A new Xist allele driven by a constitutively active promoter is dominated by Xist locus environment and exhibits the parent-of-origin effects

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The dosage difference of X-linked genes between the sexes in mammals is compensated for by genetically inactivating one or the other X chromosomes in XX females. A noncoding RNA transcribed from the $Xist$ gene at the onset of X-inactivation coats the X chromosome in cis and induces chromosome-wide heterochromatinization. Here, we report a new $Xist$ allele ($Xist^{CAG}$) driven by a CAG promoter, which is known to be constitutively active in many types of cells. The paternal transmission of $Xist^{CAG}$ resulted in the preferential inactivation of the targeted paternal X (Xp) not only in the extraembryonic, but also embryonic lineage, whereas maternal transmission ended with embryonic lethality at the early postimplantation stage with a phenotype that resembled mutant embryos carrying a maternal deficiency in $Tsix$, an antisense negative regulator of $Xist$, in both sexes. Interestingly, we found that the upregulation of $Xist^{CAG}$ in preimplantation embryos temporarily differed depending on its parental origin: its expression started at the 4- to 8-cell stages when paternally inherited, and $Xist^{CAG}$ was upregulated at the blastocyst stage when maternally inherited. This may indicate that the $Xist$ locus on Xp is permissive to transcription, but not on the maternal X (Xm). We extrapolated from these findings that the maternal $Xist$ allele may manifest a chromatin structure inaccessible by transcription factors relative to the paternal allele. This may underlay the mechanism for the maternal repression of $Xist$ at the early cleavage stage when $Tsix$ expression has not yet occurred on Xm.
Among the many long noncoding RNAs (lncRNA) implicated in gene regulation, the X-inactivational specific transcript (Xist) is one of the most extensively studied lncRNAs and its biological significance has been well described (Brockdorff et al., 1992; Brown et al., 1991; Brown et al., 1992; Marahrens et al., 1997; Penny et al., 1996). Xist RNA plays an indispensable role in X chromosome inactivation (X-inactivation) in female mammals, a process by which the dosage difference of X-linked genes between the sexes is compensated for by transcriptionally silencing one of the two X chromosomes during early development (Lyon, 1961). At the onset of X-inactivation, Xist becomes upregulated from one or the other X chromosomes and its lncRNA associates in cis with the X chromosome, from which it originates, to induce chromosome-wide heterochromatinization.

In the mouse, X-inactivation is imprinted in favor of the paternal X (Xp) in the extraembryonic lineages, which gives rise to the placenta and some extraembryonic membranes (Takagi and Sasaki, 1975), whereas it takes place in an essentially random fashion regardless of the parental origin in the embryonic lineage, which gives rise to all tissues of the fetus. Xist becomes monoallelically upregulated on Xp at the 4- to 8-cell stages and this paternal allele-specific expression is maintained in the trophectoderm and primitive endoderm of the blastocyst, both of which belong to extraembryonic lineages. Xist clouds are lost in cells that have contributed to the epiblast lineage of the inner cell mass (ICM), and the previously inactivated Xp becomes transiently reactivated prior to the subsequent inactivation of either X in differentiating epiblast cells (Mak et al., 2004; Okamoto et al., 2004). Reactivation of the inactive X has also been observed in the primordial germ cells (PGCs) of female embryos (Monk and McLaren, 1981), which takes several days across the time they enter meiosis and accompanies the loss of Xist clouds (Sugimoto and Abe, 2007). The inactive X is thought to be reactivated for the proper progression of meiosis, especially for the pairing of two homologous X chromosomes in the prophase of meiosis I (Sugimoto and Abe, 2007). Although the downregulation of Xist has commonly been observed in the ICM and PGCs, to what extent they share the mechanism for reactivation is not clear. Williams et al. previously showed that reactivation of X-linked genes sometimes precedes the loss of Xist clouds in the ICM, which implies that the downregulation of Xist is not necessarily a primary cause of reactivation, at least in the ICM (Williams et al., 2011).
Although the biological significances of X-reactivation and of the accompanying downregulation of \textit{Xist} in the ICM and PGCs have not been fully established, it is assumed that these events are associated with global reprogramming, which resets the previous epigenome in the ICM and PGCs and induces the expression of pluripotency factors including those reported to negatively regulate \textit{Xist}, such as Oct4, Sox2, and Nanog (Navarro et al., 2008). These factors may directly or indirectly downregulate \textit{Xist}. A recent study, however, showed that the deletion of binding sites for these factors present in \textit{Xist} intron 1 did not appear to affect this process in the ICMs (Minkovsky et al., 2013). A series of events in the process of reprogramming in addition to the expression of pluripotency factors may also contribute to the downregulation of \textit{Xist} by causing changes in the chromatin structure of the \textit{Xist} locus or the expression of transcription factors required for continuous \textit{Xist} expression. Although the downregulation of \textit{Xist} may not be a prerequisite for X-reactivation, as suggested by Williams et al. (Williams et al., 2011), its biological significance cannot be addressed until the effect is examined when \textit{Xist} clouds are continuously sustained in the ICMs and PGCs.

In contrast to \textit{Xist}, its antisense negative regulator, known as \textit{Tsix} is only expressed from the maternal X (Xm) from the morula/blastocyst stage until the midgestation stage in cells that maintain imprinted X-inactivation, namely, in the extraembryonic lineages (Lee, 2000; Sado et al., 2001). The targeted disruption of \textit{Tsix}, when maternally inherited, was shown to result in the ectopic upregulation of normally silent maternal \textit{Xist} and subsequent inactivation of Xm in the extraembryonic lineages of both sexes, which demonstrated \textit{Tsix}’s antagonizing effect on \textit{Xist} in cis. Xm was shown to acquire an imprint during the prophase of meiosis I, which rendered Xm resistant against inactivation in the extraembryonic lineages (Goto and Takagi, 1998; Tada et al., 2000; Takagi and Abe, 1990). On the other hand, whether Xp possesses an imprint that facilitates its inactivation in the extraembryonic lineages remains unclear. The maternal imprint by itself may be potentially sufficient enough to drive imprinted X-inactivation in these particular lineages by leaving Xp as a sole X capable of undergoing inactivation. Since Xm carrying the \textit{Tsix} deficiency has lost the ability to resist inactivation in the extraembryonic tissues, \textit{Tsix} expression apparently represents a primary output of the imprint laid on Xm. One can argue against this point of view, however, if the maternal imprint is supposed to be
responsible for the repression of maternal \(Xist\) at the 4- to 8-cell stages as well because maternal \(Tsix\) has not yet been expressed at these stages.

In this study, we generated a new \(Xist\) allele (\(Xist^{\text{CAG}}\)) that was driven by a CAG promoter, which is considered one of the strongest and most ubiquitous promoters (Niwa et al., 1991). We expected that this allele, when paternally inherited, would allow us to unanimously inactivate the targeted Xp in the embryonic lineage by the constitutive expression of \(Xist^{\text{CAG}}\) upon the choice of X-inactivation and to also sustain \(Xist\) expression in the ICM and PGCs. In contrast, when maternally inherited, we expected that this allele would result in a phenocopy of the embryo suffered from the maternal deletion of \(Tsix\) in both sexes. We found that \(Xist^{\text{CAG}}\) behaved as expected, except for the failure to sustain \(Xist\) clouds in the ICM and PGCs. Although the unexpected downregulation of \(Xist^{\text{CAG}}\) in these cells prevented further investigations into the effects of the continuous expression of \(Xist\) in cells undergoing reprogramming and X-reactivation, we identified interesting parental origin specific-differences in the timing \(Xist^{\text{CAG}}\) was upregulated in preimplantation embryos. Its expression started at the 4- to 8-cell stages when paternally inherited, whereas \(Xist^{\text{CAG}}\) was not upregulated until the blastocyst stage when maternally inherited. Here, we discussed what these results may indicate in terms of the differential regulation of \(Xist\) in preimplantation stage embryos.
RESULTS

A new Xist allele driven by a CAG promoter was introduced into the mouse

A targeting vector was constructed to replace the endogenous Xist promoter with a cassette containing a CAG promoter (Niwa et al., 1991), which is known to be active in many types of cells including germ cells (Okabe et al., 1997; Sakai and Miyazaki, 1997), and a floxed selection marker (Fig. S1A). Following homologous recombination in ES cells, those harboring the mutant allele referred to as XistCAG2L were isolated (Fig. S1B). Since the transcription initiated from the CAG promoter could essentially be terminated by the presence of polyadenylation signals located 3’ to the selection marker, we expected that the XistCAG2L allele would not produce functional Xist RNA and would behave as a null allele that was created previously (Marahrens et al., 1997). We therefore crossed the male chimeras with females heterozygous for the Tsix deletion (ΔTsix) (Sado et al., 2001) (see Materials and methods) and successfully obtained female offspring carrying the XistCAG2L allele in combination with the ΔTsix allele (Fig. S1C). They were subsequently crossed with males carrying a Pgk2-cre transgene (Kido et al., 2005), which allowed the expression of cre recombinase specifically in the spermatocyte under the control of the Pgk2 promoter. We expected that males carrying XistCAG2L in combination with Pgk2-cre thus generated would transmit the XistCAG allele to female pups via cre-mediated conversion in the spermatocytes when crossed with wild-type females. As shown in Fig. S1D, we successfully recovered females heterozygous for XistCAG, which demonstrated that spermatogenesis was not compromised by the conversion from XistCAG2L into XistCAG in the spermatocytes. The presence of respective alleles thus introduced into mice was confirmed by Southern blotting (Fig. S1C). Female embryos carrying paternally-derived XistCAG were created as such. A 197 bp portion surrounding the major transcription start site of Xist (chrX: 100,678,537-100,678,733 on mm9) was replaced with the CAG promoter in the XistCAG allele.

The X chromosome carrying the XistCAG allele was selectively inactivated in somatic cells

It was reasonable to expect the X chromosome undergoing inactivation to be confined to the wild-type X in XCAG2LX females and the mutated XCAG in XXCAG females (the maternal X precedes the paternal
one and the same is true hereafter; \( X^{CAG2L} \) and \( X^{CAG} \) refer to Xs carrying \( Xist^{CAG2L} \) and \( Xist^{CAG} \) respectively. However, since RNA expressed from the \( Xist^{CAG} \) allele lacked the first 20 nucleotides present in wild-type \( Xist \) RNA and possessed an additional unrelated sequence derived from the CAG cassette instead, we examined whether these changes affected the function and processing of RNA produced from the \( Xist^{CAG} \) allele. We derived MEFs heterozygous for the respective \( Xist \) mutations using mice with a Robertsonian translocation, \( Rb(X.9)6H \), in which the X chromosome and chromosome 9 were fused at the centromere (\( X^{Rb(X.9)} \)) (Tease and Fisher, 1991). \( X^{CAG2L}X^{Rb(X.9)} \) and \( X^{Rb(X.9)}X^{CAG} \) MEFs thus derived allowed us to easily identify the mutated X by its morphology. We carried out RNA-FISH to examine the expression of \( Xist \) in these mutant MEFs as well as control \( XX^{Rb(X.9)} \) MEFs (Fig. S2A, B). The results demonstrated that while the \( Xist^{CAG2L} \) allele was functionally null and did not produce functional \( Xist \) RNA, the \( Xist^{CAG} \) allele constitutively expressed functional RNA coating the \( X^{CAG} \) in cis.

The nature of \( Xist \) RNA expressed from \( X^{CAG} \) in MEFs was examined by Northern blotting. As shown in Fig. S2C, \( X^{CAG} \) produced apparently the same species of \( Xist \) RNA as the wild-type X, which indicated that there were no aberrant transcripts resulting from unexpected splicing or termination. The expression level of \( Xist \) in \( XX^{CAG} \) was, however, about 10-fold higher than that in \( XX \), which is consistent with the strong activity of the CAG promoter (Fig. S2C). We further compared the stability of the \( Xist \) RNA expressed from \( X^{CAG} \) with that from the wild-type X using \( XX^{CAG} \) and \( XX \) MEFs, and found that the half-life of RNA expressed from the \( Xist^{CAG} \) allele was essentially the same as wild-type \( Xist \) RNA (Fig. S2D).

Since the inactive X is known to replicate late in the S phase of the cell cycle, we examined when replication of the respective X chromosomes occurred in these MEFs. In \( XX^{Rb(X.9)} \) MEFs, the morphologically normal wild-type X replicated late in 58% of cells, whereas \( X^{Rb(X.9)} \) did so in the remaining 42%, consistent with random X-inactivation. In mutant MEFs, on the other hand, the late replicating X was confined to \( X^{Rb(X.9)} \) in \( X^{CAG2L}X^{Rb(X.9)} \) MEFs, and to \( X^{CAG} \) in \( X^{Rb(X.9)}X^{CAG} \) MEFs (Fig. S2E, F). These results demonstrated that both mutations caused a skewing of inactivation in favor of the wild-type X in \( X^{CAG2L}X \) and the mutated X in \( XX^{CAG} \).
We further examined gene expression on $X^{CAG2L}$ and $X^{CAG}$ in MEFs prepared from F1 fetuses of the JF1 strain and respective mutant mice. These MEFs allowed us to study the allelic expression of X-linked genes and the result demonstrated that the genes on $X^{CAG2L}$ were active, whereas those on $X^{CAG}$ had been uniformly inactivated in the respective mutant MEFs (Fig. S2G). Taking all these results together, we concluded that both of the mutant alleles exerted the expected effect on the inactivation pattern of the X chromosome; the $Xist^{CAG2L}$ allele rendered $X^{CAG2L}$ incompetent to undergo inactivation, while the $Xist^{CAG}$ allele constitutively produced functional RNA to silence the chromosome in cis.

**Maternal transmission of $Xist^{CAG}$ recapitulated the phenotype seen upon maternal transmission of the *Tsix* mutation**

Selective inactivation of a genetically manipulated X chromosome was shown to occur in the somatic cells of females heterozygous for $X^{\Delta Tsix}$ of a paternal origin. While these females were apparently normal, both male and female embryos conceived by them died in utero when $X^{\Delta Tsix}$ was transmitted due to the ectopic expression of maternal $Xist$ from the mutated $X^{\Delta Tsix}$ in the extraembryonic tissues after implantation (Lee, 2000; Sado et al., 2001). We set out crosses between XX$^{CAG}$ females and wild-type C57Bl/6 males to see if $Xist^{CAG}$ behaved in a manner similar to the *Tsix*-deficient allele upon maternal transmission. Although XX$^{CAG}$ females gave birth to healthy pups of both sexes, $X^{CAG}$ had been barely transmitted to them, which suggested that most of the embryos that had inherited $Xist^{CAG}$ died in utero (Fig. 1A). In a single female survivor with maternal $Xist^{CAG}$, it is possible that the imprinted Xp inactivation in the extraembryonic tissues was somehow reversed by shutting off paternal $Xist$ at the early postimplantation stage, allowing the embryo to survive despite the expression of maternal $Xist^{CAG}$. When embryos were dissected out at E7.5 from XX$^{CAG}$ females, both $X^{CAG}$ and $X^{CAG} Y$ embryos, although found at the expected ratio, were stunted with abnormal morphology, which was reminiscent of embryos carrying maternal $X^{\Delta Tsix}$ (Fig. 1A, B). The distal part of these embryos, which was enriched with the embryonic ectoderm, and their trophoblast were subsequently examined for $Xist$ expression by RNA-FISH. $X^{CAG} X$ embryos were manifested by single $Xist$ clouds in the
embryonic ectoderm, but by two clouds in a large proportion of the nuclei in the trophoblast (Fig. 1C, 2B). The single clouds in the former were most probably derived from maternal $X_{ist}^{CAG}$. In $X_{CAG}^XY$ embryos, single $X_{ist}$ clouds were found in most of the nuclei in both tissues (Fig. 1C, 2B). These results demonstrated that $X_{CAG}$, when maternally inherited, recapitulated the behavior of the X deficient for $T_{six}$. It is likely that the maternal transmission of $X_{ist}^{CAG}$ caused embryonic lethality due to functional nullisomy of the X chromosome in the extraembryonic lineage of $X_{CAG}^X$ embryos and in both embryonic and extraembryonic lineages of $X_{CAG}^Y$ embryos.

**Maternal $X_{ist}^{CAG}$ was not upregulated until the periimplantation stage**

$X_{CAG}^X$ and $X_{CAG}^Y$ embryos, although stunted, apparently had undergone gastrulation. We reasoned that if maternal $X_{CAG}$ became silenced during the preimplantation stages, these mutants would have suffered from the functional nullisomy of the X chromosome and would not have survived until the gastrulation stage. To investigate when maternal $X_{ist}^{CAG}$ became upregulated and formed the cloud in $X_{CAG}^X$ and $X_{CAG}^Y$ embryos during development, we carried out RNA-FISH using embryos recovered at earlier stages from $XX_{CAG}$ females crossed with wild-type males. The sex of each embryo was subsequently identified by Y chromosome painting (the results of Y-painting was noted visually after staining, but were not recorded as image data). $X_{ist}$ expression was examined in 15 female and 15 male 8-cell stage embryos recovered at E2.5. We found that while the nuclei of one class of female embryos contained only a single large $X_{ist}$ cloud, those of the other class contained very faint scattered signals in addition to a large $X_{ist}$ cloud (Fig. 2 A, B, and Fig.S3). Similarly, the nuclei of a half of the male embryos were negative for $X_{ist}$, those of the remaining half contained very faint scattered signals (Fig. 2 A, B, and Fig.S3). Although we could not verify the genotype of the embryos, we suspected that the very faint scattered signals represented very weak expression of $X_{ist}^{CAG}$, which contrasted well with the intense signal of the $X_{ist}$ cloud in females. When E3.5 blastocysts were examined, a small subset of the nuclei in 3 of 7 females and 6 of 11 males contained two and one $X_{ist}$ cloud(s), respectively (Fig. 2A, B). It would be reasonable to assume that they represented $X_{CAG}^X$ and $X_{CAG}^Y$ embryos, respectively. It is, therefore, likely that maternal $X_{ist}^{CAG}$ had not been fully upregulated in
the majority of the blastomeres at this stage. This contrasted well with female blastocysts carrying \textit{Xist}^{CAG} on Xp (XX\textsuperscript{CAG}), which exhibited clear single \textit{Xist} clouds in a large proportion of the nuclei (Fig. 3A). In fact, \textit{Xist}^{CAG} in every blastomere of XX\textsuperscript{CAG} embryos (n=3) had been upregulated in the same manner as \textit{Xist} on Xp in wild-type female embryos by the 8-cell stage (Fig. 3A). The prevalence of the nuclei containing an extra \textit{Xist} cloud, although increased to some extent, was still as low as 20\% in presumptive X\textsuperscript{CAG}X and X\textsuperscript{CAG}Y embryos recovered at E4.5, but reached a level comparable to that seen in the embryonic and extraembryonic tissues of E7.5 mutant embryos by E6.5 (Fig. 2B). These results demonstrated that the timing by which \textit{Xist}^{CAG} became upregulated differed depending on its parental origin and, when maternally inherited, it started to be expressed at around the blastocyst stage despite the allele being under the control of the CAG promoter. It is likely that ectopic inactivation of Xm did not take place until around the time of implantation in X\textsuperscript{CAG}X and X\textsuperscript{CAG}Y embryos. This is again reminiscent of the timing when Xm carrying the \textit{Tsix} deficiency started to be ectopically inactivated (Sado et al., 2001).

\textbf{\textit{Xist}^{CAG} on the paternal X was downregulated in the inner cell mass}

Paternal \textit{Xist} upregulated in every blastomere of the female embryos during the early cleavage stages was shown to be silenced in cells contributing to a part of the ICM (Mak et al., 2004; Okamoto et al., 2004). This accompanied transient reactivation of the hitherto inactive Xp prior to the onset of random X-inactivation taking place in the epiblast lineage. It was of interest whether the expression of \textit{Xist}^{CAG} inherited from the father, which was initiated at around the 4- to 8-cell stages, was sustained in cells allocated to the ICM in view of understanding the biological significance of \textit{Xist} downregulation in Xp reactivation and the reprogramming of cells in the ICM. Accordingly, we carried out RNA-FISH for \textit{Xist} expression in combination with immunostaining for ICM lineage-specific markers, either Nanog or Oct3/4, using blastocysts recovered from wild-type females crossed with [X\textsuperscript{CAG2L}; P\textit{gk2-cre}] males in comparison with wild-type. Among the cells positive for Nanog, about 10\% had already lost \textit{Xist} clouds in E3.5 wild-type blastocysts. Contrary to our expectation, the proportion of those negative for \textit{Xist} were comparable in XX\textsuperscript{CAG} blastocysts recovered at E3.5 (Fig. 3B, C). When
examined at E4.5, the population of cells negative for Xist RNA had become more than 40% of those positive for Oct3/4 in both cases (Fig. 3B, C). It was likely, therefore, that Xist<sup>CAG</sup> upregulated by the 8-cell stage failed to sustain its expression in a subset of ICM cells. These results suggested that paternally derived Xist<sup>CAG</sup>, although driven by the CAG promoter, was downregulated in the epiblast lineage in a manner similar to that in wild-type. Although unexpected silencing of Xist<sup>CAG</sup> precluded a further analysis for the effect of the continuous association of Xist RNA with Xp on X-reactivation and the reprogramming in the ICM, it raised an interesting possibility that a mechanism involved in the repression of the endogenous Xist promoter on Xp in the epiblast lineage might also be effective on the CAG promoter.

The expression of Xist<sup>CAG</sup> in PGCs was not sustained throughout oogenesis in X<sup>CAG-X</sup> fetuses

Reactivation of the inactive X also takes place in PGCs around the time when PGCs enter meiosis. Xist clouds are known to be lost in PGCs by E10.5 prior to reactivation of the inactive X (de Napoles et al., 2007; Sugimoto and Abe, 2007). In XX<sup>CAG</sup> female embryos, X<sup>CAG</sup> should have been uniformly inactivated in every cell including PGCs by the constitutive expression of Xist<sup>CAG</sup>. Gonads isolated from E10.5, 13.5, and 16.5 embryos were trypsinized and the cell suspension containing germ cells and the surrounding somatic cells was cytospon onto a slide for RNA-FISH. A GFP transgene driven by the Oct3/4 promoter (Yoshimizu et al., 1999) was introduced into the embryos so that germ cells could be identified by immunostaining with an antibody against GFP. As shown in Fig. 4A and 4B, Xist RNA disappeared in most of the wild-type PGCs by E10.5, which is consistent with previous studies. In contrast, more than 70% of PGCs still retained Xist RNA on X<sup>CAG</sup>, although this was lower than that of the surrounding somatic cells, in XX<sup>CAG</sup> embryos at E10.5 (Fig. 4A, B). Although we expected that Xist RNA may remain on X<sup>CAG</sup> in germ cells throughout development, the population of germ cells positive for Xist RNA declined over time to 20% at E13.5 and 0.4% at E16.5 in XX<sup>CAG</sup> embryos. Since a significant difference in the number of germ cells was not observed between wild-type and XX<sup>CAG</sup> embryos at each stage examined, the reduction in the number of germ cells positive for Xist RNA was unlikely due to elimination by cell death, but was attributable to the downregulation
of $Xist^{CAG}$. We further carried out qRT-PCR to examine the expression levels of $Xist$ RNA in PGCs isolated from E13.5 XX and $XX^{CAG}$ gonads. As shown in Fig. 4C, the relative abundance of $Xist$ RNA stably present in PGCs was markedly lower than that in the surrounding somatic cells of the gonads in both XX and $XX^{CAG}$ fetuses. This suggests that the absence of $Xist$ clouds in RNA-FISH experiments was not due to a loss of localization to the inactive X. As was the case in the ICM, a mechanism that silences the endogenous $Xist$ promoter in PGCs appeared to be effective on the CAG promoter.

**The CAG promoter was not methylated in PGCs that had lost $Xist^{CAG}$ expression**

It was unexpected that the CAG promoter could not sustain $Xist^{CAG}$ expression in the ICM and PGCs, given the fact that many transgenes driven by the CAG promoter are expressed in the ICM and PGCs. Since the unexpected silencing of transgenes is often associated with promoter methylation, we carried out bisulfite sequencing to examine the methylation levels of the 16 and 59 CpG sites in the endogenous $Xist$ promoter and CAG promoter, respectively, in E13.5 PGCs isolated from XX and $XX^{CAG}$ gonads by FACS using the GFP transgene driven by the Oct3/4 promoter. In the gonads of XX females, the $Xist$ promoter was methylated at about 40% in somatic cells surrounding PGCs, consistent with the $Xist$ promoter in somatic cells being methylated on the active X and unmethylated on the inactive X (Fig. 5A) (McDonald et al., 1998; Norris et al., 1994). Interestingly, this region was essentially unmethylated in PGCs, which suggests the possibility that $Xist$ was downregulated in PGCs by either a mechanism independent of promoter methylation if at the transcriptional level or posttranscriptional processing of $Xist$ RNA.

The $Xist$ promoter on the wild-type X was markedly methylated in the gonads of $XX^{CAG}$ females, while the CAG promoter on $X^{CAG}$ was unmethylated in somatic cells (Fig. 5B). Interestingly, as was the case in XX germ cells, the endogenous $Xist$ promoter on the wild-type X and the CAG promoter on $X^{CAG}$ were both essentially unmethylated in $XX^{CAG}$ PGCs. This suggests that the eventual downregulation of the $Xist^{CAG}$ allele in $XX^{CAG}$ PGCs was not mediated by promoter methylation. As was the case in the ICM, the $Xist^{CAG}$ allele appeared to be repressed in the same manner as endogenous $Xist$ in the PGCs of wild-type females.
DISCUSSION

The reversal of the inactive X chromosome is observed in two different phases in the mouse life cycle. It occurs in cells that have contributed to the ICM in the blastocyst and also in PGCs around the time when they enter meiosis; however, whether the underlying mechanism is common between these cell types is unknown. Since reactivation in both cases accompanied the downregulation of \( Xist \), it has been presumed that the loss of \( Xist \) RNA may be causally involved in reactivation. A previous study by Williams et al., however, showed that the reactivation of genes on the inactivated Xp sometimes preceded the disappearance of an \( Xist \) cloud in a subset of cells in the ICM, which suggested that \( X \)-reactivation was not necessarily coupled with the loss of \( Xist \) RNA in the ICM. We assumed that the \( Xist^{CAG} \) allele would allow us to address the effect of the continuous association of \( Xist \) RNA with the X chromosome on \( X \)-reactivation and the subsequent reprogramming if its expression was sustained in the ICM and PGCs. The results were as expected in somatic cells and its phenotype upon maternal transmission mimicked the phenotype caused by a maternal deficiency in \( Tsix \); however, cells in neither the ICM nor PGCs sustained an \( Xist \) cloud formed by the expression of \( Xist^{CAG} \). Although this was unexpected given the fact that the \( Xist^{CAG} \) allele was driven by the CAG promoter, which is known to be active in many types of cells including those of the ICM and PGCs, these results may imply that even the heterologous CAG promoter, when placed in the context of the \( Xist \) locus, comes to be under the control of the same regulatory mechanism as the endogenous \( Xist \) promoter. Given the fact that the CAG promoter has been replaced with a 197 bp region surrounding the major transcription start site of \( Xist \), it is likely that the element critical for the presumed regulatory mechanism for the \( Xist \) promoter resides outside the replaced region. It would be important to note that both male and female ES cells carrying \( Xist^{CAG} \) could be efficiently derived from blastocysts and \( Xist^{CAG} \) in such ES cells became upregulated to form an \( Xist \) cloud only upon induction of differentiation (Fig. S4), further suggesting that the CAG promoter behaved in a way similar to the endogenous \( Xist \) promoter.

While the transcription of \( Xist \) has been correlated with the methylation status of CpG sites in the \( Xist \) promoter in somatic cells (Norris et al., 1994), DNA methylation does not seem to be involved
in the repression of Xist at the blastocyst stage as the Xist promoter is essentially unmethylated in blastocysts and isolated ICM cells (McDonald et al., 1998). This also appeared to be the case in PGCs as the Xist promoter in E13.5 PGCs, in which an Xist cloud essentially disappeared by E10.5, was largely devoid of DNA methylation. It is tempting to speculate that the presumptive regulatory mechanism, which makes the Xist promoter transcriptionally inert in the ICM and PGCs in a manner independent of DNA methylation, also effectively operates upon the CAG promoter residing at the Xist locus and downregulates Xist<sup>CAG</sup> in the ICM and PGCs of XX<sup>CAG</sup> embryos. Among the mutated Xist alleles so far introduced into the mouse, that reported by Nesterova et al. (Nesterova et al., 2003), which causes skewed X-inactivation due to selective expression of the targeted alleles, and that reported by Sevarese et al. (Savarese et al., 2006), which is driven by a tetracyclin-inducible promoter, would be of interest to see the behavior of their heterologous promoter at the Xist locus in the ICM and PGCs. The latter allele can be upregulated in undifferentiated ES cells if doxycycline (dox) is administered, whereas the Xist<sup>CAG</sup> allele is not as long as the cells are maintained in undifferentiated state. The presence of abundant transactivator in the dox-inducible cells may explain the difference in the action between these two alleles in undifferentiated ES cells. Nonetheless, it should be mentioned that we could not formally rule out the possibility that Xist clouds were lost in the ICM and PGCs due to an alternative mechanism that abolishes the stability of Xist RNA rather than transcriptional repression.

This study also provided another important implication for the asymmetric expression of Xist between Xp and Xm in preimplantation embryos. The timing by which Xist<sup>CAG</sup> became upregulated was significantly different depending on its parental origin. Upon paternal transmission, a single Xist cloud derived from Xist<sup>CAG</sup> became evident by the 8-cell stage, which was similar to wild-type XX embryos, whereas an extra Xist cloud originating from maternally inherited Xist<sup>CAG</sup> in X<sup>CAG</sup>X and X<sup>CAG</sup>Y embryos appeared in a subset of cells at the blastocyst stage. This may be attributed to the difference in the chromatin structure at the Xist locus between Xp and Xm derived from a spermatozoon and oocyte, respectively, in the early cleavage stages of embryos. Since the paternal genome undergoes global remodeling of the chromatin soon after fertilization, it may be reasonable to
assume that this paternal genome-specific event rendered the \textit{Xist} locus on \textit{Xp} more permissive to transcription than that on \textit{Xm}. If the chromatin structure of the maternal \textit{Xist} locus needs further cell division to become similar to that of the paternal locus, this would account for the lag in the timing of maternal \textit{Xist}^{CAG} being upregulated relative to paternal \textit{Xist}^{CAG}. Such a difference in the chromatin structure, if any, may also be relevant to the monoallelic upregulation of \textit{Xist} in wild-type female embryos in favor of the paternal allele during preimplantation development (Sado and Sakaguchi, 2013). In other words, the maternal \textit{Xist} allele was repressed during early cleavage stages because it was not yet ready for transcription due to the compact chromatin structure inaccessible by transcription factors. Given that the very weak scattered signals of \textit{Xist} were observed from maternally inherited \textit{Xist}^{CAG}, one might raise an alternative possibility that the maternal X chromosome is incompetent for \textit{Xist}-coating at the early preimplantation stage. \textit{Xist}-coating detected in parthenogenetically activated embryos, however, argue against this. Recently, Fukuda et al. demonstrated that overexpression of Kdm4b, a histone demethylase for H3K9me3, can induce upregulation of \textit{Xist} from the 4-cell stage in parthenogenetically activated embryos (Fukuda et al., 2014). This suggests, in fact, that global demethylation of H3K9me3 and subsequent alteration in the chromatin structure facilitate derepression of \textit{Xist} on \textit{Xm} otherwise repressed. This is apparently consistent with the idea that maternal \textit{Xist} is repressed in the early preimplantation embryo primarily due to the chromatin structure impermissive for transcription. An earlier work demonstrated that \textit{Xist} expression occurs at the morula/blastocyst stage even in parthenogenones or gynogenones (Kay et al., 1994; Matsui et al., 2001; Nesterova et al., 2001). Although it was originally suggested that this represented erasure of the maternal imprint responsible for \textit{Xist} silencing by the morula/blastocyst stage, it could be also explained as a result of several rounds of replication or cell division, which could have made the chromatin structure at the maternal \textit{Xist} locus transcriptionally permissive.

It has been well accepted that an imprint laid on \textit{Xm} during the prophase of meiosis I makes \textit{Xm} resistant to inactivation in extraembryonic lineages (Goto and Takagi, 1998; Tada et al., 2000). Since a maternal deficiency in \textit{Tsix} leads to the ectopic expression of maternal \textit{Xist} in cis and the subsequent inactivation of \textit{Xm} in extraembryonic lineages (Lee, 2000; Sado et al., 2001), the
monoallelic expression of maternal Tsix appears to be, at least in part, responsible for the output of the imprint laid on Xm. On the other hand, one can argue against this, given that maternal Xist is repressed at the early preimplantation stages, in which Tsix has not yet been expressed. Our findings, however, suggest that the reason why the maternal Xist\textsuperscript{CAG} allele is less effectively transcribed than the paternal one at the early preimplantation stage may be because the chromatin state or structure of the Xist locus on Xm at that time is not ready for robust transcription. If maternal Xist is repressed simply due to the chromatin structure being inaccessible to transcription factors, one may not need to assume a germline imprint that prevents the upregulation of maternal Xist during the early cleavage stages. In other words, whether or not the maternal Xist is transcribed at the early preimplantation stage may not be the matter of the conventional imprint such as differential methylation but rather simply of the chromatin state or structure. Since Tsix starts to be monoallelically expressed from Xm at around the morula/blastocyst stages, Tsix would be able to block upregulation of maternal Xist thereafter if the presumptive transcriptionally incompetent chromatin structure at the Xist locus on Xm lasts for a few rounds of cleavage until the morula stage. It should be noted that the transcription of Xist\textsuperscript{CAG} appears to dominate the repressive effect of Tsix on Xm as Xist\textsuperscript{CAG} is upregulated from the blastocyst stage onward.
MATERIALS AND METHODS

Generation of mice carrying $X_{ist}^{CAG}$

The targeting vector, pCAG-ΔM20, was constructed as described in Supplemental Materials and Methods, and introduced into J1 ES cells (Li et al., 1992). Of the 464 colonies picked up, 11 harbored the expected homologous recombination ($X_{ist}^{CAG2L}$). Chimeric males were crossed with females heterozygous for the $Tsix$-deficient X (XX$^{ΔTsix}$) (Sado et al., 2001) to facilitate the transmission of Xp carrying the $X_{ist}^{CAG2L}$ allele ($X^{CAG2L}$) to female offspring as previously shown (Hoki et al., 2009; Ohhata et al., 2008; Sado et al., 2005; Sado et al., 2006). Females thus generated (X$^{ΔTsix}$X$^{CAG2L}$) were crossed with wild-type males to obtain X$^{CAG2L}$X and X$^{CAG2L}$Y. The floxed puromycin-resistance gene was removed through the male germline using a $Pgk2$-cre transgene to derive females heterozygous for $X_{ist}^{CAG}$ (XX$^{CAG}$). All mice were maintained and used in accordance with the Guidelines for the Care and Use of Laboratory Animals of Kinki University (KDAS-26-0006).

RNA-FISH and chromosome painting

A DNA probe for RNA-FISH was prepared from 1μg of pR97E1 (Sado et al., 1996), a plasmid containing an $X_{ist}$ cDNA fragment, by nick translation using the Nick Translation Kit (Abbott) and either Cy3-dUTP (GE Healthcare) or green-dUTP (Abbott).

Chromosome spreads of MEFs prepared by a standard air-dry method, and cytological preparations of blastocysts and postimplantation embryos were made as described by Okamoto et al. (Okamoto et al., 2000) and Takagi et al. (Takagi et al., 1982), respectively. RNA-FISH was performed as described previously (Sado et al., 2001). Following examination of the $X_{ist}$ signal, chromosome painting was carried out to distinguish the morphologically normal X and X$^{Rb(X, 9)}$ (Tease and Fisher, 1991) using painting probes for the chromosome X and chromosome 9 (Cambio) according to the manufacturer's instructions. The gender of blastocysts was identified using a painting probe for the Y chromosome after RNA-FISH.
Analysis of replication timing

Cells were incorporated with BrdU for 8 hours and chromosome spreads were subsequently prepared by a standard air-drying method for staining with Acridine Orange. The late replicating inactive X chromosome was identified as a pale chromosome by this method.

RNA half-life assay

MEFs were seeded onto dishes and cultured overnight. The medium was replaced with that containing 50 μg/ml of DRB (5,6-Dichloro-1-β-D-ribofuranosyl benzimidazole, Calbiochem) on the following day and cells were collected every 3 hours and lysed in ISOGEN (Wako). Five-hundred nanograms of total RNA isolated at each time point was converted into cDNA as described below. Real-time PCR was carried out for the quantification of Xist RNA at each time point using primers XistEx7F21 and XistEx7R20. The level of Xist RNA was normalized relative to that of 18S ribosomal RNA amplified using primers18SRNA1 and 18SRNA2. The abundance of the respective RNA at each time point was shown as a value relative to the abundance at 3 hours after the addition of DRB.

Allelic expression analysis by RT-PCR

Following the DNase I treatment, RNA prepared from MEFs was converted into cDNA using the Prime Script 1st strand cDNA Synthesis Kit (Takara) with random hexamer as a primer. PCR was carried out using primers specific for each of the X-linked genes, and amplified products were subsequently digested with appropriate restriction enzymes as described (Kalantry et al., 2009; Sugimoto and Abe, 2007).

Immuno-FISH

Blastocysts recovered at E3.5 and E4.5 were fixed in 4% paraformaldehyde containing 0.1% of Triton X-100 for 10 min and subjected to hybridization with an Xist probe overnight. Following serial washes, embryos were postfixied with 4% paraformaldehyde and subjected to immunostainig using antibodies
against either Nanog (Yamaguchi et al., 2009) or Oct3/4 (N-19) (Santa Cruz, sc8628), which were visualized by secondary antibodies, Alexa488-anti-rabbit IgG for Nanog or Alexa488-anti-goat IgG for Oct3/4.

Gonads isolated from E10.5, E13.5, and E16.5 female fetuses carrying an Oct3/4-GFP transgene (Yoshimizu et al., 1999) were trypsinized and the resultant suspension was subjected to a hypotonic treatment in 75 mM KCl for 10 minutes and fixed in 2% paraformaldehyde for 10 minutes on ice. Cells were cytopun for 10 minutes at 800 rpm (Cytospin 2, Shandon), permeabilized with 0.5% Triton-X100 in PBS for 5 minutes, and washed with PBS for 5 minutes. Following hybridization with an Xist probe and washing, cells were stained with an anti-GFP rat monoclonal antibody (Nacalai, 04404-26) and an Alexa488-conjugated anti-rat IgG goat antibody. Images were captured using either an Andor iXon™ CCD camera or an Olympus DP70 color CCD camera.

**Bisulfite sequencing**

Genomic DNA prepared from PGCs at E13.5 was treated with bisulfite and purified using the Bisulfast kit (TOYOBO) and EZ kit (Zymo Research), respectively. Two-round PCR was carried out using semi-nested primers. First round PCR was carried out for 25 cycles with 8 ng of DNA using primers Bx-CAG1113F and Bx-CAG1686R for the CAG promoter, and XistPr2 and XistPr3 for the Xist promoter. Second round PCR was performed for 25-30 cycles with one-twentieth of the reaction from first round PCR using primers Bx-CAG1113F and Bx-CAG1646R for the CAG promoter, and XistPr2 and XistPr4 for the Xist promoter. The PCR products were subsequently cloned into the pGEM-Teasy vector (Promega) and sequenced.

**Primers**

Primer sequences used in this study are described in Supplemental Materials and Methods.
Acknowledgments
We would like to thank Hitoshi Niwa for providing the plasmid containing the CAG promoter. We would also like to thank Hirosuke Shiura for technical advice on immune-RNA-FISH of blastocysts, Takashi Tada for a kind gift of an anti-Nanog antibody, and Minako Kanbayashi and Michiko Arii for maintaining of the mouse colonies.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Y. S. and Y. A. performed the experiments with the assistance of Y. H., analyzed the data, and edited the manuscript; H. S. and T. F. analyzed the data; S. A. and S. S. generated Pgk2-cre mice; T. S. designed the study, performed experiments and wrote the manuscript.

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REFERENCES


chromosome inactivation and reactivation in vitro and in vivo. Cell reports 3, 905-918.


Figures

A

\[ XX^{CAG} \times XY \]

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<tr>
<th></th>
<th>XX</th>
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<td>3-week</td>
<td>(46.4%)</td>
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<tr>
<td></td>
<td>(20.4%)</td>
<td>(26.5%)</td>
<td>(28.6%)</td>
<td>(24.5%)</td>
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B

C

Embryonic:

Extraembryonic:
Fig. 1. The phenotype of the embryos carrying maternal \textit{Xist}^{CAG} mimics the phenotype of the mutant embryos carrying a maternal deficiency in \textit{Tsix}.

(A) The number of pups and E7.5 embryos recovered from a cross between \textit{Xist}^{CAG} females and wild-type males. \textit{Xist}^{CAG}, although found at the Mendelian ratio when examined at E7.5, was transmitted to few live pups from the mother. (B) The gross morphology of E7.5 embryos recovered from \textit{Xist}^{CAG} females sired by wild-type males. The genotype of each embryo is indicated. (C) \textit{Xist} expression detected by RNA-FISH (green) in the embryonic and extraembryonic tissues (trophoblast) of XX, \textit{Xist}^{CAG}X, and \textit{Xist}^{CAG}Y embryos recovered at E7.5.
Fig. 2. The upregulation of Xist$^{CAG}$, when maternally inherited, was delayed relative to when it was paternally inherited in preimplantation embryos.

(A) Xist expression detected by RNA-FISH (green) in XX, XY, X$^{CAG}$X, and X$^{CAG}$Y blastocysts. Nuclei were counterstained with DAPI. Arrowheads indicate blastomeres with ectopic expression of Xist, that is, two clouds in females and one in males. (B) The percentage of nuclei showing the mono- and biallelic expression of Xist in the respective stages of embryos. n indicates the number of embryos.
examined. The number of nuclei examined in each stage is shown above each bar. The embryonic part (emb) and trophoblast (troph) were examined separately in E6.5 and E7.5 embryos. Genotype of E2.5-E4.5 was deduced based on the expression pattern of $Xist$ and that of E6.5 and E7.5 embryos was determined by PCR using a piece of the tissues between the embryonic ectoderm and trophoblast.
A
8-cell

B

XX

E3.5

Xist RNA

Nanog

Oct3/4

Hoechst

merge

E4.5

XX

E3.5

E4.5

XX

E3.5

E4.5

E3.5

E4.5
Fig. 3. *Xist* on the paternal X was downregulated in the inner cell mass

(A) *Xist* expression detected by RNA-FISH in an XX\textsuperscript{CAG} embryo at the 8-cell stage. (B) Immuno-RNA-FISH detecting *Xist* and ICM lineage markers, either Nanog or Oct3/4, in XX and XX\textsuperscript{CAG} blastocysts recovered at E3.5 and E4.5. (C) Graphs show the percentage of nuclei positive and negative for the *Xist* cloud among cells positive for Nanog (E3.5) or Oct3/4 (E4.5) in XX and XX\textsuperscript{CAG} blastocysts recovered at E3.5 and E4.5. The number of nuclei examined are shown below the graph legend.
Fig. 4. Prolonged expression of Xist in the germ cells of XX\textsuperscript{CAG} females

(A) Xist expression detected by immuno-FISH in the fetal gonads. Xist RNA (red) was detected by RNA-FISH in the gonads of XX and XX\textsuperscript{CAG} females (E10.5-16.5). Germ cells were identified by the
expression of an Oct3/4-GFP transgene, which was specifically expressed in germ cells. The nucleus indicated by an arrow in XX^{CAG} (E10.5 and E13.5) is shown at a higher magnification on the right. Xist coating was evident in these germ cell nuclei. DNA was counterstained with DAPI (blue). Germ cells were marked with dotted circles. (B) Summary of the immuno-FISH analysis in germ cells. The graph shows the prevalence of germ cells positive for Xist RNA. An asterisk indicates a significant difference (t-test, p<0.01). Error bars represent standard deviations. The numbers of cells examined were shown below the graph: blue, XX; red, XX^{CAG}. (C) qRT-PCR analysis of Xist expression in PGCs isolated from the surrounding somatic cells of E13.5 XX and XX^{CAG} gonads by FACS.
Fig. 5. Methylation levels of the Xist and CAG promoters in PGCs

The methylation status of the 16 and 59 CpG sites present in the 5' region of the endogenous Xist (A) and CAG promoters (B), respectively, was analyzed in PGCs recovered from XX and XX\textsuperscript{CAG} fetuses at E13.5 by bisulfate sequencing. PGCs isolated by FACS were used for the assay.
Supplementary Materials and Methods

Construction of the targeting vector

Construction of the targeting vector, pCAG-ΔM20, and the generation of mice carrying the *Xist* CAG2L allele were carried out as follows: a 4.6 kb EcoRI-BamHI fragment present in exon1 of the *Xist* gene was cloned into pBluescriptII SK(-), in which SalI and XhoI sites had been destroyed by double digestion and subsequent ligation of the compatible ends. A 4.4-kb EcoRI genomic fragment containing the major transcription start site of *Xist* was subsequently inserted at the EcoRI site of this plasmid in an appropriate orientation to derive pΔXS-EB9.0. PCR was carried out on genomic DNA using Mlu-Sal-21F(+)37 and AS1634F as primers, and a product of about 900 bp was digested with MluI and XhoI and used to replace the endogenous MluI-XhoI fragment present in pΔXS-EB9.0 to generate pΔM20. In parallel, pCAG-CY NotI (a gift from Hitoshi Niwa), which has a floxed pac-ECFP-pA cassette downstream of the CAG promoter, was digested with NotI to release a 4.0-kb fragment containing the CAG promoter and the floxed cassette. Following the addition of a SalI linker at both ends, this fragment was cloned at the SalI site in pΔM20 in an appropriate orientation to generate pCAG-ΔM20.
Preparation of MEFs

$X^{CAG2L}X^{Rb(X.9)}$ and $X^{CAG2L}X^{JF1}$ fetuses were obtained at E13.5 from $X^{CAG2L}X$ females crossed with either $X^{Rb(X.9)}Y$ or JF1 males. $X^{Rb(X.9)}X^{CAG}$ and $X^{JF1}X^{CAG}$ fetuses were obtained at E13.5 by crossing $[X^{CAG2L}Y; Pgk2-cre]$ males with either $X^{Rb(X.9)}X^{Rb(X.9)}$ or JF1 females. Following a removal of the head and internal organs, the carcass of each fetus placed in a 100-mm dish was minced into vary small pieces through an 18G hypodermic needle attached to a 2.5-ml syringe in 500 ul of PBS and cultured in DMEM/10% FBS for several days and passaged for expansion.

**Primer sequences**

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<th>Primer</th>
<th>Sequence</th>
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<td>5'-GGC TAA CTG GCT CGA GAA TA-3'</td>
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<td>5'-CTC CAA TTT CTG GGC TCA AG-3'</td>
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<td>Bx-CAG1113F</td>
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<tr>
<td>Bx-CAG1686R</td>
<td>5'-TTG TTT AGG AGT TGT AGG AAA AAG AAG AAG-3'</td>
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<tr>
<td>Bx-CAG1646R</td>
<td>5'-GTA TGA ATA TGG TTA GTA GAG GTT TTA GAG-3'</td>
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<td>5'-AAA TAT TCC CCC AAA ACT CCT TAA ATA A-3'</td>
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<tr>
<td>XistPr3</td>
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<tr>
<td>XistPr4</td>
<td>5'-GGT TTG TTT AAG TAG AAG ATA TAT TGA AAT-3'</td>
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Fig. S1. Introduction of a constitutively active allele of Xist into the mouse

(A) the targeting scheme for generating the XistCAG allele. The endogenous Xist promoter was replaced with a CAG-Pac ECFP-pA cassette to produce the XistCAG2L allele, which would not produce Xist RNA. In the presence of cre recombinase, the XistCAG2L allele should be converted into the XistCAG allele, which in turn expresses Xist constitutively. The locations of the probes (PE0.24, BE0.6, and ex2) used for Southern blotting in B and C are indicated. H, HindIII; Bg, BglII; B, BamHI; R, EcoRI; M, MluI. (B) Homologous recombination was confirmed by Southern blotting. 367CAG is the ES cell line harboring the correct targeting event. Genomic DNA digested with HindIII (left) and BglII (right) was probed with PE0.24 (Sado et al., 2006) and ex2 (a genomic fragment encoding Xist exon 2), respectively. (C) The presence of the respective allele was confirmed in mice. Genomic DNA digested with HindIII was probed with BE0.6 (Sado et al., 2005). (D) The number of pups recovered from a cross between wild-type females and XrCAG2L-Y males carrying a Pgk2-cre transgene.
Fig. S2. The X chromosome carrying the Xist\textsuperscript{CAG} allele was preferentially inactivated in MEFs.

(A) Representative images of RNA-FISH and subsequent chromosome painting in MEFs prepared from X\textsuperscript{CAG2L}X\textsuperscript{Rb(X.9)} and X\textsuperscript{Rb(X.9)}X\textsuperscript{CAG} fetuses. Upper panels show RNA-FISH detecting Xist RNA (red) accumulated on the X chromosome. DNA was counterstained with DAPI (blue). Lower panels show chromosome painting of the same metaphase spread examined by RNA-FISH in the upper panel.
panels using probes specific for the X chromosome (red) and chromosome 9 (green). Arrows indicate morphologically normal wild-type or mutated X, whereas arrowheads indicate X^{Rb(X.9)}. (B) The prevalence of metaphases showing the Xist coating with either morphologically normal X (wild-type X, X^{CAG2L}, and X^{CAG}) or X^{Rb(X.9)}. The accumulation of Xist RNA was observed on either X chromosome in XX^{Rb(X.9)} MEFs, reflecting random X-inactivation. In contrast, Xist RNA was found on X^{Rb(X.9)} in X^{CAG2L-X^{Rb(X.9)}} MEFs, whereas it was essentially confined to the morphologically normal X^{CAG} in X^{Rb(X.9)}X^{CAG} MEFs. A minor fraction in X^{Rb(X.9)}X^{CAG} MEFs (1.9%) showed Xist coating on both X chromosomes. This may be due to the transmigration of overexpressed Xist RNA from the Xist^{CAG} allele on X^{CAG} to the other X as previously reported by Jeon and Lee (Jeon and Lee, 2011). (C) The expression of Xist in XX and XX^{CAG} was examined by Northern blotting and quantitative RT-PCR. (D) The half-life assay of Xist RNA expressed from the Xist^{CAG} allele. XX^{CAG} and XX MEFs were treated with DRB, an inhibitor of RNA polymerase II, and RNA was collected at different time points for qRT-PCR. The amount of Xist RNA present in MEFs prepared from XX and XX^{CAG} fetuses at each time point was measured relative to 18s ribosomal RNA by quantitative RT-PCR. (E) The replication patterns of chromosomes in X^{CAG2L-X^{Rb(X.9)}} and X^{Rb(X.9)}X^{CAG} MEFs, X^{Rb(X.9)} and X^{CAG} visualized as a pale chromosome in X^{CAG2L-X^{Rb(X.9)}} and X^{Rb(X.9)}X^{CAG}, respectively (arrows), were referred to as a typical inactive X chromosome. An active counterpart is indicated by arrowheads. 5-Bromo-2-deoxyuridine (BrdU) (100 μg/ml) was incorporated into MEFs for 9 hours with Colcemid present at 100 ng/ml in the culture medium during the last hour. Chromosome spreads were stained with Acridine Orange. (F) Summary of the replication timing analysis in XX^{Rb(X.9)}, X^{CAG2L-X^{Rb(X.9)}}, and X^{Rb(X.9)}X^{CAG} MEFs, showing the prevalence of respective cells with either X^{Lab} referring to a morphologically normal X (wild-type X, X^{CAG2L}, and X^{CAG}) or X^{Rb(X.9)}, which replicated late in the S phase. (G) The allelic expression analysis of X-linked genes in MEFs derived from the F1 hybrid fetuses of JF and respective mutant mice. These MEFs allowed us to study the allelic expression of X-linked genes using restriction site polymorphisms between JF1 and laboratory strains. Six X-linked genes (Rbm3, Hprt, Fmr1, G6pd, Zfp261, and Xist) were analyzed by RT-PCR and the subsequent digestion of products with appropriate restriction enzymes. The origin of each fragment is shown on the right: X^{Lab} refers to either X^{CAG2L}, X^{CAG}, or wild-type X derived; X^{JF1} refers to the X derived from the JF1 strain.
Fig. S3. RNA-FISH for $Xist$ expression in 8-cell embryos recovered from a cross between $XX^{CAG}$ females and wild-type males.

It is likely that the very faint scattered signals (arrows) represented very weak expression of from maternally inherited $Xist^{CAG}$. An arrowhead indicates a normal $Xist$ cloud.
Fig. S4. RNA-FISH for Xist expression in X\textsuperscript{CAG}Y and X\textsuperscript{CAG}X ES cells before (d0) and after (d5) differentiation.

The Xist\textsuperscript{CAG} allele was upregulated in both male and female ES cells upon induction of differentiation. Undifferentiated ES cells were maintained in 2i medium, whereas differentiation was induced into Epiblast-like cells (EpiLCs) according to Hayashi et al (Hayashi et al., 2012).

