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Minireview

The long and the short of it: RNA-directed chromatin asymmetry in mammalian X-chromosome inactivation

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ABSTRACT

Mammalian X-chromosome inactivation is controlled by a multilayered silencing pathway involving both short and long non-coding RNAs, which differentially recruit the epigenetic machinery to establish chromatin asymmetries. In response to developmentally regulated small RNAs, dicer, a key effector of RNA interference, locally silences *Xist* on the active X-chromosome and establishes the heterochromatin conformation along the silent X-chromosome. The 1.6 kb RepA RNA initiates silencing by targeting the PRC2 polycomb complex to the inactive X-chromosome. In addition, the nuclear microenvironment is implicated in the initiation and maintenance of X-chromosome asymmetries. Here we review new findings involving these various RNA species in terms of understanding *Xist* gene regulation and the establishment of X-chromosome inactivation.

1. Introduction

In mammals, dosage compensation between XX females and XY males is achieved by inactivation of one of the two X-chromosomes during early female embryogenesis. This process of X-chromosome inactivation (XCI) results in the two X-chromosomes of each cell being packaged into contrasting yet stably maintained chromatin configurations. The role of non-protein coding RNAs in XCI was well established with the discovery of the 17.4 kb functional *Xist* transcript [1–3], followed by the description of the 40 kb regulatory *Tsix* antisense transcript in mouse [4]. Recently additional RNA factors have been described, adding to the complexity of our understanding of XCI establishment. Like *Xist* and *Tsix*, these newly described RNAs act at the level of determining contrasting chromatin conformations between the two X-chromosomes.

2. The long RNAs *Xist* and *Tsix* determine X-chromosome asymmetry

In the mammalian blastocyst, cells of the inner cell mass (ICM) have functionally indistinguishable X-chromosomes. This follows reactivation of the paternally silenced X-chromosome of the cleavage-stage embryo, which displays imprinted X-inactivation, a process which is beyond the scope of this review. From the perspective of random XCI, cells of the ICM, which can be cultured in vitro as embryonic stem (ES) cells, are considered to be in the pre-XCI state. All of the molecular events of random XCI have been successfully recapitulated by differentiating ES cells in culture, thus providing an ex vivo model system for investigating the mechanisms underlying random XCI.

In pre-XCI ES cells, *Xist* and *Tsix*, the non-coding sense and antisense transcript pair which map to the *X-inactivation centre* (*Xic*) locus, are biallelically transcribed, but *Tsix* is present in large excess over the residual level of *Xist* expression. Biallelic *Tsix* expression is correlated with active chromatin over the *Xist* gene body. This is essential for resetting the epigenetic marks prior to the onset of random XCI [5]. A recent investigation has linked the inhibition of *Xist* to the major pluripotency factors Nanog, Oct3/4 and Sox2, which are expressed specifically in ES cells and repress *Xist* transcription by binding to its first intron [6]. Upon loss of pluripotency factors and gain of differentiation-specific transactivating factors, XCI is initiated, by which *Xist* expression is upregulated on the future inactive X-chromosome (Xi), and *Tsix* becomes restricted to the future active X-chromosome (Xa).

High level transcription of *Xist* on the future Xi and coating of the transcript along the length of the chromosome leads to the adoption of a heterochromatic chromatin configuration devoid of

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RNA polymerase II (RNApolII) [7]. This is followed by hierarchical changes in the epigenetic content of the chromatin of Xi, mediated by recruitment of heterochromatin complexes. However, on the future Xa, *Tsix* expression blocks the *Xist*-mediated XCI by epigenetic silencing of the *Xist* promoter. Although the direct interaction of *Xist* RNA with the heterochromatin machinery has not yet been demonstrated, loss of *Xist* expression corresponds to the loss of recruitment of heterochromatin factors to Xi, such as the PRC2 polycomb complex responsible for trimethylation of histone H3 lysine 27 (H3K27) [8]. In addition, targeted deletion of the heterochromatin modifier EED, a PRC2 complex member which acts as a cofactor for H3K27 trimethylation, results in abnormalities in the maintenance of XCI [9]. *Xist*-mediated recruitment of the PRC2 complex, therefore, is crucial for both establishment and maintenance of XCI.

Xist regulates its well-characterized functions, such as chromosomal localization and transcriptional silencing, through defined functional sequence motifs. For example, an A-rich repeat element at the 5′ end of *Xist* is crucial for transcriptional silencing, and acts by relocating Xi into an RNApolII deficient region of the nucleus [7]. Chromatin localizing regions of *Xist* which serve to tether the RNA to the chromosome have been mapped along the entire transcript in a functionally redundant fashion [10]. No specific functional sequence motifs have previously been characterized within the *Tsix* transcript to provide mechanistic clues as to its crucial role in inhibiting *Xist* expression on Xa.

Using in vitro differentiation of female ES cells as a model system, Lee et al. have shown that on the future Xa, *Tsix* RNA interacts with DNMT3a to direct DNA methylation over the *Xist* promoter [11]. However, it is not clear whether this *Tsix*-directed methylation is a primary or secondary mechanism in *Xist* repression. On the future Xi, *Tsix* downregulation leads to the formation of a transient heterochromatic configuration over the *Xist* promoter, which paradoxically induces a high level of *Xist* transcription [11]. In the differentiation of male ES cells, functional redundancy in *Xist* promoter repression mechanisms has been demonstrated: combined mutations in *Tsix* and *Eed*, but not either single mutation, results in reactivation of *Xist* upon differentiation [12].

Post-XCI cells are characterized by contrasting histone modifications over the *Xist* promoter alleles. Navarro et al. have shown that a high H3K4 dimethylation level on Xi, but not differential H3K9 or H3K27 trimethylation, underlies the asymmetric expression of *Xist* [5]. In contrast, a study by Sado et al. suggested that H3K27 trimethylation plays a critical role in designating the active *Xist* allele [13]. However it is unclear whether these chromatin modifications are involved in the establishment of monoallelic *Xist* expression or only in its maintenance.

3. A potential role for small RNAs in establishing XCI

Although a functional role for small RNA molecules in XCI has long been suggested due to the potential for duplex formation from the overlapping sense *Xist* and antisense *Tsix* transcripts [5], it has only recently been specifically investigated. Again by exploiting the in vitro differentiation of ES cells, Lee et al. were able to demonstrate the dicer-dependent allele-specific formation of small RNA molecules during the establishment phase of XCI [14]. Despite the intriguing results of this study, many questions remain about the function of these small RNA species.

Designated as xiRNAs for their *X-inactivation centre* origin, these RNAs range in size from 25 to 42 nucleotides and originate from several regions along the *Xist* gene, including the promoter, A-repeat silencing region, and exon 7, and are detected in both sense and antisense orientations. xiRNAs are produced in male as well as female ES cells, so can be formed on Xa, and they are detected specifically during the initiation phase of XCI (between day 4 and day 10 of ES cell differentiation), but are not in pre- or post-XCI cells. Their appearance is inversely correlated with that of duplex RNAs, presumably their precursors, which are predominantly seen in pre-XCI ES cells. As duplex RNAs can be detected on both X-chromosomes in pre-XCI cells, it is thought that while the duplexes remain stable on Xi, they are specifically processed into xiRNAs on Xa, where they act locally in *cis* to silence the *Xist* promoter through directing modification of its chromatin structure. This was proposed to occur in a manner similar to the small RNA-mediated transcriptional gene silencing of centromeric repeats in fission yeast, via recruitment of the RNA-induced transcriptional silencing (RITS) complex. While this process is consistent with the *Tsix* RNA-dependent CpG methylation of the *Xist* promoter on Xa, as described above, evidence for a mammalian RITS complex is currently lacking.

Interestingly, mutation of *dicer*, encoding an endonuclease which processes precursor RNA duplexes into small RNAs, causes loss of xiRNA formation, loss of H3K27 trimethylation along the future Xi, and decreased methylation at the 5′ end of *Xist* on the future Xa, indicating that dicer affects the chromatin composition of both X-chromosomes. This was proposed to mean that the dicer-dependent RNAi pathway acts on Xi as well as on Xa, yet this interpretation raises several issues. Mammalian dicer is thought to be cytoplasmic [15], and known products of dicer cleavage are of 21–23 nucleotides, making a direct role for dicer in xiRNA generation and chromatin modification uncertain. However, there is evidence for several pathways by which dicer may act indirectly in establishing local promoter silencing on Xa and global transcriptional silencing along Xi. It has been documented that dicer defective chicken cells have heterochromatin defects at centromeres [16], indicating that dicer may have a conserved role in heterochromatin formation, although this may be an indirect effect through which loss of microRNA formation leads to general methylation defects, through regulating de novo methyltransferase levels via the transcriptional repressor Rbl2 [17]. A recent investigation has documented that genomic loss of microRNA-101 leads to overexpression of the PRC2 complex component Ezh2 in human cancer cell lines, indicating a link between dicer and PRC2 via microRNAs [18]. Nesterova et al. studied dicer-null ES cells, and also conclude that the observed effects on Xa are due to a decrease in the level of de novo methyltransferases rather than direct effects of dicer [19].

Given these experiments, it is possible that both loss of de novo methylation of the *Xist* promoter on Xa and loss of PRC2-dependent H3K27 trimethylation along Xi in dicer mutant cells could be due to such indirect effects, rather than to the loss of xiRNA generation from *Xist*. Nonetheless, the appearance of allele-specific small RNAs processed from the duplex RNAs during the initiation of X-inactivation is strongly suggestive of a functional role for these small RNAs in XCI. Importantly, given the cytoplasmic localization of dicer in mammalian cells and the pleiotropic effects of its mutation on the chromatin of both Xa and Xi, this opens up a functional role for a yet-unidentified nuclear localized duplex RNA-specific endonuclease in the generation of xiRNAs. As a nuclear localized protein, drosha could be such a candidate xiRNA processing enzyme [20].

4. A mid-length RNA adds to the complexity

A recent investigation by Lee and colleagues suggests that the transient heterochromatic configuration of the *Xist* promoter on the future Xi is mediated by the targeted recruitment of the PRC2 complex by a 1.6 kb transcript, RepA [21]. This RNA is encoded from an internal promoter on the *Xist* sense strand and covers the A-rich repeat region. RepA is expressed from both

X-chromosomes in pre-XCI cells at levels marginally higher than *Xist* but several fold lower than *Tsix*. Expression of RepA is slightly increased in post-XCI cells, but not to the same degree as *Xist* induction, suggesting differential regulation of the two promoters. Binding of RepA to PRC2 can be detected in pre-XCI cells, yet targeting of the PRC2 complex to the chromatin occurs only at the onset of XCI, when it activates the *Xist* promoter specifically on Xi. The functional structure of RepA is a double stem–loop motif, which binds directly to the Ezh2 subunit of the PRC2 complex. It was suggested that *Tsix* can also bind PRC2 using the same stem–loop motif, and that the excess *Tsix* RNA in pre-XCI cells sequesters the RepA–PRC2 complex away from the *Xist* promoter, thus neutralizing its actions prior to the onset of XCI. However, during XCI, the downregulation of *Tsix* enables the RepA–PRC2 complex to interact with and activate the *Xist* promoter through H3K27 trimethylation [21]. The RepA–PRC2 complex may be required not only to direct this promoter-specific H3K27 trimethylation, but also to enable the spreading of H3K27 trimethylation along Xi, although it is not clear how the RepA transcript, lacking chromosomal localizing signals, could direct long-range targeting of the PRC2 complex along the chromosome.

In a related study, targeted deletion of the A-rich repeat region from the *Xist* locus in mouse cells resulted in complete loss of *Xist* transcription, thus supporting a functional link between the A-rich repeat region, perhaps due to this internal RepA transcript, and *Xist* activation [22]. However, it is not yet known how the observed PRC2-dependent transient heterochromatin formation over the *Xist* promoter directs *Xist* upregulation, so the mechanisms underlying this intriguing phenomenon remain to be investigated.

Although the RepA transcript was shown to be present in both male and female ES cells before XCI, it is restricted to females, and thus to Xi, in post-XCI cells. Interestingly, the observation that the repeat A region recruits the PRC2 complex to both sense and antisense RNA strands highlights the fact that perhaps *Tsix* itself could specifically inactivate the *Xist* promoter on Xa through recruiting the PRC2 complex [21]. This suggestion is consistent with the previous observations that the *Xist* promoter on Xa is enriched with H3K27 trimethylation in post-XCI mouse embryonic tissues, and that the PRC2 component Eed is involved in *Xist* promoter repression in male ES cells. In particular, since Eed can also repress the *Xist* promoter in the absence of *Tsix*, this raises a plausible role for RepA in targeting the PRC2 complex to the *Xist* promoter on Xa [12,13].

Such a possibility then raises the important question of how does the H3K27 trimethylation mark specifically activate the *Xist* promoter on Xi, while repressing it on Xa? Interestingly, it has been shown that in ES cells, sense transcription across the *Xist* promoter determines the choice of the X-chromosome to be inactivated by maintaining hypomethylation over the promoter [19]. As such, the different chromatin contexts previously established between the two X-chromosomes at the level of DNA methylation could determine how the H3K27 trimethylation mark is

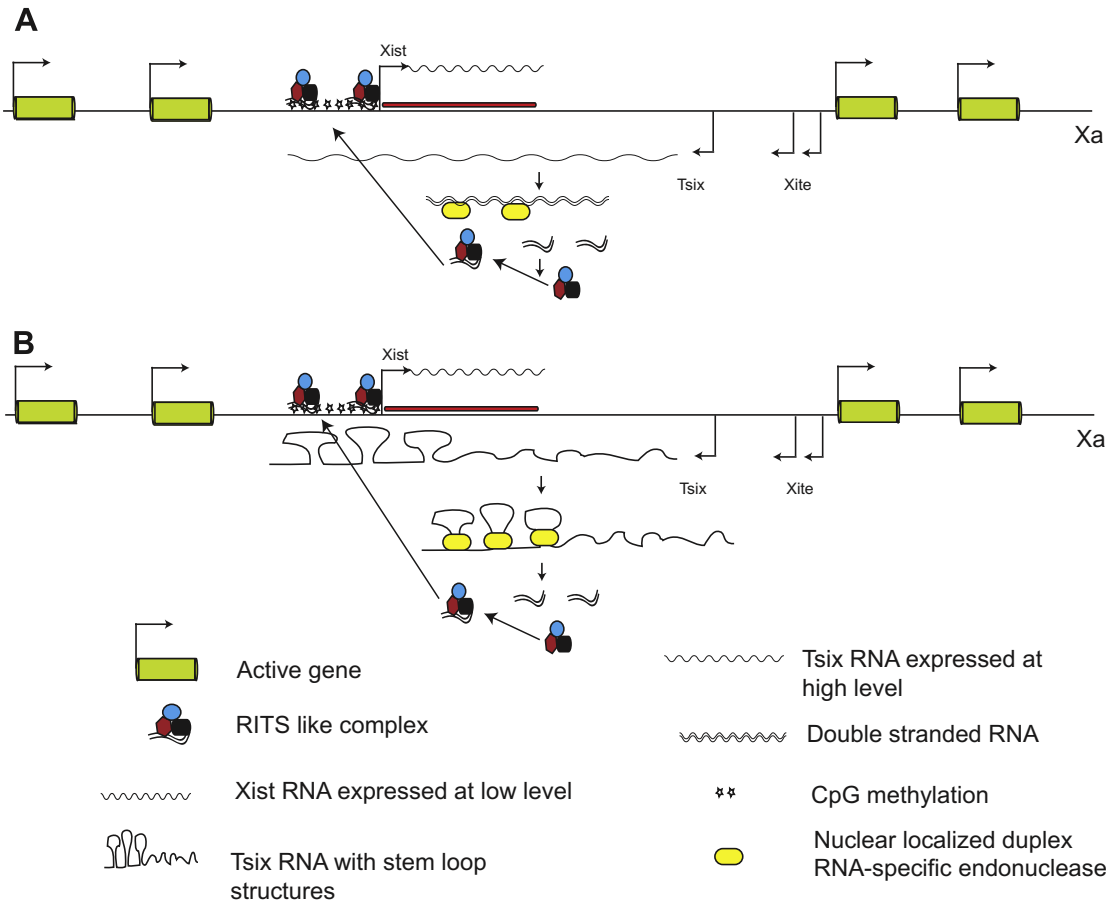

Fig. 1. Model of epigenetic silencing of the *Xist* promoter on Xa by small RNAs. (A) On Xa, transcription from both strands produces duplex RNA, which is processed by an endonuclease, such as dicer or drosha, into small xiRNA. (B) Alternatively, *Tsix* transcripts, which are produced in excess over *Xist* transcripts, could form stem–loop structures corresponding to the *Xist* promoter, which are then processed into xiRNAs in a dicer or drosha-dependent manner. In either case, the xiRNAs form part of a RITS-like complex, which is targeted to the *Xist* promoter through base pair interactions with promoter DNA sequences, leading to local methylation and silencing of the *Xist* promoter.

interpreted by the chromatin remodeling machinery. Such a scenario highlights the complex interplay between the various epigenetic marks and chromatin remodeling complexes.

5. Visualising the role of small RNA molecules in XCI

The essential process of XCI is thought to be the activation of *Xist* on the future Xi, leading to accumulation of *Xist* RNA along the chromosome, which then initiates a cascade of epigenetic changes in the chromatin of Xi through recruitment of the epigenetic machinery. However, the recent evidence described here adds further levels of complexity to this model.

Since functional roles have been suggested for small RNAs as well as for *Tsix* in the repression of the *Xist* promoter on Xa, there is a need to distinguish between the functions of these different RNA species in *Xist* repression. Localized methylation of the *Xist* promoter on Xa could arise due to base pair interactions between promoter-specific xiRNAs and the chromatin, with the resulting RNA–DNA hybrid formation leading to recruitment of the heterochromatin machinery and CpG methylation of the *Xist* promoter (Fig. 1). This model is consistent with the observation that promoter-directed siRNAs inactivate the corresponding endogenous promoter in cultured cells through directing CpG methylation [23], and with the requirement for *Tsix* RNA in directing *Xist* promoter methylation on Xa [11].

There are several ways of testing such a model. The presence of RNA–DNA hybrids, and their association with DNA methyltransferases could be detected by immunoprecipitation using Dnmt-specific antibodies before and after RNaseH digestion, or by using FRET to detect specific associations between tagged RNA and DNA sequences and Dnmts. Functional studies could be carried out in *Tsix* deficient cells by adding exogenous xiRNAs or duplex RNAs, to determine whether in this context, *Tsix* is acting primarily as a source of small RNAs, rather than as a full length transcript.

Since there remains a residual level of expression from the promoters of *Xist* and *Tsix* on Xa and Xi, respectively, one can visualize the formation of similar amounts of duplexes on both chromosomes. However, if dicer is indeed involved directly in heterochromatinization of Xi, it is unclear how the duplexes specifically on Xi would then escape dicer processing. The demonstration of a link between localization to nuclear heterochromatin compartments and gene silencing [24–26] provokes the question of whether nuclear compartmentalization might play a role in allele-specific stabilization of duplex RNA. Recent investigations have shown that during XCI, perinucleolar localization of Xi occurs shortly after transient pairing of the two X-chromosomes in differentiating

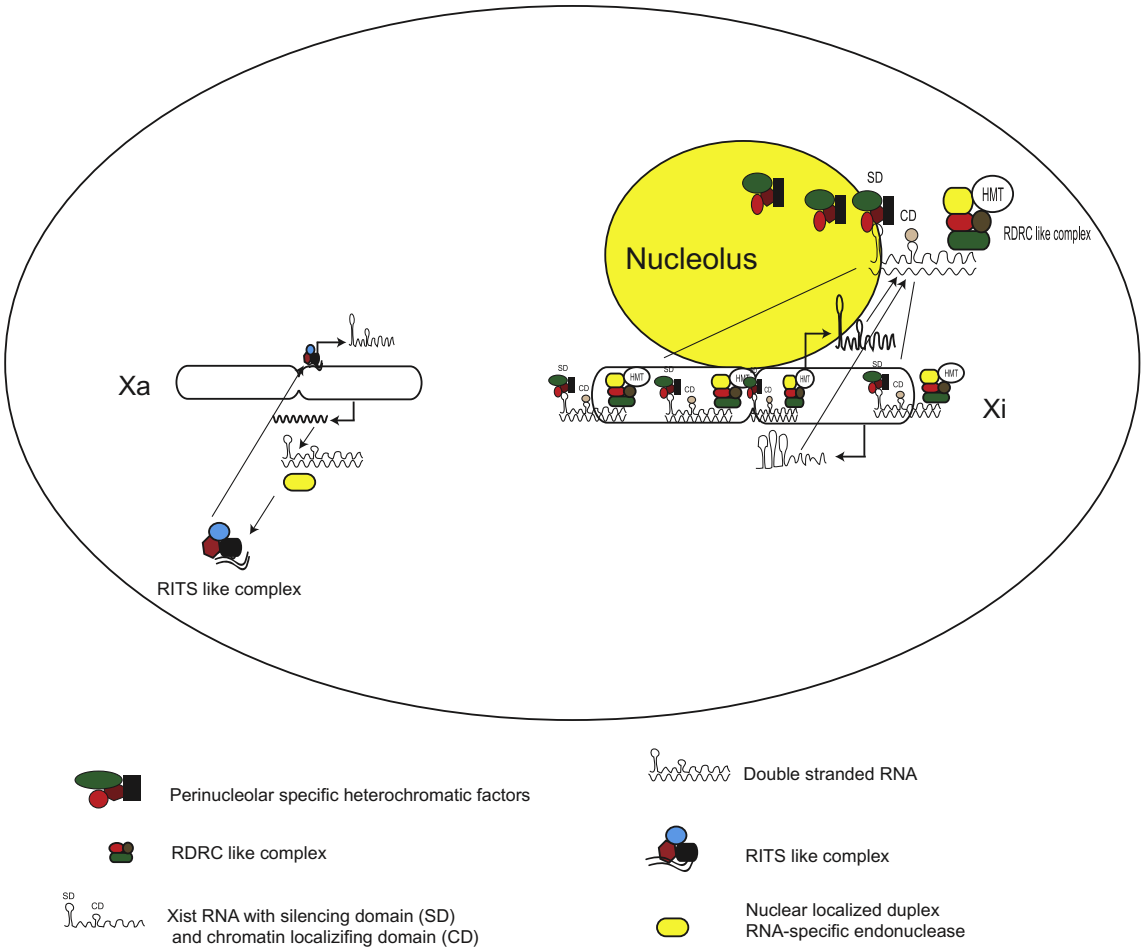

Fig. 2. Nuclear compartmentalization could contribute to the stability of duplex RNA on Xi. Following transient pairing of the X-chromosomes during XCI, Xi is targeted specifically to the perinucleolar region, where it is exposed to the heterochromatin factors required for chromosome-wide transcriptional silencing. The functional sequence motifs in the *Xist* strand of the duplex could recruit the heterochromatin machinery, and target it along Xi via the chromatin localizing signals of *Xist*. The duplexes could also attract an RDRP-like complex containing an RNA-dependent RNA polymerase, histone methyltransferase and duplex RNA-specific endonuclease. On Xi, the nuclear localized endonuclease may be prevented from processing the duplexes into xiRNAs due to their interaction with the heterochromatin factors. The RDRP complex may also synthesize further dsRNA from the *Xist* RNA to maintain a sufficient pool of dsRNA on Xi. In contrast, Xa remains in the nucleoplasm, where no heterochromatin factors are present to protect the duplexes from endonuclease processing.

female mouse ES cells [24], suggesting that this compartmentalization facilitates exposure of Xi to heterochromatin factors. As RepA interacts directly with the PRC2 complex, which is enriched in the perinucleolar region, it is possible that the RepA transcripts associated specifically with Xi have a functional role in perinucleolar targeting, and thus in stabilizing the duplexes on Xi. This is consistent with the demonstrated requirement for the A-repeat region in localization of Xi to an RNApolII deficient nuclear environment [7].

We propose that the duplex RNA could act as adaptors, facilitating the association of functional sequences within the *Xist* RNA with heterochromatin factors, while protecting the duplexes on Xi from the cleavage actions of a nuclear localized duplex RNA-specific endonuclease, such as drosha. The chromatin localizing signals of *Xist* would then guide the duplex RNA and associated factors, including the PRC2 complex, along Xi, leading to chromosome-wide trimethylation of H3K27. Since Xa remains in a different nuclear environment without access to the heterochromatin machinery, the xiRNAs on Xa may therefore have very different consequences, despite the presumed association of duplex RNA-specific endonuclease with both X-chromosomes (Fig. 2). Although the current investigation favours the formation of xiRNAs only on Xa, the data presented do not exclude the possibility that some of the duplexes on Xi could also be processed into xiRNAs. If so, these xiRNAs, rather than the duplex precursors, could be responsible for chromosome-wide targeting of the heterochromatin machinery. However, if the xiRNAs have a role in directing chromosome-wide H3K27 trimethylation, how would they achieve this only on Xi, and not on Xa where their effects are restricted to the *Xist* promoter?

We propose the following model, resembling siRNA-mediated heterochromatin assembly in fission yeast, to explain the potential xiRNA activity on Xi (Fig. 3). With the onset of XCI, RepA mediated increased expression of *Xist* over *Tsix* favours the accumulation of *Xist* along the chromosome, resulting in the formation of a heterochromatin conformation devoid of RNApolII, as well as the targeting of Xi to the perinucleolar region. The residual level of *Tsix* expression on Xi ensures the formation of duplex RNAs, and their further processing into xiRNAs by the RNAi machinery could be carried out by a nuclear localized duplex RNA-specific endonuclease. Upon synthesis, xiRNAs form part of the heterochromatin machinery, analogous to the RITS complex, and interact with *Xist* through RNA–RNA interactions. We presume that as in fission yeast, histone methyltransferases could form part of a RITS-like complex in mammals, and so play a critical role in connecting the heterochromatin machinery with *Xist* RNA. This proposal, however, is contingent on the demonstration of a mammalian RITS-like complex, and on the activity of an uncharacterized nuclear localized duplex RNA-specific endonuclease, and thus awaits experimental validation.

Given that in yeast, the RNA-directed RNA polymerase (RDRP) complex can associate directly with the RITS complex in a dicer-dependent manner, we propose that despite an initially low level of xiRNA in relation to *Xist* RNA on Xi, an RDRP-like complex associated with a duplex-specific endonuclease, could synthesize double stranded RNA from the single stranded *Xist* RNA, thus supplementing the xiRNAs on Xi to reinforce silencing [27]. These large ribonucleoprotein complexes could then spread along Xi through the chromatin localizing signals of *Xist*, thus initiating

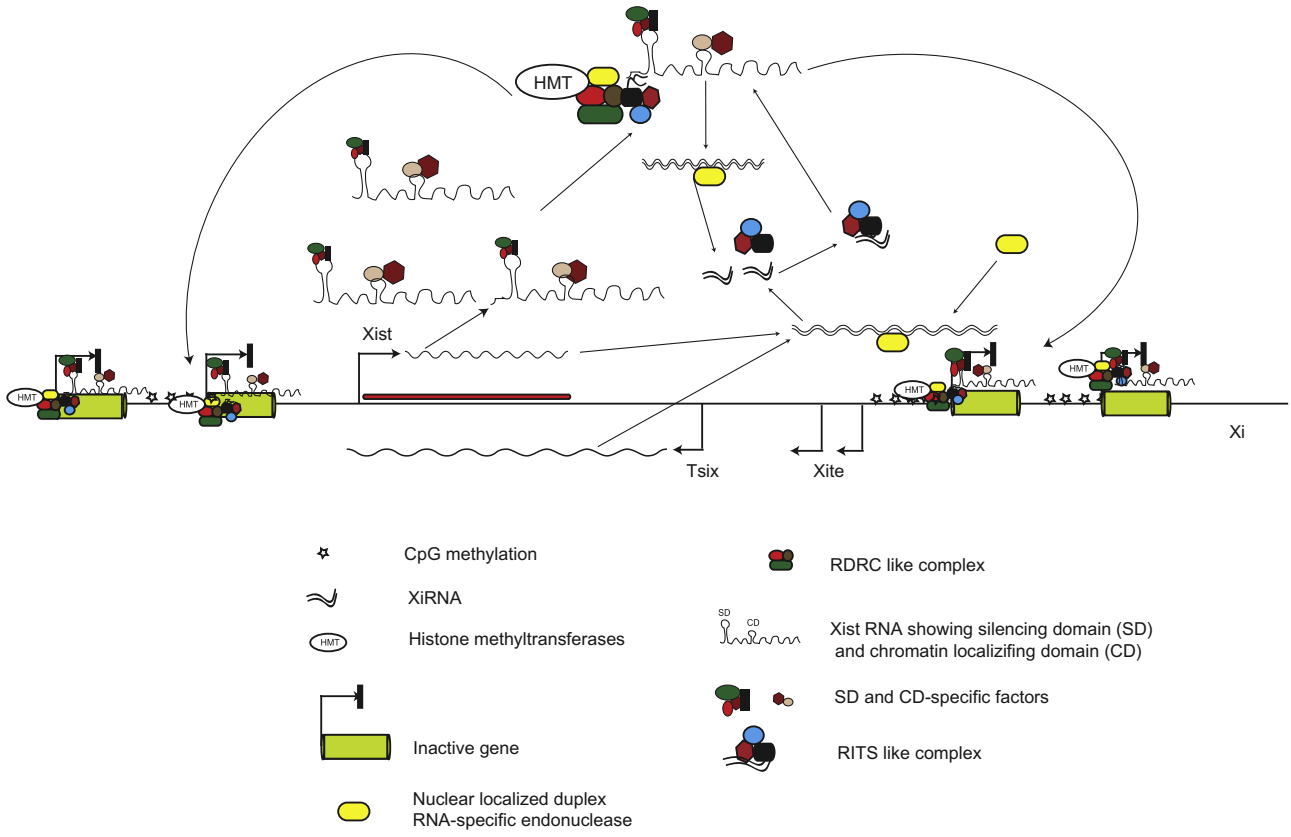

Fig. 3. Model proposing a functional role for xiRNAs in transcriptional silencing of Xi. Some of the duplexes may be processed by the nuclear localized endonuclease into xiRNAs, forming part of the RITS-like complex, which is then targeted along Xi by RNA–RNA interactions with *Xist*. The RITS complex then recruits the RDRP-like complex containing RNA-dependent RNA polymerase, histone methyltransferase and the endonuclease, thus linking RNAi to heterochromatin assembly. This complex, along with the factors associated with the A-repeat rich silencing domain of *Xist* such as the PRC2 complex, spreads transcriptional silencing along Xi through chromatin modification. In addition, the RDRP activity of this complex may also amplify small RNAs on Xi to ensure maintenance of silencing.

chromosome-wide transcriptional silencing. As the X-linked gene MeCp2 is not dosage compensated in dicer deficient clones, demonstrating compromised transcriptional silencing on Xi in the absence of dicer, it seem reasonable that such a complex interaction of factors could establish the epigenetic memory which maintains XCI through subsequent cell divisions.

Although an RDRP enzyme acting in such a capacity has not yet been characterized in mammals, a putative RDRP known as Aquarius has now been identified in mouse oocytes and preimplantation embryos. However its sequence suggests an origin via horizontal gene transfer from a virus, and it is not conserved in other mammals, making Aquarius an unlikely candidate for a critical functional role in XCI [28]. Additionally, it was shown to be expendable for RNAi silencing of exogenous transcripts in oocytes [29], although this does not strictly preclude the proposed role in XCI. Alternatively, it has recently been shown that RNApolII displays RDRP activity [30], providing another candidate in a potential RDRP-dependent xiRNA pathway in mammals. Despite the

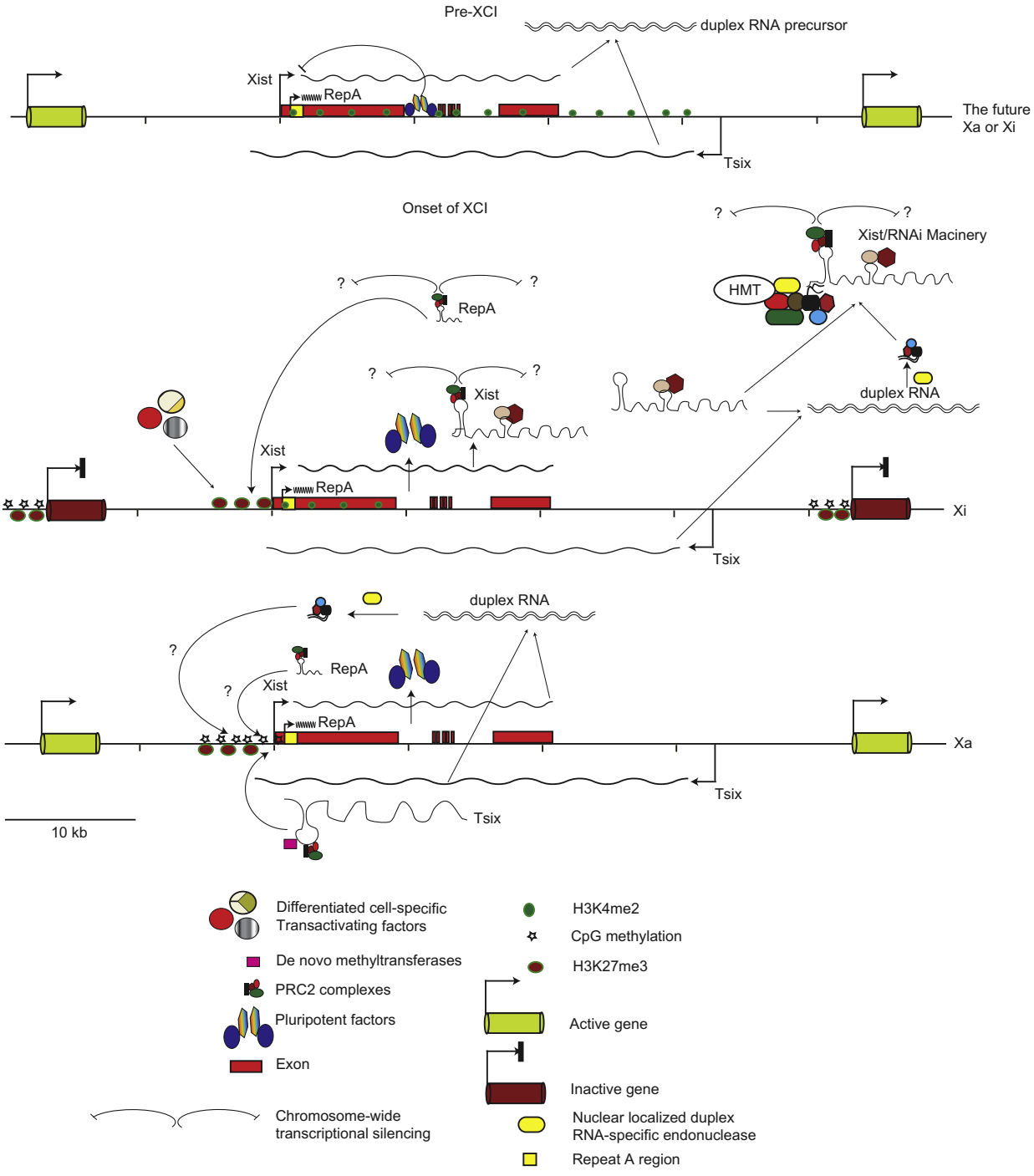

Fig. 4. Model summarizing X-chromosome status in pre-XCI cells and molecular events at the onset of XCI. On both chromosomes in pre-XCI cells, *Xist* is repressed by pluripotency factors, duplex RNAs are formed, and *Tsix* sequesters RepA–PRC2 complexes from the Xist promoter. At onset of XCI, pluripotency factors are repressed and *Tsix* and *Xist* expression are restricted to Xa and Xi respectively. On Xi, RepA activates *Xist* by directing H3K27 trimethylation to the promoter, and *Xist* RNA accumulates along Xi. On Xa, *Tsix* targets CpG methylation and H3K27 trimethylation to repress the *Xist* promoter, aided by xiRNAs and RepA.

generally RNApolII deficient nuclear environment of Xi, there must be a residual level of polymerase present to maintain transcription of *Xist* and other X-linked genes which escape chromosome-wide silencing. Clearly much experimental validation remains in confirming the presence of various factors proposed to be collaborating in establishing the transcriptional silencing of Xi.

6. Integrating RNA-dependent XCI pathways

The recent discovery of additional regulatory RNAs encoded from the *Xic* locus, including the 25–42 nucleotide xiRNAs, their duplex precursors, and the 1.6 kb RepA transcript, each of which is developmentally regulated and acting differentially on Xa and Xi, indicates that our understanding of the molecular events of X-chromosome inactivation is far from complete. The binding partners and functional consequences of the duplexes, xiRNA, and RepA at each X-chromosome remain to be fully elucidated. In addition to deciphering the details of each of these new RNA-directed pathways, the nature of the interactions between these functions and the classical *Xist*/*Tsix*-directed mechanisms of XCI are currently unclear: to what extent do the events directed by these different RNA species represent functional redundancy or molecular synergy?

As all of these RNA-dependent functions converge on chromatin remodelling pathways, it is likely that the various RNAs play distinct but complementary roles in establishing and maintaining X-chromosome asymmetry, as outlined in Fig. 4. In pre-XCI cells, *Xist* on both X-chromosomes is repressed by the pluripotency factors Oct3/4, Nanog and Sox2. Biallelic *Tsix* expression is correlated with active chromatin over the *Xist* gene body. This is essential for resetting the epigenetic marks prior to the onset of random XCI, perhaps by helping the pluripotency factors access their binding sites in the first intron of *Xist*. RepA is also biallelically expressed, but high *Tsix* expression sequesters the RepA–PRC2 complexes away from the *Xist* promoter, to block premature H3K27 trimethylation. Duplex RNAs, formed due to convergent transcription from the *Xist* and *Tsix* promoters, are detected on both chromosomes in pre-XCI cells.

At the onset of XCI and differentiation, *Xist* repression by pluripotency factors is lifted, and *Xist* and *Tsix* expression becomes restricted to the future Xi and Xa, respectively. On the future Xi, RepA recruits PRC2 to initiate local H3K27 trimethylation to activate the *Xist* promoter. This leads to accumulation of *Xist* RNA along Xi and the formation of a heterochromatic environment deficient in RNApolII. It is unclear whether *Xist* acts alone or is aided by RepA in directing PRC2-dependent silencing along Xi. Duplex precursors are processed into small RNAs on Xi, potentially through a drosha or other nuclear endonuclease-dependent RNAi pathway. The resulting xiRNAs have also been implicated in contributing to the heterochromatinization of Xi. On the future Xa, *Tsix* directs specific CpG methylation and possibly H3K27 trimethylation over the *Xist* promoter, perhaps in collaboration with the RNAi machinery. The PRC2 polycomb complex maintains *Xist* promoter repression, directed by RepA.

7. Concluding remarks

We have described possible mechanisms by which duplex RNAs, xiRNAs and RepA may participate in the local silencing of the *Xist* promoter on Xa and in the global silencing of Xi. These models incorporate the recently described nuclear compartmentalization of the two X-chromosomes into different microenvironments, and are based on mechanisms which have been described in other cellular contexts, notably RITS-RDRP-dependent heterochromatinization and transcriptional gene silencing. However such pathways, which are well documented in yeast, have not yet been conclusively demonstrated to act in mammals, and so distinguishing between these and alternative mechanisms necessarily awaits experimental verification.

Of primary concern is to determine the protein factors which make up the complex ribonucleoprotein assemblies at each *Xic* allele during ES cell differentiation, and the kinetics of assembly and spreading of these complexes along Xi. In addition, cross-species comparisons will be useful in determining whether the small and medium RNAs represent conserved mechanisms integral to the evolution of mammalian XCI, or rather these are specific adaptations unique to the mouse genome. Nevertheless, the uncovering of multiple allele-specific developmentally regulated small RNAs adds an interesting twist to the long RNA-regulated X inactivation process.

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