

Roles of Long Non-coding RNAs in X-Chromosome Inactivation

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1 Introduction

Female mammals silence the majority of genes along one of their two X chromosomes in a process termed X-chromosome inactivation (XCI). XCI likely evolved in mammals as the X and Y chromosome, once homologous autosomal pairs, diverged in sequence, largely through degeneration of the Y. This degeneration left males with only one functional copy of most X-linked genes, necessitating the development of a compensation process that would equalize X-linked gene dosage between the sexes (Livernois et al. 2012).

XCI is critical for mammalian development. Severe defects in the process are developmentally lethal, while abnormalities in X-chromosome dosage, which occur in about 1 of 500 live births, can be pleiotropic disorders, associated with forms of intellectual disabilities, infertility, and autoimmunity (Powell 2005). The importance of regulating X-linked gene dosage is underscored by the chromosomal counting process inherent to XCI. Regardless of the total number of X chromosomes an individual has, XCI ensures that one X per diploid genome remains active, with the remainder subject to inactivation, in both males and females. For example, XCI tends to silence two X's in tetraploid female cells, and only one in tetraploid male/female cell fusions (Monkhorst et al. 2008). In both cases, the ratio of one active X per diploid genome is maintained. Similarly, in humans, XCI shuts down two X's in females with three (Triple X Syndrome), and one X in males with two (Klinefelter's Syndrome); the sole X in females with Turner's syndrome remains active. These chromosomal abnormalities are often accompanied by chronic health issues (Powell 2005), indicating imperfect regulation of X-linked dosage. However, the intrinsic capability of mammalian cells, male or female, to sense and at least partially deal with abnormalities in

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X-chromosome dosage is remarkable and speaks to the physiological importance of XCI.

In addition to its role in development and human health, XCI has emerged as a paradigm for epigenetic silencing mediated by noncoding RNA (ncRNA), given the critical role of *Xist* and other ncRNAs in the process. Advances in DNA sequencing technologies have led to the identification of thousands of ncRNAs expressed by the mammalian genome, many of which are developmentally regulated and conserved (Dunham et al. 2012; Derrien et al. 2012; Cabili et al. 2011). Early studies have shown these RNAs have critical functions in a range of biological processes, including stem cell maintenance, regulation of the DNA damage response, and developmental specification (Guttman and Rinn 2012). XCI was one of the first identified gene regulatory processes in mammals with a conserved role for ncRNAs (Brockdorff et al. 1992; Brown et al. 1992). Therefore, as the importance of ncRNA-mediated gene regulation has become broadly apparent, XCI has remained a flagship model for understanding ncRNA function. In the pages below, we describe the major features of XCI, with particular focus on the diverse roles that ncRNAs play in the process.

2 XCI Overview

In the mouse, historically the field's most utilized experimental model, XCI occurs in two waves during early development. The first is termed imprinted XCI, due to the exclusive inactivation of the paternally inherited X chromosome (Takagi and Sasaki 1975). Imprinted XCI occurs rapidly after formation of the zygote, initiating at the 4-cell stage of development, and nearing completion for some paternal loci at the formation of the early blastocyst, around the 32-cell stage (Kalantry et al. 2009; Okamoto et al. 2005; Patrat et al. 2009; Williams et al. 2011). This stark parent-of-origin bias appears to be independent of the meiotic sex chromosome inactivation that occurs in the male germline (Okamoto et al. 2005), and instead is due to an imprint placed on the maternal X during oocyte maturation, which somehow blocks XCI from occurring on the chromosome (Tada et al. 2000). Cells of the extraembryonic lineage propagate a paternally derived inactive X (Xi) throughout their existence (Takagi and Sasaki 1975; West et al. 1977). In contrast, XCI is reversed in the inner cell mass (ICM) of the blastocyst, which gives rise to the embryo proper (Mak et al. 2004; Okamoto et al. 2004). Postimplantation, XCI re-occurs in the epiblast, nearing completion around embryonic gestational day (E) 6.5 (Rastan 1982). In this second wave, termed random XCI, the choice to inactivate a given X is largely random and independent from its parent-of-origin (McMahon et al. 1983). Random XCI is maintained in all cells save the germline (Sugimoto and Abe 2007), resulting in adult females who are mosaics of paternally and maternally derived Xi's.

Not all mammals share the biphasic inactivation strategy of the mouse. While rats and cows show imprinted XCI in their extraembryonic tissue (Xue et al. 2002;

Wake et al. 1976), suggesting a mouse-like biphasic inactivation strategy, other eutherian mammals examined to date—humans, horses, and mules—appear to undergo random XCI in all lineages (Moreira de Mello et al. 2010; Wang et al. 2012). In contrast, metatherians, such as the kangaroo and opossum, inactivate their paternally inherited X in all tissues (Sharman 1971; Grant et al. 2012).

3 Control of XCI via the X-Inactivation Center

Studies of balanced chromosomal translocations in the mouse mapped the location of a single X-linked region that invariably tracked with inactivation of adjoining X-linked DNA, and often led to partial silencing of the fused autosome (Lyon, M. F., Searle A. G., & International Committee on Standardized Genetic Nomenclature for Mice 1989). Because of the region's ability to inactivate neighboring DNA, it was proposed to contain the *cis*-mediated genetic signals required to

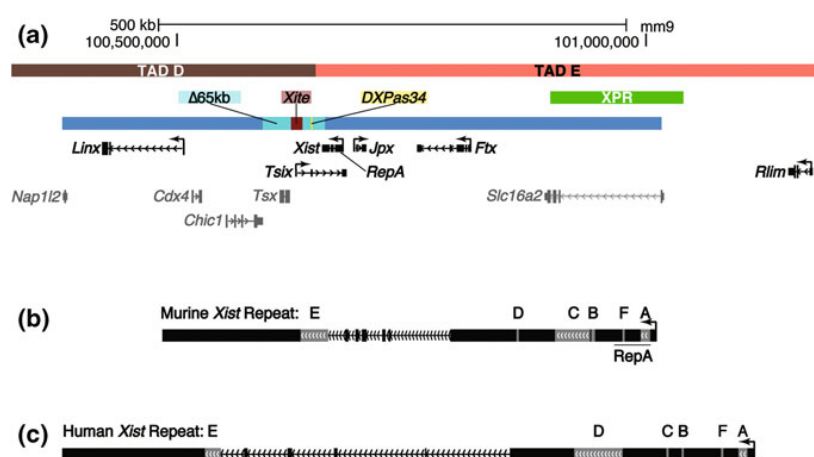


Fig. 1 *Xist* and the X-inactivation center. **a** The protein coding genes, noncoding RNAs, and regulatory elements of the murine X-inactivation center, depicted to scale relative to UCSC genome build mm9. Genes and regulatory regions in black text denote those discussed in the text with documented or proposed roles in XCI. Genes in grey text have no known roles in XCI. Exons and introns are depicted as solid bars and hashed lines, respectively. Regulatory regions are depicted as colored bars above genes. Denoted TADs are those described in (Nora et al. 2012). The large blue bar spanning the majority of Fig. 1a denotes the genomic span of bacterial and yeast artificial chromosomes that recapitulate aspects of XCI when integrated as multicopy transgene arrays into mouse cell lines (Heard et al. 1999; Lee et al. 1996). **b, c** Mouse and human *Xist* genomic loci. Exons and introns are depicted as in (a). Exonic regions in grey mark the location of the six annotated *Xist* repeats, A through F, as described in (Brockdorff et al. 1992; Brown et al. 1992; Nesterova et al. 2001). The location of the RepA transcript within the murine *Xist* locus is underlined

initiate and maintain XCI, and was termed the X-inactivation center (Xic) (Fig. 1a; (Rastan and Brown 1990)). Subsequent analysis of structurally rearranged chromosomes in humans identified a single homologous Xic, as well (Brown et al. 1991). Since then, a range of genetic and cell biological experiments have defined several features contained within the Xic that are critical for proper execution of XCI, including a surprising number of ncRNAs and regulatory elements that produce ncRNA species. At the top of this regulatory cascade is *Xist*, which stands for Xi-specific transcript. *Xist* is essential for XCI, coating the otherwise inactive chromosome from which it was expressed. Several other ncRNAs have been identified within the Xic, including *Tsix*, *Jpx*, *Ftx*, *Linx*, and *RepA*, most of which have documented roles in XCI. Also, at least two critical regulatory regions within the Xic, *DXPas34*, and *Xite*, have themselves been documented to produce RNA. Most recently, it was discovered that a large ncRNA, termed *Xact*, is expressed from the active X specifically in human pluripotent cells. Together with a complex interplay of transacting factors, many of which remain undefined, the ncRNAs and regulatory elements over the X establish a remarkably robust system of dosage compensation that is capable of delivering a single active X (Xa) per diploid genome, even in the presence of chromosomal abnormalities (Table 1).

4 *Xist*, A Long Noncoding RNA Required for XCI

One of the more striking cytological features of the Xi is the coating of the chromosome by the *Xist* ncRNA, which can be visualized under a fluorescent microscope via RNA fluorescence in situ hybridization (FISH). *Xist* was initially

Table 1 Proposed and validated functions of noncoding RNAs and regulatory elements associated with XCI

Region	Classification	Proposed/Validated Function	Seminal Reference(s)
<i>Xist</i>	NcRNA	Master regulator of XCI	(Brockdorff et al. 1992; Brown et al. 1992; Brown et al. 1991)
<i>Jpx</i>	NcRNA	<i>Xist</i> activator	(Tian et al. 2010)
<i>Ftx</i>	NcRNA	<i>Xist</i> activator	(Chureau et al. 2011)
<i>Tsix</i>	NcRNA	<i>Xist</i> repressor	(Lee et al. 1999)
<i>DXPas34</i>	Reg. Element	<i>Tsix</i> activator	(Courtier et al. 1995; Heard et al. 1993)
<i>Xite</i>	Reg. Element	<i>Tsix</i> activator	(Ogawa and Lee 2003)
<i>Linx</i>	NcRNA	<i>Tsix</i> regulator	(Nora et al. 2012)
<i>RepA</i>	NcRNA	<i>Xist</i> activator, PRC2 recruitment	(Zhao et al. 2008)
<i>LINEs</i>	DNA/RNA	<i>Xist</i> spreading, gene silencing	(Chow et al. 2010)
<i>XACT</i>	NcRNA	Xa maintenance	(Vallot et al. 2013)

identified as a candidate gene to control XCI because of its exclusive expression from the Xi and its chromosomal localization within the region defined as the Xic (Brown et al. 1991). Subsequent work defined the major characteristics of the gene in both human and mouse: It is approximately 17 kb in length, can be detected as spliced and polyadenylated, and is exclusively nuclear and untranslated (Brockdorff et al. 1992; Brown et al. 1992). Multiple spliceforms exist, some of which appear to lack polyA tails (Brown et al. 1991, 1992; Hong et al. 2000; Ma and Strauss 2005; Memili et al. 2001). Consistent with its classification as a ncRNA, *Xist* lacks conserved open reading frames, but does contain up to six regions of tandemly arrayed repetitive sequence that may be responsible for aspects of its function (Brockdorff et al. 1992; Brown et al. 1992; Nesterova et al. 2001). These regions are on the order of 100 bp to 2 kb in length, and several are clearly conserved between mouse and human (Fig. 1b, c; (Brockdorff et al. 1992; Brown et al. 1992; Nesterova et al. 2001)).

Notably, recent work has identified an Xi-specific transcript in metatherian mammals, termed *Rsx* (Grant et al. 2012). *Rsx* does not share sequence homology with *Xist*, yet, similar to *Xist*, the RNA is expressed from the Xi, appears to coat the chromosome in cis, lacks open reading frames, and is enriched for tandemly repeated sequence at its 5' end (Grant et al. 2012). This apparent functional conservation without sequence similarity suggests that ncRNA-mediated regulation of dosage compensation arose at least twice during mammalian evolution, highlighting the general utility of this regulatory strategy for the large-scale management of gene expression programs.

Genetic ablation of *Xist* demonstrated its critical role in XCI. Mouse embryonic stem cells (ESCs), which serve as a useful in vitro model because they have yet to undergo XCI, show complete, nonrandom inactivation of a wild-type over a mutant *Xist* allele during differentiation, which induces XCI in these cells (Penny et al. 1996). Similarly, maternal inheritance of an *Xist* deletion results in non-random inactivation of the wild-type, paternally inherited X in the mouse embryo. Paternal inheritance of this same deletion results in lethality due to failure of XCI in the extraembryonic lineages, where the wild-type, maternally inherited X is resistant to silencing (Marahrens et al. 1997). These studies indicate that an X-chromosome without *Xist* cannot undergo stable XCI.

While *Xist* coats the Xi in virtually every cell that contains one, the ncRNA is only required during the initiation and early maintenance of the process, at least in the mouse. Using an inducible *Xist* transgene integrated into an autosomal locus, Wutz and Jaenisch were able to show that *Xist* is only capable of gene silencing in ESCs up to 48 h postinduction of differentiation with retinoic acid. Before this time point silencing was reversible and dependent on continued expression of *Xist*, whereas afterwards XCI was irreversible even if *Xist* expression was extinguished (Wutz and Jaenisch 2000). The in vivo correlate of this time frame is unclear, but it is likely between E9.5 and 12.5, as deletion of *Xist* in mouse embryonic fibroblasts (MEFs), which are frequently derived from these developmental time points, does not result in X-reactivation (Csankovszki et al. 1999).

Other than gene silencing, coating of the Xi by *Xist* is the first documented cytological event during initiation of XCI in the mouse, and is seen as early as the four-cell stage of development (Okamoto et al. 2005). *Xist* stabilization and coating of the Xi is also observed at the onset of random XCI (Panning et al. 1997; Sheardown et al. 1997). The closely coupled timing of *Xist* coating and XCI's initiation strongly suggest a role for *Xist* in the earliest stages of XCI, including the initiation of the process.

Rigorous tests examining *Xist*'s role in initiating XCI in the mouse have thus far yielded conflicting results. To address the question, Kalantry and colleagues measured the kinetics of gene silencing during the earliest stages of imprinted XCI (Kalantry et al. 2009). They made the surprising observation that several X-linked genes exhibited indistinguishable patterns of silencing between wild-type mice and those carrying a paternally inherited *Xist* deletion at the 8- and 16-cell stage of development. At these early time points, silencing of certain genes was more affected by *Xist* loss than others, whereas all genes were affected at later time points. The results suggest imprinted XCI can initiate in the absence of *Xist* in the mouse. Moreover, they support an evolutionary model of XCI, which posits that inactivation evolved in a piece-meal fashion over the X chromosome (Lahn and Page 1999); Kalantry and colleagues found that genes whose silencing was most affected by *Xist* loss were those thought to be subject to dosage compensation for the longest amount of evolutionary time (Kalantry et al. 2009). In complete contrast, using a similar mutant allele and examining a similar set of X-linked genes, Namekawa and colleagues found that imprinted XCI did not initiate in the absence of *Xist*, suggesting the opposite conclusion reached by Kalantry and colleagues: *Xist* triggers the initiation of imprinted XCI (Namekawa et al. 2010).

Methodological differences have been proposed to explain the discrepancy between these two studies (Namekawa et al. 2010; Brockdorff 2011). The two works also used different *Xist* mutant alleles. Whereas the mutant allele used by Kalantry and colleagues removed *Xist* exons 1 through 3, the mutant allele used by Namekawa and colleagues removed *Xist* exons 1 through 6 (Kalantry et al. 2009; Namekawa et al. 2010). Nonetheless, both alleles appear to be complete for loss of *Xist* function, making this difference unlikely to account for the discrepancy between the studies.

A favored explanation is that differences between inbred mouse strains account for the differential detection of *Xist*-independent processes during the initiation of imprinted XCI. Genetic background differences often affect phenotypes of mutant mice, due to the presence of modifier alleles that associate with particular mouse strains; notable examples of this include mutational analyses of the *Apc* and *Egfr* genes (Montagutelli 2000). Whereas Kalantry and colleagues utilized F1 hybrids of *M. m. musculus* and *M. m. molossinus* mice (Kalantry et al. 2009), Namekawa and colleagues utilized F1 hybrids of *M. m. musculus* and *M. m. castaneus* mice (Namekawa et al. 2010). Therefore, differences in modifier alleles between the *M. m. molossinus* and *M. m. castaneus* subspecies could have been responsible for the differential detection of *Xist* sensitivity during the initiation of imprinted XCI. Under this assumption, the studies conducted by Kalantry and Namekawa indicate

that imprinted XCI can initiate in the absence of *Xist* over certain X-linked genes, but that the strength of *Xist*-independent initiation varies with genetic background, such that it is not detectable in *M. m. castaneus/musculus* hybrids (Kalantry et al. 2009; Namekawa et al. 2010).

Whether similar *Xist*-independent processes are involved in the initiation of random XCI is unclear. While many of the cytological features of the Xi are the same in cells subject to imprinted and random XCI (coating in *Xist* and histone H3-lysine27-tri-methylation (H3K27me3), late DNA replication, methylation of CpG islands), a major difference exists in how the future Xi is chosen between the two types of XCI. In imprinted XCI the identity of the Xi is pre-determined; in random XCI it is not. Careful quantification of cell growth and death rates during induction of random XCI via ESC differentiation showed that cells heterozygous for a mutant *Xist* only ever chose the wild-type X for inactivation (Royce-Tolland et al. 2010). This and other studies suggest *Xist* is required to trigger the initiation of random XCI in the mouse (Royce-Tolland et al. 2010; Clerc and Avner 1998; Gribnau et al. 2005; Lee and Lu 1999; Newall et al. 2001). Nevertheless, whether random XCI can initiate in the complete absence of functional *Xist* is still an open question. If it could, it would be predicted to be highly unstable in *Xist*'s absence, given that cells heterozygous for *Xist* mutations never appear to inactivate the mutant X (Penny et al. 1996; Marahrens et al. 1997; Royce-Tolland et al. 2010; Gribnau et al. 2005).

5 Spread of *Xist* Over the Xi

Xist is an unusual RNA in that it appears to coat the gene-dense regions of the Xi from which it is expressed (Chadwick and Willard 2004; Duthie et al. 1999; Mak et al. 2002). Genetic tagging experiments performed in cell fusions have shown *Xist* is retained on its chromosome of origin, suggesting the RNA spreads over the Xi only in *cis*, and cannot dissociate to bind other X's (Jonkers et al. 2008). This banded pattern of association is stable during metaphase in mouse but not in human (Duthie et al. 1999; Clemson et al. 1996). Curiously, in female MEFs expressing transgenic *Xist* from an autosomal locus, endogenously produced RNA diffuses away from its Xi of synthesis and accumulates over the integrated autosomal transgene (Jeon and Lee 2011). This phenomenon depends on a short conserved region at *Xist*'s 5' end, Repeat F (Nesterova et al. 2001; Jeon and Lee 2011). Whether *Xist* ever leaves its chromosome of synthesis in more natural settings is unclear, but these experiments indicate that diffusion is possible in certain scenarios.

Exactly how *Xist* manages to coat the gene-dense regions of the Xi is unclear. The X chromosome is significantly, and specifically, enriched in LINE repetitive elements relative to the autosomes. In mouse and human, 35 % of X-linked DNA is LINE-derived, as compared to 20 % of autosomal DNA. Other repetitive elements do not display similar enrichment levels (Fujita et al. 2011). At a minimum,

this enrichment indicates that the X chromosome provides a favorable genomic environment for LINE insertions, and further suggests insertion of these elements has been co-opted in some way to facilitate XCI. Toward the latter suggestion, LINEs were initially proposed to serve as direct conduits, or booster elements, for the spread of *Xist* over the Xi (Lyon 1998). Studies of *Xist* expression from various autosomal loci have shown that high LINE-density positively correlates with the ability of *Xist* to spread across autosomes, supporting a role for LINEs in *Xist* coating (Chow et al. 2010; Popova et al. 2006; Tang et al. 2010). These elements likely affect the propagation of *Xist* indirectly, however, as analysis of chromosome spreads indicates *Xist* is absent over the most LINE-dense regions of the Xi, associating instead with the gene-dense regions of the chromosome (Chadwick and Willard 2004; Duthie et al. 1999; Mak et al. 2002).

In addition to the role that LINE-dense regions may play in the spread of *Xist* over the Xi, mounting evidence supports an important role for the nuclear matrix in the process. Disruption of chromatin structure via DNaseI and salt extraction does not alter *Xist* localization in human cells, suggesting an indirect interaction between the RNA and the Xi, potentially via the nuclear matrix (Clemson et al. 1996). Consistent with the nuclear matrix playing a role in *Xist*'s coating of the Xi, a targeted siRNA screen identified the nuclear matrix protein Hnrnpu/SAF-A as required for the process. Knockdown of Hnrnpu/SAF-A results in destabilization of a long isoform of *Xist*, diffusion of a shorter isoform throughout the nucleus, and defective induction of XCI (Hasegawa et al. 2010). Hnrnpu/SAF-A has both RNA and DNA association domains, and it is possible that the protein serves as a direct interface between *Xist* and regions of the Xi (Hasegawa et al. 2010). In support of this model, this protein has been shown to coat the Xi in both mouse and human cells (Pullirsch et al. 2010; Helbig and Fackelmayer 2003).

A different screening approach led to the identification of SATB1 as a critical factor in the initiation of *Xist*-mediated silencing (Agrelo et al. 2009). The protein is known to be involved in the formation of chromatin loops, binding special AT-rich DNA sequences at nuclear matrix attachment regions, again implicating the nuclear matrix in *Xist*'s coating of the Xi (Alvarez et al. 2000; de Belle et al. 1998). SATB1 localizes to the area surrounding the Xi and *Xist*, rather than directly over the chromosome (Agrelo et al. 2009). Based on these properties, it has been proposed that SATB1 could anchor together the gene-poor, LINE-dense regions of the Xi, which may, in turn, condense the Xi's gene-dense regions, and facilitate the spread of *Xist* RNA over the chromosome (Tattermusch and Brockdorff 2011). Recent work has shown that the most LINE-dense regions of the Xi are located adjacent to the *Xist* coat and gene-dense regions of the chromosome, consistent with such a model (Calabrese et al. 2012).

The transcription factor YY1 has been found to tether *Xist* to its site of synthesis on the Xi (Jeon and Lee 2011). This tethering depends on YY1 binding sites in the genomic DNA, located just upstream of Repeat F in the *Xist* locus (Jeon and Lee 2011). How this local tether relates to the nuclear matrix, or the spread of *Xist* over the Xi, is unclear. Immunofluorescence analysis indicates YY1 does not form a microscopically visible coat over the Xi, suggesting it is not directly involved in

the spread of *Xist* beyond the Repeat F locus (Jeon and Lee 2011). However, siRNA knockdown of YY1 precludes *Xist* coating in MEFs, suggesting a critical role for local docking of *Xist* in the spread of the RNA over the Xi (Jeon and Lee 2011).

Multiple regions of the *Xist* RNA itself appear to mediate its ability to coat the Xi. A landmark study, in which a series of inducible *Xist* transgenes harboring various segmental deletions were inserted into the X-linked *Hprt* locus, found that no single region of *Xist* was directly responsible for its spread over the Xi (Wutz et al. 2002). In an endogenous setting, however, the spread of *Xist* is sensitive to specific disruptions. Two groups, using different antisense technologies predicted to disrupt RNA secondary structure, found that targeting of *Xist*'s Repeat C region led to visible dissociation of the RNA from the Xi (Beletskii et al. 2001; Sarma et al. 2010), indicating this region of the RNA likely plays a role in coating. Sequence inversion of a region of *Xist* that encompasses the latter half of exon 1 (Repeat D), and exons 2 and 3, results reduced Xi localization and failure of XCI in mutant carrier mice, suggesting this region may also be critical for *Xist* coating (Senner et al. 2011).

Finally, Xi coating by *Xist* is intimately linked to post-transcriptional processing of the RNA. Only spliced *Xist* coats the Xi; the intron-containing RNA does not (Sheardown et al. 1997; Panning and Jaenisch 1996). Furthermore, the induction of XCI is accompanied by an increase in the post-transcriptional stability of *Xist* and not necessarily increased rates of *Xist* transcription. *Xist* transcription rates are similar between ESCs, which do not have an *Xist*-coated Xi, and female fibroblasts, which do have one (Sheardown et al. 1997; Panning and Jaenisch 1996).

6 Post-Transcriptional Processing of *Xist*

A handful of factors have been identified as required for proper *Xist* processing, and through that role, a functional XCI response. *ASF/SF2*, an important component of the splicing machinery, binds *Xist* and is necessary for its processing and the initiation of XCI (Royce-Tolland et al. 2010). A SAGE-based expression screen for genes upregulated in female mouse embryos at the onset of XCI led to the discovery of *Upf1*, *Exosc10*, and *Eif1* as proteins required for *Xist* processing and XCI (Bourdet et al. 2006; Ciaudo et al. 2006). How these latter three genes are involved in *Xist* stabilization remains a mystery. *Upf1* and *Exosc10*, components of the nonsense mediated decay pathway and nuclear exosome, respectively, are typically involved in the destruction of RNA, not its stabilization (Houseley and Tollervey 2009). Similarly, *Eif1* has a documented role in the selection of start sites prior to translation initiation (Asano et al. 2000), but *Xist* is untranslated. Establishing an ordered pathway for *Xist* processing and retention on the Xi will likely yield critical insight into the mechanism of XCI.

7 *Xist* and the Mechanism of XCI-Induced Gene Silencing

The microscopically visible exclusion of RNA Polymerase II (Pol II) and general transcription factors from the nuclear domain occupied by *Xist* is one of the earliest observable events after the initiation of XCI (Chaumeil et al. 2006). Nevertheless, how the XCI machinery functions to inhibit Xi transcription remains a mystery. *Xist* coating is required for the accumulation of several heterochromatic marks over gene dense regions of the Xi, including H3K27me3, histone H2A ubiquitylation, histone H4-lysine20-monomethylation (H4K20me1), and incorporation of the histone variant macroH2A (Mak et al. 2002; Costanzi and Pehrson 1998; Kohlmaier et al. 2004; Plath et al. 2003; Silva et al. 2003). Induction of this heterochromatic state certainly is an important component of *Xist*-mediated gene silencing. However, both the coating of the Xi by *Xist* and the silencing of many X-linked genes are detected prior to Xi enrichment of these various heterochromatic marks, indicating they may be required to lock-in XCI-induced gene silencing rather than initiate the process. Consistent with this idea, Eed, a core component of the Polycomb Repressive Complex 2 (PRC2) that mediates deposition of H3K27me3, is only required for maintenance of XCI in differentiated extraembryonic derivatives, several cell division cycles after initiation of gene silencing (Kalantry et al. 2006). Remarkably, trophoblast stem cells (TSCs) lacking Eed lose Xi enrichment of all known heterochromatic marks, yet appear to maintain silencing of at least one X-linked locus, and still exclude chromatin modifications associated with active transcription from the genic Xi domain (Kalantry et al. 2006). These results again indicate that XCI-induced transcriptional repression can exist in the absence of enrichment for known, silencing-associated epigenetic marks.

Equally perplexing is the fact that coating of the Xi by *Xist* does not necessarily indicate the presence of a silenced X-chromosome. In human blastocysts, *Xist* coating and gene expression are co-detected at a high frequency over both X's, suggesting critical co-factors must co-localize with the RNA before gene silencing can proceed (Okamoto et al. 2012). This observation raises the intriguing possibility that some of the major players involved in the initiation of XCI during embryogenesis remain undiscovered. Similar factors would be expected to exist in mouse as well. Considering that imprinted XCI can initiate without *Xist* in certain mouse strains, but silencing is rapidly lost in *Xist*'s absence (Kalantry et al. 2009), such factors might be loaded onto the mouse X concurrently with, or prior to, spread of *Xist*, but subsequently require the RNA for stabilization and immediate maintenance of silencing. In random XCI, where Xi choice is not pre-determined, loading of *Xist* onto the future Xi may be a prerequisite for recruitment of putative silencing factors.

Additional evidence indicating *Xist* coating is separable from X-linked gene silencing comes from a study of X-reactivation in the mouse blastocyst (Williams et al. 2011). As imprinted XCI nears completion during the early stages of mouse development, cells of the epiblast reactivate their Xi before re-initiating the second

round of XCI, which randomly targets the paternal or maternal X for silencing. Quantitative analysis of gene expression via RNA FISH showed that re-activation could be detected on the Xi prior to loss of the *Xist* coat (Williams et al. 2011). Moreover, re-activation kinetics were not altered by overexpression of Nanog, which results in precocious loss of the *Xist* coat specifically in epiblast cells (Williams et al. 2011). Together, similar to the situation described above for human embryos, these results indicate that the transcriptional repression mediated by XCI and *Xist* coating of the Xi can be regulated separately in vivo.

A final piece of evidence indicating that *Xist* coating can be regulated separately from XCI-induced transcriptional repression comes from early transgenic studies of *Xist* itself. Systematic deletion of portions of the *Xist* cDNA in a transgenic mouse ESC model identified the Repeat A region as critical for the induction of gene silencing (Wutz et al. 2002). Although Repeat A mutant *Xist* was deficient in silencing, induced expression still led to *Xist* coating and accumulation of macroH2A, H3K27me3, and H4K20me1 over regions of the chromosome (Wutz et al. 2002; Kohlmaier et al. 2004; Plath et al. 2003). These data again support the notion that Xi coating by *Xist* and XCI-mediated transcriptional repression are separable events.

Contrary to what would be expected from Repeat A deletion in *Xist* transgenes, where mutant *Xist* coats the X without efficiently silencing genes (Wutz et al. 2002; Chaumeil et al. 2006; Kohlmaier et al. 2004; Plath et al. 2003), deletion of the Repeat A region from the endogenous *Xist* locus in the context of mouse development or in ESCs results in XCI failure due to a complete absence of *Xist* coating, and lack of properly spliced *Xist* RNA (Royce-Tolland et al. 2010; Hoki et al. 2009). Transcription of *Xist* appears unaltered in mutant cells (Royce-Tolland et al. 2010; Hoki et al. 2009). Together, these results indicate Repeat A is required for the post-transcriptional processing and stability of *Xist* RNA, in addition to its gene silencing properties. Inducible expression of wild-type or mutant *Xist* cDNAs from stably integrated transgenes appears to bypass XCI's post-transcriptional processing requirements, thus facilitating the identification of Repeat A as critical for *Xist*-mediated gene silencing (Wutz et al. 2002; Kohlmaier et al. 2004; Plath et al. 2003).

Beyond the requirement of Repeat A in *Xist*-mediated silencing, little is known about the mechanism by which XCI inhibits transcription. Early works showed that the nuclear domain occupied by *Xist* lacks nascent transcripts and is depleted of Pol II, general transcription factors, and splicing components (Clemson et al. 1996; Chaumeil et al. 2006; Clemson et al. 2006). Moreover, using DNA FISH to localize specific X-linked sequences relative to the mouse *Xist* domain, it was found that genes which escaped XCI were more frequently outside of the *Xist* domain than those that were subject to XCI (Chaumeil et al. 2006). Cot-1 DNA, which is primarily composed of LINE and SINE repetitive elements, also produced signal that overlapped with *Xist* RNA in FISH assays, in both mouse and human cells (Chaumeil et al. 2006; Clemson et al. 2006). Based on these data, it was hypothesized that XCI induces the formation of a repeat dense nuclear compartment, marked by *Xist*, which physically excludes Pol II and associated

transcription machinery from its occupied area (Namekawa et al. 2010; Chow et al. 2010; Chaumeil et al. 2006; Clemson et al. 2006). In such a model, genes subject to XCI enter the repeat-dense silent compartment coincident with inactivation, whereas those that escape XCI remain exterior to it, allowing them access to transcriptional machinery (Chaumeil et al. 2006).

More recent work suggests revisions to this compartmentalized view of XCI (Calabrese et al. 2012). Site-specific DNA FISH found that LINE-dense regions of the Xi are most frequently located directly adjacent to the *Xist* coat, rather than at its center, supporting previous observations that *Xist* associates with predominantly gene-dense rather than repeat-dense Xi regions (Mak et al. 2004; Chadwick and Willard 2004; Duthie et al. 1999). Also, while genes escaping XCI were frequently found outside of the *Xist* domain, so were the X-inactivated genes situated adjacent to them. In this spatial conformation, escapers were frequently expressed, but adjacent X-inactivated genes remained silent, as assessed via RNA FISH and RNA-Seq (Calabrese et al. 2012).

This latter observation is consistent with the recently described notion of topologically associated chromatin domains (TADs). TADs are (roughly) megabase-sized genomic regions that preferentially interact within themselves over surrounding DNA (Dixon et al. 2012; Nora et al. 2012). TAD location is generally consistent across cell types and differentiation states, and is often conserved between species (Dixon et al. 2012; Nora et al. 2012). Although genes contained within TADs are frequently co-regulated, differential expression within TADs also occurs (Dixon et al. 2012; Nora et al. 2012). In regards to the Xi, the nuclear position of individual TADs might largely be dictated by genes that escape XCI, which would be expected to frequently interact with transcription factories located outside of the Xi's *Xist*-dense regions. Considering the existence of TADs, it follows that X-inactivated and escaping genes present within the same or nearby TAD would be located external to the *Xist*-dense Xi domain at similar frequencies.

The observation that X-inactivated genes are not expressed, regardless of their location relative to the microscopically detectable *Xist* cloud, supports a site-specific model for XCI, where XCI-induced gene silencing is maintained independently of a singular nuclear compartment dedicated to transcriptional silencing (Calabrese et al. 2012). A collection of prior works supports this site-specific model of XCI, showing that loci across the X differentially respond to the XCI machinery in a manner that depends on both developmental and cellular context. Examining the timing of X-inactivation for individual X-linked loci during the initiation of imprinted XCI, Patrat and colleagues found that while some genes were efficiently silenced at the 4-8 cell stage, during the onset of imprinted XCI, others remained active and were not silenced until later in development, in some cases well beyond the blastocyst stage (Patrat et al. 2009). Similarly, certain genes appear more sensitive to *Xist* loss than others during the initiation of imprinted XCI (Kalantry et al. 2009), and different subsets of X-linked genes escape XCI in different cell types (Patrat et al. 2009; Calabrese et al. 2012; Carrel and Willard 2005; Cotton et al. 2011; Yang et al. 2010). Lastly, an allele-specific analysis of Pol II distribution in human somatic cells found that while most X-inactivated

genes lack Pol II association, a small number bind Pol II yet remain nontranscribed (Kucera et al. 2011). That XCI and escape can occur regardless of a gene's nuclear position, and that both processes show variability between cell types and developmental stages, suggests that the chromosome-level silencing capability of *Xist* requires some form of stably associated, developmentally regulated interface with specific regulatory sites to license the inactivation of individual loci.

Further insight into the physical mechanism by which XCI inhibits transcription has come from a quantitative analysis of chromatin states surrounding Xi regulatory elements. Recent work in F1 hybrid mouse TSCs found that X-inactivated promoters and intergenic regulatory elements maintained reduced levels of DNaseI hypersensitivity (DHS) despite excluding Pol II and other chromatin modifications associated with active transcription (Calabrese et al. 2012). This chromatin state appeared to be an epigenetic signature of XCI, as no single autosomal gene class—including autosomal Polycomb targets, lowly expressed, and nontranscribed genes—had a similar combination of DHS enrichment and Pol II exclusion. In autosomal contexts, DHS sites most frequently mark genomic locations bound by transcription factors engaged in the positive regulation of transcription (Song et al. 2011; Xi et al. 2007). The observation that X-inactivated regulatory elements still harbored detectable DHS in TSCs, albeit at reduced levels compared to the Xa, suggests they are still recognized and bound by cellular factors—these could be the transcription factors that bind cognate elements on the Xa, or unknown factors involved in XCI-induced silencing (Calabrese et al. 2012). Differentiating between these two possibilities, and determining whether cell types other than TSCs harbor similar Xi epigenetic signatures, will be important steps in understanding the mechanism of XCI.

8 Transcriptional Modulation of *Xist* as a Mechanism to Sense X-to-Autosome Ratios

The more X-chromosomes a cell has, the more it inactivates. Remarkably, however, the ratio between the number of Xa's per diploid autosomal complement remains at one, regardless of overall ploidy (Brown et al. 1992; Webb et al. 1992; Rastan 1994). These data suggest a mechanism must exist for cells to sense X-to-autosome ratios. Quantification of XCI status in diploid and tetraploid fusion ESC lines supported the presence of one to several activators of XCI present on the X chromosome, whose abundance relative to undefined autosomal loci dictated the likelihood that individual X's would undergo inactivation (Monkhorst et al. 2008). Subsequent BAC transgenic experiments identified the X-encoded ubiquitin ligase *Rnf12* (now called *Rlim*) as one of the major X-linked XCI activators (Fig. 1a; (Jonkers et al. 2009)). Overexpression of *Rlim* in male and female ESCs led to ectopic induction of XCI on one or both X's, respectively, and this induction depended on intact *Rlim* catalytic activity (Jonkers et al. 2009). *Rlim* therefore fit

the proposed build of an XCI activator: the higher the ratio of Rlim-to-autosomes, the higher the odds that any given X would be inactivated (Jonkers et al. 2009). Genetic deletion of *Rlim* resulted in complete failure of XCI in some ESC lines (Barakat et al. 2011), and no defect in others, suggesting additional XCI activators may compensate for *Rlim* loss in a strain-specific manner (Shin et al. 2010). Maternal loading of Rlim into oocytes is required for imprinted XCI in the mouse, indicating the protein is the major XCI activator during this first wave of XCI (Shin et al. 2010).

Rlim activates XCI by indirectly inducing expression of *Xist*. A proteomic screen found Rlim to interact with the autosomal transcription factor Rex1, and target it for ubiquitylation and subsequent proteolytic degradation (Gontan et al. 2012). As a result, Rex1 protein levels inversely correlate with levels of Rlim. Rex1 represses *Xist* transcription by binding to its promoter. Therefore, increasing the ratio of Rlim (X-linked) to Rex1 (autosomal) is one way that cells increase expression of *Xist*; high Rlim leads to Rex1 degradation, which in turn relieves *Xist* repression (Gontan et al. 2012). Given the need for *Xist* in the establishment of an Xi, it follows that regulated expression of the RNA is a major mechanism by which cells sense X-to-autosome ratios.

The ncRNA *Jpx* is another dose-dependent activator of *Xist* expression (Fig. 1a; (Tian et al. 2010)). Deletion of a single copy of *Jpx* in female ESCs results in a ~10-fold loss of XCI induction, an effect that can be rescued by addition of exogenous *Jpx* in *trans*. *Jpx* differs from Rlim in that it appears to activate *Xist* expression directly, counteracting the repressive effects that *Tsix* has on the locus. *Jpx* expression is induced ~20-fold during ESC differentiation, suggesting a role for the RNA in maintenance of *Xist* expression after XCI induction (Tian et al. 2010). How *Jpx* induces *Xist* expression is currently unknown.

Lastly, another ncRNA, *Ftx*, may play a partially redundant role with *Jpx* in the activation of *Xist* (Fig. 1a; (Chureau et al. 2011)). Like *Jpx*, *Ftx* is located adjacent to *Xist* in the Xic, escapes XCI, and is upregulated upon ESC differentiation. The RNA is also a miRNA precursor, an observation that may provide insight into its mechanism of action. Deletion of *Ftx* in male ESCs reduces transcription at loci across the Xic, most significantly of *Xist*, but also *Tsix*, *Jpx*, and intergenic transcription between *Jpx* and *Ftx*. Whether *Ftx* exerts its transcriptional effects in a *cis*- or *trans*-mediated manner is unclear. It is also currently unclear what role the RNA plays in a functional XCI response. ESC deletion data would predict a role in the broad regulation of ncRNA expression within the Xic (Chureau et al. 2011).

9 Transcriptional Silencing of *Xist* by *Tsix*

Just as the stabilization of *Xist* RNA on one X-chromosome is required to form an Xi, the transcriptional silencing of *Xist* on the other is required to form an Xa. In the mouse, this silencing is achieved primarily through the action of another long ncRNA, *Tsix*. As its name implies, *Tsix* is transcribed antisense to *Xist*. Its

transcription extends over the entire murine *Xist* locus, initiating about 15 kb away from *Xist*'s 3' end, and terminating about 2 kb after *Xist*'s 5' end (Fig. 1a; (Lee et al. 1999)). *Tsix* has exons and the RNA can be spliced, but splicing is not required for *Xist* silencing (Sado et al. 2006; Sado et al. 2001). Instead, transcription over *Xist*'s promoter appears to be the mechanism by which *Tsix* exerts its *cis*-mediated repressive effect (Luikenhuis et al. 2001; Ohhata et al. 2008). This transcription results in the deposition of DNA methylation and other repressive epigenetic modifications over *Xist*'s promoter that likely prevent its activation during differentiation (Ohhata et al. 2008; Sado et al. 2005). Notably, *Tsix* expression does not transcriptionally silence *Xist* in undifferentiated ESCs. Instead, its expression deposits histone H3-lysine4-dimethylation over the *Xist* locus, indicating *Tsix*'s repressive capacity is developmentally regulated (Navarro et al. 2005).

Through repression of *Xist* expression, *Tsix* plays a central role in determining which X-chromosome is chosen for silencing during random XCI. Deletion of a 65 kb region 3' to *Xist* that encompasses *Tsix*'s 5' end ($\Delta 65$ kb; Fig. 1a), or more targeted deletions that prevent *Tsix* transcription, result in nonrandom inactivation of the mutated allele in mice and ESCs (Clerc and Avner 1998; Lee and Lu 1999; Sado et al. 2001). This bias is near-absolute: *Tsix* mutant mice inactivate their mutant chromosome in 96 % of cells examined (Lee and Lu 1999; Sado et al. 2001). These studies indicate that transcription of *Tsix* plays a critical role in repressing *Xist* expression on the future Xa. Similar to the situation observed for *Xist* mutations in random XCI, *Tsix* mutants show evidence of a primary XCI defect, meaning that the mutation appears to influence choice of Xi directly, and not the maintenance of choice (Lee and Lu 1999). In the absence of *Tsix*, *Xist* expression may be more easily maintained throughout the initiation process, causing the severe inactivation bias.

In addition to its role in choice, maintained *Tsix* expression is required to prevent ectopic induction of XCI on the Xa during early mouse development. Male and female embryos with a maternally inherited *Tsix* mutation are recovered at a low frequency, between 1 and 15 % of what would be expected from normal Mendelian inheritance (Sado et al. 2001; Lee 2000). This lethality results from ectopic inactivation of the maternally inherited X in the extraembryonic lineages (Ohhata et al. 2006). ESC lines deficient in *Tsix* expression also undergo low levels of ectopic XCI upon differentiation (Luikenhuis et al. 2001; Sado et al. 2002; Morey et al. 2001; Vigneau et al. 2006). These studies suggest that continued expression of *Tsix* is required for normal Xa maintenance in both the embryonic and extraembryonic lineages. The requirement for *Tsix* in Xa maintenance, in both females and males, suggests *Xist* upregulation during the early stages of XCI is a blanket mechanism that affects all X-chromosomes lacking *Tsix* expression.

Tsix is not absolutely required for proper XCI. Surviving mouse embryos carrying a maternally inherited *Tsix* mutation are runted, but display expected XCI status and are fertile (Lee 2000). Similarly, in crosses between *Tsix* heterozygotes, *Tsix* homozygous females are recovered at only 4 % of the expected frequency, but are viable and display random XCI (Lee 2002). Female ESC populations

homozygous for this same *Tsix* mutation also are capable of proper XCI upon differentiation, but display significantly elevated levels of cells carrying two Xi's, and have high levels of cell death upon differentiation (Lee 2005). The toxicity associated with the inheritance of nonfunctional *Tsix* alleles speaks to the importance of this ncRNA in the proper regulation of XCI. That certain cells are able to establish a proper Xa-to-Xi ratio in the absence of functional *Tsix* indicates a level of stochasticity associated with XCI that appears to confer robustness to the dosage compensation process.

10 Regulation of *Tsix* Expression as a Mechanism Driving Xi Choice

The transcriptional regulation of *Tsix* is a complex process that ultimately determines choice of Xi during random XCI. Beyond *Tsix*'s core promoter, several separate regulatory regions appear to be important for expression of the RNA. The most potent of these identified thus far is the *DXPas34* enhancer, a 1.2 kb CG-rich microsatellite repeat approximately 750 bp away from *Tsix*'s transcriptional start site (Fig. 1a; (Courtier et al. 1995; Heard et al. 1993)). Deletion of *DXPas34* results in reduction of *Tsix* transcription and nonrandom inactivation of the mutated allele, similar to that observed for *Tsix* promoter deletions and truncations (Vigneau et al. 2006; Cohen et al. 2007; Debrand et al. 1999). The region likely serves as a loading site for positive regulators of *Tsix* transcription, as it has been documented to recruit a host of transcriptional regulators, including CTCF, YY1, Rex1, Klf4, and c-Myc (Donohoe et al. 2007; Navarro et al. 2008). Consistent with an enhancer function for *DXPas34*, the element displays DHS, and increases basal Luciferase activity in reporter assays (Stavropoulos et al. 2005). *DXPas34* also produces small RNA from both orientations in ESCs (Cohen et al. 2007), similar to many known enhancer elements (Kim et al. 2010).

Another important player in the regulation of *Tsix* expression is *Xite*, which stands for *X*-inactivation Intergenic Transcription Elements (Fig. 1a; (Ogawa and Lee 2003)). *Xite* marks a cluster of intergenic transcription start sites that begins upstream of *Tsix*'s basal promoter and extends to the *Tsx* gene (Ogawa and Lee 2003). Deletion of *Xite* reduces *Tsix* expression, albeit to a lesser extent than does *DXPas34* deletion, and as a consequence, *Xite* mutants show biased inactivation of the targeted allele (Ogawa and Lee 2003). Truncation of *Xite* RNA via insertion of a splice acceptor and polyadenylation sites does not bias XCI, suggesting that the RNA *per se* does not modulate *Tsix* expression (Ogawa and Lee 2003). Rather, *Xite* DNA itself appears to be an important regulator of XCI, as ESCs stably transfected with extranumerary fragments of *Xite* fail to undergo XCI upon differentiation (Lee 2005).

Most recently, a number of potential *Tsix* regulatory sites were identified in a chromosome conformation capture screen examining the spatial organization of a 4.5 Mb region of the X-chromosome that surrounds the *Xic* (Nora et al. 2012).

This work found the *Tsix* locus and all of its previously known regulators to exist within a single TAD situated upstream of *Xist*'s 3' end (TAD D, Fig. 1a). Within this TAD, several previously unknown contact sites were identified that formed significant interactions with *Tsix* or *Xite*, and showed features reminiscent of regulatory regions. Strikingly, many fell within an 80 kb transcribed region, which was termed *Linx*, for large intervening transcript in the Xic (Fig. 1a). *Linx* has features typical of a ncRNA, including nuclear retention and high levels of intron-containing transcripts. *Linx* is co-expressed with *Tsix* in the epiblast from around the time of implantation onwards, and shows frequent mono-allelism, potentially indicative of a function in XCI (Nora et al. 2012). Future experiments targeting the *Linx* locus should shed light on the potentially important biological function of this RNA.

Beyond the individual elements required for their transcription, the crucial factor driving Xi choice in random XCI is the establishment of asymmetrical expression patterns at *Xist* and *Tsix*. How this essential asymmetry is achieved is unknown. One potential clue comes from the analysis of DNA FISH patterns over the two X's in ESCs (Mlynarczyk-Evans et al. 2006). DNA FISH signals for single loci on the same chromosome can often appear as doublets due to the spatial separation of replicated alleles. Mlynarczyk-Evans and colleagues showed that, in a given ESC, the X-chromosome destined to become the Xi shows a characteristic pattern of singlets and doublets in DNA FISH assays: the Xic to be inactivated appears as a singlet, while the genic loci across the chromosome appear as doublets (Mlynarczyk-Evans et al. 2006). Remarkably, the other X, destined to become the Xa, shows the reciprocal pattern, with a doublet at the Xic and singlets across the remainder of the chromosome. These patterns depend on functional copies of *Xist* and *Tsix*, can fluctuate within the same cell, and are not the result of asynchronous DNA replication (Mlynarczyk-Evans et al. 2006). Although their physiological relevance is unclear, these DNA FISH patterns stand alone as the earliest known markers of the future Xa/Xi, differentiating the two X's prior to the induction of XCI.

Extensive microscopic analyses have revealed another physiological event with potential importance in both the sensing of X-chromosome dosage and ultimate choice of Xi: the transient homologous pairing of X-chromosomes. Shortly after induction of XCI via differentiation of ESCs, the Xic's of the two homologous X-chromosomes transiently co-localize in nuclear space (Xu et al. 2006; Bacher et al. 2006). This pairing is short-lived (about 45 min long), requires transcription and the *trans*-factors CTCF and Oct4, and can be driven by several regions within the Xic, including *Tsix*, *Xite*, and a region termed the X-pairing region (Xpr, Fig. 1a; (Xu et al. 2006; Bacher et al. 2006; Donohoe et al. 2009; Xu et al. 2007; Masui et al. 2011; Augui et al. 2007)).

The exact role of pairing in XCI remains ambiguous. Loss of pairing is seen in almost every scenario where random XCI is disrupted, including when XCI is completely inhibited, when it is nonrandom, and when it is induced on both X-chromosomes. For example, both pairing and XCI induction are disrupted by

increasing dosage of *Tsix* and *Xite* sequences via stable transfection into ESCs (Lee 2005; Xu et al. 2007). Conversely, *Tsix/Xite* deletions that result in non-random XCI also disrupt pairing (Xu et al. 2006; Bacher et al. 2006). The add-back of a 16 kb sequence that encompasses the *Tsix* promoter to these mutant cells can restore pairing but not random XCI (Bacher et al. 2006). Lastly, RNAi-mediated ablation of Oct4 results in loss of pairing with ectopic induction of *Xist* and inactivation of both X's—exactly the opposite effect of that seen in scenarios of *Tsix/Xite* overdose, and different from the nonrandom XCI observed when a single copy of *Tsix* is deleted (Donohoe et al. 2009). All together, these studies indicate an intimate link between pairing and proper execution of random XCI. However, pairing is not absolutely required for X-linked silencing, nor does the presence of pairing ensure randomness of inactivation.

In genetically normal cells, however, there is evidence to support a role for pairing in choice of Xi. Using live-cell imaging followed by fixation and RNA FISH, Masui and colleagues found that *Tsix* expression became monoallelic in differentiating ESCs shortly after release of pairing (Masui et al. 2011). Pairing may therefore play a role in the monoallelic assignment of *Tsix* transcription, and through this, choice of Xi. Considering this, and the data showing loss of pairing and XCI upon increased dosage of *Tsix* or *Xite* DNA (Lee 2005; Xu et al. 2006; Xu et al. 2007), pairing may be linked to a chromosomal counting process that requires the direct exchange of trans factors from one X to the other. The biological basis of pairing, and how it may impart monoallelic expression upon the *Tsix* locus, remains to be determined.

11 Other ncRNAs Associated with XCI

Beyond *Xist*, *Tsix*, and the ncRNAs controlling their expression within the Xic, at least three additional X-linked RNAs have potentially important roles in XCI. RepA is a 1.6 kb RNA located within the larger *Xist* that contains the Repeat A sequence (Figure S1A,B; (Zhao et al. 2008)). It was identified via immunoprecipitation of PRC2 complex components in ESCs and MEFs, followed by RT-PCR detection of associated RNA. In PRC2 immunoprecipitates, RNA from the 5' end of *Xist*, which overlapped the Repeat A sequence, was consistently detected, but the remainder of *Xist* RNA was not. Northern blots probing with Repeat A sequence subsequently identified a 1.6 kb RNA, which was termed RepA. RepA associates with Ezh2, and induction of its expression from stably integrated autosomal loci recruits the PRC2 complex. RepA is polyadenylated and may be transcribed from its own promoter or processed from a larger *Xist* transcript. shRNA knockdown of RepA is not possible without reduction of full-length *Xist* transcripts, making it difficult to unambiguously ascribe function to the shorter RNA. Nonetheless, initial results suggest RepA is a co-factor involved in *Xist* activation and recruitment of PRC2 to the Xi (Zhao et al. 2008). It is important to

note that while RepA may play an important role in both processes, redundant mechanisms are likely involved in PRC2 recruitment to the Xi; prior works have shown that overexpression of *Xist* cDNAs lacking the Repeat A region still cause H3K27me3 accumulation over the X, albeit at significantly reduced frequency relative to wild-type *Xist* (Kohlmaier et al. 2004; Plath et al. 2003).

RNA produced from full length LINE elements across the Xi may also be involved in XCI (Chow et al. 2010). RNA FISH analysis in differentiating ESCs showed a striking accumulation of LINE transcripts adjacent to, or directly overlapping with, the *Xist* domain in the early and late stages of XCI, respectively. These LINE transcripts were transcribed by Pol II and specific to the Tf- and Gf-LINE subfamilies (Ostertag and Kazazian 2001). Other classes of repetitive elements, such as SINEs, showed no such accumulation within the *Xist* domain. Furthermore, the induction of LINE transcripts was not specific to the Xi per se, but rather occurred whenever *Xist* was induced; *Xist* expression from autosomal stably integrated transgenes in male ESCs also led to localized accumulation of Gf- and Tf-LINE RNA (Chow et al. 2010).

The exact origin and function of these LINE-derived transcripts in XCI is unknown. The highly repetitive nature of full-length LINES makes it difficult to pinpoint their expression to specific chromosomal loci. Furthermore, the induction of LINE RNA appears to occur stochastically, being detected in about ~25 % of differentiated ESCs with an *Xist* domain (Chow et al. 2010). This apparent stochasticity may be due to transient induction of LINE RNAs at a specific stage of XCI, making them difficult to detect via RNA FISH in a heterogeneous population of differentiating ESCs. LINE transcripts accumulate around the time that X-linked genes become silenced, correlating LINE expression with transcriptional silencing. Moreover, low abundance sense and antisense small RNAs were also produced from at least one LINE-adjacent locus during XCI induction, potentially linking LINE-derived transcripts to RNAi-mediated processes (Chow et al. 2010).

Most recently, a long ncRNA expressed specifically from the Xa was discovered in the analysis of RNA-seq data from human ESCs (Vallot et al. 2013). *XACT* is a striking ~252 kb in length, unspliced, polyadenylated and predominantly nuclear. Similar to *Xist*, *XACT* accumulates in a cloud-like structure over its chromosome of synthesis. Unlike *Xist*, however, *XACT* coats the Xa, and is expressed in both male and female human ESCs. *XACT* expression is restricted to pluripotent cells in humans. DNA FISH, RNA FISH, and RNA-seq failed to detect *XACT* expression in the mouse, suggesting it is a human-specific ncRNA. The role of *XACT* in dosage compensation is unknown. Given its expression pattern, it likely functions as a regulator of the process specifically in undifferentiated cells (Vallot et al. 2013). The recent identification of *XACT* serves as reminder of how little is understood about XCI in humans, and the complex roles that X-linked ncRNAs play in the process across mammals.

12 Conclusions

The last 20 years of XCI research has uncovered a surprisingly large number of ncRNAs that are either required for XCI or likely play as-of-yet understood roles in the process. By virtue of these discoveries, XCI has consistently proved its value as a paradigm for understanding diverse aspects of ncRNA function in nuclear cell biology. The human genome encodes thousands of ncRNAs, many of which are expressed with high levels of tissue-specificity and are conserved across mammals, and most of which have no known function (Dunham et al. 2012; Derrien et al. 2012; Cabili et al. 2011). In many ways, XCI is a microcosm of this ncRNA universe, and knowledge gained from its study will continue to have relevance across disciplines.

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