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Meiotic sex chromosome inactivation and the XY body: a phase separation hypothesis

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Abstract

In mammalian male meiosis, the heterologous X and Y chromosomes remain unsynapsed and, as a result, are subject to meiotic sex chromosome inactivation (MSCI). MSCI is required for the successful completion of spermatogenesis. Following the initiation of MSCI, the X and Y chromosomes undergo various epigenetic modifications and are transformed into a nuclear body termed the XY body. Here, we review the mechanisms underlying the initiation of two essential, sequential processes in meiotic prophase I: MSCI and XY-body formation. The initiation of MSCI is directed by the action of DNA damage response (DDR) pathways; downstream of the DDR, unique epigenetic states are established, leading to the formation of the XY body. Accumulating evidence suggests that MSCI and subsequent XY-body formation may be driven by phase separation, a physical process that governs the formation of membraneless organelles and other biomolecular condensates. Thus, here we gather literature-based evidence to explore a phase separation hypothesis for the initiation of MSCI and the formation of the XY body.

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Author's contributions KGA and SHN conceived the concept of the manuscript. KGA, SM, PRA, and SHN reviewed the literature and wrote the manuscript.

Conflict of interest The authors declare no competing interests.

Keywords

Sex chromosomes; Germ cells; Germline; Epigenetics; Liquid–liquid phase separation; Sex body

Introduction

Mounting evidence has revealed that phase separation is a driving mechanism for the formation of biomolecular condensates, including various membraneless organelles [1, 2]. Liquid–liquid phase separation is a physical process that sees the spontaneous separation of a supersaturated liquid mixture into stable, distinct, coexisting liquid phases. In the nucleus, phase separation drives the organization of fundamental structures such as nuclear pore complexes [3], nucleoli [4], heterochromatin [5, 6], and transcription hubs associated with super-enhancers [7]. Additionally, in the germline, phase separation underlies the formation of various germline-specific biomolecular condensates [8, 9]. For example, in a 2009 study of *C. elegans*, P granules, the sites of germline-specific RNA regulation, were the first membraneless organelles shown to form from phase separation [10]. In meiosis, phase separation also drives the formation of synaptonemal complexes, which are required for the association of homologous chromosomes [11] and, during meiotic recombination, the assembly of meiotic DNA break machinery at sites of DNA double-strand breaks (DSBs) [12]. Thus, various biomolecular condensates interact to control gene expression and other cellular functions in germ cells.

Here, we gather literature-based evidence to propose a phase separation hypothesis for meiotic sex chromosome inactivation (MSCI) and the subsequent formation of the “XY body” (also known as the “sex body”), a distinct membrane-free nuclear body (Fig. 1A). An essential process in mammalian male germ cells, MSCI is a male sex chromosome-specific manifestation of meiotic silencing of unsynapsed chromatin (MSUC), a general mechanism for transcriptional silencing in both male and female meiosis [13–17]. MSUC operates as a surveillance mechanism for chromosome asynapsis after homologous autosomes have completed synapsis in the pachytene stage of meiotic prophase I. In normal male meiosis, MSUC is confined to unsynapsed X and Y chromosomes, where there is pairing and synapsis at only a small region of homologous sequence termed the pseudoautosomal region. Following the initiation of MSCI, at the onset of the mid-pachytene stage, sex chromosomes are sequestered away from recombining and transcriptionally active autosomes, and are transformed into an ellipsoidal nuclear body known as the XY body (MSCI and XY bodies are extensively reviewed in [17–24]; Fig. 1B).

Mechanistically, MSUC and MSCI are directed by DNA damage response (DDR) pathways that mediate the phosphorylation of histone variant H2AX at serine 139 (γH2AX) [23–25] (Fig. 1C). In the initial step of this mechanism, unsynapsed chromosome axes (XY axes in MSCI) are recognized by ATR, a serine/threonine-protein kinase, and TOPBP1, an ATR activator [26–28]. ATR phosphorylates H2AX at serine 139, which attracts MDC1, a γH2AX-binding partner [25]. The subsequent spread of γH2AX into a chromosome-wide domain (XY chromatin in MSCI) is directed by MDC1 in what comes to comprise an expansive feedforward mechanism: ATR and TOPBP1 are recruited to proximal

nucleosomes, H2AX is again phosphorylated, MDC1 binds γH2AX, more ATR and TOPBP1 are incorporated, and so on [25] (Fig. 1D). It is this mechanism that commences the chromosome-wide silencing of the XY.

Failure to initiate MSCI is linked to the complete arrest and elimination of male germ cells in the pachytene stage of meiotic prophase I [25, 29, 30]. The initiation of MSCI sequesters DDR signaling from autosomes to the sex chromosomes, leading to the timely progression of male germ cells through meiotic prophase I [30] (Fig. 1C). In this way, the XY body acts as a "trap" or "sink" for a diverse array of proteins [31], which facilitate the epigenetic regulation of XY chromosomes in later stages of spermatogenesis [23, 25, 32–35]. Related to this, in MSCI, the 3D chromatin organization of the sex chromosomes is spatially segregated from autosomes, and the X chromosome lacks several prominent features of higher-order chromatin organization [36–40]. Here, in summarizing such biochemical and chromatin features, we propose a hypothesis in which the sex chromosomes make for an energetically favorable environment for extensive epigenetic programming by the DDR. Then, we propose a model for the initiation of MSCI and XY-body formation in which a DDR-mediated liquid state experiences a progressive increase in ordered, less reversible interactions through the recruitment of various proteins, thereby transitioning to a gel-like state (Fig. 1B). Such a phase separation mechanism may explain how the DDR directs the regulation of the sex chromosomes in the male germline.

An overview of meiosis and the meiotic DNA damage response

In spermatogenesis and oogenesis, germ cells undergo extended, distinct periods of development that result in the formation of mature, functional gametes: sperm and eggs, respectively. One period of germ cell development stands out among the others for its length and intricacy: meiosis. In males, germ cells differentiate from a stem cell state and, following a period of premeiotic DNA replication, enter meiosis as primary spermatocytes. Now containing two complements of the diploid genome, primary spermatocytes undergo homologous recombination between maternal and paternal alleles —a biochemical mechanism that is a hallmark of sexual reproduction, ensuring accurate chromosome segregation for euploid gametes and fostering genetic diversity in offspring. The first phase of meiosis, prophase I, is followed by two quick meiotic divisions that result in four haploid round spermatids [41].

In meiotic prophase I, spermatocytes undergo the programmed induction of DNA double-strand breaks (DSBs). These DSBs are formed through the activity of SPO11, a topoisomerase II-like enzyme, and DSB-induction is essential for homologous recombination repair [42]. By resolving DSBs, homologous recombination repair promotes the proper pairing of homologous chromosomes, which in turn facilitates the shuffling of genetic material between maternal and paternal alleles [42–45]. A small portion of these homologous recombination repair events result in genetic crossovers, which ensure the proper segregation of homologs to daughter cells at the first meiotic division [42–46]. DSB repair is tightly linked to chromosome synapsis, and persistent DSBs on the unsynapsed chromosomes are thought to trigger the DDR that underlies MSUC and MSCI [23, 24, 47, 48]. Failure to repair DSBs in a timely manner triggers the developmental arrest and death

of spermatocytes [44, 49, 50]. In another form of genetic quality control, improper synapsis also triggers spermatocyte arrest and death [50–52]. Taken together, MSCI is sensitive to DNA damage and altered DSB-processing on the autosomes [51, 53]. Thus, it is posited that certain DDR proteins are available in limited quantities such that autosomal defects draw away proteins needed for MSCI, thereby disrupting the MSCI process and triggering spermatocyte death [53].

ATM- and ATR-mediated DNA damage response signaling

Meiotic prophase I corresponds to the G2 phase of the cell cycle, and the DDR pathways that function in the G2/M checkpoint in somatic cells have major functions in meiotic prophase I [54]. These pathways are driven by the ATM and ATR phosphatidylinositol 3-kinase-related protein kinases (PIKKs). ATM responds to DSBs, and ATR responds to stalled replication forks and the resected single strands of DNA that appear following DSB formation [55, 56]. In meiotic prophase I, in response to the programmed DSBs that initiate meiotic recombination, ATM-driven DDR signals cascade and propagate throughout the nucleus to facilitate homologous recombination repair [50, 57]. Accordingly, in the initial stage of meiotic prophase I, the leptotene stage, ATM-mediated accumulation of γH2AX is observed nucleus-wide [58]. ATM is typically activated and functions before ATR, both in the somatic DDR and in meiosis. As a result, ATR is subsequently required for the completion of meiotic recombination [59, 60] and MSCI [27, 61, 62]. In the next stage of meiotic prophase I, the zygotene stage, ATR-dependent γH2AX formation takes place on unsynapsed regions of autosomes and sex chromosomes; upon completion of synapsis at the onset of the subsequent pachytene stage, ATR-dependent γH2AX is confined to the unsynapsed sex chromosomes [27].

In meiotic prophase I, meiotic chromosome assembly is mediated by loop extrusion [36, 37, 63], a biochemical mechanism in which DNA is threaded through the ring-shaped, multi-subunit protein cohesin, thereby forming loops of DNA [64]. A recent study using somatic cell lines revealed a loop extrusion mechanism as the basis for ATM-dependent spreading of the γH2AX domain in response to DSBs [65]. In somatic cells, γH2AX is confined within preexisting topologically associating domains (TADs), where chromatin interacts with chromatin inside the domain at a higher frequency than that which is outside the domain [65]. Formation of γH2AX-confined TADs is mediated by rapid loop extrusion from sites of DSBs, where ATM is located (Fig. 1E). Because ATM-dependent DSB repair takes place via similar mechanisms in somatic cells and leptotene spermatocytes, it is possible—perhaps even likely—that loop extrusion underlies the initial spread of γH2AX in early meiotic prophase I. In this mechanism, the γH2AX-binding protein, MDC1, is likely recruited downstream of ATM-dependent γH2AX domain formation and, indeed, MDC1 is not required for the first wave of γH2AX domain formation in leptotene nuclei [25]. By contrast, on meiotic sex chromosomes, MDC1 may spread ATR to preexisting meiotic chromatin loops to generate a γH2AX “mega domain” that comprises the whole of the X and Y chromosomes (Fig. 1D). As discussed later in “DDR pathways and phase separation”, we propose that this ATR-dependent establishment of a γH2AX mega domain drives phase separation of the sex chromosomes during male meiosis.

Our understanding of how DDR signaling controls XY-body formation is based in part on loss-of-function mouse models for *H2ax* and *Mdc1* [25, 29, 30]. In these mutants, not a single spermatocyte has been observed to have escaped and/or to have survived defective MSCI. Thus, this surveillance mechanism in male germ cells is highly specific and potent. The mechanism that underlies the completely penetrant death of MSCI-defective spermatocytes is a mystery. One study proposed a model in which the ectopic expression of toxic Y-linked genes, such as *Zfy1* and *Zfy2*, induces cell death when MSCI is abrogated [66]. However, given that both ZFY1 and ZFY2 function in normal spermatogenesis [67–69], derepression of sex-linked genes is unlikely to be the sole mechanism inducing cell death (as discussed in [30]). Further insight comes from a recent study that developed a mouse model with a point mutation in which tyrosine 142 (Y142) of H2AX is converted to alanine (*H2ax-Y142A*); with this tyrosine converted to an alanine, phosphorylation of amino acid 142 is no longer possible [30]. Like serine 139, H2AX's tyrosine 142 is an important amino acid residue for the DDR; in *H2ax-Y142A* meiosis, the establishment of DDR signals on the XY chromatin is completely impaired, as is the case in *Mdc1*-deficient meiosis [25]. Indeed, in *H2ax-Y142A* and *Mdc1*-deficient meiosis, ATR-mediated DDR signaling is retained on autosomes [30]. The study thereby shows that the initiation of MSCI sequesters DDR factors from autosomes to the sex chromosomes at the onset of the pachytene stage, and that the subsequent formation of an isolated XY nuclear compartment, the XY body, sequesters DDR factors to permit meiotic progression from the mid-pachytene stage onward (Fig. 1C). These findings suggest that MSCI functions as a checkpoint that coordinates and orders events during the pachytene stage of meiotic prophase I, and is directly regulated by the same DDR pathway that functions in the G2 DNA damage checkpoint in somatic cells [30]. Of note, pachytene arrest and cell death were still observed in *Atr* mutant mice [27] as well as triple mutant mice deficient for all three DDR-related PIKKs: ATM, ATR, and PRKDC (also known as DNA-PKcs) [59]. Therefore, while meiotic progression is regulated by ATM and ATR, pachytene arrest and cell death per se are independent of ATM, ATR, and PRKDC, although it is possible that the sequestration of other checkpoint proteins (or a network of DDR proteins) by MSCI is required for normal meiotic progression.

The establishment of DNA damage response signals along unsynapsed axes

During the initiation of MSCI, the DDR mediates two genetically separable steps: The first is the establishment of DDR signals along the XY axes, and the second is the MDC1-dependent amplification of γH2AX through the XY chromatin [25] (Fig. 1D). ATR, TOPBP1, and the ATR-interacting protein ATRIP localize on the unsynapsed axes of the sex chromosomes [26, 70, 71], and ATR and TOPBP1 are essential for MSCI [27, 28]. Another DDR factor, BRCA1, the protein product of breast cancer susceptibility gene 1, is also a marker of unsynapsed sex-chromosome axes [72] and is required for MSCI [73]. Starting from sites of persistent DSBs on the XY axes, BRCA1 establishes and amplifies DDR signals (e.g., ATR-TOPBP1; Fig. 2) [48]. Along with BRCA1, ATR and TOPBP1 are also required for the amplification of DDR signals, along the XY axes [27, 28]. Thus, BRCA1, ATR, and TOPBP1 likely work together along the XY axes.

During MSCI, the accumulation of BRCA1 on unsynapsed XY axes precedes γH2AX domain formation; this is evident from studies of *H2ax* and *Mdc1* loss-of-function

spermatocytes, because even though γH2AX domain formation does not take place, BRCA1 accumulates normally on unsynapsed XY axes [25, 29, 30]. Since BRCA1 also accumulates at stalled DNA replication forks in somatic cells [74], the DDR signaling associated with pachytene-stage XY asynapsis resembles the replication stress response [23]. Interestingly, the order of events in MSCI differs from that of another DDR pathway in somatic cells, the "DSB response," where BRCA1 accumulates downstream of γH2AX [75]. This difference raises an interesting possibility: The order of accumulation of BRCA1 and γH2AX may be distinct between ATM- and ATR-dependent DDR pathways. If this is indeed the case, then it is likely to have consequences for loop extrusion, which presumably involves ATM, and the ultimate size of γH2AX foci or domains that assemble in response to DNA damage.

Related to this, the BRCA1-A complex, one of the major BRCA1-containing complexes, is detected along unsynapsed XY axes but not throughout the XY chromatin domain [76]. Comprised of BRCA1, RAP80, CCDC98/Abraxas, and BRCC45, the BRCA1-A complex regulates G2 checkpoint arrest in response to DNA damage in somatic cells [75]. Thus, the BRCA1-A complex could have a checkpoint function in meiotic cells that parallels its function in somatic cells. CTIP, a protein that functions in DNA end resection, is also detected on unsynapsed XY axes [76]. Although their functions in MSCI are largely unknown, the presence of BRCA1-A and CTIP suggests the existence of DNA damage and/or ongoing repair along the XY axes [77]. Indeed, their presence could be the basis for the sustained DDR that mediates signaling on the XY axes and through XY chromatin loops, thereby culminating in MSCI.

How does DDR signaling recognize unsynapsed chromatin? Although the mechanism is largely unknown, several meiosis-specific proteins are implicated. SYCP3, a component of the synaptonemal complex that forms between homologous chromosomes in meiotic prophase I, is required for the loading of BRCA1 along unsynapsed axes in female meiosis [78]. Furthermore, SMC1β, a meiosis-specific component of the cohesin complex, is required for chromosome synapsis and, consequently, for MSCI [79]. Also, the meiosis-specific HORMA domain proteins, HORMAD1 and HORMAD2, accumulate on unsynapsed axes independent of BRCA1 [48] and ATR [27]; in analyses of meiosis using loss-of-function of *Hormad1* and *Hormad2* models, DDR signaling along unsynapsed axes was attenuated [80–84]. Thus, in response to chromosome asynapsis and persistent DSBs, meiosis-specific proteins elicit ATR-mediated DDR signaling along the unsynapsed axes, and although phosphorylation of HORMAD proteins is regulated by ATR [27, 85], HORMAD proteins appear to be upstream of DDR factors (Fig. 2).

In somatic cells, ATR is activated by both the single-strand DNA-binding protein RPA, which binds ATRIP to recruit ATR, and the RAD9A-RAD1-HUS1 (9A-1-1) checkpoint clamp, which is loaded at recessed 5' ends associated with DSBs [86, 87]. Interestingly, RPA foci are detected on unsynapsed axes in meiosis, and 9A-1–1 accumulates along unsynapsed axes too. However, in analyses of spermatocytes in which *Rpa1*, *Rad9a*, and *Hus1* were conditionally deleted, MSCI appeared to take place [88–90]. Although the mechanism to activate ATR signaling in MSCI remains a longstanding question [86, 87], it is possible that persistent DSBs may provide single-strand DNA structures that result from DSB resectioning, thereby triggering the DDR [47].

Taken together, these studies establish that DDR protein networks induce MSCI through two steps of signal amplification: the first on unsynapsed axes and the second on the whole of the XY chromatin (Figs. 2 and 3).

DDR pathways and phase separation

Recent studies of 3D chromatin conformation in pachytene spermatocytes revealed striking X-chromosome structural features associated with MSCI [36–40]: X appears to lack TADs and A/B compartments, i.e., alternating states of chromatin—termed A and B—in which each state preferentially interacts with other loci of the same state. Thus, the 3D chromatin conformation of the X appears to be random in the population of pachytene spermatocytes. This raises at least a few questions. Does the DDR pathway that drives MSCI promote this apparent lack of TADs and genomic A/B compartments? If so, then how? And what could be the purpose of such an organizational scheme?

The answers may lie in phase separation, a physical phenomenon in which stable, distinct liquid phases form from the surrounding liquid environment [1, 2]. It is through liquid–liquid phase separation mechanisms that many membraneless organelles self-organize and behave as liquid droplets in the cyto- and nucleoplasm [91, 92]. Heterochromatin, for example, has been proposed to form through phase separation [5, 6], and phase separation establishes and maintains distinct forms of chromatin [93]. Therefore, it could be that an MDC1-driven, chromosome-wide DDR initiates a phase separation mechanism that culminates in XY chromatin—which is largely heterochromatic—coalescing into a self-associating, droplet-like domain that lacks several obvious features of higher-order chromatin organization such as TADs and A/B compartments. While not formally demonstrated, the possibility of phase separation of the sex chromosomes is supported by the fact that this structure does not appear to be separated from the nucleus by a membrane (Fig. 1A) [30] and by the exclusion of particular proteins such as RNA polymerase II [94].

As a basis for nuclear compartmentalization in the absence of membranes, phase separation has emerged as a key feature of biochemical processes such as transcriptional regulation and the DDR. Molecular evidence has established links between DDR signaling and phase separation. For example, poly(ADP-ribosyl)ation (PARylation) seeds phase separation and the assembly of various intrinsically disordered proteins—proteins that, on their own, lack an ordered 3D structure—at DNA break sites in somatic cells [95, 96]. Intriguingly, in MSCI, although the function of PARylation is yet to be determined, PARylation and poly(ADP-ribose) polymerase 2 (PARP2) are detected on the sex chromosomes [97] (Fig. 3). Additionally, the DDR scaffold protein 53BP1 was shown to be a major player in phase separation at DNA lesions in somatic cells [98]. In MSCI, 53BP1 is an XY marker [99] recruited to the sex chromosomes downstream of RNF8, an MDC1-interacting protein [32] (Fig. 3). Despite its localization to the sex chromosomes, MSCI occurs without obvious incident in *53bp1*-knockout mice, and *53bp1*-knockout mice are fertile [32]. Thus, in MSCI, 53BP1 could be a non-essential factor for DDR-mediated phase separation. In contrast, SUMOylation, a type of post-translational modification involving small ubiquitin-related modifier (SUMO) proteins, is the primary mechanism driving the formation of phase-separated promyelocytic leukemia nuclear bodies [100]. Interestingly, SUMOylation

is among the earliest XY modifications at the onset of MSCI [101, 102], and SUMOylation in MSCI is MDC1-dependent [25] (Fig. 3). However, the sex-chromosome substrates subject to SUMOylation are unknown. Based on a growing number of observations reported in the literature, phase separation facilitates distinct environments for DDR-related processes, possibly including MSCI.

In the pachytene stage, DSBs prefigure the localization of ATR signaling on the XY axes. Studies of *Spo11*-deficient spermatocytes support this idea: In *Spo11*-deficient spermatocytes, in which chromosomes undergo aberrant synapsis in the absence of programmed DSBs [49, 58, 103], a small number of DSBs nevertheless arise and persist [103]. These breaks colocalize with transcriptionally inactive XY body-like domains, termed "pseudo-sex bodies" because they localize outside of the XY chromosomes but are similarly enriched with γH2AX, ATR, and other DDR factors. This indicates that DSBs trigger the localization of the ATR-driven DDR signaling that gives rise to ectopic MSUC [48, 49, 58, 103]. Importantly, these ectopic γH2AX domains appear to coalesce into a single large domain, and the same is true for ectopic MSUC that recognize large asynaptic segments of autosomes [104, 105]. Thus, the γH2AX domains that form from MSUC appear to demonstrate self-assembly, a key characteristic of liquid–liquid phase separation [106].

At the onset of MSCI, the MDC1-dependent amplification of γH2AX on XY chromatin may be the major driver of phase separation. Indeed, MDC1 is enriched with intrinsically disordered regions (IDRs; Fig. 4A), regions of proteins that do not fold into fixed structures. IDRs are essential features of many proteins implicated in phase separation [107]. Furthermore, because conformational flexibility and electrostatic interactions typically underlie phase separation events [95, 108], it is possible that phase separation of the entire sex chromosomes is driven by large-scale changes in post-translational modifications such as phosphorylation of the highly basic histone H2AX. In MSCI, XY chromosomes lack functional TADs despite the presence of CTCF [39], a ubiquitous transcription factor that demarcates TADs in somatic cells by binding to insulators and domain boundaries [109]. Thus, it is possible that the spread of DDR factors is not constrained by the boundary-inducing effects of CTCF and may thereby promotes phase separation of XY chromosomes.

Apart from X-chromosome regulation in male meiosis, it has been proposed that somatic X chromosome inactivation in females occurs via a phase separation mechanism [110, 111]. However, the mechanism of female X chromosome inactivation depends on *Xist*, an X-linked long non-coding RNA. The inactive female X is folded into two separated "mega domains," an organization scheme which is distinct from that of autosomes and the active X chromosome [112–115]. In contrast, the inactive male X in male meiosis lacks two separated mega domains. It is intriguing to consider the possibility that the DDR pathway may phase-separate the male meiotic sex chromosomes, giving rise to a nuclear domain with what is an apparently near-patternless chromatin organization scheme (the structural features of meiotic sex chromosomes have been discussed in detail elsewhere [116]).

What could be the function of XY phase separation in male meiosis? Phase-separated sex chromosomes may serve as a microenvironment for the functional activity of DDR factors, chromatin remodelers, and other factors associated with MSCI while also excluding non-

essential factors. In the case of the sex chromosomes, phase separation could (1) lead to the concentration of specific factors necessary for MSCI, (2) generate specific signals within the XY chromatin by dampening those signals elsewhere, and/or (3) exclude unwanted signals or transcription factors from the XY chromatin. Importantly, phase separation-mediated MSCI could also function as a means to suppress the illegitimate recombination of unsynapsed chromosomes [19] and as a surveillance mechanism warding against other meiotic abnormalities (for example, a checkpoint to recognize ectopic asynapsis).

XY-body formation and its molecular regulators

Following the DDR-directed initiation of MSCI in the early-pachytene stage, the sex chromosomes form a distinct nuclear compartment, the XY body, in the mid-pachytene stage (Fig. 1A, B). To some extent, the XY body can be considered a heterochromatic structure, bearing epigenetic marks typically associated with transcriptional inactivation and chromatin compaction. The XY body is maintained into postmeiotic spermatids in the form of another heterochromatic nuclear body: postmeiotic sex chromatin (PMSC) [94, 104, 117]. In placental mammals and marsupials, MSCI, XY-body formation, and PMSC formation are evolutionarily conserved processes [118–121]. DDR signaling is an evolutionarily conserved mechanism to drive MSCI, and so is the deposition of H3K9me3, a histone post-translational modification typically associated with heterochromatin [118, 120].

But to describe the XY body as solely heterochromatic elides another essential fact: the XY body accumulates active post-translational modifications throughout the entire XY chromatin [32], including H4K20 monomethylation (H4K20me1) and H3K4 dimethylation (H3K4me2). This post-translational information supports “escape gene activation” in postmeiotic spermatids [32, 122]. In escape gene activation, a select set of sperm-related genes escape transcriptional repression. Such active modifications are established downstream of RNF8, an E3 ubiquitin ligase that interacts with MDC1 and mediates poly- and monoubiquitination of XY chromatin [32, 122] (Fig. 3). Downstream of RNF8-mediated ubiquitination, the histone post-translational modifications H4K20me1, H3K4me2, and H3K27 acetylation (H3K27ac), a marker of active enhancers, are deposited. Thus, RNF8-dependent open chromatin and active enhancers are enriched on the X chromosome in pachytene spermatocytes [34, 123–125]. After meiotic prophase I, the histone variant H2AZ is also incorporated into XY chromosomes [117] in an RNF8-dependent mechanism [32]. Of note, RNF8-targeted escape genes are marked with H3K9me3 in pachytene spermatocytes, presumably suppressing the genes until they are subsequently activated [126]. Together, RNF8-mediated processes function in mechanisms associated with the transcriptional activation of sex chromosome-linked genes in the postmeiotic spermatid phase of spermatogenesis. Thus, the XY body is subject to forms of epigenetic and post-translational information that are associated with both active and inactive chromatin.

Beyond those associated with DDR signaling, a variety of proteins have been identified on the sex chromosomes in meiosis (Fig. 3). This includes the histone methyltransferase SETDB1, which mediates the deposition of H3K9me3 and is recruited to the sex chromosomes downstream of the DDR [127]. This also includes several proteins known to influence the gene silencing associated with MSCI, among which are Senataxin (SETX), a

DNA repair protein [128]; AGO4, an Argonaute family member that functions in microRNA regulation [129]; SCML2, a germline-specific Polycomb protein [35, 130]; and MAPS, a male germline-specific protein [131].

There is a dynamic temporal aspect to the protein accumulation and epigenetic programming that occurs on the XY body. At the onset of MSCI, XY chromatin is enriched with SETDB1-mediated H3K9me3 [127, 132]. However, H3K9me3 is lost in the mid pachytene stage only to return when spermatocytes progress into the subsequent diplotene stage [132]. Presumably, this is due to the replacement of histones H3.1 and H3.2 with histone H3.3 [132]. Following histone eviction and replacement, H3K9me3 is deposited along with various proteins and other heterochromatic histone post-translational modifications. Concomitant with this, the testis-specific histone variant H3T—a predominant histone H3 isoform in differentiating spermatogenic cells—is excluded from the XY body [133]. Thus, independent of DNA replication, extensive histone eviction and replacement takes place, and H3.3 becomes an important substrate for H3 modifications during and after its incorporation into XY chromatin (Fig. 3).

Notably, in conjunction with SETDB1-mediated establishment of H3K9me3, a group of DDR/DNA repair proteins associated with Fanconi anemia (FA) function on the sex chromosomes to regulate H3K9 methylation. FA is a genetic disease associated with bone marrow failure, increased cancer susceptibility, and severe germline defects [134], and patients are said to have FA if they are deficient for the function of any one of > 20 FA genes. On the sex chromosomes, FA proteins function in a network to regulate the deposition of epigenetic marks such as repressive H3K9me2/3 and active H3K4me2 [33, 135]. FA proteins such as FANCB [135], FANCM, BRCA1 (FANCS) [73], BRCA2 (FANCD1) [72], FANCD2, PALB2 (FANCN), SLX4 (FANCP/BTBD12) [136], and FANCI [137] localize on the sex chromosomes in meiosis, where FA proteins function in a network to positively regulate H3K4me2 and H3K9me2 while counteracting H3K9me3 [33, 137] (Fig. 3).

In addition to these DDR-mediated processes, the Polycomb protein SCML2 regulates key epigenetic and post-translational modifications associated with the XY body. SCML2 works with USP7, another XY-body component, to suppress the ubiquitination of histone H2A at lysine 119, which is mediated by Polycomb repressive complex 1 (PRC1) [35, 130] and influences RNF8-mediated ubiquitination on the sex chromosomes (Fig. 3). SCML2 is also required for the localization of XMR, a classic marker of the XY body [138] (although the identity of this antigen is unknown so far [139]). Furthermore, SCML2 suppresses the deposition of H3K9me1 on, and the incorporation of histone variant macroH2A1 into, XY chromatin. Interestingly, in male meiosis, BRCA1 establishes various forms of protein signaling on X-pericentromeric heterochromatin, including the accumulation of macroH2A1 and the chromatin-remodeling protein CHD4 [48] (Fig. 3). Thus, the action of SCML2 on XY chromatin is mutually exclusive with that of BRCA1 on X-pericentromeric heterochromatin in male meiosis.

A closeup view of the relationship between XY-body formation and phase separation

A notable feature of the formation of the XY body is the gradual establishment of specific epigenetic states following DDR-directed initiation of MSCI (Fig. 1B and 3). In the early-to-mid pachytene stage transition, SCML2 is recruited to the XY chromatin [35, 130]. Unlike the DDR factors discussed above, SCML2 is not an early marker of DDR-mediated MSUC, as SCML2 is not detected on the pseudo-XY body in *Spo11* mutants [130]. SCML2 contains a domain comprised of 10 repeats of 28-amino-acid units enriched with basic amino acids, termed the SCML2 DNA binding (SDB) repeats [140]; the domain is made up of IDRs (Fig. 4B). Fittingly, when SCML2 was ectopically expressed in somatic cells, SCML2 self-assembled into several nuclear bodies [140] (Fig. 4C), a phenomenon associated with phase separation [106]. A fluorescence recovery after photobleaching (FRAP) assay revealed rapid recovery of SCML2 ectopically expressed in somatic cells—another phenomenon associated with phase separation. After photobleaching, it took 8.6 s to recover 50% of the plateaued intensity ($t_{1/2}$) [140] (Fig. 4C, D), a timescale that appears to be comparable to 53BP1 nuclear bodies, which are DDR-mediated phase separation condensates [141].

It is increasingly apparent that the formation and regulation of the XY body may parallel heterochromatin formation and regulation in somatic cells. Importantly, heterochromatin has been reported to form in somatic cells through a phase separation mechanism [5, 6]. Proteins in the heterochromatin protein 1 (HP1) family such as HP1β and HP1γ—both of which bind H3K9me3—associate with the XY body [142, 143]. Notably, HP1 accumulation is a relatively late event in the sequence of steps that comprise XY chromatin remodeling in meiosis; this is presumably due to the histone H3 replacement that takes place within XY chromatin in the mid-pachytene stage [132] (Fig. 1B and 3). A newly incorporated histone H3 variant, H3.3, is the likely target of H3K9me3 deposition, which in turn recruits HP1 proteins. This raises the possibility that H3K9me3-binding HP1 proteins, which accumulate later in the pachytene-diplotene transition on the sex chromosomes, further modulate the status of phase separation following the initiation of DDR-mediated phase separation in MSCI.

If indeed the XY body forms through phase separation, then what is its material state? Our understanding of the DDR-mediated initiation of MSCI lays the groundwork for a predictive framework for domain formation. Because many different proteins, including SCML2, accumulate in the XY body in a time-dependent manner, one could frame a testable hypothesis that the XY body begins in a liquid-like state, predominated by non-covalent interactions between proteins that are uniform and shorter-range in occurrence; then, over time and with the addition of various proteins, the XY body comes to acquire a gel-like state, comprised of non-uniform, longer-range non-covalent interactions. Various biomolecular condensates continue to change material states across time, such as from liquid to gel to solid states, with the mobility of components gradually decreasing, forming interaction regimes that are less and less reversible [1, 96]. The Balbiani body in oocytes is reported to undergo just such a transition [144]. Thus, it is conceivable that the gradual establishment of various proteins on the XY body change it from a liquid state to a gel state (Fig. 1B). We hypothesize that the XY body originates with DDR signaling, transitions to a network of unknown numbers of various proteins, among which is SCML2, then changes

to a stabler network that includes HP1 proteins. To test this model, researchers will need to evaluate the XY body in living cells as was done for the initial characterization of P-granule phase separation [10] and for the formation of meiotic chromosome synapses via phase separation [11]. Caveats and technical considerations associated with such approaches and associated assays have been considered in detail elsewhere [145, 146].

Our phase separation hypothesis has the potential to guide the study of MSCI through a number of instructive and testable predictions, opening researchers to the application of assays that test for XY-body formation, maintenance, and maturation. Since IDRs, noncovalent inter-protein interactions, and noncovalent intra-protein interactions are critical for phase separation in other contexts, it is important to perform experiments that test the roles of various protein domains and/or disrupt hydrophobic interactions (relevant experiments and approaches are discussed in [147]). However, care should be taken to avoid over-interpretation of resulting data, as it is increasingly clear that results from such experiments can and do sometimes support models beyond that of phase separation [148, 149].

Because the XY body persists and changes through time, it will be important to understand what factors are essential and nonessential for the maintenance and/or maturation of the XY body downstream of initiation, either dependent on or independent of phase separation. In what sequences are XY-body components deposited? And what are their structural properties, alone and in combinations with each other? How does the XY body respond to changes in interaction strengths? And what about non-protein macromolecules that potentially make up the XY body? The phase separation model allows for predictions that, for example, RNA species exert specific influences on condensation. These questions, among many others, will fuel MSCI and XY-body research for years to come.

Looking forward

In a previous study, we presented the idea that MSCI is an apt model system to dissect the roles of different DDR factors in epigenetic programming [33]; i.e., in cell biology, the MSCI model allows for the formulation of hypotheses with the potential to unlock our understanding of how proteins, epigenetic information, and/or post-translational information are regulated and interrelated in time and space. We propose to extend this MSCI model to incorporate the phenomenon of phase separation. When considering experimental design, this is useful: Given the origins of the XY body and its persistent and dynamic nature, experiments that focus on the XY body—and/or the greater nuclear system that gives rise to the XY body—have the potential to yield a better understanding of how cells regulate phase separation in a spatiotemporal manner. With such a perspective, various lines of inquiry and testable hypotheses are made available to researchers interested in MSCI and/or phase separation. Also, it is important to note that, regardless of whether or not empirical tests confirm the XY body as a biomolecular condensate that arises from phase separation, the initial perspective of a phase-separated XY body establishes a useful framing device for experimental design. Put another way, if the hypothetical phase-separated XY body is disconfirmed, the experimental design originating from the initial perspective

is likely adaptable to other hypothetical mechanisms that facilitate the sequestration and concentration of a vast sub-proteome on the meiotic sex chromosomes.

But a phase-separation mechanism should not be discounted yet. Consistent with previous reports regarding other biomolecular condensates in the germline [8, 9], compartmentalization of the XY body could serve to promote the fidelity of spermatogenesis. Of note, in pachytene spermatocytes, the XY body is adjacent to a nucleolus [150], itself a phase-separated compartment [151]. The relationship between the XY body and the XY-neighboring nucleolus is a longstanding mystery. And that is far from the only mystery concerning the XY body: for example, a recent study revealed that the dosage of sex-linked proteins in MSCI is compensated by the high translation of sex-linked proteins in pachytene spermatocytes [152], consistent with the enrichment of translational machinery on the XY body [153]. Thus, in a certain sense, the “silent” XY body “continues to speak.” And since the epigenetic and post-translational states of the XY body are largely maintained into the PMSC of postmeiotic spermatids, MSCI may prepare heritable epigenetic states [154]. Ultimately, MSCI is considered a driving force for genomic evolution [120, 155–158], and since MSCI defects are tightly associated with the hybrid sterility of heterogametic males [159, 160], such a mechanism could serve to influence speciation. Taken together, a mechanistic exploration of MSCI and the XY body addresses a role for phase separation in nuclear biology, with strong potential to reveal insights into sex-chromosome regulation and function—including, in a grander sense, sex-chromosome function in evolutionary adaptation. A phase-separation hypothesis for MSCI lights the path to a greater understanding of meiosis, epigenetics, and evolution.

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Availability of data and materials

All data relevant to this review is included in the text, references, and figures.

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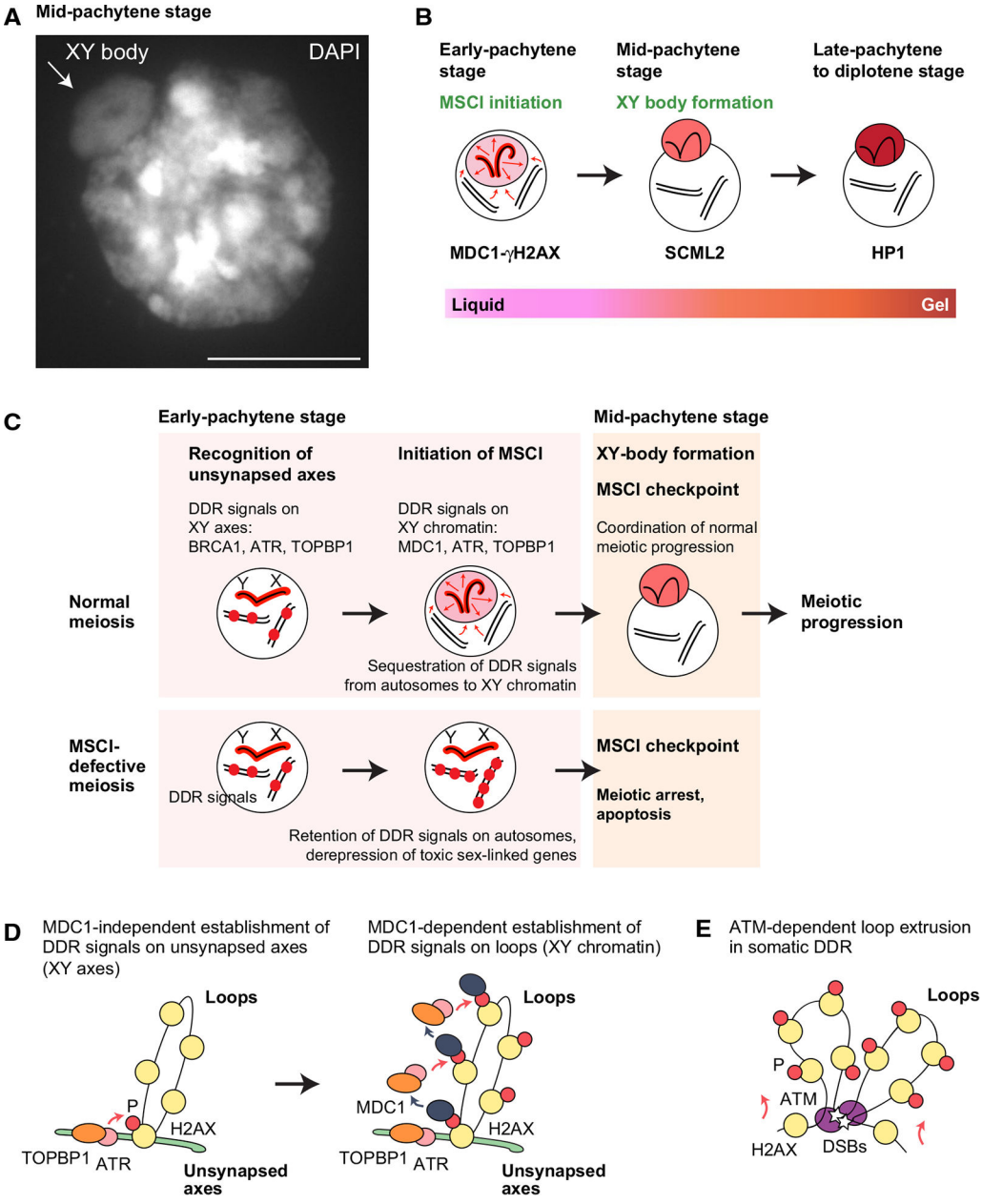


Fig. 1.

Mechanisms of MSCI initiation. **A** DAPI counterstaining of a "3D slide," i.e., a slide prepared such that the gross three-dimensional organization of chromatin is preserved [94]. The dashed circle indicates the XY body. Scale bar: 10 μm. The image is originally from Abe et al. 2020 [30]. **B** Model of phase separation of the sex chromosomes. Key proteins involved at each step of the process are shown. **C** Model of the MSCI checkpoint: the physical seclusion of DDR factors from autosomes to the XY body is a critical checkpoint in the progression of meiosis and the development of gametes. At the onset of MSCI, DDR factors (shown as red) are excluded from autosomes and sequestered to the sex chromosomes. The physical seclusion of DDR factors on/at the XY body, which may involve phase separation, is a critical step in the MSCI checkpoint in the mid-pachytene

stage of meiotic prophase I. While the MSCI checkpoint ensures meiotic stage progression in normal meiosis, the abolishment of MSCI enables the ectopic retention of DDR signals on/at autosomes; in turn, this triggers complete meiotic arrest and cell death in response to the checkpoint. **D** Model for the initiation of MSCI. ATR and its activator, TOPBP1, are recruited to unsynapsed axes in an MDC1- and H2AX-Y142-independent manner, resulting in phosphorylation of H2AX (γH2AX) on axes (left). Then, γH2AX recruits MDC1, which facilitates the progressive recruitment of ATR and TOPBP1, resulting in γH2AX and MDC1 spreading throughout loops (right). **E** Model for ATM-dependent loop extrusion in the somatic DDR. DSBs trigger the recruitment of ATM, which phosphorylates H2AX. As loop extrusion progresses, DNA passes by ATM enzymes at sites of DSBs, facilitating the phosphorylation of histones across large tracts of DNA

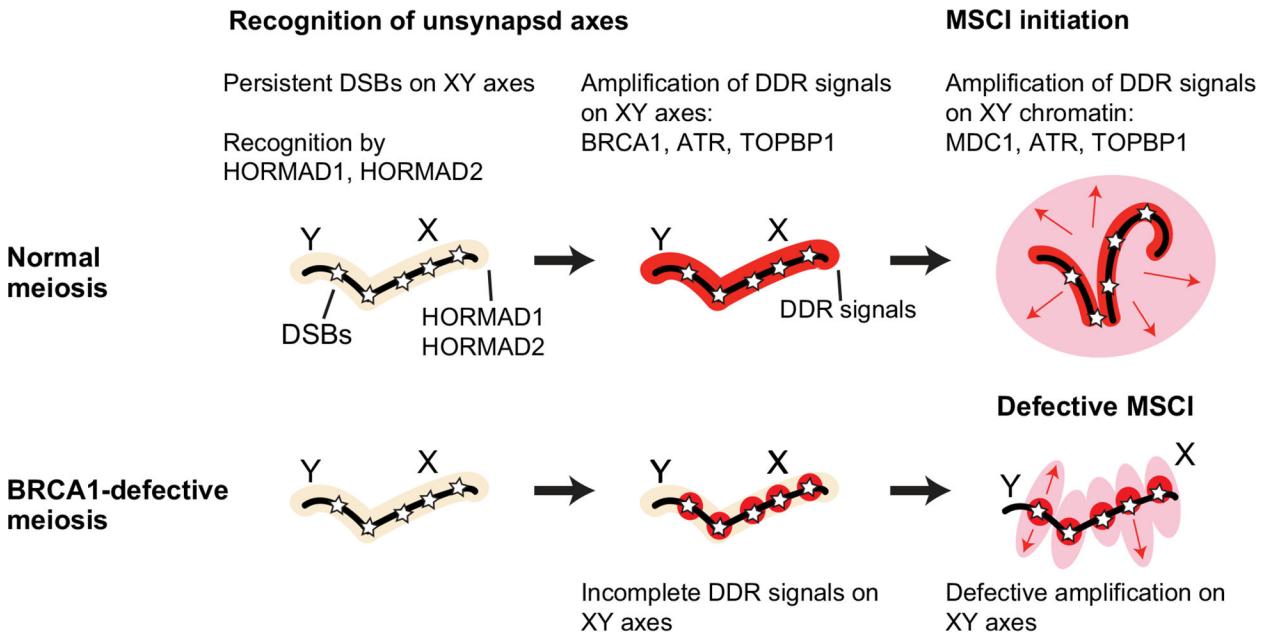
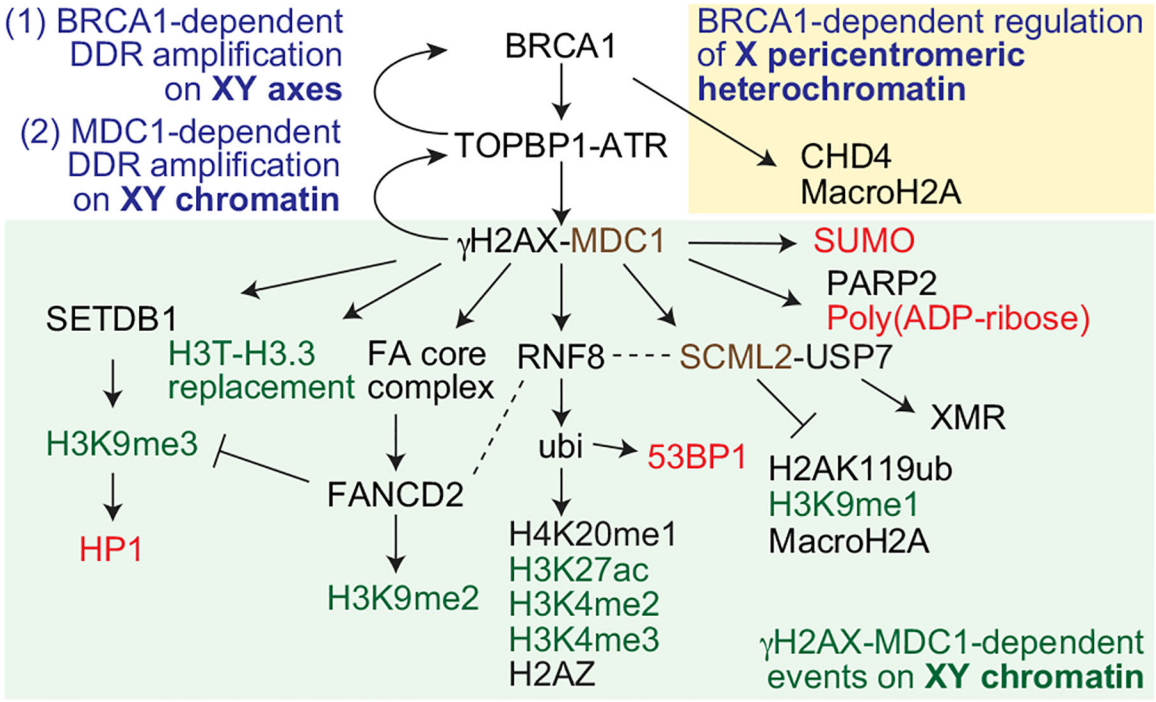


Fig. 2.

Depiction of the role of BRCA1 in meiosis. Model for BRCA1-dependent amplification of DDR signals along the XY axes followed by the initiation of MSCI



Reported key factors for phase separation in other contexts:

- SUMO
- Poly(ADP-ribose)
- 53BP1
- HP1

Possible regulators of phase separation on XY chromatin:

- MDC1
- SCML2

H3T-H3.3 replacement may impact the following histone H3 modifications:

- H3K9me1/2/3
- H3K4me2/3
- H3K27ac

---- Factors that influence each other

Fig. 3.

Model for molecular mechanisms in MSCI, including roles for DDR factors, other chromatin-associated proteins, and histone modifications. MSCI is initiated by BRCA1-dependent DDR amplification on the XY axes (1). BRCA1 has an additional function in the establishment of X pericentromeric heterochromatin (yellow box). MDC1-dependent DDR amplification subsequently takes place on XY chromatin (2; green box). Here, we focus on DDR signaling events, recruitment of chromatin factors such as HP1, and histone modifications that occur on XY chromatin. Since the formation of the XY body may involve phase separation, it should be noted that SUMO, Poly(ADP-ribose), 53BP1, and HP1 (shown in red), all of which are related to MSCI, and which are regulated downstream of MDC1, are reported to be key factors for phase separation in other contexts. MDC1 and

SCML2 (shown in brown) are possible regulators of phase separation on XY chromatin, as discussed in this review. H3T-H3.3 replacement may impact the following histone H3 modifications: H3K9me1/2/3, H3K4me2/3, and H3K27ac (shown in green). Dashed lines signify factors that influence each other. A key of color codes for various factors is shown at the bottom

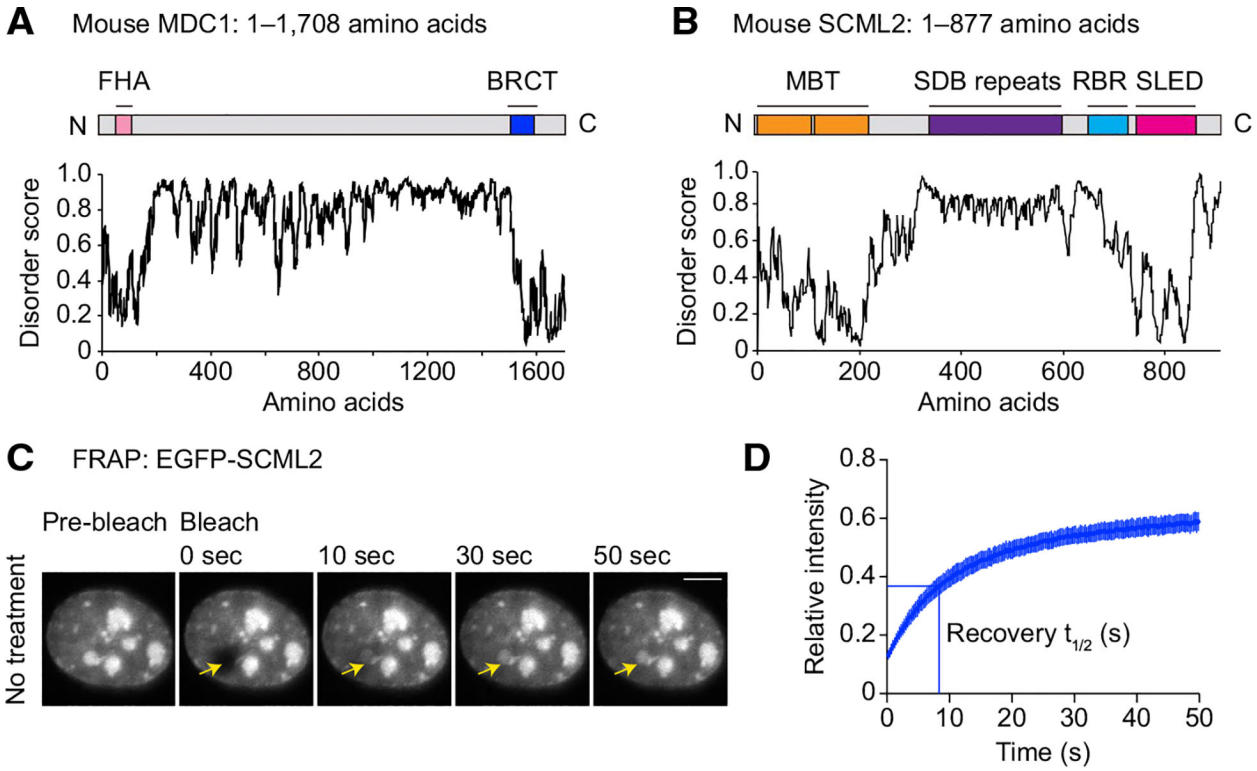


Fig. 4.

A phase separation hypothesis for the initiation of MSCI and for XY-body formation. **A**, **B** Predictions of protein disorder in MDC1 (**A**) and SCML2 (**B**; IUPRED score, Dosztányi et al., 2018 [161]). **C** Representative live images of FRAP assays in mK4 cells expressing EGFP-SCML2. Bars: 5 μm. The images are originally from Maezawa et al., 2018 [140]. Arrows show the site of photobleaching. **D** Relative intensity from FRAP assays of full-length SCML2. Recovery represents 50% of the plateaued intensity: $t_{1/2}$ (s). Error bars for FRAP curves and recovery $t_{1/2}$ represent 95% confidence intervals of the mean. The data are originally from Maezawa et al. 2018 [140]