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Supplemental Information

The *let-7*/LIN-41 Pathway Regulates Reprogramming to Human Induced Pluripotent Stem Cells by Controlling Expression of Prodifferentiation Genes

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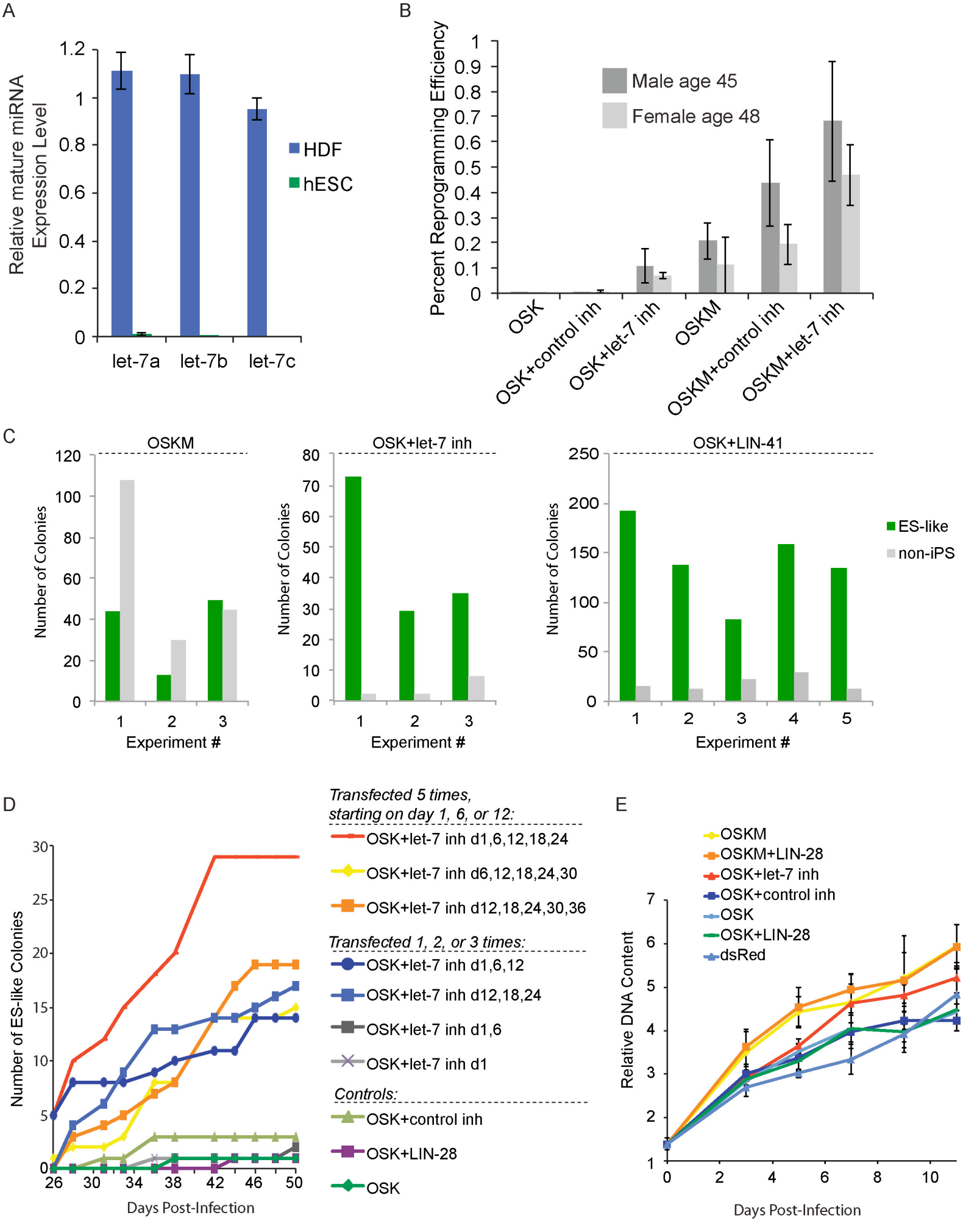
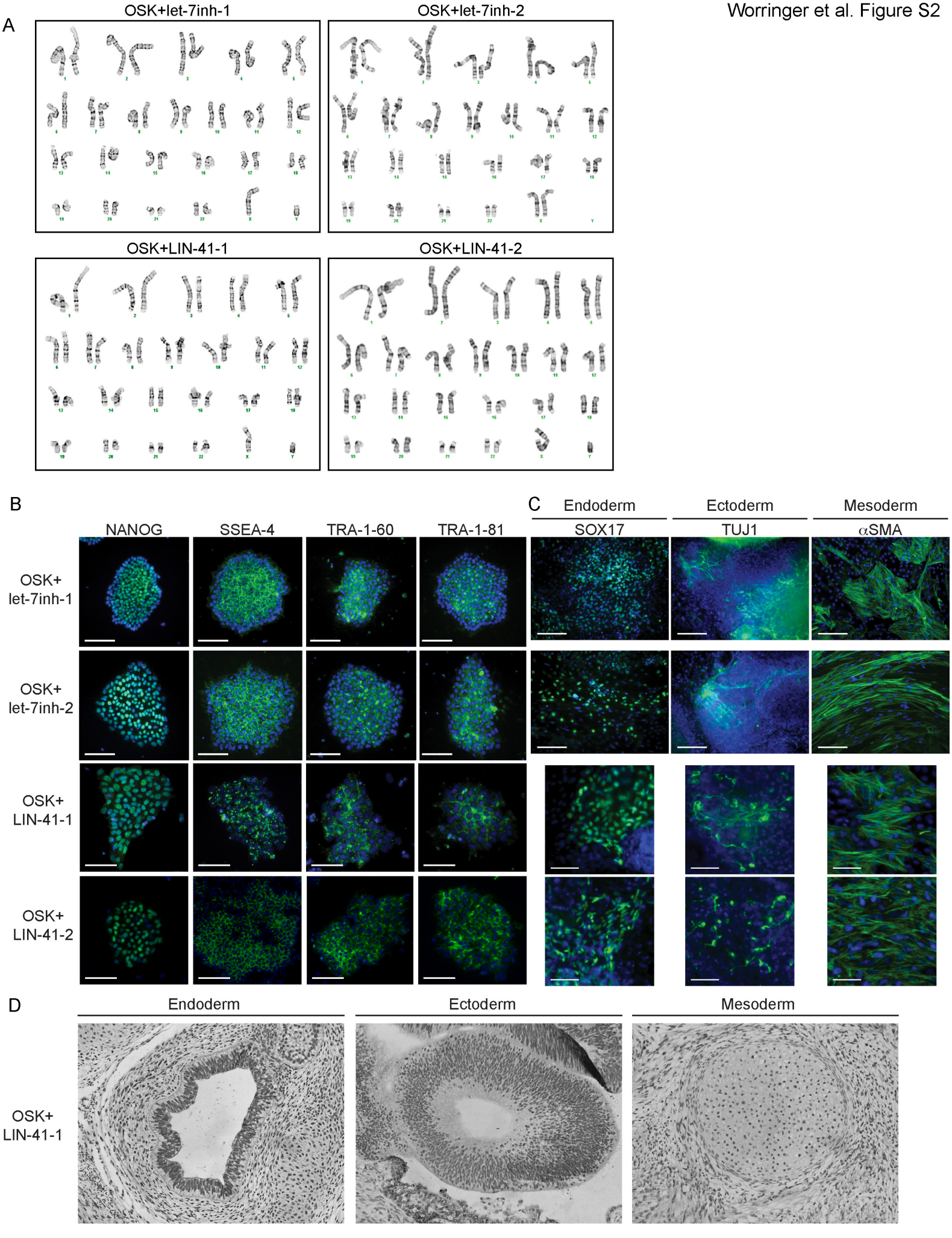
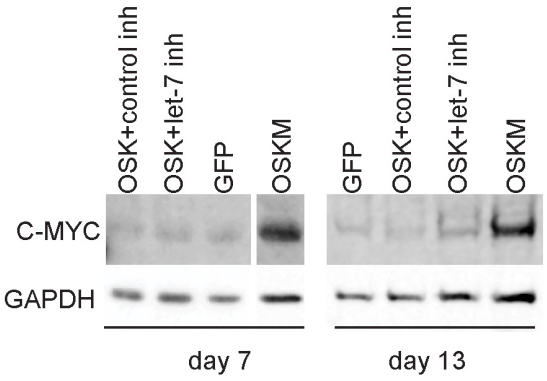

A
Relative mature miRNA Expression Level
1.2
1
0.8
0.6
0.4
0.2
0
let-7a
let-7b
let-7c
HDF
hESC
B
Percent Reprogramming Efficiency
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Male age 45
Female age 48
OSK
OSK+control inh
OSK+let-7 inh
OSKM
OSKM+control inh
OSKM+let-7 inh
C
OSKM
OSK+let-7 inh
OSK+LIN-41
Number of Colonies
Experiment #
ES-like
non-iPS
D
Number of ES-like Colonies
Days Post-Infection
Transfected 5 times, starting on day 1, 6, or 12:
OSK+let-7 inh d1,6,12,18,24
OSK+let-7 inh d6,12,18,24,30
OSK+let-7 inh d12,18,24,30,36
Transfected 1, 2, or 3 times:
OSK+let-7 inh d1,6,12
OSK+let-7 inh d12,18,24
OSK+let-7 inh d1,6
OSK+let-7 inh d1
Controls:
OSK+control inh
OSK+LIN-28
OSK
E
Relative DNA Content
Days Post-Infection
OSKM
OSKM+LIN-28
OSK+let-7 inh
OSK+control inh
OSK
OSK+LIN-28
dsRed

Worringer et al. Figure S2

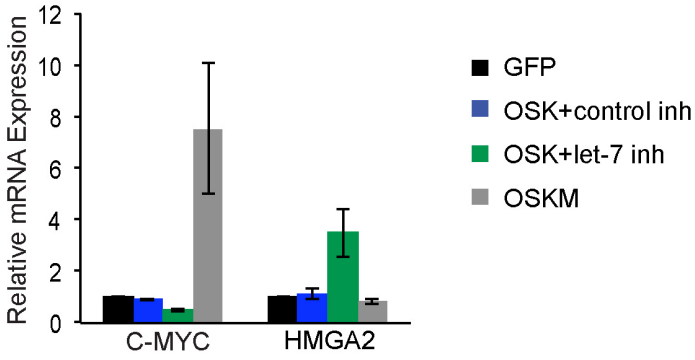


Worringer et al. Figure S3

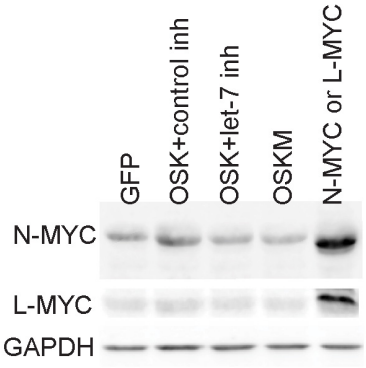
A



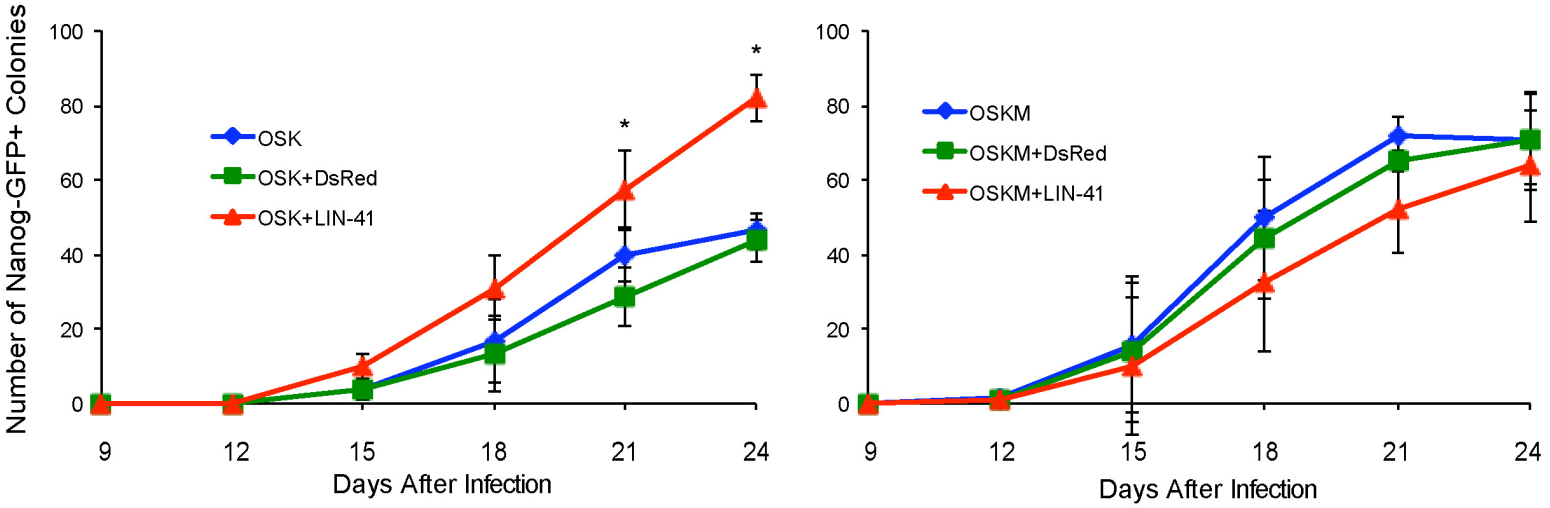
B



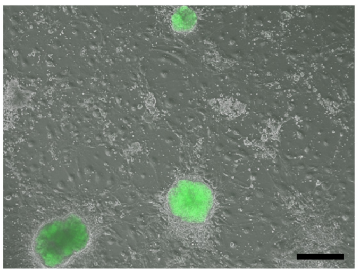
C



A


Number of Nanog-GFP+ Colonies
100
80
60
40
20
0
9
12
15
18
21
24
Days After Infection
OSK
OSK+DsRed
OSK+LIN-41
*
*
OSKM
OSKM+DsRed
OSKM+LIN-41

B



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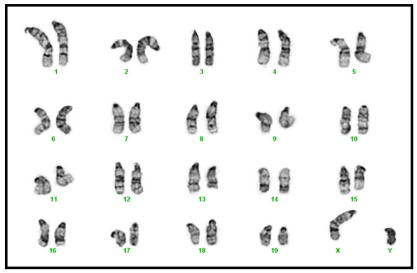
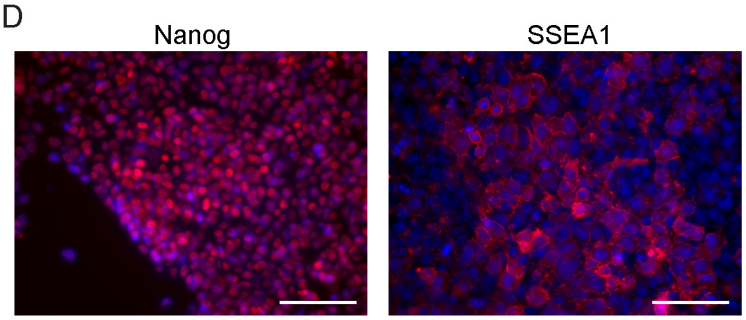
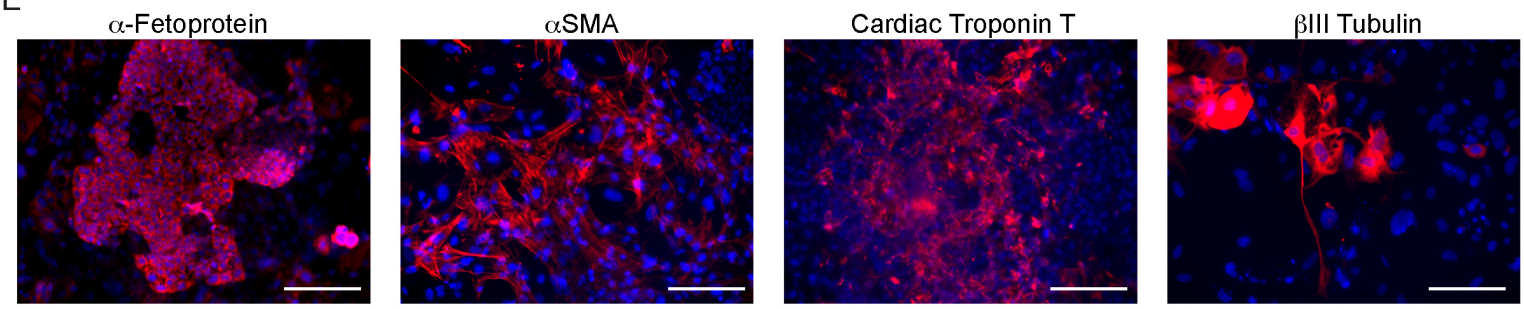


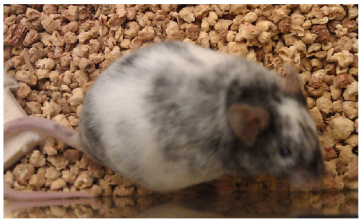
D


Nanog
SSEA1

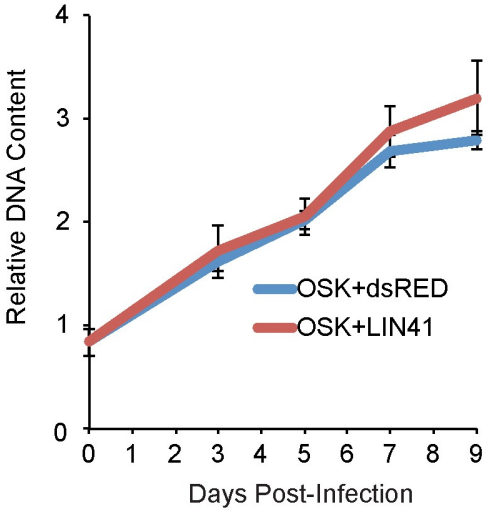
E


α-Fetoprotein
αSMA
Cardiac Troponin T
βIII Tubulin

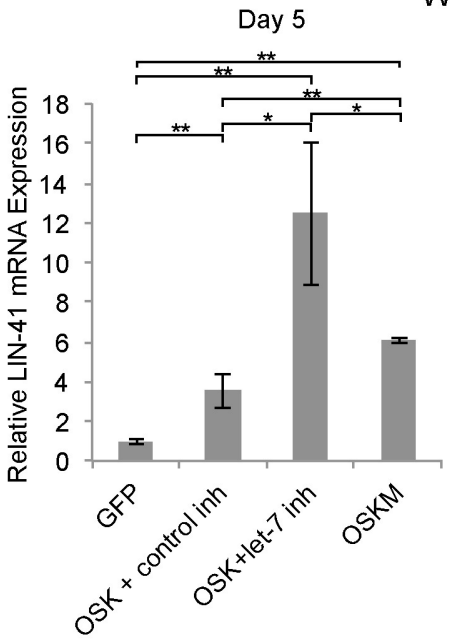
F



A



B



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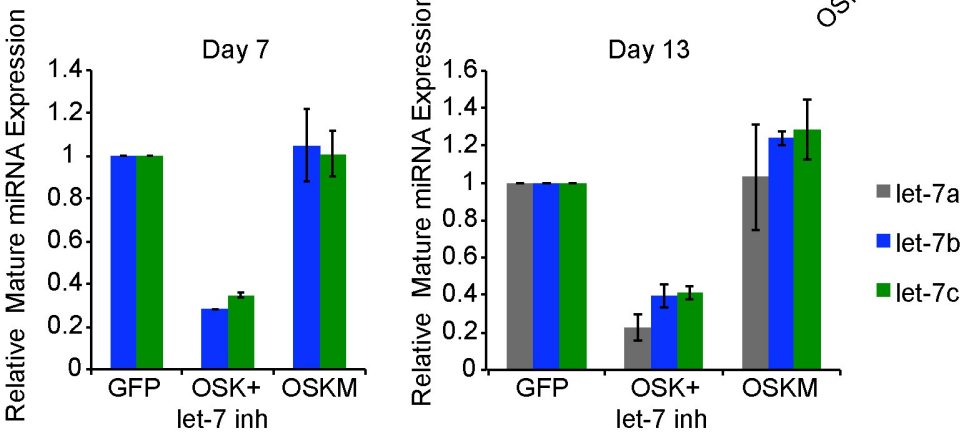
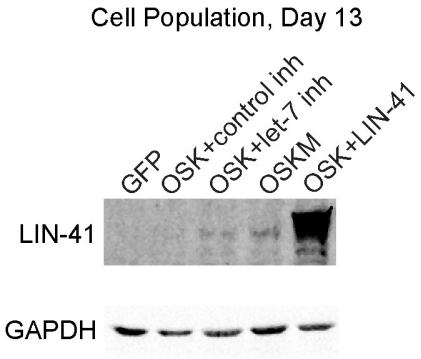


D

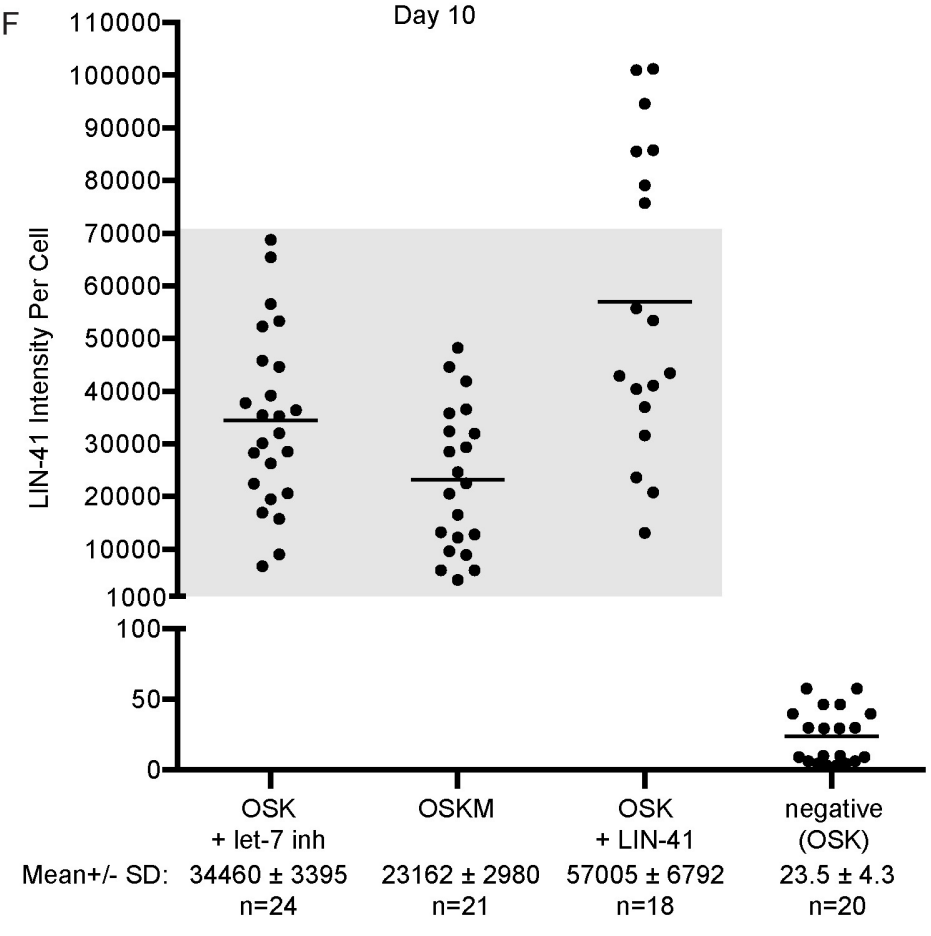


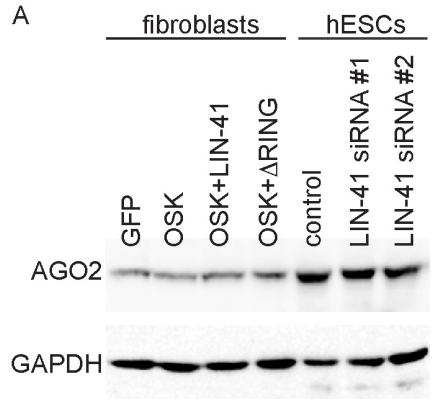
E

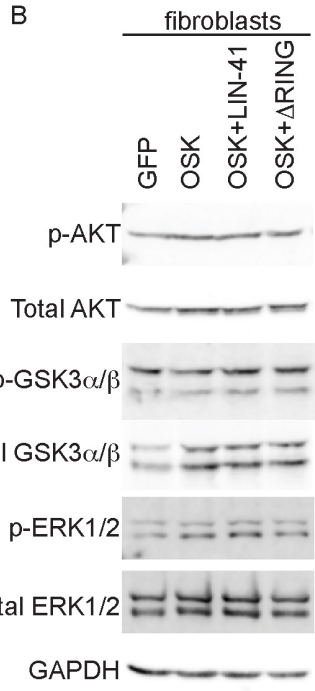
Day 10

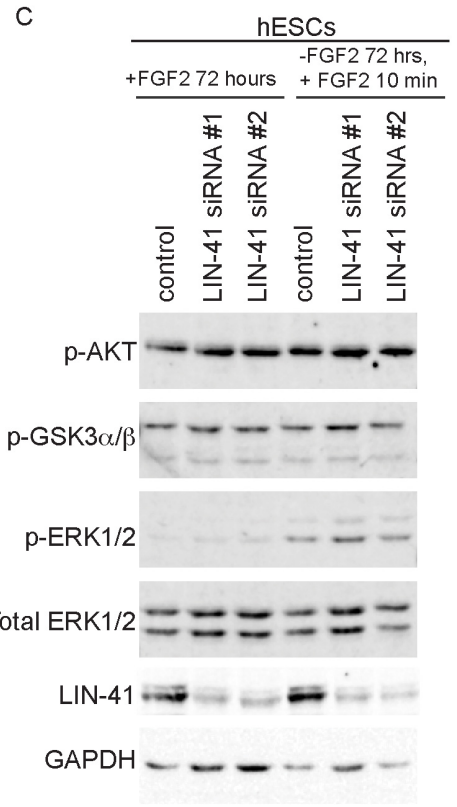
	LIN-41+ Cells	Total Cells	% LIN-41+ Cells
OSK + control inh	6	565	**1.1**
OSK + let-7 inh	20	573	**3.5**
OSKM	44	807	**5.5**
OSK + LIN-41	172	645	**26.7**

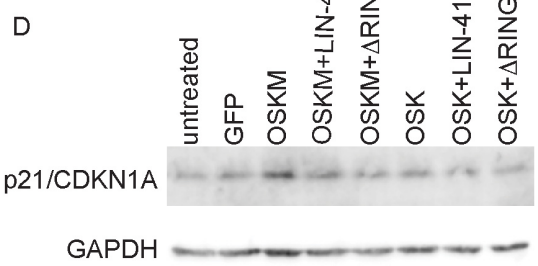
F

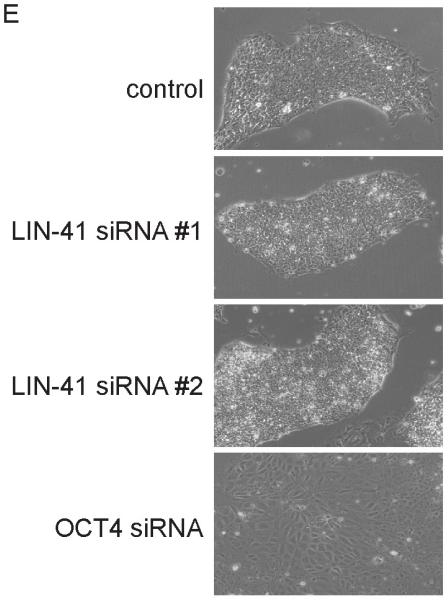


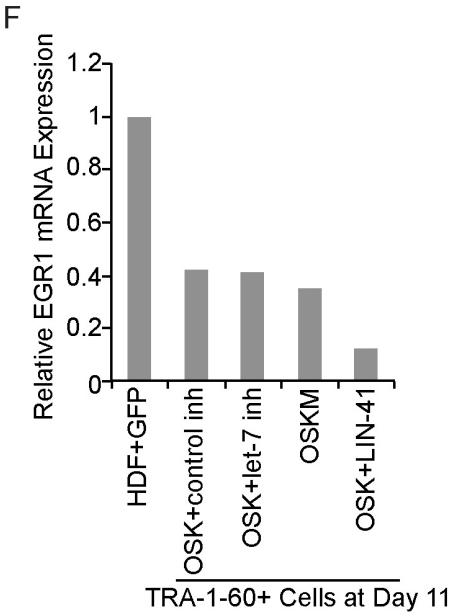


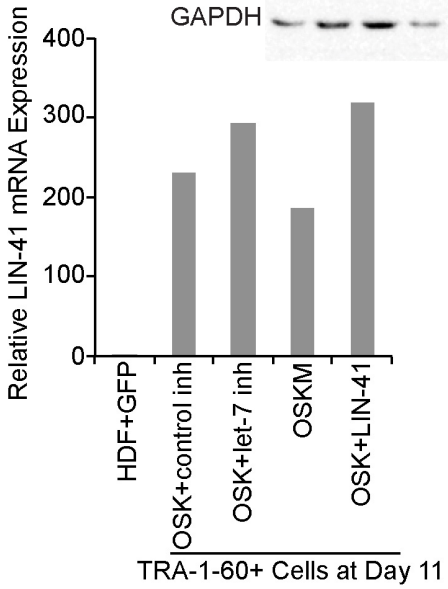








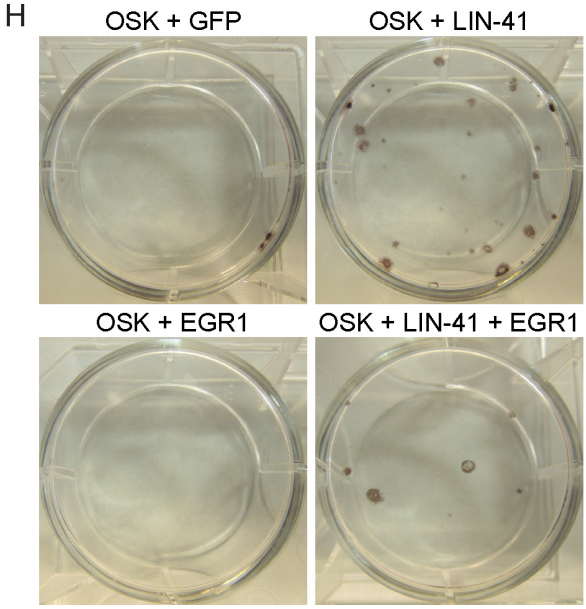




G

Predicted binding sites enriched near transcriptional start sites of genes altered >3-fold between OSK+LIN-41 and OSK

Transcription Factor	p-value	>3-fold Difference Between OSK+LIN-41 and OSK?
ZBP89	4.9E-24	No
SP1:SP3	5.3E-20	No
SP4	2.5E-19	No
EGR1	**4.0E-16**	**Yes, downregulated**
MYOG	8.1E-16	No
MATH1	5.1E-15	No
CKROX	9.3E-14	No
HTF4	4.5E-13	No
LBP1	1.2E-11	No
MZF1	1.3E-11	No



SUPPLEMENTAL FIGURE LEGENDS

Figure S1. *let-7* Inhibition Promotes Reprogramming, Related to Figures 1 and 2.

(A) Expression levels of *let-7a*, *b*, and *c* in HDFs and hESCs as measured by qRT-PCR. Expression level was normalized to miR-16. Data are represented as mean +/- SD.

(B) Two additional human dermal fibroblast lines were reprogrammed by infecting cells with the OSK or OSKM, ± *let-7* or control inh. Colonies with hESC-like morphology were then counted. Efficiency was calculated by dividing by the number of cells reseeded on day 7 of the protocol. Data are represented as mean +/- SD, n=3 for each line.

(C) HDFs were reprogrammed with OSKM (left), OSK+*let-7* inh (center), or OSKL (right) and colonies were counted and scored as having either ES-like (green) or non-ES like (gray) morphology. Plates were also stained with TRA-1-60 antibody to confirm the morphological scoring.

(D) Reprogramming as a function of the timing of *let-7* inhibition. OSK-infected cells were transfected during reprogramming and number and timing of transfections was varied as indicated in the figure. Cells transfected on days (d) 1, 6, 12, 18, and 24 produced the most colonies, and cells transfected fewer times or the same number of times but with a delayed time course of transfection resulted in fewer iPSC colonies (compare red with yellow and orange lines). One representative experiment is shown.

(E) Results from one representative cell-proliferation assay after infecting HDFs with OSK, OSKM, OSK+LIN-28, OSK+control inh, OSK+*let-7* inh, OSKM+LIN-28, or dsRED. Data are represented as mean +/- SD of 6 technical replicates.

Figure S2. Characterization of Human OSK+*let-7* inh and OSKL iPSC Lines, Related to Figures 1 and 2.

(A) iPSC lines generated using OSK+*let-7* inh and OSK+LIN-41 have normal karyotypes.

(B) Immunofluorescence of iPSCs generated with OSK+*let-7* inh or OSK+LIN-41 using antibodies to NANOG, SSEA4, TRA-1-60 and TRA-1-81 (green). DAPI (blue) stained nuclei. Scale bars, 50μm.

(C) Immunofluorescence of differentiated cells from iPSC lines generated with OSK+*let-7* inhibition or OSK+LIN-41. Antibodies to SOX17, TUJ1, and alpha smooth muscle actin (αSMA) were used (green). DAPI (blue) stained nuclei. Scale bars, 50μm.

(D) Representative images of teratomas derived from cells following reprogramming by OSK+LIN-41 showing endoderm, ectoderm, and mesoderm.

Figure S3. Assaying C-, L-, and N-MYC Protein Levels During Reprogramming, Related to Figure 2.

(A) Western blotting for C-MYC and GAPDH in HDFs at days 7 and 13 after infection with GFP, OSK+control inh, OSK+*let-7*inh, or OSKM.

(B) qRT-PCR for C-MYC and HMGA2 at day 7 of reprogramming. Levels were normalized to GAPDH. Data are represented as mean +/- SD, n=3.

(C) Western blotting for N-MYC, L-MYC, and GAPDH in HDFs at day 7 of reprogramming.

Figure S4. OSK+LIN-41 Promotes Reprogramming of Mouse Fibroblasts, Related to Figure 2.

(A) Nanog-GFP reporter-containing MEFs were reprogrammed with OSK, OSK+dsRED, and OSKL (left panel), or OSKM, OSKM+dsRED, and OSKML (right panel). Nanog-GFP+ colonies were counted every 3 days. Data are represented as mean +/- SD, *p<0.05, n=4.

(B) An image of a Nanog-GFP-positive iPSC-like colony, derived from Nanog-GFP MEFs made with retroviral OSK+LIN41. Scale bar, 200 μm.

(C) Karyotype of miPS-LIN41#16 at passage 13.

(D) Expression of pluripotent stem cell marker proteins (Nanog, SSEA1) detected by immunocytochemistry in miPS-LIN41#2 at passage 11. Scale bars, 100 μm. Secondary antibodies were labeled with AlexaFluor555 (red). Nuclei were stained with DAPI (Blue).

(E) Representative images of embryoid bodies made from miPS-LIN41#16 and stained for markers of differentiation (ectoderm: Tuj, mesoderm: cTnT, and endoderm: AFP, SMA) by immunocytochemistry. Scale bars, 50 μm. Secondary antibodies were labeled with AlexaFluor555 (red). Nuclei were stained with DAPI (blue).

(F) The OSKL iPSC line miPS-Lin41#2 line (at passage 12) is pluripotent and contributed to chimeras.

Figure S5. Assaying Proliferation and *let-7* and LIN-41 Levels During Reprogramming, Related to Figure 2.

(A) Results from one representative cell-proliferation assay after infecting HDFs with OSK+dsRED or OSKL.

(B) RT-PCR for LIN-41 after 5 days of infection with GFP, OSK+control inh, OSK+*let-7*inh, or OSKM. Data are represented as mean +/- SD, *p<0.05, **p<0.01; n=3.

(C) qRT-PCR for *let-7a, b,* and *c* expression was performed 7 days and 13 days post-initiation of reprogramming with GFP, OSK+*let-7*inh, or OSKM. Data are represented as mean +/- SD, n=2.

(D) A western blot comparing the level of endogenous LIN-41 induced in a mixed population of HDFs and reprogramming cells to the level of exogenous LIN-41 expressed by retroviral infection.

(E) Immunofluorescence was performed using an anti-LIN-41 antibody at day 10 of reprogramming. We selected random fields of DAPI stained nuclei and captured images in both the blue (DAPI) and green (LIN-41) channels. The number of nuclei and number of LIN-41-positive cells were counted.

(F) Immunofluorescence was performed using an anti-LIN-41 antibody at day 10 of reprogramming. Confocal z-stacks of LIN-41-positive and -negative cells for different reprogramming cocktails were captured using constant exposure and gain settings (Nikon). The fluorescence intensity per cell vol. was quantitated using Volocity software (PerkinElmer). Horizontal bars indicate the mean intensity for each cocktail. The shaded area indicates cells which have a level of LIN-41 comparable to that achieved by OSK+*let-7 inh*.

Figure S6. Examining Potential LIN-41 Targets, Related to Figures 5 and 6.

(A) Representative western blot of AGO2 levels upon expression of LIN-41 or ΔRING in HDFs undergoing reprogramming (left four lanes) or upon LIN-41 knockdown in hESCs (right three lanes).

(B) Representative western blots of FGF signaling mediators upon expression of LIN-41 or ΔRING in HDFs undergoing reprogramming. The FGF signaling proteins shown are p-AKT, p-GSK3α/β, and p-ERK1/2 as well as total AKT, GSK3α/β, ERK1/2, and GAPDH (control).

(C) Representative western blots of FGF signaling mediators upon LIN-41 knockdown in hESCs, when cultured in E8 medium either in the presence of FGF2 (+FGF2 72 hrs) or in the absence of FGF2 but treated with FGF2 for 10 min before harvesting the cells (-FGF2 72hrs, +FGF2 10 min). The FGF signaling proteins shown are p-AKT, p-GSK3α/β, and p-ERK1/2, as well as total ERK1/2, LIN-41, and GAPDH (control).

(D) Representative western blot of HDFs uninfected or infected with GFP, OSKM, or OSK plus LIN-41 or ΔRING. Western blotting was performed for p21/CDKN1A levels and GAPDH (control).

(E) Representative images of H14 hESCs transfected with siRNAs against LIN-41 or OCT4 and a negative control siRNA. Images were taken 72 hrs later.

(F) TRA-1-60+ cells were isolated after 11 days of reprogramming with OSK+control inh, OSK+*let-7* inh, OSKM, and OSKL, and gene expression was analyzed as described in Tanabe et al., 2013. The relative levels of EGR1 (left) and LIN-41 (right) in these samples were compared to the levels observed in GFP+ GFP-infected HDFs.

(G) List of the top ten transcription factors with enriched binding sites among genes with a greater than three-fold difference in expression level between OSKL and OSK reprograming cells, identified by Whole Genome rVISTA. Among these transcription factors, only EGR1 was downregulated in OSKL reprogramming cells.

(H) Representative NANOG staining of colonies after reprogramming with OSK+GFP, OSKL, OSK+EGR1, or OSKL+EGR1.

Table S1. RNAseq data from hESCs, Related to Figure 5.

Table S2. Predicted EGR1 targets, Related to Figure 5.

Table S3. Antibodies, Taqman probes, and primers used, Related to Figures 1-6.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell Culture and Reprogramming

Human iPSCs and ESCs were maintained on either mouse SNL feeders in hESC medium or in feeder-free conditions on matrigel in mTESR1 medium. All other cells were cultured in DMEM with 10% fetal bovine serum (FBS), glutamax, and penicillin/streptomycin. HDFs from Cell Applications were used in this study (lots 1429, 1323, and 1503). hiPSC reprogramming was carried out with retroviruses as described Takahashi et al., 2007. 0.125mL of each virus was used per well of a 6-well dish. On day 7 post-infection, the cells were trypsinized, counted, and reseeded onto SNL feeders at $2x10^4$ or $5x10^4$ per well of a 6-well dish for reprogramming with or without c-MYC, respectively. Cells were transfected with miRNA inh (20nM, Dharmacon, control inh [IN-001005-01] or let-7c inh [IH-300477-05]) (Robertson et al., 2010) on days 1, 6,

12, 18, and 24 unless otherwise indicated. Cells were transfected with siRNAs (20nM), every 3 days starting on day 2.

Generation of Mouse iPSCs

Mouse iPSC induction was performed as described with some modifications (Okita et al., 2007; Takahashi and Yamanaka, 2006). Briefly, MEFs were isolated from E13.5 embryos from Nanog-reporter mice. MEFs were seeded at 1×10^5 cells/well of a 6-well plate the day before infection with retrovirus, as described above. Three days after infection, the cells were reseeded at 1,000 cells (reprogramming with c-MYC) or 5,000 cells (reprogramming without c-MYC) per well of a 6-well plate onto SNL feeder cells. On the next day, medium was changed to mouse ESC medium (Knockout DMEM, 15% FBS, nonessential amino acids, glutamax, penicillin/streptomycin, β-mercaptoethanol) supplemented with LIF. The medium was changed every 2 days. The GFP-positive colonies were counted every 3 days starting 15 days post-infection. GFP-positive, ES-like colonies were picked at 24 days after infection and maintained in the mouse ESC medium supplemented with 2 μg/ml of puromycin.

Characterization of iPSCs

iPSC colonies were picked and passaged at least 10 times. Then, qRT-PCR for endogenous versus exogenous transgene expression using primers and probes described in Table S2 was performed to determine if transgene silencing had occurred, before injection for teratomas. Established *in vitro* differentiation methods were used to determine if the clones could differentiate into endoderm (Warren et al., 2010),

ectoderm (Takahashi et al., 2007), and mesoderm (EB differentiation). For teratoma analyses, iPSCs were grown to ~70% confluency on a 10-cm matrigel-coated dish. One-sixth of the cells, suspended in 50 μL of mTESR medium with Rock inh, were injected per testicle of a CD17 SCID mouse (Charles River). After about 2 months, the teratomas were removed, fixed with 4% paraformaldehyde (PFA) overnight at 4°C, sectioned, and stained with hematoxylin and eosin. Cell Line Genetics performed the karyotyping.

Cell Proliferation Assays

HDFs were plated starting at 3,000 cells/well of a 96-well plate. They were infected (day 0) with retrovirus as described above for reprogramming. Six replicates were performed for each condition. On days 0, 3, 5, 7, and 9, the cells were washed with 1x PBS and stored at -80°C. Cell proliferation was measured using a CyQuant Cell Proliferation Assay (Life Technologies).

Immunofluorescence

Cells were fixed and solubilized with 4% PFA/0.5% Triton X-100/1xPBS for 10 min, washed three times with 0.1% Tween-20/1x PBS, blocked with 5% BSA/1x PBS for 30 min, and incubated with the primary antibodies (Table S3) in blocking buffer supplemented with 0.2% Tween-20 for 2 hours (hrs) at 37°C or 4°C overnight. Cells were washed three times and incubated with secondary antibodies (AlexaFluor 488 or 555; 1:200) for 1 hr. After washing, vectashield with DAPI was added. For examining levels of EGR1 in individual cells, HDFs were transferred to coverslips 7 days post-

infection and on day 8 had a medium change 1 hr prior to fixation to stimulate EGR1 expression. Microscopy was performed on a Zeiss AxioObserver Microscope or on a Nikon spinning disk confocal microscope. A Pierce peroxidase detection kit was used to detect TRA-1-60.

LIN-41 IP

H1 hESCs were pelleted by 250 x gravity centrifugation, flash frozen with liquid N_2, and stored at -80°C. Cells were resuspended in 3 vol. NP-40 lysis buffer [50 mM Tris pH 8.0, 120 mM NaCl, 1 mM magnesium acetate, 0.1% NP-40, Complete Protease Inh (Roche), 1 mM DTT, and 250 U/ml RNAsin (Ambion)] and blended for 3 min. on ice with the Tissue Tearer (setting 1, Biospec Products). Insoluble material was removed by 20,000x centrifugation for 20 min. The lysate was adjusted to 5 mg/ml final concentration. 75ul Protein G Dynabeads (Life Technologies) and 25ug LIN-41 monoclonal antibody (developed in collaboration with Epitomics) were rotated for 1 hr at room temperature (RT), rinsed 4 times with lysis buffer, resuspended in 500 ul, and transferred to a fresh tube. Subsequently, peptide-blocked beads were incubated with 20ng 3X-LIN-41 peptide (NH2-RVRLTKDHYIERRVRLTKDHYIERRVRLTKDHYIER-COOH) to block the LIN-41 antibody binding sites. 1.5ml of extract was added and the samples were rotated at 4°C overnight. Beads were washed 5 times with lysis buffer for 5 min. with rotation at 4°C. Finally, the beads were resuspended in 200ul lysis buffer for RNA recovery or sample buffer for western blotting. For RNA recovery, 2 vol. of Trizol were added to the resuspended beads. The sample was vortexed, incubated for 5 min., and 200 ul chloroform was added. The sample was vortexed and incubated for 5 min.

before spinning at 14,000 rpm for 10 min. at 4°C. The aqueous layer was placed into a fresh tube. An equal vol. of 70% ethanol was added, the sample was added to an RNeasy Micro kit column (Qiagen), and the RNA was recovered per the kit protocol.

Transcriptome Analysis

To analyze gene expression upon LIN-41 knockdown, H1 hESCs were plated on matrigel with mTESR1 medium and ROCK inh (Y-27632). The next day, the medium (mTESR1 + Y-27632) was changed and the cells were transfected overnight with control or LIN-41 siRNAs (50nM final concentration) using Lipofectamine RNAimax. Medium (without Y-27632) was changed everyday. 72 hrs after transfection, total RNA was isolated using Trizol, purified using an RNeasy Micro Kit, and sequenced using Illumina HiSeq 2000. Alignment was performed on 100bp paired-end samples using Tophat 2.0.6. Reads were aligned to hg19. Duplicate reads were removed with the MarkDuplicates program, part of the Picard suite. Differential expression P-values were computed using the Defined Region Differential Seq program, part of the USeq package. We analyzed the 1,363 genes that had differential expression (FDR<0.05) between the control siRNA samples (n=3) and the LIN-41 siRNA samples (n=6) using GO-Elite (http://www.genmapp.org/go_elite/) to identify biological processes enriched among LIN-41-regulated genes (Zambon et al., 2012). We performed a similar analysis between TRA-1-60+ OSKL versus OSK reprogramming cells. We used Whole Genome rVISTA (Dubchak et al., 2013) to identify enriched predicted TF binding sites among these sets of genes.

Cloning

The LIN-41 cDNA was obtained from Thermo (clone 610064) and cloned into the retroviral expression vector pMXs by NcoI- and AvrII-mediated digestion. LIN-41 domain mutants were constructed using a Cold Fusion cloning kit (Systems Biosciences). PCRs were performed using either Phusion or KOD Xtreme enzymes. To construct the ΔRING mutant, an oligo lacking the sequence corresponding to amino acids 12-91 was cloned into the NcoI/BplI restriction sites. To construct the ΔB-box mutant, pMXs-LIN-41 was cut with XhoI/XcmI, and a PCR product lacking the B-boxes was cloned in. To construct ΔCoiled-coil, pMXs-LIN-41 was cut with XcmI/BclI, and the PCR product lacking the coiled-coil was cloned in. To construct Δ6xNHL, an oligo containing a unique MfeI site and homology to the 5'end of LIN-41 as well as the region near the SmaI site located between the Filamin domain and the NHL repeats was cloned into pMXs-LIN-41 at the NcoI/AvrII sites; the NcoI/SmaI region was then cloned into the MfeI site. To construct ΔFilamin, Δ6xNHL was cut with AfeI/AvrII, and two PCR products were made and joined using PCR and then cloned in. To make the NHL-only construct, the 6xNHL domain was PCR amplified and cloned into the NcoI/AvrII sites of pMXs-LIN-41. To make the C12AC15A mutant, a PCR product containing the relevant cysteine-to-alanine mutations was cloned into the NcoI and XhoI sites in pMXS-LIN-41. To construct 7CtoA, the C12AC15A construct was cut with NcoI/XcmI, and a four-way Cold Fusion reaction was performed using three PCR products that contained cysteine-to-alanine-producing mutations.

SUPPLEMENTAL REFERENCES

Warren, L., Manos, P.D., Ahfeldt, T., Loh, Y.H., Li, H., Lau, F., Ebina, W., Mandal, P.K., Smith, Z.D., Meissner, A., et al. (2010). Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. Cell Stem Cell 7, 618–630.