-protamine transition. One of these 67 modifications, lysine crotonylation (Kcr) is enriched on sex chromosomes, and it has been proposed that Kcr functions as a specific mark of active sex chromosome-linked genes in post-meiotic male germ cells [161,162]. In addition, a wave of hypercrotonylation accompanies spermatid elongation [161], and thus, this modification could also be relevant for the regulation of chromatin reorganization in spermiogenesis.

8. First insights into the mechanisms of chromatin reorganization during spermiogenesis

In eukaryotic cells, chromatin supports compaction and storage of DNA. Many biological processes such as transcription, replication, and recombination, but also the histone-to-protamine transition, require mechanisms to access DNA. These mechanisms often require one or more of the above-mentioned covalent histone modifications and histone replacement by histone variants. In addition, ATP-dependent chromatin remodeling complexes often participate in gaining DNA accessibility [163–166]. In this section, we will summarize the knowledge on potential components of chromatin-remodeling complexes, proteins that bind to modified histones, and molecular chaperones detected during spermiogenesis. Additionally, we will address the transient appearance of DNA strand breaks as well as the question how histones and transition proteins are degraded. The mechanisms that potentially lead to the mainly protamine-based DNA packaging in mature sperm are schematically summarized within a model (Fig. 5).

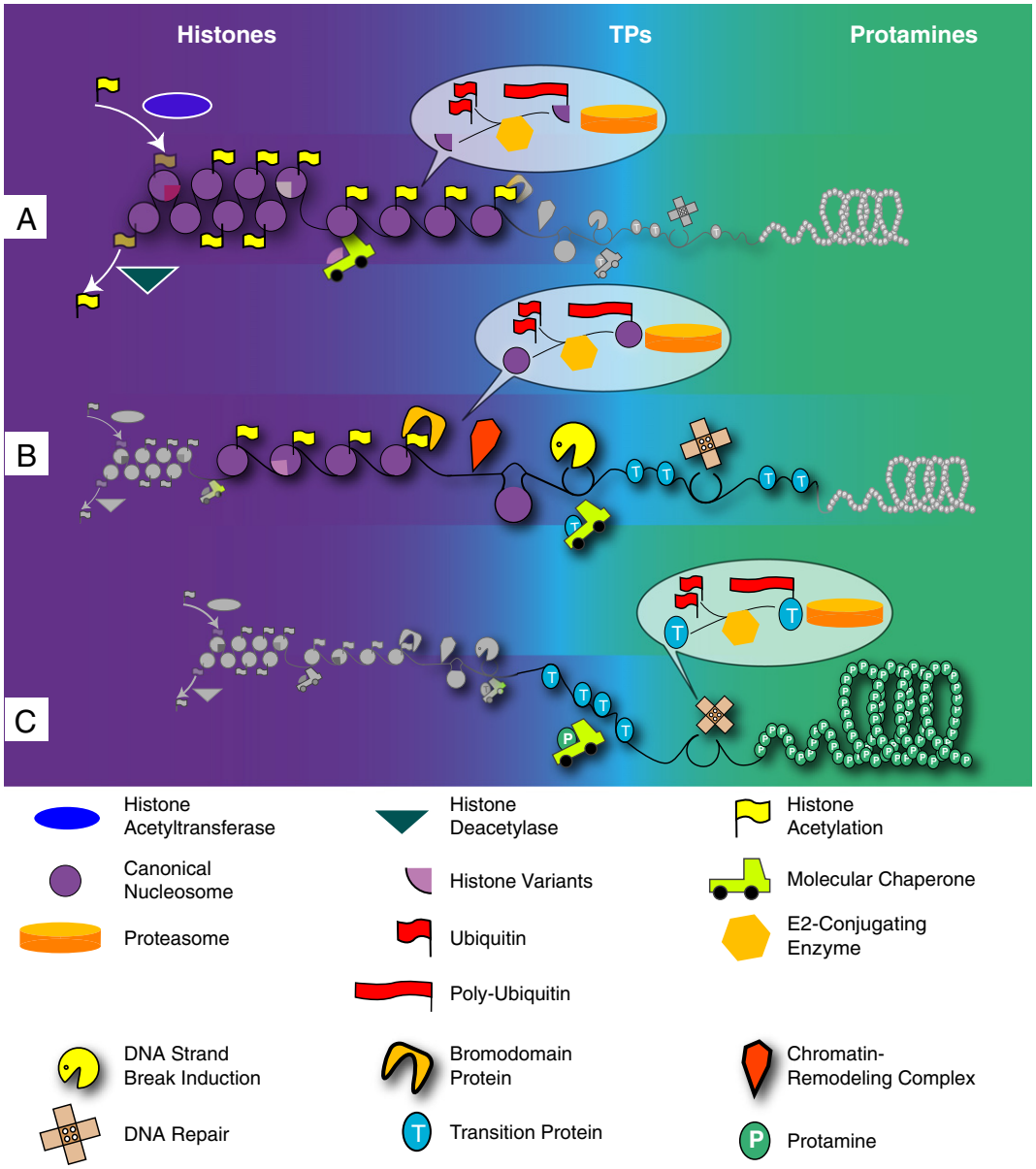

Fig. 5. Model of the regulatory mechanisms that may lead from a nucleosomal-based to a mainly protamine-based chromatin configuration. (A) Increased levels of histone acetyltransferases mediate hyperacetylation of histones to obtain a more open chromatin structure. This process might be supported by specific degradation of histone deacetylases. In parallel, chaperones participate in incorporation of histone variants to loosen the nucleosomal structure. Exchanged canonical histones in turn may undergo poly-ubiquitination and become degraded through proteasomes. (B) Binding of bromodomain proteins to acetyl-residues may facilitate recruitment of chromatin-remodeling complexes to further relax chromatin. This is accompanied by the induction of DNA strand breaks. The relaxed chromatin configuration allows replacement of histones by transition proteins, possibly with the help of molecular chaperones. Subsequently, DNA damage repair mechanisms must act to ensure chromatin integrity. (C) Finally, molecular chaperones most likely aid in replacement of transition proteins by protamines to allow tight packaging of DNA into a higher-order structure. Transition proteins may become degraded through poly-ubiquitination and proteasome activity. Note that residual histones are not depicted.

8.1. Potential components of chromatin-remodeling complexes in spermatids

To date, no ATP-dependent chromatin-remodeling complexes have been described in post-meiotic male germ cells. Nevertheless, in rat, the ATPase subunits of the ATP-dependent remodeling complex SWI/SNF, Smarca2/Brm and Smarca4/Brg-1, are highly expressed in round spermatids [167]. In addition, high expression levels of the SWI/SNF subunit Smarce1/BAF57 have been observed in round spermatids [167]. The protein Spermatogenic cell HDAC-interacting protein 1 (SHIP1) is expressed in the nuclei of spermatocytes and round spermatids before hyperacetylation of histone H4, and SHIP1 is a component of a complex with chromatin-remodeling activity [168]. Thus, SHIP1 and SWI/SNF chromatin-remodeling complexes might assist in chromatin reorganization during spermiogenesis. In addition, transition proteins themselves might help to remodel chromatin (see Section 5). However, in mice, apart from a putative role in histone removal, these remodelers may also aid in the global repression of gene transcription that occurs during this phase of spermiogenesis. Still, based on the currently available data, it appears most likely that a chromatin-remodeling complex might guide the replacement of histones by transition proteins during spermatid differentiation (Fig. 5).

8.2. "Readers" of histone modifications during spermiogenesis

Post-translational histone modifications can be recognized by "reader" proteins, and this may lead to downstream events on chromatin. For example, bromodomain-containing proteins can bind to acetylated

lysine residues of histones and are often part of chromatin-associated multiprotein complexes [169]. In mice, the testis-specific, double bromodomain-containing protein BRDT is expressed in pachytene spermatocytes and round spermatids [170–173]. Mice that express a truncated form of BRDT lacking the first bromodomain show abnormalities in post-meiotic male germ cells [174]. In addition, BRDT is able to bind acetylated histone H4 at the time of histone replacement by transition proteins [147]. Moreover, BRDT has crucial functions in the regulation of the testis-specific gene expression program [175]. In rat, BRDT interacts with Smarce1/BAF57 and exhibits acetylation-dependent, but ATP-independent chromatin-reorganization properties in round spermatids [167]. Chromatin-remodeling properties have also been shown for mouse BRDT [176]. In addition to BRDT, the bromodomain proteins BRD2 and BRD3 in mice, as well as Baz2a in rat can be detected in round spermatids [167,172]. The role of bromodomain-containing proteins during mammalian spermatogenesis has been reviewed by Berkovits and Wolgemuth [177].

8.3. Molecular chaperones during sperm maturation

The process of chromatin reorganization in spermatids most likely also requires mechanisms to incorporate histone variants, transition proteins, and protamines. In addition, mechanisms to remove histones and transition proteins have to exist. Chromatin assembly is mediated by histone chaperones and ATP-utilizing motor proteins [178]. Consequently, it has been proposed that histone chaperones function in histone removal as well as histone exchange and that they team up with ATP-dependent chromatin-remodeling factors [179]. Thus, it might be possible that molecular chaperones are required to remove histones and at the same time aid in incorporating transition proteins and protamines into spermatid chromatin. Currently, hardly anything is known about chaperones that act during spermiogenesis. The testis-specific histone chaperone tNASP in mice is already expressed in spermatocytes and binds to H1t [180]. Another testis-specific chaperone, HSPA2 (Hsp70.2), is highly expressed in post-meiotic stages and interacts with transition proteins [181]. In addition, HSPA2 (Hsp70.2) functions in meiosis, as deficient mice lack post-meiotic stages and mature sperm and are infertile [182]. Due to the essential role already in meiosis, the post-meiotic function of HSPA2 is difficult to evaluate.

In flies, chaperone proteins of the Nap family (Hanabi and Nap1) are required for proper spermiogenesis. However, the transiently expressed protein Tpl94D as well as protamines become incorporated into chromatin in both *hanabi* and *nap1* mutants [183]. Kimura has proposed that their role is more likely in cytoskeleton organization and not in histone turnover [183]. Recently, we have shown that the canonical histone chaperone chromatin assembly factor I (CAF1) is required for the stable deposition of protamines and is associated with chromatin of mature sperm. Thus, we propose that CAF1 acts as a protamine chaperone during spermiogenesis [184]. The loss of CAF1 does not affect histone removal. Thus, we hypothesize that histone removal is independent from protamine incorporation [184].

8.4. Transient DNA strand breaks during chromatin reorganization in spermatids

An additional process that occurs during chromatin reorganization in elongating spermatids of mammals and flies is the transient appearance of DNA strand breaks that probably function to eliminate free DNA supercoils formed during histone removal [19,21,23,185]. It has been proposed that in mice topoisomerase II is responsible for generating DNA strand breaks during spermiogenesis [21]. In elongating spermatids, expression of topoisomerase II beta (TOP2B) overlaps with the appearance of phosphorylated H2AX (γH2AX) foci, a marker of DNA double strand breaks. In addition, tyrosyl-DNA phosphodiesterase 1 (TDP1), known to remove TOP2B-cleavable complexes from DNA, is expressed in elongating spermatids, and DNA polymerase activity, a marker of active DNA repair, has also been detected in these cells [74]. Another well-known process that occurs in response to DNA break formation is poly(ADP-ribosyl)ation of proteins. This is mediated by members of the poly(ADP-ribose) polymerase (PARP) protein family. Poly(ADP-ribose) formation has also been observed during rat spermiogenesis, and knockout of PARP proteins or pharmacological inhibition of poly(ADP-ribosyl)ation leads to impairment of the chromatin-reorganization process in elongating spermatids [186–188]. As post-meiotic spermatids are haploid, DNA repair has to rely on non-homologous end-joining (NHEJ) or similar processes [74]. In grasshoppers, the DNA-repair protein Ku70, which has been proposed to be involved in NHEJ, is detected in round and elongating spermatid nuclei [189]. Furthermore, there is evidence that ubiquitination and SUMOylation are involved in DNA repair pathways [190]. In flies, high levels of ubiquitin and SUMO have been observed in nuclei of elongating spermatids [23]. In mouse, SUMOylation has been observed in association with (peri)centromeric chromatin in round spermatids, but is not enriched in nuclei during elongation [191]. However, increased ubiquitination of histones has been observed during this phase [157]. In yeast, the ubiquitin-conjugating enzyme RAD6 is involved in post-replication DNA repair and is able to ubiquitinate histones H2A and H2B [192,193]. The two homologues of RAD6 in mammals are HR6A and HR6B. HR6A is dispensable for male fertility, but loss of function of HR6B in mice causes defects during post-meiotic chromatin compaction [194,195]. This result strongly indicates involvement of HR6B-dependent ubiquitination in chromatin remodeling [196,197]. However, in *Hr6b* knockout mice, histone ubiquitination in germ cells does not appear to be affected [195,198]. Thus, although the phenotype of the *Hr6b* knockout spermatids strongly indicates an involvement of HR6B-dependent ubiquitination in chromatin remodeling [196], the relevant substrate remains unknown. Two other genes involved in ubiquitination, *Ube1x* and *Ube1y*, which encode ubiquitin-activating enzymes, are meiotically suppressed but reactivated in spermatids [199]. The Rad6 homolog in *Drosophila* is UbcD6 [194]. High expression levels of UbcD6 are detected in spermatid nuclei at the time of chromatin reorganization [23], providing evidence for a conserved function of RAD6 during the histone-to-protamine transition.

8.5. Ubiquitination and testis-specific nuclear proteasome subunits in spermatids

A further process that accompanies chromatin reorganization is the degradation of histones and transition proteins (Fig. 5). In eukaryotic cells, the majority of proteins are degraded by the proteasome [200]. To trigger targeting of proteasomes, proteins become poly-ubiquitinated [201–203]. Much is known about cytoplasmic proteasomes, but also proteasomes within the nucleus have been described [204]. In *Drosophila*, the testis-specific proteasome subunits Prosα3T and Prosα6T are detected in post-meiotic spermatid nuclei [23,205,206]. *Prosα6T* mutants are male sterile, exhibit a delay in histone removal, and show abnormalities during nuclear maturation and sperm individualization [206]. However, chromatin reorganization and protamine deposition occur [206]. This indicates that loss of this proteasome subunit might reduce the efficiency of the proteasome and that further subunits have to be deleted to render the proteasome non-functional. Still, these data indicate that massive histone removal during spermatid elongation may be accompanied by proteasome-mediated degradation. In mice, proteasomes localize along the outlines of the acrosomal vesicle during spermatid elongation [207], but a link between proteasome function and histone removal has not been reported. Early reports indicated that a specific protease might be associated with chromatin and mediate histone degradation during spermatid elongation in mammals [5], but these observations have not been followed up and such a protease has not been identified. Based on the current knowledge from mammals and *Drosophila*, the mechanisms that

may mediate the histone-to-protamine transition are schematically summarized in Fig. 5.

9. The possible functions of residual histones in the sperm nucleus

In human sperm, 10 to 15% of the DNA retains a histone-based nucleosomal structure [7,16,149]. In mouse sperm, only about 1% of the DNA is associated with histones [149,208]. Also the presence of non-canonical histones, especially TH2B, has been reported for human sperm [76,209]. The remaining nucleosomes are nonrandomly spread along the chromosomes, predominantly associated with centromeres and telomeres [210–213]. The persistence of a nucleosome-based chromatin structure at these regions may be important for global chromosome function, and may help to ensure that the centromeric and telomeric regions of the chromosomes are immediately recognized and appropriately organized in the zygote. Recently, it has been reported that in addition, nucleosomes might be preferentially retained at gene promoters and at loci that regulate embryo development [214,215]. It has been hypothesized that these paternally contributed nucleosome-based regions are important for normal embryogenesis [15]. Many of the remaining histones carry post-translational modifications (e.g., acetylation) that are transmitted to the early zygote and persist in the early embryo [148,211]. The injection of round spermatids into mature oocytes (ROund Spermatid Injection, ROSI) produces normal offspring, which indicates that packaging of paternal DNA in a protamine-based structure in sperm is not essential for early embryo development [216]. Still, a higher percentage of mouse ICSI embryos compared to ROSI embryos develops to term [216]. In addition, ROSI-derived early embryos display aberrant patterns of gene expression [217], and an increased frequency of embryo aneuploidy [218], which indicates that the initiation of the early developmental program is influenced by the chromatin structure provided by the paternal gamete. However, it is interesting that in certain mouse models with spermiogenesis defects, ROSI is more effective in producing offspring than ICSI [219,220]. This may be related to the further deterioration of the quality of sperm with aberrant head shapes and chromatin structure in the epididymis, as shown by a comparison between the results of ICSI with testicular sperm versus epididymal sperm in transition-protein-deficient mice [221] or in mice with partial deletions of the Y chromosome [222]. In *Drosophila*, no remaining canonical histones have been detected within mature sperm using common immunohistological staining methods [23]. Nevertheless, the presence of a small amount of canonical histones in fly sperm cannot be excluded. However, while mouse embryos activate the zygotic genome around the two-cell stage, early embryogenesis in *Drosophila* depends on a more prolonged period on maternally provided mRNAs and proteins, and zygotic gene expression can be first detected around the tenth nuclear division [223]. Thus, it appears very unlikely that residual histones within *Drosophila* sperm, should they exist, are important for early embryogenesis.

The centromere-specific histone H3 variant Cid can be detected in fly sperm [224]. This correlates well to the persistence of histones at mammalian centromeres. Lack of Cid results in gynogenetic haploid embryos as paternal centromeres fail to integrate into the gonomeric spindle of the first mitosis [224].

10. After fertilization: back to histones

The fusion of the oocyte and the sperm entails resumption of meiosis of the oocyte and formation of the male pronucleus [225,226]. Directly after the release of the sperm nucleus into the oocyte cytoplasm, protamines are quickly replaced by maternally supplied histones [208,227]. In addition, testis-specific histone variants rapidly disappear from the paternal genome after fertilization [228]. The treatment of hamster eggs with antimycin A prevents paternal chromatin remodeling, which suggests that ATP-dependent processes are required for protamine replacement [229]. It has been reported that in *Xenopus laevis*, the chaperone nucleoplasmin (NPM) is involved in histone assembly onto the paternal chromatin [230,231]. In mammals, NPM1, NPM2, and NPM3 members of the nucleoplasmin/nucleophosmin (NPM) family are expressed in oocytes, and it has been proposed that they regulate sperm chromatin decondensation [232]. Moreover, in NPM2-deficient mice, early embryogenesis is severely impaired [233]. Additionally, the *Drosophila* proteins nucleosome assembly protein-1 (dNAP-1) and DF31 as well as the human Template activating factor I (TAF-I) have been shown to promote decondensation of sperm chromatin [234–236]. In *Drosophila*, the maternal effect embryonic lethal mutation *sésame* (*ssm*) affects male pronucleus formation but not protamine removal [17,237]. *ssm* is a point mutation in the *Hira* gene that encodes the H3.3 histone chaperone HIRA. HIRA is essential for decondensation of the sperm nucleus and nucleosome assembly [238]. Eggs laid by *Hira*-deficient females lose the paternal chromosomes and produce gynogenic haploid embryos [238,239]. Recently, it has been proposed that HIRA cooperates with Yemanuclein to establish the H3.3-containing nucleosome in the male nucleus at fertilization [240]. Also in mouse zygotes, a strong enrichment of H3.3 as well as HIRA expression has been observed, which indicates that the function of HIRA in the protamine-to-histone transition may be conserved from flies to mammals [208,241].

11. Perspectives

In this review, we concentrated on chromatin reorganization during spermatogenesis and searched for the first time for common themes in the mechanisms of post-meiotic chromatin reorganization in mice and flies. Although specific aspects of meiosis and the global regulation of RNA transcription during spermatogenesis in mammals and flies remarkably differ, the sequence of events that occurs during chromatin reorganization is strikingly similar. *Drosophila* is one of the best-characterized and most-studied model organisms and well suited to answer a range of biological questions through easy genetic manipulation. While protamines in mice and humans have been studied for decades, researchers have become interested in post-meiotic chromatin reorganization in flies less than ten years ago. At present, we are still far away from a complete understanding of the whole histone-to-protamine transition.

We are convinced that comparative research in mice and flies will contribute more insights into the regulation of this process as well as answers to many other questions in male reproductive biology. Using the mouse as a model, a clear complication in the study of early steps of chromatin compaction during spermiogenesis is the concomitant gradual decrease in global gene transcription. Thus, it is difficult to assess whether a certain histone modification or chromatin component contributes to the shut-down of gene transcription or to the removal of histones or both. This question is more easily addressed in flies, which show hardly any transcription in post-meiotic stages. From the model presented in Fig. 5, it is clear that many intriguing questions remain. For example, it is obvious that hyperacetylation of histones accompanies chromatin reorganization (Fig. 5A); but it is not yet known which HATs are required for hyperacetylation and whether hyperacetylation is in part the result of degradation of HDACs. Furthermore, DNA strand breaks occur when transition proteins are deposited (Fig. 5B). Nevertheless, a very intriguing unresolved issue is why and how transient DNA breaks are formed and repaired. Molecular chaperones most likely aid in replacement of transition proteins by protamines to allow tight packaging of DNA into a higher-order structure (Fig. 5C). Finally, it is still open how and where histones and transition proteins are degraded and what the critical factor is that removes the histones in the end. We are confident that further research using both the fly and mouse model systems will help to shed light on these open questions in the biology of sperm genome compaction.

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