Inventory of supplementary items:

1. Supplementary Figure 1 and Supplementary Figure 2.
   Both figures describe the characterization of the iPS cell clones shown in Figure 1 and Figure 2.

2. Materials & Methods

Supporting online material:

Supplementary Figure 1: Establishment of iPS cell lines from fragile-X fibroblasts: Characterization of the undifferentiated iPS cells (see also figure 1 and supplementary figure 2).

A: Fragile-X (FX) iPS cells have a similar morphology to human ES cells and express markers of undifferentiated cells. **I**: FX-iPS A-12 cell line **II**: FX-iPS C-2 cell line **III**: FX-iPS C-3 cell line **IV:** iPS cells derived from differentiated-FX-ES cells (Diff.-FX-ES-iPS).

B: qRT-PCR analysis of expression of retroviral transgenes. FX-iPS cell clones and fibroblasts 7 days after transduction with the 4 factors were analyzed for expression of the PMXs retroviral transgenes.

C: qRT-PCR for markers of pluripotency in FX fibroblasts compared to FX derived iPS cell clones and two human ES cell lines (H9 and HES13). Expression of UBC gene was used as an endogenous control.

D. A DNA microarray gene expression analysis showing upregulation of many markers of pluripotency in FX-iPS cell clones vs. their parental fibroblasts.

E. Karyotype analysis of the iPS cells. Shown are the karyotypes of the FX fibroblasts and the iPS cell line that was derived from them. Both cell lines show normal diploid 46XY chromosomes.

Supplementary Figure 2: Establishment of iPS cell lines from fragile-X fibroblasts: Characterization of the undifferentiated iPS cells and their pluripotency (see also figure1 and supplementary figure 1).

A,B. Hierarchical clustering (A), and scatter plot analysis (B) of DNA microarray expression results from FX-fibroblasts, WT-fibroblasts and the iPS cell lines derived from them.

C: *In-vitro* differentiation of FX iPS cells. **I**: Cystic embryoid bodies (EBs) at day 7. **II-VI**: Images of differentiated FX-iPS cells from 8 days old EBs plated onto adherent culture tissue dishes, and stained at day 16 for markers of the three embryonic germ

layers. **II**: FoxA2 (endoderm), **III**: alpha-fetoprotein (endoderm), **IV**: Cardiac-fetal actin (mesoderm), **V**: Desmin (mesoderm), **VI**: NCAM1 (ectoderm). Nuclei were stained with Hoechst-33342 (blue).

D. *In-vivo* differentiation of FX-iPS cells. Hematoxylin and eosin staining of teratoma sections from six iPS cell clones, showing **I**: neural rosette and neuronal tissue (ectoderm), **II**: adipose, muscle and cartilage tissues (mesoderm), **III** respiratory epithelium and gut-like epithelium (endoderm): The three upper panels are from paraffin sections and the three bottom panels are from frozen cryosections .

E. Analysis of the number of the CGG repeats in FX-Fibroblasts-A and in four iPS cell clones derived from them. The size in base pairs of the DNA marker band and the number of CGG repeats are provided at the right and left sides of the gel, respectively.

F. Regulation of FMR1 gene expression and its epigenetic modifications. Shown is a schematic representation of the regulation of the FMR1 gene in FX-iPS cells. **I**: FMR1 expression and its epigenetic status in normal ES cells and somatic cells. **II**: FMR1 expression and its epigenetic status in FX-ES cells, in somatic FX cells, and in FX-iPS cells. Note that in contrast to FX-ES cells, the FMR1 gene is silent in FX-iPS cells. The silencing of the FMR1 gene in the reprogrammed iPS cells is accompanied by DNA methylation and histone modifications associated with inactive chromatin**.**

Materials and Methods

Cell culture

ES and iPS cells were cultured in standard ES cell culture media containing KnockOut DMEM (Gibco-Invitrogen) supplemented with 15% Knockout serum replacement (Gibco-Invitrogen), 2mM L-glutamine (Sigma-Aldrich), 1:100 dilution of non-essential amino acids (Gibco-Invitrogen), 1:100 dilution of ITS (insulin, transferrin and selenium) (Gibco-Invitrogen), 0.1 mM β-mercaptoethanol (Sigma-Aldrich), 8 ng/ml basic fibroblast growth factor (bFGF) (PeproTech), penicillin (50 units/ml) and streptomycin (50 ug/ml) (Gibco-Invitrogen).

GM05848 - fibroblasts from a 4 year old fragile-X patient (FX-fibroblast A), GM07072 - fetal lung fibroblasts from a 22 week old fetus with a fragile X mutation (FX-fibroblast B) and GM09497 - fibroblasts from a 28 year old fragile-X patient (FX-fibroblast C) were purchased from Coriell Institute for Medical Research and were grown in Eagle's Minimum Essential Medium (Sigma) supplemented with 15% fetal calf serum (Biological Industries), 2mM L-glutamine (Sigma-Aldrich), penicillin (50 units/ml) and streptomycin (50 ug/ml) (Gibco-Invitrogen). Wild type (WT) neonatal fibroblasts (BJ) and differentiated teratoma derived FX-ES cells (Eiges et al., 2007) were cultured on gelatin-coated dishes in DMEM (Gibco-Invitrogen) supplemented with 10% FCS (Biological Industries), penicillin (50 units/ml) and streptomycin (50 ug/ml) (Gibco-Invitrogen).

Retroviral production and iPS cell generation

For iPS cell generation, approximately $2x10^6$ 293T cells were transfected in the presence of Fugene 6 (Roche) with 4.5 μg of pMXs retroviral vectors containing either human OCT4, SOX2, KLF4 or cMYC (obtained from Addgene), and 4.5 μg of PCL-Ampho plasmid. Twenty-four hours after transfection the culture was replaced with fresh medium, and forty-eight hours after transfection the supernatant was collected, filtered through a 0.45 uM cellulose acetate filter (Whatman) and supplemented with 4ug/ml of Polybrene (Sigma-Aldrich). Virus containing medium was then transferred to a 10 cm dish containing $2x10^5$ FX-fibroblast A, FX-fibroblast B or differentiated FX-ES cells at passage number 8, 5 and 5, respectively. Seventy two hours post transfection a second round of infection was performed. Four days post infection, ES cell medium was added to the infected fibroblasts and mitomycin treated mouse embryonic fibroblasts (MEFs) were seeded onto the infected fibroblasts dish. Between 21 to 30 days post infection, ES like colonies were picked and allowed to propagate in a 12 wells plate. At this stage the culture was defined as passage 0 and passaged mechanically until passage 5, thereafter they were passaged using 0.25% Trypsin (Biological Industries). The iPS cells from FX-fibroblast C were generated as previously described in (Park et al., 2008)

Karyotype analysis

For karyotype analysis, a confluent 10 cm plate of FX-iPS #52 or FX-fibroblasts-A in log growth phase was treated with colcemid (Biological industries) give a final concentration of 100 ng/ml, then harvested with trypsin, treated with hypotonic solution and fixed. Metaphases were spread onto glass slides and stained with giemsa dyes (Sigma-Aldrich). Chromosomes were classified according to the International System for Human Cytogenetic Nomeneclture. At least 20 metaphases were analyzed per cell line.

DNA and RNA isolation and reverse transcription

Total genomic DNA was extracted using genomic DNA extraction kit (RBC) and total RNA (DNase treated) was extracted using RNAeasy Mini Kit (Qiagen) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed with random hexamer primers using ImProm-II reverse transcriptase (Promega).

Real-time PCR and PCR

Real-time PCR was carried out in triplicates using Power SYBR Green PCR Master Mix (Applied Biosystems) or TaqMan Universal PCR Master Mix (Applied Biosystems) and using 7300 Real-time PCR System (Applied Biosystems). A full description of primer sequences and annealing temperatures can be found in Table S1.

Table S1: Primer sets for PCR and Real-time PCR

	5' primer	3' primer	Temp ^{0}C	Cycles
FMR1	CAGGGCTGAAGAGAAGATGG	ACAGGAGGTGGGAATCTGA	60	40
OCT3/4	Taqman probe Hs 03005111_g1	Taqman probe Hs 03005111_g1	60	40
LIN28	Taqman probe Hs 00702808_s1	Taqman probe Hs 00702808_s1	60	40
FGF4	Taqman probe Hs00173564_m1	Taqman probe Hs00173564_m1	60	40
SOX2	GGGAAATGGGAGGGGTGCAAAAGAGG	TTGCGTGAGTGTGGA TGGGATTGGTG	60	40
NANOG	CATGAGTGTGGATCCAGCTTG	CCTGAATAAGCAGATCCATGG	60	40

REX1	TCACAGTCCAGCAGGTGTTTG	TCTTGTCTTTGCCCGTTTCT	60	40
UBC	Taqmanprobe Hs_00824723_m1	Taqmanprobe Hs_00824723_m1	60	40
GAPDH	AGCCACATCGCTCAGACACC	GTACTCAGCGGCCAGCATCG	60	40
OCT3/4 (Tg)	CCC CAG GGCCCCATT TTGGTA CC	CCC TTT TTC TGGAGACTAAAT AAA	60	40
SOX2 (Tg)	GGC ACC CCT GGCATG GCTCTT GGC TC	TTA TCG TCGACCACTGTG CTGCTG	60	40
KLF4 (Tg)	ACGATCGTGGCCCCGGAA AAG GAC C	CCC TTT TTCTGG AGACTAAAT AAA	60	40
cMyc (Tg)	CAACAACCGAAAATG CACCAG CCC CAG	CCCTTTTTCTGGAGACTA AAT AAA	60	40

Alkaline phosphatase staining and Immunocytochemistry

Alkaline phosphatase staining was performed using the Leukocyte Alkaline Phosphatase Kit (Sigma-Aldrich) according to the manufacturer's instructions. For immunocytochemistry, cells were washed twice with PBS, crosslinked with 10% formalin solution for 10 min, washed twice with PBS and blocked for one hour at room temperature with PBS containing 2% bovine serum albumin (BSA, Sigma-Aldrich) and 0.1% Triton-X-100. Primary antibody staining was performed for 1 hour in room temperature with antibodies diluted in blocking solution. The antibodies used were mouse anti human OCT3/4 (IgG, 1:200, Santa Cruz Biotechnology), goat anti human SOX2 (IgG, 1:100 Santa Cruz Biotechnology), goat anti human NANOG (IgG, 1:100, R&D Systems), mouse anti human Tra-1-60 (IgM, 1:500 Santa Cruz Biotechnology), mouse anti human FMRP (IgG, 1:100, Chemicon International), rabbit anti human alpha-fetoprotein (IgG, 1:200, Dako), rabbit anti human FOXA2 (IgG, 1:1000, Abcam), mouse anti human Desmin (1:200, IgG, Dako), mouse anti human cardiac fetal actin (1:200, IgG, Maine Biotechnology) and goat anti human NCAM1 (1:150, IgG, R&D Systems).

For immunostaining of FX iPS-C, cells were fixed in 4% paraformaldehyde for 30 min, permeabilized with 0.2% Triton X-100 for 30 min, and blocked in 3% BSA in PBS for 2 hr. Cells were incubated overnight at 4ºC with primary antibody, washed, and incubated with Alexa Fluor (Invitrogen) secondary antibody for 2 hr. OCT3/4 and NANOG antibodies were obtained from Abcam, SSEA4, TRA-1-60 and TRA-1-81 antibodies were obtained from Millipore.

DNA microarray analysis

Total RNA was extracted according to the manufacturer's protocol (Affymetrix, Santa Clara, CA) from a populations of FX fibroblasts, FX-iPSCs and normal and FX ESCs. Hybridization to the GeneChip Human Gene 1.0 ST Arrays, washing, and scanning were performed according to the manufacturer's protocol (Affymetrix), and expression patterns were compared between samples.

PCR analysis for the CGG repeat

PCR for the CGG repeats in the 5UTR of the FMR1 gene was performed using the Abbot Molecular Fragile-X kit. Approximately 60 ng of genomic DNA from each cell clone was used as a template for the PCR reaction which was performed according to the manufacture's instructions.

In vitro differentiation

For EB formation, a semi-confluent 10 cm plate of the cells was harvested using trypsin, and cell clumps were resuspended in ES cell media without bFGF, allowed to aggregate and transferred to one well of a non-adherent six wells plate. After 8 days as floating EBs, cell clumps were collected and seeded onto a 12 wells plate adherent culture dish. After an additional 8 days of growth, adherent cells were stained for the presence of markers from the three embryonic germ layers.

In vivo differentiation

For teratoma formation, a confluent 10 cm plate was harvested using trypsin. Cells were centrifuged and cell pellets were resuspended in 50uL of iPS cell medium or in a mixture of DMEM, Matrigel and collagen (ratio of 2:1:1). The iPS cells were injected either subcutaneously into the dorsal flank, or into the kidney capsule of 4 weeks old SCID-Beige male mice (The Jackson Laboratory). Tumors were dissected after 4-6 weeks. Cryosections or paraffin sections were stained with haematoxylin and eosin for histological analysis. All animal experiments were conducted under the supervision of the Hebrew University Faculty of Sciences and Animal Care and Use Committee.

DNA methylation analysis

Bisulfite treatment of genomic DNA was carried out using EZ DNA Methylation™ Kit (Zymo Research Corporation) according to the manufacturer's protocol. For pyrosequecing, the bisulfite treated DNA was amplified by HotStar Taq Polymerase (Qiagen Cat.# 203205). For 45 cycles of (95ºC 30 s; 53ºC 30 s; 72ºC 30 s). The Pyrosequencing analysis was perform by EpigenDx using the PSQ™96HS system according to standard procedures with unique set of primers that were developed by EpigenDx for the CpG sites at positions (-523) to (-384) from ATG of the FMR1 gene.

For the OCT4 analysis the bisulfite treated DNA was amplified by Faststart high fidelity taq polymerase (Roche) using the following primers: OCT4: 5' primer TTAGGAAAATGGGTAGTAGGGATTT; 3' primer. TACCCAAAAAACAAATAAATTATAAAACCT PCR products were cloned into bacteria using TOPO TA Cloning Kit (Invitrogen). Single colonies were analyzed for CpG methylation at all potential sites by direct sequencing. The methylation status of each single CpG site was found by comparison of the sequence result to the genomic sequence using QUMA software (http://quma.cdb.riken.jp/). Pyrosequencing analysis for OCT4 was perform by EpigenDx using the PSQ™96HS system according to standard procedures with assay #ADS514 for CpG sites at positions (-50) to (+96) from start codon.

Chromatin immunoprecipitation (ChIP)

Approximately $1.5x10^6$ cells were crosslinked with formaldehyde solution, lysed and the chromatin was sonicated to 200bp-1000bp DNA fragments. Chromatin was pre-cleared using 30uL of salmon sperm agarose beads (Upstate Biotechnology) for 1 hour at 4^0C. Immunoprecipitation of chromatin was performed overnight using anti- acetylated histone H3 antibody (Upstate Biotechnology), anti-methylated histone H3K4 (Upstate Biotechnology), or anti-methylated histone H3K9 antibody (Upstate Biotechnology). After the overnight incubation, crosslinking was reversed and DNA recovered using a PCR clean-up kit (Qiagen). Eluted DNA fragments were used for quantitative PCR analysis using primers for the FMR1 promoter region as well as appropriate positive and negative controls (APRT and Crystalline, see Table S2 for primer sequences).

Table S2: Primer sets for Real-time PCR

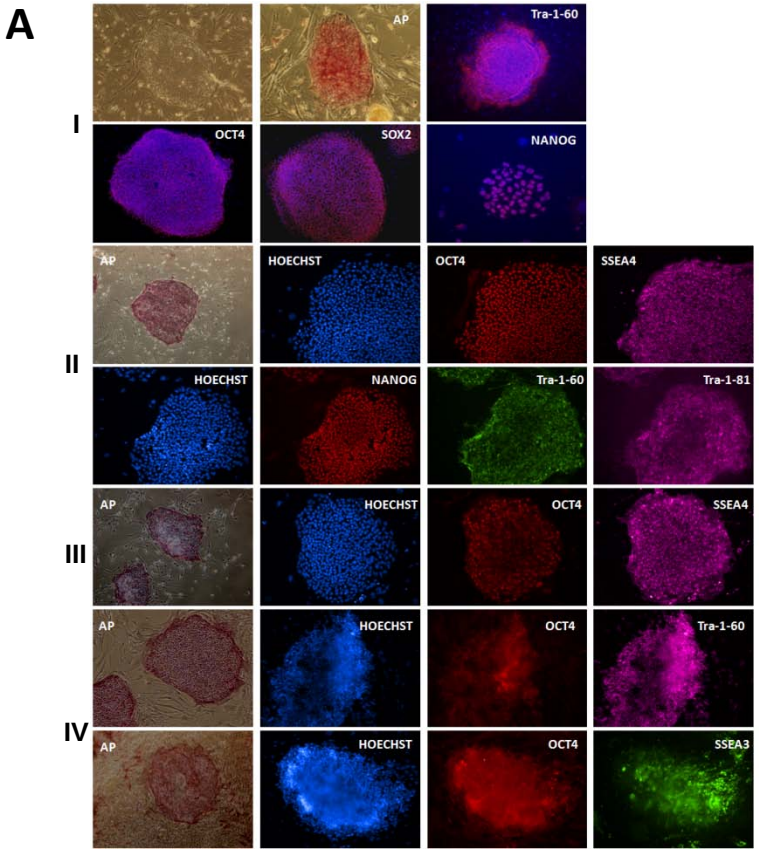
Locus	5' primer	3' primer	temp	Cycles
FMR1	AACTGGGATAACCGGATGCAT	GGCCAGAACGCCCATTTC	60^0C	40
APRT	GCCTTGACTCGCACTTTTGT	TAGGCGCCATCGATTTTAAG	60^0C	40
CRYSTALIN	CCGTGGTACCAAAGCTGA	AGCCGGCTGGGGTAGAAG	60^0C	40

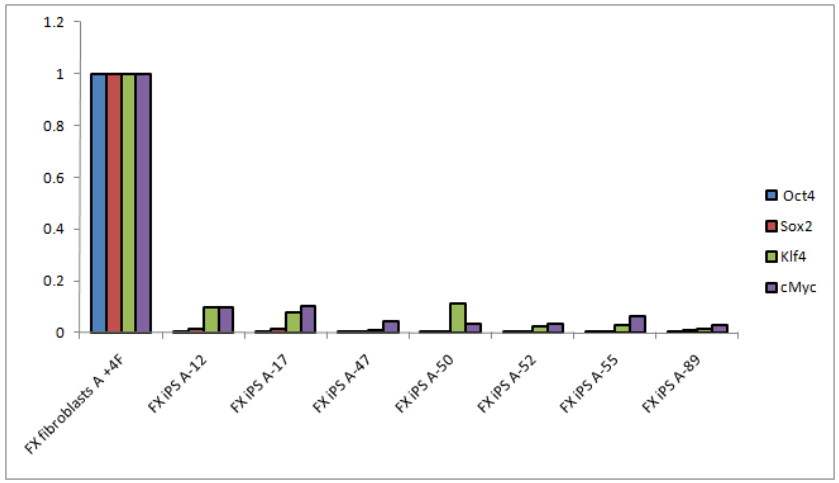
References

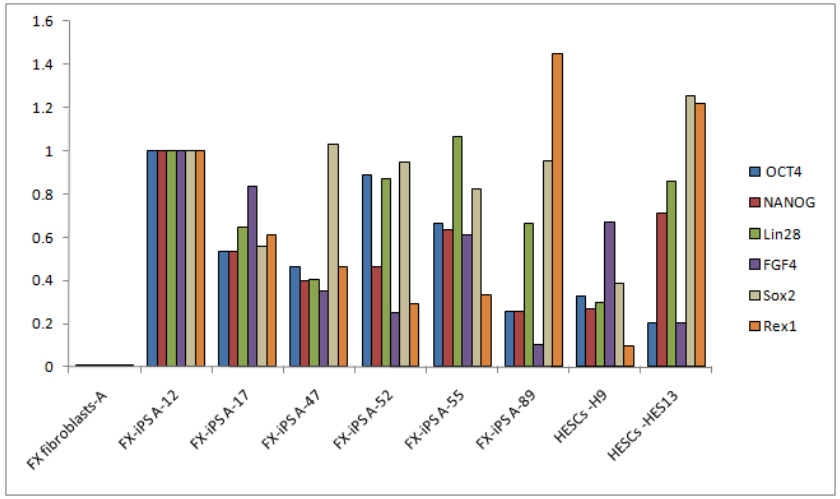
Eiges, R., Urbach, A., Malcov, M., Frumkin, T., Schwartz, T., Amit, A., Yaron, Y., Eden, A., Yanuka, O., Benvenisty, N., *et al.* (2007). Developmental study of fragile X syndrome using human embryonic stem cells derived from preimplantation genetically diagnosed embryos. Cell stem cell *1*, 568-577.

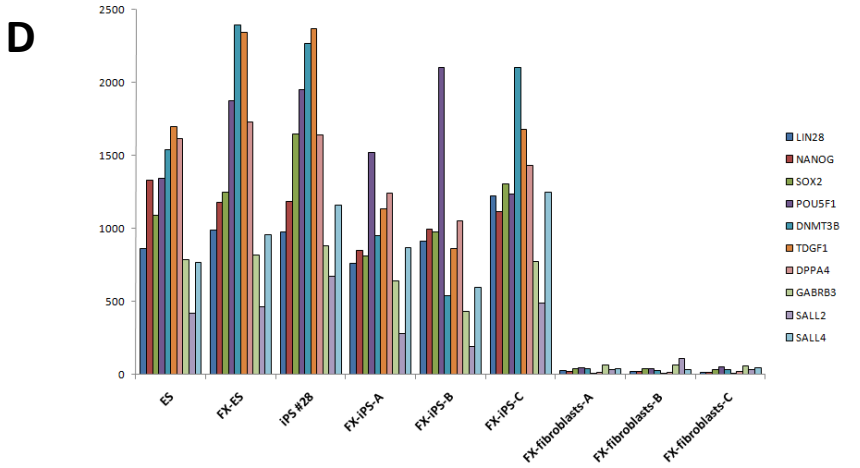
Park, I.H., Lerou, P.H., Zhao, R., Huo, H., and Daley, G.Q. (2008). Generation of human-induced pluripotent stem cells. Nature protocols *3*, 1180-1186.

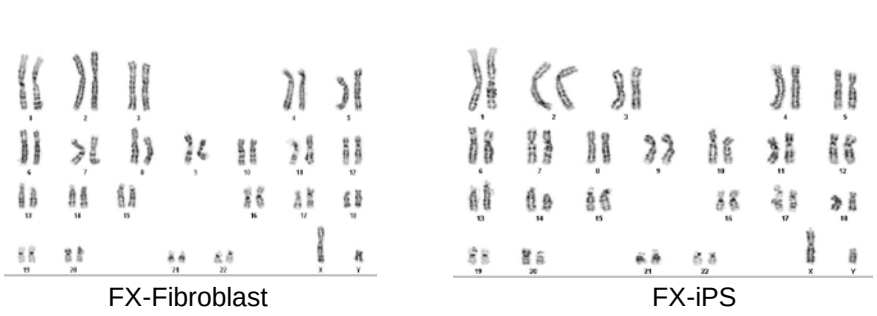
Supplementary figure 1











Supplementary figure 2