The Pluripotency Factor-Bound Intron 1 of Xist Is Dispensable for X Chromosome Inactivation and Reactivation In Vitro and In Vivo

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SUMMARY

X chromosome inactivation (XCI) is a dynamically regulated developmental process with inactivation and reactivation accompanying the loss and gain of pluripotency, respectively. A functional relationship between pluripotency and lack of XCI has been suggested, whereby pluripotency transcription factors repress the master regulator of XCI, the noncoding transcript Xist, by binding to its first intron (intron 1). To test this model, we have generated intron 1 mutant embryonic stem cells (ESCs) and two independent mouse models. We found that Xist’s repression in ESCs, its transcriptional upregulation upon differentiation, and its silencing upon reprogramming to pluripotency are not dependent on intron 1. Although we observed subtle effects of intron 1 deletion on the randomness of XCI and in the absence of the antisense transcript Tsix in differentiating ESCs, these have little relevance in vivo because mutant mice do not deviate from Mendelian ratios of allele transmission. Altogether, our findings demonstrate that intron 1 is dispensable for the developmental dynamics of Xist expression.

INTRODUCTION

To balance the expression of X-linked genes between males and females, female mammals silence one of the two X chromosomes in a developmentally regulated process called X chromosome inactivation (XCI). XCI occurs in two waves in the course of mouse embryogenesis. The earliest form of XCI begins at the two- to four-cell stage in preimplantation embryo and is imprinted, selectively occurring on the paternally inherited X chromosome (Xp) (Huynh and Lee, 2003; Kalantry et al., 2009; Namekawa et al., 2010; Patrat et al., 2009). At the preimplantation blastocyst stage, imprinted XCI is retained in the trophoderm and primitive endoderm lineages but reversed in arising pluripotent epiblast cells yielding a state with two active X chromosomes (XaXa) (Mak et al., 2004; Okamoto et al., 2004; Silva et al., 2009; Williams et al., 2011). Upon implantation, these epiblast cells establish a random form of XCI that stochastically initiates on the maternal or paternal X chromosome and is retained through the lifetime of mitotic divisions (Kay et al., 1993; Rastan and Robertson, 1985). Similarly, mouse embryonic stem cells (ESCs), which are derived from epiblast cells of the preimplantation blastocyst, undergo random XCI when induced to differentiate ex vivo. The only exception to somatic maintenance of random XCI is inactive X (Xi) reactivation in the germline, which is assumed to be essential for female fertility and occurs in primordial germ cells as they traverse the hindgut to seed the genital ridges (Chuva de Sousa Lopes et al., 2008; de Napoles et al., 2007; Sugimoto and Abe, 2007). Xi reactivation is also a feature of experimentally induced acquisition of pluripotency via transcription factor-mediated reprogramming to induced pluripotent stem cells (iPSCs), fusion of somatic cells with ESCs, or somatic cell nuclear transfer (Eggan et al., 2000; Maherali et al., 2007; Tada et al., 2001).

The cycles of XCI and Xi reactivation are associated with changes in Xist RNA coating, where cells with a Xi display coating by the noncoding Xist RNA on the Xi chromosome, and those with two active X chromosomes lack Xist RNA expression (Brockdorff et al., 1991; Brown et al., 1991). Xist’s function has been most studied in the random form of XCI in the mouse system, where it is shown to be the critical trigger of XCI. The upregulation of Xist RNA and coating of the X at the onset of random XCI immediately lead to transcriptional silencing of X-linked genes and result in the exclusion of RNA polymerase II and the recruitment of repressive chromatin-modifying protein complexes such as the Polycomb complex PRC2, which establishes an accumulation of H3K27me3 (Chaumeil et al., 2006; Chow and Heard, 2009; Plath et al., 2003; Silva et al., 2003). A stereotypic order of changes in chromatin structure culminates in heritable silencing of either the maternally or paternally transmitted X chromosome in each cell of the female adult mammal. Xist is essential for XCI to occur in cis because its deletion leads to silencing of the other X chromosome carrying an intact Xist allele, regardless of parent of origin (Marahrens et al., 1997; Penny et al., 1996). Moreover, the importance of Xist regulation for the developmental and sex-specific context of XCI is...
demonstrated by its sufficiency: overexpression of a X-linked Xist cDNA transgene in male mouse ESCs (XY:tetOP-Xist) initiates XCI and cell death due to silencing of the single X chromosome (Wutz and Jaenisch, 2000).

Xist is transcribed from a larger locus on the X chromosome that has been defined as the minimal critical region for XCI and besides housing Xist, contains other protein-coding and non-coding activators and repressors of Xist, some of which act in cis and others in trans (Rastan and Robertson, 1985; reviewed in Minkovsky et al., 2012). The best-characterized repressor of Xist is its antisense transcript, Tsix, which is highly transcribed in epiblast cells of the preimplantation blastocyst and in undifferentiated mouse ESCs/iPSCs, where Xist is repressed (Lee et al., 1999; Sado et al., 2001; Maherali et al., 2007). Deletion of Tsix leads to only slight Xist upregulation without causing precocious XCI or Xist RNA coating in self-renewing, undifferentiated ESCs. However, upon differentiation, XCI is skewed to the Tsix-deleted X in female cells heterozygous for the mutant Tsix allele (Lee et al., 1999; Lee, 2000; Luikenhuis et al., 2001; Sado et al., 2001). The effect of Tsix deletion on Xist indicates that it participates in parallel pathways with other regulators of Xist repression or activation.

Interestingly, the pluripotency factors Oct4, Sox2, and Nanog have been implicated in the control of Xist expression in pluripotent cells. Navarro and colleagues found that in mouse ESCs, Oct4, Sox2, and Nanog bind the first intron of the Xist gene (intron 1) (Navarro et al., 2008), a finding that has been recapitulated in many genomic data sets and extends to additional pluripotency regulators such as Tcf3 and Prdm14, and early developmental regulators such as Cdx2 (Figure S1A; Loh et al., 2006; Marson et al., 2008; Ma et al., 2011; Erwin et al., 2012). Such genomic regions of extensive pluripotency transcription factor co-occupancy in the ESC genome occur more commonly than would be expected by chance (Chen et al., 2008). It is thought that these genomic regions may act in distributed manner such as in ESCs, which the intron 1 region has a propensity to be in the three-dimensional proximity to the promoter of Xist and adopts a DNase hypersensitive state (Tsai et al., 2008). Additionally, pluripotency factors appear directly linked to Xist regulation. Upon Nanog deletion or inducible repression of Oct4, Xist is upregulated, and binding of the pluripotency factors to intron 1 is lost (Navarro et al., 2008). In male ESCs, which normally do not upregulate Xist, experimentally forced Oct4 repression can even induce Xist RNA coating in up to 10% of the cells (Navarro et al., 2008). Another study could not replicate Xist RNA coating upon Oct4 knockdown in male ESCs but observed biallelic XCI in differentiating female ESCs upon Oct4 depletion (Donohoe et al., 2009). A role for Nanog in Xist suppression is also supported by its expression pattern with regard to domains of X reactivation in the preimplantation blastocyst, where the restriction of Nanog expression demarcates the fraction of cells undergoing reactivation of the imprinted Xi (Silva et al., 2009). Furthermore, preimplantation embryos lacking Nanog are unable to specify epiblast cells and to lose Xist RNA, whereas forced expression of Nanog induces a more rapid loss of Xist RNA coating in developing preimplantation embryos (Silva et al., 2009; Williams et al., 2011).

Together, these findings led to the model that pluripotency factor binding to intron 1 is critical for repression of Xist in undifferentiated XaXa ESCs. However, in the experiments leading to this conclusion, cell identity and, therefore, likely the expression of many genes were modulated by experimental changes in pluripotency factor expression, which could confound the interpretation that Oct4, Nanog, and other pluripotency factors act directly on intron 1 of Xist to regulate XCI. It has also been suggested that the pluripotency transcription factors control the levels of positive and negative regulators of Xist because they are binding to Tsix and the trans-acting activator of XCI, Rnf12 (Donohoe et al., 2009; Gontan et al., 2012; Navarro et al., 2010, 2011). Accordingly, an experiment directly addressing the functional importance of binding to intron 1 showed only subtle dysregulation of XCI: in female ESCs carrying a heterozygous deletion of intron 1 of Xist, XCI remained depressed in the undifferentiated state. However, upon differentiation, Xist appeared more highly expressed from the chromosome carrying the mutation, supporting a role for intron 1 in suppressing Xist during differentiation (Barakat et al., 2011). Furthermore, deletion of intron 1 in the context of a transgene carrying the extended Xist locus moderately increased expression of Xist in undifferentiated ESCs, which was amplified by simultaneous deletion of the antisense transcript Tsix (Nesterova et al., 2011). Notably, these results were very variable between clones potentially reflecting the effect of transgene copy number and variations (Nesterova et al., 2011). Binding to Xist intron 1 has also been proposed to govern the switch from imprinted to random XCI in preimplantation development (Erwin et al., 2012). In vitro, gel shift assays suggest that the binding events between Xist’s intron 1 and the pluripotency regulator Oct4 and the trophectoderm regulator Cdx2 are direct but mutually exclusive (Erwin et al., 2012). Collectively, these findings motivated us to examine the role of Xist intron 1 further to test the model wherein pluripotency factor binding silences Xist to prevent XCI in pluripotent cells and to determine the role of the intronic region in X chromosome reactivation events, both in vivo and in vitro.

RESULTS

Generation of Conditional Xist Intron 1 ESC Lines
To further define the role of Xist intron 1, we used gene targeting to generate a conditional allele in male and female mouse ESCs. We tested the requirement of intron 1 in both sexes because male ESCs are able to undergo XCI upon forced expression of Xist, providing a sensitive background for monitoring Xist regulation independently of other X chromosomes present in a cell (Wutz and Jaenisch, 2000). By contrast, heterozygous female ESCs permit investigation of kinetics of XCI upon induction of differentiation and insight into potential effects on skewing of XCI between the targeted and wild-type (WT) chromosome.

To delineate the region of intron 1 involved in Xist repression, we inspected where pluripotency transcription factors bind within the intron 1 region as detected by published chromatin immunoprecipitation sequencing (ChiP-seq) data sets (Marson et al., 2008). We also determined the localization of pluripotency
factor DNA binding motifs and considered sequence conservation across mammals (Figure S1). We found that co-occupancy of pluripotency factors occurs in a 600 bp region within the full 2.8 kb sequence of intron 1. Most of the intron 1 sequence is not conserved in placental mammals; however, two highly conserved composite Oct4-Sox2 DNA binding motifs, which are found to stabilize a ternary Oct4-Sox2-DNA complex in the expression of many ESC-specific genes, underlie the ChiP-seq binding peaks of Oct4 and Sox2 (Figure S1; Reményi et al., 2003; Marson et al., 2008; Mason et al., 2010; UCSC genome browser phastCons elements track, http://genome.ucsc.edu). On the basis of these data, we decided to delete 800 bp of intron 1 and, subsequently, refer to this mutation as “intron 1” (Minkovsky/Plath allele; Figure S1).

We flanked the 800 bp intron 1 region with loxP sites, simultaneously inserting a hygromycin-resistance cassette (yielding a targeted allele with 3loxP sites), and, subsequently, generated experimental (1lox) and control (2lox) alleles by transient expression of Cre recombinase in hemizygously targeted male and experimental (1lox) and control (2lox) alleles by transient expression of Cre recombinase in hemizygously targeted male and male ESCs using a construct that inserts a splice acceptor-IREs/Geo cassette in exon 2 of Tsix (Nesterova et al., 2011). We targeted the disruption of Tsix to both 2lox and 1lox intron male ESCs using a construct that inserts a splice acceptor-IREs/Geo cassette in exon 2 of Tsix resulting in an early transcriptional stop (Figure 3A) (Sado et al., 2002). Correct targeting and loss of the Tsix transcript were confirmed by Southern blot (Figure 3B) and absence of FISH signal for Tsix (Figure 3C).

Upon induction of differentiation by embryoid body (EB) formation, the lack of intron 1 did not induce Xist RNA in male ESCs to a level detectable by FISH (data not shown) and yielded no Xi-like enrichment of H3K27me3 (Figures S3C and S3D), indicating that intron 1 is not an essential regulator of Xist suppression in differentiating male ESCs when all other regulators of XCI are intact. Heterozygous 1lox/WT female ESCs formed Xist RNA clouds and H3K27me3 Xi foci at comparable rates to 2lox/WT control ESCs (Figures 2C, 2D, S3C, and S3D). Xist RNA levels were also similar between undifferentiated and differentiating male and female ESCs, with or without intron 1, in RT-PCR experiments (Figure 2E). Proper differentiation was confirmed by decrease in Nanog transcript levels (Figure 2F). Furthermore, the use of Xist intron 1-spanning PCR primer pairs ruled out dramatic secondary effects of intron 1 deletion on Xist splicing (data not shown).

Next, we assessed whether XCI is skewed upon intron 1 deletion in differentiating female ESCs. The polymorphic 129/cas F1 2-1 female ESC line is known to have a baseline skewing of XCI toward the 129 allele such that approximately 70% of the cells will silence the 129 allele, due to strain-specific haplotypes (Cattanach and Isaacman, 1967). Due to the integration of the MS2 RNA tag on the intron-targeted 129 X chromosome, combined RNA-FISH for MS2 and Xist sequences can distinguish between Xist being expressed from the targeted chromosome (positive for both Xist and MS2 signals) and the untargeted X (only marked by the Xist probe) (Figure 2C; Jonkers et al., 2008). We found that, at the single-cell level, female 1lox intron/WT ESCs consistently had ~15% more cells expressing the MS2-tagged Xist than their 2lox/WT counterparts, in three of four ex vivo differentiation methods (Figures 2G and S4). This mild skewing effect in differentiating female ESCs is consistent with published results (Barakat et al., 2011).

**Genetic Interaction of Xist Intron 1 with Tsix**

Next, we investigated the possibility that the intron 1-dependent skewing of XCI in differentiating female ESCs represents a mild effect on the intron 1-deleted X chromosome at the transition to the differentiated state. We reasoned that such an effect may be more strongly revealed in the absence of other regulators of Xist and sought to assay such an effect on a “sensitized” background for Xist transcription. Tsix represents the prime candidate for a redundant Xist repressor that could compensate to repress Xist in the absence of intron 1. One study supports the view that a functional role for the intron can be uncovered in the absence of Tsix because male ESCs with randomly integrated genomic Xist transgenes lacking intron 1 and a functional Tsix allele dysregulated the expression of the transgenic Xist (Nesterova et al., 2011). We therefore performed the aforementioned analyses in male ESCs lacking intron 1 in the endogenous Xist allele on the background of a previously characterized Tsix loss-of-function mutation at the endogenous locus (Figure 3; Lee et al., 1999; Luikenhuis et al., 2001; Sado et al., 2001). We targeted the disruption of Tsix to both 2lox and 1lox intron male ESCs using a construct that inserts a splice acceptor-IREs/Geo cassette in exon 2 of Tsix resulting in an early transcriptional stop (Figure 3A) (Sado et al., 2002). Correct targeting and loss of the Tsix transcript were confirmed by Southern blot (Figure 3B) and absence of FISH signal for Tsix (Figure 3C). As expected, in the presence of intron 1 (2lox intron 1), Tsix deletion in male ESCs induced a mild transcriptional...
The upregulation of Xist RNA compared to XY:2lox/Tsix-WT ESCs in RT-PCR experiments, reaching a level found in female ESCs (Figure 3D). Upon differentiation, XY:2lox intron/Tsix-Stop ESCs further upregulated Xist transcript levels ~5-fold (Figure 3D). However, this induction was rarely correlated with a Xist RNA cloud signal detectable by RNA FISH or a Xi-like H3K27me3 accumulation (Figures 3E–3H) before and after induction of differentiation, in agreement with previous reports by Luikenhuis et al. (2001) and Sado et al. (2002). Combined deletion of intron 1 and Tsix did not alter the Xist status in undifferentiated ESCs but upon induction of differentiation, resulted in a Xist RNA cloud-like signal in FISH experiments in 3%–6% of...
cells compared to 0.2%–0.8% in differentiating XY:2lox intron/Tsix-Stop cells (Figures 3E and 3G). We did not, however, see any significant intron 1-dependent effect on Xist RNA levels by RT-PCR comparing XY:2lox intron/Tsix-Stop and XY:1lox intron/Tsix-Stop cells (Figure 3D) or an increase in the number of H3K27me3 Xi-like accumulations (Figures 3F and 3H). Thus, even though Xist RNA was induced in a slightly larger proportion of differentiating cells in the absence of both Tsix and intron 1 than in the absence of either Tsix or intron 1, this upregulation does not appear to be sufficient to mediate H3K27me3 enrichment on the targeted X chromosome, suggesting that the RNA does not efficiently coat the chromosome in these cells or that the recruitment of Polycomb proteins is affected. We conclude that these experiments reveal a subtle role of intron 1 in the control in Xist expression, which may be related to the weak skewing phenotype of XCI described above for differentiating intron 1 mutant heterozygous female ESCs (Figures 2 and 4).

In a second assay, we tested the consequence of intron 1 deletion upon modulation of global Oct4 transcript levels. We first confirmed the previously reported relationship between

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\text{Expression (units)} = \frac{\text{Xist RNA}}{\text{Gapdh RNA}}
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Figure 2. Analysis of Xist Expression in Undifferentiated and Differentiating Female and Male ESC Lines in the Presence and Absence of Intron 1
(A) Strand-specific FISH for Xist RNA (green) and Tsix RNA (red) in undifferentiated male and female ESCs of the indicated genotypes, using RNA probes. DAPI staining (blue) indicates nuclei. Representative images are shown. A male ESC line carrying a doxycycline (dox)-inducible Xist allele in the endogenous locus was used as positive control for the Xist-staining pattern, at 24 hr of dox addition.
(B) Graph summarizes the proportion of DAPI-stained nuclei with indicated patterns of Xist RNA based on an experiment as described in (A). Pairs of independent ESC clones of the given genotype were stained and counted. In each case, 500 nuclei were assessed.
(C) FISH with DNA probes targeting Xist RNA (green) and MS2 signals. *p < 0.05 by Student’s t test with 500 nuclei as control in each case, at least 500 nuclei were assessed.
(D) Graph summarizing the proportion of Nanog-negative cells in day 10 EB-differentiated female ESCs with no, one, or two H3K27me3 Xi-like accumulations. Notably, the number of cells within each H3K27me3 pattern is not statistically different (by Student’s t test) between 2lox/wt and 1lox/wt ESC lines. Values are means of counts of independent clones as shown in Figure S4D; in each case, at least 500 nuclei were assessed.
(E) RT-PCR for Xist RNA levels normalized to Gapdh expression from one representative clone of indicated ESC genotypes in the undifferentiated state (ESC) and at day 5 of RA differentiation (d5 RA). Error bars indicate SD from triplicate RT-PCR measurements in one experiment.
(F) As in (E), except that Nanog transcript levels were analyzed.
(G) Quantification of allele-specific Xist RNA cloud patterns from the experiment shown in (C) at days 6 and 10 of EB differentiation, given as mean of values from counts of two independent ESC clones of the indicated genotype. Xist expression from the 129 chromosome (targeted chromosome) is detected by both the Xist and MS2 signals, whereas Xist expression from the CAST chromosome is only detected by the Xist probe. The graph depicts the percentage of cells where the intron 1-targeted 129 chromosome is coated by Xist RNA, as identified by colocalization of the Xist and MS2 signals. *p < 0.05 by Student’s t test with 500 Xist clouds analyzed for each sample.

See also Figures S3 and S4.
the decrease of Oct4 levels and Xist RNA induction (Navarro et al., 2008; Donohoe et al., 2009). Specifically, upon Oct4 depletion in the male ZHBTc4 ESC line, in which Oct4 expression can be silenced acutely by the addition of doxycycline (Niwa et al., 2000), Xist RNA levels were induced almost 100-fold 96 hr post-induction of Oct4 repression (Figure S5A), and Xist RNA could be detected by FISH in a small number of cells (Figures S5B and S5C). Notably, we observed that Oct4 transcript levels drop

Figure 3. Xist RNA Pattern in Male ESCs Lacking Tsix and Intron 1
(A) Gene targeting and Southern blotting strategy schematic for the generation of the Tsix-Stop allele in male ESCs according to Sado et al. (2001) using the pAA2Δ1.7-targeting vector.
(B) Male 2lox and 1lox intron 1 ESC clones were targeted with the Tsix-Stop allele. A correctly targeted 2lox intron 1/Tsix-Stop male ESC clone is shown in this Southern blot analysis.
(C) Strand-specific FISH for Tsix RNA (red) in undifferentiated male ESCs of the indicated genotypes, using an RNA probe, indicates the absence of the Tsix FISH signal in Tsix-Stop-targeted clones.
(D) Graph summarizing the transcript levels for Nanog and Xist normalized to Gapdh transcript levels as determined by RT-PCR from a representative clone of each genotype in the undifferentiated state (ES) and at day 5 of RA differentiation. Control Xist RNA levels from WT undifferentiated male and female ESCs are shown on the left. Error bars indicate SD from triplicate RT-PCR measurements in one experiment.
(E) Graph summarizing the percentage of undifferentiated ESCs of the given genotype with and without a Xist RNA cloud-like pattern. Two independent male ESC clones for each genotype were analyzed by Xist RNA FISH with a RNA probe, and 500 nuclei were assessed.
(F) As in (E), except that the percentage of undifferentiated ESCs with and without a H3K27me3 Xi-like accumulation is given.
(G) Xist RNA cloud quantification as in (E), except that Nanog-negative cells were quantified upon day 10 of EB differentiation.
(H) As in (F) for H3K27me3 patterns at day 10 of EB differentiation in indicated ESC lines.
See also Figure S5.
with faster kinetics than Xist RNA levels increase, suggesting that the effect of Oct4 on intron 1 is indirect and may require efficient differentiation, which occurs at 96 hr post-Oct4 repression, as indicated by the loss of the pluripotency factor Nanog (Figure S5D). In agreement with this conclusion, siRNA-mediated knockdown of Oct4 in ESCs did not increase Xist RNA levels more than 2-fold after 72 hr, confirming a previous report by Donohoe et al. (2009) (Figure S5E). Furthermore, the absence of intron 1 did not significantly alter Xist RNA levels in female ESCs or in male ESCs lacking Tsix in Oct4 knockdown conditions (Figure S5E). These data indicate that the slight increase in Xist levels immediately upon Oct4 depletion is independent of intron 1.

### Intron 1 Acts as an Enhancer in a Reporter Assay in Differentiating ESCs

The model of pluripotency factor binding to intron 1 to repress Xist motivated us to directly assess whether intron 1 behaved as a silencer in ESCs in a reporter assay. We transfected constructs with intron 1 or control sequences upstream of a minimal promoter driving luciferase and did not see intron 1-dependent decreases in reporter activity (data not shown). The small effect of intron 1 deletion on Xist RNA levels detected in differentiating ESCs in the absence of Tsix motivated us to revisit these experiments and instead investigate whether Xist intron 1 represents a developmentally regulated enhancer that becomes active upon induction of differentiation. We therefore tested transactivation activity of intron 1 in undifferentiated and differentiating ESCs using stably integrated luciferase reporter constructs (Figure 4). Male ESCs were electroporated with hygromycin resistance-bearing constructs containing either the minimal promoter. The intronic region was broken up in three parts, with (B) representing the part bound by pluripotency factors and flanked by loxP sites as described in Figure 1 (Minkovsky/Plath allele), and (A) and (C) representing regions not bound by the pluripotency factors in ChIP-seq experiments (Figure S1). Note that (B) was concatenated 3X in the reporter construct.

(B) Three independent stable cell lines were generated by electroporation of male ESCs with the three constructs described in (A). Cells carrying the reporter constructs were selected with hygromycin, and an equal number of cells was plated and maintained in the undifferentiated state or differentiated for days 3 and 5 by LIF withdrawal and/or RA addition as indicated. After treatment, one-tenth of the cells in the well was analyzed by luciferase assay. For each reporter construct, values represent mean luminescence units normalized to values from the respective cell line in the undifferentiated state (n = 3, ± 1 SD).

See also Figure S6.

Figure 4. Enhancer Assay of Xist Intron 1

(A) Schematic representation of genomic fragments of the entire genomic intron 1 region cloned upstream of a luciferase reporter gene driven by a minimal promoter. The intronic region was broken up in three parts, with (B) representing the part bound by pluripotency factors and flanked by loxP sites as described in Figure 1 (Minkovsky/Plath allele), and (A) and (C) representing regions not bound by the pluripotency factors in ChIP-seq experiments (Figure S1). Note that (B) was concatenated 3X in the reporter construct.

(B) Three independent stable cell lines were generated by electroporation of male ESCs with the three constructs described in (A). Cells carrying the reporter constructs were selected with hygromycin, and an equal number of cells was plated and maintained in the undifferentiated state or differentiated for days 3 and 5 by LIF withdrawal and/or RA addition as indicated. After treatment, one-tenth of the cells in the well was analyzed by luciferase assay. For each reporter construct, values represent mean luminescence units normalized to values from the respective cell line in the undifferentiated state (n = 3, ± 1 SD).

See also Figure S6.
though still not to an extent where deletion affects transcription of Xist or of neighboring protein-coding genes.

Together, these ex vivo studies in undifferentiated and differentiating male and female ESCs point to a minor role for intron 1 in the regulation of Xist expression, uncovered only when another Xist repressor is deleted, and some aspect of X chromosome choice (potentially also through slight modulation of Xist RNA levels). These data do not support intron 1 as a main aspect of the mechanism of transcriptional repression of Xist, at least in this culture model.

Mice Are Normal in the Absence of Intron 1

Next, we assayed the significance of intron 1 in vivo. Our male ESCs deleted for intron 1 (1lox) were injected into C57BL/6 blastocysts. Chimeras were obtained at high efficiency and bred with C57BL/6 females to obtain germline transmission of the mutant allele. Subsequently, the 1lox intron 1 allele showed normal propagation through the maternal or paternal germline, and mice completely lacking intron 1 (crossing 1lox/1lox females with 1lox males) could be efficiently bred without any female-specific defect (Figure 5A). Because X chromosome reactivation occurs in the female germline and is likely essential for female fertility, we assayed litter size of the F2 generation of female homozygous knockout mice, and we found their litter sizes unaffected (data not shown), indicating that intron 1 is not essential in mice.

To strengthen these observations of normal transmission of the intron 1 mutation and rule out that genetic background obscured a potential intron 1 phenotype in vivo, we generated a second mouse model carrying an independent intron 1 mutation. We generated mice using previously published 129/CAST F1 female ESCs in which a larger (1.815 kb) region harboring most of intron 1 was deleted on the 129 X chromosome (Barakat/Gribnau allele) (Figure S1A; Barakat et al., 2011). Using these ESCs, we previously observed a slight upregulation of Xist RNA levels on the deleted chromosome upon induction of differentiation (Barakat et al., 2011), in agreement with results obtained using the Minkovsky/Plath allele, indicating skewing of X inactivation toward the intron 1-deleted chromosome. Importantly, this second mouse model also displayed normal Mendelian transmission of the intron 1 lox allele (Figure 5B).

To assay whether random XCI has occurred in female mice carrying a Xp lacking intron 1 and a maternally inherited WT X chromosome, and whether the lack of the intron leads to any skewing of XCI in vivo, we analyzed the allele-specific expression of Xist and two X-linked genes, Mecep2 and G6pdx, in polymorphic heterozygous females (1lox/WTCAST/E1) and a WT control (WT/WTCAST/E1). In these mice, the C57BL6 X chromosome was transmitted from the father and the CAST/E1 WT X from the mother. Allele-specific expression analysis was performed using semiquantitative RT-PCR on RNA isolated from various tissues (Figures 5C–5E). In these experiments, we used the Barakat/Gribnau mouse model described in Figures 5B and S1A. Normally, the paternal X chromosome initially undergoes imprinted XCI, which is reversed in the epiblast cells of the preimplantation blastocyst to allow subsequent random XCI. The intron 1 region has been implicated to be important for Xr reactivation in the ICM, and thus, if the absence of intron 1 prevents reactivation of imprinted XCI, we may observe nonrandom XCI in the adult mouse (Navarro et al., 2008).

However, we did not find differences in allele-specific expression pattern in the presence and absence of intron 1 in heterozygous female mice (Figures 5C–5E). As expected, the C57BL6 Xist allele is more often expressed than the CAST/E1 X, consistent with a modifier effect, likely resulting in more cells with an inactivated C57BL6 X (Cattanach and Isaacson, 1967). Because of the stochastic and clonal nature of XCI patterns in the adult mouse, variations in skewing toward Xist RNA from the C57BL/6 allele ranged from 50% to 90% (Figures 5C and 5E). Notably, we did not see a preference of Xist upregulation on the intron 1-deleted X chromosome in tissues of the adult mouse in vivo, albeit we observed slightly skewed Xist RNA levels in heterozygous-differentiating female ESCs carrying the same mutant intron 1 allele in vitro (Barakat et al., 2011). In agreement with this notion, the X-linked genes Mecep2 and G6pdx, both subject to silencing on the Xi, showed reciprocal and intron 1-independent levels of expression from the C57BL/6 chromosome compared to Xist, as would be expected from the fact that the Xist-expressing chromosome is more likely to be silent (Figures 5D and 5E). These data suggest that the paternal transmission of the intron 1 mutation does not interfere with reactivation of imprinted XCI and subsequent random XCI. A reverse cross in which the maternal allele lacked intron 1 also resulted in random XCI (data not shown). In summary, the intron 1 genomic region is dispensable in the mouse and does not critically control Xist expression and skewing of XCI in vivo.

Intron 1 Is Not Required for Loss of Xist RNA upon Reprogramming to iPSCs

Although there was no dramatic effect on XCI state in vivo, we sought to understand the requirement for intron 1 in Xist silencing associated with reprogramming to iPSCs. We have shown previously that female iPSCs derived from mouse embryonic fibroblasts (MEFs) carry two active X chromosomes, where Xist is efficiently repressed and Tsix upregulated, as seen in mouse ESCs (Maherali et al., 2007). Another study suggested that X reactivation occurs late in reprogramming at around the time pluripotency genes become expressed, again suggesting that pluripotency transcription factors could contribute to Xi reactivation and the silencing of Xist, potentially via binding to intron 1 (Stadtfeld et al., 2008). To test the role of intron 1 in the Xist-silencing process during reprogramming, we bred male mice carrying the 2lox intron 1 allele (obtained upon blastocyst injection of our male 2lox ESCs described in Figure 1, Minkovsky/Plath allele) with female mice heterozygous for a Xist knockout allele (Marahrens et al., 1997), yielding female XX:2lox intron1/Xist MEFs. Due to the presence of the Xist knockout allele, the X chromosome bearing the conditional intron 1 allele is exclusively inactivated in vivo by normal developmental mechanisms (Marahrens et al., 1998). MEFs isolated from E14.5 embryos had uniform Xist coating (Figure 6C) and were transduced with retroviruses encoding the reprogramming factors Oct4, Sox2, and Klf4, and subsequently infected with adenovirus encoding Cre recombinase at day 4 of reprogramming to efficiently delete the intron 1 region or with titer-matched empty adenovirus in control samples (Figure 6A). This
(E) Graph averaging the allele-specific expression data in (C) and (D) across all tissue and mice per genotype.

pure C57BL/6 or CAST/Ei mice as well as RNA isolated from a polymorphic C57BL/6 and CAST/Ei ESC line.

MeCP2

(D) Examination of tissues as in (C) for allelic expression of X-linked genes

Xist the tissue samples represent the relative band intensity for the C57BL/6 and CAST/Ei

1lox allele) with a WT CAST/Ei female. Panel includes controls on the left mixing pure C57BL/6 and CAST/Ei brain cDNA template in given ratios. Numbers below

mosome in organs of one female WT mouse and two littermate heterozygous 1lox/WT mice obtained by crossing a C57BL/6 male (with and without the intron 1

lation. Genotyping confirmed that Ad-Cre addition resulted in

programming efficiency for the same infected fibroblast popu-

lation. We determined the number of Nanog-expressing colonies

at day 13 after reprogramming factor introduction because

experimental setup allowed us to test the role of intron 1 in re-

programming efficiency for the same infected fibroblast popu-

lation. Genotyping confirmed that Ad-Cre addition resulted in

efficient deletion of the intron 1 region (Figure 6B). To test

whether intron 1 deletion affects the efficiency of reprogram-

ming, we determined the number of Nanog-expressing colonies

day 13 after reprogramming factor introduction because

Nanog expression has been shown to mark faithfully reprog-

rammed cells in retroviral reprogramming experiments (Maherali et al., 2007). We found a comparable number of Nanog+ colonies in the presence and absence of intron 1 (Figure 6D).

Normal, at this point of reprogramming, Xist RNA coating is just lost in Nanog+ cells (K.P. and J. Tchieu, unpublished data). In agree-

ment with this notion, an examination of all Nanog+ data using the Xist allele generated in the Plath lab (see Figure 1, Minkovsky/Plath allele).

(B) As in (A), except that mice carrying a second, independent intron 1 deletion generated by the Gribnau lab were crossed (see Figure S1 for comparison of allelic

Barakat et al., 2011).

(C) Allele-specific RT-PCR analysis of Xist RNA detecting a length polymorphism that distinguishes Xist RNA originating from the C57BL/6 and CAST X chro-

mosome in organs of one female WT mouse and two littermate heterozygous 11ox/WT mice obtained by crossing a C57BL/6 male (with and without the intron 1

1lox allele) with a WT CAST/Ei female. Panel includes controls on the left mixing pure C57BL/6 and CAST/Ei brain cDNA template in given ratios. Numbers below

the tissue samples represent the relative band intensity for the C57BL/6 and CAST/Ei Xist allele determined by comparison with the control samples.

(D) Examination of tissues as in (C) for allelic expression of X-linked genes MeCP2 (top) and G6pdx (bottom) by RFLP RT-PCR. Panel includes controls (left) from

pure C57BL/6 or CAST/Ei mice as well as RNA isolated from a polymorphic C57BL/6 and CAST/Ei ESC line.

(E) Graph averaging the allele-specific expression data in (C) and (D) across all tissue and mice per genotype ± 1 SD.

Figure 5. Transmission of the Intron 1 Mutation In Vivo

(A) Table summarizing the number and genotypes of offspring from indicated mouse crosses using the intron 1 allele generated in the Plath lab (see Figure 1, Minkovsky/Plath allele).

(B) As in (A), except that mice carrying a second, independent intron 1 deletion generated by the Gribnau lab were crossed (see Figure S1 for comparison of allelic

Barakat et al., 2011).

(C) Allele-specific RT-PCR analysis of Xist RNA detecting a length polymorphism that distinguishes Xist RNA originating from the C57BL/6 and CAST X chro-

mosome in organs of one female WT mouse and two littermate heterozygous 11ox/WT mice obtained by crossing a C57BL/6 male (with and without the intron 1

1lox allele) with a WT CAST/Ei female. Panel includes controls on the left mixing pure C57BL/6 and CAST/Ei brain cDNA template in given ratios. Numbers below

the tissue samples represent the relative band intensity for the C57BL/6 and CAST/Ei Xist allele determined by comparison with the control samples.

(D) Examination of tissues as in (C) for allelic expression of X-linked genes MeCP2 (top) and G6pdx (bottom) by RFLP RT-PCR. Panel includes controls (left) from

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experimental setup allowed us to test the role of intron 1 in re-

programming efficiency for the same infected fibroblast popu-

lation. Genotyping confirmed that Ad-Cre addition resulted in

efficient deletion of the intron 1 region (Figure 6B). To test

whether intron 1 deletion affects the efficiency of reprogram-

ming, we determined the number of Nanog-expressing colonies

day 13 after reprogramming factor introduction because

Nanog expression has been shown to mark faithfully reprog-

rammed cells in retroviral reprogramming experiments (Maherali et al., 2007). We found a comparable number of Nanog+ colonies in the presence and absence of intron 1 (Figure 6D).

Normal, at this point of reprogramming, Xist RNA coating is just lost in Nanog+ cells (K.P. and J. Tchieu, unpublished data). In agree-

ment with this notion, an examination of all Nanog+ cells for
Figure 6. The Absence of Intron 1 on the Xi Does Not Interfere with Loss of Xist RNA Coating upon Reprogramming of MEFs to iPSCs

(A) Schematic representation of the reprogramming experiment with female MEFs bearing the conditional intron 1 allele on the Xi and a Xist knockout allele on the Xa. Reprogramming was induced by infection with pMX retroviruses encoding the reprogramming factors, and the reprogramming culture was split at day 3 postinfection. Deletion of the conditional intron 1 allele was induced by delivery of 1X or 10X adenoviral particles carrying Cre recombinase, performed at day 4. Control 1X Ad Null treatment was done in parallel. At day 13 of reprogramming, efficient deletion of intron 1 was assessed by genotyping, reprogramming efficiency was determined by Nanog+ colony count, and loss of Xist RNA coating in Nanog+ cells was examined by IF/FISH. In addition, individual colonies were picked, expanded, and analyzed further.

(B) PCR genotyping for the presence of the 2lox and 1lox intron 1 alleles in reprogramming cultures at day 13, using primers pairs A and C (top panel) or B and C (bottom panel) (as in Figure 1F), indicates efficient deletion of intron 1 upon Ad-Cre treatment. “1” and “2” represent independent reprogramming samples. The asterisk marks the WT allele, which is attributed to the presence of feeder cells in reprogramming cultures.

(legend continued on next page)
the presence or absence of a Xist RNA cloud demonstrated that nearly all Nanog+ cells carrying the 2lox intron 1 allele (Ad Null-reprogramming cultures) lack a Xist RNA cloud at day 13 of reprogramming (Figures 6C and 6E). Importantly, even in the absence of intron 1 (Ad-Cre samples), Nanog+ cells displayed loss of the Xist RNA cloud (Figures 6C and 6E) and of the X-like H3K27me3 focus (data not shown). Furthermore, from the Ad-Cre-treated reprogramming cultures, 14 iPSC clones were isolated and clonally propagated and all confirmed to have lost both intron 1 and the Xist RNA cloud, demonstrating the efficient deletion of the intronic sequence early in reprogramming (Figure 6F). To ensure that the ability of an intron 1-deleted Xi chromosome to downregulate Xist was not due to intron 1-dependent events occurring within the first 4 days of reprogramming, i.e., prior to Cre-mediated deletion, we also reprogrammed MEFs carrying a germline-transmitted 1lox intron allele. These XX:1lox intron/ΔXist MEFs displayed normal Xist RNA coating before reprogramming (detectable in 95% of the cells) and lost Xist RNA in Nanog+ colonies (Figure 6G). When comparing to XX:2lox intron/ΔXist MEFs, MEFs lacking intron 1 form Nanog+ colonies with similar efficiencies (Figure 6H). Together, these studies rule out that Xist intron 1 is necessary for the downregulation of Xist in reprogramming to pluripotency.

DISCUSSION

In summary, our data argue that Xist intron 1 does not represent an essential tether-coupling repression of both Xist and XCI to the pluripotent state. ESCs lacking intron 1 do not dysregulate Xist expression in the undifferentiated state nor upon in vitro differentiation, reprogramming to the iPSC state leads to Xist repression on a Xi lacking intron 1, and mice lacking intron 1 do not display any of the gross reproductive abnormalities that would be expected if XCI were perturbed.

The deletion of intron 1 represents a clean experimental system to probe the functional role of a genomic element that displays very strong pluripotency transcription factor binding, unhampered by the secondary effects on initiation of XCI associated with global modulation of protein factors implicated in the maintenance of the pluripotent state. Although correlative binding studies were supported in part by Xist dysregulation in ESC lines with inducible deletions of the pluripotency factors Nanog and Oct4, our study cautions against extrapolating these findings to the behavior of WT ESCs and mice. In the case of the ZHBTc4 Oct4-repressable cell line, a compromised pluripotency network may result in Rnf12 upregulation followed by downregulation of the pluripotency factor Rex1, sufficient to trigger XCI in male cells independent of intron 1 (Barakat et al., 2011; Gontan et al., 2012). We also noted that ZHBTc4 ESCs lack pinpoint Tsix signal and draw a corollary between their Xist upregulation and our male ESCs deleted for Tsix (that, when differentiating, have a significantly greater number of Xist clouds upon deletion of intron 1).

In light of the two mild phenotypes (skewing effect of deleting intron 1 in female ESCs heterozygous for the allele and the slight increase in Xist clouds in Tsix and intron 1-deleted differentiating male ESCs), we hypothesize that intron 1 loss leads to mild destabilization of Xist transcriptional repression at the transition to the differentiated state, in the narrow development window of XCI initiation. Unable to capture a transcriptional difference in Xist levels at the onset of in vitro differentiation, we believe that more sensitive methods of transcript quantitation or investigation of chromatin state may address this hypothesis.

We noted a discrepancy between the ex vivo XCI-skewing phenotype and the normally occurring in vivo XCI choice in the absence of the intron. The lack of an intron 1 deletion effect in adult mice and in ESC differentiation induced by bFGF/Activin (Figure S5), which is sensitive to clonogenic skewing of XCI because of serial passage and outgrowth of few cells (unlike monolayer differentiation; Chenoweth and Tesar, 2010), suggests that Xist regulation is more robust in vivo than in vitro in the absence of the intron 1. For instance, slightly different cis-acting elements could be used in vivo and in vitro for regulating Xist expression. Thus, the cell culture-observed favoring of the intron-deleted Xist could not be organismally relevant, or the stochastic developmental nature of XCI could overshadow the effect.

It seems that the regulation of Xist, at the helm of a chromosome-wide program of gene expression, is genetically ensured by a complex multifactor mechanism. The dispensability of intron 1 for repression of Xist may be mouse specific because mice appear to be unique in the functionality of Tsix and also in the sufficiency of Xist activators such as Rnf12 to elicit Xist upregulation: addition of one copy of Rnf12 is sufficient to drive Xist expression in undifferentiated female ESCs (Jonkers et al., 2009). Other eutherians such as bovines and humans, with truncated and likely nonfunctional TSIX, may rely more on intron 1-dependent mechanisms for Xist repression (Chureau et al., 2002). Therefore, evolution of the overlapping Tsix gene and

(C) FISH of starting MEFs before introduction of pMX retrovirus displaying Xist RNA coating (left) and immunostaining/FISH images (right) of representative Nanog+ colonies in reprogramming cultures treated with Ad Null and Ad-Cre, respectively, at day 13 of reprogramming, showing Nanog expression (red), FISH for Xist RNA using a DNA probe (green), and DAPI (blue). Note that Nanog+ cells at this stage of reprogramming display a biallelic pinpoint signal when using the double-stranded DNA probe, which can be attributed to Tsix expression.

(D) Graph summarizing reprogramming efficiency by counting Nanog+ colonies at day 13 of reprogramming, showing Nanog expression (red), FISH for Xist RNA coating in resulting Nanog+ colonies at day 13 of reprogramming. All Nanog+ cells (number is given) on the reprogramming culture coverslip were counted and analyzed for the Xist signal.

(E) Graph showing the percentage of Nanog+ cells without a Xist RNA cloud at day 13 of reprogramming. All Nanog+ cells (number is given) on the reprogramming culture coverslip were counted and analyzed for the Xist signal.

(F) The graph summarizes the percentage of Nanog+ nuclei with and without Xist RNA clouds in individually expanded iPSC clones from Ad Null or Ad-Cre reprogramming cultures (200 nuclei counted for each iPSC line). Genotyping of all iPSC clones confirmed that intron 1 was deleted in all iPSCs expanded from Ad-Cre reprogramming cultures.

(G) MEFs were obtained from XX:1lox intron/ΔXist embryos and reprogrammed with Oct4, Sox2, and Klf4. Immunostaining/FISH images show the presence of normal Xist RNA coating in the starting MEFs and the absence of Xist RNA coating in resulting Nanog+ colonies at day 13 of reprogramming.

(H) Graph showing counts of Nanog+ colonies at day 13 of reprogramming for two different XX:1lox intron/ΔXist MEF preparations (A and B, from different matings) and one XX:2lox intron/ΔXist line that was reprogrammed in parallel.
the network of XCI activators in mice may have become the dominant mechanism in Xist repression.

**EXPERIMENTAL PROCEDURES**

**Generation of Mutant mESCs and Mice**

Xist intron 1 transgenic mice analyzed in Figures 5B–5E were generated from polymorphic X<sup>flk3lox</sup>/Y;lox5/lox mESC line 29, in which a 1.8 kb region of Xist intron 1 was replaced by a floxed neomycin cassette (Barakat et al., 2011). Germine transmission was verified by genotyping for the presence of the neomycin cassette integrated in the intron 1 region of Xist, and XX:lox-neo/WT females were bred to males expressing pCAGGS-Cre, to loop out the selection cassette. Loopout of the selection cassette was verified by PCR on genomic tail-tip-derived DNA. All other intron 1-mutant ESC lines and mice carrying the Plath/Minkovsky alleley were derived from a targeting construct generated by cloning the respective genomic fragments representing the 5′ and 3′ homology regions into the pCRII plasmid vector upon PCR amplification (see Table S1 for list of primers used). The 800 bp of intron sequence with a 5′ loxP site was ligated between a 2.2 kb 5′ homology arm and 3′-2.6 kb homology arm by AgeI/NotI subcloning. A positive-negative CMV-HygroTK selection cassette flanked by loxP sites was inserted into the unique NotI site. A diphertheria toxin gene (PGK-DTA) was inserted into a unique backbone EcoRI site. A total of 40 μg of plasmid was linearized by MluI digestion and electroporated into male ESCs (V6.5 line; F1 between C57BL/6 and 129Sv/Jae) and into female F1 2-1 ESCs carrying the MS2 tag in the final exon of Xist (F1 between C57BL/6 and CAST/Ei) cells cocultured with drug-resistant DR4 MEFs (Jonkers et al., 2008; Tucker et al., 1997). Hygromycin selection (140 μg/ml) was started 1 day after, and clones were screened by SpeI/KpnI digest and both 5′ and 3′ external probes. BmtI digest and 3′ external probe were used to assess allelism of targeting in F1 2-1 clones. Targeting efficiency was 30% in V6.5 and 1% in F1 2-1 cells. Two independent V6.5 and one F1 2-1 clones were expanded, electroporated with pPAC-Cre plasmid, and selected with G418 (300 μg/ml) for 8 days. Southern blot screening was performed with a 5′ probe and Xbal digest for 1lox and SpeI/ KpnI for 2lox clones. All subsequent intron 1 genotyping was performed by PCR. For intron 1/2aix-Stop double-transgenic ESC clones, XX:2lox and XY:1lox V6.5 were targeted with pAA2

**Cell Culture, Differentiation, and Reprogramming Methods**

Experimental Commission and the UCLA Animal Research Committee. Experiments were in accordance with the legislation of the Erasmus MC Animal Experiments (Promega; Table S1) and transferred to hygromycin selection (140 μg/ml) 1 day later. After serial passaging and outgrowth of stable transfecants, 1.0 × 10<sup>5</sup> or 2.0 × 10<sup>5</sup> ESCs were seeded for differentiation with and without (no LIF) RA for 3 and 5 days and harvested along with 2.0 × 10<sup>5</sup> ESCs and measured for luciferase activity with the luciferase assay system (Promega).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes six figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cej.2013.02.018.

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LICENSING INFORMATION

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