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Supplemental Information

FMR1 Epigenetic Silencing Commonly Occurs in Undifferentiated Fragile X-Affected Embryonic Stem Cells

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Fig. S1

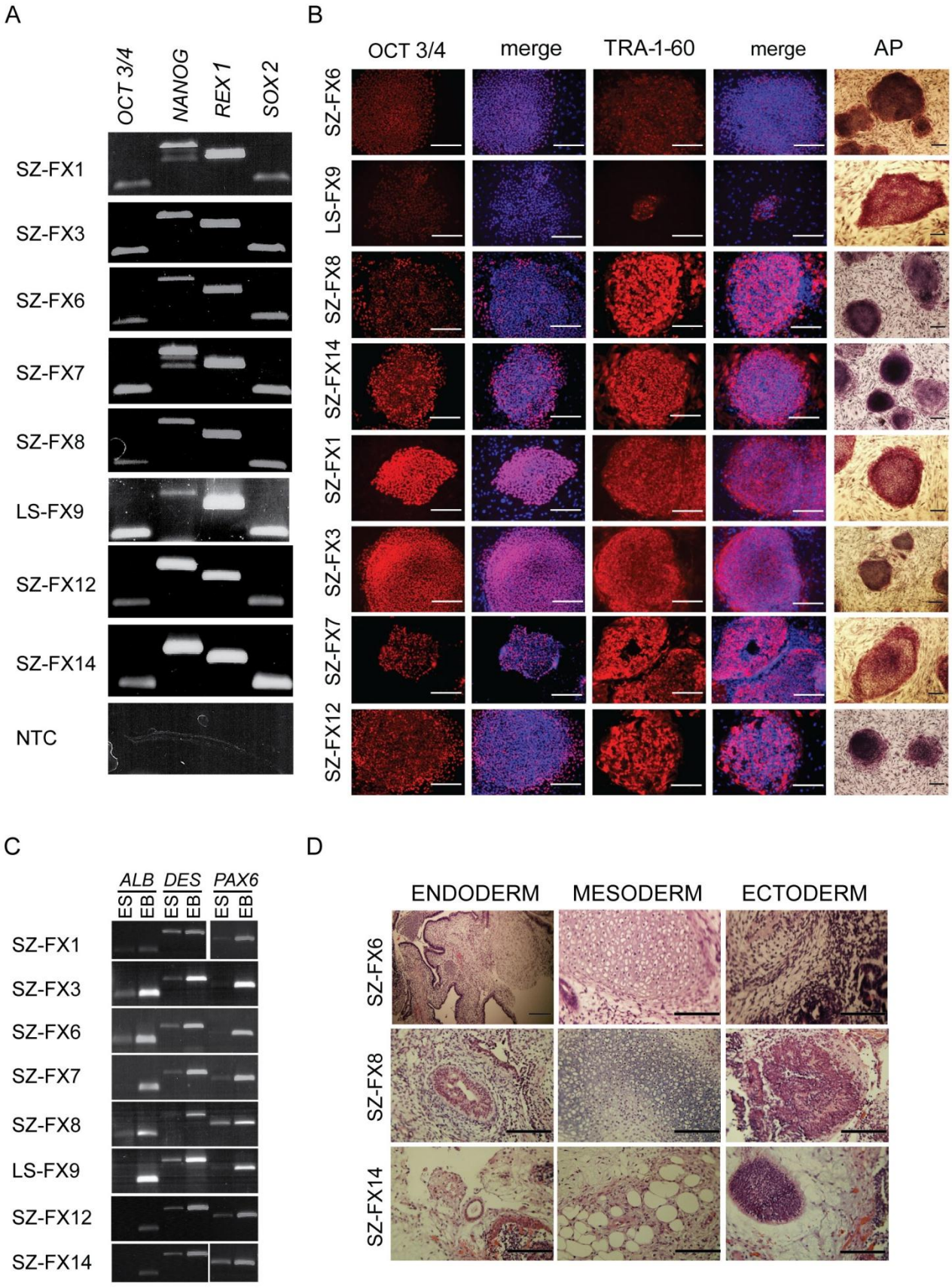
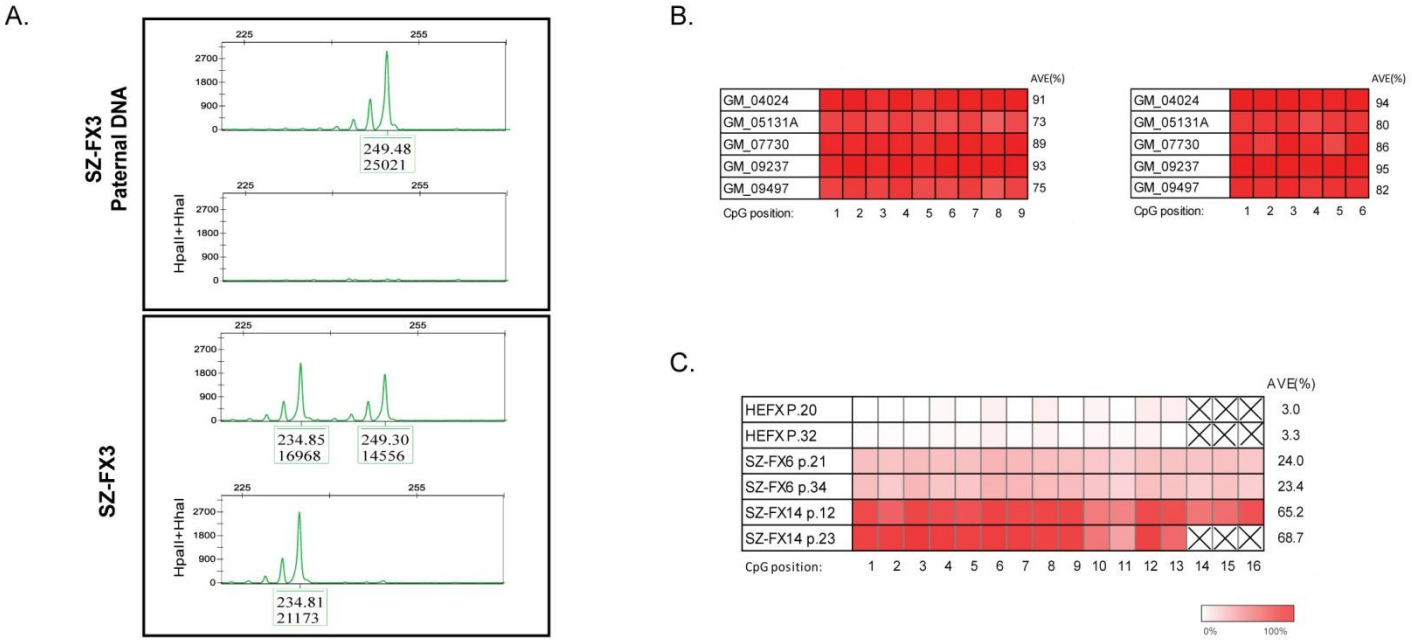

A
OCT 3/4
NANOG
REX 1
SOX 2
SZ-FX1
SZ-FX3
SZ-FX6
SZ-FX7
SZ-FX8
LS-FX9
SZ-FX12
SZ-FX14
NTC
B
OCT 3/4
merge
TRA-1-60
merge
AP
SZ-FX6
LS-FX9
SZ-FX8
SZ-FX14
SZ-FX1
SZ-FX3
SZ-FX7
SZ-FX12
C
ALB
DES
PAX6
ES
EB
SZ-FX1
SZ-FX3
SZ-FX6
SZ-FX7
SZ-FX8
LS-FX9
SZ-FX12
SZ-FX14
D
ENDODERM
MESODERM
ECTODERM
SZ-FX6
SZ-FX8
SZ-FX14

Fig. S2


A.
SZ-FX3
Paternal DNA
SZ-FX3
HpaII+HhaI
249.48
25021
234.85
16968
249.30
14556
234.81
21173
B.
AVE(%)
GM_04024
GM_05131A
GM_07730
GM_09237
GM_09497
91
73
89
93
75
CpG position: 1 2 3 4 5 6 7 8 9
94
80
86
95
82
CpG position: 1 2 3 4 5 6
C.
AVE(%)
HEFX P.20
HEFX P.32
SZ-FX6 p.21
SZ-FX6 p.34
SZ-FX14 p.12
SZ-FX14 p.23
3.0
3.3
24.0
23.4
65.2
68.7
CpG position: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
0%
100%

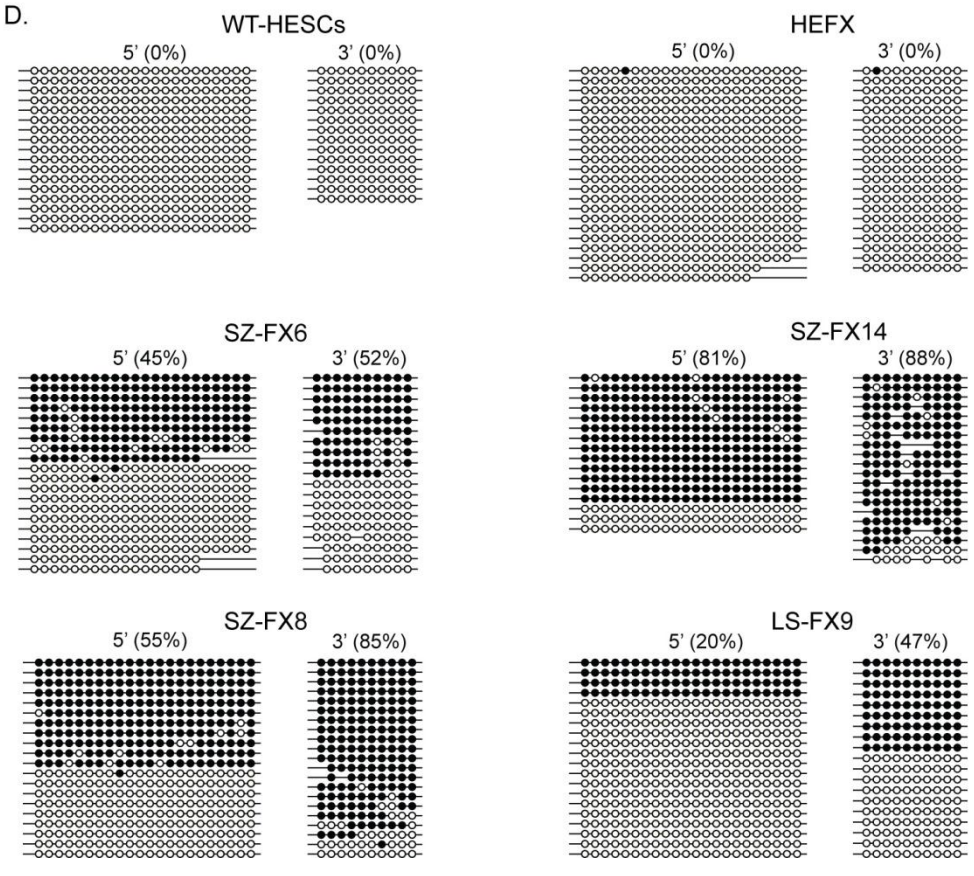

D.
WT-HESCs
5' (0%)
3' (0%)
HEFX
5' (0%)
3' (0%)
SZ-FX6
5' (45%)
3' (52%)
SZ-FX14
5' (81%)
3' (88%)
SZ-FX8
5' (55%)
3' (85%)
LS-FX9
5' (20%)
3' (47%)

Fig. S3

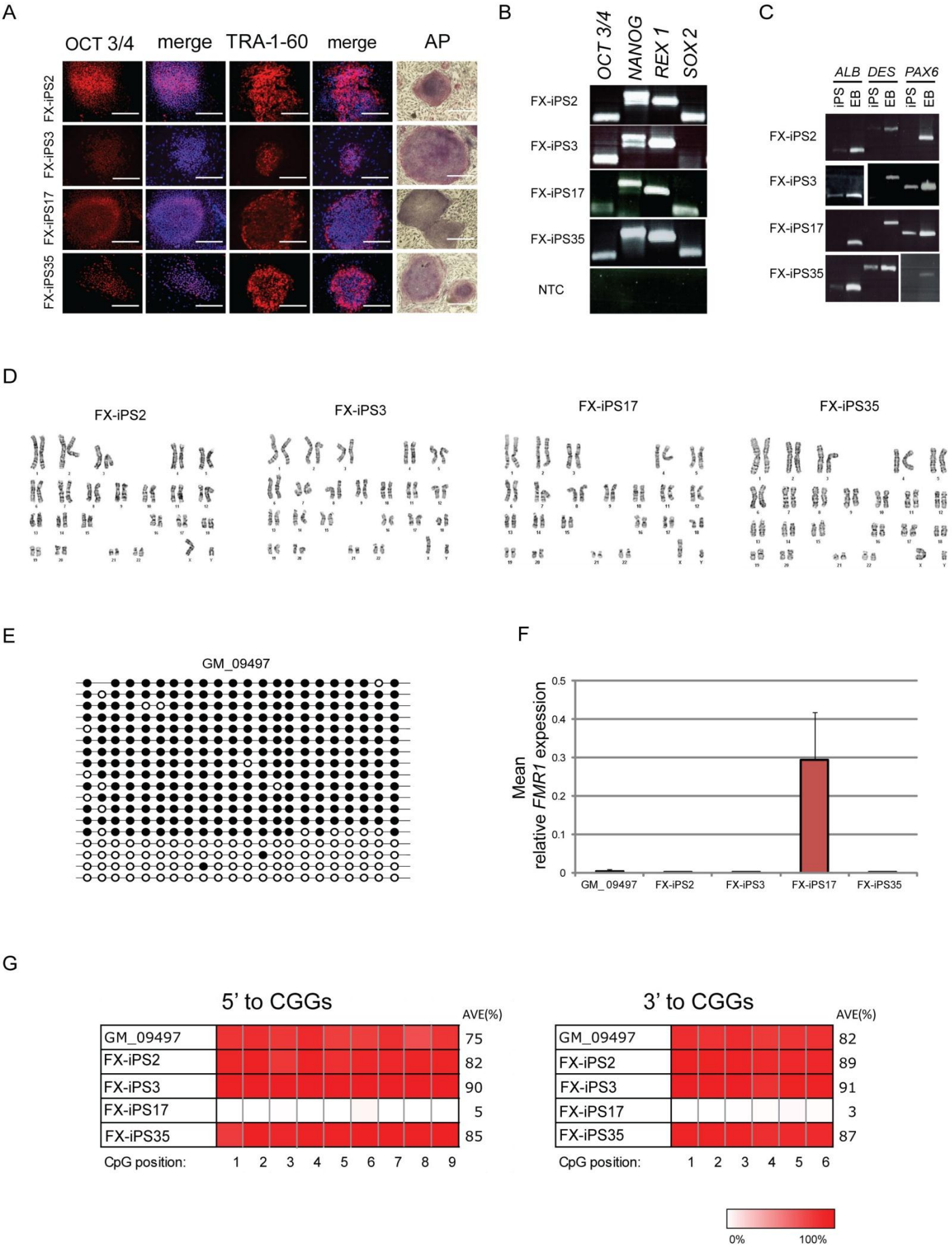

A
OCT 3/4
merge
TRA-1-60
merge
AP
FX-iPS2
FX-iPS3
FX-iPS17
FX-iPS35
B
OCT 3/4
NANOG
REX 1
SOX 2
FX-iPS2
FX-iPS3
FX-iPS17
FX-iPS35
NTC
C
ALB
DES
PAX6
iPS
EB
FX-iPS2
FX-iPS3
FX-iPS17
FX-iPS35
D
FX-iPS2
FX-iPS3
FX-iPS17
FX-iPS35
E
GM_09497
F
Mean relative FMR1 expession
0.5
0.4
0.3
0.2
0.1
0
GM_09497
FX-iPS2
FX-iPS3
FX-iPS17
FX-iPS35
G
5' to CGGs
AVE(%)
GM_09497 75
FX-iPS2 82
FX-iPS3 90
FX-iPS17 5
FX-iPS35 85
CpG position: 1 2 3 4 5 6 7 8 9
3' to CGGs
AVE(%)
GM_09497 82
FX-iPS2 89
FX-iPS3 91
FX-iPS17 3
FX-iPS35 87
CpG position: 1 2 3 4 5 6
0%
100%

FIGURE LEGENDS

Fig. S1

Characterization of FXS HESC lines: (A) Expression of *OCT4, NANOG, SOX2* and *REX1*, by RT-PCR in all newly established FXS HESC lines. (B) Staining for OCT4, TRA-1-60 and ALKALINE PHOSPHATASE activity. Scale bars stand for 200μm. (C) RT-PCR demonstrating up-regulation of *ALBUMIN*, *PAX6* and *DESMIN* gene markers, representing the three different embryonic germ layers, in fully matured embryoid bodies (EBs) and their undifferentiated cell counterparts (ES) in a representative number of FXS HESC lines. (D) Teratoma sections stained by H&E in a representative number of FXS HESC lines. Scale bars stand for 130 μm.

Fig. S2

Analysis of X-inactivation and *FMR1* methylation in FXS HESCs and primary cells of patients: (A) Skewed X inactivation (Xi) was confirmed using an established methylation-sensitive quantitative assay, as described in Kiedrowski et al., 2011. The test is based on digestion with a methylation-sensitive restriction enzyme followed by PCR amplification of a short fragment within the X-linked *ANDROGEN RECEPTOR* gene. The fragment includes a highly polymorphic region (CAG repeat) and several sites that are liable to differential methylation by Xi. While the highly polymorphic CAG repeat is used to distinguish maternal from paternal inherited X-chromosomes, the methylation-sensitive sites allow selective amplification of alleles that are exclusively present on the inactive X chromosome regardless of parental origin. Accordingly, by comparing the relative amount and fragment size of digested and undigested PCR products using capillary electrophoresis, a skewed bias from the expected 50:50 ratio between the inactive maternal or paternal X chromosomes is readily identified. Paternal DNA was used to confirm full digestion and to distinguish the maternal (carrying the *FMR1* CGG expansion) from the paternal inherited X chromosome. A representative Xi assay on SZ-FX3 is depicted in which complete X-inactivation of the maternal X chromosome is evident from the detection of a single PCR product of 234bp following digestion with a methylation-sensitive enzyme. For primer set see Table S4.
(B) Bisulfite pyrosequencing results in primary cultures (lymphocytes (GM_09237) and fibroblasts (GM_04024, GM_05131, GM_07730 and GM_09497) of 5 different patients. (C) *FMR1* methylation levels by bisulfite pyrosequencing in 3 XY FXS HESC lines along with time in culture. Bisulfite pyrosequencing results for each cell line at two different passages demonstrates that methylation levels remain stable along with time in culture. (D) Analysis

of *FMR1* methylation levels in XY WT and FXS HESC lines by bisulfite colony sequencing. Single molecule bisulfite sequencing was carried out at CpG sites located 5' (230bp, 22 CpG sites) and 3' (173bp, 10 CpG sites) with respect to the CGGs. Each line represents one molecule, with methylated and unmethylated CpGs designated by black and white circles, respectively.

Fig. S3

Characterization of FX-iPS cell clones: (A) immunostaining for OCT4 (red, merged onto Hoechst (blue)), for the cell surface marker TRA-1-60 (red, merged onto Hoechst (blue)), and for ALKALINE PHOSPHATASE activity (AP). Scale bars stand for 130 μm. (B) Expression of the undifferentiated cell specific markers *OCT4*, *NANOG*, *SOX2* and *REX1* in four FX-iPS cell line clones (FX-iPS-2, -3, -17 and -35). (C) RT-PCR demonstrating up-regulation of *ALBUMIN*, *PAX6* and *DESMIN* gene markers, representing the three different embryonic germ layers (endoderm, ectoderm and mesoderm, respectively), in fully matured embryoid bodies (EBs) as compared with their undifferentiated cell counterparts (iPS) in FX-iPS-2,-3,-17 and -35. (D) Chromosome analysis, by Giemsa staining, carried out on metaphase chromosomes of all four FX-iPS clones. (E) Bisulfite single molecule sequencing 5' to the repeats demonstrates 77% methylation levels in parental FXS fibroblasts (for methylation levels by bisulfite pyrosequencing 5' and 3' to the CGGs see Fig S2B). (F) Average values of *FMR1* mRNA levels in four FX-iPS clones as determined by 3 independent RT-qPCR experiments of a given culture. (G) Bisulfite pyrosequencing analysis for methylation levels 5' and 3' to the CGGs in the FX-iPS clones.

TABLE LEGENDS

Tables S1-S4**.** Primer sets, annealing temperatures and product sizes for qRT-PCR reactions (table S1), bisulfite analysis (table S2), ChIP experiments (table S4) and X-inactivation test (table S4).

Table S1: Primer Sets for RT-PCR reactions:

	5' Primer (sequence 5'-3')	3' Primer (sequence 5'-3')	Annealing Temp ºC	Product Size (bp)
OCT4	GACAGGGGGAGGGGAGGAGCTAGG	CTTCCCTCCAACCAGTTGCCCCAAAC	60	144
NANOG	CAGCCCCGATTCTTCCACCAGTCCC	CGGAGATTCCCAGTCGGGTTCACC	55	342, 390
REX1	CAGATCCTAAACAGCTCGCAGAAT	GCGTACGCAAATTAAAGTCCAGA	60	306
SOX2	GGGAAATGGGAGGGGTGCAAAAGAGG	TTGCGTGAGTGTGGATGGGATTGGTG	55	151
GAPDH	CCACTCCTCCACCTTTGAC	ACCCTGTTGCTGTAGCCA	62	102
ALBUMIN	TGGCACAATGAAGTGGGTAA	TCAAATGGACACTGCTGAAGA	56	131, 189
PAX6	ACCCATTATCCAGATGTGTTTG	ATGGTGAAGCTGGGCATAG	56	317
DESMIN	TCGGTATTCCATCATCTCCTG	GGTGGAGGTGCTCACTAACC	56	481
FMR1 (Exons 2-4)	CATGAAGATTCAATAACAGTTGC	CACTTTAGCTAACCACCAACAG	56	183

Table S2: Primer Sets for Bisulfite Analysis:

	5' Primer (sequence 5'-3')	3' primer (sequence 5'-3')	Annealing Temp ºC	Product Size (bp)
FMR1 5' (colony and pyro-bisulfite)	* TTGAGTGTATTTTTGTAGAAATGGG	CCTCTCTCTTCAAATAACCTAAAAA	56-59	191
FMR1 3' - colony bisulfite	GGTATTTGGTTTTAGGGTAGGTTT	TTCCAACAAACCCCAAAT	56-58	173
FMR1 3' - pyro bisulfite	AGAGGGGTTTTTAATAGGTTTTAAGTT	*CTTCCCTCCCTTTTCTTCTTAAT	59	143
FMR1 5' (sequencing primer for pyro)		CTCTTCAAATAACCTAAAAAC		
FMR1 3' (sequencing primer for pyro)	GAGAGTGTTTTGGTATTTAGG			

*Biotinilated primer for pyrosequencing

Table S3: Primer Sets for ChIP Analysis:

	5' Primer (sequence 5'-3')	3' Primer (sequence 5'-3')	Annealing Temp ºC	Product Size (bp)
HOXA9	CTCAGGAGCCTCGTGTCTTT	GTGACCAGGTGGAGGTGTGT	60	82
CRYSTALIN	CCGTGGTACCAAAGCTGA	AGCCGGCTGGGGTAGAAG	58-62	85
APRT	GCCTTGACTCGCACTTTTGT	TAGGCGCCATCGATTTTAAG	60	85
CTCF +1000	CACCAAATCACAATGGCAAC	GGCCATGTTAGGGTCTTCCT	60	98
CTCF -800	GACAGGACGCATGACTGCTA	GCACTTGAGGTTCATTTCTGC	60	89
CTCF +10kb	TTTGTGTGTGTGGCAATGAA	CTCAGTATGCCTGGGTCACA	60	162
CTCF +300	GCTAGCAGGGCTGAAGAGAA	CTGCCCTAGAGCCAAGTACC	60	91
CTCF -300 (FMR1 promoter)	AACTGGGATAACCGGATGCAT	GGCCAGAACGCCCATTTC	63	72
DMPK	CTGCCAGTTCACAACCGCTCCGAG	GCAGCATTCCCGGCTACAAGGACCCTTC	73-76	147
FXN	TCCTGAGGTCTAACCTCTAGCTGC	CGAGAGTCCACATGCTGCTCC	63-66	131

Table S4: Primer Sets for X-inactivation test (as described by (Kiedrowski et al., 2011))

	5' Primer (sequence 5'-3')	3' Primer (sequence 5'-3')	Annealing Temp ºC	Product Size (bp)
ANDROGEN RECEPTOR (AD)	HEX-GTGCGCGAAGTGATCCAGAA	CCAGGACCAGGTAGCCTGTG	59	244