REVIEW

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Novel players in X inactivation: insights into *Xist*-mediated gene silencing and chromosome conformation

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The nuclear long noncoding RNA (lncRNA) *Xist* ensures X-chromosome inactivation (XCI) in female placental mammals. Although *Xist* is one of the most intensively studied lncRNAs, the mechanisms associated with its capacity to trigger chromosome-wide gene silencing, the formation of facultative heterochromatin and an unusual 3D conformation of the inactive X chromosome (Xi) have remained elusive. Now researchers have identified novel functional partners of *Xist* in a series of breakthrough studies, using unbiased techniques to isolate *Xist*-bound proteins, as well as forward genetic screens. In addition, important insights into the 3D organization of Xi and its relation to gene expression have been obtained. In this Review, we discuss how this new information is providing a recipe for deciphering XCI mechanisms by which a multitasking RNA can structurally and functionally transform an active chromosome into uniquely organized facultative heterochromatin.

In mammals, dosage compensation between females (XX) and males (XY) is achieved through transcriptional inactivation of one X chromosome in females during early embryonic development[1]. XCI relies on expression of the X-inactive-specific transcript (*Xist*), a lncRNA that is essential for female survival[2,3]. *Xist* is monoallelically upregulated from only one X chromosome via a mechanism that involves several *cis*- and *trans*-acting factors able to sense, count and choose one X chromosome for XCI (reviewed in refs. 1,4). *Xist* RNA is expressed from and 'coats' the future Xi *in cis* and triggers a cascade of events that lead to transcriptional silencing, conformational reorganization and heterochromatinization[5,6]. *Xist* RNA is very long (15,000–17,000 nt) and is poorly conserved between placental mammals, except for a series of unique repeat regions termed the A-to-F repeats (**Fig. 1**). The size and exact number of these repeats vary greatly between species[7–9]. The most conserved A-repeat region lies at the 5′ end of *Xist*, and molecular genetics studies have shown that it plays a crucial role in X-linked gene silencing[10]. Other regions affect the ability of *Xist* to coat chromatin or to recruit chromatin-modifying factors such as Polycomb repressive complex 2 (PRC2)[10–13] (**Fig. 1**).

Since the discovery of *Xist* in the early 1990s[14,15], scientific understanding of its molecular functions has been limited by the scarce knowledge about the RNA-binding proteins (RBPs) it partners with. Candidate approaches based on RNA immunoprecipitation or band-shift assays identified PRC2, ATRX, YY1 and HNRNPU/SAF-A as *Xist* interactors[13,16–19]. However, such methods often fail to discern between specific and nonspecific RNA-protein interactions[20]. This issue is particularly critical in studies of structurally complex RNA regions (for example, the *Xist* A-repeat)[18,21] or proteins such as PRC2 components, which are notorious for their relatively nonspecific but avid RNA-binding capacity[22,23]. The recent development of unbiased, RNA-focused methods that capture RBPs[24–26] has enabled a leap forward in the discovery of novel *Xist* interactors, as well as in reassessments of previously proposed candidates.

Previous studies of differentiating mouse embryonic stem cells (ESCs), an *in vitro* model system favored by researchers investigating the initiation of XCI, showed that *Xist* has a limited developmental window of competence for gene silencing[27]. The only candidate factor for *Xist*-dependent repression was the AT-rich binding protein SATB1, identified in a mouse T cell lymphoma model[28]. However, SATB1 was found to be dispensable for the initiation of *Xist*-dependent gene silencing in the embryo[29]. Other factors previously identified as important for X-linked gene silencing were implicated in the maintenance rather than in the initiation phase of XCI: the PRC2 component EED was found to be required for maintenance of the inactive state in the trophectoderm lineage[30,31], and SMCHD1, a protein that contains a structural maintenance of chromosomes flexible hinge domain, was shown to be involved in the maintenance of Xi-linked gene repression in the embryonic lineage, probably through DNA methylation[32,33]. Only now are the functions of specific proteins in the initiation of XCI coming to light, thanks to recent forward genetic screens in mouse ESCs[34,35].

During XCI, the X chromosome is subject to major changes in its 3D organization, which ultimately result in the characteristic condensed and heterochromatic form commonly known as the Barr body[36,37]. Coating of the chromosome by *Xist* precedes the appearance of the Barr body[38].

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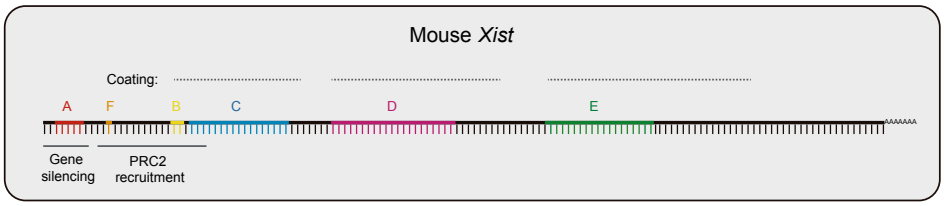

Figure 1 Structure and functions of mouse *Xist* RNA. Different regions of *Xist* RNA that are required for X-linked gene silencing, PRC2 recruitment to chromatin and *Xist* RNA coating are indicated. A-to-F repeats are highlighted by color-coding.

In fact, *Xist* rapidly induces the formation of a nuclear compartment devoid of transcriptional machinery and euchromatic marks[39], which becomes progressively enriched in repressive post-translational histone modifications such as dimethylation (me2) and trimethylation (me3) of histones (e.g., H3K27me3 and H3K9me2)[5]. X-linked genes initially reside at the edge or outside of this compartment, but they are relocated into it as they are silenced[39,40]. The recent advent of high-throughput chromosome conformation capture (3C) techniques such as 4C (3C-on-chip)[6], 5C (carbon-copy 3C)[41] and genome-wide Hi-C[26,42–45], as well as advanced 3D DNA fluorescent *in situ* hybridization (FISH) imaging[44,46], has further pinpointed the singularity of Xi topology that accompanies the silencing process.

Here we discuss recent insights into XCI, including the discovery of proteins involved in *Xist* RNA functions, as well as efforts to unravel the unique chromatin architecture of the Xi.

Identifying the *Xist* RNA-protein interactome

Recently, the *Xist* RNA-protein interactome was revealed for the first time in three independent studies that made use of novel RNA-centric techniques: comprehensive identification of RNA-binding proteins by mass spectrometry (ChIRP-MS)[24], RNA antisense purification coupled with mass spectrometry (RAP-MS)[25], and identification of direct RNA-interacting proteins (iDRiP)[26]. All three methods include a cross-linking step to preserve *in vivo Xist*-protein interactions, which are then captured with antisense oligonucleotides complementary to *Xist* and identified by mass spectrometry. Although the rationales behind these approaches are similar, there are also some important differences, which might explain discrepancies in the various interactors retrieved. These differences, which are summarized in **Figure 2a**, include the biological systems used, the cross-linking agents, the number and size of the antisense oligonucleotides used to pull down *Xist*, the proteomics approaches, and the cutoff chosen to define significant interactors.

In their ChIRP-MS study, Chu *et al.*[24] used formaldehyde as a cross-linking agent. Formaldehyde cross-links not only proteins and nucleic acids, but also RBPs and their own protein interactors[20]. They identified a total of 81 candidate proteins associated with *Xist* RNA (**Fig. 2a**). At the top of the list were HNRNPU, a previously proposed interactor[16], and HNRNPK, an RBP linked to RNA processing[47]. HNRNPK knockdown resulted in a mild effect on X-linked gene repression and a loss of Polycomb-associated marks, thus implicating HNRNPK as a potential mediator of *Xist*-dependent PRC1 and PRC2 deposition of repressive marks. A key functional feature of the study was the use of a silencing-defective *Xist* A-repeat mutant (ΔA), which allowed for comparison with the interactome of wild-type *Xist*[10]. Three *Xist*-binding proteins did not bind ΔA *Xist* RNA: SPEN (an RBP involved in transcriptional repression), WTAP (a component of N^6-adenosine–m^6A RNA-methylation machinery) and RNF20 (an E3 ubiquitin ligase involved in H2BK120 ubiquitylation). SPEN knockdown confirmed that the protein has a role in *Xist*-mediated gene silencing[24].

In their RAP-MS study, McHugh *et al.*[25] identified ten *Xist*-interacting proteins (**Fig. 2a**). Nine of these have RNA-recognition motifs (RRMs), as would be expected for a UV-cross-linking approach

Table 1 Summary of the common *Xist*-interacting silencing factors found in both proteomic and forward genetic screens

Functional class	Novel *Xist* silencing interactors	Proteomic screen			Silencing screen		Proposed functions in XCI
		ChIRP-MS[24]	RAP-MS[25]	iDRiP[26]	shRNA screen[34]	Gene-trap screen[35]	
Transcriptional repressors	SPEN/SHARP	+	+	+	+	+	SPEN: SMRT/HDAC3 recruitment and gene silencing
m^6A RNA methylation	RBM15	+	+	+	+	–	RBM15 (and RBM15B) recruits m^6A machinery in a WTAP-dependent manner to *Xist*; m^6A-modified *Xist* is recognized by YTHDC1, essential for gene silencing
	WTAP	+	–	–	+	–	
Nuclear matrix/ architecture	HNRNPU	+	+	+	–	–	HNRNPU: *Xist* coating
	MATR3	+	–	–	+	–	MATR3
	LBR	–	+	+	–	–	LBR: nuclear lamina recruitment/gene relocation to repressive compartment
Chromatin-modification-related factors	SAP18	+	–	–	+	–	SAP18
RNA processing	PTBP1	+	+	+	+	–	PTBP1
	HNRNPC	+	+	–	+	–	HNRNPC
	HNRNPK	+	–	+	–	–	HNRNPK: PRC1/PRC2 recruitment and gene silencing
	HNRNPL	+	–	+	+	–	HNRNPL
	HNRNPA0	+	–	+	+	–	HNRNPA0

The candidate proteins listed were found in at least one proteomic screen and in a forward genetic screen, except for HNRNPK, HNRNPU and LBR, which were not identified by forward genetic screens but were shown to have an effect on X-linked gene silencing in refs. 24 and 25.

that captures RBPs exclusively[20]. In contrast to ChIRP-MS and iDRiP, the RAP-MS study exploited stable isotope labeling by amino acids in culture (SILAC) as a quantitative proteomics approach[25]. SILAC is a highly sensitive approach and may be better at discerning specific interactors than standard label-free MS[48]. Functional tests showed that three proteins—SPEN, HNRNPU and LBR—were necessary for *Xist*-induced silencing[25].

Minajigi *et al.*[26] used iDRiP on a line of immortalized fibroblasts from female mice that express endogenous *Xist* RNA. XCI had already been established in this cell line, so *Xist* interactors could be considered as putative candidates for the maintenance phase of XCI, not just for initiation. Besides SPEN, which was at the top of their list, they identified several proteins, including cohesins, condesins, chromatin factors, RNA helicases and topoisomerases, that were shown to act synergistically in XCI maintenance[26].

Despite the use of distinct methodologies and different cell types, several common proteins were captured in these independent studies (**Table 1**). These include the previously identified *Xist* interactor HNRNPU[16]. The two main newly identified interactors were SPEN and RBM15, both RBPs with potential transcriptional repressor activity[49]. Numerous proteins involved in mRNA processing were also found in all three studies[24–26] (**Table 1**). Whether these are *Xist*-specific functional partners or simply general interactors of mRNAs and/or lncRNAs remains to be assessed.

With the exception of HNRNPU, proteins previously proposed as partners of *Xist*, such as PRC2 components (e.g., EZH2 and SUZ12), were not identified in these unbiased proteomics studies. JARID2, which is involved in the recruitment of PRC2 to *Xist*-coated chromatin independently of its RNA-binding domain[11,50], was also not identified. The chromatin remodeler ATRX, proposed to participate in PRC2 recruitment to the Xi[19], was identified only as a weak interactor by iDRiP[26]. Finally, the YY1 transcription factor, previously proposed to bind *Xist* RNA as well as DNA facilitating *Xist*'s association with its own locus *in cis*[17], was also not captured.

Searching for factors implicated in *Xist*-mediated gene silencing

In the quest for factors required for the initiation of *Xist*-mediated gene silencing[27], two groups recently carried out genetic screens in mouse ESCs[34,35], as *Xist* is competent to initiate gene silencing in these cells[27]. Moindrot *et al.*[34] identified a total of 225 proteins from the nucleome and 34 from ubiquitylome/SUMOylome libraries by using a dual pooled lentiviral short hairpin RNA (shRNA) strategy (**Fig. 2b**). After validating shRNA screen hits, they identified SPEN, RBM15 and WTAP as the main candidate proteins involved in *Xist*-mediated gene silencing[34]. Monfort *et al.*[35] carried out a gene-trap screen in haploid cells and identified the top 6 factors, along with 15 factors with milder effects (**Fig. 2b**). Again, SPEN was identified as a *Xist*-mediated gene-silencing factor[35].

In summary, the results of these five proteomic and forward genetic screens converged remarkably on a number of common players for *Xist*-mediated silencing, and in all cases they identified SPEN, RBM15 and WTAP—none of which had previously been suspected to have a role in XCI. In addition, a diversity of proteins related to chromatin functions (e.g., SAP18) or to chromosome structure and nuclear organization (e.g., HNRNPU, MATR3 and LBR) (**Table 1**) were also identified. In the next sections, we discuss some of these protein partners for *Xist* gene silencing in more detail.

SPEN, an RBP involved in transcriptional repression

SPEN (also known as SHARP or MINT), a homolog of the *Drosophila* Split-ends homeotic mutant[51], is a very large protein (around 400 kDa)

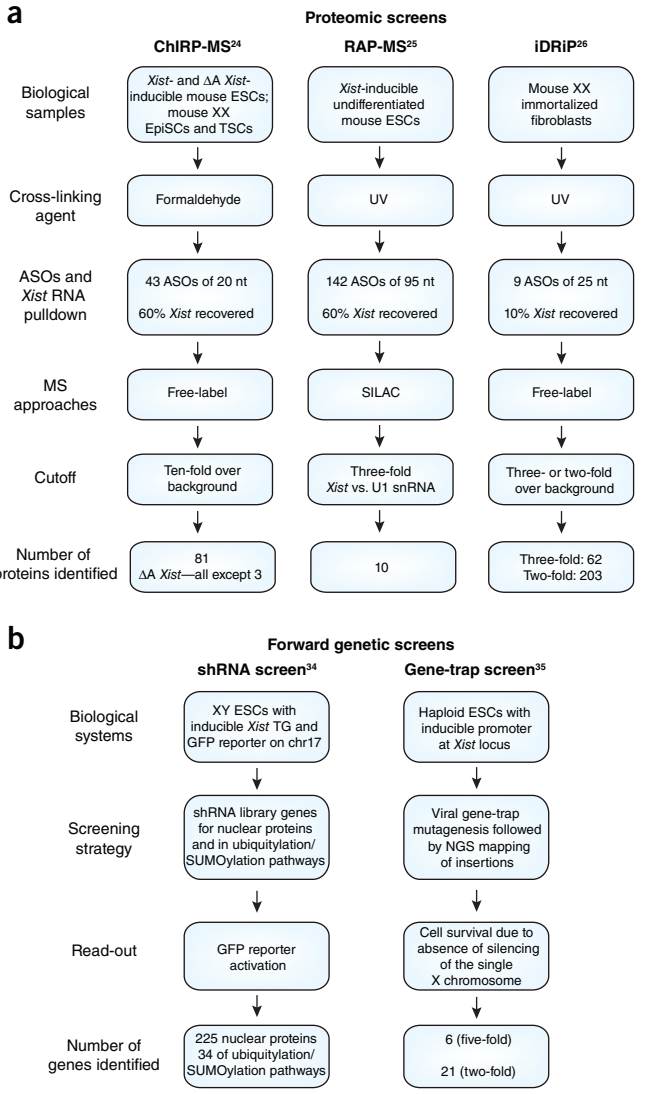

Figure 2 Proteomic and forward genetic screens used to identify *Xist* silencing partners. ESCs, embryonic stem cells; EpiSCs, epiblast stem cells; TSCs, trophoblast stem cells; UV, ultraviolet light; ASOs, antisense oligonucleotides; MS, mass spectrometry; SILAC, stable isotope labeling by amino acids in culture; snRNA, small nuclear RNA; TG, transgene; GFP, green fluorescent protein; shRNA, short hairpin RNA; NGS, next-generation sequencing.

that harbors several N-terminal RRMs, and a conserved transcriptionally repressive SPEN paralog and ortholog C-terminal (SPOC) domain at its C terminus[49]. SPEN has been implicated in RNA-directed transcriptional regulation in the context of nuclear receptor pathways[52,53]. SPEN's SPOC domain interacts with the ubiquitous transcriptional corepressors SMRT/NCOR2 and NCOR1, and it recruits histone deacetylases[49,53,54].

SPEN-*Xist* interaction was reported to be A-repeat dependent[24]. The A-repeat is composed of eight motifs that form inter-repeat double-stranded duplexes, flanked by single-stranded regions[55]. *In vitro* experiments implicated SPEN's RRMs in *Xist* A-repeat binding[24,35], and individual-nucleotide cross-linking and immunoprecipitation (iCLIP) experiments mapped this interaction to single-stranded regions upstream of the inter-repeats[55]. Consistent with its A-repeat interaction, SPEN has a clear effect on X-linked gene repression. However, loss-of-function studies showed no visible alteration of the *Xist* RNA coating on the Xi, and only a mild effect on PRC2/PRC1 recruitment[24,25,34,35]. Although McHugh *et al.*[25] reported that RNA polymerase II (Pol II) was

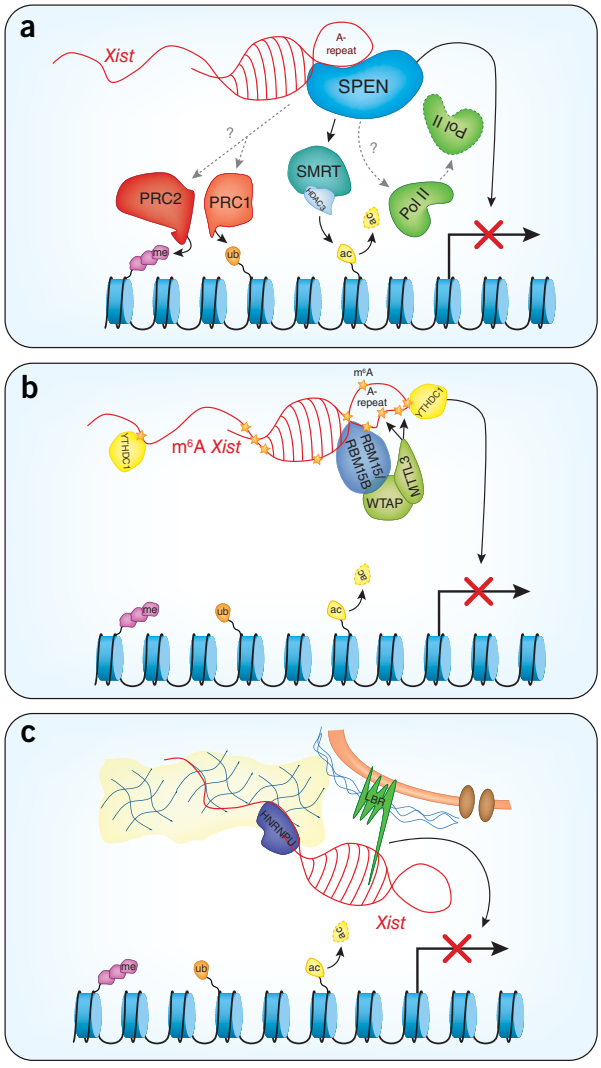

Figure 3 Functions of *Xist* RNA silencing partners for XCI. (**a**) SPEN, an RBP involved in transcriptional repression, binds directly to the *Xist* A-repeat involved in *Xist*-mediated gene silencing and interacts with the SMRT deacetylase complex responsible for the deacetylation of histones via HDAC3. This might be one of the mechanisms responsible for gene silencing. It is still unclear whether SPEN has a role in recruiting PRC1 and PRC2 or in excluding RNA Pol II from the *Xist*-coated chromosome (indicated by dashed gray arrows and question marks). (**b**) m^{6}A RNA methylation machinery: RBM15 and WTAP. RBM15 and RBM15B interact with *Xist* and recruit the m^{6}A RNA methylation machinery through interaction with WTAP. The WTAP interaction partner MTTL3 will methylate adenosine residues (marked by stars). The YTHDC1 reader binds m^{6}A residues and enables *Xist*-mediated transcriptional silencing. (**c**) Nuclear matrix/compartment: HNRNPU and LBR. *Xist* interacts with HNRNPU, a nuclear matrix protein that is essential for the *cis*-localization of *Xist* to the Xi. *Xist* also interacts with LBR, a protein that links chromatin to the nuclear envelope, which affects *Xist*-mediated gene silencing. me, H3K27me3; ub, H2AK119ub; ac, histone lysine acetylation.

no longer excluded from the *Xist*-coated repressive compartment after *Spen* depletion, Monfort *et al.*[35] found that this repressive compartment existed in *Spen*-knockout ESCs. The reasons for the discrepancy remain unclear. In any case, a failure to create a repressive compartment could not fully explain the absence of X-linked gene silencing, as previous studies showed that silencing-defective ΔA *Xist* can still induce the formation of an RNA Pol II excluded nuclear domain[39].

SPEN interacts with the SMRT corepressor deacetylase complex, which includes HDAC3 as its main catalytic unit[53,54]. McHugh *et al.*[25] found that a loss of function of either SMRT or HDAC3 resulted in XCI defects similar to those observed with *Spen* depletion. They proposed a model whereby SPEN recruits the SMRT–HDAC3 complex, leading to transcriptional silencing of X-linked genes (**Fig. 3a**). However, the *Xist*-coated chromosome is still mostly depleted of acetylated H4 in *Spen*-knockout ESCs[35], which suggests that the role of SPEN in XCI might not be limited to its interaction with the deacetylase machinery. Indeed, given its unusually large size (399 kDa), SPEN may act as a platform to recruit additional silencing or repressive factors to the *Xist*-coated chromosome. Homozygous knockout of *Spen* in the mouse is lethal from embryonic day 12.5 (E12.5)[56], which is much later than in a phenotype expected for XCI failure (between E6.5 and E12.5)[2]. Female-specific lethality with *Spen* knockout might have been masked in this study owing to the use of constitutive knockout or because of functional redundancy with other proteins. To further test the role of *Spen* in XCI initiation *in vivo*, it will be important to use maternal knockout embryos to ensure loss of the maternal pool, as well as double-knockouts for other candidate silencing factors such as *Hnrnpk*[57].

m^{6}A RNA methylation machinery: RBM15 and WTAP

Among the newly identified *Xist*-interacting proteins are two proteins that were recently linked to the *N*^{6}-adenosine (m^{6}A) RNA methylation machinery: RBM15 and WTAP (Wilms' tumor-associating protein)[58] (**Table 1**). RBM15, like SPEN, is an RBP containing a SPOC domain. However, it is a much smaller protein than SPEN, and no overlapping or redundant functions between the two proteins have been reported. RBM15's SPOC domain has been shown to interact with the histone H3K4 methyltransferase SETB1 (ref. 59) and is also linked to RNA export regulation[60]. Interestingly, RBM15 in humans (also known as OTT1) is frequently translocated to create an RMB15-MKL1 fusion protein involved in acute megakaryoblastic leukemia[61]. Recently, RBM15 and a very similar protein, RBM15B (also found to be associated with *Xist* by iDRiP[26]), were shown to interact with WTAP in a proteomic screen[62]. WTAP was initially found to be associated with the Wilms' tumor-suppressor gene product WT1 (ref. 63) and is implicated in several aspects of mRNA processing[62,64]. WTAP was recently identified as a component of the m^{6}A RNA methyltransferase complex, and it may recruit or regulate the methyltransferase function of METTL3 (methyltransferase-like 3) to target RNAs[65,66].

m^{6}A is a prevalent post-transcriptional modification of mRNA in eukaryotes that is found mostly at transcriptional start sites and 3′ untranslated regions, with a characteristic peak around stop codons[67]. The relevance of m^{6}A modification in RNA biology is unclear, but it may affect RNA splicing, RNA secondary structure and stability, and RBP recognition[67]. As in other RNAs, different regions of *Xist* are m^{6}A modified (78 adenosines in humans), including the A-repeat[58]. On the basis of the proposed interaction between RBM15 (and RBM15B) and WTAP, Patil *et al.*[58] explored the role of m^{6}A methylation in *Xist*-mediated transcriptional silencing (**Fig. 3b**). Indeed, they found that human RBM15 and RMB15B interact with METLL3 in a WTAP-dependent manner. iCLIP showed that both proteins localized in the vicinity of m^{6}A sites along human *XIST*. Furthermore, knockdown of both RBM15 and RBM15B (or of WTAP alone) resulted in a significant decrease in MTLL3 recruitment and m^{6}A levels to the *XIST*-binding sites of those proteins. In agreement with this finding, knockdown of RBM15 and RBM15B or of MTTL3 alone in mouse *Xist*-inducible ESCs resulted in a clear loss of *Xist*-mediated silencing[58]. These results thus implicated RBM15 and RBM15B in the recruitment of the m^{6}A machinery to *Xist*, and demonstrated that m^{6}A modification

is involved in *Xist*-mediated gene silencing. Using a candidate-based approach, Patil *et al.*[58] also found that YTHDC1, a known m^{6}A reader, binds m^{6}A residues within *XIST* (**Fig. 3b**). Indeed, YTHDC1 had also been captured by ChIRP-MS[24]. *Ythdc1* knockdown in mouse ESCs prevented *Xist*-mediated silencing, and forced tethering of YTHDC1 to *Xist* was sufficient to rescue X-linked transcriptional silencing in the absence of the m^{6}A machinery[58]. Although these results suggest that YTHDC1 might be a key reader of m^{6}A on *Xist*, further experiments are required to clarify the mechanistic interplay between YTHDC1 bound to m^{6}A-modified *Xist* and other silencing factors, such as SPEN, in X-linked gene silencing.

Nuclear compartment and nuclear matrix proteins: HNRNPU and LBR

Several proteins associated with the nuclear scaffold/matrix (e.g., HNRNPU) or involved in nuclear compartmentalization (e.g., LBR) were also captured as *Xist* partners (**Table 1**), although they do not interact in an A-repeat-dependent fashion[24]. HNRNPU was found in all three proteomics studies referenced in **Table 1**, in agreement with previous work suggesting that different regions of *Xist* interact with HNRNPU[16,24,68]. Indeed, HNRNPU spatially associates with *Xist* and accumulates over the Xi territory[38,69] (**Fig. 3c**). HNRNPU loss of function results in diffusion of *Xist* RNA throughout the nucleoplasm and away from the X chromosome[16,24,25], although this effect might be cell-type specific[70,71]. This suggests that HNRNPU is involved in tethering of *Xist* to the Xi (**Fig. 3c**); however, the fact that other *Xist*-interacting matrix proteins (e.g., MATR3) were also identified in the proteomic and genetic screens (**Table 1**) suggests a more general role for the nuclear scaffold in the initiation of XCI.

Another finding that links *Xist* function to nuclear structure is the discovery that *Xist* may associate with the lamin B receptor (LBR)[25,26] (**Fig. 3c**). LBR is anchored to the nuclear membrane that interacts with lamin B (also identified by iDRiP[26]) and heterochromatin[72]. LBR lacks identifiable RRMs, but it has an arginine and serine (RS) motif that recognizes three binding sites along *Xist* RNA[73]. Disruption of *Xist*-LBR interaction (either by LBR knockdown and knockout or through the deletion of key binding modules in LBR and *Xist*) led to a reduction in X-linked gene silencing for at least five X-linked genes assessed by single-molecule RNA FISH[25,73]. The effect on X-linked gene silencing correlated with the loss of interaction between the *Xist*-coated chromosome and the nuclear lamina[73]. Although no genome-wide assessment of transcriptional defects in the LBR mutant cells was done to exclude indirect effects, the artificial tethering of *Xist* to the nuclear lamina rescued both the recruitment of the Xi to the nuclear lamina and *Xist*-mediated silencing[73]. This suggests that recruitment to the nuclear lamina, mediated by *Xist*-LBR interaction, has a role in X-linked gene silencing (**Fig. 3c**). On the basis of their experimental findings, the authors proposed that the *Xist*-LBR interaction is important for efficient spreading *of Xist* RNA over active genes and for the relocation of genes closer to the *Xist*-coated repressive compartment. Although this study points to a role for nuclear positioning in *Xist*-mediated silencing, the molecular mechanisms remain to be elucidated. Also, whether the nuclear lamina alone is involved or whether other regions frequently visited by the Xi, such as the nucleolus[74,75], may be important remains an open question. Interestingly, the nucleolar protein MYBBP1 was found in two of the proteomics studies[24,26].

New insights into the 3D conformation of the Xi

The discovery that factors implicated in XCI might have a role in the 3D organization of the Xi in the nucleus fits well with results from classic cytology and electron microscopy studies that have shown that the Xi has an unique ultrastructure, distinct from euchromatin and constitutive heterochromatin[36,38]. The advent of 3C technologies and the use of DNA FISH combined with high-resolution microscopy have enabled researchers to obtain further insights into the structural organization of the Xi. Studies involving these techniques revealed that unlike the rest of the genome, which is organized into active and inactive compartments in 3D nuclear space[45,46,76], the Xi forms a compartment of its own. The few genes that are expressed from the Xi ('escapees') tend to interact with each other and with the autosomal active compartment[6]. More recently, five studies of allele-specific Hi-C in human, Rhesus macaque and mouse cellular systems with clonal XCI, researchers found that the Xi is partitioned into two 'megadomains' separated by a boundary that contains the unusual conserved *DXZ4* macrosatellite[26,42–45] (**Fig. 4a**,**b**). High-resolution 3D DNA FISH analysis of human and mouse Xi corroborated these Hi-C results[44,46].

The *DXZ4* boundary locus has several CpG-rich tandem repeats, as well as multiple CTCF-binding sites that bind exclusively on the Xi[43,74,77,78]. In humans, it also interacts with other loci, namely, FIRRE (a lncRNA previously proposed to anchor the Xi to the nucleolus)[74] and ICCE, both of which function as possible superloop anchors[42,45]. Deletion of the *DXZ4* boundary results in the loss of the bipartite Xi configuration and massive structural reorganization in both mice and humans, although overall the Xi structure remains clearly different from that of the active X chromosome (Xa)[42,44] (**Fig. 4c**). Giorgetti *et al.*[44] deleted *DXZ4* in ESCs from female mice and found that after differentiation, the absence of this locus did not affect the initiation of XCI, although the rate at which some genes escaped XCI seemed to decrease mildly. Darrow *et al.*[42] deleted *DXZ4* in human female somatic cells (retinal pigment epithelial 1 (RPE1)) in which the Xi was already stably inactive and found no obvious effect on global gene silencing or escape. Interestingly, FIRRE and ICCE loci can still interact to some extent in the absence of *DXZ4*, which suggests that *DXZ4* is not critical for such long-range interactions on the human Xi[42]. Given its conservation, deletion of *DXZ4* would be expected to result in a more severe phenotype. However, its role can be properly addressed only through an assessment of the impact of the boundary loss during the initiation and maintenance of XCI in an animal model.

Another striking feature observed on the Xi was the general lack of sub-megabase segmental organization into topologically associating domains (TADs)[26,41,44]. The recently discovered TADs are a manifestation of a level of chromosome folding that partitions genomes and often encompasses gene regulatory landscapes[41,79] (**Fig. 4b**). Internal looping between promoters and enhancers generally occurs within TADs, as do structural elements, and CTCF and cohesin have been shown to have an important role in this organization[80]. Across the Xi, a lack of segmental folding and internal looping correlates with a general reduction in cohesin binding[26,42–45] (**Fig. 4b**) and might explain observations that Xi compaction results mostly from the reorganization of chromatin domains at the megabase scale, rather than from interdomain condensation[37]. Indeed, the ~1.2-fold compaction of the Xi (as compared with the Xa) proposed by Teller *et al.*[37] was confirmed in each of the megadomains by Giorgetti *et al.*[44]. However, a few regions on the Xi exhibit TAD-like structures, and these are associated with clusters of escapees[44] (**Fig. 4b**). Most of these regions correspond to open chromatin on the Xi and tend to be associated with CTCF-binding sites. This adds to the accumulating evidence that CTCF is critically involved in TAD formation and maintenance, which may help genes escape XCI[43,44].

The distinct organization of the Xi into two megadomains seems to be dependent on *Xist* expression and its silencing function. The megadomain boundary on the *Xist*-coated X chromosome appears only in the wild type, and not in ΔA *Xist*[44]. Remarkably, deletion of *Xist* from

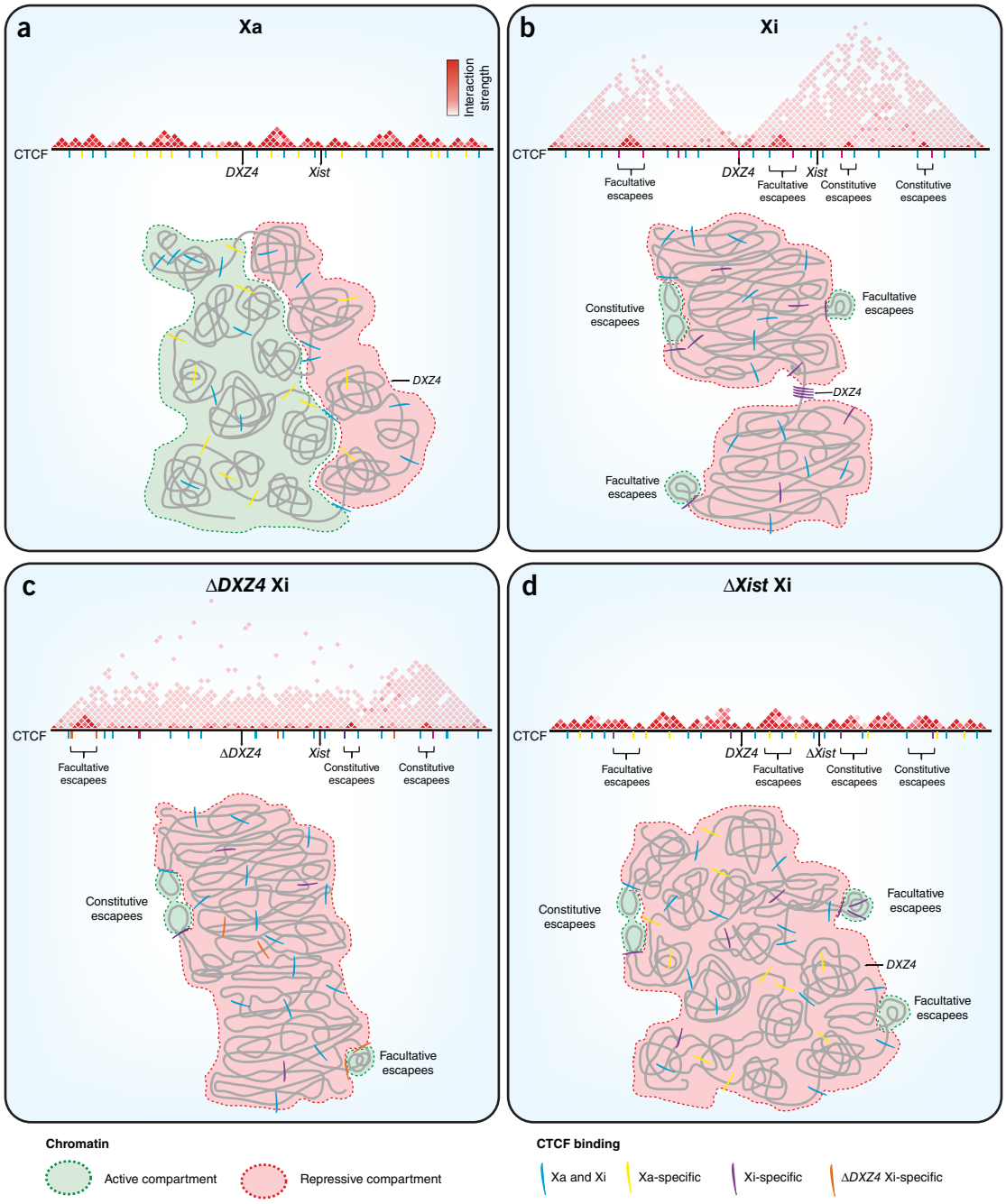

Figure 4 Distinct topology of the Xa versus the Xi, and the influence of *DXZ4* and *Xist* deletions. Typical Hi-C profiles are depicted at the top of each panel, and a schematic representation of the genome organization is shown at the bottom. (**a**) 3D conformation of the Xa showing the typical TAD organization into active and repressive compartments. (**b**) The Xi is organized into two large repressive superdomains with relatively unspecific interactions, divided by the *DXZ4* locus. A few TAD-like structures associated with facultative and constitutive escapees, which tend to cluster, loop out from the repressive domain and are enriched for Xi-specific CTCF binding. (**c**) *DXZ4* deletion leads to a loss of the bipartite organization of the Xi and merging of the two repressive domains, but the organization remains clearly distinct from that of the Xa. The few TAD-like structures, some different from those seen in the normal Xi, are associated with the presence of CTCF. *DXZ4* deletion does not result in significant changes in X-linked gene expression, with the exception of facultative escapees. (**d**) *Xist* deletion leads to the restoration of TADs to an Xa-like state, presumably accompanied by the recovery of most Xa-specific CTCF sites. This major structural rearrangement does not have a considerable effect on the reactivation of the X-linked genes.

the established Xi leads to the restoration of an Xa-like chromosome conformation state, with structured TADs[6,26] and long-range inter-TAD interactions[6], even though this seems to have little effect on X-linked gene silencing[6], presumably because of established epigenetic marks such as DNA methylation (**Fig. 4d**). These results clearly demonstrate a major role of *Xist* in defining the structure of the Xi. Minagiji *et al.*[26] proposed that this could occur via *Xist* RNA–mediated eviction of architectural proteins, namely, cohesins (e.g., SMC3) that are depleted from the Xi. However, cohesins were not found in the other *Xist*-interactome screens, and it is unclear how *Xist* could interact with and yet simultaneously repel cohesins from the Xi, given its tight interaction with the chromosome. The fact that the ΔA *Xist* mutant is unable to achieve a

Xi bipartite structure[44] suggests that A-repeat-dependent interactors such as SPEN might have a role in shaping the 3D landscape of the Xi. Also, the relevance of the nuclear position of the Xi to its 3D structure needs to be addressed. Indeed, the *DXZ4* boundary region has been reported to associate with the nucleolus[43,74]. Whether this facilitates Xi megadomain segmentation or whether nucleolar association is merely a consequence of the unusual status of *DXZ4* remains to be seen. Finally, the interaction between *Xist* and LBR might be expected to function as an important scaffold for the architectural reorganization of the Xi close to the nuclear lamina. Future work that combines techniques to explore the chromosome-wide dynamics of Xi structure and interaction with the nuclear lamina should provide further insights.

Concluding remarks

Thanks to advanced proteomics methodologies and genetic screens, the discovery of key players in XCI has opened up exciting new avenues in XCI research. The use of these approaches, combined with recent efforts to probe *Xist* RNA secondary structure[55,81,82], is enabling scientists to uncover *Xist* RNA binding modules to corresponding interacting proteins. Both the nature of the processes implicated in *Xist*-mediated gene silencing and the identity of the factors involved have been surprising, with some of the predicted suspects being indirect or downstream players, and some totally unexpected new factors being revealed, such as the m^{6}A RNA machinery. The discovery of the Xi conformational changes and chromatin alterations triggered by *Xist* RNA also demonstrate how XCI represents a powerful system for querying the relationships between chromosome organization and gene activity. An important future challenge will be to dissect the precise mechanisms by which these new actors participate in XCI, and to distinguish between the initiation of gene silencing and propagation or stabilization of the inactive state.

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