Cis- and trans-regulation in X inactivation

Joke G. van Bemmel\textsuperscript{1,2} · Hegias Mira-Bontenbal\textsuperscript{2} · Joost Gribnau\textsuperscript{2}

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Abstract Female mammalian cells compensate dosage of X-linked gene expression through the inactivation of one of their two X chromosomes. X chromosome inactivation (XCI) in eutherians is dependent on the non-coding RNA Xist that is up-regulated from the future inactive X chromosome, coating it and recruiting factors involved in silencing and altering its chromatin state. Xist lies within the X-inactivation center (Xic), a region on the X that is required for XCI, and is regulated in cis by elements on the X chromosome and in trans by diffusible factors. In this review, we summarize the latest results in cis- and trans-regulation of the Xic. We discuss how the organization of the Xic in topologically associating domains is important for XCI (cis-regulation) and how proteins in the pluripotent state and upon development or differentiation of embryonic stem cells control proper inactivation of one X chromosome (trans-regulation).

Introduction

X chromosome inactivation (XCI) is the mechanism by which female mammalian cells achieve dosage compensation of X-linked gene expression. Throughout eutherian evolution, our sex chromosomes adopted distinct fates; the X chromosome has maintained most of the original genes, whereas the Y chromosome has degenerated in a chromosome with low levels of genetic diversity, many repetitive sequences and most of the genes still present are involved in male fertility (Graves 2006). Degeneration of the Y chromosome provided a potential imbalance in X-chromosomal versus autosomal gene products. Susumu Ohno therefore predicted a twofold transcriptional up-regulation of X-linked genes (Ohno 1967), a hypothesis that was initially confirmed (Nguyen and Disteche 2006), but later contested (Lin et al. 2012; Chen and Zhang 2015). These findings indicate that dosage compensation is limited to subsets of genes, being more pronounced for highly expressed genes and genes encoding proteins acting in complexes (Deng et al. 2011). An obvious consequence of this up-regulation is that female mammalian cells would express X-linked genes at twice the level compared to autosomal genes. To compensate for these potential dosage differences, female cells inactivate one of the two X chromosomes. This XCI process occurs early in murine development in two waves. Imprinted XCI (iXCI) is initiated early during pre-implantation development in all cells of the embryo, and leads to exclusive inactivation of the paternally inherited X chromosome (Xp). In the inner cell mass (ICM) of the pre-implantation embryo, the inactive Xp is reactivated (Mak et al. 2004; Okamoto et al. 2004) followed by a second wave of XCI in the epiblast, around E5.5 of development. This second wave is random with respect to the parental origin of the X chromosome, resulting in XCI of either the maternal or paternal X chromosome. The silent state of the inactivated chromosome is mitotically inherited and maintained in the daughter cells.

Extensive work over several decades uncovered a master regulatory region critical for XCI, the X inactivation center (Xic). The Xic contains protein-coding genes, non-coding genes and their cis-regulatory elements that ensure the proper initiation and
exclusive inactivation of one X chromosome in every female cell. The main actor of XCI is Xist, which is a non-coding RNA gene, located within the Xic. Xist is up-regulated at the onset of XCI, coating the future inactive X chromosome. One of the earliest detectable events following Xist spreading is the depletion of RNA Pol II and transcription factors from the coated chromosome (Chaumeil et al. 2006). Afterwards, active histone depletion of RNA Pol II and transcription factors from the coated RNA gene, located within the Xic.

Xist onset of XCI, coating the future inactive X chromosome. One of the final steps in the silencing of the X is the establishment of repressive CpG methylation at promoters and CpG islands (Lock et al. 1987; Gendrel et al. 2013).

Female ICM-derived embryonic stem cells (ESCs) contain two active X chromosomes (Xa’s), and their differentiation in vitro results in initiation of random XCI (rXCI), thereby providing a powerful model system to study XCI. Prior to XCI initiation, Xist expression is kept at low levels by means of different genetic elements and transcription factors. The best-established negative regulator of Xist is Tsix. Similar to Xist, Tsix is also a non-coding gene, overlaps with, and is transcribed antisense to Xist. The exact mechanism by which Tsix represses Xist is however unclear, but RNA mediated recruitment of chromatin remodelers to the Xist promoter, and transcriptional interference mechanisms have been implicated (Stavropoulos et al. 2001; Luikenhuis et al. 2001; Shibata and Lee 2004; Sado et al. 2006). The factors that regulate Xist and Tsix transcription, and thus XCI, can be classified in two categories, a cis-regulatory network and a trans-regulatory network. The cis-regulatory network is embedded within the X chromosome representing the classical Xic, and is composed mainly and possibly exclusively of genetic factors, that act in cis by DNA interactions. On the contrary, trans-acting regulatory factors are diffusible, and thus can act from a distance and can be autosomally encoded or X-linked.

Recent exciting work has shed new light on the complex interplay of the different cis- and trans-acting factors in the regulation of rXCI in mouse. In this review, we describe these latest findings with respect to the regulation of rXCI, which involve all levels of gene regulation, including cis-regulatory elements, transcription factor networks, chromatin modifications, and higher-order chromatin structure.

The cis-regulatory environment and its spatial separation

The key cis-acting regulatory elements in XCI are located within the Xic, which has been delineated by genetic studies in cell lines harboring X chromosomal deletions and X to autosome translocations (Rastan 1983; Rastan and Robertson 1985; Heard et al. 1994; Lee et al. 1996). These studies revealed a ~500-kb region on the X to be required for the initiation of XCI. Close examination of this region revealed Xist, but also the presence of several other non-coding RNA genes in its close vicinity, many of which are involved in the positive or negative regulation of XCI. For most of them, it remains to be determined if they regulate Xist or Tsix expression at the RNA level via their transcript, or at the DNA level via the regulatory elements contained within them.

Two of these non-coding RNA genes, Jpx and Ftx, are located upstream of Xist (Fig. 1). Both genes escape XCI and are therefore co-expressed with Xist during differentiation (Tian et al. 2010; Chureau et al. 2011). Ftx deletion in male ES cells caused decreased expression of Xist, Jpx, and Tsix, but since this was only analyzed in male cells, cis or trans effects could not be distinguished in this study (Chureau et al. 2011). A heterozygous deletion of Jpx in female ES cells has been reported to decrease Xist up-regulation in both alleles, which could be rescued by an autosomal transgene, suggesting Jpx to act in trans (Tian et al. 2010). The reported trans-activity of Jpx has been proposed to occur by dose-dependent eviction of CTCF at the Xist promoter (Sun et al. 2013). However, other previous, as well as recent, Jpx- and Ftx-containing transgene studies did not observe any ectopic Xist up-regulation (Heard et al. 1999; Jonkers et al. 2009; Barakat et al. 2014), and a heterozygous deletion of a Jpx- and Ftx-containing region in female mESCs did not affect Xist up-regulation from the wild-type X chromosome (Barakat et al. 2014). These results suggest that Jpx and Ftx activate Xist mainly through cis-mediated mechanisms.

Just like the cis-regulators of Xist are located upstream of Xist, the positive regulators of Tsix all reside in a region upstream of the Tsix promoter. These include the RNA Tsx and the enhancer Xite (Simmler et al. 1996; Ogawa and Lee 2003; Anguera et al. 2011), as well as the more recently identified putative regulatory elements contained in Linx and Chic1 (Nora et al. 2012). Deletions of Xite and Tsx both result in mild effects in XCI and expression of Xist. Xite, which is located just upstream of Tsix, acts in cis as an enhancer of Tsix, but is itself also transcribed (Ogawa and Lee 2003). Interestingly, the putative regulatory elements within Linx and Chic1 were identified due to their long range cis-interactions with the Tsix promoter or its enhancer Xite (Nora et al. 2012). In addition to containing putative regulatory elements, the Linx gene also gives rise to a long non-coding RNA. Furthermore, Tsix transcription is found to be regulated by the transcribed DXPas34-repeat region, which is located 750 bp downstream of the Tsix promoter (Debrand et al. 1999; Stavropoulos et al. 2005; Vigneau et al. 2006; Cohen et al. 2007).

The above described localization of the cis-regulatory elements with respect to the Xist and Tsix promoters, shows a
partitioning of the cis-regulatory environment in two regions (Fig. 1), one covering the regulatory elements of Tsix, the other those of Xist. Thanks to 5C (3C-based analysis of many selected loci in parallel), which maps the frequency/probability of chromatin interactions, the Xic was found to be separated not only functionally but also spatially. The chromosome interaction map of the Xic displays a structural organization in two spatially separated domains, so called topologically associating domains (TADs) (Nora et al. 2012). Interestingly, the border of these two TADs is located exactly in between the promoters of Xist and Tsix, thereby segregating the Xist promoter and its activators from the Tsix promoter and its respective regulators.

Does this spatial segregation ensure functional separation and oppositely regulated transcription, or is the spatial organization merely a consequence of the transcriptional status of Xist and its antisense regulator Tsix? If it is causal, then what determines this organization? What delimits TADs? Is TAD border formation driven by specific features or is it rather the consequence of interactions being favored elsewhere? Several findings indicate TAD organization to be important for proper transcriptional regulation. First of all, using HiC, it became clear that the entire mouse genome as well as the human and the Drosophila genomes are organized in TADs, and that “boundaries” between them seem to be conserved between species (Sexton et al. 2012; Dixon et al. 2012; Hou et al. 2012), suggesting this organization to be functionally relevant. Second, several studies indicate that TADs can be considered as discrete units of gene regulation since genes within the same TAD tend to be transcriptionally regulated in a coordinated fashion (Nora et al. 2012; Le Dily et al. 2014) and the majority of promoter–enhancer pairs reside within TADs (Kleinjan and Coutinho 2009; Shen et al. 2012; Smallwood and Ren 2013; Nora et al. Symmons et al. 2014). Third, a 58-kb deletion of the Xist and Tsix TAD border region results in decreased spatial separation of the two domains and illegitimate interactions between sequences within the domains, which co-occurred with altered gene expression of the genes in these domains (Monkhorst et al. 2008; Nora et al. 2012).

The mechanisms behind TAD formation and the boundaries between them remain to be determined however. The genome-wide analyses by Dixon et al. showed TAD borders to be enriched for several genomic elements, suggesting a role in TAD boundary formation (Dixon et al. 2012). Especially the architectural proteins CTCF, cohesin and mediator are considered as favorite candidates to be causal in establishing topological domain structure. Distinct combinations of these architectural proteins are often but not always found at TAD boundaries, long range interacting loci, and short range intraTAD interacting loci (Li et al. 2013; Phillips-Cremins et al. 2013; Sofueva et al. 2013; Rao et al. 2014). Cleavage or depletion of the cohesin complex or CTCF was found to decrease intradomain interactions and to increase interdomain interactions but notably did not lead to a total loss of TADs (Seitan et al. 2013; Sofueva et al. 2013; Zuin et al. 2014). These results indicate that architectural proteins could contribute to TAD formation by executing an insulator function at the border and/or by mediating intra-TAD contacts, which by themselves could help to prevent inter-TAD contacts and consequently contribute to shaping the boundary.

Interestingly, the TAD border that separates the Xist and Tsix regulatory environments is bound by CTCF, but not by cohesin. A 2.3-kb deletion of the CTCF site-containing region in female mES cells resulted in improper transcriptional regulation of Xist and Tsix during differentiation (Spencer et al. 2011), suggesting that this CTCF binding site is indeed involved in the spatial separation of the regulatory elements of Xist and Tsix, even if not bound by cohesin. However, the effect of this deletion on chromatin interactions was not monitored, so it remains to be determined if this effect is really due to reduced spatial separation. In addition CTCF, often together with cohesin, is frequently bound inside the Xist and Tsix TADs. The binding sites overlap with the (putative) cis-regulatory elements and promoters of Xist and Tsix, which are all intra-TAD interacting loci. As mentioned above, these intra-
TAD CTCF- and cohesin-bound loci could also contribute to the sharpness of the boundary between the \textit{Xist} and \textit{Tsix} TADs (Giorgetti et al. 2014).

SC and Hi-C contact maps can be considered to represent the contact frequency or probability across a population, and TADs represent an average chromatin conformation within many cells. The use of FISH probes covering entire TADs and super resolution imaging showed their size and their degree of co-localization to differ from one cell to another (Nora et al. 2012). Also predictive polymer modeling of TAD conformation represented by the population data has been used to predict an ensemble of conformations, revealing the chromatin conformation within the \textit{Tsix} TAD to be highly variable between cells (Giorgetti et al. 2014). In this model, for instance, the \textit{Tsix} promoter interacts with one or more of its (putative) regulatory elements, \textit{Xite}, \textit{Chic1} and \textit{Linx} in only a certain percentage of cells. Transcriptional activity of a locus is known to vary from cell to cell, and by combining the model’s predictions with high-resolution DNA FISH and quantitative RNA FISH, a relationship was confirmed between the transcriptional activity and the chromatin conformation of the \textit{Tsix} alleles at single cell level. Notably, conformation and transcriptional activity were found to vary between the two \textit{Tsix} alleles within the same cell. Such fluctuations could be responsible for asymmetric \textit{Tsix} transcription and thus asymmetric \textit{Xist} up-regulation (Giorgetti et al. 2014).

\textbf{Trans-regulation of XCI}

\textit{Trans}-regulation of the Xic refers to the regulation of genes within the Xic by diffusible factors and can be inhibitory or activating. Years of research in the XCI field have uncovered a crucial role for several pluripotency factors in the regulation of XCI, thereby providing an important link between loss of pluripotency and XCI initiation. Several studies have implicated OCT2, NANOG, SOX2, REX1 and PRDM14 to act as inhibitors of XCI either directly, by repressing \textit{Xist} or activating \textit{Tsix}, or indirectly by repressing activators of XCI (Fig. 2) (Navarro et al. 2008; Donohoe et al. 2009; Ma et al. 2011; Navarro et al. 2011; Gontan et al. 2012; Payer et al. 2013). Removal of OCT4 from male ES cells results in differentiation and up-regulation of \textit{Xist} to similar levels as in differentiating female cells (Navarro et al. 2008), while OCT4 removal from differentiating female ES cells results in biallelic up-regulation of \textit{Xist} (Donohoe et al. 2009). OCT4, NANOG, SOX2, and PRDM14 strongly bind to \textit{Xist} intron 1, which led to the hypothesis that their repressive action on \textit{Xist} is mediated through this region. However, knockout studies deleting the \textit{Xist} intron 1 region indicated that it is dispensable for \textit{Xist} repression (Barakat et al. 2011; Minkovsky et al. 2013). Nevertheless, deletion of the \textit{Xist} intron 1 binding site together with the \textit{Tsix} positive regulatory region, DXPas34, that is also regulated by OCT4, results in de-repression of \textit{Xist}, not found with the individual mutations, suggesting that multiple redundant mechanisms are in place to repress \textit{Xist} (Donohoe et al. 2009; Nesterova et al. 2011). REX1, another pluripotency factor, also acts as an inhibitor of XCI by binding to the DXPas34 repetitive sequence associated to the \textit{Tsix} promoter, allowing proper transcription elongation of the \textit{Tsix} transcript which in turns represses \textit{Xist} (Navarro et al. 2010). In addition, REX1 was later demonstrated to directly repress \textit{Xist} by binding to the \textit{Xist} promoter and the promoter downstream region (Gontan et al. 2012). REX1-mediated repression of \textit{Xist} probably involves competition of REX1 with its paralog and \textit{Xist} activator YY1 for binding to the same sites (Makhlouf et al. 2014). REX1’s role is therefore twofold: it acts at the level of \textit{Xist} repression as well as the level of \textit{Tsix} activation to inhibit XCI in ESCs. YY1, in addition to its role as a direct \textit{Xist} activator, binds to the nucleation center located within 1Kbp from \textit{Xist} repeat A, facilitating docking \textit{Xist} RNA molecules on the Xi (Jeon and Lee 2011).

OCT4, NANOG, SOX2, and PRDM14 have also been reported to indirectly repress \textit{Xist} by inhibiting \textit{Rnf12} expression (Fig. 2) (Navarro et al. 2011). \textit{Rnf12} is an XIC activator encoding an E3-ubiquitin ligase involved in the dose-dependent degradation of REX1 (Navarro et al. 2011; Gontan et al. 2012; Payer et al. 2013). OCT4, NANOG, SOX2, and PRDM14 bind near the \textit{Rnf12} promoter, and depletion of NANOG or OCT4 results in increased \textit{Rnf12} expression (Navarro et al. 2011). PRDM14 is a transcriptional regulator that is specifically expressed in ESCs and primordial germ cells where it has been implicated in epigenetic reprogramming (Yamaji et al. 2008, 2013). Depletion of PRDM14 also results in \textit{Xist} up-regulation, which might involve loss of PRDM14 binding to the intron 1 region (Ma et al. 2011; Payer et al. 2013). PRDM14 may also act indirectly by repressing \textit{Rnf12} expression, as \textit{Prdm14}−/− ESCs show decreased binding of PRC2 to and reduced deposition of the H3K27me3 repressive mark on the \textit{Rnf12} promoter region, which correlates with a four-fold increase in \textit{Rnf12} expression. These findings indicate that several mechanisms, many of them closely linked to the pluripotency factor network, ensure proper repression of \textit{Xist} in mouse ESCs and the timed and proper initiation of XCI upon ESC differentiation.

\textit{Rnf12} is located 500 Kb upstream of \textit{Xist}. \textit{Rnf12} expression is low in mESCs but up-regulated upon differentiation, aided by decreasing levels of the pluripotent factors that repress \textit{Rnf12} expression in the pluripotent state. RNF12 over-expression results in inactivation of the single X chromosome and of both X chromosomes in male and female differentiating ESCs, respectively (Jonkers et al. 2009). \textit{Rnf12}−/− ESCs manage to inactivate one X chromosome upon differentiation, albeit at a reduced rate compared to wild-type ESCs (Barakat et al. 2011). Since \textit{Rnf12}−/− cells are technically like male cells...
RNF12 is essential for XCI in vitro (Barakat et al. 2011). In mice, the level of several pluripotency factors acting as XCI-inhibitors varies between ESCs obtained from different mouse crosses (Sharova et al. 2007). In ESCs, similarly to 2i-containing growth medium, and reduces the levels of de novo methyltransferases 3a and 3b (DNMT3a/3b), which correlates with previous reports showing global DNA hypomethylation of female ESCs (Zvetkova et al. 2005; Habibi et al. 2013). The ectopic induction of Xist in XX ESCs and concomitant XCI led to increased DNMT3a/3b expression resulting in similar DNA methylation levels in XX and XO control ESCs. Based on these observations, the authors suggest that the presence of two Xa’s inhibits exit from the naïve pluripotent state and differentiation (Schulz et al. 2014). Mechanistically, which genes on the two active X chromosomes stabilize the pluripotent state remains an open question.

### The Xic at work

Several mechanisms have been proposed to explain exclusive inactivation of a single X. Studies with heterokaryons obtained through fusion of male and female cells indicate that XCI is equally well initiated in the male and female nuclei indicating...
that all the regulatory cues involved in initiation of XCI are diffusible and cross the nuclear membrane (Barakat et al. 2014). This argues against a role for pairing, of the Xic or the X pairing regulatory regions, in the initiation of XCI (Xu et al. 2006, 2007; Bacher et al. 2006; Augui et al. 2007). XCI inhibitors including a wide range of pluripotency factors set the threshold for XCI activation (Barakat et al. 2010). Activators of XCI, including RNF12, are X-encoded and will thus be expressed at a twofold higher level in female cells compared to male cells. The higher level of X-encoded activators in female cells will overcome the threshold set by the autosomally encoded inhibitors, thereby ensuring female exclusive initiation of XCI. Xist is also activated by autosomally encoded factors, including YY1, which competes with REX1 for binding to the Xist regulatory region located downstream of the Xist promoter (Makhloof et al. 2014; Chapman et al. 2014). These autosomally encoded Xist activators, however, will be expressed at equal levels in female and male cells, and are therefore not to be considered as activators of XCI. During development or upon ESC differentiation, the drop in expression of pluripotency factors and the concomitant increase in the expression of XCI activators lead to Xist up-regulation. Initiation of XCI in female cells is most likely a stochastic process and might happen on any of the two X chromosomes in a given time span (Monkhorst et al. 2009). Exclusive initiation of XCI on one X chromosome might be facilitated by inherent differences in transcriptional activity and higher-order chromatin structure. Since fluctuations in internal TAD conformation are related to variability in transcriptional activity, stochastic interactions between the Tsix promoter and its regulatory sequences could facilitate asymmetric Tsix activity between alleles, thereby causing up-regulation of Xist from one allele and not from the other (Giorgetti et al. 2014). This does not prevent the two alleles in the same cell from adopting the same fate, and therefore fast feedback mechanisms must exist. This includes the rapid turnover of RNF12 and REX1, and the continuous requirement for one active copy of Rnf12, preventing XCI on all except one X chromosome. Indeed, the half-life of REX1 was determined to be in the order of several minutes, and also RNF12 is very unstable through auto-ubiquitination (Gontan et al. 2012). In addition, the close proximity of Rnf12 and Xist likely facilitates feedback through rapid silencing of Rnf12 upon Xist up-regulation in iXCI and rXCI (Patrat et al. 2009; Barakat et al. 2014). Finally, the close link between the presence of two Xa’s, expression of pluripotency factors, and repression of XCI puts a brake on differentiation of cells that have not yet initiated XCI. Together, all these regulatory mechanisms guarantee a robust and highly efficient XCI process.

In several mouse strains and in human, rXCI is skewed towards inactivation of the Xp or Xm (Cattanach and Williams 1972; Gale et al. 1992). Variation in regulatory elements resulting in allelic differences in transcriptional activity of Xist and Tsix could potentially offer an explanation for skewing of XCI. Small genetic differences (i.e., SNPs) might also impact on the chromatin conformation, thereby causing allelic transcriptional biases explaining skewed X-inactivation. In such a case, these SNP-induced structural variations would represent the X controlling element (Xce), which has been genetically linked to skewing and is proposed to be located within a 1.8 mb region 3’ of the Xist promoter (Chadwick et al. 2006; Thorvaldsen et al. 2012). Allele-specific chromatin conformation capture studies or DNA-FISH-based compaction analysis in hybrid cells would be needed to test this hypothesis.

**Silencing and reactivation**

The mechanisms underlying silencing of the X have been under intense scrutiny. Xist accumulation is followed by RNA Pol II and transcription factor exclusion (Chaumeil et al. 2006) and active histone mark removal (Chaumeil et al. 2002). Subsequently, PRC2 is recruited to the X that is silenced. PRC2-dependent H3K27me3 then signals PRC1 to monoubiquitylate histone H2AK119, although this order of events has been contested (Tavares et al. 2012). Xist and PRC2 take advantage of the three-dimensional structure of the chromosome to firstly silence active gene-rich regions that are in close proximity to the Xist locus in 3D, subsequently pulling gene-poor regions into the silencing compartment (Engreitz et al. 2013; Simon et al. 2013). Using probes to pull down Xist followed by mass-spectrometry (RAP-MS and ChIRP-MS), two independent studies identified proteins that interact with Xist and are necessary for its localization and/or silencing capacity (Chu et al. 2015; McHugh et al. 2015). One study revealed Xist to interact with SHARP, which recruits the SMRT co-repressor, activating HDAC3 implicated in decetylation of histones on the Xi and chromatin compaction (McHugh et al. 2015). Knockdown studies indicated that SMRT and HDAC3 are required for Xist-dependent PRC2 recruitment to the Xi. A second study describes a different set of Xist-interacting proteins, of which HnmpK and Spen specifically interact with Xist and are essential for silencing, but not localization to the Xi (Chu et al. 2015). Certain Xist interactors, such as Rmb15, Myef2, Hnmnc, etc., are found in both studies, although other Xist interactors, such as PRC2, ATRX, CTCF, and YY1, previously identified by protein pool down followed by RNA-seq (CLIP-seq) were not identified in the Xist-specific pool down experiments (Zhao et al. 2008; Jeon and Lee 2011; Sarma et al. 2014; Kung et al. 2015). This discrepancy between Xist-mediated pool down of interacting proteins versus pool down of candidate proteins followed by RNA-seq could be explained by the different methods and systems used (male vs female cells) but could also hint at a role for many different factors providing redundancy to the
system. This might involve other non-coding genes, including Firre which is X-linked but escapes XCI producing an RNA that, similar to Xist, is required for maintenance of H3K27me3 at the Xi, and nucleolar localization of the Xi (Yang et al. 2015).

These studies highlight the versatile mechanisms and robustness of the process involving non-coding RNAs and chromatin-modifying enzymes catalyzing histone modifications and CpG island methylation. XCI has therefore long been considered irreversible from the moment the Xi is established (Wutz and Jaenisch 2000), supported by studies with fibroblasts and neural progenitor cells revealing no robust reactivation after conditional knockout of Xist from the Xi, indicating that silencing is faithfully maintained through all daughter generations (Csankovszki et al. 2001; Splinter et al. 2011). This view was recently challenged by studies of Yildirim and colleagues, who deleted Xist in the blood compartment, which resulted in increased X-linked expression (Yildirim et al. 2013). Although allelic origin was not investigated, the authors suggested that Xist does play a role in maintenance of the inactive state in differentiated cells, at least in the blood compartment. Importantly, female mice develop myelofibrosis, leukemia, and other symptoms of the myeloproliferative neoplasm and meydysplastic syndrome. The authors argue that reactivation of the X chromosome leads to genome-wide expression changes and deregulation of the cell cycle, DNA replication, and hematopoietic pathways, among other genetic pathways. It thus seems that the blood lineage is plastic for the reactivation but also inactivation of the X (Agrelo et al. 2009; Yildirim et al. 2013). Recently, reactivation of the X chromosome of female mouse embryonic fibroblasts has been shown to have no clear effect on global X-linked gene expression (Bhatnagar et al. 2014). Using an RNAi interference screen during differentiation of female mouse ESCs and in differentiated cells, the authors identified 13 trans-acting XCI factors (XCIFs) that are required for proper expression of Xist and/or localization of Xist to the Xi. These XCIFs include proteins involved in cell signaling, transcription, and ubiquitination, such as AURKA, SOX5, and RNF165, respectively. A mouse knockout model of one of the XCIFs, STC1, a poorly studied gene encoding a and RNF165, respectively. A mouse knockout model of one trans-activators regulate rXCI? In addition, the mechanism by which Tsix and the other ncRNAs within the Xic regulate Xist is still being debated. Is transcription per se, i.e., activity, enough to repress or activate Xist, are the non-coding transcripts involved, or rather the regulatory elements contained within these genes and is the higher-order chromatin structure instructive or consequence in the regulation of XCI? Finally, what is the role of all the novel and previously identified Xist interactors in establishment and maintenance of the Xi, and how do these findings observed in mouse relate to human? These and many other intriguing questions are awaiting to be addressed soon.

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