Supplementary Materials and Methods

Construction of the targeting vector

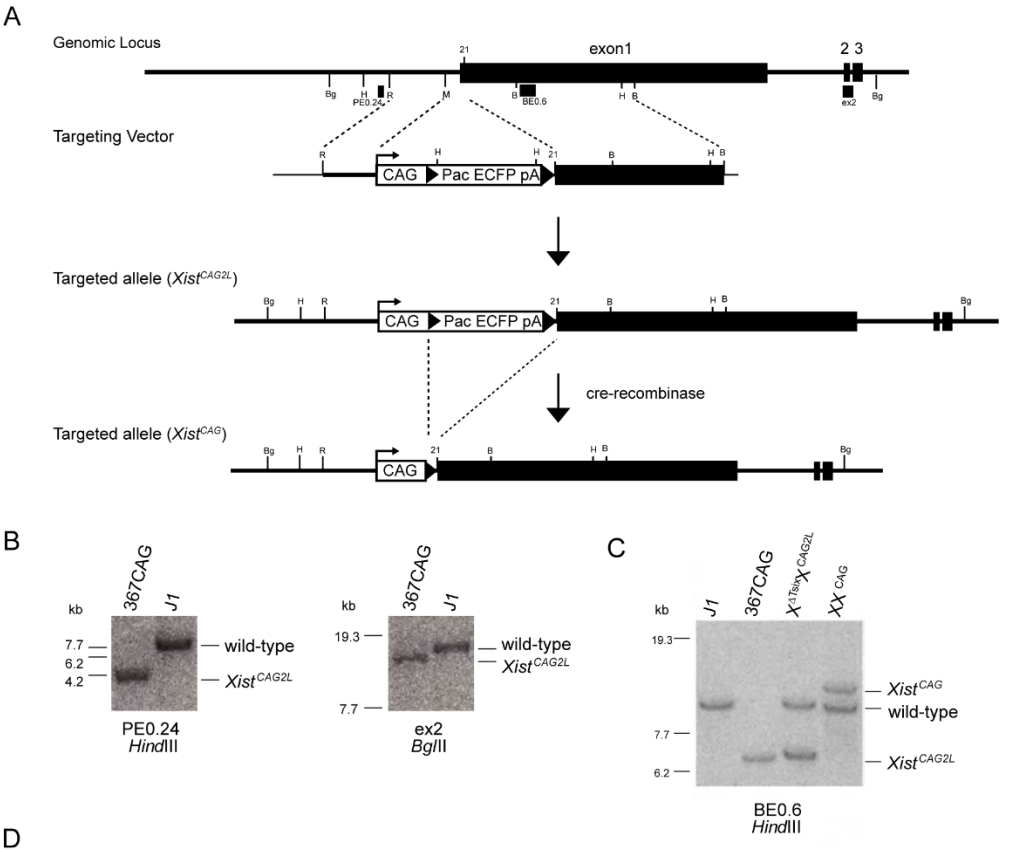
Construction of the targeting vector, pCAG-CΔM20, and the generation of mice carrying the *Xist*^{CAG2L} allele were carried out as follows: a 4.6 kb EcoRI-BamHI fragment present in exon1 of the *Xist* gene was cloned into pBluescriptII SK(-), in which SalI and XhoI sites had been destroyed by double 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-CΔM20.

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

Mlu-Sal-21F(+)37:	5'-GGC CGT GAC GCG TCG ACA TGT GCC GG TTC TTC CGT GGT-3'
AS1634F	5'-GCG TAA CTG GCT CGA GAA TA-3'
XistEx7F21	5'-GCC CAG GTC ACA TTA TGG TT-3'
XistEx7R20	5'-CTC CAA TTT CTG GGC TCA AG-3'
18SRNA1	5'-TCA AGA ACG AAG TCG GAG GTT-3'
18SRNA2	5'-GCA CAT CTA AGG GCA TCA CAG-3'
Bx-CAG1113F	5'-AAA CTA CAA CCC CCC CTA CAC CCC CCT CCC-3'
Bx-CAG1686R	5'-TTG TTT AGG AGT TGT AGG AAA AAG AAG AAG-3'
Bx-CAG1646R	5'-GTA TGA ATA TGG TTA GTA GAG GTT TTA GAG-3'
XistPr2	5'-AAA TAT TCC CCC AAA ACT CCT TAA ATA A-3'
XistPr3	5'-GTT AAT TAA TGT AGA AGA ATT TTT AGT GTT TA-3'
XistPr4	5'-GGT TTG TTT AAG TAG AAG ATA TAT TGA AAT-3'

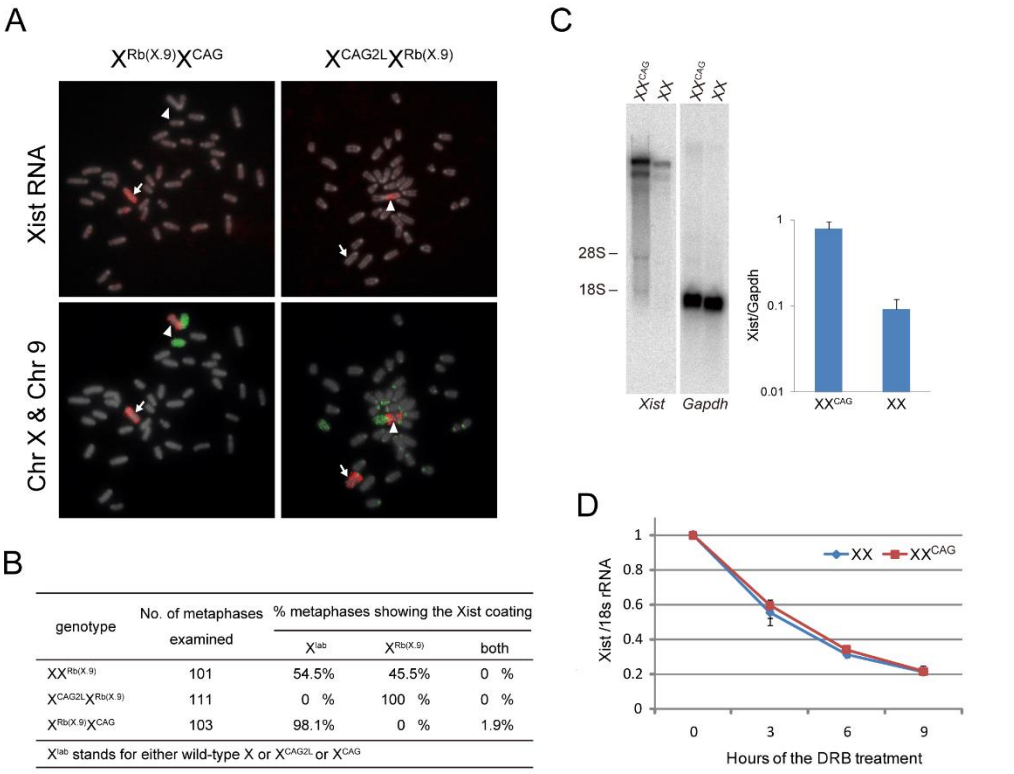


XX x [X^{CAG}Y, Pgk2-cre] (3 weeks)

	XX^{CAG}	XY	total
No. pups	67 (61.5%)	42 (38.5%)	109
			17 litters (6.4 pups/litter)

Fig. S1. Introduction of a constitutively active allele of *Xist* into the mouse

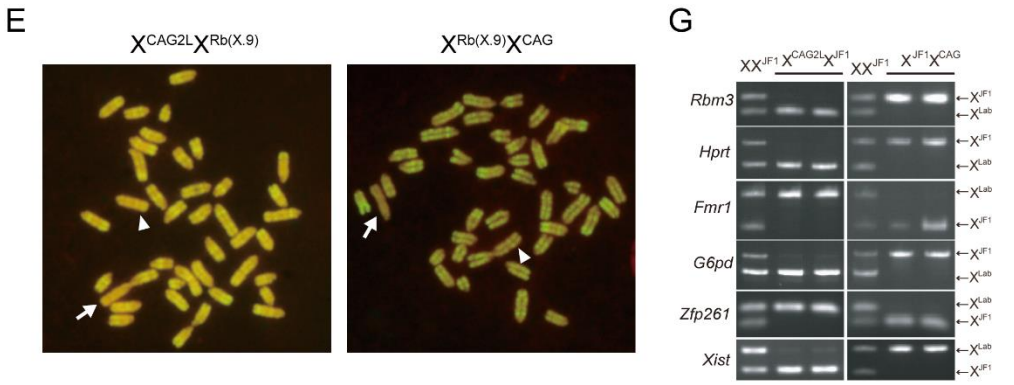
(A) the targeting scheme for generating the *Xist*^{CAG} allele. The endogenous *Xist* promoter was replaced with a CAG-Pac ECFP-pA cassette to produce the *Xist*^{CAG2L} allele, which would not produce *Xist* RNA. In the presence of cre recombinase, the *Xist*^{CAG2L} allele should be converted into the *Xist*^{CAG} 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, *Hind*III; Bg, *Bgl*II; B, *Bam*HI; R, *Eco*RI; M, *Mlu*I. **(B)** Homologous recombination was confirmed by Southern blotting. 367CAG is the ES cell line harboring the correct targeting event. Genomic DNA digested with *Hind*III (left) and *Bgl*II (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 *Hind*III was probed with BE0.6 (Sado et al., 2005). **(D)** The number of pups recovered from a cross between wild-type females and X^{CAG2L}Y males carrying a Pgk2-cre transgene.



B

genotype	No. of metaphases examined	% metaphases showing the Xist coating		
		X^{lab}	$X^{Rb(X.9)}$	both
$XX^{Rb(X.9)}$	101	54.5%	45.5%	0 %
$X^{CAG2L}X^{Rb(X.9)}$	111	0 %	100 %	0 %
$X^{Rb(X.9)}X^{CAG}$	103	98.1%	0 %	1.9%

X^{lab} stands for either wild-type X or X^{CAG2L} or X^{CAG}



F

genotype	No. of metaphases examined	late replicating X	
		X^{lab}	$X^{Rb(X.9)}$
$XX^{Rb(X.9)}$	160	58.1%	41.9%
$X^{CAG2L}X^{Rb(X.9)}$	137	0 %	100 %
$X^{Rb(X.9)}X^{CAG}$	114	100 %	0 %

Fig. S2. The X chromosome carrying the *Xist*^{CAG} allele was preferentially inactivated in MEFs. **(A)** Representative images of RNA-FISH and subsequent chromosome painting in MEFs prepared from $X^{CAG2L}X^{Rb(X.9)}$ and $X^{Rb(X.9)}X^{CAG}$ 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

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.

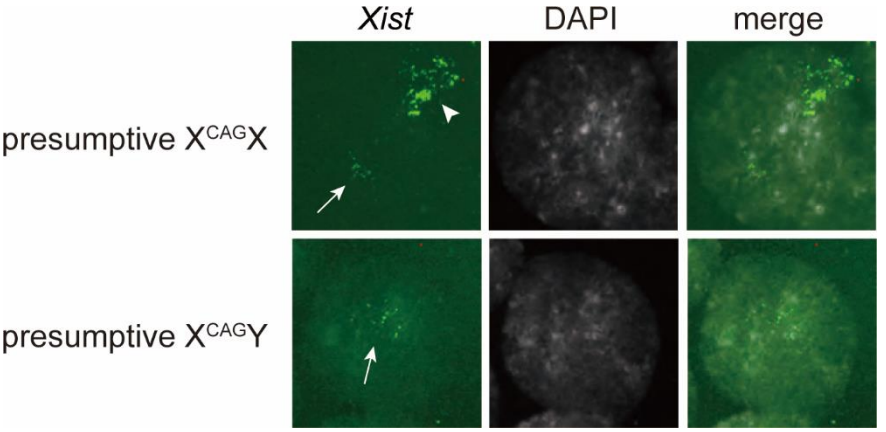


Fig. S3. RNA-FISH for *Xist* expression in 8-cell embryos recovered from. a cross between XX^{CAG} females and wild-type males.

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.

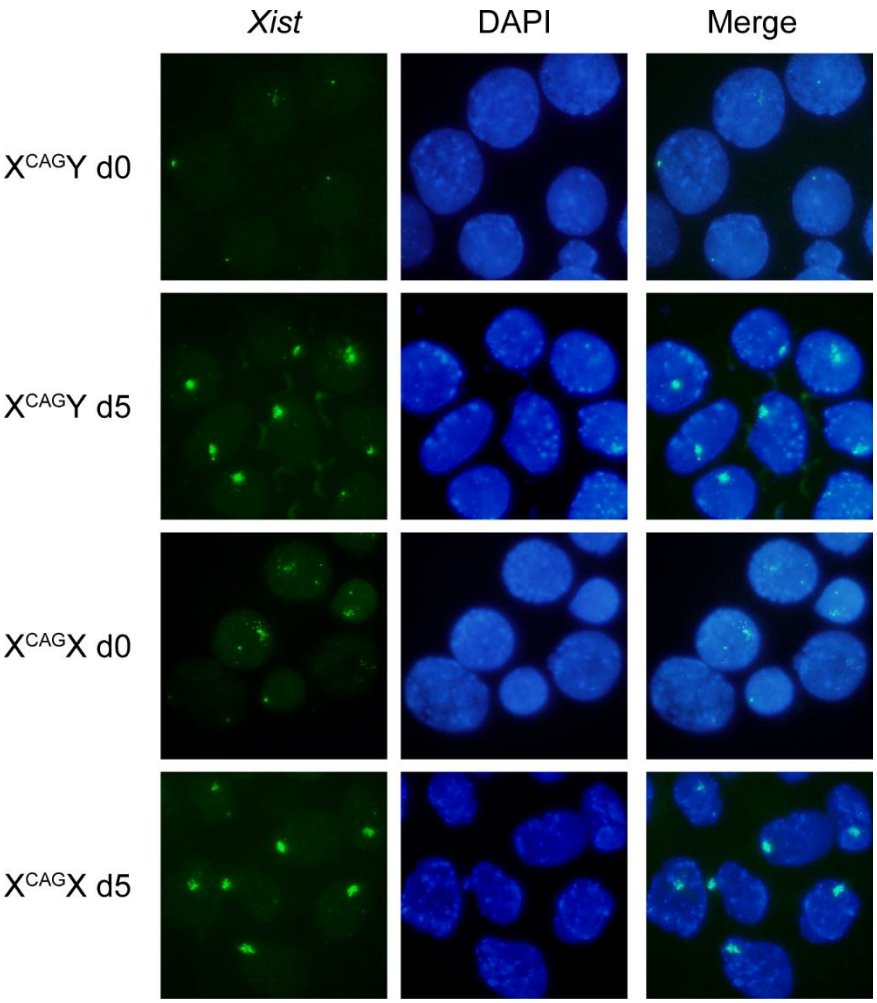


Fig. S4. RNA-FISH for Xist expression in $X^{CAG}Y$ and $X^{CAG}X$ ES cells before (d0) and after (d5) differentiation.

The *Xist*^{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).

Hayashi, K., Ogushi, S., Kurimoto, K., Shimamoto, S., Ohta, H. and Saitou, M. (2012). Offspring from oocytes derived from in vitro primordial germ cell-like cells in mice. *Science* **338**, 971-975.

Jeon, Y. and Lee, J. T. (2011). YY1 tethers Xist RNA to the inactive X nucleation center. *Cell* **146**, 119-133.

Sado, T., Hoki, Y. and Sasaki, H. (2005). Tsix silences Xist through modification of chromatin structure. *Developmental cell* **9**, 159-165.

Sado, T., Hoki, Y. and Sasaki, H. (2006). Tsix defective in splicing is competent to establish Xist silencing. *Development* **133**, 4925-4931.