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Sex chromosome inactivation in germ cells: emerging roles of DNA damage response pathways

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Abstract

Sex chromosome inactivation in male germ cells is a paradigm of epigenetic programming during sexual reproduction. Recent progress has revealed the underlying mechanisms of sex chromosome inactivation in male meiosis. The trigger of chromosome-wide silencing is activation of the DNA damage response (DDR) pathway, which is centered on the mediator of DNA damage checkpoint 1 (MDC1), a binding partner of phosphorylated histone H2AX (γ H2AX). This DDR pathway shares features with the somatic DDR pathway recognizing DNA replication stress in the S phase. Additionally, it is likely to be distinct from the DDR pathway that recognizes meiosis-specific double-strand breaks. This review article extensively discusses the underlying mechanism of sex chromosome inactivation.

Keywords

Germ cells; Meiosis; Sex chromosomes; DNA damage response; Checkpoint; Epigenetics

Introduction

Sex chromosomes are integral to sexual reproduction. In mammals, the gene-rich X chromosome is larger than the gene-poor Y chromosome. Both X and Y chromosomes are thought to have evolved from an ordinary autosome, with the large portion of Y having degenerated in the evolutionary past [1–3]. This striking difference between the X and Y chromosomes accompanies unique issues that are resolved by sex chromosome inactivation during both female and male development. In mammalian females, one of the two X chromosomes is inactivated to solve the X-linked gene dosage imbalance between XX females and XY males [4]. On the other hand, X and Y chromosomes in male germ cells are challenged by the distinct issue of heterogametic sex and undergo sex chromosome inactivation. During meiosis, homologous autosomes pair and synapse, shuffle genetic material via meiotic recombination, and faithfully segregate into haploid gametes. Without homologous partners, the X and Y chromosomes must surmount meiosis and segregate into either X-bearing or Y-bearing haploid spermatids. To this end, the X and Y chromosomes partially synapse along a small homologous region (the pseudoautosomal region) during meiosis and are transcriptionally silenced in a process called meiotic sex chromosome inactivation (MSCI) [5–10]. The silent X and Y chromosomes occupy a distinct chromatin domain called the XY body or sex body (Fig. 1). In MSCI, transcription (from the sex

chromosomes) is almost completely shut down [11, 12], except for the expression of X-linked microRNAs [13] and some genes such as non-coding RNA *Tsx* [14]. Once established, chromosome-wide silencing of sex chromosomes is epigenetically maintained throughout two rounds of meiotic divisions into both X- and Y-bearing postmeiotic spermatids [14–16]. The exception to this is a certain class of spermatid genes [11] that include multicopy genes on the sex chromosomes [17]. The silent compartment is termed the postmeiotic sex chromatin (PMSC) [11] (Fig. 1).

In the past decade, studies have begun to elucidate the significance of sex chromosome inactivation in germ cells. Failure of MSCI initiation is associated with meiotic arrest at the pachytene stage and with complete germ cell loss [18, 19], indicating that MSCI is a particularly essential step in male germ cell development. The process underlying MSCI is a general silencing mechanism that recognizes unsynapsed chromatin during meiosis [termed meiotic silencing of unsynapsed chromatin (MSUC)] in both males and females [15, 20, 21]. In normal meiosis, MSUC is confined to heterologous X and Y chromosomes in males, and conserved in mammals such as mice, horses [22], and marsupials [23]. Remarkably, the general silencing of heterologous chromatin during meiosis is also reported in a wide variety of non-mammalian organisms, including chickens (ZW sex chromosomes in females), nematodes (XO males), and fungi (unpaired DNA) [4, 5, 9], although the underlying mechanism appears to vary among the different classes of organisms.

To date, the underlying mechanism of MSCI in mammals remains unclear. Initiation of MSCI involves components of the DDR machinery present on sex chromosomes. Following the onset of MSCI, dynamic alterations of epigenetic marks occur exclusively on sex chromosomes, and several modifications are maintained in the resulting PMSC [11, 15, 16, 24]. A recent study conducted in our laboratory demonstrated that the DDR pathway is the master regulator of MSCI and of subsequent epigenetic programming of the sex chromosomes [19]. Of note, the underlying DDR pathway involved in MSCI shares a significant similarity with the DDR pathway that recognizes stalled replication forks in the somatic S phase. In addition, the former DDR pathway is genetically separable from the DDR pathway that recognizes meiotic double-strand breaks (DSBs) involved in meiotic recombination [19]. In this review article, we propose a model, which we term the DDR-adapted model, in which the DDR pathway that recognizes DNA replication stress in somatic cells is adapted to induce epigenetic silencing of sex chromosomes (Fig. 2). To clarify the underlying mechanism of MSCI, this article discusses in detail how the DDR pathway initiates MSCI.

MSCI overview: the DNA damage response pathway evokes MSCI

MSCI is an epigenetic silencing process that occurs during the meiotic prophase of spermatogenesis (Fig. 1). Initiation of MSCI is tightly associated with the progression of meiosis, a special type of cell division in which four haploid spermatids are generated from a diploid spermatocyte through reductional division [25]. In the leptotene stage of the meiotic prophase, topoisomerase-II-like Spo11 enzymes introduce DSBs that initiate meiotic recombination between homologous chromosomes. Spo11-dependent DSBs are required for proper alignment of homologous chromosomes and for synapsis [26, 27]. As a result of the action of DSBs, the entire nuclear domain is modified via the phosphorylated form of a histone variant, H2AX (γ H2AX). When homologous chromosomes, which start to synapse during the zygotene stage, complete synapsis, spermatocytes enter the pachytene stage, and γ H2AX disappears from the autosome region; however, it accumulates on unsynapsed sex chromosomes at this time.

In males, the largely unsynapsed X and Y chromosomes are modified by various DDR factors, such as γ H2AX, ATM- and Rad3-related (ATR) kinase, an ATR-activator

TOPBP1, and BRCA1 [28–33]. Due to the accumulation of these factors, it has been suggested that MSCI is initiated by DDR factors at the beginning of the pachytene stage. Supporting this notion, *H2ax* knockout mice lack γ H2AX accumulation as well as MSCI initiation [18]. However, the function of the phosphorylated form (γ H2AX) in MSCI has remained unclear.

In somatic cells, various types of DNA damage activate the DDR pathway and its downstream signaling. Both DNA damage checkpoint and homologous recombination repair machineries are regulated downstream of DDR. Nonetheless, their primary functions can be differentiated and independently understood. Homologous recombination directly repairs DNA damage, while cell cycle progression is arrested by the DNA damage checkpoint. Dysfunction of DDR pathways causes aberrant DNA repair, genomic instability, and a predisposition to cancer [34]. The mediator of DNA damage checkpoint 1 (MDC1) is a central player in the DDR pathway, particularly with regard to activation of the DNA damage checkpoint. This mediator (MDC1) directly binds to γ H2AX, and this direct interaction is required for the full activation of the DNA damage checkpoint in somatic cells [35–37]. Therefore, MDC1 transmits γ H2AX signaling to downstream factors, thereby establishing somatic DDR [38]. MDC1 also accumulates on the sex chromosome during MSCI [32, 39]. To test the role of the γ H2AX-MDC1 pathway in MSCI, our recent study utilized *Mdc1* knockout mice, clarifying for the first time that γ H2AX-MDC1 and the subsequent signaling cascade are essential determinants for MSCI [19].

Therefore, this study deepens our understanding of MSCI in the following two major ways [19]: (1) by determining that a two-step mechanism is involved in the establishment of the chromosome-wide silencing of MSCI (the initial step is the MDC1-independent recognition of the unsynapsed axes and the second step is the MDC1-dependent amplification of γ H2AX that recognizes the chromosome-wide domain of the sex chromosome), and (2) by elucidating the significant similarity between the DDR pathway downstream of the stalled replication forks in the S phase and the MSCI pathway (Fig. 2). In meiosis, another type of DDR occurs downstream of meiosis-specific DSBs, prompting us to propose that two distinct and genetically separable DDR pathways underlie meiotic recombination and meiotic silencing, as discussed in detail below.

First step of the DDR response in MSCI: activation mechanisms of DDR on unsynapsed axes

The two major kinases that phosphorylate H2AX after DNA damage are ataxia telangiectasia mutated (ATM) and ATR. In MSCI, H2AX phosphorylation is most likely mediated by ATR, because normal H2AX phosphorylation of sex chromosomes and MSCI occurs in *Atm*-deficient mice on a *Spo11* heterozygous background (*Spo11*[±]) [40]. Furthermore, ATR and its activator TOPBP1 intensely localize on the axes of the sex chromosomes and spread onto the entire chromosome-wide domain at the onset of MSCI [19, 29–31].

To discuss the DDR pathway underlying meiotic silencing, we revisited DDR pathways in somatic cells. Various types of signals, including DNA double-strand breaks, stalled replication forks, crosslinks, adducts, and eroded telomeres, can be sources of DDR activation in somatic cells [41–44]. The priming reaction of somatic DDR is phosphorylation of histone H2AX, which causes downstream factors to accumulate at the sites of DNA damage [41]. The specific type of kinase employed (either ATM or ATR) depends on the type of DNA damage and on how the damage is processed. For example, ATM kinase is activated by DNA double-strand breaks and phosphorylates H2AX around the breaks [42]. On the other hand, single-stranded DNA (ssDNA) is required for the activation of ATR kinase and the resulting phosphorylation of H2AX [42]. A recent in vitro

study revealed that, while the blunt ends of double-stranded DNA (dsDNA) or the overhangs of short ssDNA efficiently activate ATM, dsDNA molecules containing long overhangs are not efficient substrates for ATM activation. By contrast, long single-strand overhangs are preferred for ATR activation [45]. Thus, lengthened ssDNA overhangs act to switch ATM-to-ATR activation. In the context of a stalled replication fork in the S phase, ATR (but not ATM) is activated, and both ATR and its activator TOPBP1 govern the intra-S-phase checkpoint [42].

In somatic DDR, ATR activation also requires the Rad9–Rad1–Hus1 (9–1–1) complex, which is a heterotrimeric ring-shaped complex and a DNA damage-specific sensor [46]. In further support of ATR activation in MSCI, Rad1 localizes on the axes of sex chromosomes in the pachytene stage [47]. Given the specific activation of ATR, it is possible that ssDNA is present and can serve as a trigger of the DDR pathway on sex chromosomes during MSCI. Supporting this notion, replication protein A (RPA), which is an ssDNA binding protein that is required to recruit the ATR kinase complex to ssDNA [48], localizes on the axes of sex chromosomes [19, 49]. Furthermore, ATR and RPA foci specifically co-localize on the unsynapsed axes of meiotic chromosomes [50], suggesting the possible existence of ssDNA regions on unsynapsed chromatin molecules that can trigger ATR activation during meiotic silencing.

In addition to the canonical mechanism mediated by ssDNA-RPA, another form of ATR activation has recently been reported. The Fanconi anemia-associated protein, FAAP24, together with the Fanconi anemia protein, FANCM, recognizes DNA damage and recruits RPA and the ATR complex to damage sites that do not exhibit long ssDNA [51, 52]. Although localization of FAAP24 and FANCM on sex chromosomes has not been reported, other Fanconi anemia proteins, including FANCD2, BRCA2 (also known as FANCD1), and BTBD12 (also known as Slx4 or FANCP), localize on unsynapsed sex chromosomes in meiotic prophase [33, 53, 54], suggesting activation of the Fanconi anemia pathway in MSCI. Thus, FAAP24–FANCM-dependent RPA recruitment and ATR activation may be an alternative pathway for ATR activation during MSCI.

Based on deletion mutant studies of *Brca1* exon11, it has been proposed that BRCA1 recruits activated ATR to sex chromosomes undergoing MSCI [29]. *Brca1* exon11 mutants frequently exhibit aberrant localization of ATR and γ H2AX outside of sex chromosomes in the pachytene stage [29]. BRCA1 is one of the earliest markers of unsynapsed axes of sex chromosomes [29, 33]. However, some portion of the pachytene spermatocytes of *Brca1* exon11 mutants exhibit normal accumulation of ATR and γ H2AX on sex chromosomes, as well as normal initiation of MSCI [29]. This raises the possibility that BRCA1 may not be an essential factor for the activation of ATR and the initiation of MSCI. Remarkably, immediate accumulation of BRCA1 was also observed in somatic cells at the sites of stalled replication forks in the S phase [55], although the role of BRCA1 in ATR-dependent signaling remains unclear. In contrast, upon induction of DSBs following γ -irradiation in somatic cells (i.e., in the ATM-dependent pathway), BRCA1 accumulation is a late, downstream event in the γ H2AX–MDC1 signaling pathway [34]. Therefore, early accumulation of BRCA1 is a unique event that is common to MSCI and the DDR pathway downstream of DNA replication stress in the ATR-dependent pathway.

It is possible that meiosis-specific proteins are involved in the DDR pathway in MSCI. There are two homologues of meiosis-specific HORMA domain proteins in mammals, HORMAD1 and HORMAD2, and both co-localize on unsynapsed axes in the zygotene to pachytene stages [56]. Although the function of HORMAD2 has not yet been determined, HORMAD1 is needed for efficient recruitment of activated ATR to unsynapsed chromatin [57]. Another study has shown that SCP3, a component of the synaptonemal complex (a

proteinaceous structure between synapsed homologous chromosomes), is required for BRCA1 accumulation and MSUC initiation on unsynapsed axes [58]. Although it has not been determined whether the link between BRCA1 and these meiotic proteins is direct or indirect, these proteins may serve as scaffolds to support the ATR-dependent DDR pathway.

Second step of the DDR response in MSCI: chromosome-wide signal amplification

As described above, in somatic DDR, RPA recruitment is the prerequisite for recruitment and activation of the ATR complex to the site of DNA damage in both ssDNA–RPA-dependent and FAAP24–FANCM-dependent circumstances. In MSCI, RPA localization on unsynapsed axes suggests a potential role of RPA in recruiting ATR to sex chromosome axes [50, 60, 61]. However, in contrast to the limited localization of RPA on unsynapsed axes, ATR and TOPBP1 spread onto the chromosome-wide domain of sex chromosomes [29–31]. Our recent study demonstrated that this chromosome-wide spreading of the ATR complex is achieved through the action of MDC1, the binding partner of γ H2AX [19]. In *Mdc1*-deficient mice, ATR and TOPBP1 localization at the pachytene stage is restricted to the axes of the sex chromosome, while these factors spread over the chromosome-wide domain of sex chromosomes in wild-type mice [19] (Fig. 2). This result explicitly demonstrates that MSCI can be divided into two genetically separable steps. The first step occurs on the axes of the sex chromosome, where ATR and TOPBP1 are able to localize and phosphorylate H2AX in an MDC1-independent manner. In the second step, MDC1 promotes relocation of ATR and TOPBP1 as well as phosphorylation of H2AX from the axial region to the chromosome-wide domain, a process that consequently spreads the DDR signal across the entire chromosome domain of the sex chromosome.

In the somatic DDR following DSBs, ATM signaling is amplified through a positive feedback loop via γ H2AX–MDC1 interaction, after which MDC1 contributes to full activation of the DDR [62]. Our study and another recent study have demonstrated that MDC1 is also necessary for ATR-dependent γ H2AX signal amplification in MSCI and somatic DDR [19, 63]. In somatic DDR, direct interaction between MDC1 and TOPBP1 occurs specifically after DNA replication stress and activates ATR-dependent γ H2AX signal amplification via a positive feedback loop [63] (Fig. 2). Given the commonalities between MSCI and somatic DDR after DNA replication stress, MDC1 and TOPBP1 interaction likely underlies the chromosome-wide amplification of the γ H2AX signal in MSCI (Fig. 2).

Yet another layer of data supports the commonality between MSCI and somatic DDR after DNA replication stress. In somatic DDR, MDC1 interacts with the E3 ubiquitin ligase RNF8 after the induction of DSBs, and this interaction is the critical step for G2/M checkpoint control downstream of the ATM pathway [64–66]. By contrast, RNF8 is not required for activation of the ATR pathway after replication stress [63]. Consistent with this notion, *Rnf8*-deficient mice show normal accumulation of γ H2AX on chromosome-wide domains of sex chromosomes and normal initiation of MSCI, while ubiquitination of the XY body is abrogated [19, 67]. Thus, the MDC1–RNF8 interaction is essential in the ATM pathway but not in the ATR pathway. Although the role of ubiquitination on the sex chromosomes remains unclear, another E3-ubiquitin ligase (UBR2) accumulates on unsynapsed axes and is implicated in sex chromosome ubiquitination and in MSCI [59]. Given the localization of UBR2 on unsynapsed axes, UBR2 may serve to support MSCI.

The requirement of the MDC1–RNF8 interaction in the ATM pathway but not in the ATR pathway is further supported by the mechanistic difference in transcriptional silencing between the ATR- and ATM-dependent pathways. After replication stress, somatic DDR is also associated with transcriptional silencing [19]. This observation further corroborates the role of the DDR pathway, in that MDC1-dependent γ H2AX amplification is the initiation

step of chromosome-wide silencing. Additionally, in the ATM-dependent pathway in somatic cells, transcriptional silencing occurs at the site of DSBs [68]. However, the ATR-dependent pathway is likely to be independent of RNF8 because RNF8 is not required for ATR activation, and initiation of MSCI is independent of RNF8; however, the ATM-dependent pathway is partially dependent on RNF8 [68]. In spite of this mechanistic difference, transcriptional silencing is the common consequence between the ATR- and ATM-dependent pathways.

DDR-adapted model in MSUC

As discussed above, the DDR pathway that underlies MSCI shares common features with the somatic DDR pathway that occurs after DNA replication stress, but is distinct from the somatic DDR following DSBs. This notion is further supported by analyses of meiosis in *Spo11* mutant mice. In the *Spo11* mutant, autosomes cannot properly undergo synapsis without meiosis-specific DSBs. However, the γ H2AX domain, which shares features with the XY body, is formed ectopically (outside of the sex chromosome) and is called the pseudo sex body [40, 69]. Although Spo11-dependent DSBs are required for proper localization of MSUC on the sex chromosomes (i.e., MSCI), DSBs are not required to actually trigger MSUC. Additionally, MDC1 is required for pseudo-sex body formation and MSUC in the *Spo11* mutant [19]. Therefore, the DDR pathway in MSUC is genetically separable from the DDR pathway downstream of meiotic DSBs (Fig. 2).

Is MSUC independent of DSBs? A previous view held that meiosis-specific DSBs are not fully repaired and remain on unsynapsed chromosomes, acting as triggers for MSCI [6, 8]. This view is supported by cytological observations of Rad51 foci, a recombination protein that is considered to be a marker of DSBs in meiosis [69, 70]. Rad51 foci appear on unsynapsed axes at the leptotene stage at sites of DSBs, are repaired, and disappear from the synapsed axes; however, they remain on the unsynapsed axes in the zygotene stage and on the unsynapsed sex chromosomes in the pachytene stage [49]. Although Rad51 is generally understood to be a marker of DSBs in meiosis, accumulating evidence in somatic DDR strongly suggests that the ssDNA region is the critical determinant for Rad51 loading. For example, Rad51 foci appear at the site of stalled replication forks at the S phase without the concurrent presence of DSBs [71]. In addition, Rad51 binding to ssDNA can precede the formation of DSBs [72, 73]. Therefore, Rad51 foci do not always represent DSBs in somatic DDR, and it is possible that, in meiosis, Rad51 foci may represent ssDNA regions that serve as triggers for the ATR-dependent DDR pathway. Given that most Rad51 foci disappear after the zygotene stage, it may be that DSBs are repaired and DSB-derived Rad51 foci are solved. Asynapsis-associated ssDNA regions may be modified with Rad51 during the pachytene stage. In this scenario, DSBs are not essential for DDR activation on unsynapsed sex chromosomes, but unidentified chromatin architecture, possibly an ssDNA-like structure, could serve as the trigger. This theory certainly explains why the ATR signaling pathway is activated in the absence of DSBs at pseudo-sex bodies in *spo11*-mutants. Taking this information together, we propose that ssDNA-like structures mark unsynapsed chromosomes and trigger the ATR-dependent DDR response that leads to MSUC.

Furthermore, our model distinguishes two different DDR pathways between meiotic DSBs and MSUC. Of note, in *Mdc1*^{-/-} mice, the first wave of γ H2AX normally occurs in the nucleus following DSBs at the leptotene stage, but propagation of the second wave of γ H2AX (on sex chromosomes) exhibits a specific defect at the onset of MSCI in males. This suggests a further mechanistic difference between the DSBs-induced DDR pathway and the MSUC-associated DDR pathway [19]. Although the role of the DSBs-induced DDR pathway has not been fully examined, meiotic stage progression and normal chromosome synapsis in male *H2ax* and *Mdc1* mutants support the possibility that the DSBs-induced

DDR pathway may not be essential to meiotic progression [18, 19]. Indeed, female mutants of *H2ax* and *Mdc1* are fertile [18, 62].

Other DDR and repair components in MSCI

Various DDR proteins and related modifications are known to accumulate on sex chromosomes during MSCI. Chromosome-wide accumulation of these factors is determined by γ H2AX–MDC1 signaling [19], indicating that they may be downstream components of the γ H2AX–MDC1 signaling process (even if they have roles in MSCI). These factors include the heterotrimeric MRN complex (consisting of Mre11, Rad50, and Nbs1) [74], sumoylation [75], poly-ubiquitination, and ubiquitinated histone H2A [20]. However, the fact that several factors are implicated in meiotic recombination presents a potential challenge with regard to studying the role of these DDR factors in MSCI. Thus, though these proteins accumulate on sex chromosomes during MSCI, it is difficult to test their specific roles in MSCI. This group of proteins includes BRCA1 [76], BRCA2 (FANCD1) [77], the RecQ helicase Bloom's syndrome mutated (BLM) [78], and a BRCT domain containing protein BRIT1 (also known as MCPH1) [79]. On the other hand, knockout mice fertility (or lack thereof) provides important information regarding the roles of particular genes in MSCI. For example, Ku70 and 53BP1 localize on the XY body, but these knockout mice are fertile [80, 81]. Despite normal localization of the above factors on sex chromosomes during MSCI, the given knockout gene is generally not required for the critical steps of MSCI if fertility of the knockout mouse is not abrogated.

There is another class of repair genes that act in combination on sex chromosomes. Rad18 and HR6a/b localize on sex chromosomes during MSCI and are also involved in repairing DNA DSBs and in the replication bypass of DNA damage in somatic cells [82, 83]. Although *Hr6b* knockout and *Rad18* knockdown mice are subfertile and do not have essential roles in MSCI [82, 84], HR6b and Rad18 are involved in the regulation of histone modifications of sex chromosomes in later stages [84, 85]. Thus, there is a link between DNA repair machinery and epigenetic regulation of sex chromosomes. The epigenetic profiles of MSCI are reviewed in a later section.

Surveillance mechanisms in meiosis: monitoring DSBs and asynapsis

To ensure the integrity of gametes, meiotic defects such as abnormal DSB processing and synapsis defects are strictly monitored, and abnormal cells are eliminated [6, 25]. To date, the nature of the surveillance mechanism during meiosis remains obscure. However, given the central roles of ATM and ATR in checkpoint activation in somatic cells, it is likely that ATM and ATR are involved in a surveillance mechanism to monitor the integrity of meiosis [40, 86]. As discussed above, two distinct DDR pathways are activated during meiosis: the first DDR pathway responds to meiosis-specific DSBs through activation of both ATM and ATR, and the second DDR pathway responds to asynapsis through preferred activation of ATR (Fig. 2). The role of ATM is confined to meiotic DSBs formation, and a recent study revealed that ATM controls meiotic DSBs formation by Spo11 [87]. Therefore, surveillance mechanisms for DSBs and asynapsis appear to be distinct (Fig. 3). Defects in the processing of DSBs, such as *Dmc1*^{−/−} and *Msh5*^{−/−}, cause meiotic arrest and the elimination of germ cells at the mid-pachytene stage, which corresponds to testicular stage IV [86]. On the other hand, surveillance of asynapsis differs depending on the degree of asynapsis. In the case of extensive asynapsis, there is a severe defect and arrest at testicular stage IV [69]. However, if the degree of autosomal asynapsis is relatively small and limited to specific regions, as happens with Robertsonian translocation, meiosis can progress to the meiotic division phase [88], and a small region of autosomal asynapsis, such as Is1Ct, is tolerant of meiotic surveillance, resulting in fecundity [89]. This size effect of asynaptic regions can be explained by the limited amount of DDR factors involved in asynapsis surveillance (i.e., in

MSUC) [69]. Although autosomal asynapsis is known to interfere with MSCI [90], a small degree of autosomal asynapsis may not trigger a problem and does not completely disturb MSCI. Recent evidence suggests that enhanced asynapsis is associated with more severe meiotic defects [91]. Thus, surveillance of asynapsis depends on the degree of asynapsis and is likely to be less stringent compared to DSBs surveillance.

A striking difference in the checkpoint functions of somatic and meiotic cells is that the DDR pathways are activated during normal developmental processes in meiotic cells. Therefore, the integrity of the ATM and ATR pathways is also monitored by another layer of meiosis-specific checkpoints. For example, deficiency in the central components of the ATM and ATR pathways, such as ATM and MDC1, are associated with meiotic failure [62, 92]. These observations indicate the existence of another layer/mechanism of meiotic surveillance in the absence of the ATM and ATR pathways (Fig. 3).

In many species, such as *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Caenorhabditis elegans*, and mice, defects in DSB processing and/or chromosome synapsis lead to meiotic arrest at the mid-pachytene stage [93]. This monitoring system for chromosome synapsis is termed the pachytene checkpoint. In *S. cerevisiae*, *C. elegans*, and *D. melanogaster*, the pachytene checkpoint induced by unsynapsed chromosomes can be bypassed by the mutation of a pachytene checkpoint gene, *Pch2* [94–96]. However, deletion of the *Pch2* ortholog gene, *Trip13*, in mice does not bypass pachytene arrest [97, 98]. Furthermore, most of pachytene checkpoint genes identified in yeast do not have orthologs in mammals [99], suggesting that the pachytene checkpoint machinery may be species-specific.

In mammals, normal meiotic progression requires proper initiation of MSCI. If there is any defect in the silencing process, meiotic arrest and apoptosis occur at the mid-pachytene stage, which corresponds to testicular stage IV in mice, to eliminate abnormal spermatocytes [18, 19]. Additionally, in various mutants that are defective in synapsis and recombination, MSCI is not observed, and meiosis does not progress beyond the mid-pachytene stage [6, 69]. This meiotic arrest and apoptotic elimination suggest that there is a surveillance mechanism that functions as a meiotic checkpoint, monitoring a faithful MSCI (Fig. 3). However, little is understood about the molecular mechanisms involved.

A recent study identified candidate “killer” genes responsible for the elimination of abnormal meiotic cells [100]. *Zfy1* and *Zfy2* genes are located on the Y chromosome, and expression of these genes triggers elimination of meiotic cells when MSCI does not occur. Additionally, the *Zfy2* gene participates in the elimination of abnormal germ cells in the first meiotic metaphase [101]. X-linked “killer” genes are also anticipated [100, 102], and further identifications within the network may illuminate the molecular components of the mammalian pachytene checkpoint.

Epigenetic aspect of sex chromosome inactivation: from XY body to PMSC

In the course of MSCI, sex chromosomes form a distinct macrochromatin domain, termed the XY body after the mid-pachytene stage (Fig. 1). Following the initiation of MSCI in the early pachytene stage by the DDR pathway, epigenetic modifications are established on the XY body during the late pachytene stage. Hallmarks of transcriptional repression, such as di- and tri-methylated histone H3 lysine 9 (H3K9me2 and H3K9me3), heterochromatin protein1 β (HP1 β), and HP1 γ , localize on the XY body during the late pachytene stage [103, 104]. In contrast, other repressive marks, such as H3K27 methylation (mono-, di- and tri-) and trimethylation of H4K20, are largely excluded from the XY body during the pachytene and diplotene stages [24]; this exclusion of H3K27 methylation is mediated by the polycomb subunit *Scmh1* [105]. Histone replacement, another signature of epigenetic

programming, is also observed during the pachytene stage. Early in the pachytene stage, sex chromosomes are occupied by histone H3.1- and H3.2-containing nucleosomes. These nucleosomes are lost from sex chromosomes as the pachytene stage progresses; instead, H3.3-containing nucleosomes are incorporated into the sex chromosomes [24].

The XY body also accumulates various distinct modifications, the functions of which have not yet been determined. These include components of the sumoylation pathway (SUMO1, SUMO2/3, and UBE2I) and SUMO substrates (including PML and DAXX) [75, 106], a DM-domain containing protein DMRT7 [107], a testis-specific zinc-finger transcriptional factor (Ovol2/MOVO) [108], the Ran-binding protein 17 (RANBP17) [109], an Xlr gene family (Xlr6) [110], a chromatin modifier (SNF5/INI1) and a chromatin-associated protein (SIN3B) [111], and the phosphorylated extracellular signal-regulated kinase 1/2 [112]. These observations demonstrate that the XY body is highly distinct from other regions of the nuclei.

Contrary to the traditional view that MSCI and the XY body are transient and specific to the meiotic prophase, recent studies revealed that the silencing of sex chromosomes is largely maintained within each X- and Y-bearing spermatid, except for a certain class of spermatid genes that include multicopy genes on the sex chromosomes [11, 15–17]. The silent compartment of a sex chromosome is termed the PMSC [11] (Fig. 1). During the XY body to PMSC transition, several histone modifications, such as an accumulation of H3K9me2 and H3K9me3 and an exclusion of H3K27me3, are maintained within the PMSC [11, 15, 16, 24]. Thus, sex chromosome inactivation is an epigenetic process because memory is maintained through meiotic divisions. Notably, the DDR pathway provides the trigger for epigenetic programming on sex chromosomes [19], raising the possibility of a functional link between DDR pathways and epigenetic programming.

A link between the DDR pathway and epigenetic programming: regulatory mechanisms from XY body to PMSC

Currently, the manner in which the DDR pathway mediates epigenetic modifications and induces sex chromosome silencing remains obscure. However, one group of repair genes is clearly implicated in the regulation of histone modification. Rad18 and HR6a/b localize on sex chromosomes during MSCI, and deficiencies in Rad18 and HR6a/b lead to increased dimethylation of histone H3 at Lys4 and are associated with derepression of the X chromosome [84, 85].

In the somatic DDR, HP1, which associates with methylated H3K9, has been reported to accumulate at sites of DNA damage [113]. Thus, it is possible that DDR signaling enhances recruitment of HP1 to sex chromosomes and stabilizes gene silencing during MSCI following the initiation of silencing by the DDR pathway. A recent study has reported that H4K20 methylations (mono-, di-, and tri-) accumulate at sites of DNA damage and are necessary for full activation of the DNA damage checkpoint in somatic cells [114]. Local accumulations of H4K20me2 and H4K20me3 seem to be mediated by the histone methyltransferase MMSET through direct interaction with MDC1. On the XY body, H4K20me1 accumulation is observed from the mid-pachytene to the mid-diplotene stage [24]. Pr-set7 is the sole known histone methyltransferase that is responsible for H4K20me1 [115]. Therefore, Pr-set7 is likely to be regulated downstream of the DDR pathway and mediates H4K20me1 on the sex chromosomes. Intriguingly, accumulation of H4K20me1 is associated with chromatin assembly [116]. Taken together, it is possible that Pr-set7 and H4K20me1 could be the molecular link between the DDR pathway and the histone H3 replacement on sex chromosomes that occurs in the pachytene stage [24]. Increasing evidence suggests that DDR signaling regulates gene repression factors at sites of DNA

damage; however, the manner in which this is regulated and whether the same mechanisms function in MSCI remain unresolved.

In round spermatids, one regulator of HP1 and H3K9 methylation is a Y-linked multicopy gene, *Sly* [117]. *Sly* localizes on PMSC, and *Sly* knockdown decreases the level of HP1 and H3K9 methylation on PMSC, suggesting the possible role of SLY in the maintenance of postmeiotic silencing. Additionally, studies involving the large deletion of the Y-chromosome section where *Sly* is encoded show consistent results [118].

In addition to the accumulation of silent modifications on the sex chromosomes, epigenetic changes that are generally associated with active transcription are also observed on PMSC. The histone variants H2AZ and H2A.Lap1 are incorporated into PMSC [16, 119]. Furthermore, a recent study has identified a novel type of histone lysine modification (termed crotonylation) that specifically accumulates on PMSC [120]. Crotonylation accumulates on the sex chromosome-linked genes that are specifically expressed in the round spermatids, presumably protecting the spermatid-specific genes from chromosome-wide silencing [121].

The *raison d'être* of sex chromosome inactivation

Why do sex chromosomes in male germ cells need to be silenced? There are several theories as to the role of MSCI. One theory is that MSCI suppresses illegitimate recombination between unsynapsed sex chromosomes [122]. It has also been proposed that MSCI prevents aberrant transcription from the site of persistent DSBs on the unsynapsed sex chromosomes [8]. In addition, MSCI may also serve as a surveillance mechanism to monitor the proper development of meiotic cells through activation of the pachytene checkpoint, and/or MSCI may act to prevent unsynapsed sex chromosomes from being detected by the pachytene checkpoint, thereby enabling male meiotic cells to undergo meiosis [6].

The *raison d'être* of PMSC is also enigmatic. In the round spermatids, nuclear histones are displaced by transition proteins (TNPs) that lead to sperm compaction by protamines [121]. One possible role of PMSC is to protect silent sex chromosome genes from being activated during histone displacement. One view holds that PMSC serves as a precursor for imprinted X-inactivation that selectively inactivates paternally derived X chromosomes in the daughter, implicating trans-generational epigenetic inheritance from sperm to zygote [123]. In accordance with this prediction, post-meiotic silencing was discovered and shown to persist in spermatids [11, 15, 16, 23]. On the other hand, subsequent studies have revealed that gene silencing on the X-chromosomes is not continuous from MSCI to imprinted X-inactivation, so the debate regarding the pre-inactivation hypothesis is on-going [124–126] and reviewed in detail elsewhere [4]. These theories are not mutually exclusive and may represent multiple aspects of sex chromosome inactivation.

Concluding remarks

In this article, we focus on the underlying mechanisms of sex chromosome inactivation in germ cells. Recent evidence has revealed that the DDR pathway is the master regulator of MSCI and shares a significant commonality with the DDR pathway that is activated after DNA replication stress in the S phase [19]. Based on knowledge accumulated via analyses of somatic DDR, a potential DDR pathway toward MSCI initiation is discussed. An essential step in the DDR pathway is the amplification of γ H2AX by the action of MDC1, ATR, and TOPBP1. Here, we propose a DDR-adapted model in which the DDR pathway that recognizes somatic DNA replication stress is adapted to induce the epigenetic silencing of sex chromosomes (Fig. 2). We further discuss the potential role of this DDR pathway in the checkpoint response that monitors asynapsis in meiosis. Importantly, our detailed

comparison illuminates the mechanistic difference between the DDR pathway associated with MSUC and the DDR pathway that recognizes DSBs. Based on this mechanistic difference, we suggest that both DDR pathways underlie distinct checkpoint responses in meiosis: DSBs and asynapsis (Fig. 3). Another interesting aspect of MSCI is that epigenetic programming follows the initiation of MSCI via the DDR pathway. However, a missing link remains between the DDR pathway and epigenetic programming. Sex chromosome inactivation could provide a framework by which to understand the link between the DDR pathway and epigenetic programming.

Accumulating evidence has revealed a significant influence of MSCI on the evolution of the genome in terms of gene contents and expression [127]. Another interesting direction would be to explore how sex chromosome inactivation impacts the evolution of the mammalian genome and epigenome. It would be intriguing to test whether post-meiotic silencing is conserved in other species of mammals. Curiously, X-linked coding genes are largely reactivated in the round spermatids of marsupials following MSCI [126], although chromosome-wide silencing is maintained in the repeat regions of sex chromosomes in the round spermatids [23]. Therefore, sex chromosome inactivation may not be a simple process and, instead, needs to be understood on the levels of both genic and non-genic regulation. We anticipate further surprising twists in the journey toward understanding the key processes involved in sexual reproduction.

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Fig. 1.

Schematic of sex chromosome inactivation in male germ cells. Meiotic chromosome axes appear in the leptotene stage, and start to synapse at the zygotene stage. In the pachytene stage, unsynapsed X and Y chromosomes are silenced (MSCI) and form a silent compartment XY body (or sex body). Silencing of the sex chromosomes persists into post-meiotic spermatids. The DDR pathway initiates MSCI. Subsequently, histone replacement occurs and epigenetic modifications are established specifically on the sex chromosomes. In the round spermatids, sex chromosomes occupy a silent compartment, PMSC, and nuclear histones are displaced by transition proteins (TNP1 and TNP2). The *upper insets* show representative pictures of the pachytene spermatocytes and round spermatids with DAPI staining, respectively. XY body and PMSC are *circled*

Fig. 2.

The DDR-adapted model in meiotic silencing. There are two DDR pathways that underlie mammalian meiosis. **a** Spo11-dependent DSBs accompany the ATM-dependent DDR pathway and lead to meiotic recombination. After DNA end resection at DSBs, ATR is also activated on the ssDNA region. **b** Asynapsis regions are recognized by the ATR-dependent pathway, but are independent of ATM. This DDR pathway is distinct from the DSBs-associated DDR pathway and leads to meiotic silencing. MDC1 directs γ H2AX amplification and chromosome-wide spreading. **c** In somatic DDR, the pathway following DSBs is regulated by ATM and is associated with silencing, DSBs repair, and checkpoint activation. **d** In somatic DDR, the ATR-dependent pathway is activated following DNA

replication stress at the stalled replication fork in the S phase. At the stalled replication fork, ssDNA is recognized by RPA and the ATR-TOPBP1 complex is activated. This pathway is associated with silencing, DNA repair, and activation of the replication checkpoint

Fig. 3.

A model of meiotic checkpoints in mammalian meiosis. DSBs is monitored by the ATM-dependent DDR pathway, and asynapsis is monitored by the ATR-dependent DDR pathway. In addition to these surveillance mechanisms, there is another layer of monitoring mechanisms in the meiotic progression. This putative pachytene checkpoint is associated with germ cell loss at the mid-pachytene stages that correspond to testicular stage IV