The X as Model for RNA's Niche in Epigenomic Regulation

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SUMMARY

The X-linked region now known as the “X-inactivation center” (*Xic*) was once dominated by protein-coding genes but, with the rise of Eutherian mammals some 150–200 million years ago, became infiltrated by genes that produce long noncoding RNA (ncRNA). Some of the noncoding genes have been shown to play crucial roles during X-chromosome inactivation (XCI), including the targeting of chromatin modifiers to the X. The rapid establishment of ncRNA hints at a possible preference for long transcripts in some aspects of epigenetic regulation. This article discusses the role of RNA in XCI and considers the advantages RNA offers in delivering allelic, *cis*-limited, and locus-specific control. Unlike proteins and small RNAs, long ncRNAs are tethered to the site of transcription and effectively tag the allele of origin. Furthermore, long ncRNAs are drawn from larger sequence space than proteins and can mark a unique region in a complex genome. Thus, like their small RNA cousins, long ncRNAs may emerge as versatile and powerful regulators of the epigenome.

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1 INTRODUCTION

It has become very fashionable to study long noncoding RNA (ncRNA). Given that only 1%–2% of the mammalian genome carries protein-coding information, genomicists have oft pondered the relevance of the remaining 98% which, as a whole, bear little phylogenetic conservation among any except for the most closely related species. It is by now clear, however, that the vast intergenic space is anything but quiescent. An astounding 70%–90% of our nucleotides are apparently transcribed (Kapranov et al. 2007b; Mercer et al. 2009). The mouse and human transcriptomes contain 80,000–180,000 RNAs (Carninci et al. 2005; Wheeler et al. 2008), the majority of which lack conserved open reading frames and belong to a rising class of transcripts formerly thought to be “junk,” ranging in size from 50 nt to >100 kb. Affectionately dubbed “macroRNA,” they are distinct from catalytic and structural RNAs, as well as the well-known small RNAs of the RNA-interference pathway (Cam et al. 2009; Cech 2009; Ghildiyal and Zamore 2009; Moazed 2009; Sharp 2009), and can be antisense, promoter-associated, or intergenic (Claverie 2005; Katayama et al. 2005; Kapranov et al. 2007a; Kapranov et al. 2007b; He et al. 2008; Guttman et al. 2009; Khalil et al. 2009; Mercer et al. 2009). Whether they possess function or are merely incidental biproducts of genome activity remains unknown, but many are clearly developmentally regulated and linked to genes with undisputed function.

Although the occurrence of genome-wide transcription has sparked widespread interest in long ncRNA only recently, such RNAs have captivated investigators in some fields for some time. Epigeneticists have long been drawn to the mystique surrounding RNA behemoths, beginning with the discovery of the 2.6-kb H19 RNA in the field of genomic imprinting (Brannan et al. 1990; Bartolomei et al. 1991) and the 17-kb XIST RNA for X-chromosome inactivation (XCI) (Borsani et al. 1991; Brown et al. 1991a; Brockdorff et al. 1992; Brown et al. 1992). Genes subject to imprinting are expressed from only one of two alleles in a manner dependent on parent of origin, and such genes are almost always clustered within domains and coordinately regulated *in cis* by a single (sometimes two) “imprinting centers” (Edwards and Ferguson-Smith 2007; Wan and Bartolomei 2008). Likewise, XCI—the mechanism of dosage compensation in mammals—is a domain phenomenon that extends to include the entire X-chromosome (Lyon 1961; Wutz 2003; Lucchesi et al. 2005; Masui and Heard 2006; Wutz and Gribnau 2007; Payer and Lee 2008). During XCI, almost all of the ~1000 protein-coding genes on one of two X-chromosomes become transcriptionally inactivated *in cis* by a single control region known as the

“X-inactivation center” (Cattanach and Isaacson 1967; Rastan and Robertson 1985). Because the nature of XCI is also allelic, silencing must be coordinately controlled to take place only on genes of one chromosome. Thus, imprinting and XCI are unified by the fact that a small control region exerts long-range effects on chromatin and by the allelic nature of the regulatory process.

The existence of two macroRNAs for two quintessentially “epigenetic” phenomena brought early suspicion that RNA may be central to the control of epigenetic inheritance. The subsequent discoveries of the regulatory antisense RNAs, *Airn* for the imprinted *Igf2r* locus (Wutz et al. 1997) and *Tsix* for XCI (Lee et al. 1999a; Lee and Lu 1999)—themselves even longer ncRNAs—raised that level of suspicion. The emerging understanding that *Tsix* RNA may help target DNA methylation by *Dnmt3a* and other chromatin modifiers, either directly or indirectly, to the *Xist* promoter suggested that RNA may be a recruiting tool for chromatin modifiers on the X (Navarro et al. 2005; Sado et al. 2005; Sun et al. 2006). That RepA RNA directly binds and targets Polycomb proteins to the X then argued that RNA may be a direct mechanism for recruiting specific enzymatic activities *in cis* (Zhao et al. 2008). The antisense *Airn* and *Kcnqlot1* RNAs have also been hinted to play similar roles at imprinted loci, by direct or indirect means (Nagano et al. 2008; Pandey et al. 2008). Such findings have brought recognition of RNA’s potential in regulating our epigenomic landscape *in cis* and led to further ruminations about the possible roles of pervasive noncoding transcription in our genomes.

This article will develop the concept of RNAs as effectors of choice by drawing examples from the XCI paradigm. To date, at least seven distinct noncoding genes have been found within the X-linked region loosely defined as the “X-inactivation center” (*Xic*), a 100–500-kb domain with few, if any, protein-coding genes (Brown et al. 1991b; Lee et al. 1996; Simmler et al. 1996; Willard 1996; Lee et al. 1999b; Chureau et al. 2002). Why have long ncRNAs come to dominate the *Xic* landscape? RNA’s supremacy seems to imply that this nucleic acid polymer offers something that proteins do not (Lee 2009). Herein, I will argue that RNA is ideally suited to epigenetic inheritance, particularly in cases that demand allelic regulation (*cis* effects) and action at a single locus (singularity).

2 THE UNIQUE CHALLENGES OF XCI

XCI presents a number of interesting challenges. Two X-chromosomes sharing the same nucleus must be treated in diametrically opposite ways during early development. There is first the problem of chromosome counting—the determination of whether it possesses one (XY) or two

Xs (XX) and whether it should therefore initiate XCI. Genetic analyses have shown that an X:autosome ratio of 1 or greater triggers the XCI cascade (Kay et al. 1994; Lyon 1999; Avner and Heard 2001; Boumil and Lee 2001) and that noncoding elements of the *Xic* influence the X:A ratio (Clerc and Avner 1998; Morey et al. 2004; Lee 2005; Donohoe et al. 2009; Monkhorst et al. 2009).

There is second the problem of X-chromosome choice, which implies the existence of a random selection mechanism that chooses the active X (Xa) and the inactive X (Xi) in a mutually exclusive manner (Lee 2002). The mutually exclusive nature of choice necessitates communication between the Xs *in trans* to ensure that a cell does not adopt the lethal XaXa or XiXi fate. This second step of XCI requires the action of noncoding genes as well.

Finally, silencing factors must be recruited to the future Xi in a colinear fashion, spreading along the chromosome in a strictly *cis*-limited manner and without *trans* effects on homologous loci of the future Xa. It is widely believed that inactivation begins at the *Xic* before spreading outwardly to encompass the rest of the X-chromosome. During XCI, the Xi becomes distinguished from Xa by its enrichment for Polycomb proteins, the SET domain protein HP1, variant histones such as macroH2A, and a number of distinct chromatin modifications (Plath et al. 2002; Wutz 2003; Lucchesi et al. 2005; Payer and Lee 2008). How heterochromatinization and gene silencing are coordinated along 1000 genes of the X remains a largely unsolved problem,

but there is widespread belief that Xist RNA is the messenger of the *Xic*, that it spreads along the X, and that this action is directly responsible for reprogramming the chromosome's gene expression state.

3 XCI'S REPERTOIRE OF NCRNA REGULATORS

At least seven noncoding loci have been identified within the 100- to 500-kb region in or around the *Xic* (Brown et al. 1991b; Lee et al. 1996; Simmler et al. 1996; Willard 1996; Lee et al. 1999b; Chureau et al. 2002) (Fig. 1A). Several have been shown to play vital roles during XCI (Fig. 1B). The 17-kb Xist RNA is transcribed only from the Xi and its accumulation on the X is thought to induce chromosome-wide silencing by binding and spreading silencing complexes throughout the X *in cis* (Brockdorff et al. 1992; Brown et al. 1992; Clemson et al. 1996; Penny et al. 1996; Marahrens et al. 1997). Embedded within the 5' end of the *Xist* locus is a repeated motif known as "Repeat A" (Brown et al. 1992), previously shown to be essential for Xist RNA's silencing function (Wutz et al. 2002). It is now known to encode a separate transcription unit, named "RepA." RepA RNA directly binds Polycomb proteins and recruits them to the *Xic* in an action that paradoxically activates *Xist* expression and the initiation of XCI (Zhao et al. 2008).

The actions of RepA and Xist RNAs are controlled by Tsix, a 40-kb ncRNA that is antisense to both RNAs (Lee et al. 1999a; Lee and Lu 1999; Lee 2000; Luikenhuis et al.

Figure 1. The X-inactivation center, its noncoding genes, and their functions during the XCI cascade. (A) More than seven noncoding genes have been found within or around the *Xic*. *Xist*, *RepA*, *Tsix*, and *Xite* have ascribed function. The regions responsible for X-chromosome pairing, counting/choice, and spreading/silencing are indicated. (B) Steps of XCI and the factors known to regulate them.

2001; Sado et al. 2001). *Tsix* bears a crucial relationship to *Xist* (Fig. 2). In undifferentiated cells (pre-XCI), *Tsix* is expressed on both Xs. During this stage, *Xist* RNA is expressed at extremely low “basal” levels. At the onset of cell differentiation, *Tsix* persists only on the chromosome selected to become Xa. It is the persistence of *Tsix* RNA that prevents the up-regulation of *Xist* on Xa. Conversely, the down-regulation of *Tsix* on Xi creates a permissive state for *Xist* transactivation. Whether *Tsix* RNA will persist during cell differentiation is controlled by the upstream locus, *Xite*. *Xite* harbors a developmentally regulated enhancer that maintains expression of *Tsix* on Xa and prevents inactivation of Xa (Ogawa and Lee 2003; Stavropoulos et al. 2005). Thus, the ncRNAs have a Yin-yang relationship to each other, with RepA and *Xist* RNAs promoting the initiation of XCI, and *Xite* and *Tsix* RNAs blocking that action.

Several other ncRNAs have been identified, including Jpx/Enox (Chureau et al. 2002; Johnston et al. 2002), Ftx (Chureau et al. 2002), and the bidirectional transcripts (Cohen et al. 2007) associated with a repeat element called “DXPas34,” located at *Tsix*’s 5’ end (Courtier et al. 1995; Debrand et al. 1999). Small RNAs of 21–42 nucleotides are also observed in this region, presumably cleaved from long *Xist*/*Tsix* RNA duplexes (Ogawa et al. 2008). Although some ncRNAs remain uncharacterized, several have well-defined roles and their mechanisms of action have come into sharper focus during the past years. The discrete roles of four noncoding genes—*Xite*, *Tsix*, *RepA*, and *Xist*—will be discussed in the next sections.

4 RNA IN PAIRING, COUNTING, AND CHOICE

In the widely used embryonic stem (ES) cell model, XCI is triggered directly by decreasing Oct4 levels and the onset of

cell differentiation (Monk and Harper 1979; Navarro et al. 2008; Donohoe et al. 2009). Evidence suggests that “counting” involves interaction between Oct4 and a 15-kb region encompassing *Xite* and the 5’ end of *Tsix* (Fig. 1A) (Clerc and Avner 1998; Morey et al. 2004; Lee 2005; Donohoe et al. 2009). This same region has also been shown to function during “choice.” Its mutually exclusive nature implies interchromosomal communication to ensure that each cell acquires exactly one Xa and one Xi (Marahrens 1999; Lee 2005). Recent studies have proposed that homologous X-chromosome “pairing” may mediate communication *in trans* and, in doing so, help achieve correct counting and choice of Xa and Xi (Bacher et al. 2006; Xu et al. 2006; Xu et al. 2007; Donohoe et al. 2009). Time course analyses have indicated that the Xs make very brief contact ($t_{1/2} < 30$ –60 min) shortly after the onset of cell differentiation and just before the up-regulation of *Xist* RNA (Xu et al. 2007). When pairing is disrupted genetically or pharmacologically, cells adopt either the XaXa or XiXi phenotype and they die or arrest in culture. Thus, pairing achieves counting and the mutually exclusive choice of Xa and Xi by providing a platform for inter-chromosomal communication and by breaking symmetry between two X-chromosomes (Bacher et al. 2006; Xu et al. 2006; Nicodemi and Prisco 2007b).

Unlike homologous pairing during meiosis and in somatic cells of fruitflies, X-X pairing does not involve whole chromosomes—it occurs only at the *Xic*. Not surprisingly, pairing elements coincide with those responsible for counting and choice in the 15-kb region including *Xite* and *Tsix* (Xu et al. 2006) (Fig. 1A). Deleting 3.7 kb of sequence at the 5’ end of *Tsix* on both X-chromosomes abrogates pairing. For *Xite*, deleting even just one allele severely compromises pairing potential in female cells. *Tsix* and *Xite* are not only required but are also sufficient to induce pairing. When

Figure 2. Yin-Yang relationship between *Tsix* and *Xist* RNAs. *Tsix* bears a critical relationship to *Xist* expression. In undifferentiated cells (pre-XCI), *Tsix* is expressed from both Xs at high levels and *Xist* RNA is expressed at low basal levels (~5 copies). At the onset of cell differentiation, *Tsix* persists only on the chromosome selected to become Xa. It is the persistence of *Tsix* RNA that prevents the up-regulation of *Xist* on Xa. Conversely, the down-regulation of *Tsix* on Xi creates a permissive state for *Xist* transactivation and spread of the RNA along the X to initiate chromosome-wide silencing. *Tsix* is eventually turned off on Xa once the pattern of XCI is fixed.

inserted at ectopic locations in the genome, *Tsix* and *Xite* direct pairing of the X with the chromosome into which they are inserted (Bacher et al. 2006; Xu et al. 2006). Unexpectedly, pairing can be induced by 1–3-kb fragments that carry very diverse sequences, and even sequences of very low complexity recapitulate pairing. For example, a 1.6-kb element (*DXPas34*) carrying a 34-nucleotide motif repeated 29 times induces X-autosome interactions when multimerized on autosomes (Xu et al. 2007).

How do elements of such diverse nature and low complexity drive two chromosomes together? Two interesting features are shared by most if not all pairing-competent sequences (Xu et al. 2007). First, they bind Ctfp protein, an 11-Zn finger protein involved in many aspects of epigenetic regulation (Lobanenkov et al. 1990; Bell et al. 1999; Ohlsson et al. 2001; Kim et al. 2007). Ctfp has a large number of binding sites within *Tsix* and *Xite* loci and is thought to function as a transcriptional activator of both genes (Chao et al. 2002; Xu et al. 2007). Ctfp interacts with Oct4, a pluripotency factor thought to trigger counting as Oct4 levels fall during cell differentiation, and knocking down either Ctfp or Oct4 leads to an abrogation of pairing in ES cells (Donohoe et al. 2009; Xu et al. 2007).

Sequences sufficient for pairing also share transcriptional competence, a fact that raises the intriguing possibility of an RNA requirement. Despite their diversity of sequence and low sequence complexity, nearly all pairing-competent fragments show Pol-II promoter activity (Sado et al. 2001; Ogawa and Lee 2003; Stavropoulos

et al. 2005; Cohen et al. 2007; Xu et al. 2007). Inhibiting Pol-II transcription by a 2.0–4.0 h actinomycin D treatment completely inhibits X-X pairing, indicating that new transcription is necessary for X-chromosome coupling (Xu et al. 2007). In contrast, inhibiting Pol-II for as little as 0.5 h disrupted new pair formation but did not affect cohesion of previously formed pairs. Time course analysis shows that cohesion of the previously formed pairs decayed with a half-life of ~0.5–1.0 h. Combined, these data show that pairing is very transient and that an RNA, or the act of transcribing it, is crucial for formation of new X-chromosome pairs.

It is therefore proposed that a complex of RNA and protein mediates X-X pairing (Fig. 3). In undifferentiated pre-XCI cells, Oct4 and Ctfp proteins would dimerize and bind *Tsix* and *Xite* to activate expression of the ncRNAs. Falling Oct4 levels during differentiation would then trigger trans-association of the *Xic*'s in an RNA-dependent manner. The *trans*-interactions would then lead to physical differences between two Xs and signal one to become Xa and the other Xi. For example, proximity of *Xic*'s might result in the irreversible shift of protein factors such as Oct4 and Ctfp—which are initially bound to both *Xic*'s—from one *Xic* (future Xi) to the other (future Xa) (Xu et al. 2006; Xu et al. 2007; Donohoe et al. 2009). Although this idea has yet to be tested, computational modeling suggests that, under certain conditions, the shift of factors to one chromosome could create greater thermodynamic stability (Nicodemi and Prisco 2007a; Nicodemi

Figure 3. Symmetry break mediated by pairing and *Tsix*. Adapted from (Anguera et al. 2006) and (Lee 2009): In the pre-XCI state, the Xs are epigenetically equivalent, both expressing *Tsix* at high levels and *Xist* at low basal levels. Falling levels of Oct4 during cell differentiation triggers X-X pairing. Pairing is mediated by Ctfp, *Tsix*, and *Xite* and enables cross-talk to achieve correct counting and mutually exclusive Xa and Xi fates. Transcription factors such as Ctfp, Oct4, and others (red, green circles), which were previously randomly distributed between the two Xs, shift to bind one X, a state that is thermodynamically favored. The chromosome that retains the transcription factors maintains *Tsix* expression and becomes Xa. Loss of factor binding to the future Xi enables RepA to target PRC2 and H3-K27me3 to the 5' end of *Xist*, an event that leads to *Xist*'s transcriptional activation. *Xist* RNA spreads along the future Xi and recruits PRC2 to it to initiate chromosome-wide silencing.

and Prisco 2007b; Nicodemi et al. 2008). This shift of activators would then result in allele-specific persistence of *Tsix* on the future Xa, which in turn would establish the necessary X-chromosome asymmetry. In this way, the pairing of X's makes possible both the counting of X's and the mutually exclusive choice of Xa and Xi at the onset of cell differentiation.

5 SYMMETRY BREAK BY TSIX RNA

Such a model suggests that pairing generates asymmetric *Tsix* expression and that *Tsix* must therefore function as a molecular switch for XCI. Accordingly, *Tsix* is one of the most heavily mutagenized genes in the history of mouse genetics. Toward understanding its mechanism of action, multiple knockouts, knock-ins, and transgene manipulations have been created to delete, truncate, overexpress, and transplant parts or all of the antisense RNA (Lee et al. 1999a; Lee and Lu 1999; Lee 2000; Luikenhuis et al. 2001; Sado et al. 2001; Stavropoulos et al. 2001; Sado et al. 2002; Morey et al. 2004; Shibata and Lee 2004; Lee 2005; Ohhata et al. 2006; Sado et al. 2006; Xu et al. 2007; Ohhata et al. 2008; Shibata et al. 2008). Abrogating *Tsix* expression results in constitutively elevated *Xist* expression, whereas its forced expression prevents *Xist* up-regulation. Such analyses have shown that not only does *Tsix* participate in the most upstream steps of XCI (counting, choice, pairing), but it also plays an active role during cell differentiation. Indeed, it is *Tsix*'s binary state during cell differentiation that breaks X-chromosome symmetry (Fig. 3).

In the pre-XCI cells, both alleles of *Tsix* are expressed, with the consequence that both *Xist* alleles are uninduced (very low level, basal expression state). Time course analyses of chromatin structure using chromatin immunoprecipitations (ChIP) have revealed dynamic changes during cell differentiation. Several of the analyses show that *Tsix* regulates *Xist* by modulating the chromatin state of the *Xist* promoter (Navarro et al. 2005; Sado et al. 2005; Navarro et al. 2006; Sun et al. 2006). One study followed allelic differences in female ES cells during XCI and found that *Xist* promoter's configuration mirrored that of *Tsix*'s transcriptional state (Sun et al. 2006). When *Tsix* is expressed in pre-XCI cells, both *Xist* alleles are maintained in an open chromatin state. During cell differentiation, loss of *Tsix* on the future Xi results in a closed chromatin conformation along the 40-kb *Tsix/Xist* locus. The repressive chromatin strangely correlates with *Xist* transactivation, as the 5' end of *Xist* becomes H3-K27 trimethylated (H3-K27me3) just prior to the 100-fold induction of *Xist* transcription (see next section for further discussion). These unexpected results suggested that *Xist* expression may, paradoxically, be enhanced by repressive chromatin.

On the future Xa, cell differentiation leads to continued expression of *Tsix*, which in turn maintains the domain in an open state that is enriched for H3-K4 methylation and H4 acetylation and depleted of H3-K27 trimethylation. This "open" chromatin configuration occurs on the repressed *Xist* allele. RNA ChIP in ES cells suggests that *Tsix* RNA complexes with the de novo methyltransferase, Dnmt3a, and facilitates CpG methylation and silencing of the *Xist* promoter (Sun et al. 2006), consistent with in vivo analysis in *Tsix*-deficient and Dnmt3a-deficient mice (Sado et al. 2004; Sado et al. 2005). Interestingly, small RNAs can be generated from longer *Tsix:Xist* RNA duplexes in vivo, a finding that raises the possibility of RNA-directed DNA methylation and transcriptional gene silencing (Ogawa et al. 2008). Consistent with this idea, Dicer-deficient ES cells, which have markedly reduced small RNA production, have reduced *Xist* DNA methylation and significantly increased *Xist* RNA levels (Nesterova et al. 2008; Ogawa et al. 2008; Kanellopoulou et al. 2009). These results lead to a working hypothesis in which *Tsix* RNA maintains the activity of the X in two ways—first by directing euchromatic modifications to the *Xist* locus and paradoxically blocking *Xist* activation, and second by recruiting Dnmt3a activity to CpG-methylate and stably silencing the *Xist* promoter (Fig. 4).

Therefore, *Tsix*'s binary state—"off" on Xi and "on" on Xa—underlies the asymmetry of XCI. A crucial aspect of the mechanism may be that the chromatin states of *Tsix* and *Xist* are intimately locked together, such that when *Tsix* is euchromatic, *Xist* is also; when *Tsix* is heterochromatic, *Xist* is likewise. This lock-step relationship elicits the obvious question of why their expression patterns do not also mirror each other—and in fact are even diametrically opposed. It is in this paradox, we believe, that one of the challenges of XCI is met. We suppose that *Tsix* and *Xist* thrive in opposite chromatin environments, with *Tsix* being activated by open chromatin, and *Xist* being repressed by it. This unusual structure-function relationship serves XCI exceptionally well, when considered from the standpoint that *Xist* must escape inactivation on the X that it ultimately silences. A gene that is activated by heterochromatin and remains robust in it would be singularly suited to both initiate and maintain the Xi state.

6 POLYCOMB PROTEINS TARGETED AND REGULATED BY RNA

Here we consider how *Xist* is induced and initiates chromosome-wide silencing. One of the first changes to occur during the silencing step is the recruitment of Polycomb repressive complex 2 (PRC2). PRC2 contains four

Figure 4. Long ncRNAs recruit chromatin modifiers in a *cis*-limited and locus-specific manner. The example of PRC2 recruitment by RepA RNA is shown.

subunits (Eed, Ezh2, Suz12, and RbAp46/48) and belongs to the Polycomb group (PcG) family of proteins that have genome-wide roles in the maintenance of stem-cell identity, proper developmental progression, and etiology of diseases such as cancer (Ringrose and Paro 2004; Schwartz and Pirrotta 2008). How Polycomb complexes are recruited to specific genomic locations remains unclear. *Drosophila* PcG complexes are known to contain sequence-specific DNA binding proteins such as Zeste, Pipsqueak (PSQ), and Pho, but whether mammalian complexes also contain such DNA-binding proteins remains somewhat controversial (Ringrose and Paro 2004; Schwartz and Pirrotta 2008). The possibility of an RNA cofactor was initially raised by the curious observation that PcG complexes become

unstable when treated with RNase (R. Paro, personal communication). Experimental evidence first came to light with the discovery of HOTAIR for human *HOX* gene regulation (Rinn et al. 2007) and RepA for XCI (Zhao et al. 2008).

XCI has provided a convenient model to study Polycomb regulation, as PRC2 is localized *en masse* to the X almost immediately following Xist RNA accumulation (Plath et al. 2003; Silva et al. 2003; Kohlmaier et al. 2004). As discussed in the previous section, one of the first changes to follow Tsix down-regulation is appearance of a PRC2 and H3-K27me3 domain at the 5' end of *Xist* (Sun et al. 2006; Zhao et al. 2008). PRC2 is brought there *in cis* by RepA RNA, a 1.6 kb ncRNA originating from the 5' end

of *Xist*, precisely in the region showing H3-K27me3 activity. RepA consists of 7.5 tandem repeats of two stem-loop structures (Wutz et al. 2002), and is transcribed in the same orientation as *Xist*. RNA gel shift analysis shows that RepA RNA directly interacts with PRC2 through its 28-nt stem loop structures and binds the catalytic subunit, Ezh2 (Zhao et al. 2008). RepA is apparently sufficient to recruit PRC2, as when an inducible *RepA* transgene is placed at an ectopic location, increased recruitment of PRC2 can be observed. Importantly, this recruitment is observed only when *RepA* is transcribed (transgene expression induced by doxycycline treatment). When a transcribed RepA RNA is knocked down by shRNA, PRC2 recruitment *in cis* is significantly disrupted. Taken together, these experiments show that RepA functions as a ncRNA and directly targets PRC2 to the *Xic*.

RepA-mediated PRC2 recruitment and H3-K27 trimethylation of the *Xist* promoter are believed to create the “heterochromatic state” (Sun et al. 2006) (discussed in the previous section) that is required for transcriptional induction of *Xist*. Indeed, knocking down RepA RNA and PRC2 components (Zhao et al. 2008) or knocking out the Repeat A motif in mice (Hoki et al. 2009) results in the inability to activate *Xist* expression. In undifferentiated ES cells, RepA RNA is biallelically expressed and binds PRC2 without catalyzing H3-K27 trimethylation. During cell differentiation, the down-regulation of Tsix RNA on the future Xi allows the RepA-PRC2 complex to load onto the *Xist* chromatin and induce H3-K27me3, an event that would then lead to activation of the *Xist* promoter, accumulation of *Xist* RNA, and its spread along the X. RNA immunoprecipitations (RIP) show that *Xist* RNA can also bind PRC2, presumably via the Repeat A motif, which it shares with RepA. Because *Xist* RNA greatly exceeds the length of RepA and contains a number of putative chromosome-binding domains (Wutz et al. 2002), *Xist* RNA can perform what RepA RNA cannot—the spreading of PRC2 along the rest of the X-chromosome.

In the course of characterizing the RepA-PRC2 interaction by RNA EMSA, it was discovered that the antisense strand of RepA—i.e., Tsix—could bind PRC2 (Zhao et al. 2008). Similarly, RIP analysis in ES cells indicated that Tsix RNA occurred in a complex with PRC2. These unexpected findings led to the idea that Tsix RNA might act as a competitive inhibitor for PRC2. Several mechanisms could be envisioned. First, if Tsix and RepA bound PRC2 in a mutually exclusive manner, Tsix RNA could directly compete with RepA RNA for PRC2. Because Tsix is present in vast molar excess over RepA RNA (~100:1) in undifferentiated ES cells (Shibata and Lee 2003), formation of a Tsix-PRC2 complex would be stoichiometrically favored over a RepA-PRC2 complex. Alternatively, Tsix RNA could

block loading of the RepA-PRC2 complex to chromatin, or interfere with PRC2’s catalytic activity—both of which would be consistent with the fact that H3-K27me3 on *Xist* is not observed until Tsix RNA is down-regulated (Zhao et al. 2008). Whether PRC2 binds both sense and antisense strands at the same time or mutually exclusively is not known. Pretreating RIP samples with ribonucleases showed that the RNAs were destroyed in the presence of RNase I and RNase V1, which digest ssRNA and dsRNA respectively, but digesting with RNase H and DNase I did not affect RIP pulldowns. Therefore, RNA bound by PRC2 possess both single-stranded (ss) and double-stranded (ds) character and is not annealed to DNA. The bound transcript(s) may be a single strand of RNA with intramolecular basepairing (secondary structure) or an RNA duplex formed by RepA and Tsix RNAs. The existence of such duplex RNA is supported by *in vivo* RNase protection assays in ES cells (Ogawa et al. 2008).

Taken together, these results suggest that ncRNAs play three roles during the initiation of the silencing step (Fig. 3): (1) In undifferentiated ES cells and on the future Xa of differentiating cells, high levels of Tsix RNA prevent the initiation of XCI by interfering with the RepA-PRC2 complex. (2) When Tsix is down-regulated on the future Xi, RepA RNA productively engages PRC2, targets the Polycomb activity to the *Xist* promoter, and triggers the 100-fold up-regulation of *Xist* RNA by trimethylating H3-K27 to create a patch of heterochromatin at the 5’ end of *Xist*. (3) *Xist* then binds PRC2 and spreads PRC2 and its H3-K27 trimethylase activity along the X to initiate chromosome-wide silencing.

6.1 The Advantages of Long ncRNA

The X-linked region that is now the X-inactivation center was once dominated by protein-coding genes (Duret et al. 2006). Comparative genomic analysis suggests that ncRNAs infiltrated the region (and transformed the original coding residents) only within the last 120–150 million years with the rise of eutherian mammals and the evolution of random XCI. The marsupial mammal, which uses an imprinted form of XCI (Sharman 1971), completely lacks an *Xic* and its noncoding genes (Duret et al. 2006; Davidow et al. 2007; Hore et al. 2007; Shevchenko et al. 2007). Clearly, ncRNAs of the *Xic* established themselves very quickly and effectively within a short space of time. Does RNA’s preeminence here hint at similar strategies elsewhere in the genome, especially considering the degree of noncoding transcription that is now known to occur generally? For epigenetic regulation, there may be two simple reasons to favor RNA over protein (and small molecules). I contend that RNAs deliver superior *cis*-control of allelism

and make possible the targeting of a unique genomic location (Lee 2009).

Unlike proteins and small RNAs, large ncRNAs are stably tethered to the site of transcription and therefore tag the allele of origin. Because a long ncRNA remains bound via RNA polymerase to its parent locus during the act of transcription, the large RNA presents itself as a sequence-specific tag for the locus. The proteome cannot serve this function, as memory of allelic origin is always lost once mRNA detaches from the locus and exits the nucleus to be translated to protein. The ncRNA tags must be lengthy, with a 5' "business end" that binds its target protein(s) once the RNA is synthesized and exposed, and a transcriptionally lagging 3' "tethering end" that remains associated with the transcription machinery and chromatin (Fig. 4). With the potential to be locus-specific "beacons," long ncRNA may be the missing link between chromatin modifier and genomic target. It is probably no coincidence that the Repeat A motif is at the proximal terminus of both RepA and Xist RNAs. This strategic position enables the RNAs to bind PRC2 co-transcriptionally and tether the proteins to the X *in cis*. If the RNA is then destabilized by degradation signals at the 3' end that would be revealed by Pol-II as transcription is completed, the RNA could be prevented from diffusing away from the site of synthesis. In this manner, long ncRNAs such as RepA and Xist RNA could function monoallelically and act strictly *in cis*.

Large ncRNAs are drawn from larger sequence space than proteins and can therefore achieve greater sequence specificity. The diversity of sequences specified by RNA is a direct function of its length, composition, and nucleotide permutation. RNA sequence space exceeds that of the proteome, as there is virtually no limit as to how long a macroRNA can be. A single transcription unit can identify a unique position in the genome. In contrast, transcription factors and other DNA-binding proteins recognize DNA motifs of a limited number of basepairs and the number of possible binding sites is therefore generally $\gg 1$, enabling a transcription factor to operate within a large regulatory network. For instance, the transcription factor and chromatin insulator, Ctf, binds a 20-bp motif that appears thousands of times in the genome, and the pluripotency factor, Oct4, likewise binds an 8-bp motif with many genomic repetitions (Kim et al. 2007; Chen et al. 2008). On the other hand, the Tsix and RepA ncRNAs occur only once, specifically at the *Xic*. Therefore, whereas transcription factors tend to function within a large gene network, long ncRNA can specify a unique address in the genome.

The concept of an "RNA guide" to target chromatin modifiers takes RNA away from its well-recognized role as

intermediary between genotype (DNA) and phenotype (protein) and shifts it toward a dynamic role in epigenome control. RNA-mediated targeting need not be restricted to the X. PRC2 has also been associated, either directly or indirectly, with autosomal ncRNAs such as HOTAIR (Rinn et al. 2007; Khalil et al. 2009) and Kcnqt1ot1 (Pandey et al. 2008). Other chromatin modifiers, such as the *de novo* DNA methyltransferase Dnmt3a (Sun et al. 2006) and the H3-K9 methylase G9a (Nagano et al. 2008), may also be regulated by long ncRNA, as suggested by RNAChIP. Further work is required to determine if there is direct RNA-protein interaction or if the proteins are recruited by the underlying chromatin, of which RNA may be one component. An RNA-mediated mechanism may explain why many chromatin modifiers lack a sequence-specific DNA-binding activity but often harbor RNA-binding motifs (Denisenko et al. 1998; Bernstein and Allis 2005; Bernstein et al. 2006).

With an ever-growing number of long ncRNAs being discovered across the genome (Claverie 2005; Kapranov et al. 2007a; Kapranov et al. 2007b; Guttman et al. 2009; Mercer et al. 2009), it is easy to imagine that RNA guidance may become a recurrent strategy in epigenomic regulation. Interchromosomal associations have been similarly proposed to underlie allelic choice at autosomal loci (LaSalle and Lalande 1996; Spilianakis et al. 2005; Lomvardas et al. 2006), begging the question of whether transcription and RNA guide these *trans*-interactions. Furthermore, many genes harbor antisense RNA (Katayama et al. 2005; He et al. 2008), raising the possibility of RNA-mediated recruitment of chromatin modifiers at those loci as well. On the X-chromosome, ncRNA's full range of function has yet to be explored fully, given that many *Xic*-linked ncRNAs remain uncharacterized.

As we discover more examples of regulation by long ncRNA, one might predict that the large transcripts will eventually rival small RNAs and proteins in their versatility as epigenetic regulators. This article has argued that long ncRNAs are particularly well-suited to allelic regulation and to any action that requires locus-specific targeting and communication *in cis*. Thus, the vast intergenic sequence space that was once widely thought to harbor "junk" may actually be primary determinants of phenotypic variation within and among species. Closely related organisms (e.g., humans versus chimps, mice versus rats), and even individuals of a species, are virtually identical in their "ORF-omes" but can vary significantly in their transcriptomes. For example, differences in coat color and disease susceptibility have been attributed to a retrotransposon near the *Agouti* gene in mice (Waterland and Jirtle 2003; Blewitt et al. 2006). Human cancer and metastasis have been associated with loss of regulation by long ncRNAs, including MALAT1 for lung adenocarcinomas,

GTL2 in neuronal tumors, and H19 for Wilms tumor (Lin et al. 2007; Edwards et al. 2008; Wan and Bartolomei 2008; Wilusz et al. 2008). Interestingly, many of these RNAs operate *in cis*. Before long, we may be looking into the ncRNA world, as a matter of routine, for answers to many questions relating to health and disease.

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