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eXIST with matrix-associated proteins

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Abstract

X-chromosome inactivation has long served as an experimental model system for understanding the epigenetic regulation of gene expression. Central to this phenomenon is the long, non-coding RNA Xist that is specifically expressed from the inactive X chromosome and spreads along the entire length of the chromosome *in cis*. Recently, two of the proteins originally identified as components of the nuclear scaffold/matrix (S/MAR-associated proteins) have been shown to control the principal features of X-chromosome inactivation; specifically, context-dependent competency and the chromosome-wide association of Xist RNA. These findings implicate the involvement of nuclear S/MAR-associated proteins in the organization of epigenetic machinery. Here, we describe a model for the functional role of S/MAR-associated proteins in the regulation of key epigenetic processes.

Organization of the nucleus

One of the most controversial ideas in the field of cell and molecular biology is the nuclear matrix, which was originally defined as a biochemical nuclear component that resists extensive extraction with detergent and high salt after treatment with nuclease [1]. The constituents of this insoluble material include a wide spectrum of proteins, ranging from structural components of the nuclear envelope to factors involved in most of the basic molecular processes operating in the nucleus; DNA replication and repair, transcription, and pre-mRNA splicing [1]. Because the extraction procedure might induce the rearrangement of nuclear proteins and the artefactual formation of the macromolecular complex, the precise molecular identity and physiological relevance of the nuclear matrix are not known; especially, the mesh-like filamentous structures observed by electron microscopy have not been explained [2,3].

The identification and characterization of A+T-rich DNA sequence elements that remain bound to the extracted nucleus [4], known as scaffold-associated regions or matrix attachment regions (S/MARs), led to another overlapping but distinct area of research, showing that eukaryotic chromosomes are functionally organized into higher-order structures consisting of distinct loop and matrix-attached domains [5]. Subsequent studies have identified proteins that preferentially associate with these DNA elements and more than 500 experimentally validated S/MARs (159 in humans) and nearly 100 S/MAR-binding proteins (27 in humans) have so far been identified from different organisms (<http://>

smartdb.bioinf.med.uni-goettingen.de/SMARTDB/browse/index.html) [6]. Although the classical view of the static matrix or scaffold has been challenged and is being revised [7], it is widely accepted that gene expression is strongly influenced by higher-order chromatin structures and the three-dimensional organization of the cell nucleus [8,9].

Recently, two of the bona fide S/MAR-binding proteins, SATB1 and SAF-A/hnRNP U, have been shown to be crucial for the epigenetic control of gene expression mediated by the long nuclear-retained non-coding RNA Xist [10,11]. In this Opinion, we summarize the unexpected link between the S/MAR-associated proteins and X chromosome inactivation (XCI) and discuss the implications of recent findings in terms of the mechanisms that control the localization and fate of several nuclear-retained non-coding RNAs.

X-chromosome inactivation and X chromosome inactive-specific transcript (Xist): an enigma

In female mammals, one of the two X chromosomes is epigenetically silenced to overcome the gene dosage discrepancy between the two sexes, equalizing the expression of X-linked genes with that of males, who carry only one X chromosome [12–14]. XCI was first identified from a study of the mottled coat color of female mice that were heterozygous for X-linked pigmentation genes [15]. Analysis of animals carrying X-autosome translocation led to the identification of the X-inactivation center (Xic), a central region of the X chromosome that is essential for the establishment of inactive X chromosome (Xi) [16]. A major milestone in XCI came with the discovery of a long, non-coding RNA X chromosome inactive-specific transcript (Xist) that is specifically transcribed from the Xic of Xi [12–14]. The identification of Xist stimulated a series of molecular genetics experiments that clearly demonstrated the requirement of Xist for the formation of the Xi [12–14]. It is of particular interest that Xist RNA is retained in the nucleus and spreads along the entire length of Xi [17], which is different from the majority of the RNA polymerase II-transcribed mRNAs that are immediately exported to the cytoplasm after processing [18]. Histone-modifying complexes such as PRC1 and PRC2 are transiently localized to the X chromosome during the formation of Xi, and specific epigenetic markers are subsequently incorporated on Xi [12–14]. It is currently speculated that the function of Xist is to recruit epigenetic modification machinery to the entire length of the chromosome *in cis* [12–14]. This, in turn, induces changes of the chromatin structure leading to accumulation of the Xi-specific proteins necessary for the formation of Xi. Once established, Xist is dispensable for the maintenance of Xi [12–14], a process that is regulated by other chromatin-related factors, including structural maintenance of chromosomes flexible hinge domain-containing 1 (Smchd1) [19].

Although the basic scheme for the formation of Xi is understood, two important aspects of XCI remained unanswered until recently. First, the formation of Xi can be induced only in particular cell types in an early developmental context such as embryonic cells or ES cells [12–14], and the regulation of this limited competency was unknown. Second is the factor(s) that regulate(s) characteristic chromosomal localization and/or spreading of Xist RNA: how does Xist RNA escape nuclear export and specifically coat the Xi [17]? An earlier study indicated that reduced binding of Xist RNA with RNA export factors is partially responsible for the nuclear retention of Xist RNA [20]; however, this cannot fully explain the Xi-restricted spreading of Xist RNA. Interestingly, recent studies have provided important clues concerning the involvement of S/MAR-associated factors (SATB1 and SAF-A/hnRNP U) in the cell type- or cell stage-specific competency of XCI as well as the Xi-restricted distribution of Xist non-coding RNA (ncRNA).

SATB1: a competence factor for X inactivation

The special A+T-rich sequence-binding protein 1 (SATB1) was originally identified as a factor that specifically recognizes a unique structural feature of A+T-rich sequence S/MARs [21]. SATB1 is expressed predominantly in thymocytes, and mice lacking SATB1 show severe defects in T-cell maturation during the early stage of postnatal development [22]. SATB1, which can act as a transcriptional repressor, interacts with a variety of chromatin-remodeling and -modifying factors including nucleosome re-modeling and histone deacetylase (NURD), chromatin accessibility complex (CHRAC) and ATP-dependent chromatin assembly and remodeling factor (ACF) [23]. SATB1 also epigenetically regulates the expression of malignant-associated genes in breast cancer cells [24].

The link between SATB1 and X-inactivation was demonstrated by an elegant cell-based mutation study [11] that made use of transgenic male mice that conditionally expressed Xist RNA from the X-chromosome [25]. Upon the induction of forced Xist expression, embryos were destroyed and expression of X-linked genes was examined. X-linked genes were suppressed when Xist RNA was induced during early development; however, this suppression was less clear when Xist RNA was induced at later stages of embryonic development [25]. Using this mouse model system, the authors showed that Xist has the ability to induce X chromosome-silencing in immature hematopoietic precursor cells but not in other adult cells [25]. Furthermore, immortalized lymphoma cell lines established from the male mice were subjected to extensive passage to induce spontaneous mutations that reduced sensitivity to the Xist-induced cell death. Subsequent microarray analysis identified a series of genes that were differentially expressed in the Xist-resistant lymphoma cells and SATB1 was identified as one of these competence factors. Further, the role of SATB1 has been confirmed by both loss- and gain-of-function studies [11]. The SATB1-mediated competency for XCI is redundantly regulated by a specific form of SATB2, which might account for the moderate phenotypes caused by gene targeting of SATB1 [11]. It would be intriguing to study whether the function of SATB1 can also be fulfilled by other S/MAR binding proteins.

Studies on the intra-nuclear distribution of SATB1 and Xist RNA have provided important clues regarding the molecular interactions between these two molecules. SATB1 forms a cage-like structure in the nuclei of T-helper 2 cells [26] and Xist RNA aligns this SATB1 network [11]. SATB1 is known to induce compaction of the cytokine-containing genomic locus into dense loops anchored by S/MAR at their base, and such a structural change is essential for the activation of the target cytokine genes located in the loop regions [26]. Because SATB1 is recruited to the Xist RNA transcription site upon the induction of transgene expression [11], it is possible that these two molecules interact with each other. X chromosomes coated with Xist RNA might thus be organized into a compact structure through their interaction with SATB1, which enables the efficient formation of the silencing complex and the subsequent establishment of Xi (Figure 1) [27]. This model is in accord with earlier reports indicating that Xist RNA induces the translocation of gene-rich regions of the chromosome into the core silent domain-containing non-genic repeat elements and Xist RNA [28,29]. However, SATB1 is not involved directly in the formation of the core Xist silencing domain because the condensed signal of Xist RNA was observed in SATB1 $-/-$ thymocytes [11].

hnRNP U/SAF-A/Sp120: a chromosomal localization factor of Xist RNA

The heterogeneous nuclear ribonucleoprotein U (hnRNP U) was originally characterized as one of the 20 proteins ranging in size from 34 kDa (A1) to 120 kDa (U) that efficiently cross-link to nuclear heterogeneous RNAs [30,31]. The same gene was characterized as an

S/MAR-binding factor and named scaffold attachment factor A (SAF-A) [32] and scaffold protein 120 kDa (SP120) [33]. Two domains of SAF-A/hnRNP U, SAF-box [34] and RGG-box [31], have been functionally characterized to bind DNA and RNA, respectively, in a non-competitive manner [35].

The SAF-A/hnRNP U-EGFP fusion protein was used to study the localization of SAF-A/hnRNP U in living cells, which was found to be enriched on the inactive X chromosome through its RGG-box [36]. Such a localization pattern was not observable by conventional immunohistochemical methodology using antibodies that recognize endogenous SAF-A/hnRNP U [30,33]. However, autoimmune antibodies that specifically recognize SAF-A/hnRNP U have recently been shown to decorate Xi [37]. It is thus possible that cells contain several post-translationally modified forms of SAF-A/hnRNP U, and the antibody recognizes only a specific modified form of the protein. Addition of the EGFP tag possibly influences the post-translational modification as well as its structural conformation. The mobility of SAF-A/hnRNP U-EGFP in the Xi territory is much slower compared to the other nuclear compartments, suggesting that SAF-A/hnRNP U acts as a platform to immobilize Xist RNA [38]. Direct experimental evidence supporting this idea came from screening a custom siRNA library targeting abundant RNA-binding proteins [10]. In cells lacking SAF-A/hnRNP U, Xist RNA was dissociated from the X chromosome and distributed diffusely throughout the nucleoplasm (Figure 2a) [10]. SAF-A/hnRNP U interacts with Xist RNA, utilizing its RGG-box, and both DNA- and RNA-binding activities of SAF-A/hnRNP U are required for the chromosomal localization of Xist [10]. On the basis of these results, we speculate that Xist RNA is tethered to the S/MAR-rich sequences on the X chromosome through its interaction with SAF-A/hnRNP U (Figure 2c). This interaction is rather specific to SAF-A/hnRNP U, because knockdown of the closely related molecule hnRNP U-like did not induce the mislocalization of Xist RNA [10].

In mitotic cells, Xist RNA is released from the chromosome and is distributed diffusely in the nucleoplasm [17]. A recent study showed that the chromosomal localization of Xist RNA in mitotic cells is negatively regulated by Aurora B kinase (Aurkb) [39], a mitotic kinase that regulates chromosomal segregation and cytokinesis. Similar to Xist RNA, SAF-A/hnRNP U is excluded from the mitotic chromosomes (Figure 2b). We speculate that SAF-A/hnRNP U is phosphorylated by Aurkb or other mitotic kinases during mitosis and, in turn, disrupts the association of the SAF-A/hnRNP U-Xist RNA complex with mitotic chromosomes (Figure 2d). In contrast, it should be noted that two earlier studies using SAF-A/hnRNP U-EGFP protein or autoimmune antibodies against SAF-A/hnRNP U showed that SAF-A/hnRNP U does localize on the mitotic chromosomes [34,37]. As discussed above, specific post-translational modifications of SAF-A/hnRNP U might result in different structural conformations that are specifically visualized by a distinct detection method. Specific arginine methylation of SAF-A/hnRNP U by PRMT1 arginine methyltransferase might also be physiologically relevant, although this modification is constitutive rather than regulatory [40]. Post-translational modifications of SAF-A/hnRNP U thus could provide another layer of complexity for the regulation of Xist RNA localization.

LINE hypothesis and the involvement of S/MAR-associated proteins

One of the striking implications for the interaction between S/MAR-associated factors and Xist RNA is that these proteins might provide a molecular rationale for the chromosomal specificity of Xist-mediated inactivation. In X:A translocations, inactivation originating from Xic spreads into the autosomal segment to a limited extent [16], which has resulted in the “way station” model wherein certain booster elements specific to the X chromosome promote the efficient spreading of the Xic-originated inactivation signals along the entire length of the X-chromosome [41]. Consistently, Xist RNA coats the chromosomal segments

derived from the X chromosome but not the autosomal segments in the X:A hybrid chromosome [42]. The way station model was later extended, and it was suggested that the extraordinarily high frequency of a repeat element long interspersed nuclear element 1 (LINE1) (Box 1) in the X chromosome is responsible for the efficient spreading of the inactivation signal [43]. Interestingly, the regions on human Xi that include genes that escape XCI contain relatively low levels of LINE1, giving further support for this hypothesis [44]. Several recent studies examining the spatial organization of the X chromosome have proposed possible roles for C₀t-1 repeat elements (Box 1) in XCI [28,29]. During the early stages of XCI in human and mouse cells, Xist RNA creates a core silencing compartment consisting of gene-sparse, repeat-enriched chromosomal segments and the gene-rich regions are subsequently recruited to the silencing compartment in a Xist-dependent manner [28,29]. Furthermore, a recent elegant study showed that an X-chromosome that undergoes XCI transiently transcribes young LINE1, which in turn might facilitate the local spreading of silencing at later stages of XCI [45]. All of these results are in accord with the LINE hypothesis, in that the competence of Xist-mediated silencing associates with confined levels of LINE1. Intriguingly, LINE1 contains experimentally confirmed S/MAR sequences that interact with SATB1 *in vivo* [46]. We thus emphasize that S/MAR sequences might function as booster elements in the X chromosome, providing entry sites for SATB1 and SAF-A/hnRNP U to facilitate the formation of the inactivation core complex and the subsequent chromosome-wide silencing. SATB1 preferentially binds to the so-called ATC sequence, which contains a stretch of A or T with asymmetric distribution of G/C residues [21,46]. These sequences are not reported for SAF-A/hnRNP U-binding elements, [32–34], suggesting the possibility that SATB1 and SAF-A/hnRNP U might occupy distinct regions along the X-chromosome. At the chromosomal level, SAF-A/hnRNP U and SATB1 show differential distribution; SAF-A/hnRNP U is non-randomly distributed at the pericentromeric DNA [47], whereas SATB1 displays a cage-like distribution, at least in T-cells [26]. Whatever the case, the identification of the X-chromosomal regions that associate with SATB1, SAF-A/hnRNP U and other S/MAR-associated factors during X-inactivation might provide an important insight into how the X chromosome is preferred over the autosome for Xist-mediated chromosomal inactivation (Figure 2c).

Box 1

Repeat elements

The genome of higher eukaryotes contains a number of repeat elements, which was originally recognized by study of a fraction of C₀t-1 DNA showing the differential kinetics of re-hybridization of denatured DNAs [69]. The repeat elements are largely divided into transposable elements and non-transposable tandem arrays of short sequences, such as SatIII (Satellite III) in the human genome (<http://www.girinst.org/repbse/index.html>). The transposable elements are further divided into three families: DNA transposons, retrotransposons with a long terminal repeat (LTR) and non-LTR type retrotransposons. Long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs) belong to the family of non-LTR-type retrotransposons and constitute a large fraction of the noncoding region of the mammalian genome. Most LINEs and SINEs have lost the ability to transpose due to deletions of essential sequence elements for retrotransposition; however, certain ‘young’ LINEs, which are supposed to have emerged relatively recently during evolution, are active and transcribed into RNAs under specific conditions [45]. Although most of these repeat elements have long been considered junk DNA, recent studies have demonstrated that small RNAs derived from

these repeat sequences control the establishment and maintenance of heterochromatin in lower eukaryotes and in plants [70].

S/MAR-associated proteins and lncRNA-mediated epigenetic gene regulation

Large-scale, genome-wide transcriptome analysis has identified an unexpectedly large number of long non-protein-coding RNAs (lncRNAs) transcribed from a wide region of the mammalian genome [48]. While the physiological roles of these lncRNAs largely remain to be elucidated, several studies indicate that many lncRNAs epigenetically repress target gene expression through interaction with epigenetic modifiers [48]. Such lncRNAs include antisense Igf2r RNA (Air) [49], Kcnq1 overlapping transcript 1 (Kcnq1ot1) [50,51], HOTAIR [52,53], lincRNA-p21 [54] and ANRIL [55]. Because approximately 20% of intergenic lncRNAs are associated with histone methyl transferase PRC2 [56], gene regulation through epigenetic modification might be one of the principal roles of lncRNAs [48]. Functionally characterized lncRNAs involved in epigenetic processes typically regulate multiple genes *in cis* surrounding their transcription sites [48]. Consistently, RNA *in situ* hybridization signal of Air RNA spreads from the site of transcription and covers the neighboring chromosomal region that, while a small area, resembles the Xist RNA [49]. This is the case also for Kcnq1ot1 RNA, which does overlap with the genomic region that contains imprinted genes regulated by this lncRNA [57]. Taking the close similarity between the mode of action of Xist and other epigenetic-related lncRNAs into account, we propose that S/MAR-binding proteins might also be involved in the stabilization of these lncRNAs around their transcription sites and their spreading in epigenetically controlled genomic regions (Figure 3). Interestingly, clusters of S/MAR sequence elements are mapped to imprinted genomic regions [58,59], and binding of differentially methylated regions of imprinted Igf2 genes to the matrix is tissue-specific and allele-specific [60]. Again, it will be crucial to map the genomic regions that interact with S/MAR-associated proteins, such as SAF-A/hnRNP U and SATB1, to determine whether they interact with known imprinted regions in the genome.

Epilogue: Do other nuclear lncRNAs meet the nuclear scaffold/matrix?

Several recent studies have demonstrated that a fraction of cellular RNAs are retained within the mammalian cell nucleus and localize to specific sub-nuclear compartments [61,62]. Examples of such nuclear-retained lncRNAs include paraspeckle-localized NEAT1 and CTN-RNA, nuclear speckle-associated MALAT1/NEAT2, telomere-localized TERRA and others that associate with imprinted gene loci [61,62]. A group of lncRNAs such as Gomafu [63] and GRC-RNAs [64] remain nuclear-localized even after prolonged treatment with salt and DNase, indicating that these lncRNAs are tightly associated with specific sub-nuclear structures, similar to Xist RNA [17] and nuclear poly(A)⁺ RNAs [65]. The fate of transcribed RNA is regulated by associated proteins recruited either co-transcriptionally or post-transcriptionally [18]. We predict that protein components like S/MAR-associated proteins, including SAF-A/hnRNP U, serve to control the sorting of various RNA polymerase II-transcribed products to functionally appropriate destinations (Figure 3). Conventional homology searches and secondary structure prediction algorithms fail to identify conserved sequence elements common to all of the nuclear-retained lncRNA, indicating that they utilize independent nuclear retention mechanisms. One of the problems that complicates the identification of nuclear retention elements is that they tend to be distributed redundantly throughout the length of the transcript, as has been shown for Xist [66], MALAT1 [67] and Gomafu [68]. Therefore, it is possible that each lncRNA associates with a distinct set of proteins, and specific combinations or ‘codes’ of these RNA-binding

proteins, including S/MAR-binding proteins, determine the final location of the transcripts. Determining how the structural architecture of the nucleus modulates nuclear lncRNA distribution and function is anticipated to keep several laboratories engaged in the coming years.

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Figure 1.

The role of SATB1 in organizing the entire Xi chromosome into a core silent domain. **(a)** The central region of the X chromosome containing Xic and highly clustered Xist entry sites (presumably S/MAR, see Figure 2) could form the core repressive compartment, along with Xist and other associated chromatin-modifying factors. SATB1 accomplishes chromosome-wide transcriptional silencing by interacting simultaneously with Xist RNA and S/MAR. **(b)** Xist RNA distribution at Xic and the formation of the core repressive compartment is independent of SATB1 levels in the cell; however, the Xi in SATB1-depleted cells fails to organize into a highly compact structure.

Figure 2.

The effect of SAF-A/hnRNP U knockdown on the localization of Xist RNA on Xi and models depicting the involvement of SAF-A/hnRNP U in the spreading of Xist RNA on the X chromosome. **(a)** Xist RNA-FISH (green) and SAF-A/hnRNP U immunolocalization (magenta) analysis in the SAF-A/hnRNP U-depleted cultured cell line Neuro2A shows the dissociation of Xist RNAs from the Xi. Note that the cells that express SAF-A/hnRNP U (magenta) continue to show localization of Xist RNA in Xi. **(b)** Localization of SAF-A/hnRNP U in the interphase and mitotic cell. Note that SAF-A/hnRNP U is excluded from the condensed chromosome during mitosis, and this distribution is in contrast to the localization on condensed chromosomes detected by other methods, as reported earlier [34,37]. SAF-A/hnRNP U could thus exist in different structural conformations, which are specifically recognized by distinct detection methods. **(c)** Xist localization on the X-chromosome and autosome. Xist RNAs are either tethered on SAF-A/hnRNP U already deposited on the S/MAR elements or initially form RNP complexes with free SAF-A/hnRNP U and are subsequently recruited to the chromosome. In the autosome, the number of S/MAR elements that can serve as entry sites for SAF-A/hnRNP U is below the threshold

required to induce the higher-order assembly of the SAF-A/hnRNP U-Xist RNP complex, thus failing to establish an RNP-coated chromosome. **(d)** Possible link between the post-translational modification of SAF-A/hnRNP U and release of Xist RNA from the mitotic chromosome. Xist RNA is released from the X chromosome during mitosis [17]. Because the mitotic kinase Aurkb negatively regulates the chromosomal localization of Xist RNA [39], phosphorylation of SAF-A/hnRNP U by Aurkb could inhibit the interaction of SAF-A/hnRNP U with S/MAR, leading to dissociation of the RNP complex from the chromosome. The photographs are reproduced courtesy of Y. Hasegawa (RIKEN ASI, Japan).

Figure 3.

The fate of protein-coding mRNAs and lncRNAs. In the case of protein-coding genes **(a)**, transcribed mRNAs (broken curve) are released from the transcription site post 3' cleavage and post polyadenylation and immediately exported to the cytoplasm through their interaction with export factors such as NXF1/NXT1 (orange circles). Nuclear lncRNAs **(b)** (unbroken curve) are retained in the nucleus. These lncRNAs associate with nuclear scaffold/matrix proteins (purple stars) that might tether these transcripts at their transcription sites. The balance between export factors and retention factors presumably controls the fate of these RNAs.