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A lifelong duty: how *Xist* maintains the inactive X chromosome

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Abstract

Female eutherians transcriptionally silence one X chromosome to balance gene dosage between the sexes. X chromosome inactivation (XCI) is initiated by the lncRNA *Xist,* which assembles many proteins within the inactive X chromosome (Xi) to trigger gene silencing and heterochromatin formation. It is well established that gene silencing on the Xi is maintained through repressive epigenetic processes, including histone deacetylation and DNA methylation. Recent studies revealed a new mechanism where RNA-binding proteins that interact directly with the RNA contribute to the maintenance of *Xist* localization and gene silencing. In addition, a surprising plasticity of the Xi was uncovered with many genes becoming upregulated upon experimental deletion of *Xist*. Intriguingly, immune cells normally lose *Xist* from the Xi suggesting that this *Xist*-dependence is utilized *in vivo* to dynamically regulate gene expression from the Xi. These new studies expose fundamental regulatory mechanisms for the chromatin association of RNAs, highlight the need for studying the maintenance of XCI and *Xist* localization in a gene- and cell-type-specific manner, and are likely to have clinical impact.

Keywords

Xist; lncRNA; X-inactivation; PTBP1; Xi reactivation

Introduction

X-chromosome inactivation (XCI) is established in all cells early in embryonic development and is maintained for the whole lifespan of an individual [1-7]. To trigger the formation of the inactive X chromosome (Xi) in the embryo, the long noncoding RNA (lncRNA) *Xist* becomes expressed on either the maternally or paternally inherited X chromosome

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Author's Note

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Declaration of Interest

The authors declare no conflict of interest.

[1,3-7]. Without *Xist*, XCI cannot be initiated, and the increased expression of X-linked genes causes early embryonic lethality in mice [8-10]. Similarly, dysregulation of XCI in female human pluripotent stem cells (hPSCs) correlates with an altered differentiation potential [11-13]. Recent work in hPSCs showed that the absence of XCI severely alters the transcriptome and proteome across the entire genome, due to increased expression of X-linked transcription factors and regulators of translation [14,15]. Although the importance of XCI during development is well established, it is less clear how critical the process is for adult homeostasis. Addressing this challenge is not trivial as the Xi in somatic cells is not only controlled by *Xist* and its interacting proteins but also by multiple repressive chromatin layers that are difficult to disassemble, leading to the belief that the Xi is extremely stable throughout life and that *Xist* may not be critical for Xi maintenance [16,17]. However, recent studies have revealed that not all genes on the Xi are controlled by the same epigenetic layers, such that some are more malleable than previously thought and become upregulated upon loss of *Xist* [18-22]. Intriguingly, this plasticity particularly affects genes that normally escape complete silencing on the Xi [22,23]. Moreover, *Xist*, long thought to coat the Xi in all differentiated cells *in vivo*, loses its localization to the Xi in specific cell types of the immune system [20,21,24], opening the door to the tuning of X-linked gene expression. These new findings have critical implications for disease susceptibility as well as the development of new treatment strategies of X-linked disorders, which underscores the importance of understanding Xi maintenance.

In this review, after briefly considering the regulation of XCI initiation, we discuss how gene silencing on the Xi is maintained. We will focus on the recent discovery of a new maintenance mechanism that involves the RNA-binding proteins Polypyrimidine Tract Binding Protein 1 PTBP1, Matrin 3 MATR3, CUGBP Elav-like family member 1 CELF1, and TAR DNA-binding protein 43 TDP-43, which are typically known for their role in RNA processing, but are now shown to play a general role in controlling RNA localization and function in the nucleus. We then introduce the emerging principle that *Xist* is not only critical for the initiation of XCI but also for the maintenance of silencing for many genes on the Xi, and discuss how the *Xist*-dependence is exploited *in vivo*.

Initiation of X-chromosome inactivation by *Xist*

To achieve XCI, *Xist* recruits diverse proteins, including RNA-binding proteins, transcriptional repressors, and architectural and heterochromatin proteins (Figure 1a). These factors ensure that the RNA stays localized on the X chromosome it is transcribed from, induces gene silencing, and alters the chromatin environment and the three-dimensional organization of the X chromosome to form the Xi compartment, also known as the Barr body [25-36]. Most of the *Xist*-interacting proteins are recruited through one of the six repeat arrays in the RNA termed A – F [1,3-7,28] (Figure 1a), which are conserved across eutherians but can differ in copy number between species [37,38]. For instance, mouse *Xist* has a C-repeat expansion and the B-repeat in human *XIST* is split into two elements (B and Bh) [38]. The A repeat interacts with the transcriptional repressor SPEN (also called SHARP (silencing mediator for retinoid or thyroid-hormone receptors SMRT/histone deacetylase 1 HDAC1 Associated Repressor Protein)), which is critical for the silencing of virtually all genes on the Xi [25,26,30,36] (Figure 1a). The B and C

repeats bind hnRNP-K, which recruits the polycomb repressive complexes (PRC) 1 and 2 that deposit the histone marks H3K27me3 and H2AK119Ub1 [32,39], as well as the architectural chromatin protein Structural Maintenance of Chromosomes flexible Hinge Domain Containing 1 SMCHD1 [27,40] (Figure 1a), to regulate chromatin reconfiguration and compaction [29,35]. After gene silencing has occurred, DNA methylation is established at CpG islands of many genes on the Xi through SMCHD1-dependent and independent action of the DNA methyltransferase DNMT3B [41,42]. DNA methylation in particular has long been recognized as a key factor in XCI maintenance [16]. While most genes on the Xi are completely silenced, 3-7% of mouse and 20-30% of human X-linked genes remain partially expressed, lack the repressive epigenetic marks described above, and are referred to as escape genes [23].

A recently identified aspect of XCI initiation is that *Xist* distributes to only approximately 50 sites along the Xi [29,43-45]. At each of these 50 sites, two RNA molecules are tethered to chromatin with high affinity, locally confining their movement [29,34]. Where exactly the pairs of *Xist* molecules bind on chromatin is currently not defined. Specific *Xist*-interacting proteins such as the RNA and DNA binding protein SAF-A (also termed hnRNP-U) are involved in anchoring *Xist* on chromatin [46-50], whereas others are critical for the coupling of the two *Xist* molecules [34]. At each *Xist* hub, many protein molecules are recruited via RNA-protein and extensive protein-protein interactions to form supramolecular protein complexes (SMACs) [29] (Figure 1b). In contrast to *Xist* molecules, which persist for minutes to hours at these sites [29,34], most protein components of SMACs exhibit very short residence times in the range of seconds [29]. Therefore, SMACs are highly dynamic structures that allow most constituent proteins to rapidly bind and dissociate, which creates a local concentration gradient of these proteins around each *Xist* hub [29] (Figure 1b). Mechanistically, this is achieved by fleeting, low-affinity interactions between disordered regions found in *Xist*-interacting proteins as shown by the deletion of the intrinsically disordered domain of SPEN [29]. As a result, it has been posited that “free” SPEN proteins silence genes across the X without requiring continuous association with the RNA [29]. Importantly, the SMAC model of *Xist* action may provide a general mechanism for lncRNAs and other RNA species to achieve large regulatory effects even if they are very lowly expressed, via concentrating a highly dynamic pool of proteins [29].

A requirement for PTBP1, MATR3, CELF1, and TDP-43 for XCI maintenance

Experimental manipulations have identified a developmental switch where *Xist*-mediated silencing rapidly transitions from a fully Xist-dependent and reversible process to a largely *Xist*-independent and irreversible process [17]. Surprisingly, this switch occurs before the deposition of DNA methylation at CpG islands of genes on the Xi [41]. Consequently, additional mechanisms must contribute to the maintenance of gene silencing in the absence of *Xist*. Recent work has shed light on one such mechanism [31]. It was found that the proteins PTBP1, MATR3, TDP-43, and CELF1 proteins, which normally function in RNA processing, bind to the E-repeat of *Xist* [31] (Figures 1a, 2a). Since this sequence harbors a large number of putative binding sites for each of these proteins, many protein copies likely bind each *Xist* molecule [31]. The deletion of the E-repeat does not disrupt the initial *Xist* spread across the X or the initiation of gene silencing. Instead, the maintenance of gene

silencing and *Xist* localization is impacted [31] (Figure 2a). These defects develop when XCI normally transitions to the *Xist*-independent stage, that is, when the experimentally induced loss of *Xist* from the Xi should have only minor consequences. Thus, E-repeat-dependent proteins shape the Xi-compartment in a way that enables it to maintain an epigenetic memory of gene silencing independently of *Xist*. This requires self-association of and interactions between these proteins, and is independent of their role in RNA processing [31] (Figure 2a). Together, these findings suggest that the composition of the Xi-compartment changes over time and that these changes are essential for the developmental switch to *Xist*-independence. Consistent with this idea, SPEN and CELF1 levels have been shown to increase over time in the Xi or *Xist*-SMACs [29,31], and proteomics studies have uncovered changes in the *Xist*-interactome with differentiation [22,25].

The mechanism of action of the XCI memory proteins described above remains unclear and will be an exciting problem to tackle in the future. One observation has been that CELF1 can remain enriched on the Xi in a PTBP1, MATR3, and TDP-43-dependent manner after *Xist* is deleted from the Xi in differentiated cells [31]. The CELF1 Xi enrichment upon *Xist* deletion appears to be stable only in a subset of cells and only for a short time (maybe for a few cell divisions) [31], suggesting that *Xist* is normally continuously needed to reinforce the protein interactions in the Xi-compartment. Regardless, it will be interesting to explore if the silencing protein SPEN can remain transiently localized together with the memory proteins in the Xi-compartment after *Xist* depletion in differentiated cells. This could explain the long-standing observation that silencing is maintained in the absence of *Xist* before the establishment of DNA methylation early in differentiation [17]. Intriguingly, in addition to the silencing protein SPEN, PTBP1 and MATR3 have been identified as essential for maintaining the repression of a candidate gene on the Xi in adult human cells, supporting a critical role of these E-repeat binding proteins in the maintenance of XCI [22] (Figure 3a). Intriguingly, another E-repeat binding protein, Cip1-interacting zinc finger protein CIZ1 (Figure 1a), is also important for *Xist* localization [34], However, it does not appear to function together with PTBP1, MATR3, TDP-43, and CELF1 in maintaining gene silencing [22,31] (Figure 3a).

Overall, these studies identified an unanticipated role for a group of well-studied RNA-binding proteins in tethering RNAs to chromatin and controlling nuclear compartment functions, which does not require their RNA processing functions [31]. Excitingly, this emerging function of these proteins may not be limited to *Xist* (Box 1) (Figures 2b, c).

Xist's contribution to the maintenance of the inactive X chromosome

Classic experiments that focused on a few X-linked genes suggested that *Xist* is no longer required in somatic cells [16,17,51]. Interference with DNA methylation in *Xist*-deleted cells partially upregulated genes on the Xi, particularly when combined with histone deacetylase inhibition, consistent with the redundancy of Xi maintenance through various epigenetic layers [16]. Consequently, a common feature of Xi reactivation studies is that multiple inhibitors are combined [52-54]. However, an exciting recent study reassessed the role of *XIST* in the adult human B cell line GM12878 by applying various genomics approaches to define chromatin modifications and expression state across the Xi [22].

The authors found that *XIST* is critical for maintaining the silencing of a surprisingly large number of X-linked genes, as a quarter of genes on the Xi were upregulated upon experimental deletion of the RNA [22] (Figure 3b). These *XIST*-dependent genes carry lower methylation levels at their promoters on the normal Xi than genes resistant to *XIST* deletion [22] (Figure 3b). Genes known to escape XCI are enriched among these *XIST*-dependent genes [22]. Escape genes are normally expressed from the Xi, but often at lower levels than on the active X chromosome (Xa) [23,55]. Accordingly, escape genes have an active epigenetic state, unlike silenced genes on the Xi [56-59], which is consistent with them requiring continuous silencing from *XIST* [22]. The silencing environment created by *XIST* likely is explained by constant recruitment of the transcriptional repressor SPEN, as it has been shown to be required for the dampening of escape genes on the Xi [22,26] (Figure 3a). Since escape from XCI is more prevalent in humans than mice [60], genes on the human Xi may have a greater reliance on *XIST* than in mice. Nevertheless, recent studies using conditional *Xist* knockout approaches in mice have revealed a limited re-establishment of expression from the Xi [54,61,62]. Interestingly, the tissue-specific deletion of *Xist* in mice is typically well tolerated except for two tissues where induced *Xist* loss has dramatic consequences. These include blood, where loss of *Xist* in hematopoietic stem cells is oncogenic, and the gut, where *Xist* loss increases tumor burden upon exposure to chronic stress [61,62]. It is currently unknown why there are variable consequences of *Xist* deletion across different tissues, but tissue-specific escape from XCI may provide an explanation [61]. Additionally, recent studies suggest that gene repression on the Xi is balanced by upregulation of genes on the Xa in a tunable manner, via a process known as X chromosome upregulation [63]. Therefore, it remains possible that the upregulation of genes on the Xi results in dampening of their counterparts on the Xa, which could make most cell types robust to the consequences of XCI defects [63].

A link between escape genes and dependence on *XIST* may also exist in hPSCs. Normally, hPSCs carry an Xi with its classic epigenetic hallmarks, yet in most cell lines *XIST* eventually becomes repressed by *de novo* DNA methyltransferases [64]. *XIST* loss is typically followed by the re-expression of a subset of previously silenced genes on the Xi and loss of CpG island methylation at the affected genes [12,14,65]. This Xi-erosion occurs most often close to XCI escapees [65]. Thus, the degradation of the Xi-compartment appears to spread from genes already evading multiple layers of XCI repression, which are now known to be dynamically regulated by *XIST*. Intriguingly, genes proximal to escapees are also the earliest to become re-expressed during the reactivation of the Xi during mouse induced pluripotent stem cell iPSC reprogramming [66,67]. Since boundary elements surrounding escape genes are required to prevent activation of adjacent silenced genes [68], the erosion of boundaries could explain this consistent order of reactivation.

Xist localization is disrupted in immune cells

The aforementioned examples of *XIST*/*Xist* loss are either experimentally induced or represent culture-induced abnormalities. Although it was long thought that coating of the Xi by *Xist* is maintained in all somatic cells, recent studies have described an unusual distribution of the RNA in human and mouse quiescent lymphocytes, where it is dispersed throughout the interphase nucleus instead of being localized to the Xi [20,21,69] (Figure

3c). The mechanism underlying the redistribution of *XIST*/*Xist* in lymphocytes is currently unclear. However, *XIST*/*Xist* re-localizes to the Xi upon stimulation of lymphocytes and re-entry into the cell cycle (Figure 3c), requiring the RNA-interacting proteins Yin Yang 1 YY1 and SAF-A [20,21,69].

Curiously, in dividing cells, *XIST*/*Xist* is normally released from the Xi and disperses across the nucleocytoplasm in mitosis [70]. SAF-A is one of the proteins implicated in controlling the localization of the RNA in this process [47,48,50,71]. For instance, a recent paper demonstrated that the mitotic dispersal of *XIST* can be prevented by the inhibition of the cell cycle regulator aurora B kinase [48], which phosphorylates SAF-A to release the protein, together with *XIST*, from mitotic chromosomes [48] (Figure 3d). Although the question of how exactly *XIST* is anchored on chromatin is complex and might be highly cell type-specific [46-50,71], similar regulatory mechanisms may be at play to control SAF-A and/or other *XIST*/*Xist* chromatin anchors in quiescent lymphocytes to modify the localization of the RNA.

The deregulation of *XIST*/*Xist* localization in lymphocytes is particularly intriguing, given the recently discovered *XIST*-dependence of some genes on the Xi [22]. Indeed, a comparison of female and male lymphocytes showed that the dispersion of *XIST*/*Xist* is accompanied by an increase in the expression of X-linked immune regulators in female cells [21]. Allelic studies are required to systematically define which genes on the Xi become derepressed upon loss of the RNA from the Xi.

Together these findings show that *Xist* localization can be dynamically regulated in normal cells and that the cell type-specific dispersion of *Xist* results in XCI deregulation, which in lymphocytes increases the expression of X-linked immune regulators. Thus, *Xist*-dependence of some genes combined with the control of *Xist* localization on the Xi provides a mechanism to achieve the cell-type specific regulation of a subset of X-linked genes that would not be achievable if they were regulated via multiple repressive chromatin mechanisms.

Consequences of partial X chromosome reactivation due to *Xist* loss

It is well established that females mount stronger immune responses than males, which results in faster clearance of pathogens but also in increased susceptibility to autoimmune diseases [72]. This difference has been linked to XCI escape in females [72], and the aforementioned studies [20-22,69] suggest that the deregulation of *XIST* in lymphocytes potentiates the expression of escapees in females. These new studies of normally occurring dysregulation of XCI maintenance indeed support a link to the female-specific development of autoimmune diseases. For instance, it is well established that elevated levels of the X-linked escapee *TLR7* enhance the formation of CD11c+ atypical B cells (ABCs), a cell type that greatly expands in a female-specific manner with age and in patients with autoimmune diseases [22]. ABCs lack proper *XIST* localization and express elevated levels of X-linked genes in female patients with autoimmune diseases, including systemic lupus erythematosus (SLE) [18-20,22] (Figure 3c). Intriguingly, there is also a causal link between *XIST* deregulation and cell fate specification, because the differentiation of stimulated naïve

B cells into ABCs is enhanced when *XIST* is experimentally deleted in cultured cells [22]. Together, these findings open the door for a better understanding of the sexual dimorphism of human diseases, which might lead to new treatment opportunities, and suggest that the dispersal of *XIST* could be a useful biomarker for detecting autoimmune disorders. The latter idea is particularly important for diseases such as SLE, where diagnosis is often extensively delayed, resulting in worse patient outcomes [73].

The finding that the Xi is more plastic than previously thought may have implications for approaches that aim to reactivate the Xi to present a cure for heterozygous X-linked diseases such as the neurodevelopmental disorder Rett syndrome (RTT) caused by mutations in the X-linked gene methyl CpG binding protein 2 *MECP2* [74]. When a deleterious copy of the X-linked gene *MECP2* is expressed from the active X chromosome in RTT patients, reactivating the wildtype copy that is silenced on the Xi may rescue disease phenotypes [74]. Strengthening this idea, it was found that the induction of *Mecp2* expression in mutant mice after the onset of symptoms can reverse disease symptoms [75]. Therefore, several studies have attempted to disrupt the Xi in mice, typically via deletion of *Xist* and/or inhibition of DNA methylation, and assess the activation of genes on the Xi as well as the physiological consequences [54,61,62]. Since escape from XCI (and therefore likely *XIST*-dependence) is more predominant in human than in mouse cells [23,55], similar studies need to be performed in human models of these X-linked diseases [76] to assess if Xi reactivation processes are well-tolerated in human tissues. The targeted demethylation and reactivation of specific genes on the Xi through programmable epigenetic editing represents an alternative strategy [77].

Conclusion

The study of XCI continues to provide fundamental insights into RNA biology, gene regulation and nuclear organization and is beginning to yield a better understanding of human diseases. Accordingly, elucidating the mechanisms that underlie the delocalization of *XIST* in lymphocytes and other immune cell types [20,24,69] may pave the way for the development of therapeutic approaches that prevent the loss of *XIST* from the Xi and upregulation of *XIST*-dependent genes. It is intriguing that *XIST* is also more dispersed in human pre-implantation embryos [78] and germ cells [79] where X chromosome dampening occurs [79,80], indicating that the modulation of *XIST* localization is exploited at different developmental stages to achieve a cell-type specific output of X-linked genes. In general, a deeper understanding of escape from the action of *Xist*-SMACs is an important focus, particularly as these genes appear to contribute disproportionately to sex-biased disorders [81]. Finally, the recent insights into the function of MATR3, PTBP1, SAF-A, CELF1 and TDP-43 in the control of RNA localization and chromatin organization suggest that further studies of these proteins will reveal tremendous insights into the extended molecular structures that surround genes and how RNA-protein interactions segregate nuclear compartments and ultimately gene function.

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with each other to form a protein assembly that is dispensable for XCI initiation, but necessary for XCI maintenance.

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Box 1:

A broader role for PTBP1 and MATR3 in the control of RNA localization, chromatin organization, and nuclear compartmentalization.

Xist studies have revealed numerous mechanisms that are also used by other RNAs. This now also extends to the PTBP1, MATR3, TDP-43 or CELF1-dependent control of RNA localization on chromatin. Various recent studies suggest that these E-repeat binding proteins, in particular PTBP1, mediate the chromatin localization of diverse RNAs such as Line L1 retrotransposon transcripts, lncRNAs and pre-mRNAs. Specifically, a recent publication showed that PTBP1, MATR3, TDP-43 and homologs of CELF1 densely bind antisense L1 sequences contained in mRNAs [82]. lncRNAs of the asynchronous replication and autosomal RNA (ASAR) family, which spread from their transcription locus *in cis* to control chromosome-wide replication timing, contain antisense L1 sequences that are required for the function and chromatin association of ASARs [83]. Together, these findings suggest that PTBP1, MATR3, TDP-43 and CELF proteins and their homologs may contribute to the chromatin association of ASARs and mRNAs containing antisense L1s, a function that may have been coopted by *Xist* during its evolution. Another example of *Xist* repurposing transposon-related mechanisms can be seen in the A-repeat of *Xist*, which is derived from an insertion of the endogenous retrovirus K (ERVK) [84]. Intriguingly, another study identified polyadenylated but incompletely spliced transcripts from protein-coding genes that are densely bound by PTBP1, associated with chromatin, and absent from the cytoplasm as mature mRNAs [85]. For one such gene, *Gabbr1*, it has been shown that its transcripts are released from chromatin upon depletion of PTBP1, suggesting that PTBP1 mediates its chromatin anchoring [85] (Figure 2b). Similarly, PTBP1 is also required for the localization of the lncRNA pyrimidine-rich noncoding transcript PNCTR in a peri-nucleolar compartment [86] (Figure 2c). The chromatin association of RNAs often directly impacts chromatin architecture [49]. *Xist* is a classic example for this type of regulation as the Xi becomes compacted and reorganized relative to the active X, through various architectural and heterochromatin regulators [29,35]. Similarly, thousands of other RNAs are maintained on chromatin through their interaction with RNA-binding proteins, such as SAF-A and MATR3, to prevent the compaction of active chromatin regions [49]. Moreover, MATR3 can bind the architectural proteins CCCTC-binding factor CTCF and ohesion [87] and thereby may directly link chromatin organization to chromatin-associated RNA molecules. Further studies of PTBP1, MATR3, TDP-43 and CELF1 and their homologs are therefore likely to reveal exciting new insights into the chromatin association of RNAs and the control of functional nuclear compartments.

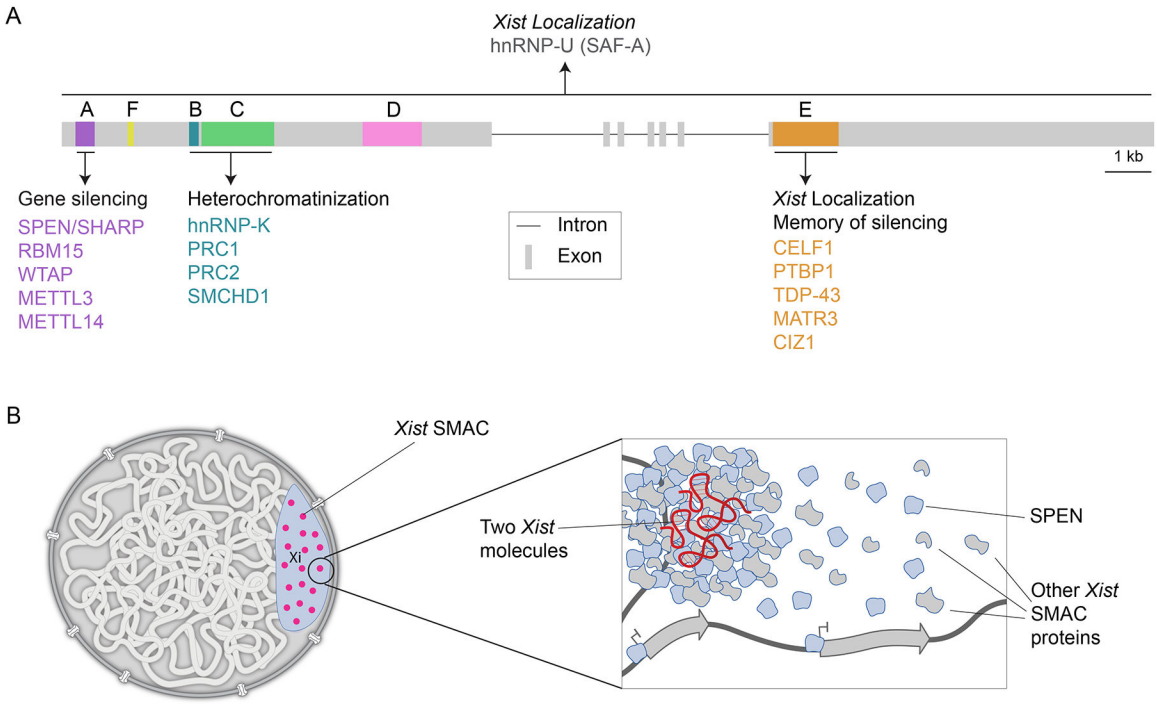

Figure 1. Xist structure and mechanisms of action

A) Diagram of the mouse genomic *Xist* locus with its exons and introns and the location and key binding partners of the *Xist* repeat elements A-F. Some proteins such as scaffold attachment factor A (also called hnRNP-U : heterogeneous nuclear ribonucleoprotein U) SAF-A bind across the RNA. (kb=kilobase).

B) *Xist* silences X-linked genes by seeding supra-molecular complexes (SMACs). Left: Depiction of a nucleus with the Xi and its 50 *Xist*-SMACs that are locally constrained within the Xi. Right: Depiction of one Xist-SMAC highlighting that it is formed by two RNA molecules and a large number of proteins that bind to the RNA and undergo extensive protein-protein interactions. The dynamic behavior of most protein constituents of SMACs is thought to generate protein gradients, allowing free proteins to act on genes on the X chromosome to initiate and maintain their silencing and induce the heterochromatin state of the Xi.

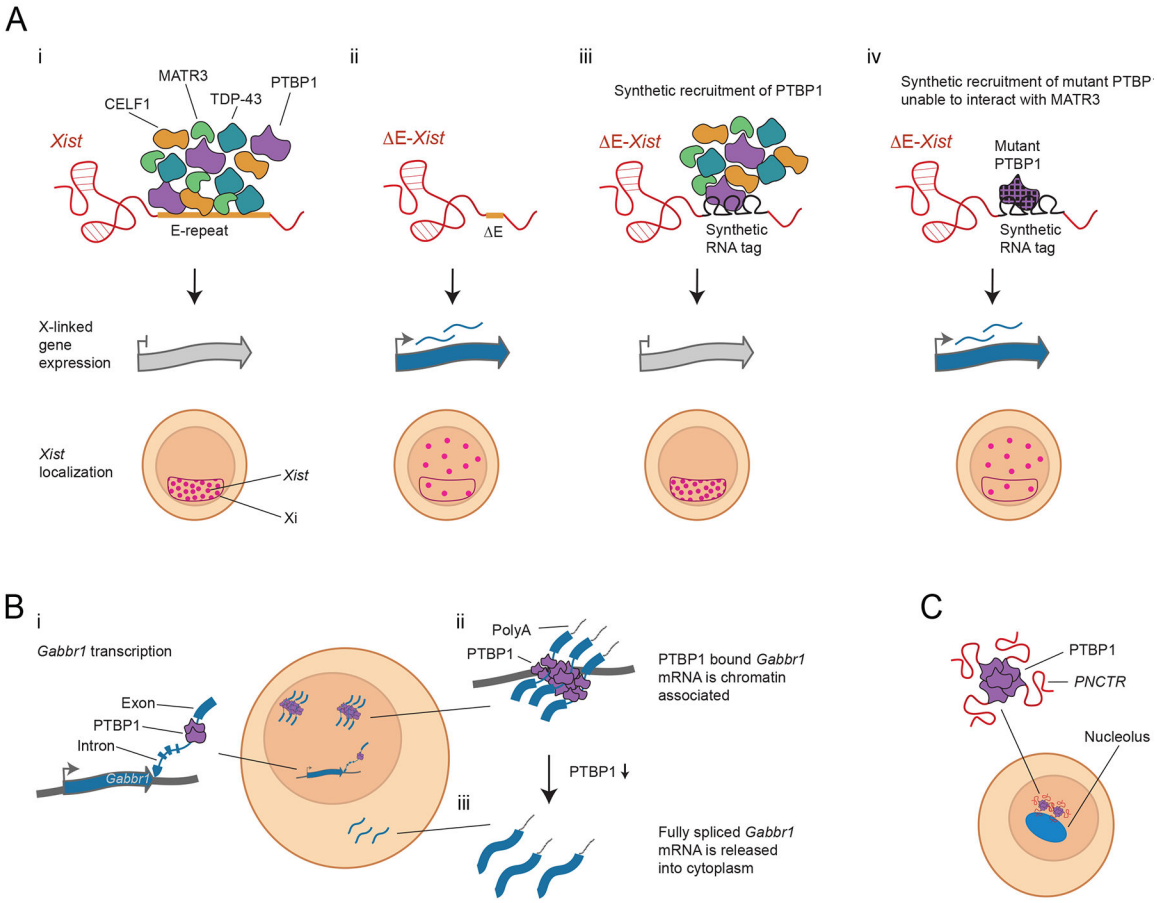

Figure 2. Stabilization of the Xi by the Xist E-repeat binding proteins PTBP1, MATR3, TDP-43 and CELF1

A) i) The E-repeat binding proteins PTBP1, MATR3, TDP-43 and CELF1 bind to the E repeat in wildtype *Xist*, a 1.4kb region that contains a large number of sequence motifs for each of these proteins (top row). The proteins also interact with each other. In differentiating female mouse embryonic stem cells expressing wildtype *Xist*, the RNA exhibits coating of the Xi and gene silencing occurs (second and third row). ii) If the E repeat is deleted, PTBP1, MATR3, TDP-43 and CELF1 no longer bind to *Xist*. These cells initiate *Xist* coating and genes begin to silence, but after initiation of XCI, *Xist* becomes dispersed through the nucleus, and genes on the Xi reactivate. iii) If the E repeat is deleted but multiple PTBP1 molecules are artificially tethered to the mutant RNA, MATR3, TDP-43 and CELF1 can be indirectly recruited and silencing of X-linked genes and *Xist* coating of the Xi are retained. This result also holds when either MATR3, TDP-43, or CELF1 are artificially tethered to *Xist* [31]. iii) If a PTBP1 mutant that prevents the interaction with MATR3 is artificially tethered to Xist, the protein complex no longer forms and *Xist* fails to maintain its localization and silencing is not maintained. This result also holds if MATR3 is artificially tethered to *Xist* but contains a mutation preventing it from interacting with PTBP1 or blocking its self-interaction [31], supporting the importance of protein-protein interactions.

B) PTBP1 regulates the chromatin association of the *Gabbr1* mRNA. i) The *Gabbr1* transcript is transcribed from its locus. ii) The fully transcribed *Gabbr1* mRNA is polyadenylated with most introns spliced out, but PTBP1 binding prevents splicing of one intron. The PTBP1-associated transcript is chromatin associated (although the specific location is currently unknown). iii) Following reduction of PTBP1 (by knockdown or neural differentiation), the intron is spliced and the transcript is released into the cytoplasm [85]. **C)** PTBP1 also maintains the localization of the lncRNA *PNCTR* to the peri-nucleolar compartment [86].

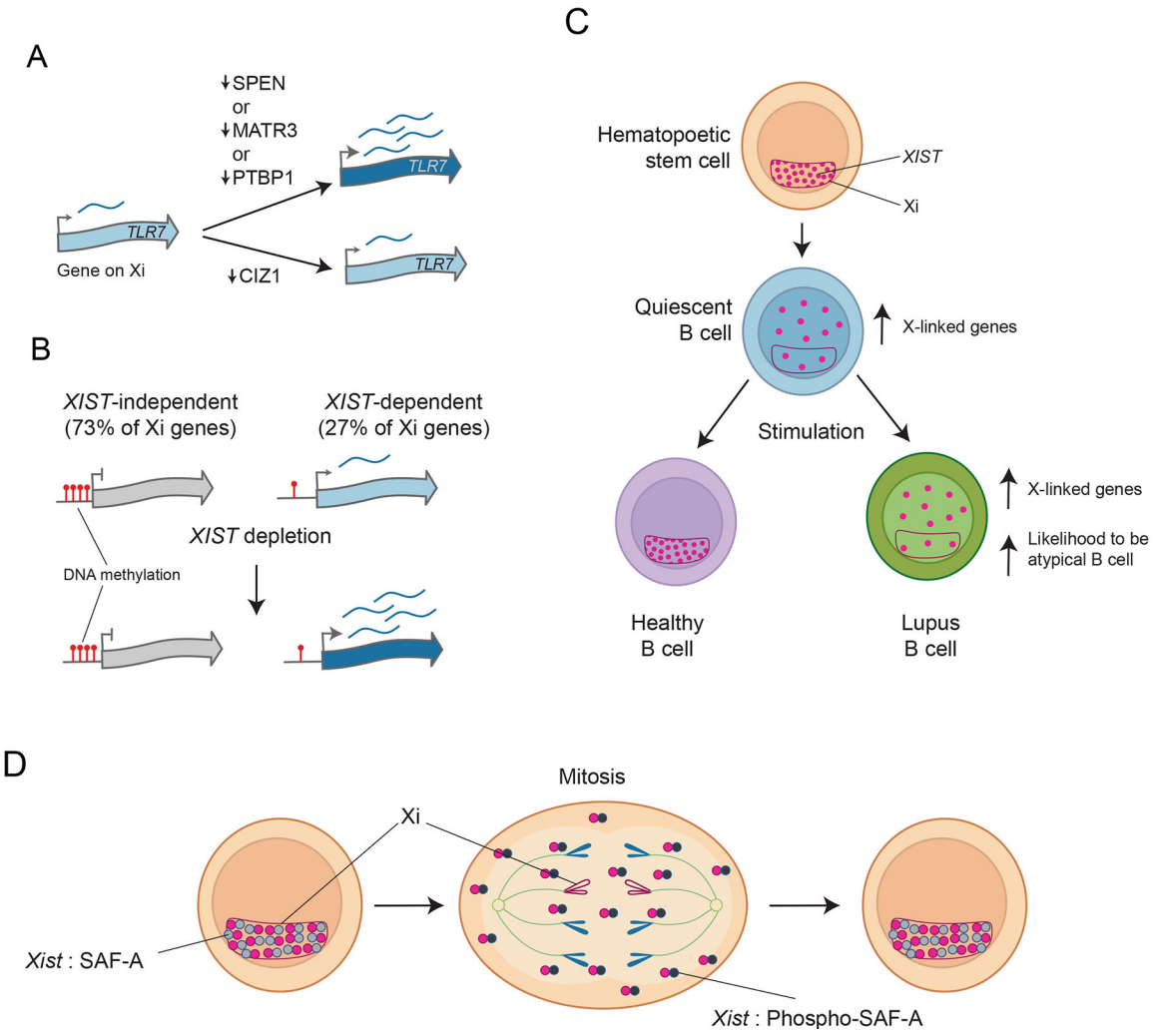

Figure 3. Loss of *XIST*/*Xist* impacts Xi maintenance and differentiation potential

A) Similar to the transcriptional repressor SPEN, PTBP1, and MATR3 are required to maintain the repression of the inflammatory gene *TLR7* on the Xi in the human B cell line GM12878 (which has an Xi-localized *XIST*). Like PTBP1 and MATR3, CIZ1 binds the E-repeat of *XIST*, but is not required to maintain silencing of Toll-like receptor 7 *TLR7* [22,31], consistent with the E-repeat acting via two distinct pathways.

B) Requirement of *XIST* for Xi maintenance. The deletion of *XIST* in the human B cell line GM12878 showed that the majority of genes on the Xi are redundantly silenced by *XIST* and various layers of epigenetic regulation such as DNA methylation. However, the removal of *XIST* is sufficient to upregulate some genes on the Xi. These *XIST*-dependent genes often escape XCI and are lowly methylated in unperturbed B cells [22]. One example of an *XIST* dependent escape gene is *TLR7*.

C) Changes in *XIST* localization in lymphocyte development. *XIST* associates with the Xi in hematopoietic progenitor cells, forming its well-known territory over the Xi, but becomes delocalized from the Xi in quiescent B cells, distributing across the nucleus. Upon stimulation and re-entry into the cell cycle, B cells from healthy individuals regain the proper localization of *XIST*, while *XIST* remains delocalized in B cells from SLE (Lupus)

patients [18]. SLE B cells display higher expression of *XIST*-dependent immune-regulatory genes on the X chromosome, including *TLR7*, and have an increased likelihood of forming atypical B cells (ABCs) [22].

D) During the normal cell cycle, *XIST* dissociates from the Xi in mitosis and one known regulatory mechanism is the aurora B kinase-mediated phosphorylation of SAF-A, which leads to the dissociation of *XIST*-SAF-A complexes [48]. In the new cell cycle round, newly transcribed *XIST* recoats the Xi. The dynamic localization of *Xist* is therefore a process that occurs in every dividing cell type, and therefore has the potential to be a regulatory or a disease mechanism in tissues beyond the hematopoietic system.