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Supplemental Information

X Chromosome of Female Cells Shows Dynamic Changes in Status during Human Somatic Cell Reprogramming

Kun-Yong Kim, Eriona Hysolli, Yoshiaki Tanaka, Brandon Wang, Yong-Wook Jung, and In-Hyun Park

Supplemental Figure Legends

Figure S1. SNPs and qPCR analysis of secondary iPSC clones generated from fibroblast-like cells differentiated from mono-allelic iPSCs. (A) *GRPR* SNP in secondary iPSC clones derived from dfD551-iPSCK1 fibroblast cell lines showed that iPSC clones showing express the same or opposite allelic specificity as parental fibroblasts, both alleles are produced. **(B)** The expression levels of XCI related (*XIST*, *EZH2*, *RNF12*) and X linked genes (*MECP2*, *HPRT*) in mono- or bi-allelic iPSCs. Relative expressions are normalized against β-*ACTIN* and bars indicate mean ± S.E.M of three independent assays with three of mono-iPSC and bi-iPSC clones. Statistical significance was analyzed with Student's t-test; $^{*}p < 0.01$. **(C-E)** secondary iPSC clones isolated from fibroblast-like cell lines differentiated from mono-allelic RTT-iPSCs express MECP2 on X chromosome in a same or opposite allelic specificity as parental fibroblasts, or express from both alleles.

Figure S2. Decrease of XCI markers during the reprogramming. (A-C) Loss of H3K27me3 foci during reprogramming. RTT3, IMR90 and WI38 fibroblasts were reprogrammed and fixed at the given days. Cells were then stained for H3K27me3 (red) and DAPI (blue). GFP represents the expression of retrovirus-mediated reprogramming factors. Arrows indicate H3K27me3 foci. Scale bar: 20 μm. **(D)** Expression of XCI-related (*XIST*, *RNF12*) and pluripotent genes (*NANOG*, *LIN28a*, *REX1*) during reprogramming. **(E)** Change in expression of pluripotent genes (*LIN28a* and *REX1*) in cells isolated using a combination of markers given below at different reprogramming stages. Relative expression of the given genes was calculated by normalized against β-*ACTIN* after qPCR. Error bars represent mean ± SD in triplicate reactions.

Figure S3. Analysis of X chromosome status in secondary iPSCs derived from LNS-syndrome fibroblasts with a mutation in *HPRT*. (A) Stable maintenance of inactive X

chromosome after drug selection. HAT-(fLNS-HAT) or 6TG-(fLNS-6TG) selected LNS fibroblasts were cultured with drugs for two weeks, and cultured with either drug. fLNS-HAT cells do not show resistance ot 6TG, and fLNS-6TG cells do not show resistance to HAT. **(B)** *GYG2* SNP analysis of secondary iPSC lines derived from df6TG-3($Xa^{HPRT-}Xi^{HPRT+}$) that was differentiated from 6TG-iPSC3 having 6TG-resistance and (C) SNP in *GYG2*. Secondary iPSC clones with C, T, or C/T SNPs were isolated, showing the generation of iPSC with X chromosome state converted. **(C)** Drug resistance of iPSC clones derived from fLNS-HAT ($Xa^{HPRT+}Xi^{HPRT-}$) that has an A SNP in *MAOA* gene on X chromosome. Clones with resistance to HAT (HAT-iPSC1) or to 6TG (HAT-iPSC2) were generated. The corresponding SNP in *MAOA* gene is shown in left column. Among HAT-resistant clones, iPSC clones with two active X chromosomes were isolated (HAT-iPSC5). Crystal violet staining was performed in iPSCs after HAT or 6TG selection for 14 days. **(D)** Drug resistance of secondary iPSCs derived from fibroblast-like cells (dfHAT-iPSC1, $Xa^{HPRT+}Xi^{HPRT-}$) differentiated from HAT-iPSC1 that is resistant to HAT. secondary iPSC clones resistant to HAT, or 6TG were isolated. Crystal violet staining of secondary iPSCs was performed after 14 days of drug selection. **(E)** SNP analysis of *GYG2* and *MAOA* genes present on X chromosome in secondary iPSCs derived from dfHAT-1. **(F)** Representative H3K27me3/OCT4 staining images of secondary iPSC derived from dfHAT-1. Secondary dHAT1-iPSC2 that is resistant to 6TG has H3K27me3 foci, while secondary dHAT1-iPSC6 expresses both alleles of *GYG2* and *MAOA* and has no H3K27me3 foci. Scale bar: 20 μm. **(G)** Southern blot analysis to identify the integration of provirus. Genomic DNA from each of 6TG-iPSC clones derived from 6TG selected fibroblasts were digested with EcoRI and SpeI, and hybrizided with *GFP*, *OCT4* and *SOX2* probes.

Figure S4. Tracing X chromosome status in nascent iPSC clones. (A) Representative images of H3K27me3/OCT4 staining with iPSC clones at the given passages. After picked up and passaged, RTT3-iPSC11 clone maintains the inactive X chromosome marker H3K27me3, while RTT3-iPSC13 clone loses the marker. White rectangular boxes on the upper right corner of RTT3-iPSC13 figures represent the magnified images of cellular border showing the presence and absence of H3K27me3 foci. Nuclei were counterstained with DAPI (blue). Scale bar: 20 μm. **(B)** Magnified images of H3K27me3 staining with Detroit-iPSCN1 clones at passages 2 and 3 as related with Figure 4A. White dotted line indicates the boundary between H3K27me3 foci positive and negative area. **(C)** FISH analysis for *XIST* RNA in mono-allelic RTT3-iPS11and bi-allelic RTT3-iPS13 clone at passages 10 and 12. Scale bar: 10 um. **(D-F)** Quantification of the percentage of established iPSC clones showing H3K27me3 foci-negative cells during passages as shown in Figure S4A. A total of ten RTT3-iPSCs, six HAT-iPSCs and six 6TG-iPSC clones were picked and expanded to trace the change of H3K27me3 status. Black, H3K27me3 foci-positive clones; Dark gray, clones mixed with H3K27me3 foci-positive and negative cells; Light gray, H3K27me3 foci-negative clones.

Figure S5. Gain of H3K27me3 foci in differentiated cells undergone XCI and a model of change in X chromosome status. (A) Induction of XCI-linked *RNF12* and concurrent decrease of pluripotency markers in cells differentiated from bi-allelic iPSC clones. Mono-allelic cells and H1 (ESCs) were used for control. β–*ACTIN* was used to normalize the changes in triplicate reactions. **(B)** Appearance of H3K27me3 foci-positive cells in fibroblast-likes cells differentiated from IMR-iPSC3 and RTT3-iPSC43 iPSC clones having no H3K27me3 foci. 6TG-iPSC6 cells do not show H3K27me3 foci after differentiation, suggesting that they are Class III iPSC. Arrows indicate H3K27me3 foci. Scale bar: 20 μm. **(C)** SNPs and analysis of iPSC clones

generated from 6TG drug selected fLNS-6TG cells. **(D)** Modeling showing the dynamics in X chromosome status in human somatic cell reprogramming. In addition to previously shown retention of the inactive X chromosome (XCI retention), here we showed that the inactive X chromosome becomes re-activated by overexpression of reprogramming factors (rXCR, reprogramming-XCR), marked by repression of XCI-genes (*XIST*, *RNF12*), loss of H3K27me3 foci and expression of genes that are located in the inactive X chromosome. Completion of reprogramming induces a random XCI. In some established iPSC clones, iPSC clones without the inactive X chromosome marks arise that include Class I iPSC having two active X chromosomes as well as Class III iPSCs having on active and one eroded X chromosome.

Figure S1

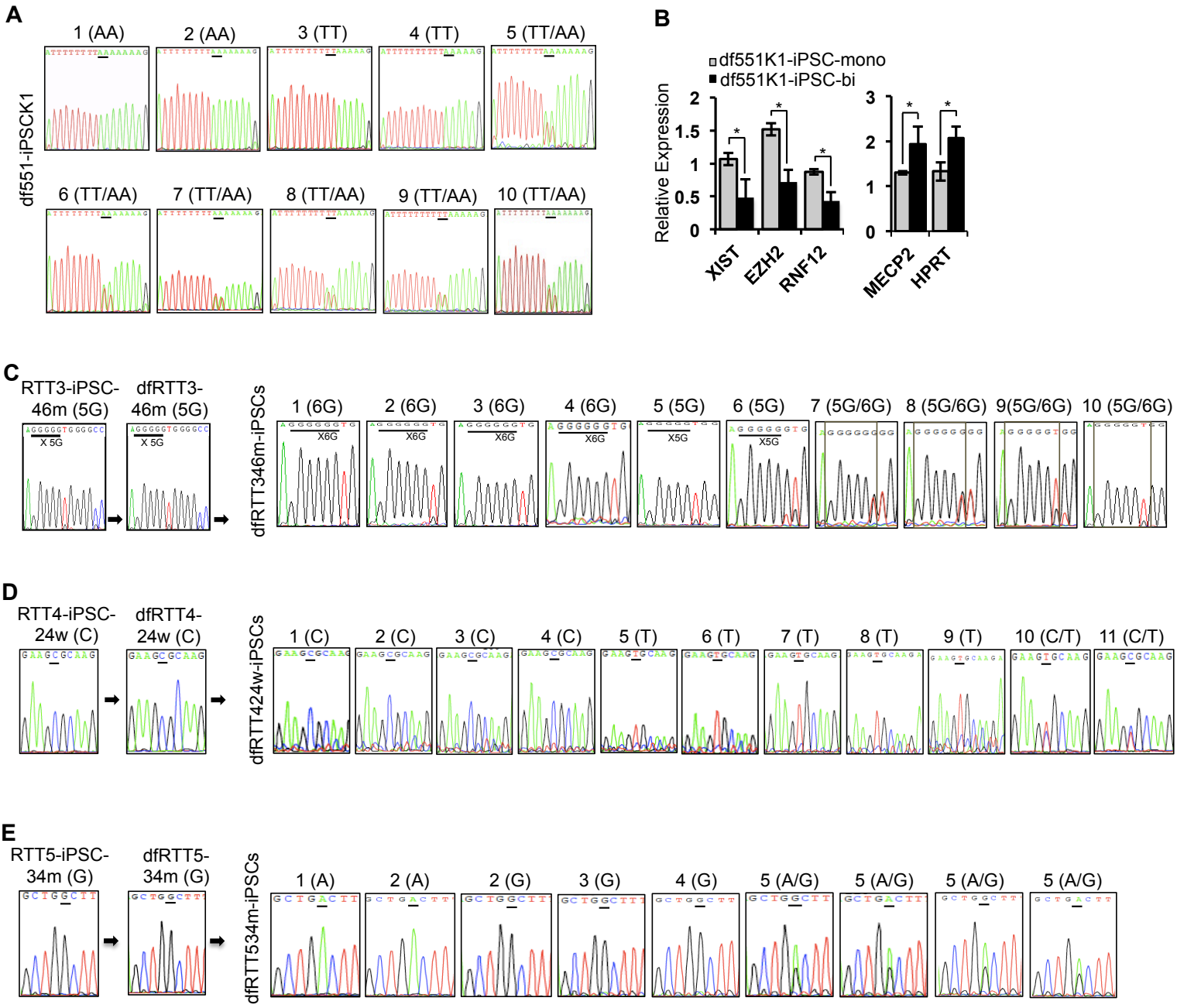
A
1 (AA)
2 (AA)
3 (TT)
4 (TT)
5 (TT/AA)
df551-iPSCK1
6 (TT/AA)
7 (TT/AA)
8 (TT/AA)
9 (TT/AA)
10 (TT/AA)
B
df551K1-iPSC-mono
df551K1-iPSC-bi
Relative Expression
XIST
EZH2
RNF12
MECP2
HPRT
C
RTT3-iPSC-46m (5G)
dfRTT3-46m (5G)
dfRTT346m-iPSCs
1 (6G)
2 (6G)
3 (6G)
4 (6G)
5 (5G)
6 (5G)
7 (5G/6G)
8 (5G/6G)
9(5G/6G)
10 (5G/6G)
D
RTT4-iPSC-24w (C)
dfRTT4-24w (C)
dfRTT424w-iPSCs
1 (C)
2 (C)
3 (C)
4 (C)
5 (T)
6 (T)
7 (T)
8 (T)
9 (T)
10 (C/T)
11 (C/T)
E
RTT5-iPSC-34m (G)
dfRTT5-34m (G)
dfRTT534m-iPSCs
1 (A)
2 (A)
2 (G)
3 (G)
4 (G)
5 (A/G)
5 (A/G)
5 (A/G)
5 (A/G)

Figure S2

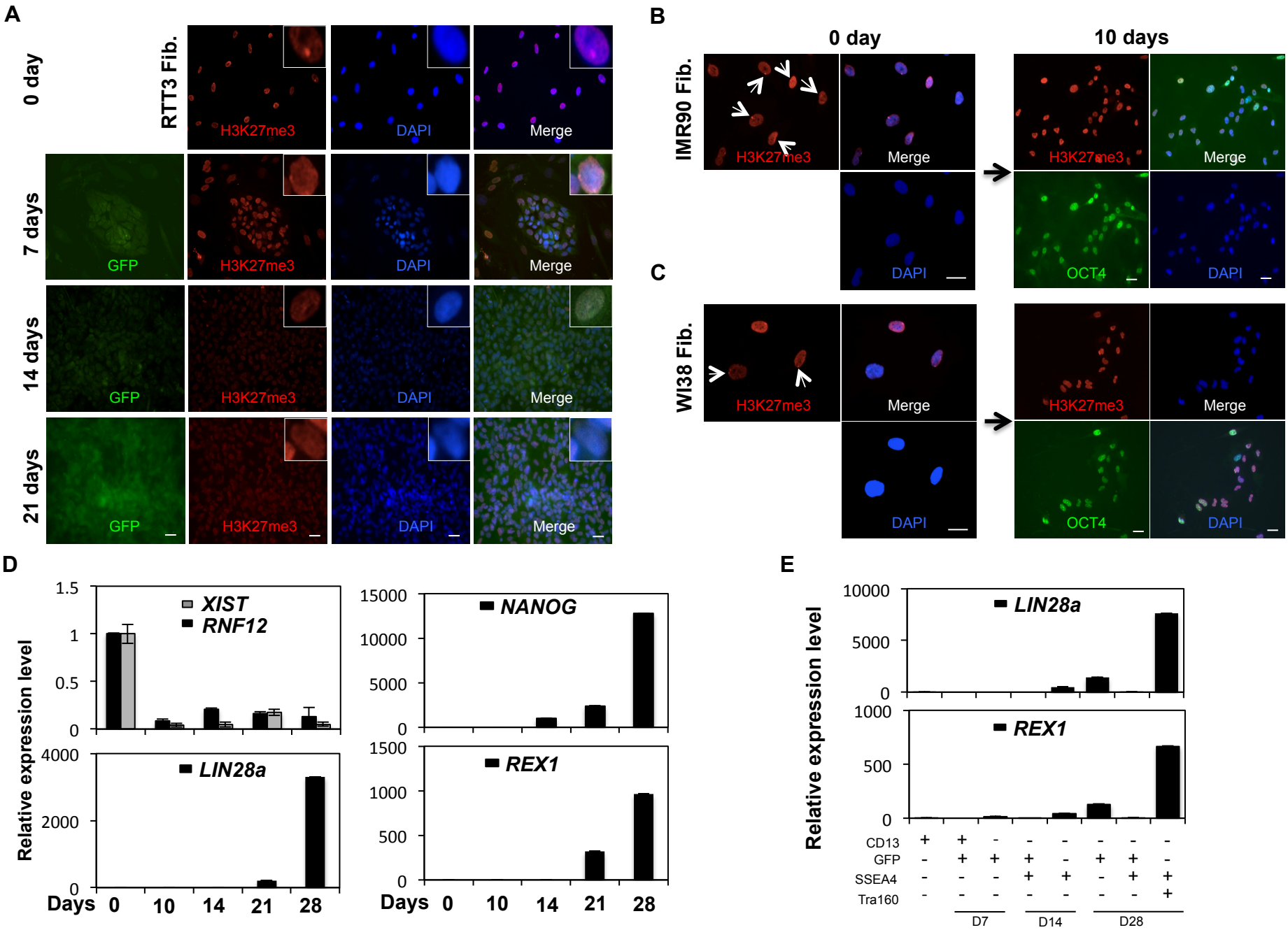
A
0 day
RTT3 Fib.
H3K27me3
DAPI
Merge
7 days
GFP
14 days
21 days
B
0 day
10 days
IMR90 Fib.
OCT4
C
WI38 Fib.
D
XIST
RNF12
NANOG
LIN28a
REX1
Relative expression level
Days 0 10 14 21 28
E
CD13
GFP
SSEA4
Tra160
D7
D14
D28

Figure S3

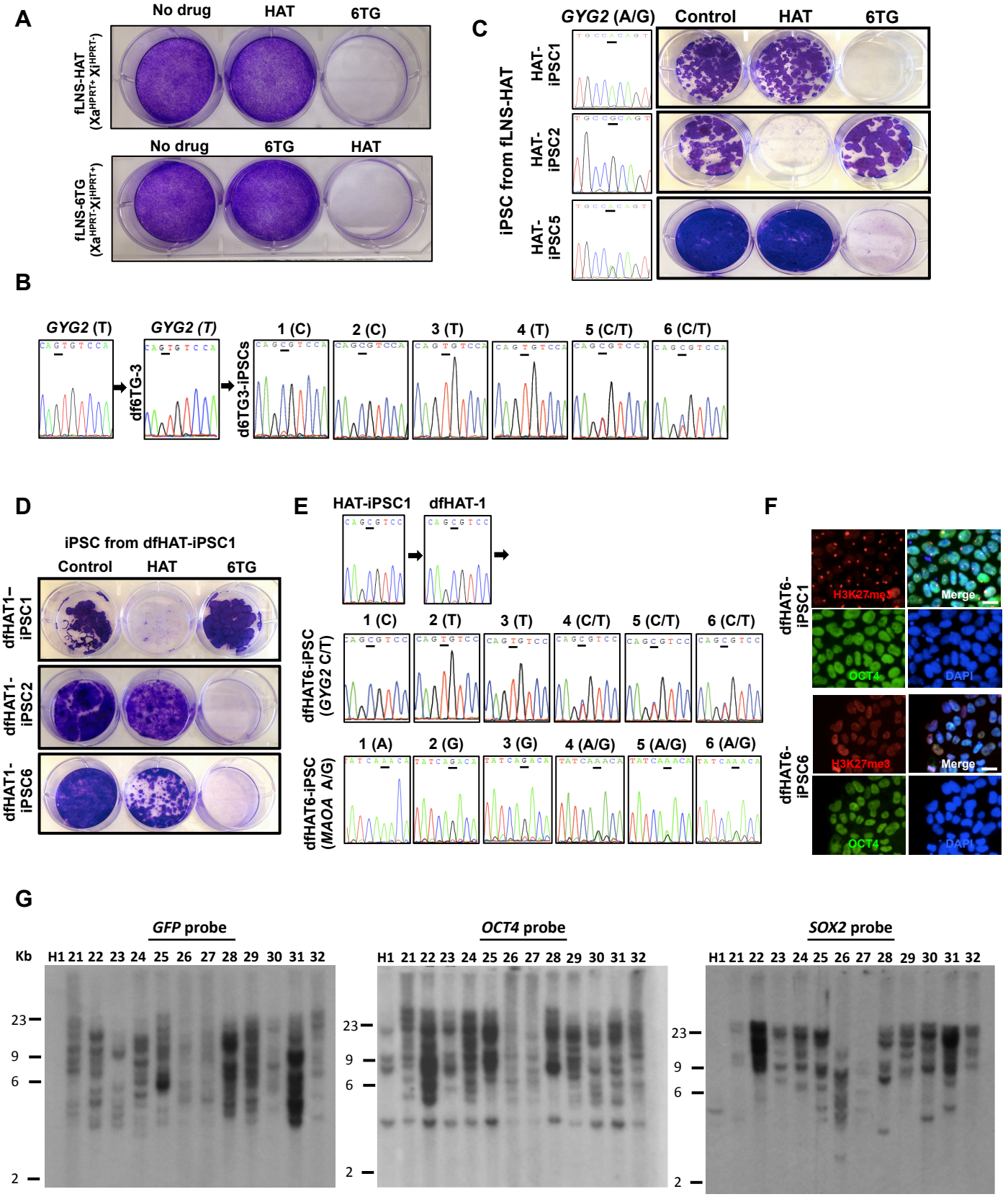
A
No drug
HAT
6TG
fLNS-HAT
(Xa^HPRT+ Xi^HPRT-)
No drug
6TG
HAT
fLNS-6TG
(Xa^HPRT- Xi^HPRT+)
B
GYG2 (T)
GYG2 (T)
df6TG-3
d6TG3-iPSCs
1 (C)
2 (C)
3 (T)
4 (T)
5 (C/T)
6 (C/T)
C
GYG2 (A/G)
Control
HAT
6TG
iPSC from fLNS-HAT
HAT-iPSC1
HAT-iPSC2
HAT-iPSC5
D
iPSC from dfHAT-iPSC1
Control
HAT
6TG
dfHAT1–iPSC1
dfHAT1-iPSC2
dfHAT1-iPSC6
E
HAT-iPSC1
dfHAT-1
1 (C)
2 (T)
3 (T)
4 (C/T)
5 (C/T)
6 (C/T)
dfHAT6-iPSC (GYG2 C/T)
1 (A)
2 (G)
3 (G)
4 (A/G)
5 (A/G)
6 (A/G)
dfHAT6-iPSC (MAOA A/G)
F
dfHAT6-iPSC1
H3K27me3
Merge
OCT4
DAPI
dfHAT6-iPSC6
H3K27me3
Merge
OCT4
DAPI
G
GFP probe
OCT4 probe
SOX2 probe
Kb
H1 21 22 23 24 25 26 27 28 29 30 31 32
23
9
6
2

Figure S4

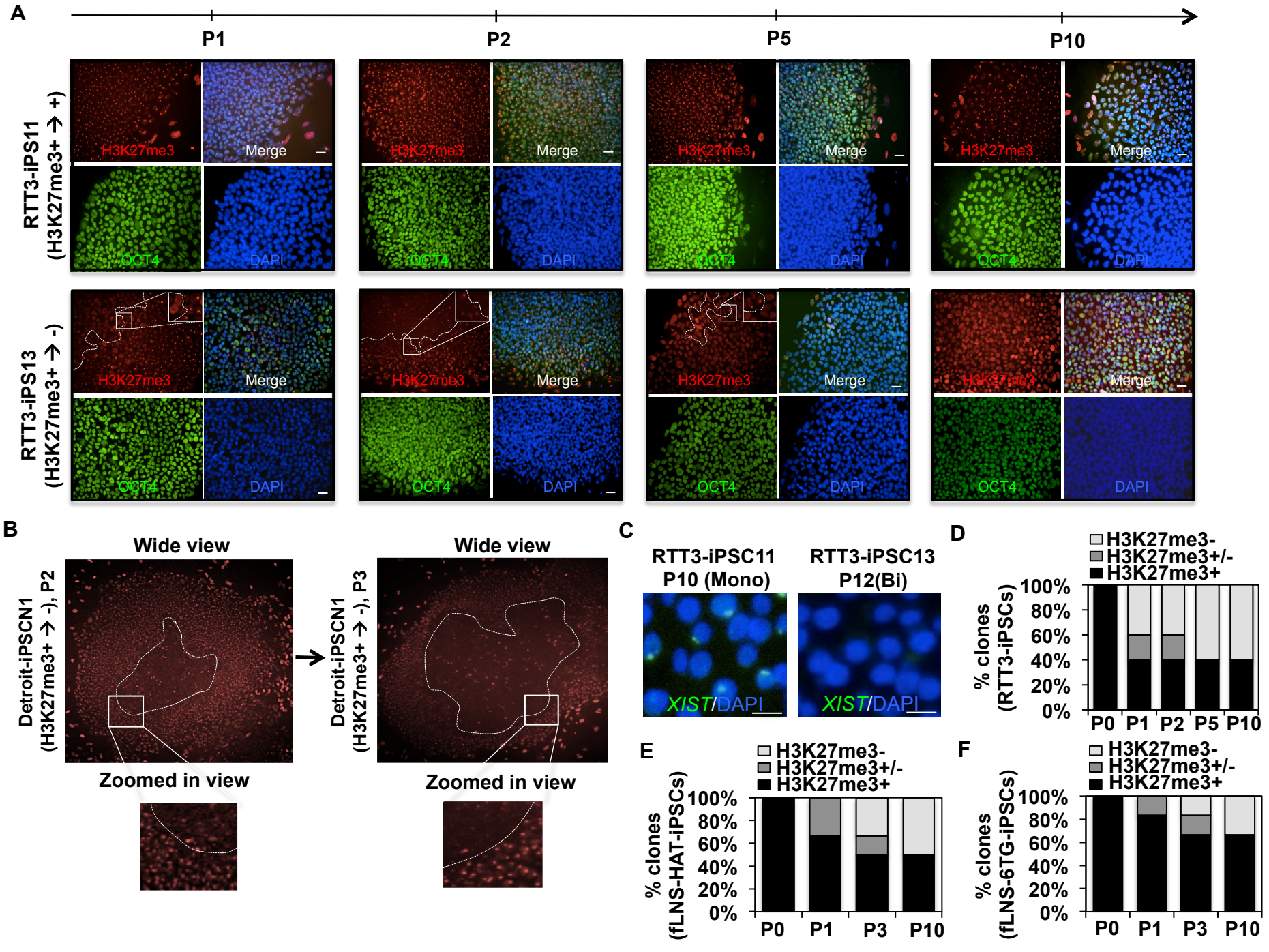
A
P1
P2
P5
P10
RTT3-iPS11
(H3K27me3+ → +)
RTT3-iPS13
(H3K27me3+ → -)
H3K27me3
Merge
OCT4
DAPI
B
Wide view
Detroit-iPSCN1
(H3K27me3+ → -), P2
Detroit-iPSCN1
(H3K27me3+ → -), P3
Zoomed in view
C
RTT3-iPSC11
P10 (Mono)
RTT3-iPSC13
P12(Bi)
XIST/DAPI
D
H3K27me3-
H3K27me3+/-
H3K27me3+
% clones
(RTT3-iPSCs)
100%
80%
60%
40%
20%
0%
P0
P1
P2
P5
P10
E
% clones
(fLNS-HAT-iPSCs)
P0
P1
P3
P10
F
% clones
(fLNS-6TG-iPSCs)
P0
P1
P3
P10

Figure S5

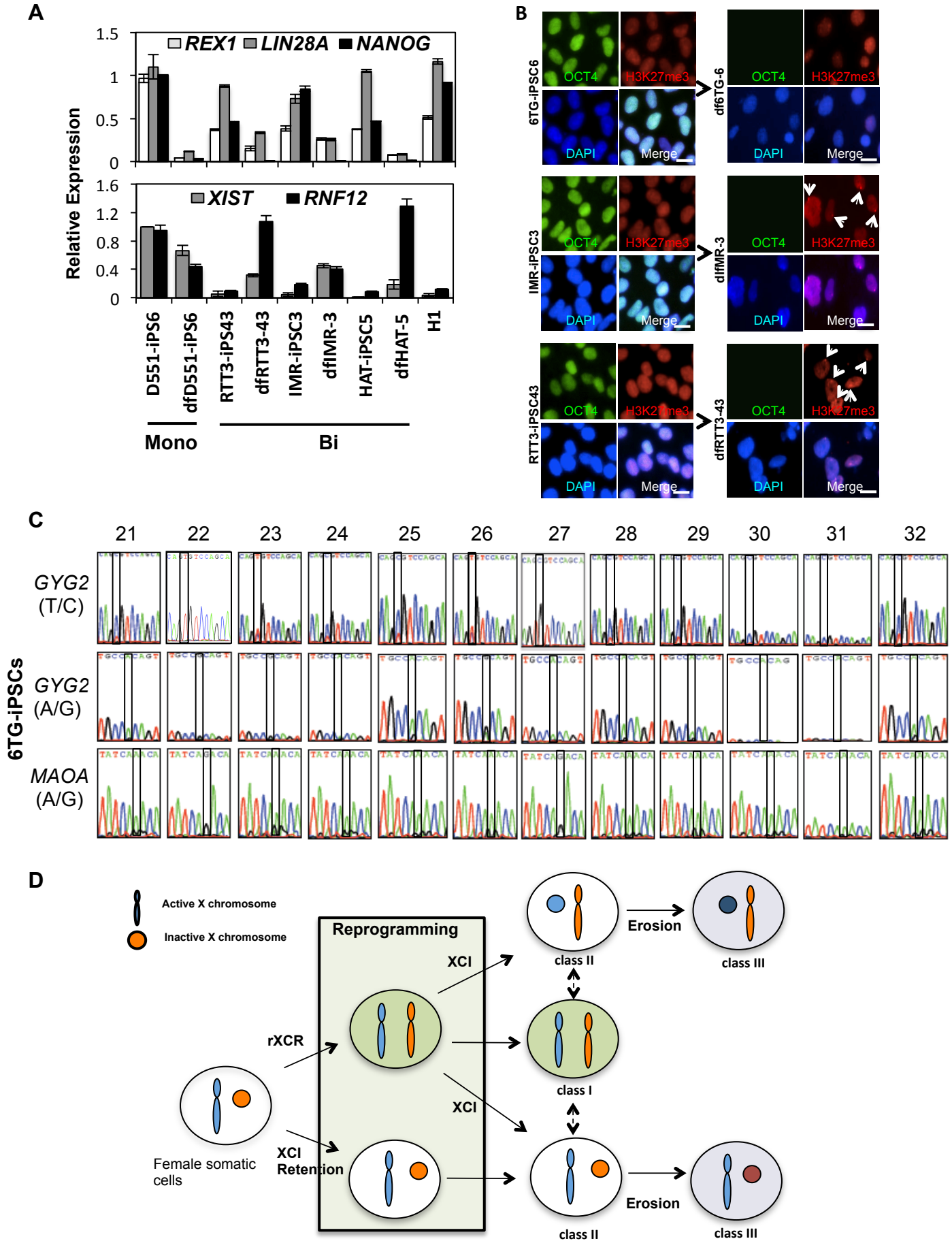
A
REX1
LIN28A
NANOG
XIST
RNF12
Relative Expression
D551-iPS6
dfD551-iPS6
RTT3-iPS43
dfRTT3-43
IMR-iPSC3
dfIMR-3
HAT-iPSC5
dfHAT-5
H1
Mono
Bi
B
6TG-iPSC6
df6TG-6
IMR-iPSC3
dfIMR-3
RTT3-iPSC43
dfRTT3-43
OCT4
H3K27me3
DAPI
Merge
C
6TG-iPSCs
GYG2 (T/C)
GYG2 (A/G)
MAOA (A/G)
21
22
23
24
25
26
27
28
29
30
31
32
D
Active X chromosome
Inactive X chromosome
Reprogramming
XCI
rXCR
XCI
XCI Retention
Female somatic cells
class II
Erosion
class III
class I
class II
Erosion
class III

Table S1. List of primers used for RT-qPCR

Gene	Forward	Reverse
MECP2	CCAGGACTTGAGCAGCAGCG	CGGGAAGCTTTGTCAGAGCCC
XIST	CGGTACGTTGAAGTTAGGGAATG	GTGCTGTATAATCCAATGGGTAG
EZH2	ACCGGTTGTGGGCTGCACAC	TGCAGCGGCATCCCGGAAAG
RNF12	ACCGATTGGATCGAGAAGAAGC	TGTAGTCGTCTCAGCAACTCT
NANOG	TGAACCTCAGCTACAAACAG	TGGTGGTAGGAAGAGTAAAG
REX1	GCGTCATAAGGGGTGAGTTTT	AGAACATTCAAGGGAGCTTGC
ACTIN	TGAAGTGTGACGTGGACATC	GGAGGAGCAATGATCTTGAT
***OCT4* total**	AGCGAACCAGTATCGAGAAC	TTACAGAACCACACTCGGAC
***OCT4* endo**	CCTCACTTCACTGCACTGTA	CAGGTTTTCTTTCCCTAGCT
***OCT4* ecto**	CCTCACTTCACTGCACTGTA	CCTTGAGGTACCAGAGATCT
***SOX2* total**	AGCTACAGCATGATGCAGGA	GGTCATGGAGTTGTACTGCA
***SOX2* endo**	CCCAGCAGACTTCACATGT	CCTCCCATTTCCCTCGTTTT
***SOX2* ecto**	CCCAGCAGACTTCACATGT	CCTTGAGGTACCAGAGATCT
***MYC* total**	ACTCTGAGGAGGAACAAGAA	TGGAGACGTGGCACCTCTT
***MYC* endo**	TGCCTCAAATTGGACTTTGG	GATTGAAATTCTGTGTAACTGC
***MYC* ecto**	TGCCTCAAATTGGACTTTGG	CGCTCGAGGTTAACGAATT
***KLF4* total**	TCTCAAGGCACACCTGCGAA	TAGTGCCTGGTCAGTTCATC
***KLF4* endo**	GATGAACTGACCAGGCACTA	GTGGGTCATATCCACTGTCT
***KLF4* ecto**	GATGAACTGACCAGGCACTA	CCTTGAGGTACCAGAGATCT

Table S2. List of primers used for allelic expression and for probe for Southern Blot

Gene	Forward	Reverse
MECP2 for RTT3 RTT4	AGGTAGGCGACACATCCCT	CTTACAGGTCTTCAGGACCTT
MECP2 for RTT5	AGAAACGGGGCCGAAAGCCG	CGGGAAGCTTTGTCAGAGCC
GRPR	AGCCCTGTTAAATGGTCGTG GCC	ATGGTGGAATGGCACCCTGGATGA
HPRT	TGTGGCCATCTGCCTAGTAA	CAGCCAACACTGCTGAAACA
GYG2	CAGCACGCCATGGAACACGGCA	AGGCTGAATCCGCAC ACGG CC
MAOA	ACTCCCTGCTTAGCTCTGTGGG	GTGGGCACCCTTGTGGGCCGAC
GFP **probe**	GGTGAGCAAGGGCGAGGAGC	CAGGGTGTCGCCCTCGAACTTC
OCT4 **probe**	TGGAGGTGATGGGCCAGG	CCGGGTTTTGCTCCAGCT
SOX2 **probe**	GGCCCGCAGCAAACTTCG	GGGCCAGCAGCCCGCCGG

Supplemental Experimental Procedures

Cell Culture and Reprogramming.

Reprogramming was performed as previously described (Kim et al., 2011). In short, retrovirus expressing OCT4, SOX2, KLF4 and c-MYC were generated in 293T cells by transfecting 0.25 ug VSVG, 2.25 ug GAG-POL, and 2.5 ug pMIG vector expressing each of four genes in 10 cm plate. X-tremeGENE 9 (Roche) was used for transfection following manufacturer's instruction. 1 x 10^5 cells in one well of 6-well plate were infected with retrovirus expressing reprograming factors with MOI=5 in the presence of 10 ug/ml protamine sulfate. Five days after infection, cells were plated on irradiated MEFs and replenished with human ESC medium every day. When were formed, iPSCs were picked up by 10 uL Pipetman onto plate pre-plated with MEFs and maintained according to standard protocol (Park et al., 2008).

Isolation of cells undergoing reprogramming

Following reprogramming with pMIG retrovirus expressing reprogramming factors, cells were harvested at day 7, 14, and 28 by treating with accutase. Cells at 7 days after reprogramming were stained in FACS buffer (1% FBS in 1XPBS) for 30 minutes at 4°C with antibodies recognizing CD13 (BD Pharmigen cat# 555394), and SSEA4 (R&D cat# FAB1435A). Cells at 14 days and 28 days after reprogramming were stained for CD13 (BD Pharmigen cat# 555394), SSEA4 and TRA160 (BD Pharmigen cat# 560193). Stained cells were sorted according to the combined expression of cell surface markers and retroviral GFP expression using Yale Stem Cell Center FACS Core.

Gene Expression and SNP Analysis

Gene expression analysis was performed with real-time PCR using iQSYBR Green Supermix (BioRad) with primers listed in Table S1. The data were analyzed by using comparative threshold cycle (CT) method and normalized to β-*ACTIN*. All qPCR assays were performed in

triplicates. To identify SNPs, X-linked *GRPR*, *MAOA*, *GYG2* and *MECP2* genes were amplified from cDNA by PCR using primers in Table S2 and subjected to Sanger sequencing using the Keck DNA Sequencing Facility at Yale School of Medicine or restriction digestion.

Immunostaining

iPSCs, or cells undergoing reprogramming were fixed for 10 min at room temperature with 4% paraformaldehyde in PBS, and permeabilized by using 0.2% Triton X-100 in PBS for 5 min. After blocking with 3% BSA blocking buffer in PBS, cells were incubated with primary antibodies for OCT4 (Ab19857, Abcam), H3K27me3 (#9733, Cell Signaling Technologies), and/or MECP2 (C-17, Santa Cruz) for 2hr at RT or overnight at 4 °C in blocking solution. Cells were then incubated with Alexa-488 (A11008, Invitrogen) and Alexa-546 (A21422, Invitrogen) labeled secondary antibodies and stained with DAPI.

FISH for *XIST* and *ATRX*

RNA FISH was carried out as described previously (Tchieu et al., 2010). Briefly, iPSCs were on gelatin coated coverslips and permeabilized by means of sequential transfer into twice ice-cold PBS for 30 sec, ice-cold CSK buffer (100mM NaCl, 300 mM sucrose, 3mM MgCl2 and 10 mM pH 6.8 PIPES and RNase-inhibitor RNasin) for 30 sec, and then CSK buffer containing 0.2% Triton X-100 buffer for 15 min, followed twice with CSK buffer for 30 sec each. Cells were then fixed with 4% formaldehyde in PBS for 15 min and dehydrated through sequential changes of 70%, 85% 95% and 100% EtOH for 3 min each, followed by air drying before hybridization of *XIST* and *ATRX* probe. For the *XIST* and *ATRX* probe, double-strand shorter DNA probes were generated form G1A plasmid (Clemson et al., 1996) and BACs (RP11-1145J4 and RP11-42M11). Probes were labeled with fluorescein-12-dUTP using the Prime-It Fluor Labeling kit (Stratagene). The probe was then hybridized with the prepared sample at 37 °C overnight in a humidified chamber.

Western Blot

Cells were lysed directly in RIPA lysis buffer (20 mM pH 7.5 Tris-HCl, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate and protease inhibitor cocktail) and western blot analysis was performed with antibody recognizing the C-terminus of MECP2 (C-17, Santa Cruz). β-ACTIN was used as loading control.

Southern Blot

The genomic DNA was isolated from individual iPSC clones according to the method previously published (Laird et al., 1991). Total 5 μg of genomic DNA was digested overnight with EcoRI and SpeI according to a standard protocol, separated in 0.8% gel, and transferred to Nylon membrane. *GFP, OCT4* and *SOX2* probes were generated by performing PCR in pMSCV vectors using primers listed on Table S2. PCR products were purified using Qiagen PCR purification kit and labeled with α-^{32P}-dCTP according to the Prime-it II random primer labeling kit manual (Agilent). Blots were hybridized (MyracleHyb, Stratagene) overnight to detect the presence of the integrated provirus encoding *GFP, OCT4*, or *SOX2.*

SUPPLEMENTAL REFERENCES

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