Stem Cell Reports

Supplemental Information

Stable X Chromosome Reactivation in Female Human Induced Pluripotent Stem Cells

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Supplemental Experimental Procedures

hiPS cell generation and culture

To generate iPSC, $1x10^5$ human fibroblasts from lines X12 (p12), X15 (p4), and 47,XXX (p14) were seeded per well of a 6-well plate. The next day, cells were transduced with a polycistronic lentiviral reprogramming vector (Warlich et al., 2011) for 16 hours in the presence of 4 µg/ml polybrene (Millipore). Cells were further cultured in fibroblast medium at 20% O_2. At day 4 post-transduction, $5x10^4$ cells were seeded on irradiated MEFs in fibroblast medium. One day later, medium was changed to standard human ESC medium. From day 2 to 9 medium was supplemented with 2 mmol/l valproic acid (Calbiochem) as described by Warlich et al. (2011). iPSC colonies were picked between day 22 to 25 and further propagated on MEFs. Human iPSC lines growing on conventional/standard medium were passaged weekly using collagenase IV (1 mg/ml, Invitrogen) on γ-irradiated MEFs. All analysis for characterization of hiPSC was performed prior to cryopreservation.

All hESC and hiPSC cells were maintained in human ESC culture medium, consisting of DMEM/F12 (Gibco-Invitrogen) supplemented with 20% knock-out serum replacement (Gibco-Invitrogen), 2mM L glutamine, 1: 50 units of penicillin/streptomycin/glutamine, 0.1 mM MEM-non-essential amino acids (PAA Laboratories GmbH), 0.1mM β-mercaptoethanol (Sigma), and 10 ng/ml bFGF (Invitrogen) filtered through a 0.22µm filter (Corning). For differentiation experiments, hiPSC cells were differentiated in suspension culture for 1 week in EB medium, consisting of IMDM-glutamax, 15% foetal calf serum, 100 U ml penicillin, 100 mg ml streptomycin, 1% non-essential amino acids (all Invitrogen), 37.8 ml l monothioglycerol (Sigma) and 50 mg/ml ascorbic acid (Sigma).

NHSM medium and culture conditions were as described by Gafni et al., 2013. Wis-NHSM medium containing knockout DMEM (Invitrogen), 20% knockout serum (invitrogen), human insulin (Sigma, 12,5µg ml-1 final concentrstion), 10 µg recombinant human Lif (Peprotech), 8 ng ml-1 recombinant bFGF (Peprotech) and 1 ng ml-1 recombinant TGF-B1 (Peprotech), 1 mM glutamine (Invitrogen), 1% nonessential amino acids (Invitrogen), 0.1 mM beta-mercaptoethanol (Sigma), penicillin-streptomycin (Invitrogen) and small molecule inhibitors: PD0325901 (1µM, ERK1/2i, Axon Medchem); CHIR99021 (3µM, GSKβi, Axon Medchem); SP600125 (10µM, JNKi, TORCIS) and SB203580 (10 µM, p38i, Axon Medchem) Y-27632 (5µM, ROCKi, Axon Medchem) and protein kinase C inhibitor G06983 (5 µM, PKCi, TOCRIS). Naïve hiPSC clones were grown on γ irradiated MEFs on gelatin coated plates and passaged by single-cell trypsinization (0.05% + EDTA) every 4 days.

RT-PCR and single cell RT-PCR

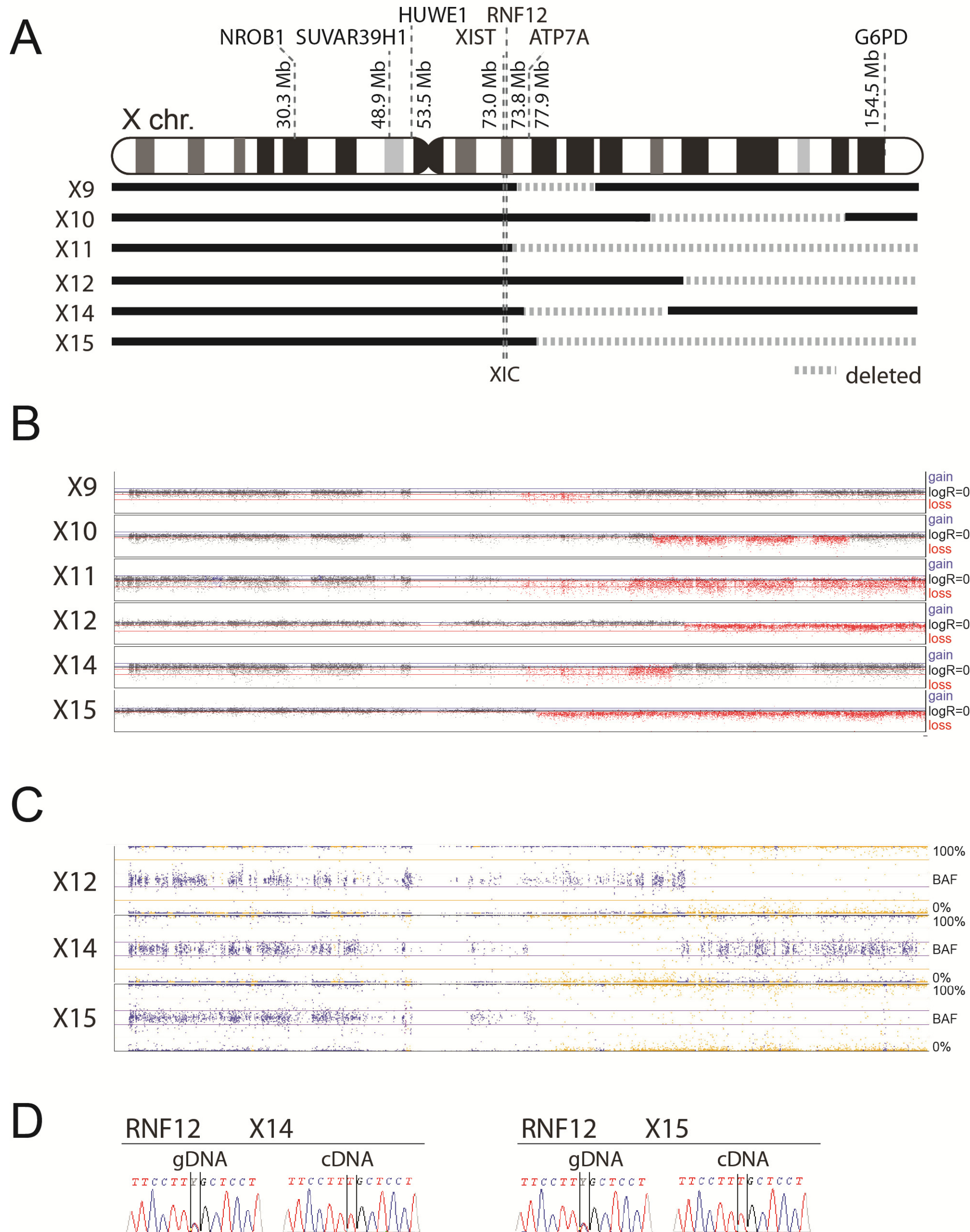
For single cell RT-PCR, cDNA was prepared with gene specific outer primers (a combination for four different genes per cell line), using the one-step RT-PCR kit (Invitrogen), according to manufacturer's instructions in a total volume of 16 µl. After an initial amplification using the outer primers, nested PCR was performed using the internal primers, and Phusion PCR polymerase (Fynnzyme). PCR products

were precipitated, and digested with the indicated restriction enzymes (New England Biolabs) to distinguish expression from the different alleles. RFLPs in *XIST*, *SUVAR39H1*, *G6PD*, and SNPs in *HUWE1*, *NROB1* and *ATP7A* were identified by PCR amplification and Sanger sequencing, followed by allele specific expression analysis using cDNA specific primer sets described in **Supplementary Table 2**.

HUMARA analysis

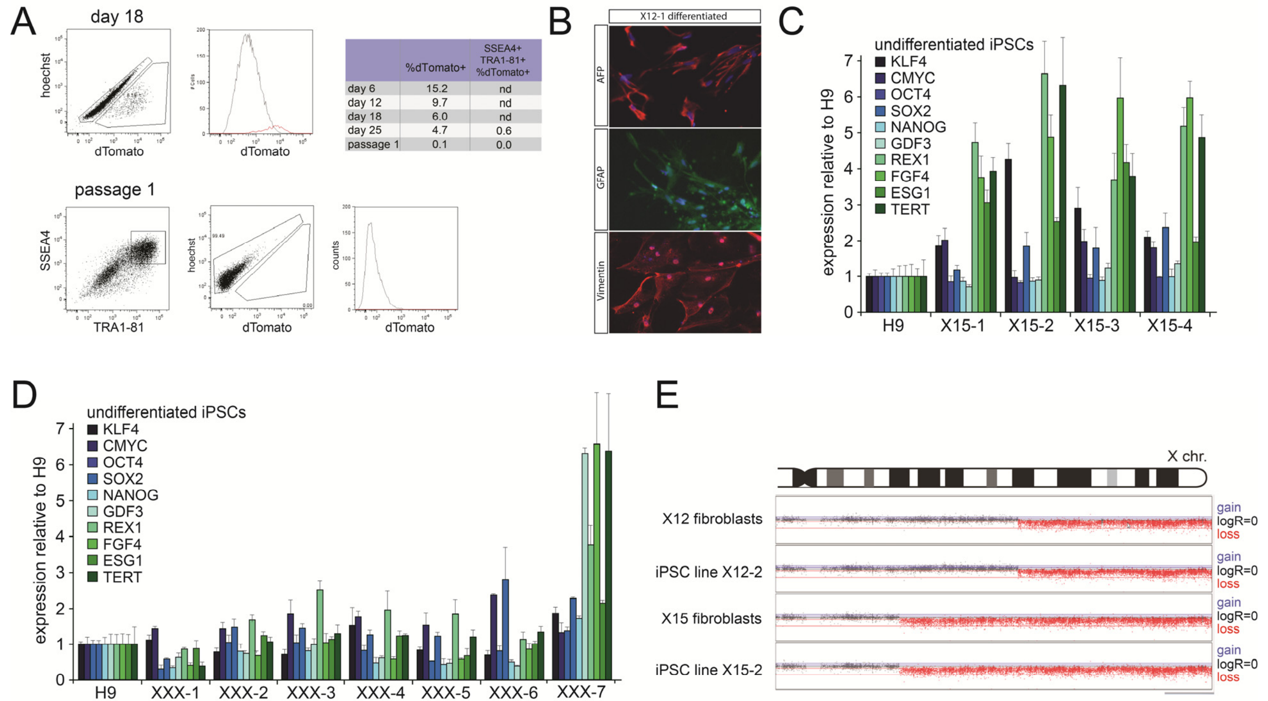
To determine skewing of XCI, allele specific methylation of the Androgen receptor gene promoter was quantified using the HUMARA assay (Human Androgen Receptor Assay) (Allen et al., 1994); 40ng of genomic DNA was double digested with DdeI and HpaII (New England Biolabs) at 37°C in an overnight reaction. 1μl of reaction product was used as a template for PCR using Phusion polymerase (Fynnzyme) using FAM-labelled primers GCTGTGAAGGTTGCTGTTCCTCAT and TCCAGAATCTGTTCCAGAGCGTGC. PCR products were visualized on a sequencer.

Supplemental Experimental Figures



***Supplemental Figure S1**: SNP Array of fibroblasts used to generate hiPSCs*

A) Schematic representation of the human X chromosome. Deleted areas are indicated with dashed lines. **B)** SNP Array analysis showing the deleted areas in fibroblast cell line X9, X10, X11 X12, X14, and X15. Every dot is representing a probe along the X chromosome. Deleted areas are indicated in red. **C)** SNP Array data showing loss of heterozygosity (areas marked in yellow, with B allele frequency (BAF) of 100% or 0%) in the deleted regions in fibroblast cell line X12, X14, and X15. **D)** Allele specific expression analysis of a SNP in *RNF12* indicates completely skewed XCI in X14 and X15 fibroblasts.



	%dTomato+	SSEA4+ TRA1-81+ %dTomato+
day 6	15.2	nd
day 12	9.7	nd
day 18	6.0	nd
day 25	4.7	0.6
passage 1	0.1	0.0

***Supplemental Figure S2**: Gene expression analysis of hiPS cell lines*

A) Transduced cells show a gradual loss of dTomato expression (right table). A small fraction of the cells is dTomato positive 18 days after transduction (left panels), but this expression is lost after picking of iPS clones (bottom panels and table). No dTomato/*SSEA4/TRA1-81* triple positive cells were detected at p1 (bottom panels and table). **B)** **I**mmunocyochemistry on day 8 differentiated EBs plated on chamber slides prior to fixation. Pictures show cells positive for *AFP* (Rhodamine red), *GFAP* (FITC) and *vimentin* (Rhodamine red, DAPI is in blue). **C,D)** qRT-PCR analysis of pluripotency factors in X15 hiPSC lines (1-4, **C**), and 47,XXX hiPSC lines (1-7, **D**) The expression of the same factors in the hESC line (H9) that served as a control was set at 100%. **E)** SNP Array analysis showing the deleted areas in fibroblast cell lines X12 and X15 and in hiPSC lines X12-2 and X15-2. Every dot is representing a probe along the X chromosome. Deleted areas are indicated in red.

X12-pool P0

SUVAR39H1 \ XIST	A	B	AB	no	total
A	13.5%	2.1%	1.0%	9.4%	26.0%
B	15.6%	0.0%	0.0%	4.2%	19.8%
AB	6.3%	0.0%	0.0%	6.3%	12.5%
no	30.2%	2.1%	1.0%	8.3%	41.7%
total	65.6%	4.2%	2.1%	28.1%	

X12-23 P3/4

SUVAR39H1 \ XIST	A	B	AB	no	total
A	3.1%	5.2%	1.0%	7.3%	16.7%
B	4.2%	2.1%	1.0%	17.7%	25.0%
AB	0.0%	2.1%	1.0%	3.1%	6.3%
no	3.1%	2.1%	1.0%	45.8%	52.1%
total	10.4%	11.5%	4.2%	74.0%	

X12-15 P3/4

SUVAR39H1 \ XIST	A	B	AB	no	total
A	4.2%	1.0%	1.0%	1.0%	7.3%
B	43.8%	6.3%	2.1%	25.0%	77.1%
AB	9.4%	2.1%	0.0%	2.1%	13.5%
no	2.1%	0.0%	0.0%	0.0%	2.1%
total	59.4%	9.4%	3.1%	28.1%	

X12-24 P3/4

SUVAR39H1 \ XIST	A	B	AB	no	total
A	4.2%	11.5%	8.3%	2.1%	26.0%
B	0.0%	0.0%	0.0%	0.0%	0.0%
AB	13.5%	35.4%	16.7%	7.3%	72.9%
no	0.0%	0.0%	0.0%	1.0%	1.0%
total	17.7%	46.9%	25.0%	10.4%	

X12-19 P3/4

SUVAR39H1 \ XIST	A	B	AB	no	total
A	1.0%	1.0%	0.0%	7.3%	9.4%
B	15.6%	9.4%	0.0%	35.4%	60.4%
AB	5.2%	1.0%	0.0%	6.3%	12.5%
no	2.1%	3.1%	0.0%	12.5%	17.7%
total	24.0%	14.6%	0.0%	61.5%	

X12-2 P25

SUVAR39H1 \ XIST	A	B	AB	no	total
A	5.7%	5.7%	5.7%	9.1%	26.1%
B	0.0%	0.0%	0.0%	0.0%	0.0%
AB	19.3%	11.4%	15.9%	12.5%	59.1%
no	5.7%	2.3%	3.4%	3.4%	14.8%
total	30.7%	19.3%	25.0%	25.0%	

X12-20 P3/4

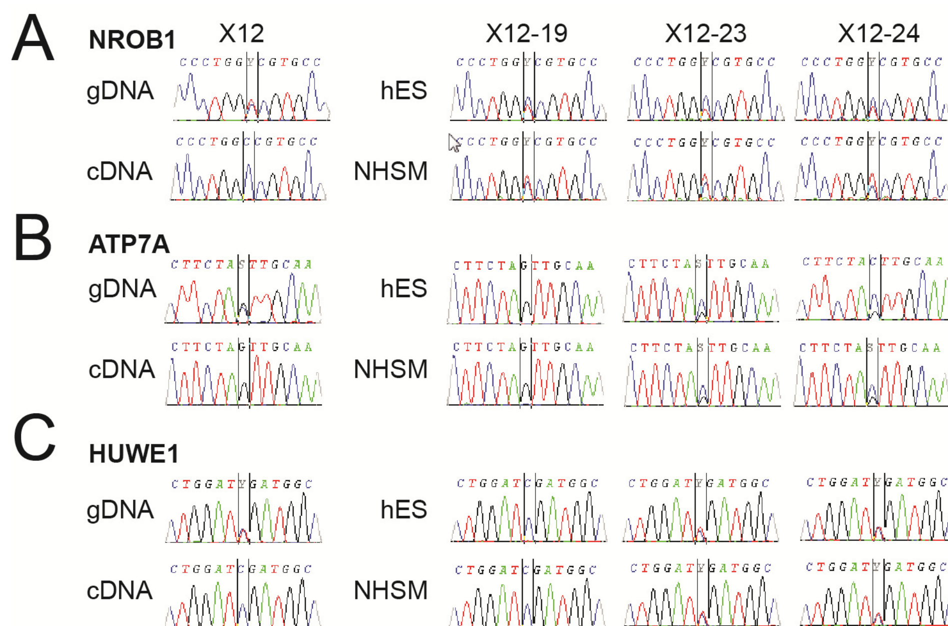
SUVAR39H1 \ XIST	A	B	AB	no	total
A	7.3%	2.1%	4.2%	5.2%	18.8%
B	19.8%	5.2%	3.1%	6.3%	34.4%
AB	13.5%	2.1%	3.1%	3.1%	21.9%
no	12.5%	5.2%	2.1%	5.2%	25.0%
total	53.1%	14.6%	12.5%	19.8%	

X12-4 P25

SUVAR39H1 \ XIST	A	B	AB	no	total
A	9.1%	2.3%	3.4%	1.1%	15.9%
B	0.0%	0.0%	0.0%	0.0%	0.0%
AB	40.9%	12.5%	23.9%	6.8%	84.1%
no	0.0%	0.0%	0.0%	0.0%	0.0%
total	50.0%	14.8%	27.3%	8.0%	

Supplemental Figure S3: Single cell allele specific expression analysis of XIST and SUVAR39H1

Quantification of the allele specific expression analysis of *SSEA1+/TRA1-81+* sorted cells from hiPSC lines at different time points (passages) after reprogramming. Allele specific expression of *XIST* and *SUVAR39H1* was assessed by digestion with MfeI and MspI distinguishing between both parental alleles as shown in **Figure 4D** (A= expression form allele A only, B=expression from allele B only, AB=bi-allelic expression and no=no expression detected) . Each part of the table shows the percentage of cells (total N=96) with specific expression characteristics.



D

	HUWE1	ATP7A	NROB1
gDNA X12	T/C	G/C	C/T
cDNA X12	C/C	G/G	C/C
X12-19 hES	C/C	G/G	C/T
X12-19 NHSM	C/C	G/G	C/T
X12-23 hES	T/C	G/C	C/T
X12-23 NHSM	T/C	G/C	C/T
X12-24 hES	T/C	G/C	C/T
X12-24 NHSM	T/C	G/C	C/T

Supplemental Figure S4: Allele specific expression analysis of X-linked genes in X12 hiPSC clones.

A,B,C) Sanger sequencing results of three X-linked genes (**(A)** *NROB1*, **(B)** *ATP7A* **(C)** *HUWE1*), on genomic DNA (gDNA), RNA isolated from X12 fibroblasts (cDNA), and RNA isolated from three different iPSC clones (X12-19, X12-23 and X12-24) grown in human ES (hES) and NHSM medium. **D)** Overview of the results obtained in **(A-C)**.

nr	code	source	karyotype	deleted	cell type	MLPA/array/ FISH	array	HUMARA	FISH
9	GM10254	Corriel	46,X,del(X)(q21.1q21.3)	77.5-91.0 Mb	lymphoblastoid		+	76%	-
10	GM09332	Corriel	46,X,del(X)(q22q26)	102.8-140.6 Mb	lymphoblastoid		+	50%	-
11	GM13277	Corriel	46,X,del(X)(q13)	75.0-156.0 Mb	lymphoblastoid		+	-	+
12	GM07148	Corriel	46,X,del(X)(q22.3)	108.9-156.0 Mb	fibroblast		+	100%	+
13	GM06563	Corriel	45,X[36]/46,X,r(X)(p22.3q24)[14]	#	fibroblast		-	96%	-
14	GM03923	Corriel	46,X,del(X)(q21.1q22)	78.7-103.7 Mb	fibroblast		+	98%	+
15	GM03827	Corriel	46X,del(X)(q21)	80.5-156.0 Mb	fibroblast		+	100%	+
16	86E1373	Erasmus MC	46,XX control		fibroblast		-	60%	-
17	EA1512	Erasmus MC	47,XXX		fibroblast		+	-	-

Supplemental Table S1: Overview of collected cell lines harboring X chromosomal deletions

This table provides an overview of the collected cell lines that were analyzed in this study. Shown is the source and code, the cell type and the outcome of the MLPA and HUMARA studies on the primary cells. Cells that were subjected to a SNP array analysis are also indicated (# mosaic, <30% detectable in MLPA and array).

hu_KDR–Flk-1-F	CTGGCATGGTCTTCTGTGAAGCA
hu_KDR–Flk-1-R	AATACCAGTGGATGTGATGGCGG
hu_PAX6-F	TTTGCCCGAGAAAGACTAGC
hu_PAX6-R	CATTTGGCCCTTCGATTAGA
hu_GATA2-F	TGACTTCTCCTGCATGCACT
hu_GATA2-R	AGCCGGCACCTGTTGTGCAA
hu_AFP- F	GAAACCCACTGGAGATGAACA
hu_AFP- R	CTGCAGCAGTCTGAATGTCC
hu_Alb1-F	GCTACGGCACAGTGCTTG
hu_Alb1-R	CAGGATTGCAGACAGATAGTC
hOCT3/4-S1165	GAC AGG GGG AGG GGA GGA GCT AGG
hOCT3/4-AS1283	CTT CCC TCC AAC CAG TTG CCC CAA AC
hSOX2-S1430	GGG AAA TGG GAG GGG TGC AAA AGA GG
hSOX2-AS1555	TTG CGT GAG TGT GGA TGG GAT TGG TG
h NANOG-S	CAG CCC CGA TTC TTC CAC CAG TCC C
h NANOG-AS	CGG AAG ATT CCC AGT CGG GTT CAC C
hGDF3-S243	CTT ATG CTA CGT AAA GGA GCT GGG
hGDF3-AS850	GTG CCA ACC CAG GTC CCG GAA GTT
hREX1-RT-S	CAG ATC CTA AAC AGC TCG CAG AAT
hREX1-RT-AS	GCG TAC GCA AAT TAA AGT CCA GA
hFGF4-RT-S	CTA CAA CGC CTA CGA GTC CTA CA
hFGF4-RT-AS	GTT GCA CCA GAA AAG TCA GAG TTG
h ESG1-S40	ATA TCC CGC CGT GGG TGA AAG TTC
h ESG1-AS259	ACT CAG CCA TGG ACT GGA GCA TCC
hTERT-S3234	CCT GCT CAA GCT GAC TCG ACA CCG TG
hTERT-AS3713	GGA AAA GCT GGC CCT GGG GTG GAG C
hKLF4-F	TGA TTG TAG TGC TTT CTG GCT GGG CTC C
hKLF4-R	ACG ATC GTG GCC CCG GAA AAG GAC C
human c-MYC-F	GCG TCC TGG GAA GGG AGT TCC GGA GC
human c-MYC-R	TTG AGG GGC ATC GTC GCG GGA GGC TG
SUVAR39 2192 nest for	AGGCACTGGGTAGAGCACCT
SUVAR39 2710 nest rev	TTTTATTGATGCCCACTCCA
hXIST SNP1 nest for	AATGGGCAAAGTGGTTATGC
hXIST SNP1 nest rev	AGGCCCCTTTCTCAAACTGT
SUVAR39-F	GCATAGGGTTGAGGGGTGTA
SUVAR39-R	TTTGTGCTCACCCTGGTTC
XIST SNP1 nested int F 6033	TTTCTTGGCCTCCCAATATG
XIST SNP1 nested int R 6610	CAGGAACCGGGACAAACA

ATP7A (gDNA)	AACACGATTGTTTTGGCTTAATCTCC
ATP7A (gDNA)	AGTGCAATAAACACAAACAGCATAGGG
ATP7A (cDNA)	TGGTTATGGACCACCACTTTGC
ATP7A (cDNA)	TTTTGCCCTTTGCTATATGTTCCAG
HUWE1 (gDNA)	TTTGCTGGGTAGAATTAAAGCTCTGC
HUWE1 (gDNA)	GGGCAAATACACAACACATAGAAAACG
HUWE1 (cDNA)	TGCTACCCGTGAAGTCCTTGG
HUWE1 (cDNA)	GCTCTGCATGGCCTGTACTTCC
NORB1 (gDNA)	TTTACCCCCTGGCCTCTGC
NORB1 (gDNA)	AGAGACGCGGCTGGTGGAT
NORB1 (cDNA)	ATGCTGACGAGCGCAAAGC
NORB1 (cDNA)	CTTGGTGCGTCATCCTGGTG
NORB1 (cDNA)	ATGCTGACGAGCGCAAAGC
NORB1 (cDNA)	CGGCACGTCCGGGTTAAAG
FAM-labelled primer1	GCTGTGAAGGTTGCTGTTCCTCAT
FAM-labelled primer2	TCCAGAATCTGTTCCAGAGCGTGC

Supplemental Table S2: Primers used in this study

A description of the primers that were used in the experiments described in this manuscript.