**RESEARCH COMMUNICATION**

*Tsix-mediated epigenetic switch of a CTCF-flanked region of the $X\textit{ist}$ promoter determines the $X\textit{ist}$ transcription program*

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Initiation of $X$ inactivation depends on the coordinated expression of the sense/antisense pair $X\textit{ist}/T\textit{six}$. We show here that a precisely defined $X\textit{ist}$ promoter region flanked by CTCF is maintained by $T\textit{six}$ in a heterochromatic-like state in undifferentiated embryonic stem (ES) cells and shifts to a pseudoeuchromatic structure upon $T\textit{six}$ truncation. We further demonstrate that the epigenetic state of the $X\textit{ist}$ 5‘ region prior to differentiation predicts the efficiency of transcriptional machinery recruitment to the $X\textit{ist}$ promoter during differentiation. Our results provide mechanistic insights into the $T\textit{six}$-mediated epigenetic regulation of $X\textit{ist}$ resulting in $X\textit{ist}$ promoter activation and initiation of $X$ inactivation in differentiating ES cells.

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Histone modifications participate in the establishment of local chromatin structures that, in particular, render gene promoter permissive or not for the subsequent assembly and/or activation of the transcriptional machinery [Mellor 2005]. Among such marks, covalent modifications of Lys 4, Lys 9, and Lys 27 of the histone H3 tail [H3K4, H3K9, and H3K27, respectively] are known to play crucial roles. H3K4 methylation and H3K9 acetylation have been extensively associated with active regions of the genome (euchromatin) while H3K9 and K27 methylation participate in the establishment and maintenance of silent domains (heterochromatin). The relationship between euchromatin and heterochromatin, dictated in part by covalent modifications of histones but also of CpG sites on DNA [Jaenisch and Bird 2003], provides an elegant support for the information required to establish heritable epigenetic states of gene expression programs during development [Turner 2002, Rasmussen 2003].

One of the most relevant paradigms for such epigenetic regulation is provided by $X$ inactivation, in which a single $X$ chromosome is randomly chosen in females to be transcriptionally silenced at the onset of epiblast differentiation. Once established, this silent state is inherited through cell division and lineage commitment. Initiation of $X$ inactivation depends on the noncoding $X\textit{ist}$ RNA, which coats the $X$ chromosome in cis and induces gene silencing and heterochromatin formation [Heard 2005]. The regulation of $X\textit{ist}$ expression is therefore an essential event of the X-inactivation process, thought to involve both post-transcriptional [Panning et al. 1997; Sheardown et al. 1997; Ciabo et al. 2006] and transcriptional [Navarro et al. 2005; Sun et al. 2006] mechanisms.

Embryonic stem (ES) cells recapitulate random $X$ inactivation at the onset of cell differentiation and have proved an excellent model for the study of this epigenetic process [Chaumeil et al. 2002]. In undifferentiated female ES cells, both $X$ chromosomes transcribe low levels of $X\textit{ist}$ RNA. As the cell differentiates, one $X$ allele per diploid set of autosomes is up-regulated, inducing $X$ inactivation in cis, while the second $X$ allele of females and the single $X$ allele of males are turned off. An intriguing characteristic of the $X\textit{ist}$ gene is its complete overlapping by a noncoding antisense transcription unit, $T\textit{six}$, which represses $X\textit{ist}$ RNA accumulation in cis [Ogawa and Lee 2002; Rougeulle and Avner 2004]. Studies of $T\textit{six}$ mutations in both female [Debrand et al. 1999; Lee and Lu 1999; Luikenhuis et al. 2001] and male [Morey et al. 2004; Vigneau et al. 2006] ES cells indicate that an $X$ chromosome in which $T\textit{six}$ transcription has been disrupted systematically up-regulates $X\textit{ist}$ expression at the onset of ES cell differentiation, whether in male or female ES cells. This indicates that $T\textit{six}$ ensures the randomness of $X\textit{ist}$ up-regulation in females and programs $X\textit{ist}$ for silencing in males. In agreement with this, insertion of an inducible promoter to force $T\textit{six}$ expression during female ES cell differentiation abolishes the possibility of the mutated allele to up-regulate $X\textit{ist}$ [Stavropoulos et al. 2001]. $T\textit{six}$ therefore determines the potential of $X\textit{ist}$ to be up-regulated at the onset of differentiation.

Recently, we and others have shown that $T\textit{six}$ has complex chromatin remodeling activities within the $X\textit{ist}/T\textit{six}$ locus. $T\textit{six}$ triggers H3K4 dimethylation within the overall locus but represses increased accumulation across the $X\textit{ist}$ promoter [Navarro et al. 2005]. In addition, male mouse embryonic fibroblasts (MEFs, which in contrast to females do not express $X\textit{ist}$) derived from $T\textit{six}$ mutants show aberrant chromatin conformation at the $X\textit{ist}$ promoter, characterized in particular by high levels of H3K4 dimethylation [Sado et al. 2005].

Given [1] the dramatic effect that $T\textit{six}$ abolishment has both on $X\textit{ist}$ chromatin modification and expression levels and [2] the involvement of chromatin conformation in the establishment and maintenance of specific gene expression programs during differentiation, we hypothesized that $T\textit{six}$ regulation may induce different epigenetic states at the $X\textit{ist}$ promoter on the future inactive and active $X$ chromosomes to determine $X\textit{ist}$ expression programs. Our analysis exploiting wild-type and $T\textit{six}$-truncated ES cells demonstrates that $T\textit{six}$ represses the euchromatinization of a CTCF-flanked region of the $X\textit{ist}$ promoter, precluding transcriptional $X\textit{ist}$ up-regulation during differentiation. In contrast, $T\textit{six}$ truncation gen-

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...ners a stable pseudoeuchromatic state at the Xist 5’ region that preempts transcription apparatus assembly at the Xist promoter and initiation of X inactivation. These conclusions are in striking contrast to those of a recent study (Sun et al. 2006), where it was suggested that down-regulation of Tsix induces a heterochromatic state at Xist, paradoxically followed by transcriptional activation of Xist.

Results and Discussion

Tsix triggers H3K9 trimethylation and DNA methylation to the Xist 5’ region

Antisense transcription across the Xist promoter was previously shown to repress increased levels of H3K4 dimethylation around this specific region (Navarro et al. 2005). In order to map precisely the region affected and to assess whether other epigenetic marks are similarly controlled by Tsix, we have undertaken a systematic analysis of the Xist promoter region [Fig. 1A] in wild-type and mutant male ES cells in which Tsix transcription is ec-

topically terminated before it overlaps the Xist transcription unit (Luikenhuis et al. 2001). We show that Tsix truncation leads to a consistent increase in H3K4 dimethylation specifically in the −1– to +1.5-kb region spanning the Xist promoter [Fig. 1B]. Importantly, this effect is not restricted to H3K4 dimethylation but is also observed for other active histone modifications since this region, devoid of H3K4 trimethylation and H3K9 acetylation in wild-type ES cells [Fig. 1C,D; Supplementary Fig. 1B], is highly enriched for both marks upon truncation of Tsix [Fig. 1C,D]. This clearly demonstrates that Tsix transcription across the Xist promoter represses the accumulation of active histone marks within the −1- to +1.5-kb interval in undifferentiated ES cells. The maximum of active histone marks enrichment corresponds to positions 1 kb downstream from the Xist P1 promoter. This could be linked to the presence of a control element at this position and may explain why the recent analysis of a single position just 5’ of P1 failed to detect such an enrichment [Sun et al. 2006].

Strikingly, the gain of the three active marks tested in the mutant was accompanied by the virtually complete loss of H3K9 trimethylation around the Xist 5’ region [Fig. 1E]. Importantly, this effect is not restricted to histone methylation since DNA methylation, known to partially mark the Xist promoter in ES cells (Sado et al. 1996), was also lost after Tsix truncation [Fig. 1F].

Our results indicate that, in ES cells, Tsix blocks the euchromatization of the Xist 5’ region by triggering negative epigenetic marks, possibly through a mechanism similar to that used by Xist RNA to induce X-chromosome-wide heterochromatinization (Bernstein and Allis 2005), involving the recruitment of repressive enzymatic complexes to the Xist 5’ region (such as H3K9 and DNA methyltransferases together with histone deacetylases and/or H3K4 demethylases). The recent finding of biochemical interaction between Dnmt3a, a de novo DNA methyltransferase, and Tsix RNA [Sun et al. 2006] supports this idea.

Global euchromatic effects of Tsix transcription on chromatin conformation of the Xist/Tsix locus

In the overall Xist/Tsix locus, the effect of Tsix on H3K4 dimethylation was shown to be distinct from its effect at the Xist promoter [Navarro et al. 2005]. It was tempting to speculate that similar regulation would apply to the other modifications controlled by Tsix within the Xist 5’ region. To address this specific issue, additional positions upstream of and downstream from the inserted transcriptional stop signal were analyzed by chromatin immunoprecipitation (ChIP) [Fig. 2A]. The levels of H3K4 trimethylation [Fig. 2C] and H3K9 acetylation [Fig. 2D] are substantially reduced in the mutant within the first few kilobases immediately downstream from the transcriptional stop site. Within Xist itself [Fig. 2, primers c, d, and e], however, the levels of these two modifications are found, in both wild-type and mutant ES cells, to be as low as the levels seen within the hotspot of H3K9 and K27 methylation located 5’ to Xist [Fig. 2, primers a and b; Heard et al. 2001; Rougeulle et al. 2004], a region known to be devoid of euchromatic marks.

In addition, the high levels of H3K9 trimethylation that are detected across the overall Xist/Tsix locus with the exception of the Tsix promoter [Fig. 2E, Supplemen-
CTCF as a candidate protein to constrain the repressive epigenetic effects mediated by Tsix to the Tsix 5′ region exclusively

We have demonstrated that Tsix oppositely affects the Xist 5′ region and the overall Tsix transcription unit. This suggests that an insulation of the Xist 5′ region, capable of limiting the spreading of H3K9 trimethylation and/or CpG methylation to the overall Xist/Tsix region, may be occurring. Importantly, the region showing variation of chromatin modification levels appears to be precisely defined and restricted to the −1- to +1.5-kb interval of the Xist promoter region [Fig. 3A].

In mammals, CTCF has been shown to be able to insulate specific regions of the genome and to define distinct chromatin domains. CTCF binds to regions of transition between X-inactivated genes and genes escaping X inactivation [Filippova et al. 2005] and acts at the CTG repeats of the DM1 locus to constrain H3K9 methylation and prevent its spreading [Cho et al. 2005]. Like the CTG repeats at the DM1 locus, the Xist 5′ region can be viewed as an island of negative epigenetic marks embedded within a region of euchromatin-associated histone modifications. We therefore searched for CTCF binding on both sides of the −1- to +1.5-kb interval. Using two independent antibodies against CTCF [Supplementary Fig. 2A,B], we were able to immunoprecipitate CTCF at the predicted positions in both undifferentiated female [Fig. 3B] and male ES cells [Fig. 3C, dotted line]. The binding profile of CTCF was found to be altered in Tsix-truncated cells [Fig. 3C, plain line]. In all chromatin preparations analyzed, the binding over site c1 was systematically noted to be higher in mutant than in wild-type cells. Although more variability was observed in CTCF binding at the c2 site, higher levels in the mutant than in the wild-type were never observed. Interestingly, the modification of CTCF-binding profile occurring upon truncation of Tsix leads to a profile similar to that of female MEFs, in which Tsix is transcriptionally si-
Tsix truncation leads to inappropriate transcriptional up-regulation of Xist in differentiated male ES cells

In undifferentiated ES cells, Xist expression is significantly down-regulated through repression of the transcription machinery assembly at the Xist P1 promoter (Navarro et al. 2005). This correlates with the finding that Tsix induces, across this specific region, the accumulation of epigenetic marks associated with inactive chromatin and represses the enrichment for active histone modifications. The truncation of Tsix, which completely remodels the chromatin architecture of the CTCF-flanked Xist 5’ region (Fig. 1) has, however, been shown to have no direct influence on the efficiency of transcription preinitiation complex (PIC) recruitment to the Xist P1 promoter in undifferentiated cells (Navarro et al. 2005). One possible explanation could be that the simultaneous enrichment at the Xist promoter for both H3K27 trimethylation (Sun et al. 2006; P. Navarro C. Chureau, S. Vigneau, P. Avner, P. Clerc, and C. Rougelle, in prep.) and euchromatin-associated marks resulting from Tsix mutation generates a bivalent structure reminiscent to that described at other noncoding loci, which represses expression in ES cells but poises it for activation on differentiation (Bernstein et al. 2006). The recent finding of ectopic Xist RNA accumulation and X inactivation in differentiated Tsix mutant male ES cells (Vigneau et al. 2006) suggests that, upon truncation of Tsix, the Xist promoter is indeed primed to undergo transcriptional up-regulation.

To test this hypothesis, wild-type and mutant male ES cells were induced to differentiate and levels of RNA Polymerase II and TFIIB [Fig. 4A] measured at several different promoters. As expected, in both wild-type and mutant cells, PIC recruitment to the Oct3/4 promoter was repressed after 4 d of retinoic acid treatment, while only minor variations were observed at the β-actin and ArpoP0 promoters. The down-regulation of Tsix that takes place during differentiation (Lee et al. 1999) appears to be regulated at the level of PIC recruitment, as an 80% reduction in RNA Polymerase II and TFIIB binding at the Tsix promoter was observed in both wild-type and mutant cells after 4 d of differentiation. In wild-type cells, the levels of PIC recruitment to Xist P1 promoter were either unchanged [TFIIB] or slightly reduced [RNA Polymerase II].

In striking contrast, RNA Polymerase II and TFIIB binding to P1 were significantly increased after 4 d of differentiation in Tsix-truncated cells [Fig. 4A]. At P2, however, the second Xist promoter located 1.5 kb downstream from P1, no change in PIC binding was observed, confirming that only P1 is developmentally regulated (Navarro et al. 2005) This elevation in PIC recruitment to the Xist P1 promoter leads to significant differences in Xist transcription levels as evaluated both by RNA Polymerase II distribution within the Xist first kilobases (from position 0.5 to 2.5) [Fig. 4B] and by levels of primary unspliced Xist transcript measured using quantitative intronic RT–PCR [Fig. 4C]. These results demonstrate that Xist transcription is clearly up-regulated in differentiating Tsix mutant male ES cells, and this correlates with ectopic X inactivation (Vigneau et al. 2006).

Conclusions

We demonstrate here that Tsix induces a number of epigenetic marks within the Xist/Tsix region, which result
in a CTCF-flanked Xist 5' region enriched for H3K9 trimethylation and DNA methylation, embedded within a larger euchromatic domain enriched for H3K4 dimethylation and protected from H3K27 trimethylation. Under these conditions, male ES cells are unable to transcriptionally up-regulate the Xist promoter at the onset of differentiation. In contrast, Tsix truncation leads to elevated H3K4 di/trimethylation and H3K9 acetylation at the Xist 5' region prior to cell differentiation. Strikingly, under this primed state for activation, Tsix-truncated male ES cells efficiently up-regulate Xist transcription through stimulation of PIC recruitment to the Xist P1 promoter during differentiation, with concomitant ec-topic X inactivation [Vignea et al. 2006]. It therefore appears that the chromatin modifications induced by Tsix over the Xist promoter are sufficient to determine the transcriptional fate of Xist at the onset of cell differentiation. We conclude that Tsix mediates the counting process of X inactivation, which precludes high Xist up-regulation in males, through the epigenetic repression of the Xist promoter.

This study has further consequences for our understanding of X-inactivation regulation in female ES cells, where Tsix is repressed first on the future inactive X [Lee et al. 1999]. In this context, the initial monoallelic Tsix repression in a specific time window of differentiation will induce the establishment of a euchromatic architecture at a single Xist promoter region, allowing monoallelic PIC recruitment and participating to monoallelic Xist RNA up-regulation and X inactivation. We propose that asymmetric Tsix silencing, which might be regulated through the activity of Tsix control regions [Stavropoulos et al. 2005], achieves choice through the epigenetic activation of a single Xist promoter. On the second Xist promoter in female and on the single X in male cells, the repressive chromatin conformation, initially maintained by continuous transcription of Tsix, is subsequently propagated by Tsix-independent mechanisms. This is supported by the fact that in male MEFs, the inactive Xist promoter is devoid of active histone marks although Tsix is silenced [Supplementary Fig. 3]. Our findings demonstrate a crucial role for Tsix in programming the Xist expression pattern through modifications of chromatin structure of a precise CTCF-flanked Xist 5' region. These results illustrate the extraordinary epigenetic potential of noncoding antisense transcription units, whose number in the genome is surprisingly higher than previously thought [Kiyosawa et al. 2003; Numata et al. 2003]. Interestingly, recruitment of repressive histone marks by an antisense RNA has also been suggested to occur in the imprinted cluster on mouse chromosome 7 [Lewis et al. 2004; Umlauf et al. 2004]. Whether other antisense transcription units epigenetically control the expression of their sense counterpart through histone and DNA modifications will be key to our understanding of the epigenome regulation.

Materials and methods

Cell culture

Cells were cultured and differentiated as previously described [Navarro et al. 2005; Vignea et al. 2006]. Chromatin and RNA of undifferentiated and differentiated Ma1L and Ma2L cell lines were prepared and analyzed in parallel.

ChIP

ChIP assays were performed as described [Navarro et al. 2005] with the exception of sonication, which was performed using a Bioruptor (Di-agende) according to the manufacturer’s instructions. Ten micrograms to 20 µg of chromatin were used for each immunoprecipitation. The following antibodies were used at the indicated dilutions: TBIB [1/50, Santa Cruz Biotechnology], CTCF [1/50, Santa Cruz Biotechnology], RNAPolII [1/100, Upstate Biotechnology], tri-meK4 [1/100, Upstate Biotechnology], tri-meK27 [1/500, Upstate Biotechnology], and tri-meK4 [1/250, Abcam].

Methyl-CpG DNA immunoprecipitation (MeDIP)

MeDIP assay was performed as described [Weber et al. 2005]. Briefly, genomic DNA from unfixed cells was fragmented by sonication, and 4 µg of denatured DNA were incubated with 10 µL of monoclonal antibody against 5-methylcytidine [Eurogentec] in MeDIP buffer (10 mM Na-phosphate at pH 7, 0.14 M NaCl, 0.05% Triton X-100) for 2 h with overhead shaking at 4°C. Immunocomplexes were recovered using protein G-Sepharose beads (Sigma) and washed three times with 1 mL of MeDIP buffer. The immunoprecipitated DNA was eluted in 250 µL elution buffer [50 mM TrisHCl at pH 8, 10 mM EDTA, 1% SDS] for 15 min at 65°C. After proteinase K (Eurbio) treatment, the immunoprecipitated DNA was phenol/chloroform-extracted and ethanol-precipitated. DNA pellets were resuspended in 60 µL of H2O and 5 µL were used for real-time PCR quantification.

Real-time PCR analysis of ChIP and MeDIP assays

The immunoprecipitated DNA and a 1/100 dilution of the input DNA were analyzed by real-time PCR using SYBR Green Universal Mix and an ABI Prism 7700 [Perkin-Elmer Applied Biosystems] as previously described [Navarro et al. 2005].

Quantitative RT–PCR

Random-primed RT was performed at 42°C with SuperScript II reverse transcriptase [Invitrogen] using 4 µg of DNAse-treated [Roche] RNA isolated from cell cultures with RNable [Eurbio]. Control reactions lacking enzyme were verified negative. We used Arpo P0 transcript levels to normalize between samples. All the primer sequences are provided as Supplementary Figure 4.

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