



Xist drives spatial compartmentalization of DNA and protein to orchestrate initiation and maintenance of X inactivation

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Abstract

X chromosome inactivation (XCI) is the process whereby one of the X chromosomes in female mammalian cells is silenced to equalize X-linked gene expression with males. XCI depends on the long noncoding RNA Xist, which coats the inactive X chromosome in *cis* and triggers a cascade of events that ultimately lead to chromosome-wide transcriptional silencing that is stable for the lifetime of an organism. In recent years, the discovery of proteins that interact with Xist have led to new insights into how the initiation of XCI occurs. Nevertheless, there are still various unknowns about the mechanisms by which Xist orchestrates and maintains stable X-linked silencing. Here, we review recent work elucidating the role of Xist and its protein partners in mediating chromosome-wide transcriptional repression, as well as discuss a model by which Xist may compartmentalize proteins across the inactive X chromosome to enable both the initiation and maintenance of XCI.

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Overview

X chromosome inactivation (XCI) is a developmental process during which one of the two X chromosomes in female placental mammals is randomly silenced to ensure dosage balance in gene expression between males and females. XCI has emerged as a mechanistic

paradigm for studying epigenetics, gene regulation, three-dimensional (3D) nuclear structure, and long noncoding RNA (lncRNA) biology.

The Xist lncRNA orchestrates random XCI by spreading across one of the two X chromosomes [1,2] and recruiting dozens of chromatin-modifying proteins, DNA methylation enzymes, transcriptional repressors, and RNA-binding proteins (RBPs) to the future inactive X chromosome (Xi) [3–7]. During this process, Xist triggers large-scale remodeling of the Xi, including tethering the entire chromosome to the nuclear periphery [8–10], chromosome-wide compaction [11,12], loss of topologically associating domains [13], and establishment of a unique 3D silencing compartment [11,13–16]. Ultimately, Xist induces stable and heritable chromosome-wide transcriptional silencing that is maintained in an Xist-independent manner [17,18].

The mechanisms underlying the functions mediated by Xist have been at the center of intensive research efforts over the last several decades. In this review, we discuss recent mechanistic insights into how Xist initiates transcriptional silencing, spreads across the X chromosome, and establishes epigenetic maintenance of the silent state. We describe an emerging model for Xist RNA in driving spatial compartmentalization of various proteins on the Xi and the potential roles of this process in the initiation and maintenance of transcriptional silencing.

The Xist lncRNA orchestrates XCI

Xist was initially identified as the only gene that is expressed exclusively from the Xi [19,20]. Based on this unusual expression pattern, it was immediately suspected to be an essential regulator controlling XCI, a hypothesis that has since been confirmed by several lines of evidence, including: (i) deletion of Xist before random XCI disrupts initiation of XCI *in vivo* [21,22] and (ii) induction of Xist expression is sufficient to initiate XCI even in contexts where this process does not normally occur, such as in mouse embryonic stem cells, in male cells, or when it is expressed on an autosome [18,23].

Xist expression is required within a critical developmental period for proper establishment of XCI. During

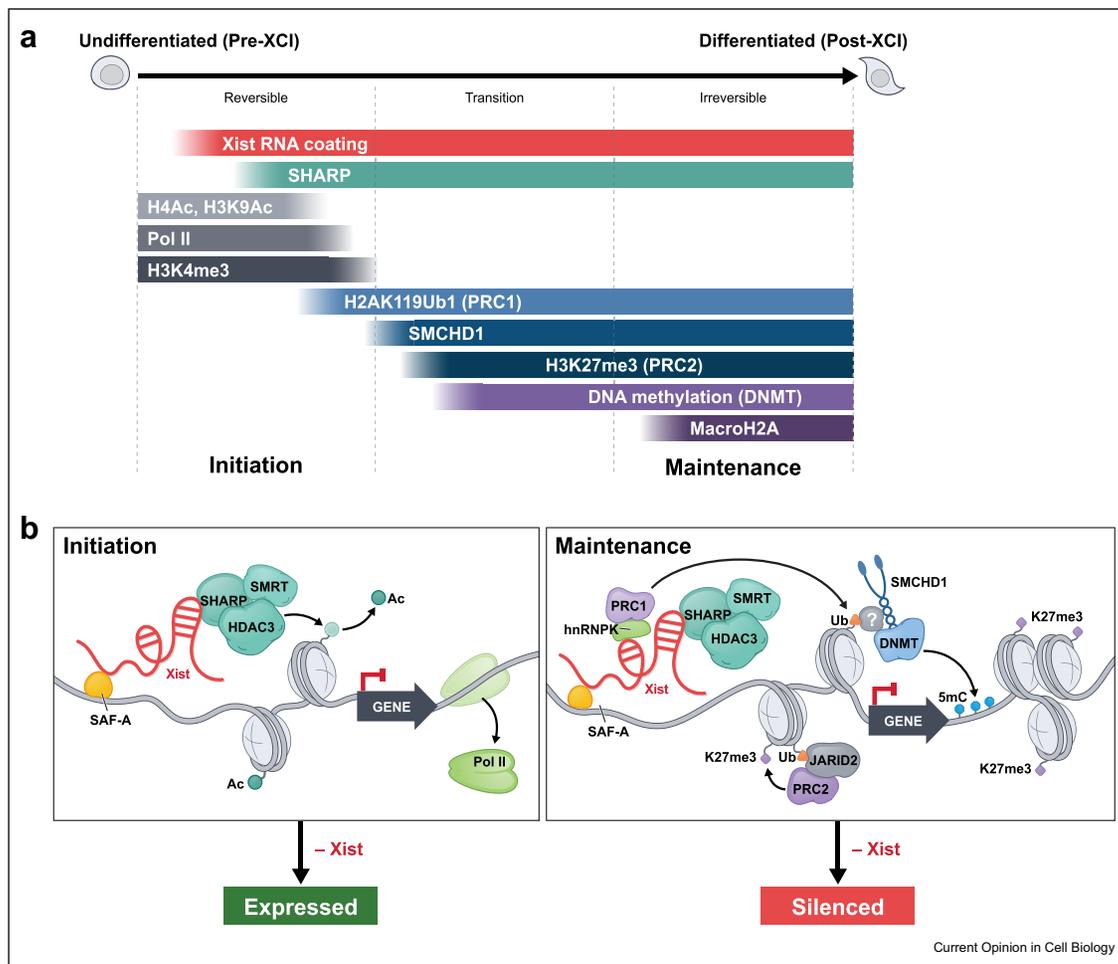
this window, removal of the Xist RNA leads to reversal of the silencing phenotype [18], yet following this initiation phase, the X chromosome becomes stably repressed and the loss of Xist expression does not lead to dramatic reactivation of X chromosome expression [17,18,24]. Accordingly, XCI can be divided into two stages—initiation of silencing and maintenance of silencing—each of which is associated with distinct chromatin modifications (Figure 1).

How Xist initiates transcriptional silencing on the X chromosome

To mediate XCI, Xist acts as a molecular scaffold to recruit proteins to the Xi. Recent studies have identified approximately a dozen RBPs that bind directly to Xist

[3,5] and interact with many more auxiliary proteins that are also involved in XCI [5,7]. Notably, among these RBPs is SHARP (SMRT/HDAC-associated repressor protein; also known as Spen) [3–6]—a critical repressive protein that binds to Xist and interacts with the SMRT co-repressor [25], which is known to activate the histone deacetylase activity of the HDAC3 complex [26]. HDAC3 requires interaction with the deacetylation activation domain of the NCoR/SMRT complex to initiate a conformational change that enables its catalytic activity [27]. Genetic knockout [28], perturbations [3], and drug treatment [29] have all shown that the histone deacetylase activity of HDAC3 is required for silencing on the Xi. In contrast, other HDACs, including other members of the class I HDAC family, appear to be

Figure 1



Protein recruitment and chromatin modifications during the initiation and maintenance of XCI. (a) X chromosome inactivation can be separated into two phases—initiation and maintenance. These stages correspond to precise timepoints during differentiation and have specific molecular characteristics, including chromatin modifications and structural changes on the Xi. (b) Xist recruits a variety of regulatory complexes to the Xi. Initiation (left): Xist is tethered to the X chromosome through SAF-A, a DNA and RNA-binding protein. Xist interacts with SHARP, which recruits SMRT and HDAC3, to evict RNA polymerase II (Pol II) and silence transcription. Removal of Xist during initiation leads to reactivation of gene expression from the Xi. Maintenance (right): Several architectural and chromatin modifying proteins are recruited to the Xi. For example, Xist interacts with hnRNP-K to recruit PRC1 and deposit H2AK119ub1, which in turn recruits PRC2 and its associated H3K27me3 histone modification. H2AK119ub1 is also required for recruiting SMCHD1 and DNMT enzymes through an unknown intermediate (indicated with a question mark). During this stage, Xist is dispensable for continued transcriptional silencing.

dispensable for this function [3,28]. Importantly, histone deacetylation has long been noted as one of the earliest chromatin modifications on the Xi [30,31]. Nevertheless, it remains unclear whether the interaction between Xist and SHARP directly recruits HDAC3 to the Xi or if the Xist–SHARP interaction activates HDAC3 that is already prebound to the X chromosome [3,28,32].

SHARP interacts with a highly conserved region at the 5' end of Xist called the A-repeat, a tandem repeat containing 7–8 copies of a GC-rich sequence [33]. This region forms a multivalent interaction with SHARP, such that a single copy of Xist binds to multiple SHARP proteins. Although the exact stoichiometry of the SHARP complex on a single molecule of Xist *in vivo* is unknown, recent biochemical studies have suggested that there might be four proteins bound per Xist molecule [34].

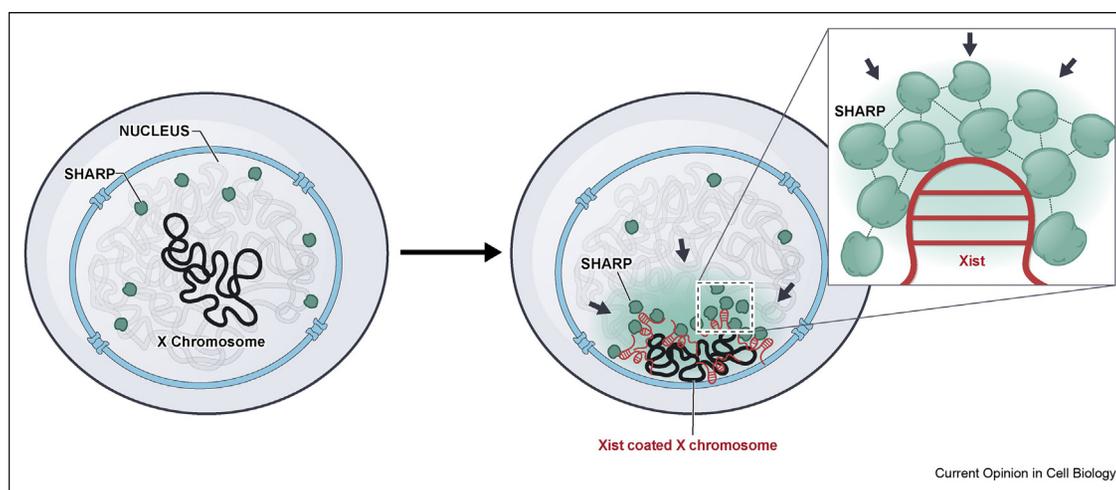
Several studies have shown that the interaction between Xist and the SHARP/SMRT/HDAC3 complex (Xist–SHARP) is required to evict RNA polymerase II from the Xi [3–6]. Nevertheless, this molecular interaction alone cannot explain how the Xist–SHARP complex leads to deterministic silencing of the entire X chromosome during mammalian development as its stoichiometry is not high enough to silence each gene individually. Specifically, there are ~200 copies of Xist within each cell [35] and the complex would need to localize across the >167 million base pairs of the X chromosome (~1 copy/megabase of DNA) to achieve complete silencing. One possible explanation is that the complex can sample multiple sites and activate

HDAC3 that is already present across the chromosome. Another possibility is that multivalent interactions formed between Xist and SHARP increase the spatial concentration of the silencing complex and allow it to act preferentially on the X chromosome, even when not directly bound to an Xist molecule. This process may be facilitated by cooperative interactions between the large intrinsically disordered regions (IDRs) contained within SHARP (Figure 2). Although a recent study showed that the Spen paralog and ortholog C-terminal (SPOC) domain of SHARP is sufficient to repress transcription when tethered to Xist, the level of silencing achieved by this domain alone is lower than that observed for full-length SHARP [32]. Moreover, we note that this SPOC domain is also responsible for recruiting SMRT to the Xi, and SMRT itself contains many IDRs, which may similarly promote the formation of a high concentration compartment.

How Xist and its silencing complex spread across the inactive chromosome

High-resolution studies mapping Xist localization over time in both inducible Xist systems and endogenous female differentiation have shown that Xist initially localizes to sites on the X chromosome that are in 3D spatial proximity to its transcription locus and are largely enriched for inactive genes [1,2,36]. Because these inactive regions tend to be abundant in L1 elements, there is also a correlation between early Xist spreading and L1 sites. Indeed, these L1 elements appear to be among the earliest silenced features on the X chromosome [11]. Nevertheless, L1 concentration does not appear to be required for initial spreading as Xist still

Figure 2



SHARP, and its associated SMRT/HDAC3 complex, are recruited to the Xi by direct binding of SHARP to the A-repeat of Xist. The repetitive nature of this region enables a single copy of Xist to bind to multiple copies of the SHARP protein. In this way, induction of Xist expression leads to dynamic assembly of a high local concentration territory of SHARP over the Xi. As a result, SHARP may be able to silence the entire X chromosome despite the Xist RNA being expressed at sub-stoichiometric levels relative to its target sites on the Xi. Such preferential recruitment of SHARP to the Xi may be reinforced by self-association of the IDRs contained within the SHARP protein.

accumulates at regions that do not possess L1 elements [1].

Several studies have identified an RNA and DNA-binding protein called SAF-A (scaffold attachment factor A; also known as hnRNPU) as a critical factor for tethering Xist to chromosomal DNA [37]. While SAF-A appears to be localized broadly across chromatin throughout the nucleus (rather than being enriched specifically on the Xi) [37], it may act to specifically localize Xist on the X chromosome by sequestering the RNA on chromatin close to its transcriptional locus through 3D diffusion. Recent studies have also shown that binding of the nuclear matrix protein CIZ1 (Cip1-interacting zinc finger protein 1) to the E-repeat of Xist is crucial for its proper localization to the Xi [38,39].

Since early Xist accumulation occurs at transcriptionally inactive regions, the initial Xi compartment that forms following Xist spreading is depleted of Pol II. Although actively transcribed genes initially appear to loop out of this region, over time they are relocated into the Xi compartment and undergo silencing [1,11]. Importantly, repositioning of active genes into this silencing compartment is dependent on the A-repeat region of Xist [1,11].

The A-repeat of Xist has also been implicated in positioning the Xi at the nuclear periphery through its interaction with Lamin-B receptor (LBR) [3,10]—a transmembrane protein that acts to anchor chromatin to

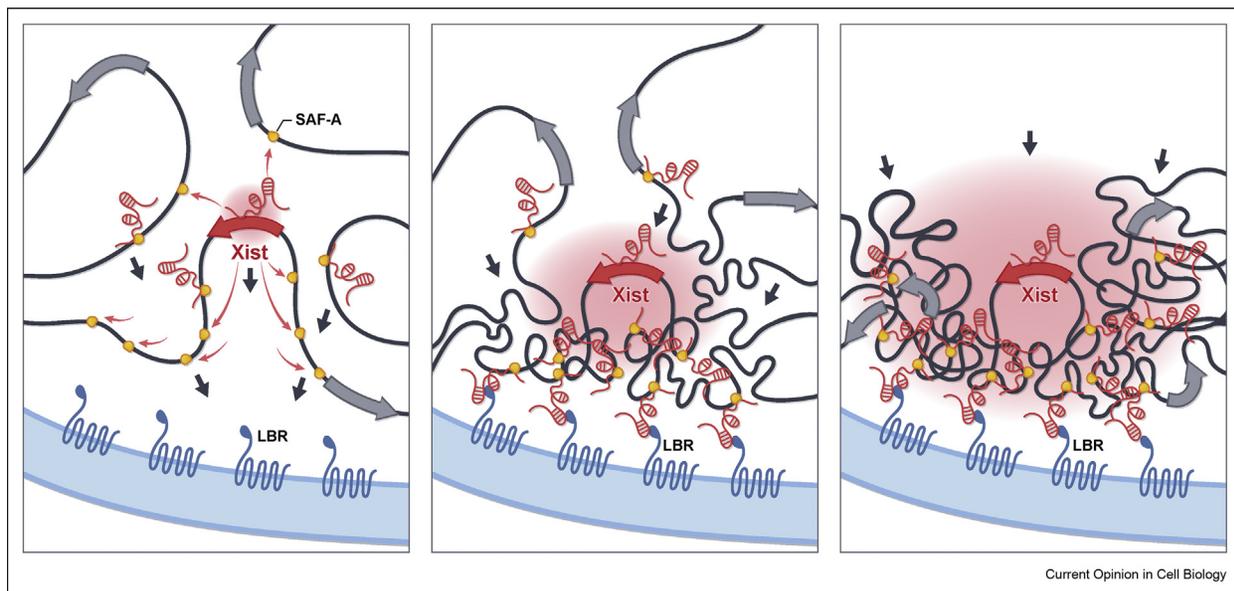
the nuclear lamina [40]. One of the earliest observations about the Xi was that it is tethered at the nuclear lamina [8,9]. However, it was unclear what, if any, functional significance this association might play until it was recently shown that the deletion of LBR disrupts Xi association with the nuclear lamina, and by doing so precludes Xist spreading to active genes across the X chromosome [10]. Together, these observations suggest a model for how Xist localizes specifically on the future Xi and spreads across the chromosome over time (Figure 3).

How Xist establishes an epigenetically heritable state that maintains inactivation

Xist triggers a cascade of histone modifications and DNA methylation on the Xi that are deposited throughout the process of XCI. For example, Polycomb repressive complex 1 (PRC1) is recruited to the Xi through a direct interaction between Xist and hnRNPK, which interacts with the noncanonical Pcgf3/5 components of the PRC1 complex [41]. PRC1 deposits H2A ubiquitylation marks (H2AK119ub1) across the Xi, leading to subsequent recruitment of the PRC2 complex and its associated H3K27me3 modifications [42].

The PRC1 complex has been implicated as a key regulator of XCI because Pcgf3/5 loss of function leads to a significant reduction in X-linked silencing [43]. Pcgf3/5 may contribute to maintenance of silencing because Pcgf3/5 knockout embryos show lethality in females at mid-gestation stages—several days after initiation of

Figure 3



Xist spreads across the Xi by initially diffusing to DNA sites that are in close 3D proximity to its transcription locus (red arrows), where the RNA is sequestered by binding to SAF-A on chromatin (left). Xist-bound DNA regions are repositioned to the nuclear lamina by binding to the Lamin-B receptor (LBR, black arrows), leading to structural changes on DNA that enable other sites to come into close proximity to the Xist transcriptional locus (middle). This alters the accessibility of DNA such that Xist is able to spread across the entire chromosome in later stages of XCI (right).

XCI has already occurred [42**]. Moreover, it has been shown that deletion of other components of the PRC1 complex do not impact proper initiation of silencing on the X chromosome [44,45].

PRC1 might play a role in maintenance of XCI, at least in part through its ability to recruit SMCHD1 to the X chromosome. Specifically, PRC1-mediated deposition of H2AK119ub1 has been shown to be critical for recruiting SMCHD1—a cohesin family protein recently shown to be essential for mediating structural changes on the Xi [14,15]. Independently, mutations in SMCHD1 have been shown to cause defective X-linked silencing and female embryonic lethality at mid-gestation [46,47]. Loss of SMCHD1 also reduces DNA methylation levels across the X chromosome [48]. Nevertheless, it remains unclear the full extent of how PRC1 might contribute to X-linked silencing mechanisms and whether SMCHD1 mediates its functional role in XCI through structural changes, recruitment of DNA methylation, or other possible mechanisms.

DNA methylation (methylation of cytosines within CpG dinucleotides) has long been suspected to play a key role in the maintenance of XCI [49] as it is generally associated with transcriptional repression, especially when clustered within promoter regions, and is known to be recognized by several silencing proteins [50]. DNA methylation is deposited by *de novo* methyltransferases (DNMT3A/B) and propagated after cell division through a maintenance methyltransferase (DNMT1) [50]. In this way, DNA methylation is maintained across the cell cycle through a self-reinforcing epigenetic mechanism [50].

The proposed role for DNA methylation in maintenance of XCI is based on three key lines of evidence. First, DNA methylation is known to be enriched on the Xi [51,52] and is specifically deposited on the Xi in an Xist-dependent manner by the DNMT3B *de novo* methyltransferase [48]. Second, genetic and pharmacological disruptions of DNA methylation in somatic cells have shown that specific genes on the X chromosome can undergo variable levels of reactivation from the Xi [29,53–56]. Finally, the mechanism of propagation of DNA methylation through cell division provides a simple epigenetic memory mechanism that could explain how silencing is propagated in an Xist-independent manner [50].

Still, there are several lines of evidence that raise doubts about whether DNA methylation is the only component required for maintenance of XCI. Treatment of various human somatic cell lines with drugs that lead to demethylation (e.g. 5-azacytidine) does not cause reactivation of most genes on the Xi [17,57]. This is true even when coupled with genetic deletion of Xist [17]. Furthermore, genetic deletion of DNMT3A/B does not

impair the propagation of the silenced state in mouse embryos [58], and human patients with immunodeficiency, centromeric region instability or facial anomalies (ICF) syndrome (caused by a loss of function mutation in both copies of the DNMT3B gene) maintain normal XCI despite global hypomethylation across the Xi [59]. Finally, the transition to the maintenance state of XCI appears to occur before establishment of DNA methylation [48]. Together these results indicate that other factors are likely to be important for maintaining transcriptional silencing on the Xi.

A possible role for protein compartmentalization and phase separation in the maintenance of XCI

Recent studies have shown that proteins involved in many critical transcriptional processes, including chromatin regulation and transcription, undergo liquid-liquid phase separation (LLPS) [60]. These observations have led to a model whereby LLPS in the nucleus could explain the dynamic formation of membrane-less compartments that coordinately regulate different aspects of gene expression. A central tenant of this model is that cooperative interactions between high local concentrations of nucleic acids and proteins—particularly those with IDRs—can lead to the formation of phase-separated bodies that act to compartmentalize molecular components and biochemical functions in cells [60]. For example, many RBPs contain low-complexity domains that facilitate self-aggregation, and in several cases, RNA has been shown to facilitate this phase separation process by increasing local concentrations of such RBPs [61,62] or by directly participating in heterotypic multivalent interactions through RNA sequence repeats [63].

Several observations suggest that such concentration-dependent phase transition may lead to compartmentalization of silencing components on the Xi. (i) Many of the RBPs that interact with Xist are known to contain IDRs (e.g. Ptbp1, SAF-A, SHARP) [64], and several of these have been shown to individually undergo RNA-mediated phase-separation *in vitro* [65–67]. (ii) Xist contains repetitive sequences that are known to form direct multivalent interactions with these RBPs [34,68], suggesting a molecular mechanism for how Xist can initiate compartmentalization and phase transitions *in vivo*. (iii) Molecules within the inactive compartment diffuse rapidly, with properties that are consistent with diffusion of liquids [69]. (iv) The Xist-coated nuclear compartment appears to be stable even on removal of DNA, suggesting that it consists of RNA-protein interactions [70]. (v) There is a critical developmental time point at which Xist-mediated silencing transitions from a reversible to an irreversible process [18]. Such sharp transitions are a physical characteristic of molecular phase-transition events [71].

In such a model, induction of Xist expression leads to high concentration of the RNA over the Xi. The various RBPs that interact with Xist then preferentially localize within this compartment to achieve a high local concentration. Upon achieving high local concentration, these RBPs can interact with each other to undergo LLPS and, in this way, localization of various silencing proteins could be stabilized even in the absence of the Xist RNA (Figure 4). This model could explain why Xist is essential for initiating transcriptional repression, but is dispensable for maintaining X-linked silencing. Consistent with this idea, a recent study has shown that deletion of Ptbp1 (or its multivalent binding site on Xist)—a protein that is known to undergo phase separation *in vitro* [62,72]—does not disrupt initiation of XCI, but impacts compartment formation and maintenance of silencing on the Xi [73,74].

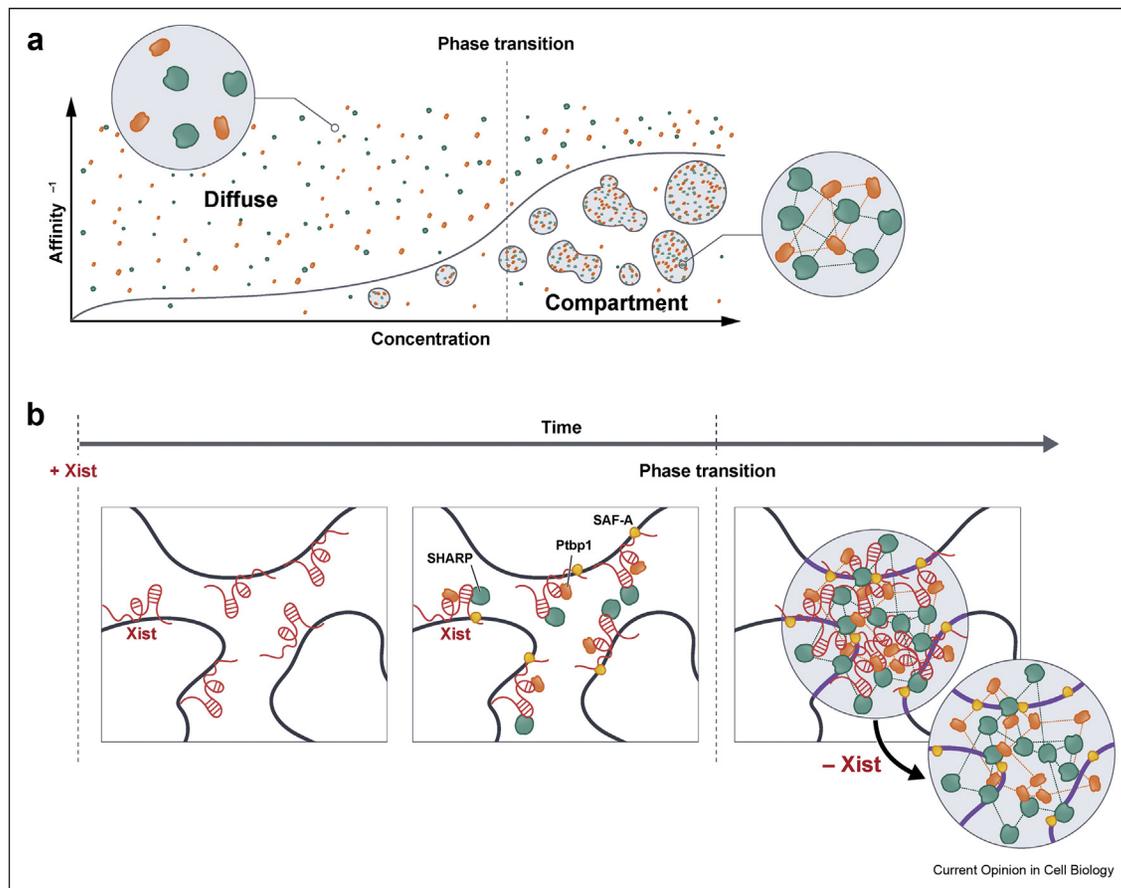
For this compartment to play a role in maintenance of silencing, it must be propagated across cell divisions.

Although it remains unknown how this might occur, we note that various histone modifications and DNA methylation are stably associated with the Xi through cell division [50,75,76] and that these modifications may act to seed re-establishment of the silencing compartment following mitosis. In addition, studies of transcription factors have shown that certain proteins can be retained on mitotic chromosomes to mark specific nuclear locations (referred to as ‘mitotic bookmarking’) [77–79]. A mitotic bookmarking mechanism might similarly act to retain one or more of the proteins involved in XCI—or the phase-separated compartment itself—on the Xi across cell divisions.

Concluding remarks

The recent identification of proteins that interact with Xist has provided a framework for dissecting many of the molecular mechanisms of Xist function and XCI in greater detail. Nevertheless, there are still many open questions that need to be addressed. For example, it

Figure 4



A liquid-liquid phase separation (LLPS) model of XCI. (a) Molecules can transition from diffuse to compartmentalized based on the local concentration and affinity of the molecular components in 3D space. In this model, small changes in concentration can cause phase transitions that promote self-reinforcement of molecular components. (b) Such an LLPS model may explain many of the observed properties during the maintenance of XCI. In this model, Xist recruits a number of intrinsically disordered proteins to the Xi, including SHARP and Ptbp1, that interact and lead to a high spatial concentration of silencing complexes. After a phase transition event, these proteins can form self-reinforcing interactions with each other, thereby enabling protein enrichment within the spatial compartment on the Xi independently of the Xist RNA. In this way, transcriptional silencing may be maintained in the absence of Xist.

remains unclear what molecular components are required for maintenance of XCI and how this process occurs. Answering these will not only fill a fundamental gap in our understanding of XCI, but it may also provide new insights into broader aspects of epigenetic silencing beyond the X chromosome. Furthermore, although the proposed model of LLPS on the Xi is an intriguing hypothesis for the mechanism underlying formation and maintenance of the silent state, this hypothesis remains to be functionally tested. We expect that new molecular and cell biology tools will enable exploration of this hypothesis and provide other critical insights into this essential developmental process.

Conflict of interest statement

Nothing declared.

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- ** of outstanding interest

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