Supporting Information

Bhatnagar et al. 10.1073/pnas.1413620111

SI Materials and Methods

Single-Nucleotide Primer Extension Assay. A single-nucleotide primer extension (SNuPE) assay for *Pgk1* was carried out using a TaqMan SNP genotyping assay (Applied Biosystems) according to the manufacturer's specifications. The following primers and reporters were used for the assay: 5′-CCGGCCAAAATTGATGCTTTCC-3′, 5′-CAGTCCCAAAAGCATCATTGACAT-3′, 5′-CACTGTCCAAACTAGG-3′, and 5′-CACTGTCCACACTAGG-3′. The data are plotted as the function of ΔRn for each sample, which represents the reporter fluorescence for each allele (VIC/FAM) normalized to the passive reference dye.

Imprinted Gene Analysis. Mouse embryonic fibroblasts (MEFs) from strain C57BL6 (CAST 7), provided by M. Bartolomei (University of Pennsylvania, Philadelphia), were cultured in DMEM supplemented with 10% (vol/vol) FCS and 10% (vol/vol) nonessential amino acids. Analysis of imprinted genes was performed using MEFs isolated from the C57BL/6 (CAST7) strain, which contains chromosome 7 from the *Mus castaneus* (Cast) strain in a C57BL/6 background, as previously described (1). Briefly, total RNA was extracted and cDNA synthesis was carried out as described above. For PCR amplification, the cDNA was added to Ready-To-Go PCR Beads (GE Life Sciences) together with 0.3 μM gene-specific primers (Table S2). Expression of the imprinted gene was analyzed by allele-specific restriction enzyme digestion (StcI for *Ascl2*, StuI for *Kcnq1ot1*, MnlI for *Peg3*, and FauI for *Zim1*), and digested PCR products were resolved by polyacrylamide gel electrophoresis.

1. Rivera RM, et al. (2008) Manipulations of mouse embryos prior to implantation result in aberrant expression of imprinted genes on day 9.5 of development. *Hum Mol Genet* 17(1):1–14.

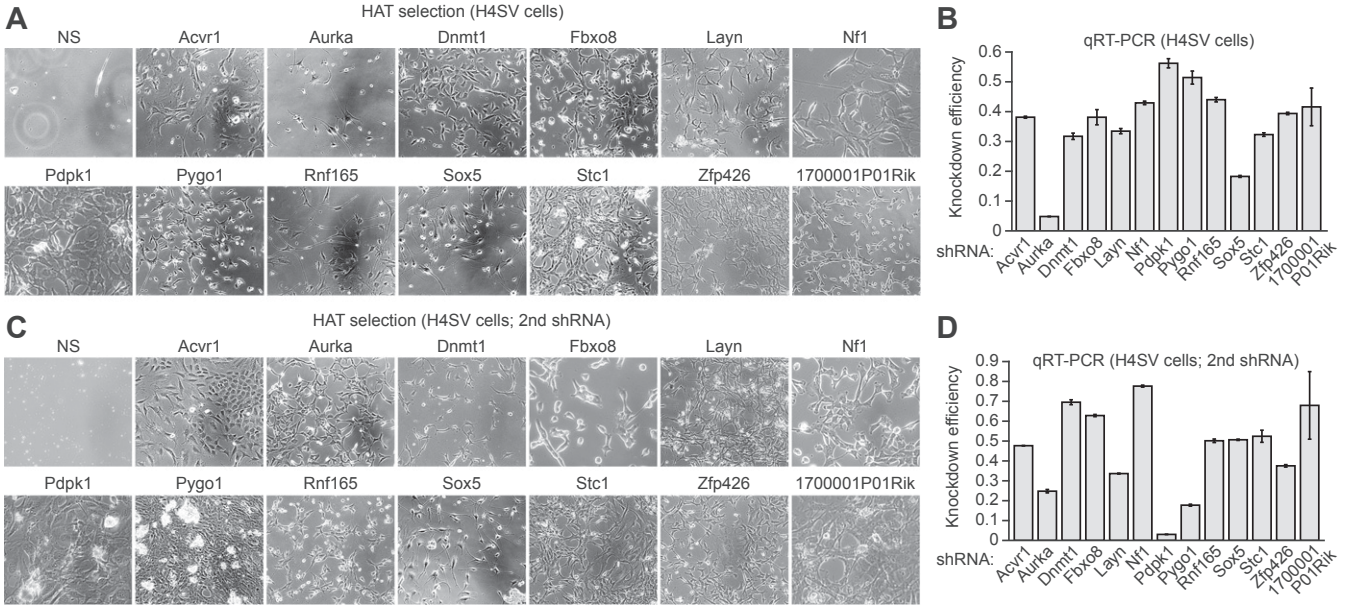

Fig. S1. shRNAs targeting an X-chromosome inactivation factor (XCIF) reactivate the inactive X chromosome (Xi)-linked *Hprt* gene and decrease mRNA levels of the targeted gene. (*A*) Bright-field images showing growth of the 13 XCIF KD H4SV cell lines following selection in hypoxanthine–aminopterin–thymidine (HAT) medium. (*B*) Quantitative real-time RT-PCR (qRT-PCR) analysis monitoring target gene expression in the 13 XCIF KD H4SV cell lines expressing the shRNA identified in the primary screen. For each gene, knockdown efficiency was determined relative to the level of target gene expression in the control cell line expressing a nonsilencing (NS) shRNA, which was set to 1. Error bars indicate SD. (*C*) Bright-field images showing growth of the 13 XCIF KD H4SV cell lines, expressing a second, unrelated shRNA to that shown in *A*, following selection in HAT medium. (*D*) qRT-PCR analysis monitoring target gene expression in the 13 XCIF KD H4SV cell lines expressing a second, unrelated shRNA to that shown in *B*. Error bars indicate SD.

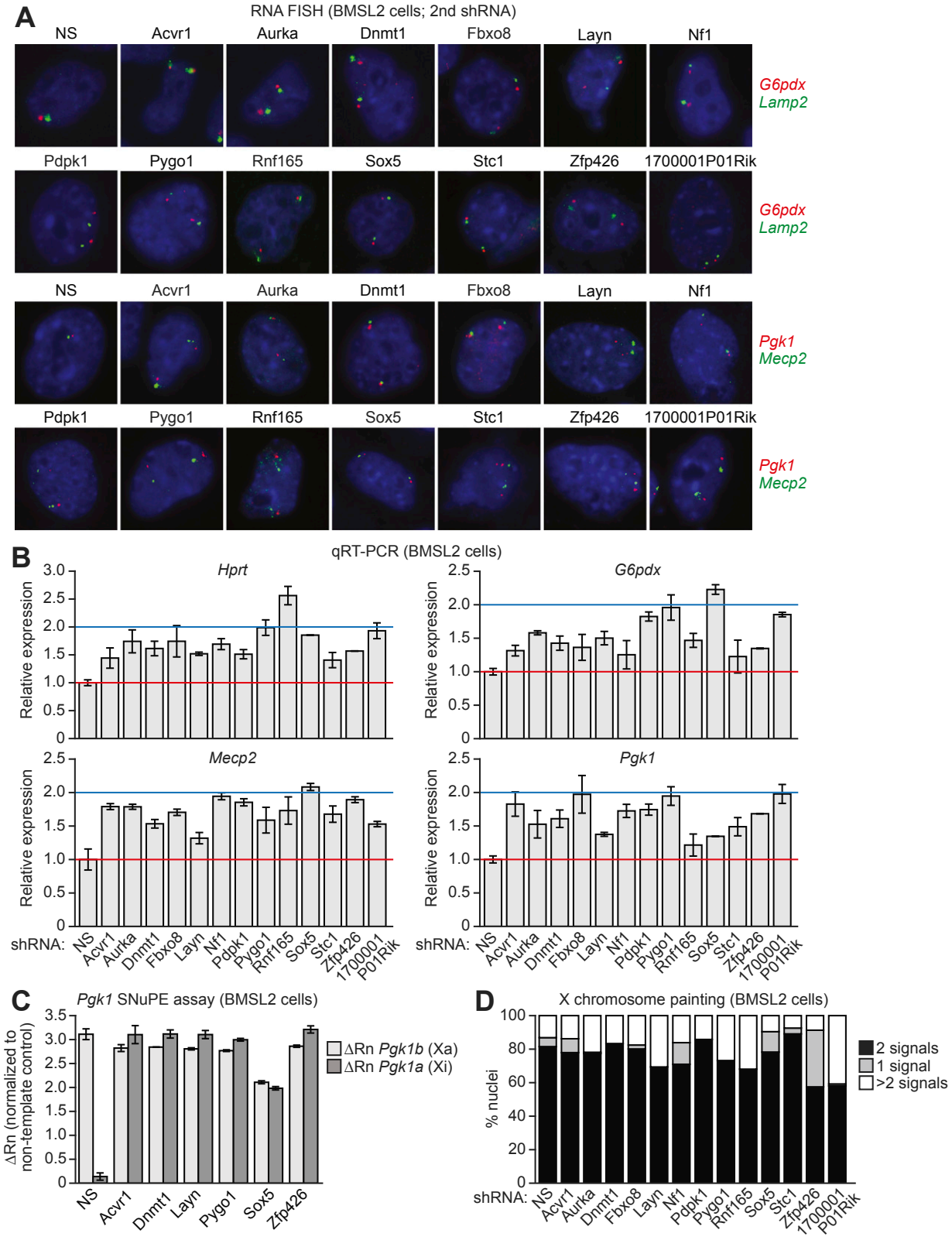

Fig. S2. Additional RNA FISH images and control experiments related to Fig. 1. (*A*) Representative two-color RNA FISH images showing expression of *G6pdx* (red) and *Lamp2* (green; *Upper*) and *Pgk1* (red) and *Mecp2* (green; *Lower*) in each of the 13 XCIF KD BMSL2 cell lines. DAPI staining is shown in blue. (*B*) qRT-PCR analysis monitoring expression of *Hprt*, *G6pdx*, *Mecp2*, and *Pgk1* in each of the 13 XCIF KD BMSL2 cell lines. The results were normalized to that obtained with the NS shRNA, which was set to 1 (red line). A twofold increase in expression is indicated by the blue line. (*C*) In BMSL2 cells, the Xi and Xa encode two distinguishable *Pgk1* alleles, *Pgk1a* and *Pgk1b*, respectively, which differ by a single-nucleotide polymorphism within the mRNA. Allele-specific expression of the Xi- and Xa-linked *Pgk1* genes in six representative XCIF KD BMSL2 cell lines was analyzed using a SNuPE assay. The data are plotted as the function of ΔRn

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for each sample, which represents the reporter fluorescence for each allele (VIC/FAM) normalized to the passive dye. The results show that, in each of the six XCIF KD BMSL2 cell lines, the Xi-linked *Pgk1a* gene was reactivated. (*D*) X-chromosome painting experiments in the 13 XCIF KD BMSL2 cell lines. The results show that the X-chromosome content of the XCIF KD BMSL2 cell lines was similar to that of the control BMSL2 cell line expressing a NS shRNA. Thus, the substantially increased biallelic expression of X-linked genes observed by RNA FISH in the XCIF KD cell lines cannot be explained by differences in X-chromosome number.

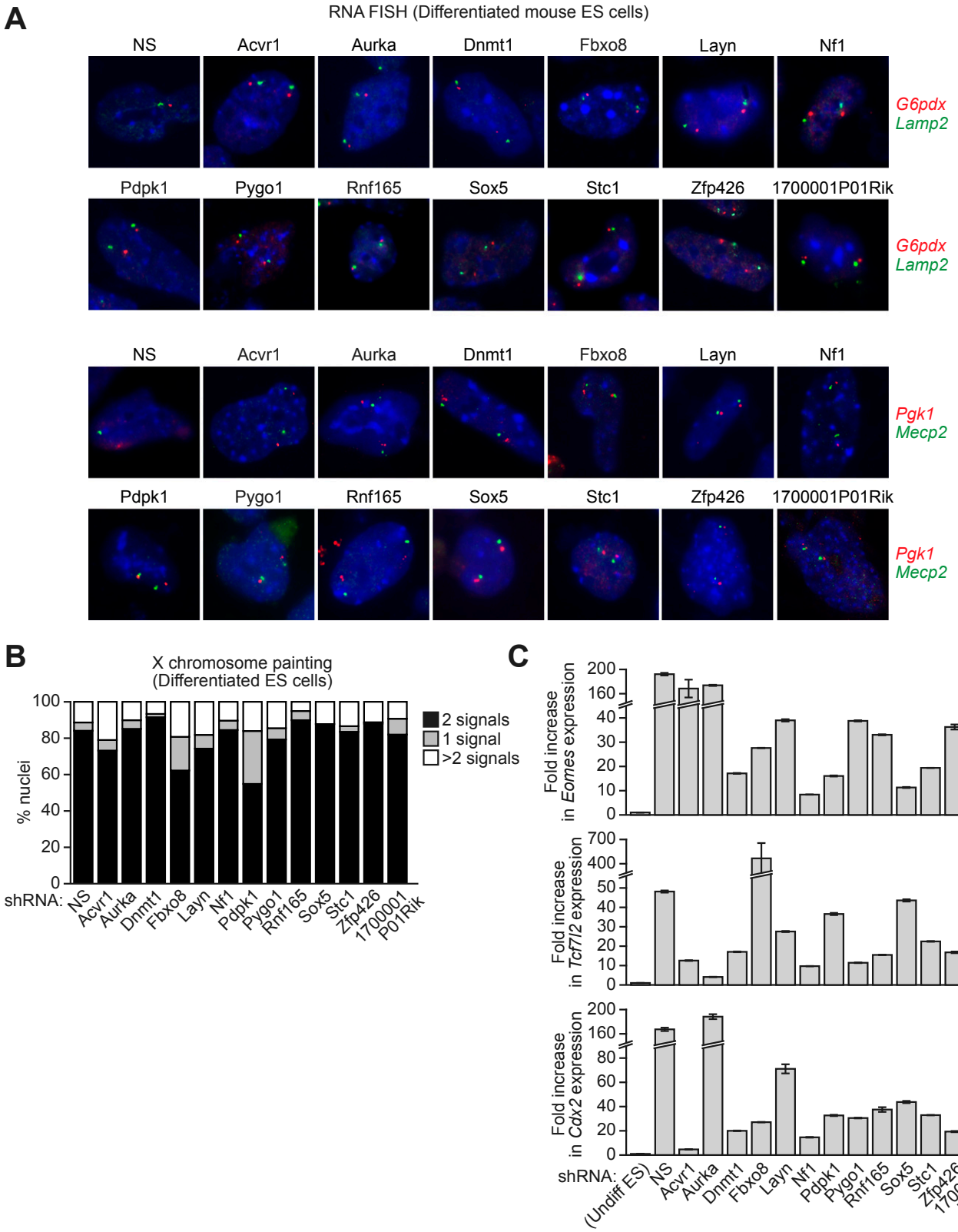


Fig. S3. Additional RNA FISH images and control experiments related to Fig. 2. (*A*) Representative two-color RNA FISH images monitoring expression of *G6pdx* (green) and *Lamp2* (red; *Upper*) and *Pgk1* (green) *Mecp2* (red; *Lower*) in the 13 XCIF KD ES cell lines following differentiation. DAPI staining is shown in blue. (*B*) X-chromosome painting experiments in the 13 XCIF KD ES cell lines following differentiation. (*C*) qRT-PCR analysis monitoring expression of *Eomes*, *Tcf7l2*, and *Cdx2* in the 13 XCIF KD ES cell lines following treatment with RA. As a control, expression of each gene in undifferentiated ES cells is shown and was set to 1. Error bars indicate SD.

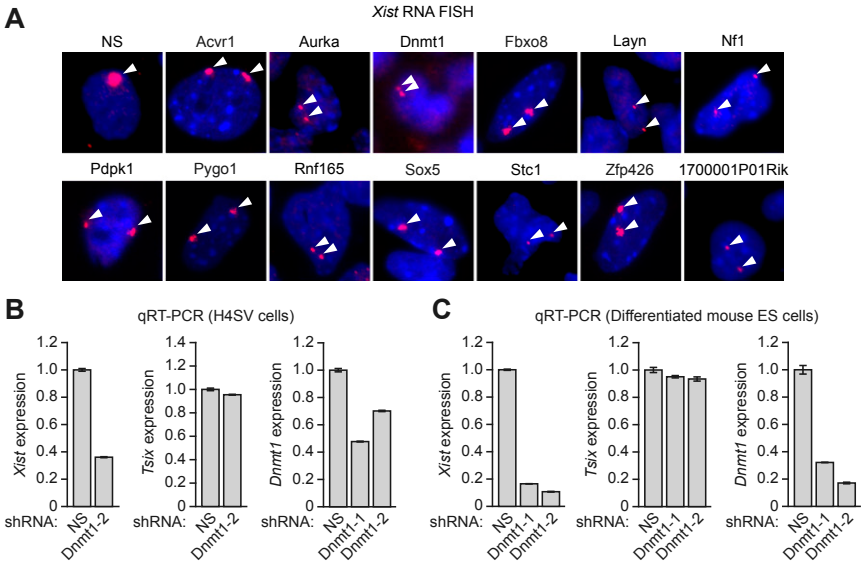


Fig. S4. RNA FISH images and control experiments related to Fig. 3. (*A*) RNA FISH images. In each of the 13 XCIF KD ES cell lines following differentiation, the majority of cells that lost the typical *Xist* localization pattern lacked a detectable *Xist* signal (Fig. 3*B*). However, some cells that had lost the typical *Xist* localization pattern contained two small *Xist* signals, reminiscent of undifferentiated ES cells. Examples of this latter localization pattern are shown here. Nuclear signals are indicated in red and denoted by arrowheads; DAPI staining is shown in blue. (*B*) qRT-PCR analysis monitoring expression of *Xist* (*Left*), *Tsix* (*Center*), and *Dnmt1* (*Right*) in H4SV cells expressing a NS or one of two Dnmt1 shRNAs (Dnmt1-1 or Dnmt1-2). For *Xist* and *Tsix* expression, a second, unrelated Dnmt1 shRNA to that used in Fig. 3*H*. Expression was normalized to that obtained with the control NS shRNA, which was set to 1. Error bars indicate SD. (*C*) qRT-PCR analysis monitoring expression of *Xist* (*Left*), *Tsix* (*Center*), and *Dnmt1* (*Right*) in differentiated ES cells expressing a NS shRNA or one of two Dnmt1 shRNAs (Dnmt1-1 or Dnmt1-2). Expression was normalized to that obtained with the control NS shRNA, which was set to 1. Error bars indicate SD.

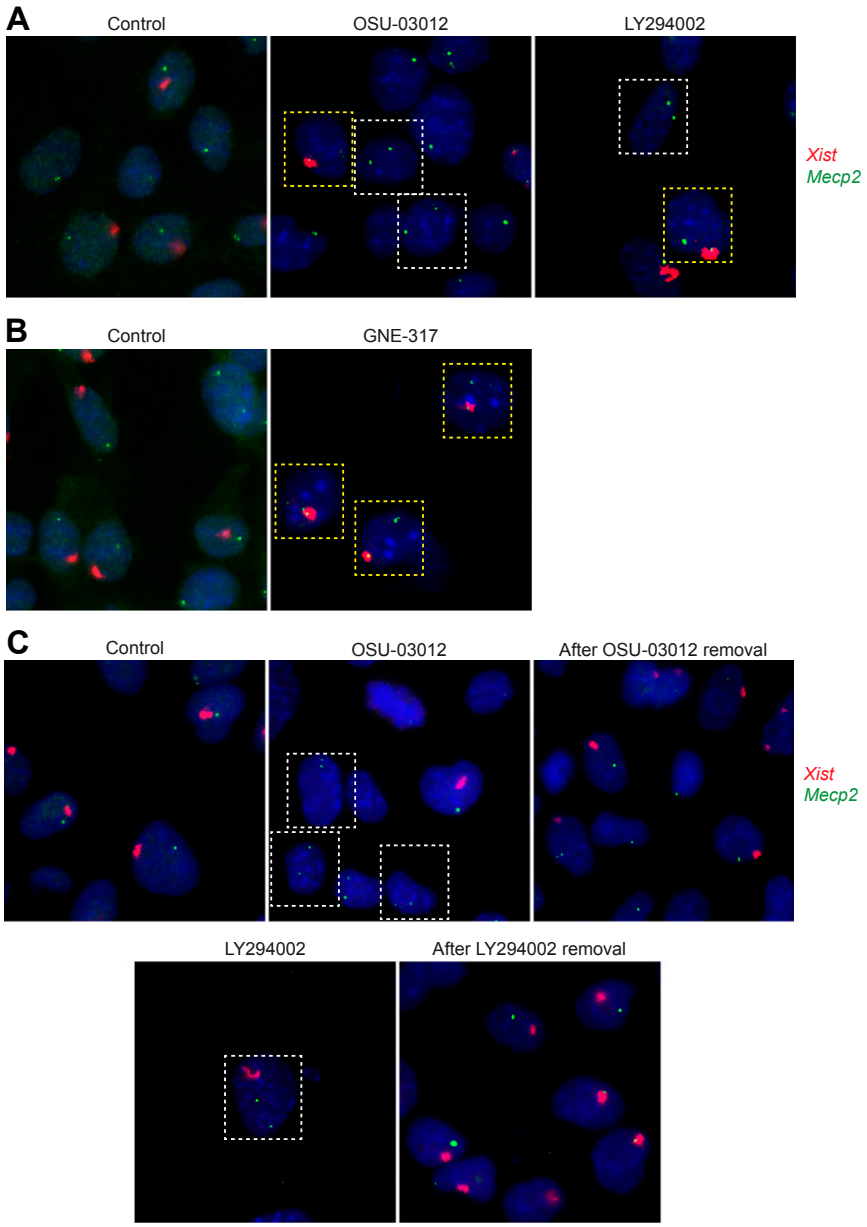


Fig. S5. Additional RNA FISH images related to Fig. 4. (*A* and *B*) Two-color RNA FISH monitoring expression of *Xist* (red) and *Mecp2* (green) in differentiated ES cells treated with DMSO (control), OSU-03012 (4 μM), or LY294002 (10 μM) (*A*), and in BMSL2 cells treated with DMSO or GNE-317 (5 μM) (*B*). The yellow boxes indicate cells with colocalizing *Xist* and *Mecp2* signals; the white boxes indicate cells with biallelic expression of *Mecp2* and complete loss of the *Xist* signal. (*C*) Two-color RNA FISH monitoring *Xist* (red) and *Mecp2* (green) expression in BMSL2 cells treated with DMSO (control), OSU-03012 (2.5 μM), or LY294002 (8 μM), and at least 6 d following removal of the inhibitor. The white boxes indicate cells with biallelic expression of *Mecp2*.

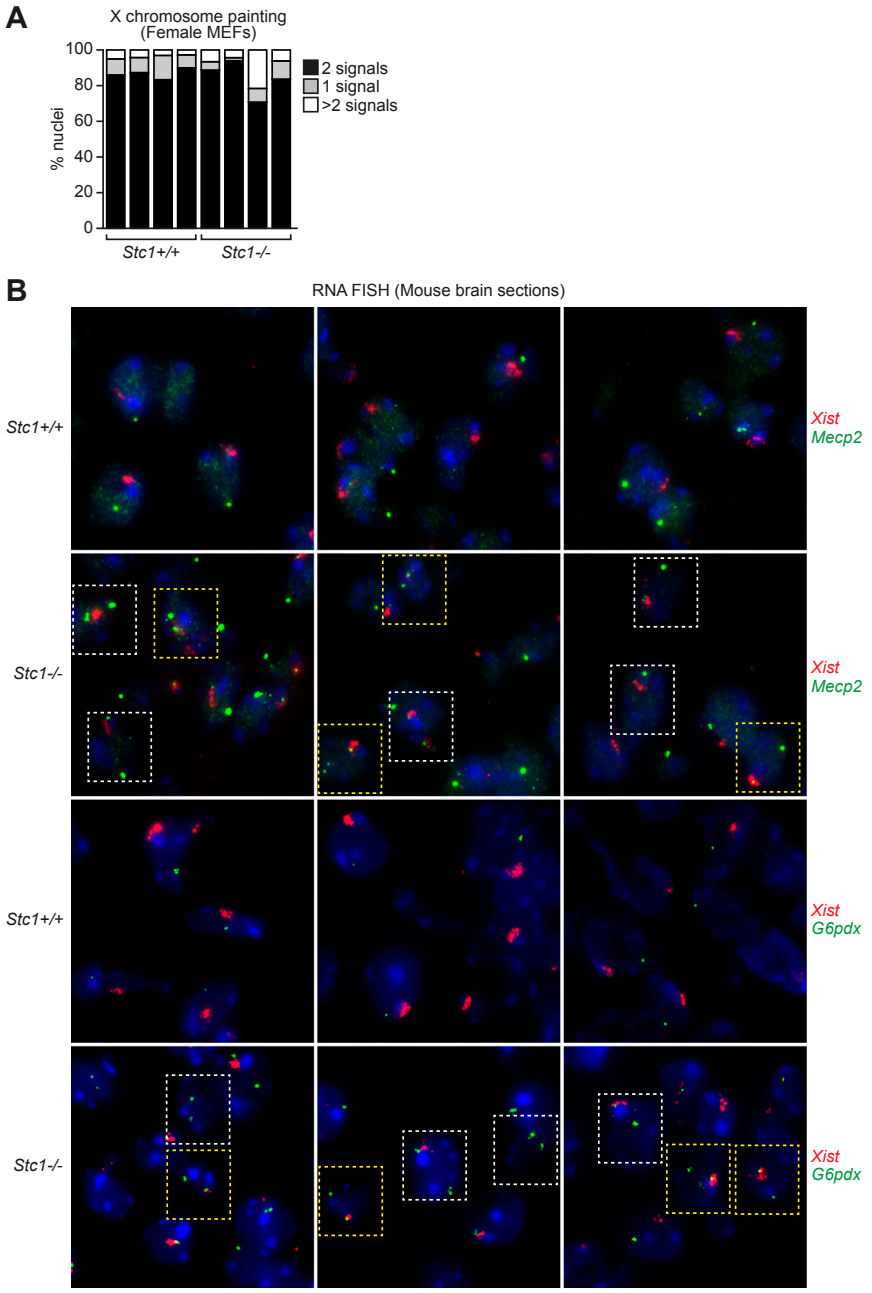
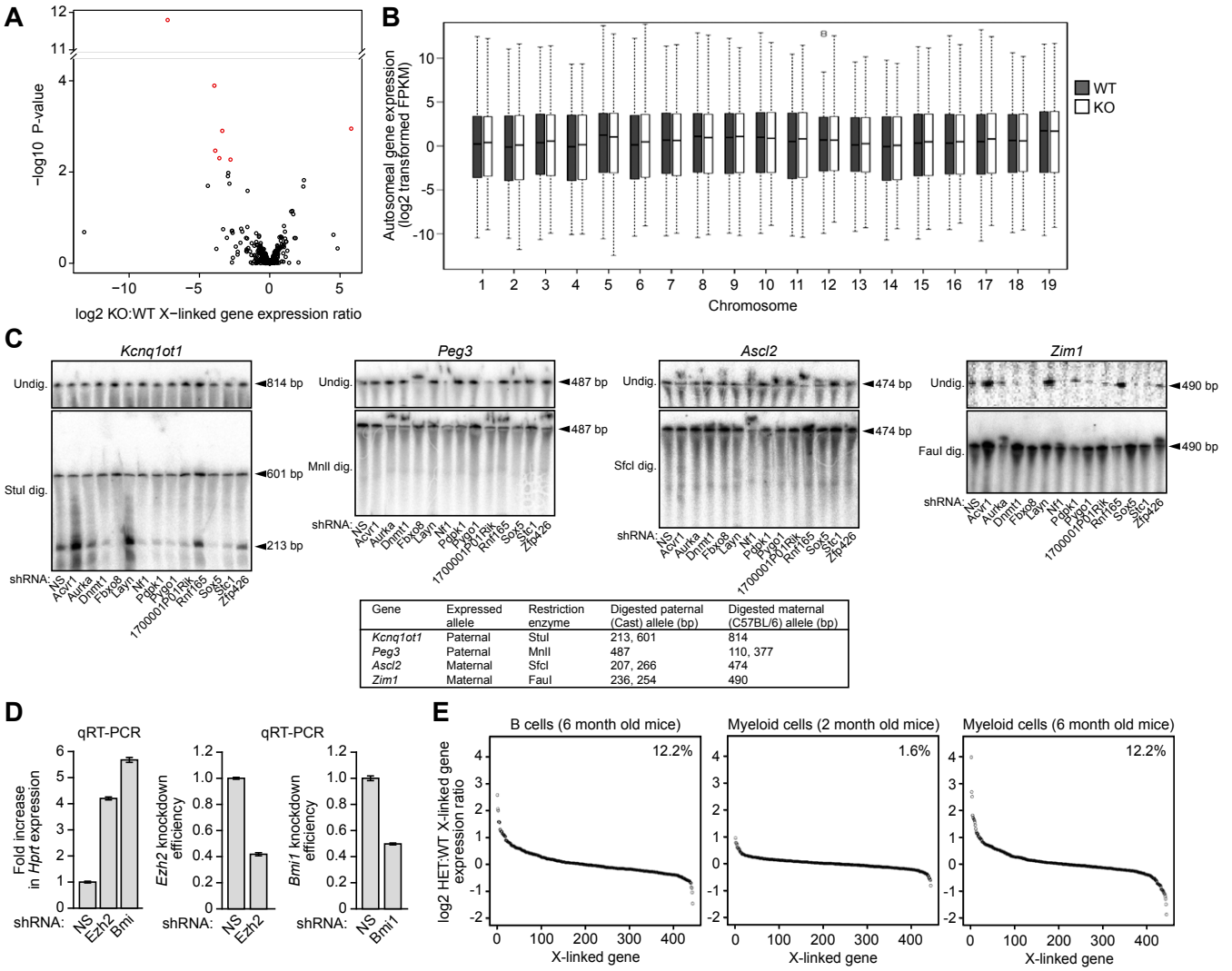


Fig. S6. Control experiment and RNA FISH images related to Fig. 5. (*A*) X-chromosome painting experiments in female *Stc1*$^{+/+}$ and *Stc1*$^{-/-}$ MEFs. The results show that the X-chromosome content of *Stc1*$^{-/-}$ MEFs was similar to that of *Stc1*$^{+/+}$ MEFs. Thus, the substantially increased biallelic expression of X-linked genes observed by RNA FISH in the *Stc1*$^{-/-}$ MEFs cannot be explained by differences in X-chromosome number. (*B*) Defective XCI in cortical neurons from brain sections of female *Stc1*$^{-/-}$ mice. Two-color RNA FISH monitoring expression of *Xist* (red) and *Mecp2* or *G6pdx* (green) in cortical neurons from adjacent 5-μm brain sections of female *Stc1*$^{-/-}$ and *Stc1*$^{+/+}$ mice ($n = 3$ per genotype, stage P1). The boxed regions denote cells with two *Mecp2* or *G6pdx* signals; the yellow boxes indicate cells with colocalizing *Xist* and *Mecp2*/*G6pdx* signals. All cells in the regions shown represent neurons that, based on anatomical landmarks, are present in posthybridized sections.



Gene	Expressed allele	Restriction enzyme	Digested paternal (Cast) allele (bp)	Digested maternal (C57BL/6) allele (bp)
Kcnq1ot1	Paternal	StuI	213, 601	814
Peg3	Paternal	MnlI	487	110, 377
Ascl2	Maternal	SfcI	207, 266	474
Zim1	Maternal	FauI	236, 254	490

Fig. S7. Additional experiments and data analyses related to Fig. 6 and *Discussion*. (*A*) Volcano plot showing distribution of $\log_2$-transformed ratio of X-linked gene expression in MEFs isolated from *Stc1*$^{-/-}$ (KO) and *Stc1*$^{+/+}$ (WT) embryos ($n = 3$ per genotype). The genes are plotted against negative transformed log of *P* value. The red circles represent genes with a more than twofold change in expression and $P < 0.01$. The results show that the similarity of X-linked gene expression between female *Stc1*$^{+/+}$ and *Stc1*$^{-/-}$ MEFs was statistically significant. (*B*) Box plots displaying changes in autosomal gene expression [$\log_2$-transformed fragments per kilobase of exon per million fragments mapped (FPKM)] in *Stc1*$^{-/-}$ and *Stc1*$^{+/+}$ MEFs. The boxed areas span the first to the third quartile. The whiskers represent 15th and 85th percentiles; samples falling outside these percentiles are shown as circles. (*C*) XCIFs are not required for repression of imprinted genes. Primary female mouse embryonic fibroblasts from the strain C57BL/6 (CAST7), which contains chromosome 7 from *Mus castaneus* (Cast), were transduced with shRNAs against each of the XCIFs and analyzed for allele-specific expression of four genes located on chromosome 7 that are either paternally expressed (*Kcnq1ot1* and *Peg3*) or maternally expressed (*Ascl2* and *Zim1*). Expression of the two alleles can be distinguished by allele-specific restriction enzyme digestion following gene-specific RT-PCR. The sizes of the undigested and digested bands are indicated, and the sizes of the predicted digested fragments are shown in the table (*Lower*). If knockdown of an XCIF results in reactivation of the normally silenced allele, a mixture of the maternal and paternal allele-specific digestion patterns would be observed. The results show that, in all 13 XCIF KD cell lines, all four genes displayed only the expected allele-specific expression pattern, indicating that the XCIFs are not required for repression of the imprinted genes. (*D*) Requirement of Polycomb subunits EZH2 and BMI1 for repression of the X-linked *Hprt* gene. (*Left*) qRT-PCR analysis monitoring *Hprt* expression in BMSL2 cells expressing an Ezh2 or Bmi1 shRNA or, as a control, a NS shRNA. (*Right*) qRT-PCR analysis confirming target gene knockdown in mouse ES cells expressing an Ezh2 (*Left*) or Bmi1 (*Right*) shRNA. Error bars indicate SD. (*E*) Analysis of available datasets from Yildirim et al. (1) showing the distribution of $\log_2$-transformed ratio of X-linked gene expression in hematopoietic cells from female heterozygous (HET) *Xist* mutant mice and wild-type (WT) mice. The data were downloaded from Gene Expression Omnibus (GSE43961), normalized by RMA, and filtered by detection above background (DABG) (cutoff *P* value of <0.0001) using Bioconductor package xps. The percentage of X-linked genes up-regulated >1.5-fold is shown.

1. Yildirim E, et al. (2013) Xist RNA is a potent suppressor of hematologic cancer in mice. *Cell* 152(4):727–742.

Table S1. Summary of the 13 XCIFs

Mouse gene symbol	Human gene symbol	Gene name	Chromosome, mouse (human)	Biological process
Acvr1	ACVR1	Activin A receptor, type 1	2 (2)	Signal transduction
Aurka	*AURKA*	Aurora kinase A	2 (20)	Cell cycle regulation
Dnmt1	*DNMT1*	DNA methyltransferase (cytosine-5) 1	9 (19)	Chromatin modification
Fbxo8	*FBXO8*	F-box protein 8	8 (4)	Unknown/ubiquitin-dependent protein catabolic process
Layn	*LAYN*	Layilin	9 (11)	Unknown/receptor for hyaluronic acid
Nf1	*NF1*	Neurofibromatosis 1	11 (17)	Signal transduction
Pdpk1	*PDPK1*	3-Phosphoinositide–dependent protein kinase 1	17 (16)	Signal transduction
Pygo1	*PYGO1*	Pygopus 1	9 (15)	Transcriptional regulation
Rnf165	*RNF165*	Ring finger protein 165	18 (18)	Unknown
Sox5	*SOX5*	SRY-box containing gene 5	6 (12)	Transcriptional regulation
Stc1	*STC1*	Stanniocalcin 1	14 (8)	Cell metabolism
Zfp426	*ZNF426*	Zinc finger protein 426	9 (19)	Transcriptional regulation
1700001P01Rik	*C17orf98*	RIKEN cDNA 1700001P01 gene	11 (17)	Unknown

Table S2. List of primers used for qRT-PCR and RT-PCR analysis, cDNA synthesis, ChIP assays, and mouse genotyping

	Forward primer, 5′→3′	Reverse primer(s), 5′→3′
qRT-PCR		
Actin	TTGCCGACAGGATGCAGAA	GCCGATCCACACGGAGTACTT
Acvr1 (mouse)	GGCCAGCAGTGTTTTTCTTC	TTCCCCTGCTCATAAACCTG
ACVR1 (human)	TCAGGAAGTGGCTCTGGTCT	CGTTTCCCTGAACCATGACT
Aurka (mouse)	TAGGATACTGCTTGTTACTT	CCTCCAACTGGAGCTGTA
AURKA (human)	TGGAATATGCACCACTTGGA	ACTGACCACCCAAAATCTGC
Bmi1	AAATCAGGGGGTTGAAAAATCT	GCTAACCACCAATCTTCCTTTG
Cdx2	GCCAAGTGAAAACCAGGACAAAAGAC	GCTGCTGTTGCTGCTGCTGCTTC
Dnmt1 (mouse)	GGAAGGCTACCTGGCTAAAGTCAAG	ACTGAAAGGGTGTCACTGTCCGAC
DNMT1 (human)	GTGGGGGACTGTGTCTCTGT	TGAAAGCTGCATGTCCTCAC
Eomes	CCTGGTGGTGTTTTGTTGTG	TTTAATAGCACCGGGCACTC
Ezh2	CTAATTGGTACTTACTACGATAACTTT	ACTCTAAACTCATACACCTGTCTACAT
Fbxo8 (mouse)	GCTGAGCCATTTTCTTCTCG	ATGATGGTTTCTGGCCACTC
FBXO8 (human)	CAAGGGTTGTGGAGAGTGGT	ATGTCAATGCCTCCTTGGAC
Gapdh	ATGGCCTTCCGTGTTCCTAC	ATAGGGCCTCTCTTGCTCAG
G6pdx	TCAAAGCACACGCCCTCTT	TAGCGCACAGCCAGTTTCC
Hprt	AAGCTTGCTGGTGAAAAGGA	TTGCGCTCATCTTAGGCTTT
Layn (mouse)	GCAAGGAGAGTGGATGGGTA	ACTTGTGATGCTGTGCTTGC
LAYN (human)	CTACAGGCCGTGCTGCTG	CTGACTAGCTGGCCTCCATC
Mecp2	CATGGTAGCTGGGATGTTAGG	GCAATCAATTCTACTTTAGAGCG
Nf1 (mouse)	GTAGCCACAGGTCCCTTGTC	CTGAGAACAAGTACACAGAGAGTGA
NF1 (human)	AATTCTGCCTCTGGGGTTTT	GCTGTTTCCTTCAGGAGTCG
Oct4	CTCACCCTGGGCGTTCTCT	AGGCCTCGAAGCGACAGA
Pdpk1 (mouse)	GGTCCAGTGGATAAGCGAAA	TTTCTGCACCACTTGTGAGC
PDPK1 (human)	GACTCTTCCGTGCGTTCTTC	GAGGAGAAAGGTGACCCACA
Pgk1	ATGTCGCTTTCCAACAAGCTG	GCTCCATTGTCCAAGCAGAAT
Pygo1 (mouse)	TAATGTCAGCGGAACAGGAC	TTATCTGGGCTTCCGAGTTG
PYGO1 (human)	ATCCTGGCTTTGGAGGCTAT	GTGGCCCAAAGTTAAAAGCA
Rnf165 (mouse)	ATGCCTCCAGCTACAGCCTA	GCCCAATGCTAACTGAGAGC
RNF165 (human)	AGGGAGAGCTGGAAAAGGAG	AGCCCTCCCTGGTTTAGTGT
Sox5 (mouse)	GTGGAAGAGGAGGAGAGTGAGA	AAATTCCTCAGAGTGAGGCTTG
SOX5 (human)	AGGGACTCCCGAGAGCTTAG	TTGTTCTTGTTGCTGCTTGG
Stc1 (mouse)	AAGTCATACAGCAGCCCAATCA	CCAGAAGGCTTCGGACAAGTC
STC1 (human)	TGATCAGTGCTTCTGCAACC	TCACAGGTGGAGTTTTCCAG
Tcf7l2	AAAACAGCTCCTCCGATTCC	TAAAGAGCCCTCCATCTTGC
Tsix	CAATCTCGCAAGATCCGGTGA (TSIX2F)	TCAAGATGCGTGGATATCTCGG (P422R)
Xist (nonstrand specific)	CCCTGCTAGTTTCCCAATGA	GGAATTGAGAAAGGGCACAA
Xist (strand specific)	GATGCCAACGACACGTCTGA (XIST2281F)	AAGGACTCCAAAGTAACAATTCA (XIST2424R)
XIST (human)	ACGCTGCATGTGTCCTTAGTAGTC	ATTTGGAGCCTCTTATAGCTGTTTG
Zfp426 (mouse)	ATGACCTTTCGCTCATGGAC	GGCAAGCTTTGCTTTAGTGC
ZNF426 (human)	CTGAGGTGGGTGGATCACTT	CTCTGCTTCCTGGGTTCAAG
1700001P01Rik (mouse)	GCTGATGTCAACTGTTTCC	CGCAGAATCTTCCACCCT
C10orf98 (human)	TCGGGCAAGGACAAAGATAC	CGATGGCTATGAAGGGAAAA
RT-PCR		
Mecp2 (first round)	CCGATCTGTGCAGGAGACCG	TGGGGTCCTCGGAGCTCTCGGGCT
Mecp2 (second round)	GACCCGGGAGACGGTCAGCA	AGCTCTCGGGCTCAGGTGGAGGT
Ascl2	TGAGCATCCCACCCCCCTA	CCAAACATCAGCGTCAGTATAG
Kncq1ot1	ATTGGGAACTTGGGGTGGAGGC	GGCACACGGTATGAGAAAAGATTG
Peg3	ATGCCCACTCCGTCAGCG	GCTCATCCTTGTGAACTTTG
Zim1	CTTCAAGCAGAGCACAAAGC	GTGGCACACGAAAGGTTTCTC
cDNA synthesis		
Xist	AGAGCATTACAATTCAAGGCTC (XIST2688R)	
Tsix	GATGCCAACGACACGTCTGA (TSIX2R)	
Gapdh	TGTGAGGGAGATGCTCAGTG (GAPDR)	
ChIP		
Xist (promoter)	TAAAGGTCCAATAAGATGTCAGAA	GGAGAGAAACCACGGAAGAA
Xist (exon 2)	GTGCTCCTGCCTCAAGAAGAA	GCACTCTTCACTCCTCTAAATCCAG
Mouse genotyping		
Dnmt1$^{+/+}$	CTTGGGCCTGGATCTTGGGGATC	GGG CCAGTTGTGTGACTTGG
Dnmt1$^{-/-}$	GGGAACTTCCTGACTAGGGG	GGGCCAGTTGTGTGACTTGG
Stc1$^{+/+}$	AGCGCACGAGGCGGAACAAA	AGAGAGCCGCTGTGAGGCGT
Stc1$^{-/-}$	AAAAGCCAGAGGTGCAAGAA	TATGATCGGAATTCCTCGAC
SRY	TTGTCTAGAGAGCATGGAGGGCCATGTCAA	CCACTCCTCTGTGACACTTTAGCCCTCCGA

Table S3. Oligo ID numbers for shRNAs obtained from Open Biosystems/Thermo Scientific

Gene	Oligo ID
Acvr1	V2MM_75565
	V2MM_76215
Aurka	V2MM_188005
	V2MM_71909
Bmi1	V2MM_10594
	V2MM_2034
Dnmt1	V2MM_46797
	V2LMM_43170
Ezh2	V2MM_35988
	V2MM_30422
Fbxo8	V2MM_36526
	V3LMM_494067
Layn	V2MM_130482
	V2MM_214085
Nf1	V2MM_194180
	V2HS_76027
Pdpk1	V2MM_75859
	V2MM_72465
Pygo1	V2MM_110610
	V2MM_110609
Rnf165	V2MM_172866
	TRCN0000135474
Sox5	V2MM_6385
	V2HS_94936
Stc1	V2MM_22454
	V2MM_26886
	TRCN0000109921
Zfp426	V2MM_31994
	TRCN0000085016
1700001P01Rik	V2MM_100177
	V2MM_205788

Table S4. cDNAs used to prepare RNA FISH probes

Gene	Clone no.*	Ref.
G6pdx	BAC clone RP23-13D21	1
Lamp2	BAC clone RP24-173A8	1
Mecp2	Fosmid clone WI1-894A5 or WI1-1269o10	
Pgk1	BAC RP23-404E5	
Xist	—	2

*Obtained from the BACPAC Resources Center.

1. Patrat C, et al. (2009) Dynamic changes in paternal X-chromosome activity during imprinted X-chromosome inactivation in mice. *Proc Natl Acad Sci USA* 106(13):5198–5203.
2. Shin J, et al. (2010) Maternal Rnf12/RLIM is required for imprinted X-chromosome inactivation in mice. *Nature* 467(7318):977–981.

Other Supporting Information Files

Dataset S1 (XLS)