Tsix transcription across the *Xist* gene alters chromatin conformation without affecting *Xist* transcription: implications for X-chromosome inactivation

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X-chromosome inactivation (XCI) is highly dynamic during early mouse embryogenesis and strictly depends on the *Xist* noncoding RNA. The regulation of *Xist* and its antisense partner *Tsix* remains however poorly understood. We provide here the first evidence of transcriptional control of *Xist* expression. We show that RNA polymerase II (RNAPolII) preinitiation complex recruitment and H3 Lys 4 (H3-K4) methylation at the *Xist* promoter form the basis of the *Xist* expression profiles that drives both imprinted and random XCI. In embryonic stem (ES) cells, which are derived from the inner cell mass where imprinted XCI is reversed and both Xs are active, we show that *Xist* is repressed at the level of preinitiation complex (PIC) recruitment. We further demonstrate that *Tsix*, although highly transcribed in ES cells, is not itself responsible for the transcriptional down-regulation of *Xist*. Rather, *Tsix* induces efficient H3-K4 methylation over the entire *Xist/Tsix* unit. We suggest that chromatin remodeling of the *Xist* locus induced by biallelic *Tsix* transcription renders both *Xist* loci epigenetically equivalent and equally competent for transcription. In this model, *Tsix*, by resetting the epigenetic state of the *Xist/Tsix* locus, mediates the transition from imprinted to random XCI.

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X-chromosome inactivation (XCI), the transcriptional silencing of one X chromosome in female mammals, is dynamically regulated during early development. In mice, X-inactivation is first imprinted in all the cells of the cleavage-stage embryo, with exclusive inactivation of the paternal X-chromosome (Huynh and Lee 2003; Mak et al. 2004; Okamoto et al. 2004). This initial form of imprinted X-inactivation is labile, however, and at the blastocyst stage, the paternal X is reactivated in cells of the inner cell mass (ICM) (Mak et al. 2004; Okamoto et al. 2004). This results in both X chromosomes being active during a short time window. Subsequently, random X-inactivation is initiated, and either the paternal or the maternal X can be chosen for inactivation. In contrast, X-inactivation remains imprinted in extra-embryonic tissues (Takagi and Sasaki 1975). The remarkable and initially unsuspected plasticity of XCI during early development implies the resetting, at the implantation

stage, of the epigenotype of both Xs to confer on them an equal probability of inactivation.

The initiation of X-inactivation is controlled by the X-inactivation center (*Xic*), a complex X-linked locus responsible for the counting and choice mechanisms that ensures the inactivation of a single X in female cells and an absence of inactivation in male cells (for review, see Rougeulle and Avner 2003). The *Xist* gene, which lies within the *Xic*, produces an essential noncoding RNA that coats the X chromosome (Clemson et al. 1996) and triggers gene silencing in *cis* (Penny et al. 1996; Marahrens et al. 1997). *Xist* expression must be tightly regulated in order to ensure the dynamics of XCI, as only high levels of *Xist* RNA can induce X-inactivation. Indeed, in preimplantation embryos, *Xist* expression is imprinted and high *Xist* RNA levels are produced from the paternal X only (Kay et al. 1994). Drastic changes in *Xist* expression pattern take place in the ICM, where *Xist* becomes biallelically expressed. At this stage, *Xist* expression is strongly down-regulated, presumably to avoid the coating and inactivation of both X chromosomes. At the onset of random XCI, *Xist* is up-regulated specifically on

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the future inactive X (Borsani et al. 1991), irrespective of its parental origin. Two aspects of *Xist* regulation, the levels of *Xist* RNA and its chromosomal origin, appear therefore to be crucial for X-inactivation.

Very little is known about the transcriptional control of *Xist* expression, and current models for the up-regulation of *Xist* during random X-inactivation are centered on the stabilization of the transcript (Panning et al. 1997; Sheardown et al. 1997). *Xist* transcription is thought to initiate from two distinct promoters, P1 and P2 (Johnston et al. 1998), separated by 1.5 kb (P2 being downstream of P1). The functional significance of these two isoforms is related to the existence of a repeat element lying between both promoters that is essential for the silencing activity of *Xist* RNA (Wutz et al. 2002). Transcribing *Xist* from P1 rather than from P2 would be expected to radically change the silencing properties of the *Xist* RNA. Somewhat surprisingly, it was shown that transcripts initiating from P2, and therefore devoid of the repeat element, are in large excess compared to those initiating from P1. This has led to the suggestion that P2 is the major *Xist* promoter (Johnston et al. 1998). However, the activity of the P1 and/or P2 promoters may change in the course of imprinted and/or random X-inactivation, and the possibility that the regulation of *Xist* expression is mediated by transcriptional mechanisms remains open.

One of the most exciting characteristics of the *Xist* gene is its complete overlapping by a noncoding antisense transcription unit, *Tsix* (Debrand et al. 1999; Lee et al. 1999; Mise et al. 1999). *Tsix* has been shown to be a crucial *cis*-acting repressor of *Xist* up-regulation, and a tight correlation between antisense down-regulation and *Xist* RNA accumulation during random X-inactivation was established (for review, see Rougeulle and Avner 2004). Importantly, both deletion of the major *Tsix* promoter and truncation of the antisense transcription unit lead to a complete bias in random X-inactivation towards the mutated allele (Debrand et al. 1999; Lee and Lu 1999; Luikenhuis et al. 2001). This strongly suggests that *Tsix* is involved in the chromosomal origin of *Xist* expression during random X-inactivation. Interestingly, the phenotype of *Tsix* deletions cannot be reverted by inserting back a cDNA expressing a spliced variant of *Tsix* (Shibata and Lee 2004). This points to the possibility that the act of *Tsix* transcription itself is required for the function of *Tsix*. Whether *Tsix* modulates *Xist* transcription or acts at the level of *Xist* RNA stabilization or both remains an open question, however. It has also been proposed that *Tsix* is responsible for the paternal-restricted expression of *Xist* during imprinted X-inactivation, although the role of *Tsix* in the early, reversible imprinted *Xist* expression has yet to be clearly addressed (Lee 2000; Sado et al. 2001).

Histone modifications are involved in the epigenetic regulation of gene expression (Sims et al. 2003). By reconfiguring the chromatin template and establishing a local chromatin structure, such epigenetic modifications make promoter regions permissive or not for the subsequent assembly of the preinitiation complex (PIC), com-

posed of RNA polymerase II (RNAPolII) and the general transcription factors, and/or for the activation of transcription (Lee and Young 2000). Interestingly, differential histone modifications are associated with the patterning of expression of several imprinted genes (Xin et al. 2001; Fournier et al. 2002; Lewis et al. 2004; Umlauf et al. 2004; Vu et al. 2004). In these cases, the paternal and maternal alleles are distinguished by selective enrichment of the promoter region by a set of epigenetic marks associated with either active (e.g., methylation of H3 Lys 4 [H3-K4]) or silent (e.g., H3-K9 and K27 methylation) chromatin. Although some data exist concerning the chromatin features of the *Xist* locus (Kimura et al. 2002; Rougeulle et al. 2003, 2004), a more systematic approach is clearly needed to assess the role of histone modifications in the regulation of *Xist* expression.

To gain insight into the epigenetic and transcriptional aspects of *Xist* regulation, we focused on the chromatin structure of the locus in relation to PIC recruitment at the *Xist* and *Tsix* promoters. Our detailed chromatin immunoprecipitation (ChIP) analysis exploited three cell types representative of different X-inactivation status. Trophoblast stem (TS) cells (Tanaka et al. 1998) carry an inactive X chromosome of paternal origin and can be used as a model system to study imprinted X-inactivation (Mak et al. 2002). In TS cells, *Xist* expression is restricted to the paternal allele, whereas *Tsix* is expressed from the maternal one (S. Pichard, D. Arnaud, C. Chureau, E. Heard, C. Ciaudo, C. Rougeulle, and P. Avner, in prep.). Mouse embryonic fibroblasts (MEFs) have already undergone random X-inactivation and can be used to analyze stable X-inactivation as established in somatic cells. In female MEFs, *Xist* is expressed only from the inactive X, while both *Tsix* alleles are silenced. Finally, embryonic stem (ES) cells, derived from the ICM of the blastocyst where imprinted X-inactivation is reversed and both Xs active, have been used extensively to study the initiation of random X-inactivation. In ES cells, both *Xist* and *Tsix* are biallelically expressed, although *Tsix* transcripts are in large excess compared to *Xist* transcripts (Shibata and Lee 2003).

Our analysis provides the first evidence of transcriptional control of *Xist*. We show that the strong monoallelic *Xist* expression required to achieve X-inactivation, whether imprinted or random, is driven by monoallelic recruitment of the transcriptional apparatus and H3-K4 dimethylation to the *Xist* P1 promoter. Conversely, in ES cells, the essential down-regulation of *Xist* expression appears in a large part to be mediated through the repression of PIC recruitment to the *Xist* P1 promoter. In contrast to current models, we demonstrate that the function of *Tsix* in ES cells is not to directly repress *Xist* transcription but rather to induce H3-K4 methylation over the entire *Xist/Tsix* locus. We propose that the role of such chromatin remodeling activity is to erase pre-established epigenetic marks associated with imprinted X-inactivation, in order to render both *Xist* homologs epigenetically equivalent and equally competent for random X-inactivation. This provides a new role for *Tsix* in the transition from imprinted to random X-inactivation.

Results

Monoallelic Xist expression is marked by allelic H3-K4 dimethylation but not by H3-K9 and K27 methylation

In female TS cells, *Xist* is expressed from the paternal, inactive X (Mak et al. 2002), whereas *Tsix* expression comes from the maternal, active X (S. Pichard, D. Arnaud, C. Chureau, E. Heard, C. Ciaudo, C. Rougeulle, and P. Avner, in prep.). To address whether methylation of H3-K4 and/or H3-K9 and H3-K27 over the promoters is involved in the allelic expression of both *Xist* and *Tsix* in imprinted X-inactivation, we used a female TS cell line carrying a paternal X chromosome of 129/Sv origin and a maternal X chromosome whose *Xic* region is derived from a *Mus musculus musculus* strain that carries the *Pgk.1a* allele. We exploited single nucleotide polymorphisms (SNPs) to distinguish the paternal (inactive) and maternal (active) X in the ChIP assay. Two allelic primers pairs were successfully designed for the *Xist* P1 promoter (position X1) and the *Tsix* promoter (position X11), respectively (Fig. 1A). As shown in Figure 1B, promoters of expressed *Xist* and *Tsix* alleles on the paternal and maternal X chromosomes respectively are enriched for H3-K4 dimethylation. However, and in contrast to other imprinted genes, the silent maternal *Xist* allele is not found enriched for either H3-K9 dimethylation or H3-K27 trimethylation, compared with the active one (Fig. 1C,D). The silent, paternal *Tsix* allele displays sig-

nificant enrichment for H3-K27 trimethylation, but not for H3-K9 dimethylation.

A similar pattern of histone modifications at the *Xist* promoter is detected in cells displaying random XCI, such as MEFs. Enrichment for H3-K4 dimethylation over the *Xist* promoter is specific to female cells compared with males (which do not express *Xist* as they have a single active X chromosome) (Fig. 1B). Similarly to TS cells, the silent *Xist* promoter in male MEFs is enriched neither for H3-K9 di- nor for H3-K27 trimethylation (Fig. 1C,D). Promoter-restricted H3-K4 dimethylation therefore appears to be the epigenetic mark for monoallelic *Xist* expression in both imprinted and random X-inactivation. Importantly, neither H3-K9 dimethylation nor H3-K27 trimethylation is specifically associated with the silent *Xist* allele on the active X chromosome. It is formally possible, however, that other, still unidentified repressive modifications mark the silent *Xist* promoter.

Intriguingly, as previously reported by others (Kimura et al. 2002), the promoter region of *Tsix* is enriched for H3-K4 dimethylation in MEFs despite the fact that *Tsix* is not expressed in such cells (Fig. 1B). The function of such enrichment remains elusive but might be related to the choice process occurring at the onset of random X-inactivation, in which *Tsix* is known to be involved (Lee et al. 1999; for review, see Rougeulle and Avner 2004). Alternatively, the function of H3-K4 methylation at the *Tsix* promoter could be related to the counting process, i.e., the mechanism that ensures the inactivation of the

Figure 1. ChIP analysis of histone modifications within the *Xist/Tsix* region in female TS cells and in MEFs. (A) Schematic representation of the *Xist/Tsix* locus. *Xist* exons are represented by black boxes, and *Tsix* exons are represented by white boxes. Dotted arrows indicate the positions of the primer pairs used in the ChIP analysis. (B–D) Analysis of H3-K4 dimethylation (B), H3-K9 dimethylation (C), and H3-K27 trimethylation (D) in female TS cells and MEFs. In TS cells, two allelic primers (X1, black bars, and X11, gray bars) allowed us to distinguish the paternal allele (Xi) from the maternal (Xa). White bars (XEx5) correspond to a primer pair detecting both the paternal and maternal alleles. As both X chromosomes in female MEFs are of 129/Sv origin, they cannot be distinguished using allelic primers. All graphs were obtained after normalization with position XEx5 located in the body of *Xist*.

appropriate number of X chromosomes in each cell. Recently, Morey et al. (2004) showed that a counting element lies within a 20-kb region that surrounds the antisense promoter. The presence of H3-K27 trimethylation at the *Tsix* promoter in MEFs (Fig. 1D) is likely to be related to the silencing of *Tsix*.

Monoallelic Xist expression in imprinted and random X-inactivation is mediated by allelic PIC recruitment to the Xist P1 promoter

To address whether the differential pattern of H3-K4 dimethylation at the expressed and silent *Xist* alleles reflects a transcriptional control of *Xist* expression, we performed a systematic analysis of RNAPolII and TFIIB binding to the *Xist/Tsix* region in TS cells and in MEFs.

In TS cells, the distribution of RNAPolII and TFIIB parallels that of H3-K4 dimethylation, with a binding specific to the active *Xist* and *Tsix* promoters on the paternal and maternal chromosomes, respectively (Fig. 2A,B). This indicates that monoallelic expression of both *Xist* and *Tsix* in imprinted X-inactivation is mediated through monoallelic PIC recruitment to the corresponding promoters. Similarly, high levels of monoallelic *Xist* expression in random X-inactivation is driven by efficient recruitment of the transcriptional apparatus to a single allele, as shown in MEFs (Fig. 2C,D). The specific accumulation of general transcription factors at the *Xist* promoter region in female cells and its absence in males strongly suggest that recruitment occurs specifically on the inactive X. This further indicates that the lack of

Xist expression in males results from the absence of transcription rather than from a post-transcriptional mechanism, in agreement with a previous report of DNA methylation at the *Xist* promoter on the active X (Norris et al. 1994). Similarly, there is no enrichment for basal transcription factors at the *Tsix* promoter in male and female MEFs.

Interestingly, in both TS cells (data not shown) and MEFs (Fig. 2C,D), the transcription machinery is mostly detected at the P1 promoter compared with P2. Binding of TFIIB appears 10-fold enriched over P1 compared with P2 in MEFs, and this is accompanied by a twofold enrichment of RNAPolII (Fig. 2C,D). The greater enrichment noted for TFIIB than for RNAPolII likely reflects the presence, at P2, of RNAPolII that has in fact initiated transcription at P1. In support of this interpretation, we detected, at a position located between P1 and P2, RNAPolII levels similar to that seen at P2 (data not shown). These data, which contrast with previous observations (Johnston et al. 1998), strongly suggest that P1 is the major *Xist* promoter, at least in cells expressing high levels of *Xist*.

We conclude that efficient monoallelic *Xist* expression in imprinted and random X-inactivation is regulated at the level of PIC recruitment to the P1 promoter, and associated with allelic distribution of H3-K4 dimethylation.

In undifferentiated ES cells, Xist down-regulation is mediated by the repression of PIC recruitment to the Xist promoter

Undifferentiated ES cells are unique in the expression pattern of the *Xist/Tsix* locus, with both genes being

Figure 2. Binding of the transcription machinery to the *Xist/Tsix* region in female TS cells and in MEFs. (A,B) Binding of RNA polymerase II (A) and TFIIB (B) in female TS cells. As in Figure 1, the positions corresponding to the *Xist* P1 promoter (X1) and the *Tsix* promoter (X11) are in black and gray, respectively. Allelic primers for X1 and X11 allowed us to distinguish the maternal and paternal alleles. All the other positions (in white) detect both paternal and maternal alleles. Male TS cells exhibit a profile similar to that of the maternal X chromosome of female TS cells (data not shown). (C,D) Similar analysis in female and male MEFs. The graphs show the fold-enrichment value of each position compared with position X3.

expressed from each individual X chromosome. While it has been shown that *Tsix* transcripts are in a 10- to 100-fold molar excess compared with *Xist* (Shibata and Lee 2003), it is unknown whether this difference in abundance of the RNAs is due to transcriptional or post-transcriptional mechanisms. To address this issue, we monitored the binding of the transcription machinery to the *Xist/Tsix* region in ES cells.

The distribution of RNAPolIII reveals a strong bias in transcription towards *Tsix* (Fig. 3A), as only the *Tsix* promoter displays high levels of RNAPolIII accumulation. All the positions tested within the overlapping *Xist/Tsix* region (positions X1, X5, XEx1b) show RNAPolIII levels similar to that observed at positions transcribed only in *Tsix* orientation (X9, X6), indicating that in ES cells, RNAPolIII specifically associated with the transcription of *Xist* cannot be detected by ChIP. Similarly, TFIIB binding shows an extreme skew towards the antisense promoter, although low but significant levels of TFIIB binding are detected at both *Xist* P1 and P2 promoters (Fig. 3B). These observations imply that the excess of *Tsix* transcripts compared with *Xist* transcripts is in large part supported by a differential distribution of RNAPolIII within the region, which is in turn regulated by a differential efficiency of PIC assembly on the promoters.

The extremely low levels of TFIIB binding detected at the *Xist* promoters further indicates that, in ES cells, reduced levels of *Xist* expression result from repression

of PIC recruitment. The efficiency of PIC assembly at P1 is therefore dramatically different between cells in which X-inactivation has occurred (such as TS cells and MEFs) and cells in which X-inactivation has yet to occur (ES cells). Using quantitative RT-PCR, we determined the relative levels of expression of *Xist* and *Tsix* in female TS, ES cells, and MEFs (Fig. 3C, black bars). As expected, in TS and MEFs female cells, the ratio is skewed towards *Xist*. In contrast, ES cells show a skew towards *Tsix*, in agreement with previous observations (Shibata and Lee 2003). Calculation of the ratios of TFIIB binding to *Xist* P1 promoter versus *Tsix* promoter (Fig. 3C, gray bars) indicates that cells expressing predominantly *Xist* (after imprinted or random X-inactivation) show a bias of TFIIB binding towards the *Xist* P1 promoter. Conversely, cells expressing predominantly *Tsix* (a configuration specific to ES cells) show a bias of TFIIB binding towards the *Tsix* promoter. This comparative analysis is strongly supportive of the *Xist/Tsix* balance being regulated at the level of PIC recruitment to *Xist* and *Tsix* promoters.

Tsix is not responsible for the transcriptional repression of *Xist*

Given the distribution of the transcription machinery in ES cells and the known role of *Tsix* as a negative regulator of *Xist* accumulation, it is tempting to speculate

Figure 3. *Xist* down-regulation in undifferentiated ES cells is regulated by repression of PIC recruitment to the P1 *Xist* promoter. (A,B) Binding of RNA polymerase II (A) and TFIIB (B) in female ES cells (LF2). Identical results were obtained in male ES cells (data not shown). Graphs show the fold-enrichment value of each position compared with position X3. (C) Comparative analysis of the *Xist/Tsix* transcripts ratio (black bars) with the ratio of TFIIB binding to *Xist* P1 promoter versus *Tsix* promoter (gray bars) in female TS cells, ES cells, and MEFs. The RNA levels of *Xist* and *Tsix* were determined by real-time PCR using primers XcR1 for *Xist* and X6 for *Tsix*, and standardized against the mRNA levels of *Arpo P0*.

that *Tsix* transcription across the *Xist* promoter is itself sufficient to repress high levels of PIC recruitment in ES cells. To address this issue, we used two previously characterized mutations that result in an absence of *Tsix* transcription (Luikenhuis et al. 2001; Morey et al. 2004). We exploited mutations on an XY background in order to overcome the need for polymorphisms, a potential limitation in the number of positions that can be analyzed. Male ES cells have been shown to be competent for *Xist* up-regulation and X-inactivation, as shown in male cells carrying *Xic* transgenes (Lee et al. 1996) or a 65-kb deletion 3' to *Xist* (Morey et al. 2004). Identical results were obtained in mutant female ES cell lines (Supplementary Figure S1D,E).

One mutant ES cell line, Ma2L, carries a splice acceptor site and a transcriptional stop signal located some 4 kb downstream of the *Tsix* promoter (Luikenhuis et al. 2001). The control cell line, Ma1L, contains a single loxP site at the equivalent position. Detailed profiling of

RNAPolII in the *Xist/Tsix* region of the Ma2L ES cell line confirmed the efficiency of the introduced stop signal (Fig. 4A). The amount of RNAPolII at positions located upstream of the transcriptional stop (positions cTA and X11) is identical in both wild-type and mutant cells, showing that truncating the *Tsix* unit has no effect on the initiation of *Tsix* transcription. At position X24, which lies 700 bp downstream of the stop signal, the levels of RNAPolII were increased in the mutant, probably reflecting the accumulation of RNAPolII at the ectopic 3' end of *Tsix*. The other positions tested, whether lying within or outside the *Xist* transcription unit, show a virtually complete loss of RNAPolII, confirming our previous conclusion that in ES cells the *Xist/Tsix* region transcribes almost exclusively *Tsix*.

Strikingly, neither RNAPolII nor TFIIB (Fig. 4B) are enriched at the *Xist* promoters upon truncation of *Tsix* transcription, as would have been expected if *Tsix* were repressing *Xist* at the transcriptional level. This indi-

Figure 4. Transcriptional and chromatin analyses of *Tsix*-truncated undifferentiated male ES cells. Ma2L cells (white bars) carry a transcriptional stop signal 4 kb downstream of the *Tsix* promoter. Ma1L (black bars) is the corresponding control cell line. (A) ChIP analysis of RNA polymerase II distribution within the *Xist/Tsix* region in Ma1L and Ma2L cell lines. The position of the stop signal is indicated in the graph. (B) ChIP analysis of TFIIB binding to the *Xist* P1 and P2 promoters and the *Tsix* promoter in Ma1L and Ma2L. (C) Extensive ChIP analysis of H3-K4 dimethylation within the *Xist/Tsix* locus in Ma1L and Ma2L. The graphs show the fold-enrichment value of each position compared with position X3. The schematic map below the graph shows the position of the primers used and of the stop signal.

cates that *Tsix* is not directly responsible for the repression of PIC recruitment at P1. To confirm this, we used a second cell line, CK35Δ65, that carries a 65-kb deletion 3' to *Xist* encompassing the *Tsix* promoter (Morey et al. 2004). Similarly to the *Tsix* truncation, deletion of the antisense promoter does not result in increased levels of RNAPolII or TFIIB at P1/P2 *Xist* promoters (Supplementary Fig. S1B,C). Our results clearly indicate that although *Tsix* is highly transcribed in ES cells, neither *Tsix* transcription nor *Tsix* transcript is responsible for repressing *Xist* transcription, ruling out an often-cited model to mechanistically explain the effects of *Tsix* on the expression level of *Xist*. Importantly, this implies the existence of *Tsix*-independent mechanisms that repress *Xist* transcription specifically in ES cells. Our result furthermore provides an explanation for the failure of undifferentiated ES cells to induce strong *Xist* RNA accumulation and X-inactivation upon *Tsix* invalidation (Debrand et al. 1999; Lee and Lu 1999; Luikenhuis et al. 2001).

Tsix transcription induces complex modification of H3-K4 dimethylation within the *Xist/Tsix* locus

Given the low levels of PIC recruitment to the *Xist* P1 promoter in ES cells (Fig. 3A,B) and the association of H3-K4 dimethylation with the active *Xist* promoter in TS cells and in MEFs (Fig. 1B), we monitored the levels of H3-K4 dimethylation within the *Xist/Tsix* locus in ES cells, with the expectation of finding low levels of this epigenetic mark associated with the poorly active *Xist* promoter. The data presented in Figure 4C (black bars) were obtained with male ES cells, but a similar profile was observed in female ES cells (data not shown). Surprisingly, significant enrichment for H3-K4 dimethylation was detected at the *Xist* 5' region. In contrast to MEFs and TS cells however, H3-K4 dimethylation is not restricted to the *Xist* promoter region but is associated with the entire *Xist/Tsix* unit (Fig. 4C, black bars). Interestingly, we found that H3-K4 dimethylation spreads from the 5' extremity of *Tsix* (position X11) toward the 3' end (position X9) in a gradient reminiscent of *Tsix* RNA levels described by others (Shibata and Lee 2003). We conclude that in ES cells, the *Xist* locus is in a primed state for transcription, characterized by a global enrichment for H3-K4 dimethylation.

We show here that in ES cells (1) the *Xist/Tsix* locus is mainly transcribed in the *Tsix* orientation (Fig. 3A), (2) H3-K4 dimethylation spreads over the entire *Tsix* transcription unit (Fig. 4C, black bars), and (3) deleting 65 kb 3' to *Xist* (CK35Δ65) results in a specific and efficient loss of H3-K4 dimethylation within *Xist* (Morey et al. 2004). Altogether, these data point to a function for *Tsix* in the deposition of H3-K4 dimethylation within the *Xist/Tsix* region.

To confirm this, we analyzed the pattern of H3-K4 dimethylation in the Ma2L mutant ES cells that carry a truncated *Tsix* allele (white bars in Fig. 4C). Strikingly, blocking *Tsix* transcription leads to an almost complete loss of H3-K4 dimethylation at positions located down-

stream of the introduced transcriptional stop signal, whereas levels of dimethylated H3-K4 were found to be mostly unchanged at upstream positions (Fig. 4C). This result indicates that *Tsix* induces H3-K4 dimethylation within the region, probably through the recruitment of histone methyltransferase activity by either the RNAPolII transcribing *Tsix* or the *Tsix* RNA itself.

Intriguingly, *Tsix* appears to have a dual role in the regulation of H3-K4 dimethylation at the *Xist* promoter, since the absence of *Tsix* transcription (or transcript) induces elevated levels of dimethylation (and also trimethylation) (data not shown) over this region (positions X1 and XP1.2 in Fig. 4C). Interestingly, this increase concerns not only the *Xist* promoter itself but also an extended region that corresponds to the only spliced exon of *Tsix* known to overlap *Xist* RNA (Sado et al. 2001; Shibata and Lee 2003). We conclude that *Tsix* induces complex modification of H3-K4 dimethylation within *Xist*, demonstrating for the first time that *Tsix* remodels the chromatin structure of the *Xist* locus.

Discussion

Transcriptional regulation of Xist expression

We have reported here major variations in the PIC recruitment activity of the *Xist* P1 promoter that correlate with the X-inactivation status of the corresponding chromosome. The level of transcription basal machinery associated with the P1 promoter in cell types expressing high levels of *Xist* RNA, in which XCI has already occurred (female MEFs and TS cells) is much greater than the level detected in cells expressing low levels of *Xist*, in which XCI has yet to occur (ES cells). This indicates that *Xist* expression is regulated transcriptionally by the selective recruitment of the transcriptional apparatus to its promoter (Fig. 5). Since high levels of *Xist* expression were previously shown to be supported by the stabilization of the transcript, it is of clear interest to address whether or not these mechanisms act together. Because transcription and RNA processing are tightly coupled events (Proudfoot 2000), it is tempting to speculate that PIC assembly at the *Xist* promoter might also be linked to the recruitment of *Xist* RNA stabilization factors.

Intriguingly, the strong variation in the binding of the transcription machinery that we detected at the *Xist* P1 promoter in the different cell types studied was not observed for P2. Our results indicate first that unlike P1, the activity of P2 is not developmentally regulated, and second, that P1 is the major *Xist* promoter, at least in cells expressing high levels of *Xist*. This latter observation contradicts earlier conclusions based on the RNA steady-state-level measurement of the two putative isoforms produced by these promoters (Johnston et al. 1998). This apparent paradox can be attributed to more efficient transcription from P2 than from P1, regulated either at the level of promoter clearance or at the elongation step. An alternative explanation is suggested by preliminary measurement by quantitative RT-PCR of *Xist* RNA at several positions both up- and downstream

Figure 5. A model of the regulation of the *Xist*/*Tsix* region during early mouse development. Developmental stages are shown on the left, and the status of the *Xist*/*Tsix* region in terms of transcription and chromatin at each stage is shown on the right. (A) In the pre-implantation embryo, the segregation of PIC together with H3-K4 dimethylation over the paternal and maternal *Xist* and *Tsix* promoters ensures paternal *Xist* and maternal *Tsix* expression, respectively. Bold arrows represent high levels of expression, dotted arrows represent low levels of expression. (B) At the implantation stage, an unknown ICM-specific repressive mechanism of PIC recruitment at P1 *Xist* promoter avoids biallelic X-inactivation (represented by a question mark and a transparent PIC at the *Xist* promoter). In the meantime, biallelic *Tsix* activation erases the histone methylation pattern by inducing high levels of H3-K4 dimethylation across the region (pink arrows), rendering both *Xist* alleles epigenetically equivalent. (C) At the onset of random X-inactivation, monoallelic down-regulation of *Tsix* results in an increase of H3-K4 dimethylation over the corresponding *Xist* promoter region. The absence of ICM-specific *Xist* repression allows stable binding of the transcriptional apparatus, inducing high levels of monoallelic *Xist* expression and X-inactivation. (D) In the female epiblast, other epigenetic marks lock in the expression profile of *Xist* and the silencing of *Tsix* transcription.

of P2. We observed a strong influence of the reverse transcription (RT) reaction temperature on the RT efficiency over the 5' extremity of *Xist* RNA (data not shown). This suggests the existence of secondary structures of *Xist* transcripts that might have been responsible for artifactual quantification of molecules bearing this region. The *Xist* 5' region is known to be rich in DNA repeats such as the so-called A repeat responsible for the silencing properties of *Xist* RNA.

The correlation in ES cells between low *Xist* RNA levels and the presence of limited quantities of RNAPolII and TFIIB at the *Xist* P1 promoter (note that other general transcription factors such as TFIIA, TFIIIE, TFIIF, and TFIIFH gave similar results) (Supplementary Fig. S2) suggests either the presence of a repressor or the lack of a specific activator in such cells. Importantly, the repression of P1 in ES cells might be part of the mechanism allowing the reactivation of the paternal X chromosome in the ICM. Our analysis of *Tsix* mutant cells reveals that *Tsix* is not directly responsible for the repression of PIC assembly at the *Xist* promoters, despite *Tsix* being highly transcribed in ES cells. This implies firstly that a *Tsix*-independent mechanism represses *Xist* transcription specifically in ES cells (Fig. 5B), and secondly that the elevation in *Xist* RNA levels resulting from *Tsix* invalidation in undifferentiated ES cells (Supplementary Fig. S1A) must be due to mechanisms other than PIC

recruitment, affecting for example the stability of *Xist* RNA. Our results are in agreement with previous observations indicating that undifferentiated ES cells lacking *Tsix* do not accumulate high enough levels of *Xist* RNA to initiate X-inactivation (Debrand et al. 1999; Lee and Lu 1999; Luikenhuis et al. 2001; Sado et al. 2001). The molecular elucidation of the transcription factor network achieving the repression of PIC recruitment specifically in ES cells will provide invaluable information regarding X-inactivation.

The chromosomal origin of Xist expression is based on allelic H3-K4 dimethylation and PIC recruitment

H3-K4 methylation has emerged as an important mark in the epigenetic programming of the genome, as highlighted by the acquisition of H3-K4 dimethylation in reprogrammed somatic genomes (Kimura et al. 2004). We found that H3-K4 dimethylation also appears to be involved in the allelic programming of *Xist* expression, as it is the only mark tested to display a differential distribution that follows monoallelic *Xist* expression. The allelic pattern of H3-K4 dimethylation correlates with the binding of transcription basal machinery to the promoter. Thus, in both imprinted and random X-inactivation, monoallelic *Xist* expression is achieved by transcriptional mechanisms likely based on the epigenetic

distinction of both alleles through H3-K4 dimethylation (Fig. 5A,D). However, whether allelic PIC recruitment is a cause or a consequence of allelic H3-K4 dimethylation remains to be determined. Interestingly, and in contrast to other examples of monoallelically expressed genes, neither H3-K9 dimethylation nor H3-K27 trimethylation is associated with the silent *Xist* allele, even in cells with imprinted XCI. The absence of such repressive marks might be important for the plasticity of *Xist* expression, which must shift from imprinted to random at the implantation stage of development, and it is in agreement with the role of *Tsix* in reprogramming H3-K4 methylation. However, we cannot exclude the possibility that other marks contribute to the epigenetic repression of the silent *Xist* promoter on the active X chromosome. Among them, DNA methylation is unlikely to be involved, since the *Xist* promoter region was shown to be hypomethylated in preimplantation embryos (MacDonald et al. 1998).

Tsix triggers H3-K4 dimethylation within Xist

In ES cells, the entire *Tsix* unit is enriched for H3-K4 methylation, and our results have clearly established that *Tsix* is responsible for such histone modification, providing the first demonstration of a function of *Tsix* in regulating chromatin conformation of the *Xist* locus. This is reminiscent of the situation in *S. cerevisiae*, where a link between H3-K4 methylation and transcription elongation was demonstrated (Bernstein et al. 2002; Krogan et al. 2003; Ng et al. 2003). Such transcription-dependent H3-K4 methylation is mediated by the Set1 histone methyltransferase (HMT) through its association with the elongating RNAPolII (Krogan et al. 2003; Ng et al. 2003). We suggest that the association of an as yet unknown HMT-containing complex with the *Tsix* transcribing polymerase is responsible for the methylation of the overall region in ES cells (Fig. 5B,C). Alternatively, it is formally possible that H3-K4 dimethylation is triggered by the *Tsix* transcript itself through interaction between the RNA and an HMT-containing complex, similarly to the proposed function of *Xist* RNA in directing H3-K9 dimethylation and H3-K27 trimethylation to the inactive X chromosome (Heard 2004). That no H3-K4 methylation can be detected within the body of *Xist* in TS cells, despite maternal transcription of *Tsix*, might be due either to the low transcriptional and thereby methylation activity of *Tsix* or to the absence of the HMT responsible for such a modification, or a key cofactor controlling its accessibility, in such cells.

Tsix is usually described as a negative regulator of *Xist* expression. This clearly contrasts with the role of *Tsix* in the deposition of a histone modification specific for active chromatin within *Xist*, which suggests that *Tsix* transcription might also be involved in the transcriptional potential of *Xist* in ES cells. We propose that, at the implantation stage of development, biallelic activation of *Tsix* is responsible for the erasure of the allelic H3-K4 methylation mark that characterizes imprinted *Xist* expression (Fig. 5). The enrichment of the *Xist* locus

in H3-K4 dimethylation will result in both *Xist* homologs being epigenetically equivalent and equally competent for transcription and random X-inactivation. This provides a new function for *Tsix* in the reversion of imprinted *Xist* expression. The absence of a functional *TSIX* in human, which correlates with the absence of imprinted XCI in this species (for review, see Rougeulle and Avner 2004), is in agreement with our model.

Does Tsix down-regulation mark the future inactive X chromosome by restoring promoter-restricted H3-K4 dimethylation pattern on a single Xist allele?

The analysis of *Tsix*-truncated ES cell lines revealed that H3-K4 methylation of the *Xist* promoter region is not regulated solely by the transcription-dependent global methylation of the region, but that additional mechanisms are involved in which *Tsix* acts as a repressor. Because the region concerned corresponds to the only spliced exon of *Tsix* known to overlap *Xist* RNA (Sado et al. 2001; Shibata and Lee 2003), one might hypothesize that an RNA duplex between spliced transcripts of *Tsix* and *Xist* is responsible for the negative regulation of H3-K4 dimethylation within the *Xist* promoter region through targeting of the RNA interference (RNAi)-dependent heterochromatinization machinery. This notion is supported by the recent identification of the RNA-induced transcriptional silencing (RITS) complex that is believed to recruit components of the RNAi-mediated heterochromatinization pathway to genomic loci by interaction involving nascent-transcript and short interfering RNAs (siRNAs) produced by DICER (Verdel et al. 2004). Interestingly, DNA methylation, which can be induced by siRNAs, was shown to characterize to a certain extent the *Xist* promoter on both alleles in ES cells (Sado et al. 1996).

The down-regulation of *Tsix* during random XCI was shown to occur first on the future inactive X (Lee et al. 1999), and this was proposed to contribute to the choice process (Lee and Lu 1999). Our finding that disruption of *Tsix* transcription in ES cells results in increased levels of H3-K4 dimethylation over the *Xist* promoter region in *cis* provides novel insights into the mechanism of action of *Tsix* in the choice process of random X-inactivation: Monoallelic down-regulation of *Tsix* at the onset of random XCI will mark epigenetically the future active *Xist* promoter (Fig. 5C). The combined loss of the ICM-specific *Xist* repressive mechanism will result in allelic recruitment of the transcription machinery. The action of *Tsix* in the choice process of X-inactivation is therefore likely to be dual, with persistent *Tsix* transcription on the future active X repressing *Xist* RNA accumulation, while *Tsix* down-regulation epigenetically marks the future inactive X for monoallelic *Xist* expression.

In conclusion (Fig. 5), we demonstrate here that *Xist* transcription is a highly regulated event participating in both the expression level and the chromosomal origin of *Xist* RNA. Importantly, we show that *Tsix* has no effect on the transcriptional regulation of *Xist*. Rather, *Tsix* acts as a chromatin remodeler responsible for complex

regulation of H3-K4 dimethylation within *Xist* in ES cells. We propose that the chromatin-related function of *Tsix* is crucial for the transition from imprinted to random *Xist* expression. Biallelic activation of *Tsix* at the implantation stage of mouse development erases epigenetic marks associated with imprinted XCI and renders both *Xist* loci epigenetically indistinguishable and competent for random X-inactivation. Finally, our results suggest that the known role of *Tsix* in random choice is in part mediated by the establishment, on the future inactive X, of a chromatin structure characteristic of monoallelic *Xist* expression.

Material and methods

Cell culture

MEFs were prepared from 13.5-d-old embryos and cultured in Dulbecco's modified Eagle medium (DMEM, GIBCO), 10% fetal calf serum (FCS, ES cell grade, GIBCO). ES cell lines were grown in DMEM, 15% FCS, and 1000 U/mL LIF (Chemicon). Female LF2 ES cells were cultured on gelatin-coated plates in the absence of feeder cells. Male Ma1L and Ma2L ES cells (a kind gift from R. Jaenisch, Whitehead Institute for Biomedical Research, Cambridge, MA) were cultured on Mitomycin C-treated male embryonic fibroblast feeder cells. Female TS cells (F3 cell line) were grown on male fibroblast feeder cells in RPMI 1640 medium (GIBCO), 20% FCS (Biowest), MEM sodium pyruvate 1 mM (GIBCO), 2-mercaptoethanol 100 μ M (GIBCO), FGF4 25 ng/mL (RD systems), and Heparin 1 μ g/mL (Labosi). Feeder cells from Ma1L, Ma2L, and F3 cell cultures were removed by adsorption before chromatin and RNA extractions.

ChIP

ChIP assays were carried out essentially as described (Heard et al. 2001), with the following modifications: Cross-linking was performed on cells resuspended in DMEM, 10% FCS for 15 min in the presence of 1% formaldehyde at room temperature. TFIIB (C-18, Santa-Cruz), RNAPolII (7C2, Euromedex), and H3 di-meK4, di-meK9 and tri-meK27 (Upstate Biotechnology) antibodies were used respectively at dilutions of 1/50, 1/500, 1/100, 1/100, and 1/500.

The immunoprecipitated (IP) 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). Each PCR was run in triplicate to control for PCR variation. The primer sequences are provided as Supplemental Material. To standardize between experiments, we calculated the percentage of immunoprecipitation by dividing the average value of the IP by the average value of the corresponding input, both values first being normalized for dilution factors.

Quantitative RT-PCR

Total RNA isolated from cell cultures using RNable (Eurobio) was DNase-treated (Roche) and routinely verified by electrophoresis. Random-primed reverse transcription was performed at 42°C using Superscript II reverse transcriptase (Invitrogen). Control reactions lacking the RT enzyme were systematically verified for the absence of product. Quantitative real-time PCR measurements using SYBR Green Universal Mix were per-

formed in triplicate, and *Arpo P0* transcript levels were used to normalize between samples.

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***Tsix* transcription across the *Xist* gene alters chromatin conformation without affecting *Xist* transcription: implications for X-chromosome inactivation**

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