12-1-2017

Heritable Sperm Chromatin Epigenetics: A Break to Remember

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Running Title:

DDR signaling forms sperm chromatin epigenetics

Summary Sentence:

DNA repair pathways are involved in normal establishment of the mature sperm chromatin structure as a carrier of heritable epigenetic information to the next generation.

Keywords:

Histone modifications, topoisomerase, epigenetics, PARP, ADP-ribose, spermiogenesis, sperm, chromatin, gametogenesis, male infertility, reprogramming, spermatid, spermatogenesis, testis.

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Grant support:

This work was supported by a grant from the Utah Agricultural Experiment Station, Utah State University (UTA01166) to RGM.

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ABSTRACT

Sperm chromatin not only has a unique structure to condense and protect the paternal DNA in transit, but also provides epigenetic information that supports embryonic development. Most of the unique sperm nuclear architecture is formed during the sweeping postmeiotic chromatin remodeling events in spermiogenesis, where the majority of nucleosomes are removed and replaced by protamines. The remaining histones and other chromatin proteins are located in structurally and transcriptionally relevant positions in the genome and carry diverse posttranslational modifications relevant to the control of embryonic gene expression. How such postmeiotic chromatin-based programming of sperm epigenetic information proceeds, and how susceptible the process is to modulation by exogenous factors are key questions for understanding the inheritance of acquired epigenetic marks through the male germ line. We propose that transient DNA strand breaks mediated by topoisomerase 2 beta and the subsequent activation of DNA damage response pathways result in defined posttranslational modifications of histones in spermiogenesis. These pathways, likely along with others, may contribute to chromatin remodeling in elongating spermatids, influence chromatin-based intergenerational inheritance of epigenetic information, and may be defective in pathologies of abnormal male gametogenesis and infertility.

1. INTRODUCTION

There is now accumulating evidence of non-genetic (epigenetic) inheritance through the male germ line allowing individual ancestors to transfer information about their environment to their progeny [1–7].

Four distinct but interconnected molecular pathways that permit epigenetic information transfer from sperm to progeny have been discovered so far: (i) Coding and non-coding RNA, as well as tsRNA [8–13] in sperm, (ii) DNA methylation of the sperm genome ([14], reviewed in [15]), (iii) gene positioning and sperm nuclear architecture [16–18], and (iv) sperm chromatin components including histones and other DNA binding proteins [19–22].

How exactly germ cells obtain clues to environmental factors and encode them in some form of epigenetic information remains enigmatic at this time. However, the influence of environmental factors on the epigenome of male germ cells appears to be most impactful if it happens during a developmental phase when these cells are epigenetically reprogrammed. It has been established that male germ cells undergo at least two of such major epigenetic programming phases, namely during the prenatal differentiation of primordial germ cells, and during differentiation of germ cells into spermatogonia for the execution of spermatogenesis [3,23–25]. An additional window of opportunity likely also exists in the adult male, where continuous spermatogenesis is a life-long continuous process that could provide sensing of environmental cues and translation into epigenetic messages carried by the mature sperm to the next generation. [26,27]. Focusing on the emerging role of sperm chromatin proteins in intergenerational epigenetic inheritance, a review of recent data yields overwhelming evidence that certain chromatin-borne epigenetic signatures in mature sperm are important to embryonic development. How these signatures are established during spermatogenesis is poorly understood,

in part because they depend to an unknown extent on complex and not yet well characterized chromatin remodeling events [28]. Particularly the postmeiotic chromatin remodeling events, where spermatids develop into sperm, are probably among the most dramatic ones in cell biology and the question arises how sperm epigenetic information survives these events or may even be dependent on them. Here, we review emerging concepts and vehicles of sperm chromatin-based epigenetic inheritance and explore the potential influence of DNA damage repair (DDR) pathways on epigenetic programming of sperm in the context of postmeiotic chromatin remodeling events.

2. SPERM CHROMATIN AS A CARRIER OF EPIGENETIC INFORMATION

2.1 Spermiogenesis and the origin of sperm nucleosomes

Sperm are both, highly adapted to ensure safe transport of the paternal genome complement to the egg as well as epigenetically configured to support embryonic programming and totipotency of the zygote. To accommodate both of these functions, the protamine-based chromatin and compact nuclear architecture of sperm is uniquely organized and very different from the nucleosomal nuclear organization of the archetypal cell type [29]. Sperm DNA is not superspiralized but relaxed and associated with protamines that are cross-linked by disulfide bonds to form comparatively large toroid-like loops [17,30], which are physically very stable. To accomplish that chromatin change, the majority of histones are temporarily replaced by transition proteins (TP1 and TP2) and then finally by protamines (PRM1, PRM2) during the postmeiotic phase of spermatogenesis, termed spermiogenesis [31]. Only a small portion of the genome remains nucleosomal (approximately 2 % to 15 % in mice and humans, respectively [32,33]). Initial investigations revealed that at least a portion of the remaining nucleosomes are retained in

defined telomeric and pericentromeric chromosome regions of mammalian sperm, where they have important structural roles [33,34]. Besides their structural functions, histones often also carry posttranslational modifications (PTM) that are able to control gene activity, and therefore have the potential to transmit epigenetic information to the offspring [35]. Until recently, sperm were not thought to provide much histone-mediated epigenetic information because the majority of histones have been replaced by protamines during spermatid differentiation and the remaining ones were thought to be restricted to non-coding repetitive sequences in pericentromeric and telomeric heterochromatin.

Newer studies then found that another portion of the remaining sperm histones are located also elsewhere in the genome, but their exact positions, their functions, and their posttranslational modifications have been controversially discussed. Results of individual studies varied considerably, which may in part be due to differences in experimental details of the general technical approach used to map histones to the sperm genome [36,37], which is based on sequence analysis of DNA bound to nucleosomes separated from protaminated chromatin by limited micrococcal nuclease (MNase) digestion of sperm nuclei followed by chromatin immunoprecipitation (ChIP-seq) [38]. Some studies using this approach found sperm histones to be enriched in intergenic regions rather than in promoter regions [36,37,39] while other reports place histones at gene promoters, imprinted gene clusters, microRNA, HOX gene clusters and binding sites of the chromatin insulator protein CCCTC-binding factor (CTCF) [19–21,37]. There is some consensus that a portion of the sperm histones maps to DNA in GC-rich, hypomethylated promoter regions and first exons in most genes in human and murine sperm [36,37,40–42]. The potential association of residual sperm histones with promoters of specific genes and other genomic regions relevant to embryonic development gave rise to the concept

that such sperm histones may regulate gene expression in the early embryo [19–21,43–45,37,41]. Supporting this hypothesis, a direct link between the location and number of histones in sperm gene promoter regions and the expression of these genes in resulting 2-cell embryos could be shown using two mouse models with altered poly(ADP-ribose) metabolism [41]. Further supporting the concept that sperm chromatin carries a rich source of gene regulatory information, Jung and colleagues recently found that most promoter regions in mouse sperm are flanked by active histone modifications when they used transposase Tn5 instead of MNase processing to map accessible open chromatin states. They also used ChIP-seq analyses of MNase-digested sperm chromatin to demonstrate that adult enhancer regions carrying gene activating histone marks are already specified in sperm, and that CTCF and cohesin are important factors that organize the three-dimensional (3D) structure in sperm [22]. CTCF and cohesin are also involved in the formation of topologically associating domains (TADs) of chromosomes that form a 3D structure of mammalian nuclei, including sperm [46,47,16] and of nuclear lamina-associated domains (LADs) [48]. TADs in sperm are unique in that they are characterized by extra-long-range interactions and frequent inter-chromosomal interactions in addition to having conserved TADs found in somatic cells [16,49]. Pioneering, microscopy-based investigations have long shown that sperm exhibit a nuclear architecture that is very distinct from that found in somatic cells. In human and murine sperm, pericentromeric heterochromatin is clustered in the center of the nucleus, and telomeres with their subtelomeric repeats are located near its surface [50,51]. These heterochromatic sequences appear to be important for the specific and systematic arrangement of chromosomes during meiosis to form the specific sperm nuclear structure which has long been considered to constitute an additional layer of epigenetic information in itself [18,50,52–55]. The unique nuclear structure may therefore explain a subset of the unique TADs

found in sperm. The extent to which the sperm TAD structure is inherited by the embryo is unclear as the high-order structures of both the paternal and maternal genomes in zygotes and two-cell embryos are not well defined but are later gradually re-established through development [49].

Given the important roles of heterochromatic, nucleosomal regions in sperm nuclei, it is not surprising that genome-wide nucleosome mapping also suggests that a large portion of sperm nucleosomes are preferentially located at repetitive sequences throughout the genome, e.g. SINE, LINE, and retrotransposons [39]. These findings, which appear also to be in line with other studies [56,57] have been disputed [58], because histone enrichment in repetitive sequences may be overestimated by certain computational approaches to map high-throughput sequencing reads to the genome. While this may be true, the overall result that sperm nucleosomes are preferentially retained in repetitive sequences [59] is consistent with results from other groups who used histone immunostaining or fluorescence in situ hybridization (FISH) of sperm nuclei. These investigations support the hypothesis that sperm histones tend to co-localize with repeat-enriched constitutive heterochromatin blocks such as found in pericentromeric regions [37,57,60]. All of these findings are, however, not inconsistent with the overwhelming evidence that at least a portion of sperm histones occupy gene regulatory and other genomic regions in a way that is expected to direct early embryonic gene expression, because these regions represent only a comparatively small fraction of the genome.

In addition to histones and CTCF, other DNA binding proteins capable of genome regulation and transcriptional regulation, such as transcription factors and the transactive response DNA binding protein (TDP-43) have been detected in mature sperm [19,22,37,61,62]. The roles of these proteins in sperm mediated epigenetic inheritance have yet to be determined.

2. 2. Testicular histone variants as carriers of epigenetic information.

Canonical histone genes (H2A, H2B, H3 and H4) are clustered in the mammalian genome and expressed from these clusters solely in S-phase to permit histone incorporation into newly replicated DNA. In addition, non-canonical variants of all canonical histones except for H4 are present in mammals, where they are expressed from individual genes throughout the cell cycle [7,63]. Male germ cells express a large number of non-canonical histone variants, including H2AFX (also known as H2A.X) [64], H2A.Z, TH2A [65], H3.3 [66–68], H3T [69], three H2AL and two H2BL variants [57], TH2B [70], as well as macroH2A.1, macroH2A.2 and their subtypes [71,72] (reviewed in [28,63]). Histone H1 has 11 variants, of which H1t [73,74], H1T2 [75] and HILS1 [76] are selectively expressed in the testis (reviewed in [77]). Expression of testicular histone variants is regulated temporally and spatially during germ cell development. They fulfill various structural and gene regulatory functions during spermatogenesis, and may contribute to epigenetic mark formation in response to environmental clues [63].

Structurally, histone variants deviate from canonical histones by small protein sequence alterations that changes the stability of the nucleosomes they are incorporated in. A change in nucleosomal stability goes along with a changed degree of chromatin condensation and a changed accessibility of the DNA for binding factors, which has consequences for transcription and chromatin reorganization during later phases of spermatogenesis. Some functions of testicular histone variants appear to be limited to germ cell development, without directly affecting subsequent embryo development. For example, H2AL1 and H2AL2 disappear rapidly from the paternal genome after fertilization [78], and it is unknown whether they convey any epigenetic information to the embryo. A recent report proposed functions of H2AL2 in the

loading of transition proteins onto the DNA in elongating spermatids where protamines are recruited subsequently to facilitate histone eviction from the DNA [79].

TH2B is a testis-specific histone variant that replaces most of the canonical H2B histones during the meiotic prophase of spermatogenesis [80]. TH2B destabilizes nucleosomes in spermatids because its carboxyterminal surface reduces nucleosomal binding affinity to DNA. As a consequence, incorporation of TH2B is thought to facilitate the transition of nucleosomes to transition proteins and protamines [80,81]. This raises the question whether selective placement of TH2B could be involved in epigenetic memory formation. Some TH2B is found in mature sperm [82] and an interesting open question is whether TH2B is mostly eliminated alongside with canonical H2B from the elongating spermatid nucleus or whether it is able to selectively escape nucleosome removal during spermiogenesis, with possible epigenetic consequences.

In sperm, histone variant H3.3 is enriched at promoter regions (CpG islands, CGI) of genes that are highly transcribed during spermiogenesis, while canonical histones H3.2 and H3.1 are less enriched at these regions, suggesting that they were replaced by H3.3 during gene transcription events in round spermatids [40,83].

In summary, DNA replication-independent expression of non-canonical histone variants and their insertion into the chromatin of actively transcribed genes, where they substitute canonical histones, could be one of the mechanisms of sperm chromatin mark formation [67]. If that is the case, then a form of histone variant-based epigenetic information could exist in sperm chromatin to represent a memory of gene activity that occurred during spermatogenesis and that could be transmitted to the next generation to inform progeny. However, mechanisms underlying the sensing of environmental influences (diets, toxicant exposures) by germ cells and how gene expression could be altered in response to such environmental cues are currently still unclear.

2.3. Posttranslational modifications (PTM) of sperm histones with the potential to control gene expression.

Sperm histones carry various PTMs that can regulate chromatin functions and gene expression. PTM in the testis include mono-, di- and trimethylation of lysine residues, acetylation, phosphorylation, ubiquitination, ADP-ribosylation [84], crotonylation [85] and others (reviewed in [28,62]). Most of these histone PTM are regulated by a complex interplay of enzymes either adding or removing defined molecular groups to histone N-terminal domains. The acetylation of histones is generally associated with gene activation and transcription [86]. Constitutive heterochromatin, e.g. as it is found in pericentromeric, telomeric and repeat-rich genomic regions, is marked by trimethylation of lysine 9 on histone H3 (H3K9me3), a silencing histone modification, and by DNA methylation. Both marks persist in heterochromatic regions during fertilization [87,88]. Di- and trimethylated lysine 4 on histone H3 (H3K4me2 and H3K4me3), both generally considered activating histone marks, are enriched at certain developmental promoters and paternally expressed loci [20]. Genes important for developmental regulation and cellular homeostasis concurrently carry both, H3K4me2 and H3K27me3 chromatin marks [21]. The simultaneous presence of markings with apparently contradictory function in the same region, e.g. silencing H3K27me3, in addition to activating H3K4me3, marks “bivalent” promoters of developmental genes expressed in embryonic development [20,21]. Both markings can occur on the same nucleosome, but typically not on the same histone N-terminal tail within one histone [20,89].

During *Xenopus* spermiogenesis, the activating H3K4me2 and H3K4me3 marks are removed from a subset of developmentally important genes to prevent their promiscuous expression in

early embryogenesis [90], further highlighting the importance of sperm chromatin epigenetics for embryo development. Generally, H3K4me2 is an activating mark that is found on meiotic sex chromosomes and elsewhere throughout the spermatid genome and that has been suggested as an important mediator of heritable epigenetic memory in mammals [20,21,91]. H3K27me3 marks are mediated by the polycomb repressor protein complex PRC2 [92]. This mark, which is recognized and bound by a second complex, PRC1, is important for Trithorax- and Polycomb-mediated gene silencing, and paternal diet affecting H3K27 methylation in sperm may represent one mechanism by which an epigenetic signature forms due to environmental factors [93].

Dietary methyl (C1) donors, such as folate, methionine and choline, are known players important for methylation of DNA in spermatogenesis [94] and variations in the availability of those metabolites change the DNA methylation component of the sperm epigenome. How deficient or excessive uptake of these methyl donor changes body-wide and importantly germ cell-specific methylation of histones is not understood [95–99]. Targeted research needs to address the question to what extent dietary C1 donors affect the sperm chromatin-borne epigenome, specifically the methylation of histones, in addition to the sperm DNA methylome [95]. The importance of sperm histone methylation patterns for the regulation of embryonic development was recently demonstrated in male transgenic mice with H3K4 demethylase KDM1A overexpression. Progeny of such animals with reduced sperm H3K4 dimethylation were developmentally impaired and had reduced survival rates [100]. Similar results were obtained from a different group who used a heat-inducible testicular KDM1A overexpressing mouse model [101]. In summary, while the exact mechanism remains unclear, pathways that modulate the activity of H3K4me3 or H3K27me3 demethylating enzymes during spermatogenesis in response to environmental cues may be able to affect the sperm epigenetic

program and consequently embryonic gene expression. The activity of poly(ADP-ribose) (PAR) polymerase (PARP) enzymes in response to DNA strand breaks is an example for a DDR pathway with a modulating influence on the establishment of histone methylation marks that can also respond to environmental cues (see also 3.1. below). Given the apparent importance of H3K4 and H4K27 methylation in sperm, it is puzzling that the paternal genome, but not the maternal genome, appears to become depleted of H3K4me3 peaks in zygotes [102]. In addition, Zheng et al. observed global erasure of H3K27me3 marks from sperm after fertilization, accompanied by inheritance of distal H3K27me3 from oocytes downstream of gene transcription start sites [103]. More investigations are needed to understand the roles of H3K4 and H3K27 methylation in sperm and after fertilization. An attractive, but untested hypothesis is that these histone modifications could be involved in directing the rapid DNA de- and remethylation events that occur during and shortly after fertilization to direct gene expression later in development. Besides sperm histones, which provide potential epigenetic information in the form of at least 26 different modifications, protamines have recently also been shown to bear at least 11 PTM, which may add a whole new layer of sperm chromatin-borne epigenetic information [35]. The exact functions of these remain to be elucidated.

3. DNA DAMAGE RESPONSE PATHWAYS IN SPERMATID CHROMATIN REMODELING

Mouse embryos derived from injection of round spermatids into oocytes develop into adult mice at much lower frequencies than embryos generated by the injection of sperm, which highlights how functionally important the mature sperm chromatin structure is [104,105]. The process that leads to the typical protamine-based chromatin structure during spermiogenesis in mammals

requires extensive nucleoprotein exchange (see 2.1) and controlled DNA strand breaks. These breaks accommodate the DNA conformational change from the superspiralized, histone-based nucleosomal conformation to a linear, relaxed one stabilized by protamines. [7,63,82,106–112]. DDR pathways that are associated with controlled DNA strand breaks are therefore emerging as major factors driving the necessary nucleoprotein exchange during chromatin remodeling in the elongating spermatid (see also Figure 1).

3. 1. Topoisomerase II beta (TOP2B) provides DNA relaxation and triggers a DNA damage response

Because homologous recombination is not an option for repairing the DNA double strand breaks (DSBs) that occur during chromatin condensation in haploid spermatids, free DNA breaks could theoretically only be repaired by the alternative, more error-prone pathway of non-homologous end joining (NHEJ), or remain unrepaired until after fertilization. Classical NHEJ repair is not functional in spermatids, and the effectiveness of the alternative NHEJ pathway in repairing DSBs in spermatids is unclear [113].

While such DNA DSBs may exist as rare events in spermatids, the majority of them seem to occur as a result of type II topoisomerase activity to allow for DNA relaxation and reorganization of chromatin loops [114–117]. In this process, which in spermiogenesis is mediated by topoisomerases, including the topoisomerase variant 2β (TOP2B), DNA ends remain covalently bound to the enzyme during the decatenation reaction (Figure 1a-c) [107,114,115,118]. It is unclear whether TOP2B is able to perform DNA relaxation in a sequence-specific way. In somatic cells, the enzyme preferentially binds to DNA sites that are supercoiled or arranged in a four-way junction, which is a conformation of DNA in torsional

stress, such as during transcription [119]. Such sites are mostly AT- rich and occur throughout the mammalian genome, particularly in nuclear matrix- and scaffold- attachment sites [120]. In elongating spermatids, binding of TOP2B to a given genomic locus therefore could be driven by torsional stress in supercoiled DNA during or prior to the histone-to-protamine exchange and occur near nuclear matrix attachment sites [107,121]. An interesting observation is that elongating spermatids become positive in the Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay, which recognizes only free, but not protein-bound DNA ends, suggesting that a large portion of these breaks are not the result of normal TOP2B activity [122]. Both single and double strand breaks are present in elongating spermatids, raising the possibilities that TOP2B activity may either be abortive at times, and that a tyrosyl-DNA phosphodiesterase (likely TDP1) may remove TOP2B from the DNA, forming a true DNA break [107,108,121]. The excision of stalled TOP2B by TDP1 from the DNA, followed by NHEJ as a potential alternative repair pathway, has been shown in somatic cells, but not in spermatids so far [108] (Figure 1c). Alternatively, SPO11, an endonuclease responsible for creating breaks during the meiotic recombination process in spermatocytes, may also form DNA strand breaks in elongating spermatids [121]. In somatic cells, TOP2B creates DNA single strand breaks in silent promoters to activate poly(ADP-ribose) polymerase 1 (PARP1), which facilitates the replacement of the silencing histone H1 by high mobility group B protein, which then allows transcription to proceed [123,124]. Whether this mode of TOP2B/PARP1 interaction also exists in elongating spermatids is not clear, but the activation of PARP enzymes in elongating spermatids has been shown [125,126].

In somatic cells PARP1 and PARP2 enzymes are rapidly recruited to DNA single and double strand breaks, where their catalytic domains become immediately activated. Activated

PARP enzymes cleave NAD^+ to synthesize poly(ADP-ribose) (PAR) as a first signal that DNA strand breaks are present (black arrow in Figure 1a) [127] (reviewed in [128]). In somatic cells, PAR formation mediates subsequent recruitment of MRE11, NBS1 and ATM to the DNA lesion (see further below) [129]. PAR is a highly electronegative biopolymer that is attached to target proteins like PARP enzymes themselves, histones and other chromatin proteins as a large posttranslational modification. The strong electronegative charge of PAR reduces or fully neutralizes the ability of target proteins to bind DNA and leads to a profound local chromatin decondensation required for DNA repair and transcription [130,131]. Subsequently, PAR is degraded by the catabolic enzyme PAR glycohydrolase (PARG), which is also expressed in spermatids [109,132]. The concerted activity of PARP and PARG enzymes thus results in a rapid local consumption of NAD^+ and turnover of PAR near DNA strand breaks that facilitates chromatin decondensation as well as histone displacement, which has been studied extensively in somatic and germ cells such as spermatocytes [133–136] (Figure 1a, 1b). The DNA strand breaks that activate PARP in elongating spermatids could also stem from other sources than TOP2B, such as SPO11, however, it could be demonstrated that PAR formation by PARP negatively regulates TOP2B activity both in vitro and in elongating mouse spermatids in vivo. The activities of PARP1 and PARP2 therefore seem to curb TOP2B activity by providing a negative feedback loop [125]. PAR degradation by PARG is necessary to remobilize TOP2B to complete its DNA unknotting activity and the combined activity of PARP1, PARP2, PARG and PAR appears to be important for the regulation of TOP2B activity [125]. After completion of TOP2B-mediated DNA decatenation that is necessary to achieve the chromatin structure in mature sperm (Figure 1d), TOP2B may remain bound to loops of nucleosomal DNA between toroids of protaminated DNA where it could play a role in sperm chromatin fragmentation and unpacking of the paternal

genome after fertilization [17,137,138]. Altering PAR metabolism in elongating spermatids using genetic or pharmacologic intervention results in abnormal histone retention in sperm, deviant sperm head shaping and altered epigenetic regulation of embryonic gene expression in 2-cell embryos, highlighting the importance of PARP-mediated chromatin remodeling in the establishment of sperm chromatin [41,60,82,109,126,139–141]. PARP activity is modulated by various endogenous and exogenous substances and pathways, e.g. the presence of endogenous PARP inhibitors (e.g. vitamin D3 metabolites, certain other vitamins, and unsaturated fatty acids [142,143], reviewed in [142]), and by the availability of NAD^+ as the substrate. In humans, blood NAD^+ levels vary according to nutritional vitamin B3 (niacin, nicotinic acid and nicotinamide) intake [145–148], since vitamin B3is the main dietary precursor of NAD^+. Whether a niacin uptake-dependent modulation of PARP activity in spermiogenesis occurs is an open question. Furthermore, in somatic cells, ADP-ribosylation of histone N-terminal tails influences other histone modifications such as H4K16ac and H3K27me3 on the same nucleosomes in somatic cells either by direct competition for the same amino acid or indirectly by steric hindrance [84,139,141,149,150] (see Figure 2). Further research is necessary to determine whether PARP activity has any influence on histone modifications in spermiogenesis as well.

Interestingly, PARP is also directly involved in the regulation of the activity of the protein demethylases KDM5B and KDM4D. These enzymes remove methylation marks at H3K4me3 and H3K9me3, respectively. In somatic cells, PARP activity in the vicinity of DNA strand breaks inhibits these demethylases [151–153]. According to Krishnakumar and Kraus [154], PARP1 binds to promoter regions of active promoters where KDM5B binds as well, where it ADP-ribosylates KDM5B and thus inhibits KDM5B from removing H3K4me3 in somatic cells. By inhibiting this demethylase, PARP therefore maintains the active H3K4me3 mark to keep

genes active in these cells. The regulation of other KDM enzymes by PARP1 may be different as Gong and colleagues reported recently that PARP1 recruits KDM5A to DNA DSBs in somatic cells to help removing H3K4me3 in the vicinity and to silence genes affected by DNA strand breakage [155]. While interactions of PARP with any of these histone-modifying enzymes in spermatogenesis are likely, they are hypothetical at this time. In addition, PARP1 in somatic cells also directly ADP-ribosylates H3K27 as a posttranslational modification that competes with methylation of this lysine residue [84,149], but to-date no comparable insights are available for male germ cells. In summary, DNA strand breaks that occur naturally in elongating spermatids trigger DNA damage response signaling, including PARP activation.

3. 2. Activation of the ATM/ATR pathway by TOP2B to phosphorylate H2AFX

The ataxia telangiectasia mutated (ATM) signaling network is an important part of DNA repair pathways that are activated by TOP2B activity in spermatids, as well as by other sources of DNA double strand breaks [108,113,156]. As discussed above, the classical NHEJ pathway is likely not present in spermatids, but the alternative Parp1/XRCC1 dependent NHEJ pathway is active in these cells to recruit ATM and Rad3-related protein (ATR) [113,156]. It was shown in both somatic and testicular cells that the serine/threonine-protein kinase ATM and ATR interact to phosphorylate the histone variant H2AFX (H2A.X, in phosphorylated form also known as gamma-H2AX) in response to DNA DSBs (Figure 1b) [157]. The ATM/ATR-mediated H2AFX phosphorylation spreads along the DNA for hundreds of thousands of base pairs surrounding the break, where the modification is bound by MDC1 (mediator of DNA damage checkpoint 1) [158]. Phosphorylated H2AFX as well as the histone variant H2A.Z (H2AFZ) are also essential in DNA damage response signaling in somatic cells [159–161]. Transient PAR/NAD^+ turnover

mediated by PARP1/2 and PARG activity after DNA strand breakage is critical for activation of the ATM signaling pathway, and absence of PARP activity impairs ATM/ATR phosphorylation [162,163] (Figure 1b).

Interestingly, the activities of several proteins involved in DDR pathways that are discussed here are also essential for the progression of meiosis, inactivation and epigenetic programming of the sex chromosomes (meiotic sex chromosome inactivation, MSCI) and chromatin remodeling that takes place during male meiosis [91,164]. For example, SPO11, an enzyme that creates DNA double strand breaks for genetic recombination, activates the ATM pathway in pachytene spermatocytes. ATM provides a negative feedback regulation to limit SPO11 activity, and thus the number of DNA strand breaks formed by SPO11 during meiosis, which renders *Atm*$^{-/-}$ testes sterile due to meiotic recombination defects [165]. Phosphorylation of H2AFX by ATR, and to a lesser extent by ATM, is a hallmark of XY body formation as part of the MSCI where several DDR enzymes colocalize to silence sex chromosomal gene expression ([166] reviewed in [167]). The interplay of ATR with other proteins such as ATM, BRCA1, MDC1 and the topoisomerase II-binding protein (TOPBP1) is a central mechanism that promotes meiotic sex chromosome silencing by H2AFX phosphorylation. In mice, genetic disruption of several DDR proteins therefore mostly leads to a halt of meiotic progression, which illustrates their importance in meiotic surveillance [166–168]. H2AFX phosphorylation is also involved in H2AFZ deposition, as outlined in the next section, which could have an influence on embryonic gene expression.

3. 3. Functions of H2AFZ in the DNA damage response in spermatids

In mammalian somatic cells, spermatocytes, and round spermatids H2AFZ is inserted into the chromatin to replace H2A and macroH2A in nucleosomes. The canonical H2A is exchanged

after its ubiquitination by RNF8 in an interaction with MDC1. In male meiosis, H2AFZ is enriched in facultative heterochromatin of inactivated sex chromosomes [169], where it is involved in the activation of genes that escape transcriptional silencing due to MSCI [91]. Insertion of H2AFZ depends at least partially on the binding of phosphorylated H2AFX by MDC1 [158] (Figure 1b) but the precise mechanism has not been elucidated in spermatids. In somatic cells, MDC1 recruits the NuA4 complex with its TIP60 (Tat-interacting protein of 60 kDa) and p400 motor ATPase subunits to the DNA strand break [170] (Figure 1c, panel i). The p400 motor ATPase exchanges canonical H2A/H2B dimers for H2AFZ/H2B dimers in nucleosomes in the vicinity of the DNA strand break, where H2AFZ and H4 subsequently become acetylated by the TIP60 histone acetyl transferase subunit. Whether the scenario is the same in spermatids is not known. Comparable to PAR-mediated local chromatin decondensation, incorporation of H2AFZ into nucleosomes and the subsequent acetylation step by TIP60 and the interacting protein EPC1 locally opens the chromatin structure [7,63,170,171] (Figure 1c, panel i). In somatic cells, H2AFZ-containing nucleosomes are located directly at transcriptional start sites and where they are involved in transcriptional control [172]. H2AFZ also promotes recruitment of DNA repair factors and ubiquitination of chromatin components by the RNF8 (RING finger protein 8) ubiquitin ligase [170].

In line with expectations extrapolated form somatic cells, recent analyses of knock-out animals with functionally disrupted TIP60 function showed reduced histone acetylation in spermatids as well as impaired elongation and condensation of spermatids [171]. Therefore, certain protein factors involved in DDR in somatic cells also fulfill important functions in epigenetic remodeling during meiosis and the subsequent spermatid development. The interplay

of TIP60 and EPC1 is essential for histone acetylation, particularly of H4, which is a prerequisite for the exchange of nucleosomes for protamine [171], as outlined in the next section.

3. 4. Histone H4 Acetylation

Global lysine residue hyperacetylation of core histones, including H4, is an important, but not completely understood step preceding nucleosome eviction during spermiogenesis in various species, e.g. flies, roosters and mammals [173–180]. The proteins MDC1,TIP60 and EPC1 seem to account for most of the observed global histone acetylation observed in round spermatids [171]. Besides the acetylation of H3 (H3K122 and H3K64) and other histones, the increasing acetylation of H4 in lysine residues K5, K8, K12 and K16 adds negative charges to the histone, which destabilizes the nucleosome and weakens its binding to DNA [181]. While the net result is likely a general chromatin decondensation, the individual acetylation marks of H4 also serve specific functions in spermatid gene expression. In addition to histone acetylation, the acylation of histones with different types of lysine posttranslational modifications other than acetylation, such as propionylation, butyrylation, crotonylation and others, have been linked the regulation of gene expression and the metabolism of acyl-CoA in somatic cells [182,183]. Spermatid gene expression is regulated by both, histone acetylation and butyrylation marks [184]. H4 marked with both H4K5ac and H4K8ac are bound by the first bromodomain (BD1) of the testis-specific bromodomain protein BRDT [185–187], a protein that stimulates the transcription of certain spermatogenesis-specific genes by binding directly to their transcriptional starts sites. While histone butyrylation generally directly stimulates transcription, butyrylation of H4K5 prevents the binding of BRDT, which leads to a dynamic regulation of genes marked with both, acetylation and butyrylation of H4 in their promoter regions [184]. BRDT also functions in

455 transcriptional repression during spermatogenesis, where it interacts with HDAC1, PRMT5 and TRIM28 [188] and in RNA processing [189].

The interaction of BRDT with acetylated H4K5 and H4K8 is essential for successful histone removal and sperm development as deletion of BD1 results in deformed sperm with excessive histone retention [190]. How BRDT binding of H3K5ac and H3K8ac contributes
460 mechanistically to histone eviction is still unclear, but a direct mechanical 'squeezing' of chromatin fibers by polymerizing BRDT proteins has been proposed [191] (Figure 1c, panel ii). Dhar and colleagues reported that this process involves binding of other proteins including beta actin and SMARCE1, which is a subunit of the SWI/SNF family of ATP-dependent chromatin remodelers [192]. In humans, strong H4 acetylation already appears in early round spermatids
465 long before nucleosome eviction [193], indicating that additional events besides H4K5ac/H4K8ac binding may be required for BRDT-mediated histone eviction.

The bromodomain protein BRD4 and likely other proteins, have also been proposed to have important roles in the removal of nucleosomes from spermatid chromatin [194] (Figure 1c, panel iii). Other H4 acetylation events include H4K12 acetylation, but its involvement in histone
470 removal for protamine deposition is unlikely. Genome-wide mapping showed that H4K12ac remains present at least in part in human mature sperm, where it is enriched in binding sites of the chromatin insulator CTCF (CCCTC-binding factor) and in the promoter regions of genes that are later expressed in 8-cell embryos and blastocysts, suggesting a role of the histone mark in epigenetic inheritance [195].

475 In contrast, H4K16 acetylation is an event in elongating spermatids that is common to all mammals and that occurs just prior to histone eviction. The H4K16ac mark is formed independently from H4K5 and H4K8 acetylation specifically in response to DNA strand breaks

[91]. H4K16ac is created by the acetyltransferase KAT8 (MYST1, MOF), which is phosphorylated by the DNA DSB-activated ATM/ATR pathway. All protein factors of this pathway, which also involves the kinase TSSK6 [196] colocalize with phosphorylated H2AFX in the vicinity of DNA strand breaks in somatic cells [197] (Figure 1b and 1c, panel ii). As discussed above (3.2), the TOP2B-mediated DNA torsional relaxation activates PARP and ATM/ATR signaling, which provides a hypothetical but plausible link between DDR signaling and histone H4K16 acetylation in elongating spermatids.

Although MDC1, TIP60 and EPC1 seem to account for most of the observed H4 acetylation, not all histone acetyl transferases (HAT) that are responsible for H4 hyperacetylation may have been conclusively identified. Deletion of Chd5 (chromodomain helicase DNA binding protein 5) in mice resulted in reduced expression of the GCN5-related N-acetyltransferase 8 (GCN5-related) family member 5 (Nat8f4 in mice, 1700019G17Rik) and in reduced H4 acetylation in elongating spermatids [111,198], indicating that NAT8F4 may also be involved in the acetylation process. Chd5 binds to unmodified H3 and H3K27me3 nucleosomes [199–201] and may act as a multifunctional key orchestrator of nucleosome acetylation and eviction prior to transition protein and protamine deposition (Figure 1c, panel iii) [111].

After highly acetylated histones, which also carry the H4K16ac mark, have been removed from chromatin, they are eliminated by a large and unique proteasome complex ("spermatoproteasome") [202]. A spermatoproteasome comprises a spermatid-specific alpha subunit (PSMA8), the catalytic beta subunits of immunoproteasomes and an activator protein PA200 (Figure 1c). The activator protein binds acetylated histones with a bromodomain-like domain, and mice lacking PA200 have defects in both, acetylation-dependent degradation of

canonical histones and histone disappearance during sperm head condensation [202,203] (Figure 1c, panel ii).

Overall, the DNA strand break-dependent H4K16ac mark may be emerging as the possible final and crucial negative charge needed to tip the balance towards histone binding of a remodeling complex via BRDT or another bromodomain protein, and the eviction of nucleosomes, underscoring the importance of DNA damage response pathways in the chromatin remodeling process in elongating spermatids. The observation that assisted reproduction in humans by round spermatid injection into oocytes (ROSI, ROSNI) is possible, but inefficient, likely because of the immature epigenetic information in round spermatids, illustrates that the defined eviction of histones is important for correct epigenetic programming of sperm, [204,205].

3. 5. Epigenetic marks of genotoxic agent exposure in sperm

External mutagens that elicit DNA repair in germ cells have an impact on the epigenome of sperm [63,206–208] and on testicular gene expression of progeny [209]. Besides purely mutagenic effects of DNA damaging agents that are considered rare events based on unsuccessful DNA repair, DNA repair events themselves leave epigenetic marks in chromatin domains and gene loci even after successful repair. This has been demonstrated in rodents treated with bleomycin, etoposide and cis-platinum (BEP) to emulate a testicular cancer chemotherapy regimen. In that study, BEP treatment led to DNA hyper- and hypo-methylation, partly in preferred loci, suggesting either an interference with normal methylation patterning or abnormal repair of damaged patterns during spermatogenesis [207]. Furthermore, epimutations, i.e.

epigenetically abnormal gene regulation, can cause genetic mutations if such epimutations affect DNA repair enzymes [210], likely contributing to transgenerational epigenetic inheritance. Nutritional parameters and genotoxic insults are closely interconnected. For example, metabolic syndrome is associated with hyperglycemia, hyperinsulinism and hyperlipidemia causing elevated oxidative stress (reviewed by Rani et al. [211]). Interesting but unknown is whether oxidative stress and resulting DNA damage caused by metabolic syndrome affects selective chromatin regions in spermatids, e.g. by triggering DNA repair events in Dad's germ cells during spermatogenesis, similar to the ones shown in Figure 1, with epigenetic consequences to the sperm chromatin, and ultimately the offspring.

4. AFTER FERTILIZATION

The significance of sperm epigenetic information hinges on its potential to exert long-term consequences on the offspring. After fusion of sperm and oocyte, the sperm nucleus is rapidly unpacked, protamines are efficiently removed from the DNA and replaced by maternally provided histones that are hypomethylated or acetylated and lack silencing marks [87]. At the same time, there is genome-wide DNA demethylation in the male, but not in the female pronucleus. Differentially methylated regions of paternally imprinted genes are the exception as they remain methylated in the male pronucleus [212]. Persisting sperm-associated histones apparently remain at their original loci in the male PN throughout this extensive process [213]. Such persistence is important because core histones present in sperm retain their posttranslational modifications [19–21], similar to differentially methylated DNA regions of imprinted genes that survive the global DNA demethylation in the 1-cell stage. In *Xenopus*, paternal H3K4me2/3 and H3K27me3 influence embryonic gene expression, further supporting this theory [90].

In somatic cells, gene silencing histone PTM (such as H3K9me2, H3K9me3) and DNA methylation were found to promote each other [214]. In contrast, activating histone PTM (such as H3K4 methylation) generally prevent *de novo* DNA methylation and preserve an unmethylated DNA state. This seems highly relevant prior to the first cell division in mammals when the DNA of the paternal PN becomes largely demethylated except for some regions, e.g. imprinted genes and regions associated with H3K9me2. The maternal genome in contrast is protected from this active DNA demethylation step in the zygote by PGC7/STELLA [215,216]. As the preimplantation embro develops differentiated tissues, the DNA of both chromosome sets is methylated to program cells for pluripotency and for differentiation. In view of this extensive de- and re-methylation of the paternal DNA during the zygotic chromatin remodeling, it is a plausible hypothesis that residual sperm histones bound to gene regulatory sequences may direct embryonic DNA methylation patterns according to the nature of their PTM and position in the genome, which would have delayed effects on embryonic transcription.

Preimplantation embryos are initially transcriptionally silent and use maternally provided RNA until their genome becomes activated during the maternal-to-embryonic transition (e.g. in mid-late 2-cell stage in murine embryos [217]). It is therefore also theoretically possible that the retention or elimination of histones in the sperm nucleus, which upon fertilization becomes the paternal pronucleus, could also immediately affect embryonic gene expression based on their activating or silencing PTM. Experimental evidence obtained in mice shows that changing the numbers and positions of sperm nucleosomes in gene promoters correlates highly significantly with altered expression of corresponding genes in 2-cell embryos sired by such sperm, providing some support of this hypothesis [41].

5. CONCLUSIONS

While other mechanisms that were not discussed here may exist as well, current evidence supports the idea that DNA damage signaling and DNA repair events in elongating spermatids, as well as transcription in round spermatids, are essential steps in sperm epigenetic programming to support embryonic development. Similar to other phases of the mammalian germline life cycle, in which major epigenetic programming occur, namely during primordial germ cell (PGC) development and after fertilization in the early embryo (reviewed in [218,219]), chromatin remodeling in spermiogenesis represents a potential window of opportunity for the acquisition of epigenetic marks in response to the environment. Interestingly, and comparable to spermiogenesis, successful epigenetic reprogramming in PGC development and programming of the zygote also depend on DNA repair pathways involving strand breaks and the resulting DNA damage response signaling [220–223]. A major difference between the reprogramming events in PGC, the zygote and the spermatid is that there is no known DNA demethylation in spermiogenesis but, on the other hand, spermatid maturation is the time of one the most dramatic chromatin protein reorganization events in mammalian biology.

ACKNOWLEDGEMENTS

We realize that there is a large body of excellent relevant work that was not discussed in this review due to space limitations and we apologize to all those whose work could not be cited here for this reason.

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FIGURE LEGENDS

Fig. 1: Proposed model of DNA damage repair (DDR) signaling involvement in chromatin remodeling and nucleoprotein exchange in elongating spermatids.

Please see also detailed discussion of this figure in the main body of the text. Processes shown in panels (a)-(d) may be successive or concurrent events. Events that have been demonstrated only in somatic cells and are considered hypothetical in spermatids are marked with an asterisk* (a) TOP2B activity initiated by DNA torsional stress or other, currently unknown factors, potentially including the formation of DNA strand breaks by nucleases like SPO11, results in PARP1/PARP2 activation. Modification of PARP enzymes and histones with PAR results in partial core histone and H1 removal from the site [224] and PAR recruits diverse DNA repair factors including ATM. After recruitment by PARP, KDM5A removes H3K4me3 in somatic cells; whether this also occurs in spermatids has not been investigated. Histones may be already partly acetylated such as in H4K5ac and H4K8ac. (b) During and after TOP2B-facilitated DNA

decatenation, PARP-mediated activation of the ATM pathway results in KAT8 and H2AFX phosphorylation and H4K16ac formation as the last step of histone H4 hyperacetylation necessary for histone eviction and replacement by transition proteins. The deacetylase SIRT1, which normally counteracts H4 acetylation in somatic and earlier germ cells, is likely no longer present at this time. PARP, auto-modified with PAR, is gradually removed from the site of TOP2B activity. MDC1 binds to phospho-H2AFX and recruits RNF8 to ubiquitinate H2A histones. (c) Panel i: MDC1 recruits the TIP60 (KAT5) histone acetyltransferase complex that also replaces H2A/H2B for H2AFZ/H2B in nucleosomes. This is an event that was also taking place earlier in spermatocytes. The TIP60 complex also acetylates ATM to enhance phosphorylation of histones including H2AFX and H2AFZ. Histones acetylated at H4K16 by MYST1/KAT8 are removed from the DNA by a complex containing BRDT, or a different bromodomain protein like BRD4, replaced by transition proteins or protamines, and degraded (panel ii). Alternatively, or additionally, histones are bound by CHD5 and acetylated by NAT8F4 for removal (panel iii). TDP1 may remove stalled TOP2B covalently bound to DNA, creating a strand break that could be repaired by a non-homologous end joining pathway or remain unrepaired. (d) The final sperm chromatin structure is based on protamine-based toroid structures with nucleosomal linker chromatin domains carrying epigenetic information.

Figure 2) A model of epigenetic modifier regulation by DNA strand break-dependent poly(ADP-ribose) metabolism.

TOP2B activity relaxes DNA in elongating spermatids in a decatenation reaction and for reorganization of chromatin loops. This step triggers PARP1/2 activation and resulting poly(ADP-ribose) (PAR) formation provides a feedback loop to regulate TOP2B activation

[123–125]. PARP activation is necessary for activation of the ATM/ATR kinase pathway [162,163], leading to phosphorylation of H2AFX [157] and H4K16 acetyltransferase MYST1 (KAT8, MOF) [197]. Direct ADP-ribosylation by PARP1 in H3K27 position (H3K27-ADPR) may compete with H3K27 methylation [84,225] but PARP1 also interacts with histone demethylases of the KDM family either through PARP enzyme-bound PAR chains or through direct protein interaction at PARP binding sites in the genome. For instance, KDM5A is recruited by PARP1 and activated to remove H3K4me3 from promoter sites as part of DNA damage-mediated gene silencing [155]. In contrast, KDM5B has been reported to be inhibited by PARP1 and excluded from DNA strand breaks to maintain H3K4me3 and transcription near sites of DNA damage repair [154]. These may be mutually exclusive pathways or they could depend on the activity of the PAR digesting activity of poly(ADP-ribose) glycohydrolase (PARG). KDM4D, which removes H3K9me2 associated with promoters of retinoic acid receptor-dependent genes heterochromatin, is inhibited by PARP1, unless PARG is present to allow for retinoic acid-dependent gene expression [151]. Events that have been demonstrated only in somatic cells are considered hypothetical in spermatids and are marked with an asterisk*.

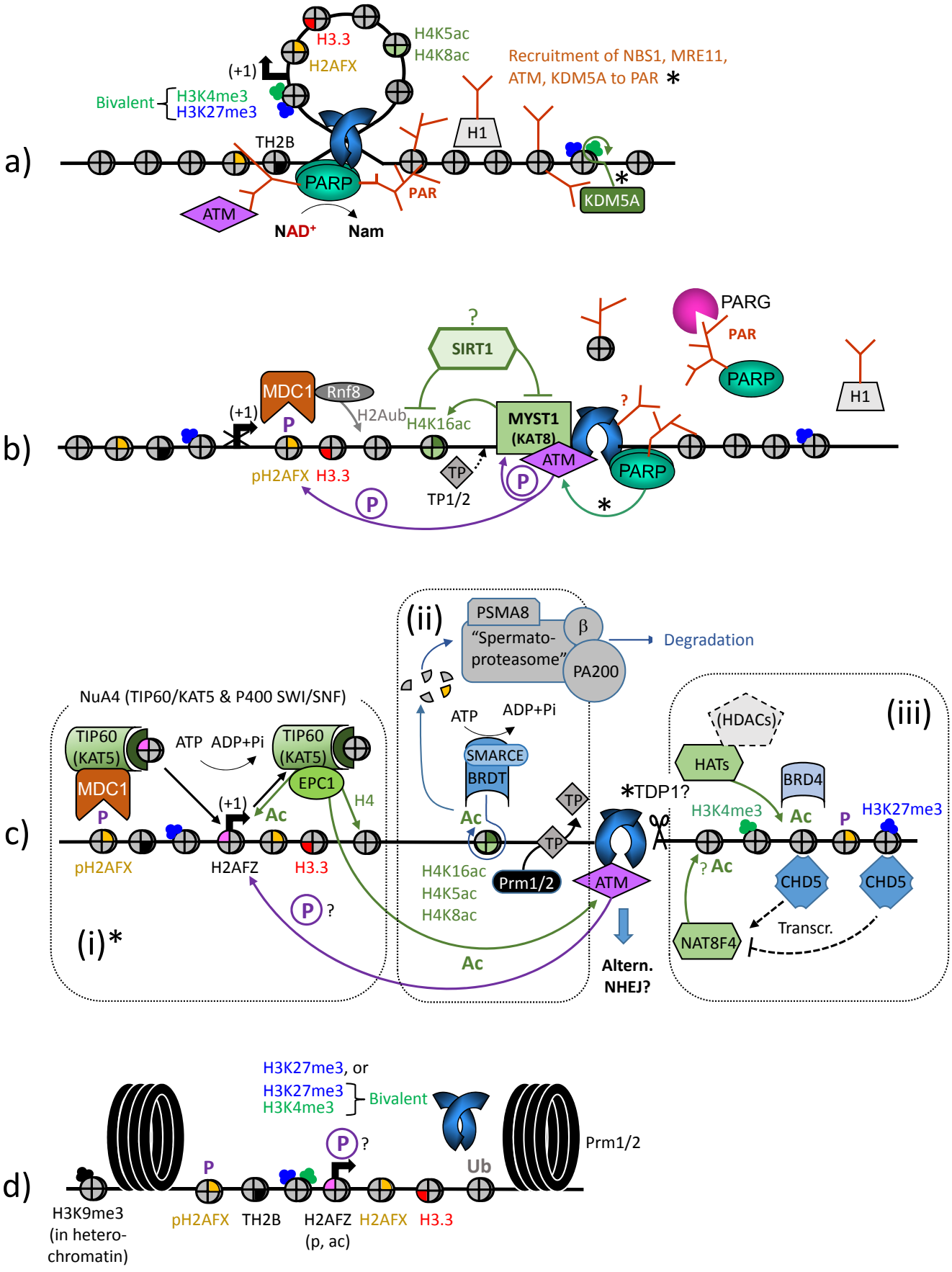
a)
H3.3
H2AFX
(+1)
Bivalent
H3K4me3
H3K27me3
H4K5ac
H4K8ac
Recruitment of NBS1, MRE11, ATM, KDM5A to PAR *
TH2B
H1
PARP
PAR
ATM
NAD+
Nam
KDM5A
b)
?
SIRT1
PARG
PAR
PARP
H1
MDC1
Rnf8
(+1)
P
H2Aub
H4K16ac
MYST1 (KAT8)
ATM
PARP
pH2AFX
H3.3
TP
TP1/2
P
*
c)
NuA4 (TIP60/KAT5 & P400 SWI/SNF)
TIP60 (KAT5)
MDC1
P
ATP
ADP+Pi
TIP60 (KAT5)
EPC1
(+1)
Ac
H4
pH2AFX
H2AFZ
H3.3
P
?
(i)*
(ii)
PSMA8
"Spermato-proteasome"
β
PA200
Degradation
ATP
ADP+Pi
SMARCE
BRDT
Ac
TP
TP
H4K16ac
H4K5ac
H4K8ac
Prm1/2
ATM
*TDP1?
Ac
(iii)
(HDACs)
HATs
BRD4
H3K4me3
Ac
P
H3K27me3
? Ac
CHD5
CHD5
NAT8F4
Transcr.
Altern. NHEJ?
d)
H3K27me3, or
H3K27me3
H3K4me3
Bivalent
P
?
Ub
Prm1/2
P
H3K9me3
(in hetero-chromatin)
pH2AFX
TH2B
H2AFZ
(p, ac)
H2AFX
H3.3

Figure 1

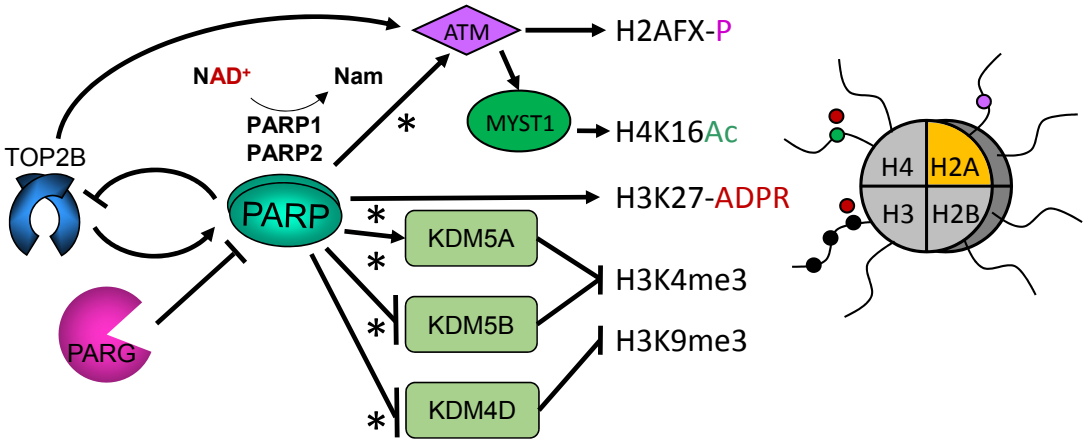
ATM
H2AFX-P
NAD+
Nam
PARP1
PARP2
MYST1
H4K16Ac
TOP2B
PARP
H3K27-ADPR
KDM5A
H3K4me3
KDM5B
H3K9me3
PARG
KDM4D
H4
H2A
H3
H2B

Figure 2