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Supplemental Information

Tsix RNA and the Germline Factor, PRDM14, Link X Reactivation and Stem Cell Reprogramming

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SUPPLEMENTAL FIGURES

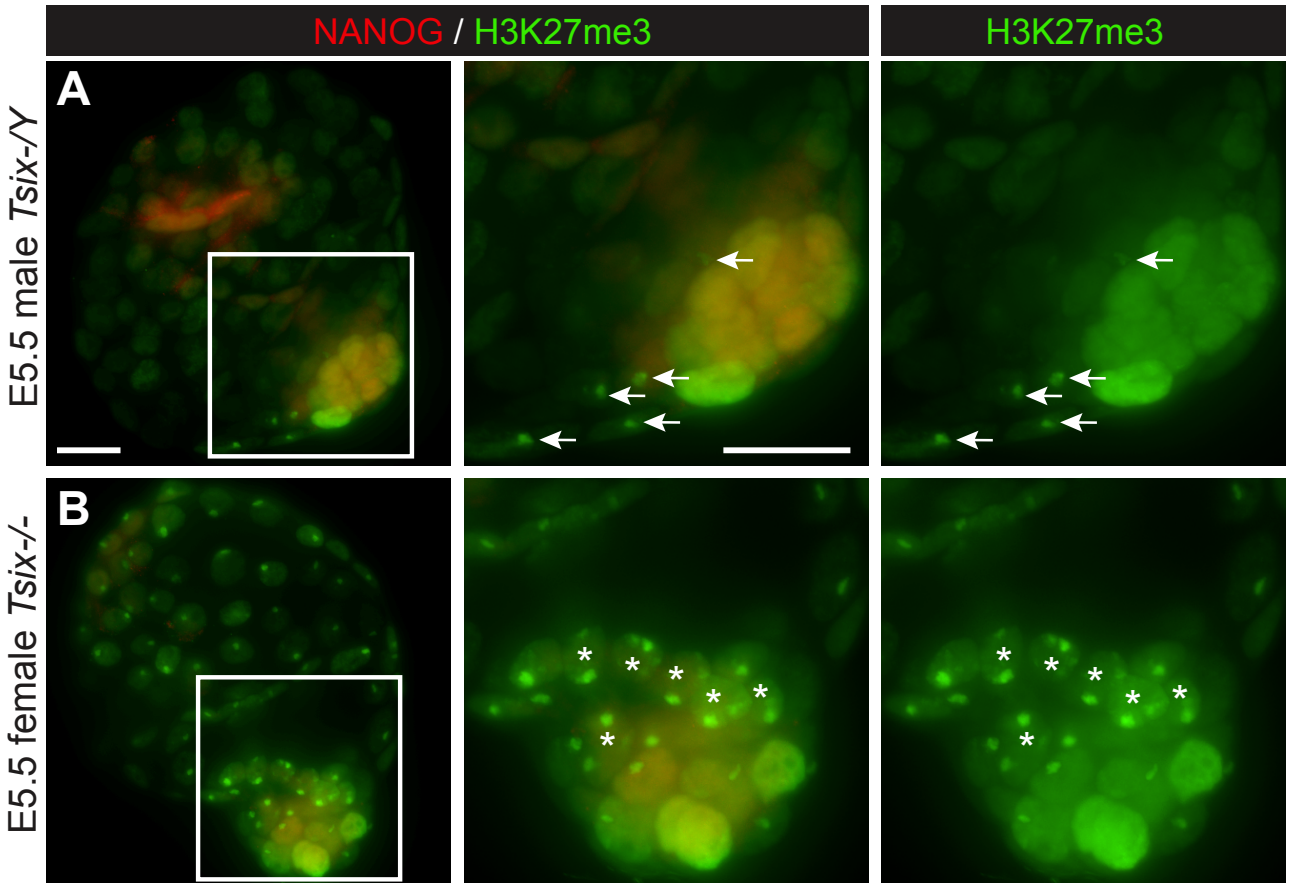

Figure S1, related to Figure 1. Ectopic XCI in *Tsix*-mutant E5.5 diapause embryos

(A) Male *Tsix*$^{-/Y}$ embryo displaying H3K27me3(green)-positive spots (arrows) suggesting ectopic XCI in some NANOG(red)-negative cells. The boxed ICM region is shown in a close-up in the middle and right panel. Projection of z-series (full embryo). Scale bars = 20 μm

(B) Female *Tsix*$^{-/-}$ embryo with two H3K27me3-spots in several extraembryonic cells (stars, PE and/or TE cells). Projection of 20 μm thick z-stack (partial embryo).

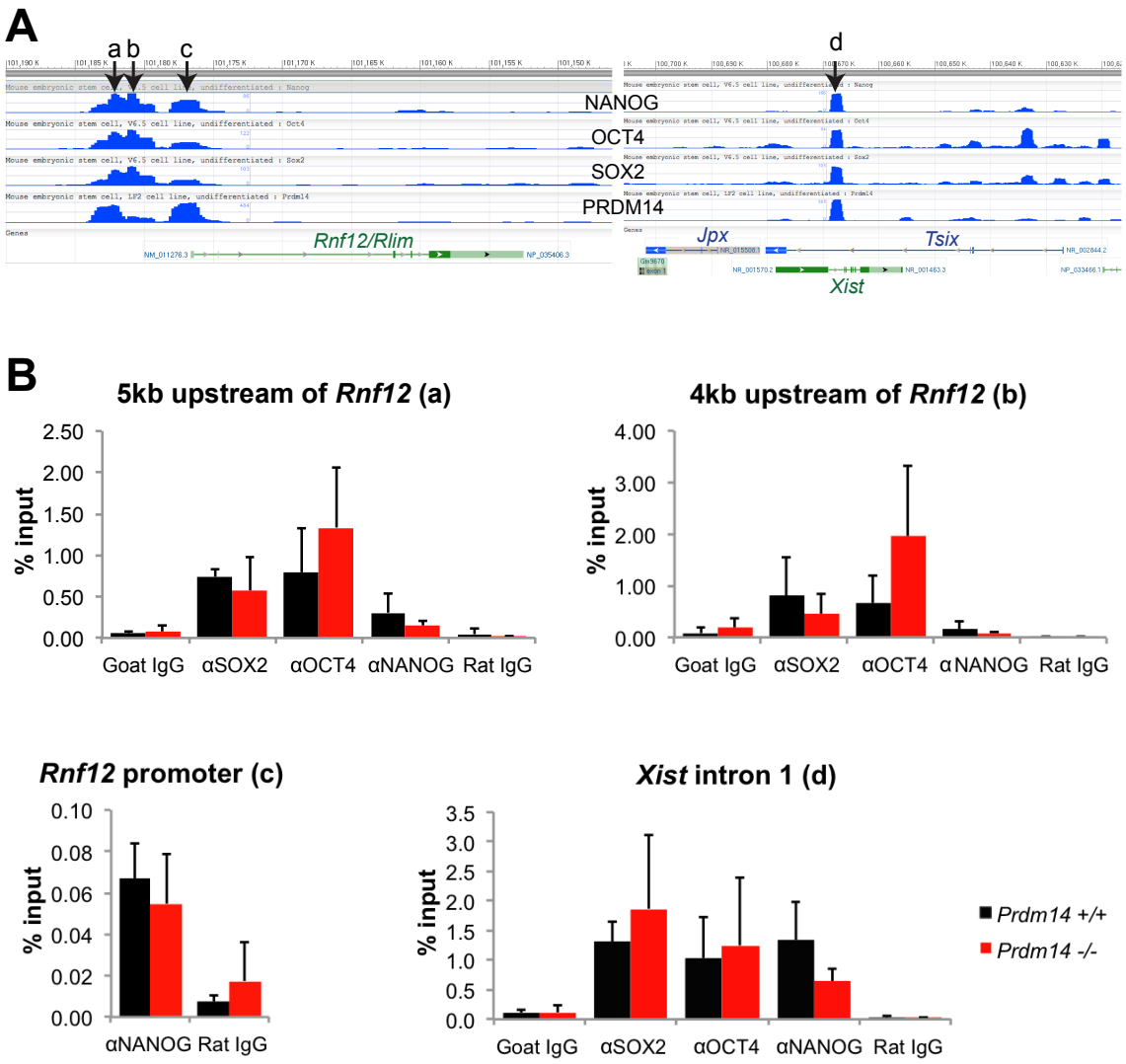

Figure S2, related to Figure 7. Pluripotency factor binding across the *Rnf12* locus and the *Jpx/Xist/Tsix* region

(A) ChIP-seq data (also shown in Figure 7B) for binding of NANOG, OCT4, SOX2 (Marson et al., 2008) and PRDM14 (Ma et al., 2010) along the *Xic*, retrieved via the NCBI epigenomics database. Arrows indicate binding sites assayed in (B).

(B) ChIP-qPCR for pluripotency factor binding 5' of *Rnf12* (a, b, c) and at *Xist* intron 1 (d) in undifferentiated *Prdm14*$^{+/+}$ (black) and *Prdm14*$^{-/-}$ (red) ESCs. Error bars = SD. Goat IgG = negative control for αOCT4 and αSOX2. Rat IgG = negative control for αNANOG.

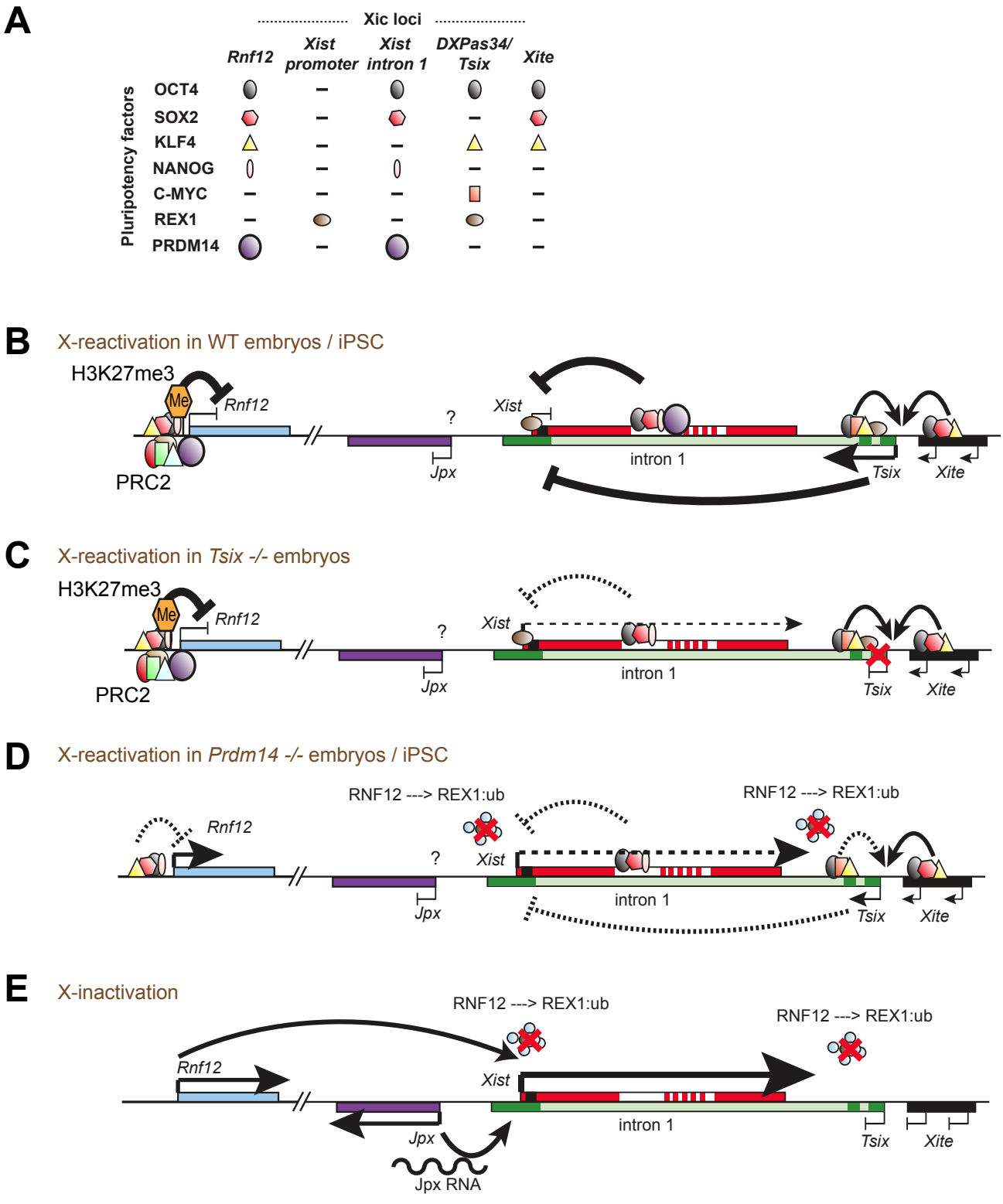
A
Xic loci
Rnf12
Xist promoter
Xist intron 1
DXPas34/ Tsix
Xite
Pluripotency factors
OCT4
SOX2
KLF4
NANOG
C-MYC
REX1
PRDM14
B
X-reactivation in WT embryos / iPSC
H3K27me3
Me
Rnf12
PRC2
?
Jpx
Xist
intron 1
Tsix
Xite
C
X-reactivation in Tsix -/- embryos
H3K27me3
Me
Rnf12
PRC2
?
Jpx
Xist
intron 1
Tsix
Xite
D
X-reactivation in Prdm14 -/- embryos / iPSC
RNF12 ---> REX1:ub
RNF12 ---> REX1:ub
Rnf12
?
Jpx
Xist
intron 1
Tsix
Xite
E
X-inactivation
RNF12 ---> REX1:ub
RNF12 ---> REX1:ub
Rnf12
Xist
Jpx
Jpx RNA
intron 1
Tsix
Xite

Figure S3, related to Figure 7. Extended model for molecular mechanisms of XCR

(A, B) XCR and XCI are controlled by multiple mechanisms. The master regulator *Xist* is the target of opposing factors. Several pluripotency factors bind to regulatory regions of *Rnf12*, *Xist* and *Tsix*, where they act either as activators or repressors. One of those factors is PRDM14, which acts repressively by binding to *Xist* intron1 and by recruiting PRC2 to *Rnf12*, which PRC2 silences through establishing H3K27 tri-methylation. *Tsix*, which is activated by pluripotency factors, in turn represses *Xist,* at least in part by recruiting PRDM14 to *Xist* intron 1. In addition to these repressive forces, the lack of the expression of *Xist*-activators RNF12 and Jpx contribute to the *Xist* "off" state during XCR. The question mark indicates that nothing is known about the regulation of *Jpx*.

(C) In the absence of *Tsix*, PRDM14 dissociates from *Xist* intron 1. During XCR in blastocysts, *Xist*-repression is thereby delayed. However, in *Tsix*$^{-/-}$ mutant pluripotent stem cells, *Xist* is not upregulated due to the lack of RNF12 and Jpx and by repression through other pluripotency factors.

(D) In *Prdm14*-mutant cells/embryos, *Rnf12* gets de-repressed and its protein ubiquitinates REX1, another *Xist* and *Tsix* regulator, resulting in REX1 degradation. Due to other repressive pluripotency factors this is not sufficient to lead to upregulation of *Xist* in *Prdm14*$^{-/-}$ ESCs. However, in *Prdm14*$^{-/-}$ blastocysts and during iPSC reprogramming this leads to a reduced XCR-efficiency.

(E) In cells before or after XCR, the lack of pluripotency factor-driven repressive mechanisms and the expression of the *Xist* activators RNF12 and Jpx lead to full upregulation of *Xist*.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Expression analysis by qPCR

ESC and iPSC RNA was isolated and DNase I-treated using RNeasy Mini kit (Qiagen). RNA was then reverse transcribed with Oligo(dT)$_{15}$ primers (Promega) and Superscript III reverse transcriptase (Invitrogen). QPCR on cDNA of at least three biological replicates each for *Prdm14*$^{+/+}$ and *Prdm14*$^{-/-}$ ESCs was performed on an Bio-Rad iCycler using iQ SYBR Green Supermix (Bio-Rad). Ct values were normalized to Gapdh in order to calculate expression changes (Pfaffl, 2001).

Stable transfection of ESCs

2x10^{7} EL 16.7 and *Tsix*$^{-StopTST}$ (Ogawa et al., 2008) mouse ESCs were electroporated with 30 mg of linearized pCAGGS plasmid vector containing recombinant PRDM14 fused to C-terminal Flag and Hemagglutinin tags (PRDM14-FH) (Yamaji et al., 2013), in PBS using GenePulser II (Bio-Rad) as described (Anguera et al., 2011). Cells were selected using media supplemented with 1μg/ml Puromycin (Gibco). PRDM14-FH expression was confirmed by Western Blot using anti-HA tag antibody (Sigma, H-6908).

Chromatin immunoprecipitation

EL 16.7, *Tsix*-Stop, *Prdm14*$^{+/+}$ or *Prdm14*$^{-/-}$ mouse ESCs were trypsinized and cross-linked with 1.1% formaldehyde. 5x10^{6} cells were subjected to chromatin immunoprecipitation as described previously (Jeon and Lee, 2011) using specific antibodies (see below). Enrichment was quantified using a Bio-Rad iCycler.

List of antibodies used for immunostainings

Antibody	Dilution
Alexa Fluor 488/555/647 secondary antibodies (Invitrogen)	1:500
goat anti-GATA4 (Santa Cruz, sc-1237))	1:50
mouse anti-H3K27me3 (Abcam, ab6002)	1:100
rabbit anti-NANOG (Novus Biologicals, NB100-588)	1:750
rat anti-NANOG (eBioscience, 14-5761)	1:200
mouse anti-SSEA1 (Developmental Studies Hybridoma Bank, University of Iowa, MC-480)	1:200

Primer list for quantitative RT-PCR

Target	Primers	Primer Sequence
Dnmt3b[1]	Dnmt3bBP1F	CTC GCA AGG TGT GGG CTT TTG TAA C
	Dnmt3bBP1R	CTG GGC ATC TGT CAT CTT TGC ACC
Dnmt3l[1]	Dnmt3lBP1F	CCA GGG CAG ATT TCT TCC TAA GGT C
	Dnmt3lBP1R	TGA GCT GCA CAG AGG CAT CC
Gapdh[1]	GapdhBP1F	ATG AAT ACG GCT ACA GCA ACA GG
	GapdhBP1R	CTC TTG CTC AGT GTC CTT GCT G
Gata6[1]	Gata6BP1F	CAC AGT CCC CGT TCT TTT ACT G
	Gata6BP1R	GTG GTA CAG GCG TCA AGA GTG
Jpx[2]	Jpx76+	TTA GCC AGG CAG CTA GAG GA
	Jpx255-	AGC CGT ATT CCT CCA TGG TT
Nanog[1]	NanogBP1F	CTT TCA CCT ATT AAG GTG CTT GC
	NanogBP1R	TGG CAT CGG TTC ATC ATG GTA C
Oct4[1]	Oct4BP1F	GAT GCT GTG AGC CAA GGC AAG
	Oct4BP1R	GGC TCC TGA TCA ACA GCA TCA C
Rnf12[3]	Rnf12 ex4-5 for	GGT CCA CCA CCA CAG AGC
	Rnf12 ex4-5 rev	TGA CCA CTT CTT GTT GTA TTT CC
Sox2[1]	Sox2BP1F	CAT GAG AGC AAG TAC TGG CAA G
	Sox2BP1R	CCA ACG ATA TCA ACC TGC ATG G
Tsix[4]	TsixBP6F	TGG GTC ATT GGC ATC TTA GTC
	TsixBP6R	CCC AGG GTG TCT GAT CTC TT
Xist[5]	XistBP2F	CCC GCT GCT GAG TGT TTG ATA TG
	XistBP2R	CAG AGT AGC GAG GAC TTG AAG AG

The primer sequences have been previously described in [1](Kurimoto et al., 2006), [2](Sun et al., 2013), [3](Barakat et al., 2011), [4](Sugimoto et al., 2007) and [5](Shibata and Lee, 2003).

List of antibodies used for ChIP experiments

Antibody	Isotype Control	Amount per Reaction (mg)
Anti-Histone H3K27me3 rabbit polyclonal (Active Motif, cat# 19155)	Rabbit IgG (Abcam, ab46540)	5
Anti-Sox2 (Y-17) goat polyclonal antibody (Santa Cruz, sc-17320)	Normal goat IgG (Santa Cruz, sc-2028)	5
Anti-Oct3/4 (N-19) goat polyclonal antibody (Santa Cruz, sc-8628)	Normal goat IgG (Santa Cruz, sc-2028)	5
Anti-Mouse Nanog clone eBioMLC-51 rat monoclonal antibody (eBioscience, cat# 14-5761)	Rat IgG2A (Abcam, ab18450)	2.5

Primer list for genomic loci tested on ChIP experiments

Amplicon	Primers	Primer Sequence
β-actin promoter[1]	b_actin_prom_F	CCG TTC CGA AAG TTG CCT T
	b_actin_prom_R	CGC CGC CGG GTT TTA TA
5' *Rnf12*	RNF12_P14_3F	AGC GCC AGC TCG GAG ACG TA
	RNF12_P14_3R	GGC CTG TGA AGC TGG GAG CG
4kb upstream to *Rnf12*[1]	RNF12_4kb_F	CAG CCT CTG GCT CTA CCA GT
	RNF12_4kb_R	GTG ACC TGC TGG GGA GAA TA
5kb upstream to *Rnf12*[1]	RNF12_5kb_F	GCC TGT CAA ACG TCC TGT TTA
	RNF12_5kb_R	GGA GGT TGT GGG AGA AAC AA
H3K27me3 upstream to *Rnf12*	RNF12_K27_F	CTC CCA AAT GAC CCT TCC CC
	RNF12_K27_R	TGA GAG GAC TGC AAG AAG GC
Xist Intron 1[2]	XIn1_F	AAC CCT TTT AAG TCC ACT GTA AAT TCC
	XIn1_R	TAG AGA GCC AGA CAA TGC TAA GCC
Xist Intron 1 (Mus-specific)	Xin1_ASP_F	CTA GAG AGC CAG ACA ATG CT
	Xin1_ASP_R2 (Mus)	TTT TGC ATT TGC CTT TTG AA
Xist Intron 1 (Cas-specific)	Xin1_ASP_F3	AAA TGT TTC CTT TTG AAG CA
	Xin1_ASP_R1 (Cas)	TTT TGC ATT TGC CTT TTG AT

[1] Primer sequences for regions 4 kb and 5 kb upstream to *Rnf12* transcription start site as well as control non-binding *β-actin* promoter region were adopted from (Navarro et al., 2011).

[2] *Xist* intron 1 primer pair was described in (Navarro et al., 2008).

SUPPLEMENTAL REFERENCES

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