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Chromosome silencing mechanisms in X-chromosome inactivation: unknown unknowns

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Summary

Fifty years ago, Mary Lyon hypothesised that one of the two X chromosomes in female mammalian cells is inactivated at random during early embryogenesis and that the inactive X is then stably maintained through all subsequent cell divisions. Although Lyon's hypothesis is now widely regarded as fact, we should not forget that her conceptual leap met with considerable resistance from the scientific establishment at the time – a common response to new ideas. Taking this point as a theme, I discuss our current understanding of the molecular mechanism of chromosome silencing in X-chromosome inactivation and focus on topics where new findings are challenging the prevailing view.

Key words: Chromatin, Epigenetics, X inactivation, Xist, X chromosome

Introduction

X-chromosome inactivation (XCI) is the process that has evolved in mammals to equalise the dosage of X-linked genes in XX females relative to XY males. Cells of early XX mammalian embryos silence a single X chromosome. Once established, chromosome silencing is stable and heritable through subsequent cell divisions, representing a classical example of epigenetic regulation.

Mary Lyon proposed her XCI hypothesis 50 years ago (Lyon, 1961). Her idea raised two major questions: how do cells of the early embryo appropriately regulate XCI such that only one of the two X chromosomes in female cells is selected; and what is the mechanism for the stable and heritable silencing of genes along the entire chromosome? With the discovery in 1991 of X inactive specific transcript (Xist), the master regulator of XCI (Brown et al., 1991), these questions translate broadly into: what mechanisms underlie the developmental regulation of *Xist* expression; and how does Xist RNA coating trigger chromosome-wide silencing?

This year has seen a number of reviews of the field published to celebrate the fiftieth anniversary of Mary Lyon's landmark discovery (Morey and Avner, 2011; Pontier and Gribnau, 2011; Wutz, 2011). In trying to conjure up something original to say, I found myself reflecting on the now infamous quote from the US politician Donald Rumsfeld regarding 'known knowns', 'known unknowns' and 'unknown unknowns'. Although often ridiculed, Rumsfeld's quote in fact makes some sense and one could certainly apply it to scientific research. In this case, the definition of known knowns and known unknowns is fairly self explanatory: questions for which we are certain of the answer and questions that we know represent gaps in our knowledge, respectively. The definition of unknown unknowns is less obvious, but we can generalise two categories: things that we have never conceived of; and things we think we know but are in fact incorrect and are therefore in reality unknown. The latter can be especially difficult to uncover because of the belief that we already know the answer, which deters serious examination of the alternatives. There are, however, tell-tale signs that can help – small clues, an accumulation of nagging inconsistencies. The natural tendency is to try to rationalise these observations in the context of the dogma or, failing that, to ignore them or set them aside in the hope that they will one day make sense. This is unfortunate because such inconsistencies can be the catalysts of progress, the exception to the rule that can trigger a leap of the imagination, dismantling established dogma and heralding a new era of understanding.

With this in mind I have focused this review on selected known knowns by turning the spotlight on inconsistencies that challenge the prevailing view or dogma. As it is not possible to cover all aspects of the XCI field I have confined my discussion to questions relating to the mechanism for stable and heritable gene silencing. I will discuss observations that challenge the following ideas: that cycles of X inactivation and X reactivation in preimplantation embryos are solely attributable to the modulation of Xist RNA expression; the prevailing view that Xist-mediated silencing is solely attributable to the A-repeat, a domain located at the 5′ end of the transcript; and the view that Polycomb group (PcG) repressors are recruited to the inactive X chromosome (Xi) via direct interaction with Xist RNA.

Xist RNA dependency for X-chromosome silencing

When *Xist* first appeared on the scene, the field discussed two competing models for Xist-mediated chromosome silencing: first, that *Xist* produces a functional non-coding (nc) RNA that recruits factors required for heterochromatin formation; and second that the active *Xist* locus somehow compartmentalises the chromosome, for example by tethering it to a repressive location at the nuclear periphery (Brockdorff et al., 1992; Brown et al., 1992). The former idea, that Xist RNA directly mediates silencing, has since achieved general acceptance and is considered to be a known known (Fig. 1A). A key experiment that underpins this belief is the demonstration that a short tandem repeat region at the 5′ end of Xist RNA, called the A-repeat, is the only sequence absolutely required for chromosome silencing. The introduction of mutations that interfere with the potential of this sequence to form an RNA stem-loop structure ablates silencing function (Wutz et al., 2002).

There are, however, inconsistencies that challenge this known known. It has been demonstrated that *Xist* evolved by pseudogenisation of the autosomal gene ligand of numb protein 3 (*Lnx3*, or *Pdzrn3*), which is protein coding in vertebrates other than mammals (Duret et al., 2006; Elisaphenko et al., 2008). *Lnx3* is X-linked in marsupial mammals but it has unexpectedly retained protein coding potential, demonstrating that marsupials do not have a direct homologue of the Xist ncRNA (Duret et al., 2006). Because marsupial mammals also inactivate one of the two X

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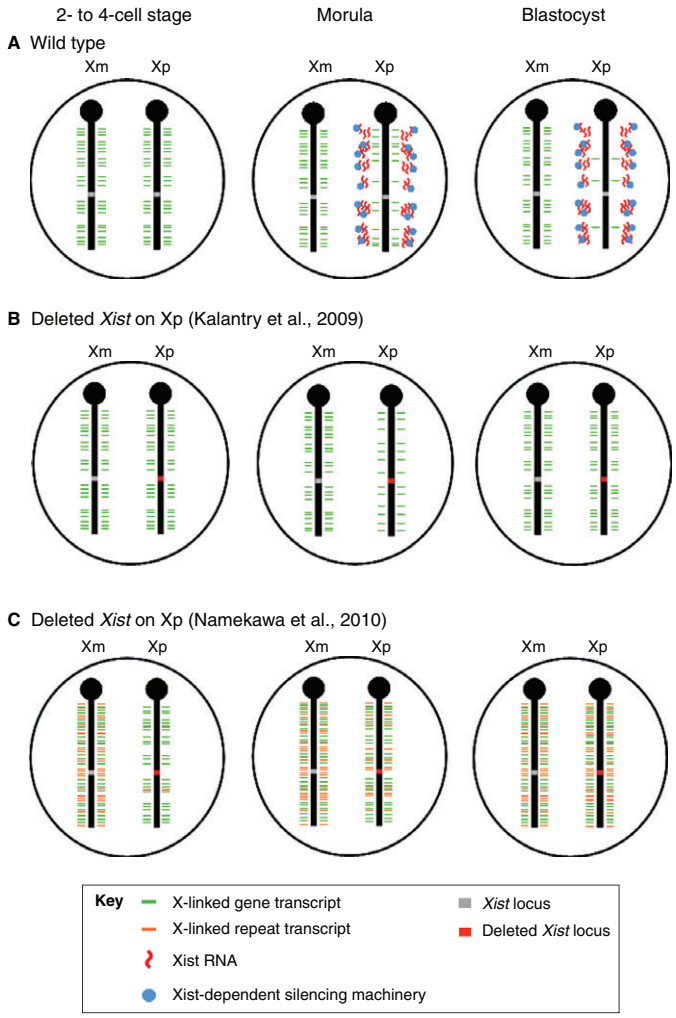

Fig. 1. Xist RNA-dependent and -independent silencing in mouse preimplantation embryos. (**A**) Schematic of the established model of X-chromosome inactivation (XCI), in which maternal (Xm) and paternal (Xp) genes on the X chromosome show equivalent transcription at the 2- to 4-cell stage of development, when *Xist* expression from Xp commences. At the morula stage, Xist RNA has coated the future inactive X (Xi) and the associated silencing of genes has occurred. Genes closest to the *Xist* locus (grey box) are preferentially silenced. At the blastocyst stage, there is extensive silencing of X-linked genes. (**B**) XCI in XX embryos with a deletion of *Xist* (red box) on Xp (Kalantry et al., 2009). Repression of at least some Xp-linked genes is detected at the morula stage. By the blastocyst stage, Xp repression is no longer detectable, indicating a requirement for Xist RNA only in the maintenance of imprinted XCI. (**C**) XCI in XX embryos with a deletion of *Xist* on Xp (Namekawa et al., 2010). No detectable repression of Xp-linked genes occurs at the morula stage. However, the repression of Xp-linked repeat sequences from 2-cell to morula stages is observed. Xp repeat silencing is then lost by the blastocyst stage.

chromosomes in females [exclusively the paternal X chromosome (Xp)], the absence of an Xist ncRNA homologue has been interpreted to indicate the existence of an *Xist*-independent XCI mechanism. This mechanism could conceivably operate in combination with Xist RNA in eutherian mammals. An alternative scenario is that an Xist-like cis-acting ncRNA has evolved independently, perhaps also by pseudogenisation, in marsupial mammals.

A more direct challenge to the idea that Xist RNA mediates silencing has come from a study of X-linked gene expression in XX mouse preimplantation embryos (Kalantry et al., 2009) (see Fig. 1B). Previous studies had demonstrated that XCI, which at this stage occurs exclusively on Xp, is initiated de novo starting at approximately the 2- to 8-cell stage, when paternal *Xist* is first upregulated (Kay et al., 1993; Nesterova et al., 2001; Okamoto et al., 2005; Okamoto et al., 2004; Patrat et al., 2009). Using RNA fluorescence in situ hybridisation (FISH) to detect nascent transcripts for a panel of X-linked genes, Kalantry et al. (Kalantry et al., 2009) determined the proportion of cells with monoallelic versus biallelic expression at different stages of preimplantation development. They made the surprising observation that 8- to 16-cell embryos have similar levels of monoallelism in wild-type (WT) and *Xist* null embryos, suggesting that initial gene silencing might be Xist RNA independent (Fig. 1B). By the blastocyst stage, there was clearly less monoallelic expression in *Xist* null embryos, leading to the suggestion that Xist RNA is important for the maintenance, but not the initial establishment, of silencing. Their analysis of allelic expression using single-nucleotide polymorphisms (SNPs) that occur between different mouse strains and a *GFP* transgene on Xp lent further support to this conclusion (Kalantry et al., 2009).

In a separate study, Namekawa et al. (Namekawa et al., 2010) also analysed gene silencing in *Xist* null XX preimplantation mouse embryos, but in this case found no evidence for Xist RNA-independent silencing of Xp genes at the 8- to 16-cell stage. Similar to the Kalantry et al. study, their conclusions were based on the analysis of nascent RNA signals, SNPs and an Xp-located *GFP* transgene. The reason for the observed difference is unclear. Both studies used the same *Xist* null allele, so this is unlikely to be a factor. The panel of genes analysed was not identical so it is possible that the selected loci were unrepresentative. Kalantry et al. (Kalantry et al., 2009) suggested that genes that show the greatest degree of Xist RNA-independent silencing belong to a class that might have been subject to dosage compensation early during the evolution of mammalian sex chromosomes, and it follows that under-representation of this class in the Namekawa et al. study (Namekawa et al., 2010) could account for the differences observed. However, the results obtained for alpha thalassemia/mental retardation X-linked (*Atrx*) and ATPase Cu^{2+} transporting alpha (*Atp7a*), which were analysed in both studies, were converse, suggesting that technical differences might have contributed to the observed differences, at least in part. For example, the analysis of X-linked alleles using SNPs measures steady-state levels of RNAs and thus does not record allelic expression in real time. Additionally, working with small amounts of material, as is the case for early preimplantation embryos, increases the probability of bias due to limited template availability at the reverse transcription step. Nascent RNA FISH is, in theory, a more informative assay but also has its limitations; notably, the probability of detecting RNA foci is a function of transcription rate, which of course differs from gene to gene and varies also with developmental stage. Thus, when expression is seen from only one of two alleles, this could indicate that the gene is subject to XCI or that levels of the nascent transcript are at a detection threshold.

It is possible that these discrepancies could be resolved by anticipated developments in next-generation sequencing. Specifically, new sequencing technologies promise to enable the direct sequencing of DNA/cDNA from single cells (Clarke et al., 2009; Eid et al., 2009). Analysis of SNPs in interspecific crosses could then give a readout of allelic levels for a large and fully

representative sample of genes. Moreover, if this could be applied to nuclei isolated from single cells, thereby eliminating the contribution of cytoplasmic RNAs, one would obtain a readout that closely approximates to transcriptional events in real time.

Although Namekawa et al. (Namekawa et al., 2010) found no evidence for Xist RNA-independent gene silencing, they did report Xist RNA-independent silencing of intergenic repetitive sequences on the Xp, which was apparent from the 2-cell stage onwards (Fig. 1C). Using Cot-1 DNA as a probe for RNA FISH analysis, a 'hole' was seen to be associated with the paternal but not the maternal X chromosome, both in WT and *Xist* null embryos. Cot-1 DNA preferentially labels transcripts that contain common dispersed repeat elements (SINEs and LINEs) and, indeed, the results were verified with SINE- and LINE-specific probes and also by staining for RNA polymerase II (RNAPII).

These findings challenge the dogma that XCI is solely attributable to Xist RNA-mediated silencing and reawaken an earlier debate that centred on the idea that the Xp might enter the zygote in a pre-inactivated state (Huynh and Lee, 2003; Okamoto et al., 2005). A key issue, though, is whether this effect is specific to the X chromosome or whether it reflects a general asymmetry of repeat transcription from paternal and maternal genomes in early preimplantation embryos. In support of the latter possibility, it has been demonstrated that PcG repressor proteins show ectopic localisation to constitutive and interstitial heterochromatin domains on the arms of all paternal, but not maternal, chromosomes in early mouse zygotes, up to morula stage (Puschendorf et al., 2008). Presumably, this difference is in some way a reflection of de novo chromatin assembly that occurs in the male, but not the female, pronucleus. It should be possible to test whether repeat silencing occurs on the Xp only, or more widely across the paternal genome using RNA-seq to analyse embryos from interspecific crosses. SNPs present in common repeats should allow a quantitative analysis of the relative expression levels of maternal and paternal alleles. It is worth considering that the Xist RNA-independent silencing of X-linked genes in early preimplantation embryos observed by Kalantry et al. (Kalantry et al., 2009) might also be linked to epigenetic asymmetry of the paternal and maternal genomes rather than to regulation of the Xp. This also would be revealed by high-throughput genome-wide analyses of nascent transcription, as described above.

In summary, the prevailing view that Xist RNA is solely responsible for silencing of the Xp in early mouse preimplantation embryos is challenged by recent findings. The mechanism of Xist RNA-independent silencing, whether of repeat sequences or genes, is currently unknown.

The role of *Xist* in X-chromosome reactivation

Just as there are challenges to the idea that XCI is mediated solely by Xist RNA, recent observations indicate that switching Xist RNA off might not be the only factor that mediates the developmentally regulated X-chromosome reactivation that occurs in the inner cell mass (ICM) of preimplantation mouse embryos and in developing XX primordial germ cells (PGCs).

Studies utilising a conditional null allele of *Xist* have demonstrated that *Xist* is not required for the maintenance of XCI in differentiated somatic cells (Csankovszki et al., 1999). However, by creating and using an inducible *Xist* transgene system, Wutz and Jaenisch have demonstrated that X-chromosome reactivation occurs if Xist RNA is switched on and then off in mouse ES cells and during early stages of ES cell differentiation (Wutz and Jaenisch, 2000). The idea that X reactivation requires extinction of Xist RNA expression in pluripotent cell types, but not in fully differentiated cells, is a further example of a known known. This principle has been extrapolated to explain the X-chromosome reactivation that occurs in the ICM (Fig. 2A) and PGC lineages during the normal development of female embryos (de Napoles et al., 2007; Mak et al., 2004; Okamoto et al., 2004). It has also been used to explain the X reactivation that occurs when somatic cells are reprogrammed experimentally via cloning or cell fusion with pluripotent cells, or as a result of reprogramming by induced pluripotent stem cell (iPS) technology (Do et al., 2008; Maherali et

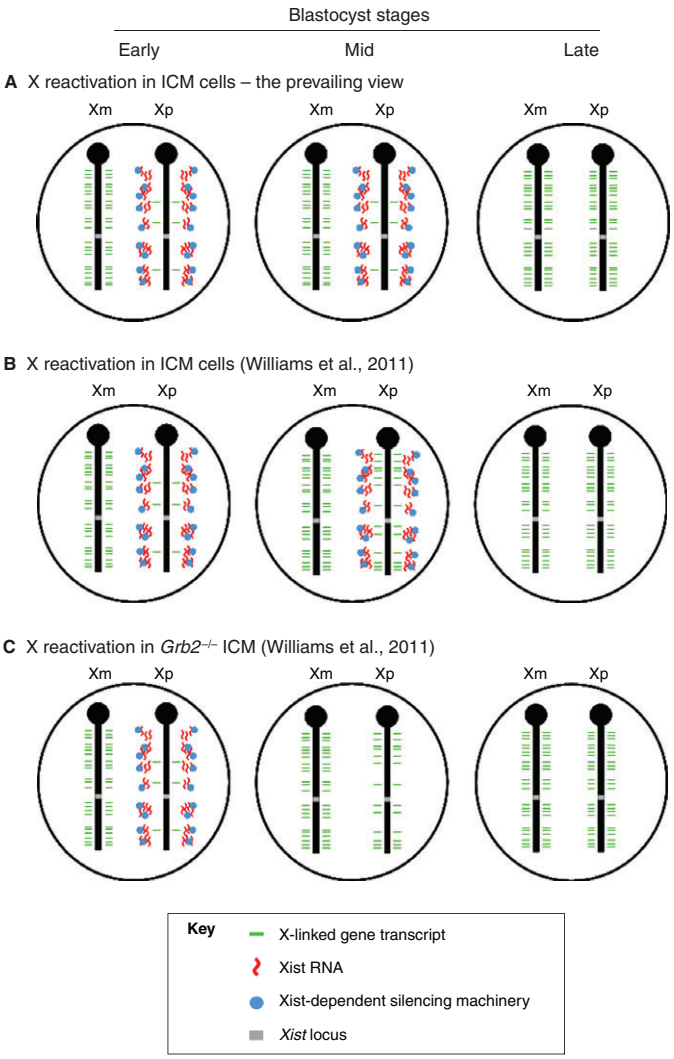

Fig. 2. Xp gene reactivation in XX ICM cells. (**A**) The prevailing view of Xp reactivation in mouse development, during which genes on the Xp are reactivated in the inner cell mass (ICM) cells of late stage blastocysts owing to *Xist* repression mediated by pluripotency factors (the *Xist* locus is represented by a grey bar). (**B**) Reactivation of at least some Xp-linked genes in mid-stage blastocysts when Xist RNA domains are still present (Williams et al., 2011). (**C**) This pattern is also observed in *Grb2*$^{-/-}$ XX blastocysts, in which ICM cells fail to form the primitive endoderm lineage and exhibit high expression of pluripotency factors and the premature loss of Xist RNA expression at the mid-blastocyst stage, indicating that the repression of Xp Xist RNA and gene reactivation may be uncoupled. In both B and C, Xp gene expression patterns in late stage blastocysts are extrapolated, as data for this stage have not been published.

al., 2007; Nolen et al., 2005). In all these cases, the observed X reactivation is accompanied by the loss of Xist RNA expression in the setting of a pluripotent cell nucleus. Indeed, there is good evidence that pluripotency-associated transcription factors, such as Nanog and Oct4 (Pou5f1), directly repress *Xist* (Navarro et al., 2008; Navarro et al., 2010).

The idea that X reactivation in pluripotent cells occurs in response to loss of Xist RNA expression is, however, challenged by some inconsistencies. Williams et al. examined X reactivation in the mouse ICM using RNA FISH for nascent transcripts of several X-linked genes (Williams et al., 2011). Unexpectedly, they observed the transcription of previously silenced Xp genes at the mid-blastocyst stage prior to the loss of detectable Xist RNA coating (Fig. 2B). It is plausible that early gene reactivation occurs at the mid-blastocyst stage because of a partial reduction in both Xist RNA levels and associated repressive chromatin marks, although it can be argued that this is unlikely because experiments using growth factor receptor-bound protein 2 (*Grb2*) null blastocysts, in which high Nanog expression in the ICM drives the premature loss of Xist RNA, show similar reactivation kinetics, as determined by nascent RNA FISH for X-linked genes (Fig. 2C).

There remain some caveats associated with these findings. Dynamism in cell allocation in the ICM could mean that a given cell can express and extinguish Xist more than once during the course of blastocyst development. There is the added factor that the initial silencing of Xp varies from gene to gene, as might transcription rates during preimplantation development, complicating the interpretation of both nascent RNA FISH-based and SNP-based expression analyses. Thus, similar to the examples discussed above, the development of methods to assess genome-wide allele-specific nascent transcription at the level of single cells will help to resolve these questions.

A recent study has found that cells of early human preimplantation embryos express XIST RNA both from Xp and the maternal X chromosome (Xm) at the same time, and, moreover, that this early XIST RNA expression is not coupled to the silencing of X-linked genes, as assessed by nascent RNA FISH assays (Okamoto et al., 2011). This seems to support the idea that XIST RNA coating in early embryos is not necessarily linked to chromosome-wide gene silencing. However, XIST RNA domains in human blastocysts are atypical, as compared with those of somatic cells, appearing to be relatively diffuse and weak, indicating that a factor required for proper in cis localisation of XIST RNA might be lacking. This study also examined Xist RNA expression in rabbit preimplantation embryos, where the authors also found the expression of both Xp and Xm *Xist* alleles, but, in marked contrast, this was accompanied by gene silencing on both alleles. Presumably, both human and rabbit embryos establish appropriate monoallelic *XIST*/*Xist* expression patterns at a subsequent developmental stage.

Although these observations collectively challenge the view that X reactivation in pluripotent cells requires the switching off of Xist RNA expression, set against this are the experiments carried out using inducible *Xist* transgenes in mouse ES cells (Wutz et al., 2002). Further studies are needed to reconcile these disparate findings.

Functional domains in Xist RNA

Although there are suggestions that Xist RNA-independent mechanisms contribute to silencing the Xi, there is little doubt that Xist RNA-mediated silencing remains central to this process. New findings, however, indicate that we might need to reappraise prevailing views on the mechanism of Xist-mediated silencing. As mentioned above, it is generally believed (a known known) that the only region of Xist RNA that is absolutely required for silencing is a single conserved tandem repeat, termed the A-repeat, which is located at the 5′ end of the Xist transcript. The rest of the Xist RNA is thought to mediate its localisation in cis along the chromosome, a function that appears to be attributable to multiple elements dispersed throughout the transcript that function redundantly or additively (Fig. 3A). These conclusions come from experiments that made use of a doxycycline-inducible *Xist* transgene located on the single X chromosome in XY ES cells (Wutz et al., 2002). When WT Xist RNA was induced in this system, it coated and silenced the single X chromosome and thereby triggered rapid cell death. Conversely, when the A-repeat was deleted from the transgene, its induction resulted in the X chromosome being coated with the mutant Xist RNA, albeit less prevalently, and treated cells survived. Although the deletion of other regions of Xist RNA did not fully rescue cells in this study, viability occurred over a continuous range. This might reflect the

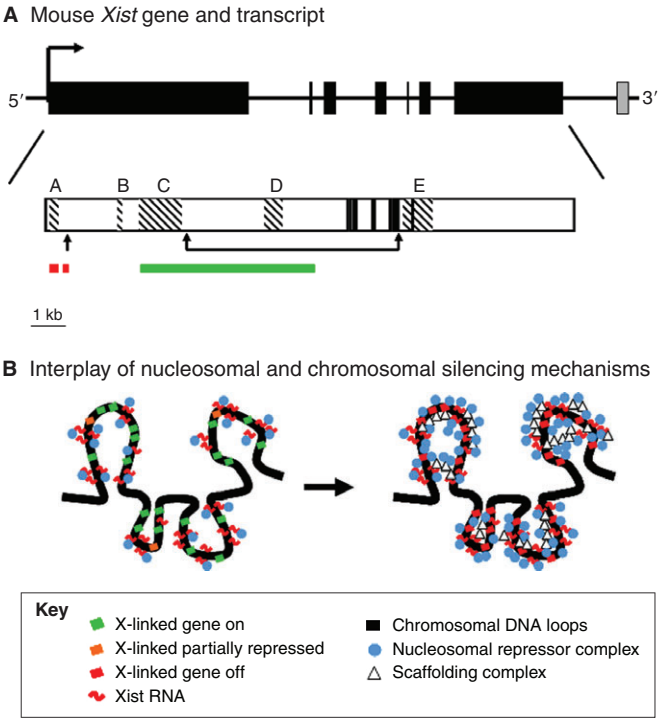

Fig. 3. A revised view of the functional domains of Xist RNA. (**A**) The mouse *Xist* gene (the infrequently used exon 8 is shown in grey) and, below, a representation of mature Xist RNA, indicating the regions that comprise tandem repeats A-E. The arrow on the left indicates the location of a small insertion into Xist RNA (*Xist*^{INS}), recently reported by Hoki et al. (Hoki et al., 2011). The bracket with arrows indicates the breakpoints of the Xist RNA inversion (*Xist*^{INV}) reported by Senner et al. (Senner et al., 2011). Red bars indicate domains implicated in Xist RNA-mediated silencing (the A-repeat and *Xist*^{INS} insertion site), and the green bar indicates the region suggested to have a major role in localising Xist RNA in cis. (**B**) Model illustrating the interaction of nucleosomal repressor complexes and scaffolding complexes in XCI, which proposes that nucleosomal repressors (e.g. histone methyltransferases/demethylases) are sufficient to partially silence some genes in the vicinity of primary Xist RNA-binding sites, and that scaffolding complexes (e.g. SATB1, LINE-1 repeats) are required to polymerise and stabilise silencing complexes over larger domains, thereby bringing about chromosome-wide silencing.

role of other Xist RNA elements in localisation, but does not rule out additional elements contributing to Xist RNA-mediated chromosome silencing.

More recent studies suggest that sequences other than the A-repeat contribute to the silencing function of Xist RNA. The inducible transgene system reported by Wutz et al. (Wutz et al., 2002) was used to show that A-repeat-deleted RNA can recruit PcG repressors to the X chromosome in differentiating ES cells, albeit less efficiently than does WT Xist RNA (Kohlmaier et al., 2004; Schoeftner et al., 2006). PcG recruitment did not occur in cells that had been differentiated for ~9 days, consistent with Xist RNA being unable to establish chromosome silencing in differentiated cell types (Wutz and Jaenisch, 2000). Interestingly, the transient expression of this A-repeat-deleted *Xist* transgene during early differentiation imparted a 'memory' to the chromosome that allowed PcG recruitment to occur in response to re-expression of the transgene at 9 days of differentiation, indicating that Xist RNA can modify chromosome/chromatin structure in the absence of repeat-A, and, moreover, that this modification is stable and heritable once transgene expression is switched off.

In a more recent study, Pullirsch et al. (Pullirsch et al., 2010) showed that the A-repeat-deleted Xist RNA induces chromosome-wide histone H4 hypoacetylation and H3K4 hypomethylation, which are both associated with gene silencing. In addition, weak silencing of at least a subset of X-linked genes was observed. Interestingly, H4 hypoacetylation, but not H3K4 hypomethylation, persisted following the transient induction of the A-repeat-deleted Xist RNA, indicating that this epigenetic mark could be part of the memory that confers the ability to recruit PcG repressors in more differentiated cells. Presumably, these novel functions must de facto map to Xist RNA elements other than the A-repeat.

These new observations highlight the limitations of assaying silencing by X-linked *Xist* transgenes indirectly, based on lethality/viability in XY ES cells. The partial or unstable inactivation of many genes or the inactivation of a limited subset of genes on the single X chromosome might, for example, be insufficient to trigger significant levels of cell death. Whether or not such effects occur might in turn be modulated by the binding of mutant Xist RNA in cis. Deletions that compromise in cis localisation clearly cannot be assessed in terms of silencing activities. In addition to these considerations, variability in the extent of cell lethality could also arise as a consequence of *Xist* transgene upregulation failing to occur in all cells within a culture or by cell selection events within a culture that produce a stochastic or variable response to a specific transgene.

The observation that A-repeat-deleted *Xist* transgenes modulate chromatin modifications across the chromosome independently of global silencing challenges our preconceptions about the role of these histone modifications in gene silencing. A priori, we might have anticipated that chromosome-wide H4 hypoacetylation, loss of H3K4 methylation and the establishment of the PcG-associated histone modifications H3K27me3 and H2AK119u1 would together cause significant gene silencing. However, because the analyses are carried out using immunofluorescence assays, we cannot precisely define the sites at which the chromatin modifications occur. For example, although Pullirsch et al. (Pullirsch et al., 2010) observed chromosome-wide H4 hypoacetylation following the expression of A-repeat-deleted *Xist* transgenes, several large X-chromosome domains with significant H4 acetylation were retained. Additionally, the Xist RNA-dependent enrichment of PcG proteins and their associated histone modifications is significantly reduced in the absence of the A-repeat. A possible scenario is that A-repeat-deficient RNA nucleates silencing at a limited subset of chromosomal sites but is deficient in its capacity to propagate the spread of nucleosomal modifications to genes across the entire chromosome (see Fig. 3B). This view would be consistent with accumulating evidence that proteins that modulate higher order chromosomal organisation, such as Satb1, Smchd1 and hnRNPU (Agrelo et al., 2009; Blewitt et al., 2008; Hasegawa et al., 2010), and/or the chromosomal distribution of LINE-1 elements (Chow et al., 2010; Popova et al., 2006; Tang et al., 2010), play a key role in Xist RNA propagation and/or chromosome silencing. It will be interesting to extend the analysis of inducible *Xist* transgenes, both WT and mutant, applying RNA-seq of nuclear RNA to quantify allelic expression using SNPs in interspecific hybrid cell lines. This will provide highly informative data, both on the degree of silencing at individual genes and on the distribution of silenced genes relative to chromosomal features such as scaffold attachment sites and LINE-1 repeats.

Further evidence that Xist RNA domains other than the A-repeat are important for X-chromosome silencing comes from a recent study by Hoki et al. (Hoki et al., 2011), who describe compromised X-chromosome silencing in mouse embryos that carry the $Xist^{IVS}$ allele, which contains a 16 nucleotide insertion of unrelated sequence ~0.9 kb into *Xist* exon 1 (Fig. 3A). $Xist^{IVS}$ RNA is transcribed in females and appears to coat the X chromosome in cis, leading to the establishment of at least some XCI-associated histone modifications, such as PcG protein-mediated H3K27me3 and histone H4 hypoacetylation. However, silencing of X-linked genes is compromised in both extra-embryonic and embryonic lineages, resulting in embryo lethality between the mid- and late gestation stages. The fact that lethality in $Xist^{IVS}$ mutant embryos occurs at a later stage of development than it does in mouse embryos carrying *Xist* null alleles demonstrates that $Xist^{IVS}$ is a hypomorphic allele. In future studies, it will be important to determine whether it is the establishment or maintenance of silencing that is compromised in $Xist^{IVS}$ mutants, and also what features of Xi might be affected. $Xist^{IVS}$ appears to identify a previously unrecognised functional domain within Xist RNA. However, the possibility cannot be ruled out that the insertion functions at a distance to disrupt the correct folding of the A-repeats or the interaction of the A-repeats with key silencing factors.

In addition to revisiting the mapping of Xist RNA silencing elements, it might also be necessary to reconsider our conclusions regarding the Xist elements that are involved in the cis localisation of Xist RNA. Senner et al. (Senner et al., 2011) have reported another novel allele of mouse *Xist*, $Xist^{INV}$, which is transcribed at normal levels but shows compromised localisation in cis. $Xist^{INV}$ is also hypomorphic relative to *Xist* null alleles, with female embryo lethality occurring at ~E9.5-10.5. Analysis of gene silencing and chromosome-wide histone modifications indicates that partial chromosomal silencing does occur with this allele, most notably of those genes in relatively close proximity to the *Xist* locus. The mutation was produced by inverting sequences ~5 kb into *Xist* exon 1 through to exon 5 (Fig. 3A). Thus, in addition to reversing sequence elements located in WT Xist RNA, the mutation introduces sequence from the opposite strand, both from introns and exons (Fig. 3A). For this reason, it is difficult to pinpoint which features of $Xist^{INV}$ RNA are responsible for its compromised localisation.

Other evidence, however, indicates that exon 1 of *Xist* is crucial for its in cis localisation. Specifically, screening with peptide nucleic acid (Beletskii et al., 2001) or locked nucleic acid (Sarma

et al., 2010) oligonucleotides demonstrates that probes designed to bind to the C-repeats, but not to other regions of Xist RNA, disrupt its localisation. Additionally, a recent study has shown that the nuclear scaffold protein heterogeneous nuclear ribonucleoprotein U (hnRNPU) is important for Xist RNA localisation and that hnRNPU might directly bind to Xist RNA exon 1 (Hasegawa et al., 2010). Specifically, UV cross-linking and immunoprecipitation experiments have shown that hnRNPU interacts with Xist RNA through its RNA-binding RGG domain, with highest enrichment seen for a central region of Xist exon 1. These results represent the first clear example of a protein with a defined RNA-binding domain that is involved directly in Xist RNA function. Taken together, these experiments indicate that sequences located centrally in Xist exon 1 play a key role in the localisation of Xist RNA in cis (Fig. 3A). This is not inconsistent with the findings of Wutz et al. (Wutz et al., 2002), who also found evidence that exon 1 sequences contribute to Xist RNA localisation.

Xist RNA recruits PcG repressor proteins

It is thought that silencing by Xist RNA involves the recruitment of chromatin modifying factors, some of which may interact directly with Xist RNA. To date, the best-studied candidates are the repressor proteins that belong to the major PcG complexes, PRC1 and PRC2. Although there is evidence that PcG repressors are direct targets of Xist RNA, there are confounding observations that also need to be considered.

It is well established that PcG proteins are enriched on the Xi, as are the histone modifications that these complexes are known to catalyse: H2A mono-ubiquitylation (H2AK119u1) and H3K27 tri-methylation (H3K27me3). Historically, this link was discovered through the analysis of mouse embryos that carry a mutation in the core PRC2 protein Eed. In *Eed* mutants, a specific deficiency in development of the extra-embryonic trophectoderm is observed in XX but not XY embryos (Wang et al., 2001). Analysis of an X-linked *GFP* transgene in *Eed* mutant embryos indicated that the stochastic reactivation of Xp occurs in trophoblast cells (this lineage is subject to imprinted X inactivation of Xp), indicating that XCI is compromised in this mutant. Subsequent experiments demonstrated a spectacular enrichment of Eed and of its associated PRC2 proteins on the Xi in extra-embryonic (Mak et al., 2002) and embryonic (Plath et al., 2003; Silva et al., 2003) lineages. Moreover, this enrichment was shown to drive the X-chromosome-wide increase in H3K27me3 (Silva et al., 2003).

Prior studies had established that H3K27me3 catalysed by PRC2 is required for the recruitment of the second major PcG repressive complex, PRC1, via its binding of the chromodomain of the core PRC1 protein polycomb (Fischle et al., 2003; Min et al., 2003; Wang et al., 2004). Consistent with this, it was found that core PRC1 proteins are enriched on Xi (de Napoles et al., 2004), an observation that contributed to the discovery that PRC1 is a ubiquitin E3 ligase that catalyses the histone modification H2AK119u1. Analysis of metaphase mouse Xi chromosomes demonstrated that PRC1, PRC2 and associated histone modifications localise to specific X-chromosome bands and that these bands correlate with gene-rich, Giemsa-light bands, a localisation pattern that mirrors the chromosomal localisation of Xist RNA (de Napoles et al., 2004; Duthie et al., 1999; Mak et al., 2002; Silva et al., 2003). This provided the first hint that the recruitment of PcG complexes to Xi could be directly linked to Xist RNA. Further support for this notion came from studies that demonstrated that the enrichment of PcG proteins and its associated histone modifications occurs rapidly in response to the onset of Xist RNA expression (Kohlmaier et al., 2004; Plath et al., 2003; Schoeftner et al., 2006) and is rapidly lost when Xist expression is extinguished (Mak et al., 2004).

The aforementioned findings were considered to provide good evidence for Xist RNA directly recruiting PcG complexes, but formally it was also possible that the effect was indirect, i.e. that Xist triggered a modification of the underlying chromatin that then allowed PcG recruitment to proceed (Fig. 4). A key experiment that swung the argument in favour of direct recruitment was the observation that the silencing-deficient A-repeat-deleted *Xist* transgenes recruit PcG proteins, albeit less efficiently than do WT *Xist* transgenes (Kohlmaier et al., 2004; Plath et al., 2003; Schoeftner et al., 2006). Thus, direct recruitment of PcG proteins by Xist RNA joined the annals of known knowns. The finding that PcG recruitment is reduced in response to A-repeat-deleted Xist RNA led to the hypothesis that the A-repeats are important for PcG recruitment, an idea that has garnered support from biochemical studies that demonstrate the interaction of A-repeats with the PRC2 subunits Ezh2 and/or Suz12 in vitro (Kaneko et al., 2010; Kanhere et al., 2010; Zhao et al., 2008). Collectively, these observations have spawned a new and highly active area of investigation into a wider role for ncRNAs in PcG recruitment and chromatin regulation (Khalil et al., 2009; Rinn et al., 2007; Zhao et al., 2010).

So are there any inconsistencies to consider? Again, the answer is yes. Preimplantation mouse embryos express Xist RNA from the 2-cell stage onwards but enrichment of PRC2 proteins is undetectable prior to the 16-cell or morula stage (Okamoto et al., 2004; Puschendorf et al., 2008). This cannot be attributed simply to an absence of core PcG complexes as genome-wide H3K27me3 is seen at all stages. Similarly, the expression of inducible *Xist* transgenes in mouse ES cells that have been differentiated for 9

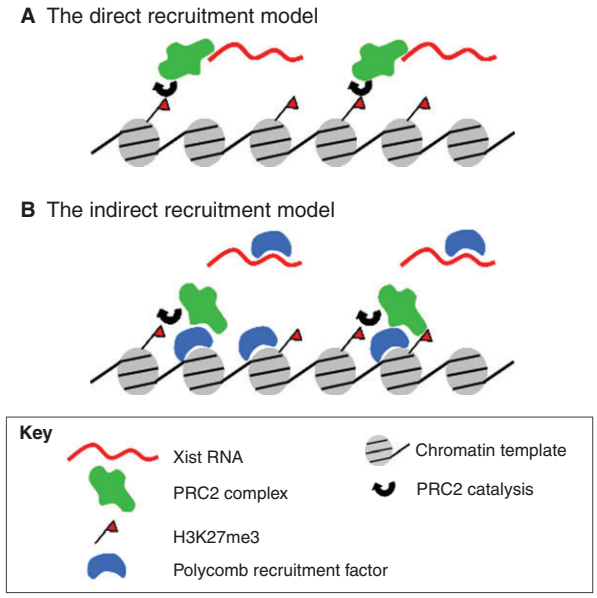

Fig. 4. Direct and indirect models of PRC2 recruitment by Xist RNA. (**A**) The direct model, in which Xist RNA interacts with a core PRC2 protein (Ezh2 or Suz12), facilitating the enrichment of PcG complexes and of H3K27me3 on Xi. (**B**) The indirect model, in which Xist RNA interacts with, and concentrates a factor on, Xi chromatin. This factor provides a recognition site for the recruitment of PRC2 and the subsequent enrichment of H3K27me3.

days does not lead to PcG protein recruitment to the Xi, again in a cellular context in which abundant active PRC2 complex is present (Kohlmaier et al., 2004; Schoeftner et al., 2006). One possible explanation is that a PcG co-factor is responsible for the Xist RNA-mediated recruitment of PcG complexes and that this factor has a restricted developmental expression pattern. Alternatively, developmentally regulated post-translational modification of PcG proteins or their co-factors, or even the post-transcriptional modification of Xist RNA, could define the context that allows PcG proteins to bind Xist RNA. Candidate co-factors for mediating PRC2-RNA interactions include the proteins Jarid2 and polycomblike 2 (Pcl2, or Mtf2), both of which are present at higher levels in ES cells and during early differentiation time points than in more differentiated cells (Casanova et al., 2011; Landeira et al., 2010; Li et al., 2010; Peng et al., 2009; Shen et al., 2009). Indeed, the knockdown of *Pcl2* in XX mouse ES cells significantly reduces the recruitment of PRC2 to the Xi (Casanova et al., 2011). However, Pcl2 and its homologues are known to modulate PRC2 recruitment at sites other than the Xi and, moreover, neither Pcl2 (or its closest homologues) nor Jarid2 proteins have a defined RNA-binding domain.

There are two further inconsistencies to consider. First, the demonstration that the transient expression of silencing-deficient *Xist* transgenes imparts a memory that allows PRC2 recruitment to occur when these transgenes are re-expressed after 9 days of differentiation (Kohlmaier et al., 2004; Schoeftner et al., 2006) is not easily explained in the context of the direct recruitment of PcG complexes by Xist RNA. Second, PRC1 proteins can be recruited to the Xi in a PRC2-deficient background (Schoeftner et al., 2006), demonstrating that there must be an H3K27me3-independent recruitment pathway. Thus, we need an explanation not only for PRC2 recruitment by Xist RNA, but also for the recruitment of PRC1.

What, then, of the evidence that supports the direct recruitment of PcG complexes by the A-repeat of Xist RNA (Kaneko et al., 2010; Kanhere et al., 2010; Zhao et al., 2008)? It should first be noted that none of the core PRC2 proteins has a known RNA-binding domain. Also, in relation to evidence for the role of A-repeats in PRC2 binding, it should be remembered that inducible *Xist* transgenes that lack this element do recruit PRC2, albeit less efficiently. Moreover, evidence for a direct interaction with the A-repeats has been obtained for both the Ezh2 (Kaneko et al., 2010; Kanhere et al., 2010; Zhao et al., 2008) and Suz12 (Kanhere et al., 2010) subunits of PRC2. Although it is possible that both interactions do occur in vivo, invoking Occam's razor would suggest this to be unlikely. The published evidence that Ezh2 and/or Suz12 interact with Xist and other ncRNAs relies heavily on in vitro experiments that use recombinant single subunits of PRC2. Thus, it is plausible that in one or both cases the interactions occur via surfaces that are masked in the holocomplex and therefore might not be physiologically significant. Moreover, RNA immunoprecipitation (RIP) experiments aimed at verifying such interactions in vivo were carried out either in the absence of cross-linking (Kanhere et al., 2010; Zhao et al., 2008), raising concerns about non-specific associations, or using formaldehyde cross-linking, which does not discriminate between direct and indirect interactions (Kaneko et al., 2010). A more convincing test for Xist RNA-PcG interactions would be UV cross-linking, in which only very close associations between nucleic acids and proteins are detectable. Derivative methods, such as cross-linking immunoprecipitation (CLIP) (Ule et al., 2003) or cross-linking and analysis of cDNAs (CRAC) (Granneman et al., 2009), have been developed to analyse the interactions between specific proteins and defined RNAs or RNA populations, and have the added advantage that they provide detailed information on the RNA elements involved in the interaction.

Although none of the above arguments rules out direct interaction between PRC2 proteins and Xist RNA, the weight of inconsistencies suggests that we should remain open to other possibilities. Indeed, the recent finding that A-repeat-deleted Xist RNA does, to some degree, induce chromosome-wide histone modifications (Pullirsch et al., 2010), means that the pivotal argument in favour of direct recruitment no longer stands. It follows, therefore, that indirect recruitment models cannot be ruled out.

Conclusions

In this review, I have focused on selected topics that relate to the question of how Xist RNA mediates chromosome silencing, our understanding of which is in a state of flux. I selected topics for which there are inconsistencies in the data that might suggest the prevailing view, or known known, might in fact be an unknown unknown. I have not discussed known unknowns at length, for example that we still need to identify key players in Xist RNA-mediated silencing. Given the number of reviews on XCI coming out on this fiftieth anniversary, I have no doubt that these and other issues will be well aired.

My scientific take-home message is that it has become clear that we need to better understand the interplay of pathways that modify chromatin structure at the level of the nucleosome with pathways that influence the spatial organisation of chromosome loops and domains in the context of the interphase nucleus. My philosophical take-home message is that our understanding of this enigmatic process continuously evolves and that we should at all times be aware of our assumptions (known knowns) and keep an open mind. I hope, and believe, that the next 50 years of XCI research will be as surprising and interesting as the last.

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