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Epigenetic gene regulation by noncoding RNAs

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Functional noncoding RNAs have distinct roles in epigenetic gene regulation. Large RNAs have been shown to control gene expression from a single locus (*Tsix* RNA), from chromosomal regions (*Air* RNA), and from entire chromosomes (*roX* and *Xist* RNAs). These RNAs regulate genes in *cis*; although the *Drosophila roX* RNAs can also function in *trans*. The chromatin modifications mediated by these RNAs can increase or decrease gene expression. These results suggest that the primary role of RNA molecules in epigenetic gene regulation is to restrict chromatin modifications to particular regions of the genome. However, given that RNA has been shown to be at the catalytic core of other ribonucleoprotein complexes, it is also possible that RNA also plays a role in modulating changes in chromatin structure.

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Abbreviations

ChIP	chromatin immunoprecipitation
Eed	embryonic ectoderm development
ES	embryonic stem
ICE	imprint control element
IF	immunofluorescence
MSL	male-specific lethal
RNAi	RNA interference
RNP	ribonucleoprotein
roX	RNA on the X
Xa	active X chromosome
Xi	inactive X chromosome
Xist	X inactive-specific transcript
Xm	maternal X chromosome
Xp	paternal X chromosome

Introduction

Epigenetic gene regulation refers to heritable changes in gene expression without alteration of the DNA sequence. Functional noncoding RNAs are implicated in regulating several epigenetic phenomena. Many examples of RNA-dependent silencing require gene products that are also necessary for RNA interference (RNAi), including post-transcriptional and transcriptional gene silencing in *Arabidopsis* and *Drosophila*, quelling in *Neurospora*, and silencing of mating type loci and centromeres in *Schizosaccharomyces pombe* (reviewed in [1–4]). Mammalian dosage compensation and genomic imprinting provide examples of epigenetic gene regulation in which noncoding RNAs are used to establish monoallelic expression from specific regions of the genome. In flies, an RNA-containing complex directs transcriptional activation, indicating that noncoding RNAs can stimulate transcription as well as silence gene expression.

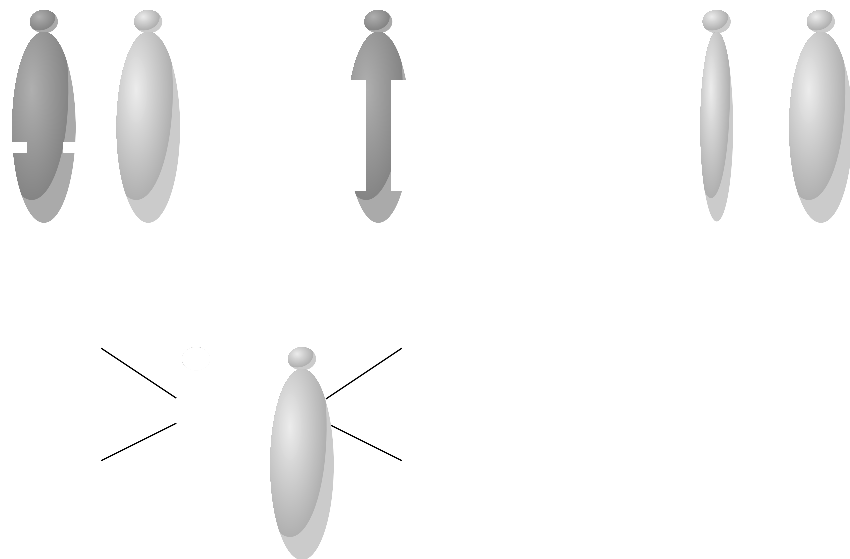
In this review, we will focus on recent advances in understanding the roles of noncoding RNAs in genomic imprinting and dosage compensation in flies and mammals.

Dosage compensation in fruit flies

In *Drosophila*, gene expression from the X chromosome is equalized by doubling the transcription rate from the single X chromosome in XY males relative to XX females [5]. In male flies, the male-specific lethal (MSL) complex spreads hyperactive chromatin bidirectionally from 30–40 chromatin entry sites located on the X chromosome (Figure 1a) [6]. The MSL complex contains six proteins: MSL1, a novel acidic protein; MSL2, a Ring-finger protein; MSL3, a chromodomain protein; MLE, an RNA helicase; MOF, a chromodomain-containing histone acetyltransferase; and JIL-1, a protein kinase. Two noncoding RNAs encoded on the X chromosome, *roX1* and *roX2*, are also components of the MSL complex. This complex covalently modifies histone tails through the activities of MOF, which acetylates histone H4 at Lys16 (H4Ac16), and the JIL-1 protein kinase, which displays histone H3 kinase activity *in vitro* [7,8]. Spreading of the MSL complex requires the ATPase activity of the MLE helicase and the acetyl transferase activity of MOF [9,10]. Thus, the MSL ribonucleoprotein (RNP) complex functions in both chromatin remodeling and in *cis* chromosome coating.

roX1 and *roX2* are functionally redundant for dosage compensation, but 5% of males survive in the absence of both *roX* RNAs [11,12•]. Males lacking the *roX* RNAs exhibit a disruption of MSL2 localization and a loss of H4Ac16 enrichment on the X chromosome [12•]. In addition, MSL2 relocalizes to and appears to activate autosomal regions in *roX*-null males [12•]. These data suggest that a partial MSL complex retains some chromatin remodeling activity. *roX* RNA is required for assembly of the MSL complex at the appropriate chromatin entry sites on the X chromosome and for proper dosage compensation.

Figure 1



Two of the 30–40 chromatin entry sites that direct the MSL complex to the X chromosome occur at the *roX* genes [6]. A recent report [13••] revealed that *roX* RNAs compete for limiting MSL proteins to form functional MSL complexes, and that the concentration of MSL complexes at a *roX* gene determines the extent of *cis* spread from that gene. It had previously been observed that an MSL complex that contains an *roX* RNA expressed from an autosomal transgene could spread either in *cis* along the autosome or in *trans* along the X chromosome [6,12•,14]. Park *et al.* [13••] found that the extent and frequency of spreading of the MSL complex in *cis* from an *roX* autosomal transgene increased when the endogenous *roX* genes were disrupted. Furthermore, overexpression of the MSL complex proteins MSL1 and MSL2 also increased *cis* spread from a *roX* autosomal

transgene. These results suggest that the concentration of MSL proteins is limiting for *cis* spread of the MSL complex. Park *et al.* suggest that the rate of MSL complex assembly is slow when MSL proteins are limiting, giving the *roX* RNA time to diffuse away from the site of synthesis and assemble into a functional MSL complex on a *trans* chromatin entry site. When MSL proteins are not limiting, assembly of a functional MSL complex would be rapid, facilitating spread of this complex in *cis* from the site of synthesis. Thus, in wild-type male flies, the concentration of MSL proteins might determine whether the complex spreads in '*cis*' from a chromatin entry site within a *roX* gene or in '*trans*' from one of the remaining chromatin entry sites on the X chromosome.

The presence of the *roX* RNAs in the MSL complex suggests that an RNA may be uniquely suited for some functions of this complex. The chromatin entry sites that mark the X chromosome appear to correspond to DNA sequence elements [15]. The recognition of these chromatin entry sites could occur, at least in part, through base-pairing between the *roX* RNAs and DNA. In addition, the *roX* RNAs are transcribed from and act in *cis* on the male X chromosome, and therefore might provide specificity by recruiting MSL proteins to the appropriate chromosome [16]. Within the MSL complex, the *roX* RNAs might serve as only a scaffold to bind MSL proteins, which function in coating and silencing the X chromosome. Alternatively, conformational rearrangements within the *roX* RNAs might also be required for chromosome coating. Thus, RNAs may enhance the targeting and the processivity of a chromatin-remodeling complex.

Xist RNA regulates dosage compensation in mammals

Dosage compensation in mammals is accomplished by the transcriptional silencing of one X chromosome in XX females, through a process known as X-inactivation (reviewed in [17]). X-inactivation is a random process in primates and the mouse embryo; the maternal or paternal X chromosome has an equal probability of being inactivated in every cell. As in *Drosophila*, a RNP complex that spreads in *cis* along the X chromosome appears to accomplish dosage compensation in mammals (Figure 1b). The *Xist*/*XIST* RNA regulates X-inactivation in mouse and humans, respectively [17]. When X-inactivation is initiated *in vivo* in the developing female embryo, or *in vitro* by differentiating female mouse embryonic stem (ES) cells, *Xist* RNA spreads from its site of transcription to coat the entire chromosome that will become the inactive X (Xi; [17]). The spread of *Xist* RNA correlates tightly with the spread of transcriptional silencing along the Xi. Once silenced, the identity of the Xi is stable and maintained clonally.

The initiation of X-inactivation can be recapitulated in undifferentiated mouse ES cells by overexpression of an *Xist* cDNA transgene, indicating that undifferentiated ES cells possess all the requisite enzymatic activities for X-inactivation [18]. During this phase of X-inactivation, transcriptional silencing is reversible and *Xist*-RNA-dependent. After differentiation, transcriptional silencing is *Xist*-RNA-independent, consistent with previous observations that *Xist* plays only a minor role in Xi maintenance in somatic cells [19]. Ectopic expression of *Xist* can no longer initiate silencing in differentiated ES cells [18]. These data suggested that gene silencing by *Xist* RNA must occur within a specific developmental context.

A recent study, however, suggests that some differentiated cells are indeed capable of undergoing *de novo* chromosomal inactivation in response to *XIST*. A genomic transgene of human *XIST* was introduced into adult male HT-1080 fibrosarcoma cells [20•]. In one transgenic cell line, ectopic expression of human *XIST* induced transcriptional silencing of linked genes, revealing that in some cell types *XIST* RNA can induce gene silencing even after differentiation. The autosome containing the transgene acquired features of heterochromatin, including late replication, Barr body formation, and histone H4 hypoacetylation. This cell line still displayed the differentiated phenotype. Somatic cells such as HT-1080 that are susceptible to inactivation by *XIST* RNA might contain an enzymatic activity required for the initiation of silencing that is not typically abundant in differentiated cells, or the chromatin states in these cells might be more flexible, or both. These cell lines could provide a useful *in vitro* model for the initiation of X-inactivation in humans.

To identify functional domains within *Xist* RNA, Wutz *et al.* [21••] performed a thorough deletion analysis of *Xist* RNA using transgenes in male mouse ES cells. Over 40 different *Xist* cDNA transgene deletions were analyzed by introducing them individually into the same site on the single X chromosome. Ectopic expression of a wild-type *Xist* cDNA transgene from an inducible promoter led to cell death, and this was most likely to be caused by silencing of the single X chromosome by *Xist* RNA. It was found that *Xist* RNA consists of separable domains for silencing and coating the chromosome in *cis*. The transcriptional silencing activity of *Xist* RNA can be attributed to a domain at the 5′ end of the RNA that shows sequence conservation in all species in which *Xist* has been analyzed. An *Xist* RNA deleted for this 5′ domain coats, but does not silence, the X chromosome. This cisR7om re2?dlenth25507.22lltwosom41(d)ugh 24ft9(-loops.-type)]TJ/

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observations [22]. The separable motifs within *Xist* RNA probably recruit protein complexes to mediate coating and/or silencing of the X chromosome.

The chromatin modifications observed on the Xi provide some clues as to what proteins *Xist* RNA recruits. The Xi acquires many characteristics of constitutive heterochromatin, including hypoacetylation and methylation of histone H3, replication late in S phase, methylation of CpG dinucleotides, and hypoacetylation of histone H4 [17]. During the initiation of X-inactivation in differentiating female mouse ES cells, the earliest observed chromatin modifications occur on histone H3, including hypoacetylation and methylation of histone H3 Lys9 (H3-K9) and hypomethylation of H3-K4, as observed by immunofluorescence (IF) and chromatin immunoprecipitation (ChIP) [23••,24••]. These modifications immediately follow the spread of *Xist* RNA, and appear to precede or coincide with transcriptional silencing of linked genes. Thus, one function of *Xist* RNA could be to recruit a protein complex with H3-K9 deacetylase and/or methyltransferase activity. The role of H3-K9 methylation in the initiation and/or maintenance of X-inactivation has yet to be addressed.

The Polycomb-group protein Eed (embryonic ectoderm development) was the first protein shown to have a functional role in X-inactivation [25••]. X-inactivation is imprinted in the extra-embryonic tissues of the mouse, such that the paternal X chromosome (Xp) is inactivated in every cell. Mice homozygous for mutations in *eed* fail to maintain X-inactivation in extra-embryonic cells, as observed by reactivation of an Xi-linked green fluorescent protein (GFP) transgene. Eed has been shown to interact with Enx1, another Polycomb-group protein [26,27]. Eed and Enx1 accumulate on the Xi of mouse cells derived from an extra-embryonic lineage, remaining associated with the Xi throughout mitosis, although *Xist* RNA is released [28•]. Complexes containing EED and EZH2, the human homologs of Eed and Enx1, respectively, exhibit H3 methyltransferase activity *in vitro* [29••,30••]. EED–EZH2 complexes methylate both H3-K9 and H3-K27 *in vitro*; however, H3-K27 appears to be the preferred site for modification. Thus, *eed* mutant mice might fail to maintain imprinted X-inactivation, owing to a lack of H3-K9 and/or H3-K27 methylation of the Xi, although these modifications have not yet been shown to be enriched on the Xi in wild-type extra-embryonic cells [29••] (see also Update). The H3-K9 methylation of the Xi observed in differentiating female ES cells is not mediated by the methyltransferases Suv39 [31••] or G9a [32]. It has been suggested that an Eed–Enx1 complex mediates the H3-K9 methylation observed at the onset of random X-inactivation in differentiating female mouse ES cells [29••] (see also Update). However, Eed and Enx1 did not accumulate on the Xi in somatic cells, consistent with the observation that *eed* mutant mice did not appear to be defective in any aspect of random X-inactivation in the embryo proper [25••,28•] (see also Update). It would be of interest to determine if H3 methylated at K27 is enriched on the Xi during random X-inactivation, and what role the methylation of H3 plays in the initiation and/or maintenance of X-inactivation (see also Update).

Using IF and ChIP, Heard *et al.* [23••] detected an enrichment of H3-K9 methylation across a 100 kb region upstream of the *Xist* promoter in both undifferentiated female and male mouse ES cells. The enrichment of H3-K9 methylation at this region is retained on the Xi and spreads shortly after *Xist* RNA coats the chromosome. Heard *et al.* [23••] proposed that this region serves as a nucleation center for the spread of *Xist* RNA along the X chromosome, analogous to the chromatin entry sites on the *Drosophila* X chromosome. However, this DNA sequence is not required for *Xist*-RNA-mediated silencing when *Xist* is expressed at high levels from an inducible, autosomal cDNA transgene [18]. These data indicate that either the 'hot spot' of H3-K9 methylation upstream of *Xist* is not absolutely required for gene silencing by *Xist* RNA, or that high levels of *Xist* RNA can induce a hot spot of H3-K9 methylation in a sequence-independent manner.

More recently, BRCA1, a breast and ovarian cancer tumour suppressor protein, has been implicated in maintenance of random X-inactivation and was shown to be required for *XIST* RNA coating of the Xi (see also the review by Starita and Parvin, this issue) [33•]. BRCA1 and *XIST* RNA co-localize on the Xi during S phase in multiple female human cell lines. Interestingly, in BRCA1-deficient female cells *XIST* RNA was expressed but did not coat an X chromosome; and reconstitution of these cells with wild-type BRCA1 restored *XIST* RNA coating of the X chromosome. Conversely, inhibiting BRCA1 synthesis in female cells by RNAi resulted in the loss of Xi coating by *XIST* RNA and in the reactivation of some genes on the Xi. How BRCA1 promotes *XIST* RNA coating of the Xi is currently unknown. Intriguingly, BRCA1 has been shown to interact with RNA helicase A, the human homolog of *Drosophila* MLE, which is required for chromosome coating by the MSL complex [9,34,35]. (For a review on BRCA1, see Starita and Parvin, this issue.)

Xist RNA appears to be part of a RNP chromatin-remodeling complex analogous to the MSL complex in *Drosophila*. Like the MSL complex, the *Xist* RNP may require molecular motors such as helicases for movement along the chromosome. In addition, chromatin modification enzymes might facilitate *cis* spreading of *Xist* RNA. Chromosome coating by an *Xist* RNP could be achieved, in part, by a reiterative cycle that includes modifying flanking histones followed by binding to the modified histone products. *Xist* RNA contains several domains that

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contribute to chromosome coating [21••]. The identification of proteins and enzymatic activities associated with these RNA domains will illuminate the molecular mechanisms of coating and silencing.

The noncoding RNA *Tsix* negatively regulates *Xist*

Tsix is transcribed in the antisense direction through the *Xist* locus and plays a crucial role in dictating which X chromosome will become the active X (Xa) and the Xi [36–38]. *Tsix* loss-of-function mutations have different effects on random and imprinted X-inactivation [37,38, 39•–41•].

In female mouse ES cells, *Tsix* is expressed from both X chromosomes before and during differentiation, but not after it. Deletion of *Tsix cis*-regulatory elements or insertion of a polyadenylation cassette downstream of *Tsix* abolished antisense transcription through the *Xist* locus and resulted in through the *Xist* locus and resulted in nonrandom X-inactivation in differentiating ES cells, with the mutant X chromosome becoming the Xi in most cells [38,39•–41•]. These data indicate that expression of *Tsix* promotes choice as the Xa in random X-inactivation.

Several imprinted genes are overlapping and transcribed in opposite orientations (Table 1) [42]. In murine extra-embryonic cells *Tsix* and *Xist* are oppositely imprinted, with *Tsix* expressed exclusively from the maternal X chromosome (Xm) and *Xist* expressed exclusively from the Xp [37]. Imprinted X-inactivation was unaffected in female mice that inherited the *Tsix* loss-of-function mutations on the Xp; the Xp became the only Xi in all extra-embryonic cells. By contrast, mice that inherited the *Tsix* mutations on the Xm inactivated both the Xm and Xp in female extra-embryonic cells and the single Xm in male extra-embryonic cells [37,41•]. Thus, *Tsix* is required for the Xm to become the Xa in imprinted X-inactivation. ES cells with reduced *Tsix* expression show elevated levels of *Xist* expression relative to wild-type cells, suggesting that *Tsix* functions to lower *Xist* RNA steady-state levels [38,40•,41•,43]. Several molecular mechanisms for *Tsix* regulation of *Xist* have been proposed [17,44]. Antisense transcription through the *Xist* locus might decrease the rate of *Xist* transcription. Alternatively, *Tsix* RNA could destabilize *Xist* RNA by a variety of post-transcriptional mechanisms. A *Tsix*–*Xist* RNA duplex might induce RNAi and degradation of both transcripts. The formation of a *Tsix*–*Xist* RNA duplex could also interfere with the structure of *Xist* RNA, thereby preventing the formation of a functional *Xist* RNP. *Xist* RNA may be less stable when it is not part of a functional RNP complex, as observed for the *roX* RNAs in the absence of the MSL complex [45]. Before splicing, *Tsix* RNA is over 40 kb and complementary to the entire *Xist* RNA; however, several smaller spliced isoforms having limited complementarity with *Xist* RNA have been observed [41•,43]. Interestingly, all of the identified spliced isoforms of *Tsix* RNA overlap the A repeats of *Xist* RNA. *Tsix* RNA might thus prevent proper folding and function of the *Xist* RNA ‘silencing’ domain, although this model has yet to be tested.

Table 1

Imprinted sense–antisense gene pairs in mice.

Chromosome	Gene	Antisense gene
2	*Nesp*	*Nespas*
	Gnasx1	*Gnas*
6	*Copg2*	*Copg2as*
7	*Ube3a*	*Ube3aas*
	Zpf127	*Zpf127as*
	Kvlt	*Kvltas*
	Igf2	*Igf2as*
17	*Igf2r*	*Air*
X	*Xist*	*Tsix*

In contrast with mouse Tsix, human TSIX is expressed throughout human embryonic development [46]. Moreover, TSIX RNA is co-expressed with XIST RNA on the Xi in human cells [46]. Together with the observation that human extra-embryonic tissues do not exhibit strict imprinted X-inactivation of the Xp [47,48], these data suggest that human TSIX does not function in the same manner as mouse Tsix.

Gene silencing by the noncoding RNA *Air*

The genes *Igf2r*, *Slc22a2* and *Slc22a3* comprise one of a growing group of imprinted gene clusters in which antisense RNAs are implicated in the regulation of monoallelic expression (Table 1). *Igf2r*, *Slc22a2* and *Slc22a3* are imprinted genes expressed exclusively from the maternal allele [49]. *Air*, which exhibits imprinted expression exclusively from the paternal allele, overlaps *Igf2r* and is transcribed in the antisense direction through this locus (Figures 1c and 2a). *Air* does not overlap *Slc22a2* and *Slc22a3*. The promoter for *Air* is found within the imprint control element (ICE), a DNA element required to regulate imprinting of this gene cluster [50]. The ICE is methylated on CpG dinucleotides on the maternal allele, and is unmethylated on the paternal allele [51]

A paternally inherited ICE deletion abolished transcription of *Air* and disrupted paternal-specific silencing of *Igf2r*, *Slc22a2* and *Slc22a3*, resulting in the expression of these genes from both the paternal and maternal alleles in mutant mice (Figure 2b) [49,52]. To determine whether imprinting is disrupted owing to abolishing *Air* transcription or to deleting a critical genomic sequence, Sleutels *et al.* [53••] truncated the *Air* transcript by inserting a polyadenylation cassette downstream of the *Air* transcriptional start site, and downstream of the second exon of *Igf2r* (*Air*-T; Figure 2c [53••]). The truncation of *Air* did not disrupt the maternal-specific methylation of the ICE or the paternal-specific expression of *Air*. Mice with a

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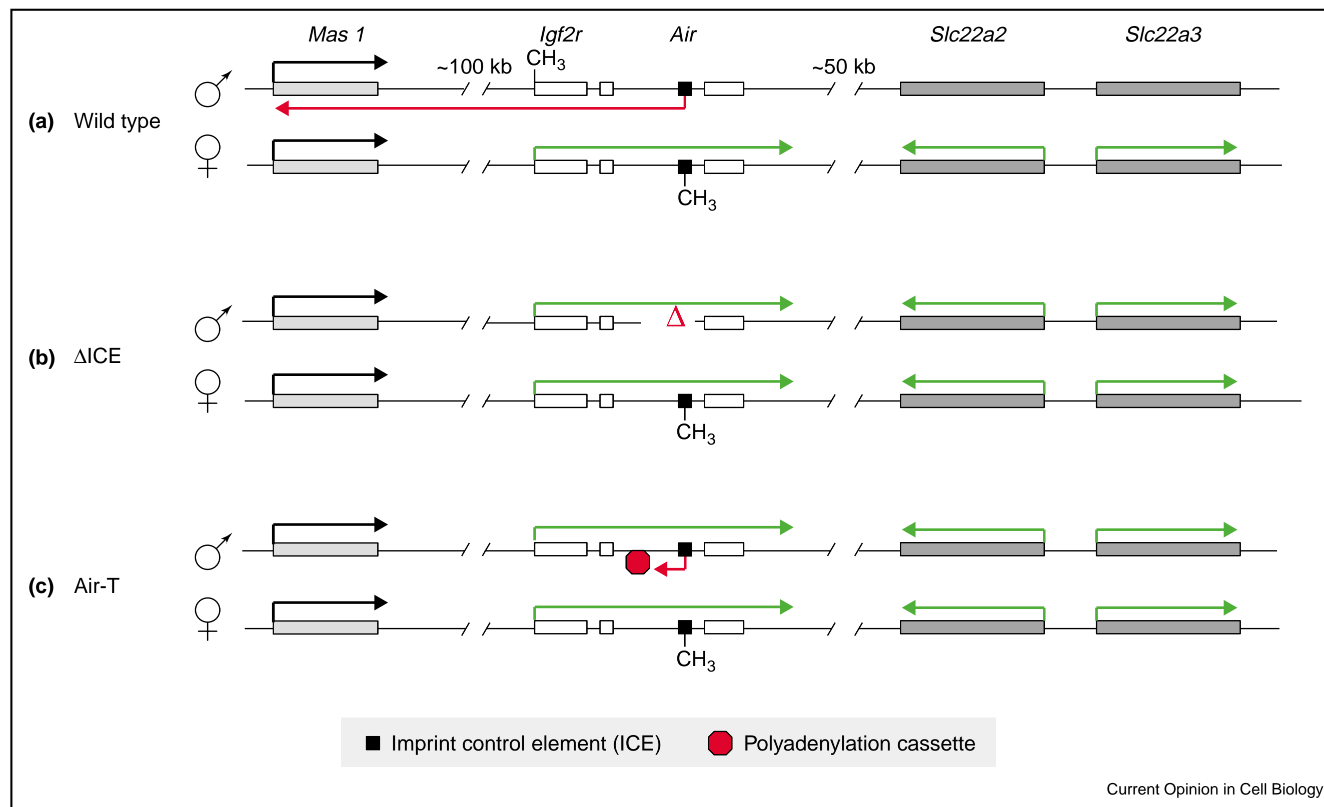
Figure 2



Mutational analysis of the *Igf2r* imprinted gene cluster. **(a)** In wild-type mice, the imprinted genes *Igf2r*, *Slc22a2* and *Slc22a3* are expressed exclusively from the maternal allele (green arrows) and *Air* is expressed exclusively from the paternal allele (red arrow). The *Mas 1* gene is upstream of *Igf2r* and partially overlaps *Air*, although *Mas 1* is not imprinted. **(b)** Mice that inherit a 3.7 kb deletion in the ICE on the paternal allele are not able to repress *Igf2r*, *Slc22a2* and *Slc22a3* expression on this allele. The defect in imprinting might result from the loss of *Air* expression on the paternal allele (due to deletion of the *Air* promoter) or from the deletion of a different *cis*-regulatory element. **(c)** Mice that inherit a paternal *Air-T* allele express a truncated version of *Air* owing to the insertion of a polyadenylation cassette. These mice display the same phenotype as mice inheriting a paternal ICE deletion, owing to de-repression of *Igf2r*, *Slc22a2* and *Slc22a3* on the paternal allele. This observation revealed a role for antisense transcription in bi-directional imprinting of a gene cluster. CH_3, DNA methylation.

maternally inherited *Air*-T allele were identical to wild-type mice. By contrast, mice with a paternally inherited *Air*-T allele displayed the same phenotype as mice with the paternally inherited ICE deletion, owing to de-repression of *Igf2r*, *Slc22a2* and *Slc22a3* on the paternal allele. The *Igf2r* promoter, which is methylated on the paternal allele in wild-type mice, is de-methylated on the *Air*-T paternal allele. Therefore, transcription of *Air* beyond the second exon of *Igf2r* is required for the imprinted repression of *Igf2r*, *Slc22a2* and *Slc22a3*.

Sleutels *et al.* propose two models of how *Air* RNA causes gene silencing [53••]. Antisense transcription across the *Igf2r* locus could repress the *Igf2* promoter and induce a silent chromatin state. Chromatin modifications associated with the silent state could spread bidirectionally in a limited manner into *Slc22a2* and *Slc22a3*. In this way, *Air* and *Tsix* could use a similar mechanism, employing an antisense transcription to silence an overlapping gene at the transcriptional level. Alternatively, *Air* RNA might recruit an enzymatic complex and function as part of a RNP to coat and silence flanking chromatin, analogous to gene silencing by *Xist* RNA.

Conclusions

Noncoding RNA plays a crucial role in several instances of epigenetic gene regulation. In genomic imprinting and dosage compensation, noncoding RNAs generally act in *cis* to regulate one allele of a gene pair. Several imprinted clusters encode antisense RNAs, and these antisense transcripts are implicated in regulating changes in chromatin structure over small genetic distances. Antisense transcripts might destabilize and/or inactivate a complementary functional transcript, as suggested for *Tsix* regulation of *Xist*. Alternatively, the process of antisense transcription *per se* through a gene locus could repress an overlapping gene, which in turn might repress other genes within an imprinted cluster. By contrast, during

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mammalian and *Drosophila* dosage compensation, functional noncoding RNAs can regulate up to thousands of genes in *cis*. The RNP complexes that regulate dosage compensation appear to modulate gene expression by nucleating and spreading chromatin modifications. The intracellular concentrations of RNP components may determine the localization of chromatin modifications and chromosome coating, as observed for the *Drosophila* MSL complex. The identification of *Xist*-RNA-interacting proteins that support the silencing and chromosome coating functions of the *Xist* RNP is anxiously anticipated. A recent analysis of the mouse transcriptome identified several hundred noncoding RNA candidates, suggesting that the significance of noncoding RNA in biological processes such as epigenetic gene regulation is only beginning to be appreciated [54].

Update

Two recent reports reveal that Eed and Enx1 are transiently enriched on the Xi at the onset of X-inactivation [55••,56••]. In addition, H3 methylated at Lys27 is enriched on the Xi, and the Eed–Enx1 complex is required to establish this histone modification. Xist expression is both necessary and sufficient for the recruitment of the Eed–Enx1 complex and for the methylation of H3-K27.

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This study examined 48 different *Xist* RNA deletion mutants for silencing and coating activities. The authors found that coating and silencing were mediated by separable *Xist* RNA domains. One RNA deletion mutant could coat but not silence the X chromosome. Mutational analysis

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revealed that the silencing domain consists of 7.5 repeated units corresponding to two RNA stem-loops. RNAs containing both the silencing domain and one of several nonoverlapping domains could coat and silence the chromosome. Thus, several redundant domains, including the silencing domain, contribute to the coating function of *Xist* RNA.

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