Stem Cell Reports, Volume *3*

Supplemental Information

Manipulation of KLF4 Expression Generates iPSCs Paused at Successive Stages of Reprogramming

Ken Nishimura, Tetsuo Kato, Chen Chen, Lalhaba Oinam, Emi Shiomitsu, Daisuke Ayakawa, Manami Ohtaka, Aya Fukuda, Mahito Nakanishi, and Koji Hisatake

Figure S1

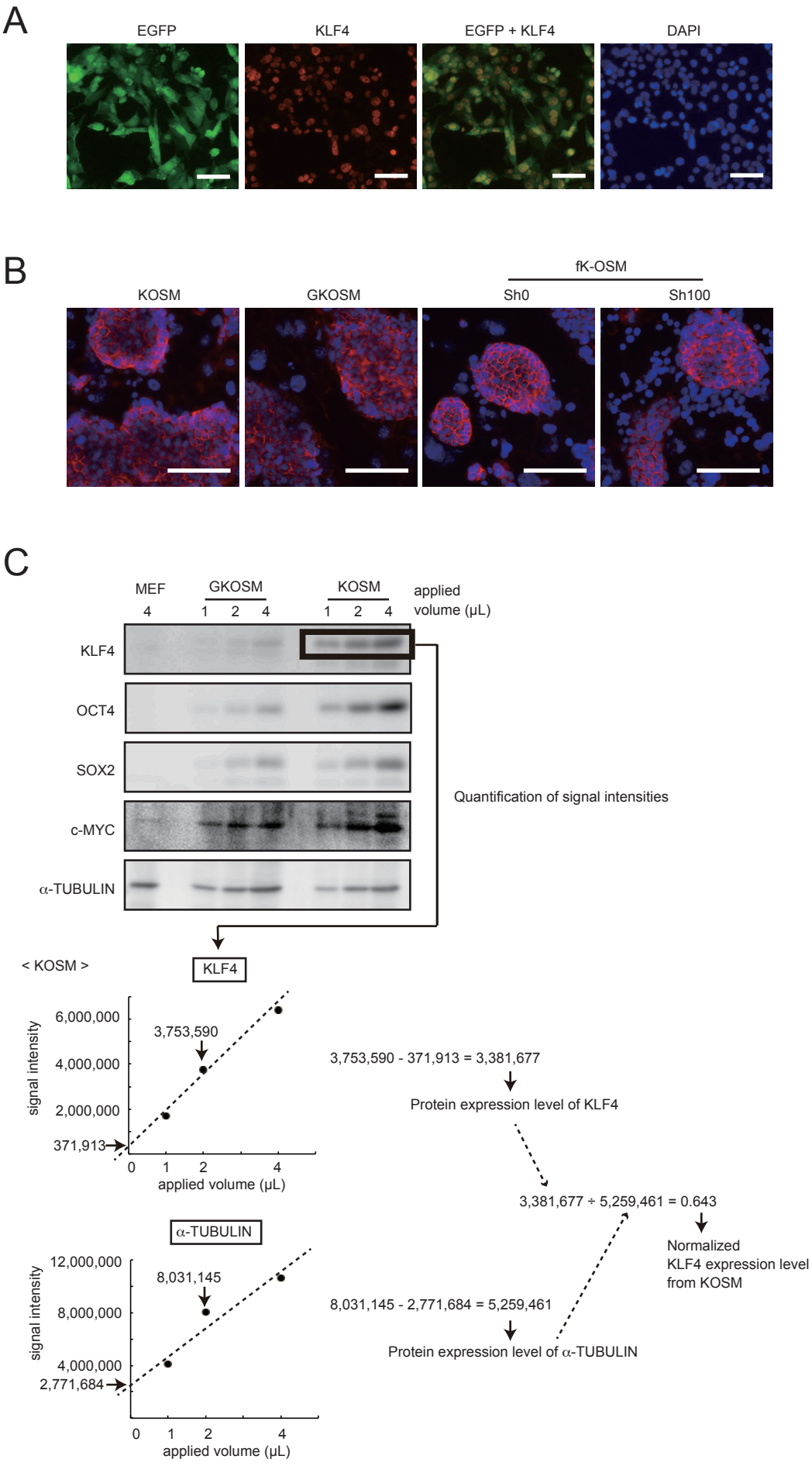

A
EGFP
KLF4
EGFP + KLF4
DAPI
B
fK-OSM
KOSM
GKOSM
Sh0
Sh100
C
MEF
GKOSM
KOSM
applied volume (μL)
4
1 2 4
1 2 4
KLF4
OCT4
SOX2
c-MYC
α-TUBULIN
Quantification of signal intensities
< KOSM >
KLF4
signal intensity
6,000,000
4,000,000
2,000,000
371,913
3,753,590
0 1 2 4
applied volume (μL)
3,753,590 - 371,913 = 3,381,677
Protein expression level of KLF4
3,381,677 ÷ 5,259,461 = 0.643
Normalized
KLF4 expression level
from KOSM
α-TUBULIN
signal intensity
12,000,000
8,000,000
4,000,000
2,771,684
8,031,145
0 1 2 4
applied volume (μL)
8,031,145 - 2,771,684 = 5,259,461
Protein expression level of α-TUBULIN

Figure S1. (Related to Figures 1 and 3)

(A) Infection of MEFs with SeVdp(GKOSM) and monitoring of the gene expression from the vector. MEFs were infected with SeVdp(GKOSM), and expression of EGFP and one of the reprogramming factors (KLF4) was detected at day 2 by the green fluorescence and immunostaining, respectively. Scale bars, 100 μm. (B) Cell morphology of mouse iPSCs produced by several SeVdp vectors. Mouse iPSCs colonies induced by SeVdp(KOSM), SeVdp(GKOSM) or SeVdp(fK-OSM) (in the presence or absence of 100 nM of Shied1) were stained for β-CATENIN at day 30. Scale bars, 100 μm. (C) Quantitative western blots to determine the relative protein levels of reprogramming factors. MEFs were infected with SeVdp(GKOSM) or SeVdp(KOSM) and collected one day after infection to prepare cell extract. The cell extracts were applied onto the SDS-PAGE gel and subjected to western blotting, and the signals were analyzed by LAS4000 and quantified using ImageQuant TL software. The applied volumes and the signal intensities from each western blot were plotted to obtain the linear regression line. Pilot experiments were performed to determine the appropriate amounts of cell extracts that showed a linear correlation with the obtained signals. Then the relative expression level of each factor was calculated from the intensity of the signal that falls within the linear range. The protein expression level of each reprogramming factor was normalized against that of α-TUBULIN, which was determined in the same quantitative manner. The quantifications were biological triplicates.

Figure S2

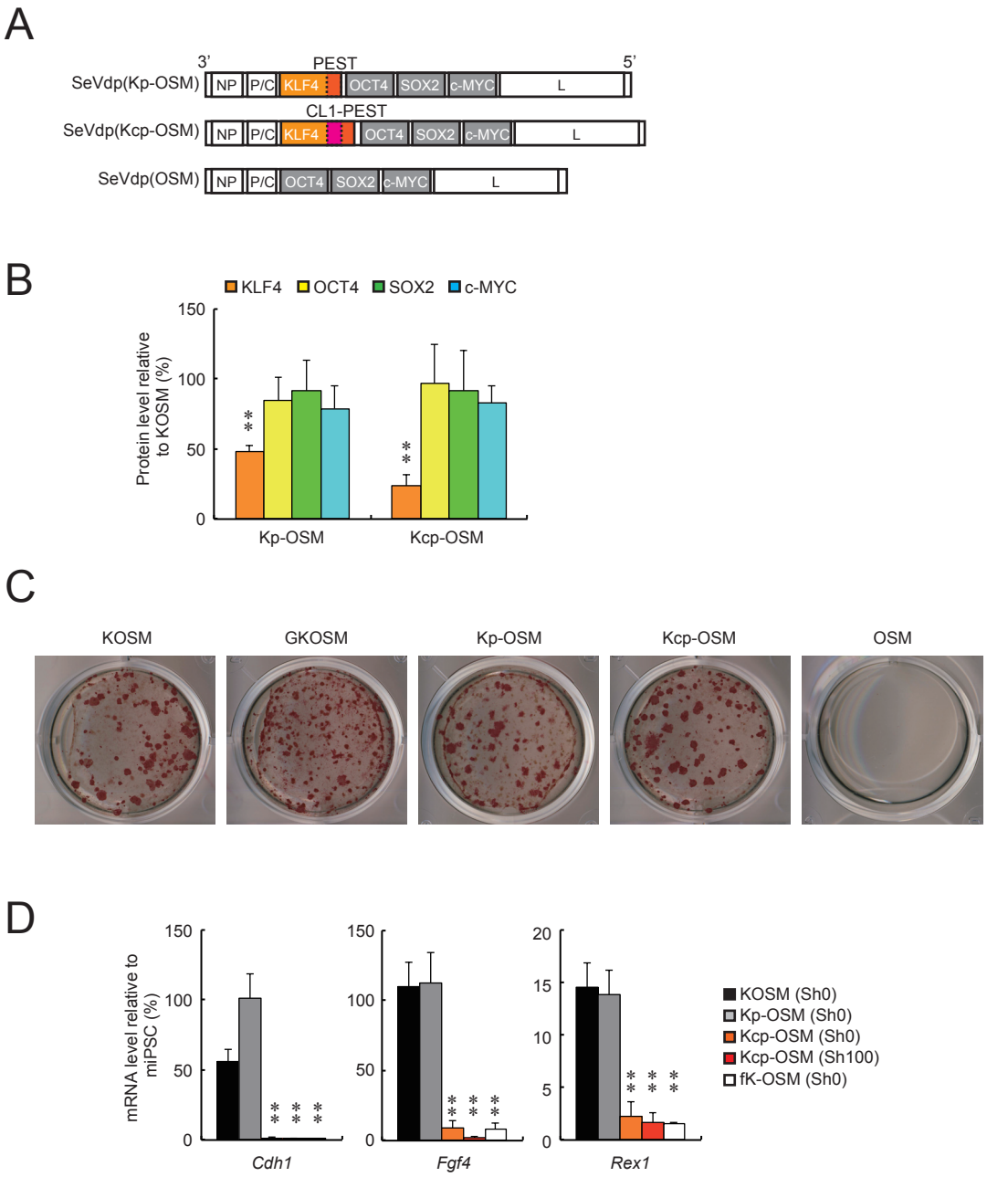
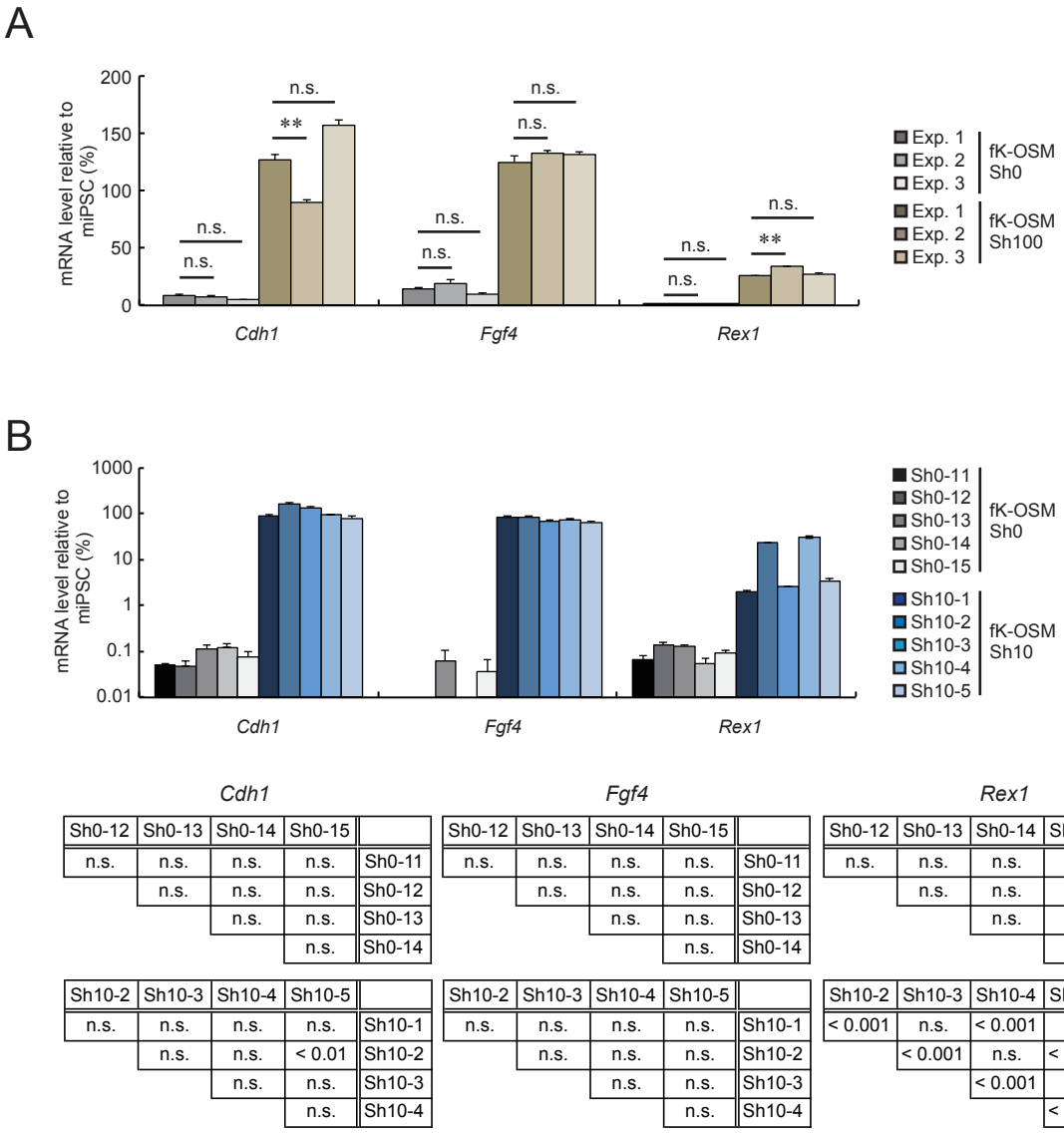


Figure S2. (Related to Figure 2)

(A) Structures of the SeVdp-derived vectors. In SeVdp(Kp-OSM) and SeVdp(Kcp-OSM), KLF4 is tagged C-terminally with PEST or CL1-PEST. In SeVdp(OSM), there are only three reprogramming factors; namely, OCT4, SOX2 and c-MYC. (B) Protein levels of reprogramming factors in MEFs infected with SeVdp(Kp-OSM) or SeVdp(Kcp-OSM). Cell extracts were prepared a day after infection with the indicated vector. Protein expression levels are indicated relative to those in SeVdp(KOSM)-infected cells. Data represent means ± SEM of three independent experiments. $^{**}P < 0.01$ versus SeVdp(KOSM). (C) Alkaline phosphatase (+) colonies induced by each vector. MEFs infected with the indicated vector were stained for alkaline phosphatase at day 13. (D) Expression of ES cell markers in reprogrammed iPSCs. The mRNA levels of *Cdh1*, *Fgf4* and *Rex1* in the cells infected by the indicated vector in the presence or absence of 100 nM Shield1 were determined by quantitative RT-PCR at day 16. Data represent means ± SEM of three independent experiments. $^{**}P < 0.01$ versus SeVdp(KOSM).

Figure S3



Cdh1

Sh0-12	Sh0-13	Sh0-14	Sh0-15	
n.s.	n.s.	n.s.	n.s.	Sh0-11
	n.s.	n.s.	n.s.	Sh0-12
		n.s.	n.s.	Sh0-13
			n.s.	Sh0-14

Sh10-2	Sh10-3	Sh10-4	Sh10-5	
n.s.	n.s.	n.s.	n.s.	Sh10-1
	n.s.	n.s.	< 0.01	Sh10-2
		n.s.	n.s.	Sh10-3
			n.s.	Sh10-4

Fgf4

Sh0-12	Sh0-13	Sh0-14	Sh0-15	
n.s.	n.s.	n.s.	n.s.	Sh0-11
	n.s.	n.s.	n.s.	Sh0-12
		n.s.	n.s.	Sh0-13
			n.s.	Sh0-14

Sh10-2	Sh10-3	Sh10-4	Sh10-5	
n.s.	n.s.	n.s.	n.s.	Sh10-1
	n.s.	n.s.	n.s.	Sh10-2
		n.s.	n.s.	Sh10-3
			n.s.	Sh10-4

Rex1

Sh0-12	Sh0-13	Sh0-14	Sh0-15	
n.s.	n.s.	n.s.	n.s.	Sh0-11
	n.s.	n.s.	n.s.	Sh0-12
		n.s.	n.s.	Sh0-13
			n.s.	Sh0-14

Sh10-2	Sh10-3	Sh10-4	Sh10-5	
< 0.001	n.s.	< 0.001	n.s.	Sh10-1
	< 0.001	n.s.	< 0.001	Sh10-2
		< 0.001	n.s.	Sh10-3
			< 0.001	Sh10-4

fK-OSM Sh0

fK-OSM Sh10

Figure S3. (Related to Figure 3)

(A) Reproducibility of reprogramming by SeVdp(fK-OSM). SeVdp(fK-OSM)-infected cells were cultured in the presence or absence of Shield1. The cells were collected at day 27 (Exp.1), day 24 (Exp.2) or day 26 (Exp.3), and the mRNA levels of *Cdh1*, *Fgf4* and *Rex1* were determined by quantitative RT-PCR. Data represent means ± SEM of three independent PCR reactions. n.s., not significant; $^{**}P < 0.01$ versus Exp. 1. (B) Homogeneity of partially reprogrammed clones generated by SeVdp(fK-OSM). Partially reprogrammed cells were induced by SeVdp(fK-OSM) in the presence (Sh10-1 to 5) or absence (Sh0-11 to 15) of 10 nM Shield1, and individual colonies were isolated and expanded for analyses. The mRNA levels of *Cdh1*, *Fgf4* and *Rex1* in each clone were determined by quantitative RT-PCR at day 56. Data represent means ± SEM of three independent PCR reactions. *P*-values of individual data sets are listed in the tables. n.s., not significant.

Figure S4

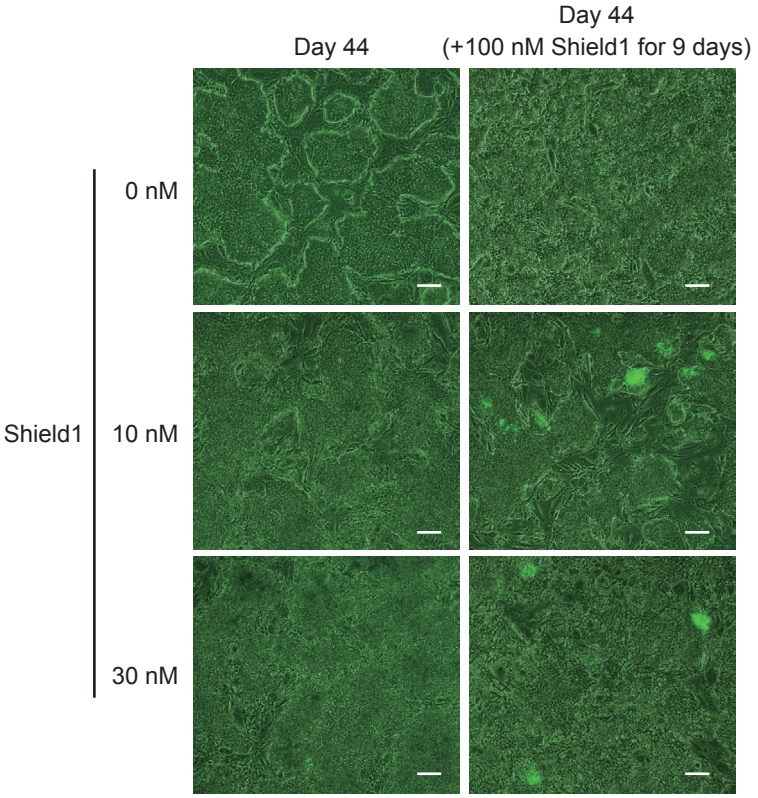


Figure S4. (Related to Figure 4)
Paused iPSCs were generated in the presence of the indicated concentration of Shield1, and 100 nM Shield1 was added to the cells at day 35. The NANOG expression was observed by GFP fluorescence from the *Nanog*-GFP reporter transgene at day 44. Scale bars, 100 μm.

Figure S5

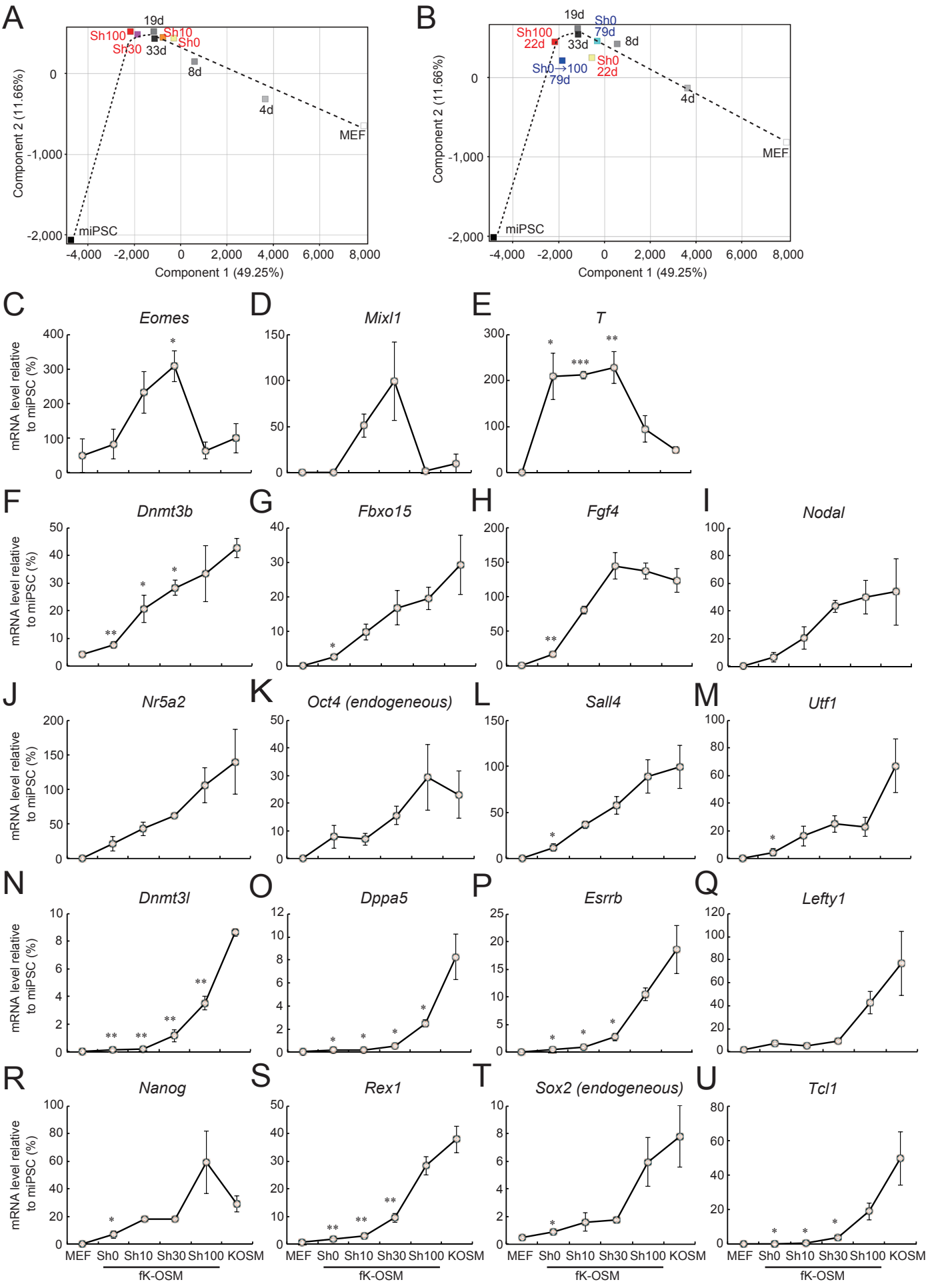

A
B
19d
Sh100
Sh10
Sh30
33d
Sh0
8d
4d
MEF
miPSC
Component 2 (11.66%)
Component 1 (49.25%)
Sh0 79d
22d
Sh0→100 79d
C
Eomes
D
Mixl1
E
T
F
Dnmt3b
G
Fbxo15
H
Fgf4
I
Nodal
J
Nr5a2
K
Oct4 (endogeneous)
L
Sall4
M
Utf1
N
Dnmt3l
O
Dppa5
P
Esrrb
Q
Lefty1
R
Nanog
S
Rex1
T
Sox2 (endogeneous)
U
Tcl1
mRNA level relative to miPSC (%)
MEF Sh0 Sh10 Sh30 Sh100 KOSM
fK-OSM

Figure S5. (Related to Figure 5)

(A, B) Principal component analysis (PCA) of the paused iPSCs. The expression profiles of the differentially expressed genes (DEGs) between MEFs and miPSCs ($P < 0.05$) were used for the PCA. PCA of SeVdp(KOSM)-reprogrammed MEFs collected at day 4, 8, 19 and 33 is compared to paused iPSCs generated by SeVdp(fK-OSM) in the presence of 0, 10, 30 or 100 nM of Shield1 collected at day 22 (A) or iPSCs that had paused and then resumed reprogramming (B). Sh0→100 79d; MEFs were reprogrammed by SeVdp(fK-OSM) in the absence of Shield1, and 100 nM Shield1 was added to the cells at day 25, which were further cultured until day 79. (C-U) Gene expression profiles of mesendodermal genes and pluripotency-related genes in paused iPSCs. SeVdp(fK-OSM)-infected cells were reprogrammed in the presence of indicated concentrations of Shield1, and the cells were collected at day 27. The mRNA levels of mesendodermal genes (C-E) and pluripotency-related genes (F-U) were determined by quantitative RT-PCR. Data represent means ± SEM of three independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus SeVdp(KOSM).

Figure S6

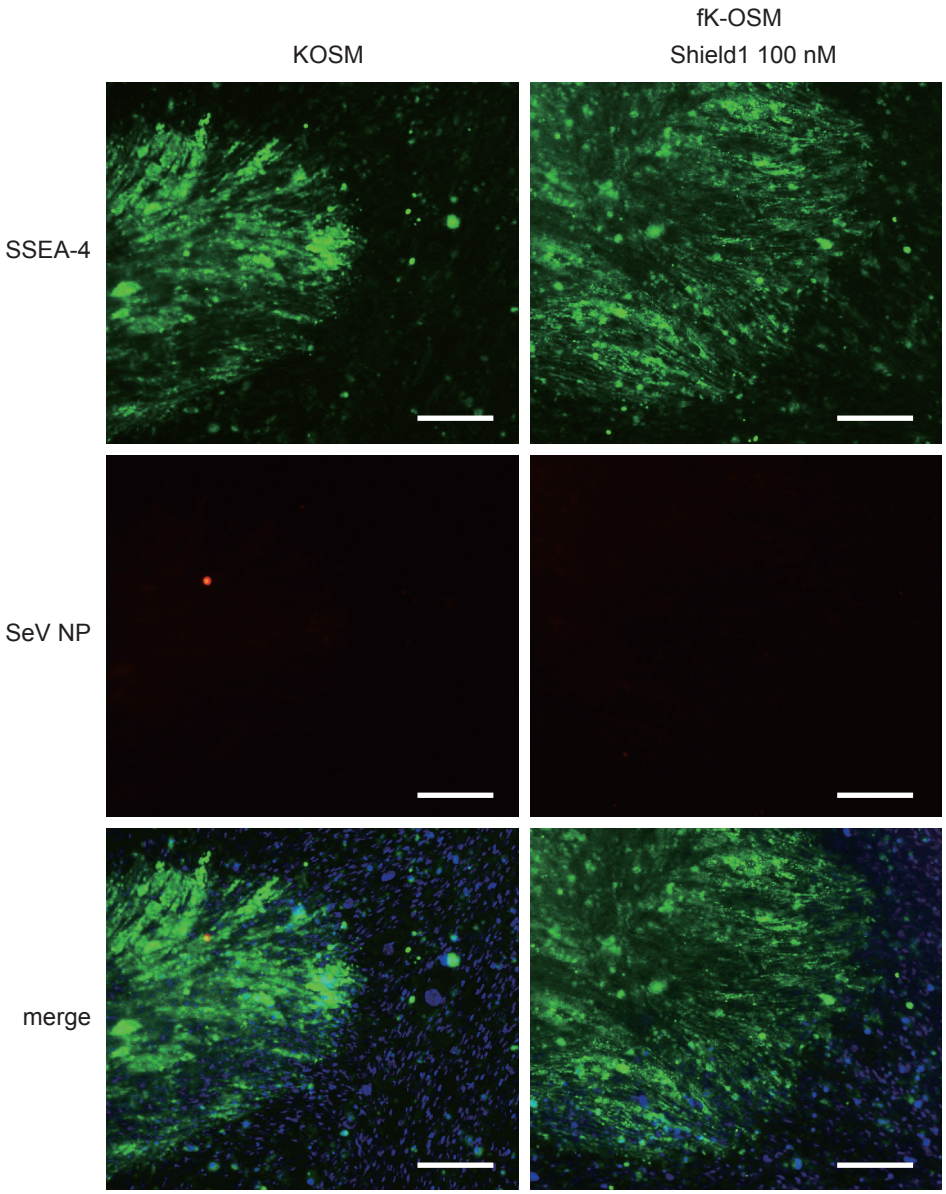


Figure S6. (Related to Figure 6)
Human iPSCs generated by SeVdp(KOSM) or SeVdp(fK-OSM) in the presence of 100 nM Shield1 were treated three times with L527 siRNA, and the cells were stained for SSEA-4 and SeV NP at day 44. Scale bars, 100 μm.

Table S1. Number of alkaline phosphatase (+) or *Nanog*-GFP (+) colonies in mouse or human iPSC production
Related to Figures 1, 2 and 6.

< mouse iPSCs >

Alkaline Phosphatase

		KOSM	GKOSM	fK-OSM	K-fOSM	KO-fSM	KOS-fM
Shield1	0 nM	705±221	712±170	986±331	9±2*	40±9*	2±1*
	100 nM	n.d.	n.d.	693±232	16±3*	339±119	85±38

Nanog-GFP

		KOSM	fK-OSM	K-fOSM	KO-fSM	KOS-fM
Shield1	0 nM	63±14	0±0**	1±1*	10±5*	0±1*
	100 nM	n.d.	30±12	3±1*	37±11	9±5*

< human iPSCs >

Alkaline Phosphatase

		KOSM	GKOSM	fK-OSM
Shield1	0 nM	36±5	63±5*	19±4
	100 nM	n.d.	n.d.	25±5

Colony numbers were counted 16 days after infection. Data represent means ± SEM of three independent experiments.
*P < 0.05, **P < 0.01 versus KOSM in the absence of Shield1.
n.d., not done

Table S2. Primer sequence for quantitative RT-PCR analyses
Related to Figures 1, 3, 4, 5 and 6.

• For mouse genes

Gene	Sequence
Cdh1	ACGTCCCCCTTTACTGCTG
	TATCCGCGAGCTTGAGATG
Cdh2	ATCAACCCCATCTCAGGACA
	CAATGTCAATGGGGTTCTCC
Dnmt3b	CCACATTTGCTGGAGGATG
	TACCAAAGCAAGGGGAAGG
Dnmt3l	GAACCGACGGAGCATTGAAG
	TCCCTCAAACAAGGGGTGCCG
Dppa5	CCAGTCGCTGGTGCTGAAATATCTG
	GCCCGAATCTTGTTGTTTTGAGAGC
Eomes	TCCAAGACTCAGACCTTCACC
	AGGGACAATCTGATGGGATG
Esrrb	TGGCAGGCAAGGATGACAGA
	TTTACATGAGGGCCGTGGGA
Fbxo15	CTCATCTGTCACGAAGCAGC
	AGGTCACCGCATCCAAGTAA
Fgf4	GCAGCGAGGCGTGGTGAGCATCTT
	CCCCTTCTTGGTCCGCCCGTTCTT
Lefty1	ACAGCTGGAGCTGCACA
	GTTCTCGGCCCACTTCATC
Mixl1	TGGAGCTCGTCTTCCGACA
	AATGACTTCCCACTCTGGCG
Nanog	ACCTGAGCTATAAGCAGGTTAAGAC
	GTGCTGAGCCCTTCTGAATCAGAC
Nodal	ACATGTTGAGCCTCTACCGAG
	GTGAAAGTCCAGTTCTGTCCG
Nr5a2	CAAAGTGGAGACGGAAGCC
	ATCGCCACACACAGGACATA
Oct4 (endogeneous)	CCAACGAGAAGAGTATGAGGC
	GTGCTTTTAATCCCTCCTCAG
Rex1	TTGATGGCTGCGAGAAGAG
	ACCCAGCCTGAGGACAATC
Sall4	CACGAAAGGCAACCTGAAG
	GGAGCTCCTTGGAAAACACC
Snail1	TGTGTCTGCACGACCTGTG
	AGTGGGAGCAGGAGAATGG
Snail2	ACACATTGCCTTGTGTCTGC
	GCCCTCAGGTTTGATCTGTC
Sox2 (endogeneous)	AGAGAAGTTTGGAGCCCGAG
	ATCTGGCGGAGAATAGTTGG
T	AAGGCGCCTGTGTCTTTCAG
	CTCACGATGTGAATCCGAGG
Tbp	GGGGAGCTGTGATGTGAAGT
	CCAGGAAATAATTCTGGCTCA
Tcl1	GGAGAAGCACGTGTACTTGGA
	TGACTGGGGGACATAGCTTC
Tgfb3	GAAGGCTGCACTCAGGAGAC
	TGAGGACACATTGAAACGAA
Thy1	TCGCTCTCCTGCTCTCAGTC
	TTATTCTCATGGCGGCAGTC
Twist1	CCCACACCTCTGCATTCTG
	CTGTCAGTGGCTGATTGGC
Utf1	GTCCCTCTCCGCGTTAGC
	GGGGCAGGTTCGTCATTT
Zeb1	TGCCAGCAGACCAGACAGTA
	TTCGGATCATGGTTTTGCTC
Zeb2	GCTAACCCAAGGAGCAGGTAAC
	TGAACTGTAGGACCCAGAATGA

• For human genes

Gene	Sequence
CDH1	GAGACAGTTTCGCTCCATCG
	AGCTTGGGCAACATAGCAAG
FGF4	ACCTTGGTGCACTTTCTTCG
	AAAAAACACACCCGCAGAAC
REX1	GCTCCTACACTTTGTGATACCG
	TCACTGACCTTCCTGTTGGA
TBP	TCTCATGTACCCTTGCCTCC
	GGCACTTACAGAAGGGCATC

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Production of SeVdp vectors

The SeVdp vector genomic cDNA was constructed as described (Nishimura et al., 2011). First, we changed the order of reprograming factors in SeVdp(*c-Myc*/*Klf4*/*Oct4*/*Sox2*) cDNA to the order of KLF4, OCT4, SOX2 and c-MYC to construct SeVdp(KOSM) cDNA. Using the SeVdp(KOSM) cDNA, we constructed the following vector cDNAs. The DNA fragment encoding the EGFP gene was amplified by polymerase chain reaction (PCR) using pEGFP-1 (Takara Bio) as a template. The amplified EGFP expression cassette containing transcription initiation and termination signals for Sendai virus (SeV) was inserted between the P/C and KLF4 genes of SeVdp(KOSM) vector to create SeVdp(GKOSM) cDNA. The DNA fragment of the destabilization domain (DD) derived from an FKBP12 mutant was amplified by PCR from pPTuner vector (Takara Bio) and inserted into the N-terminus of the coding region of each reprogramming factor (KLF4, OCT4, SOX2, or c-MYC) to create a fusion protein using In-Fusion HD Cloning Kit (Takara Bio). These cDNAs were used to construct vector cDNAs of SeVdp(fK-OSM), SeVdp(K-fOSM), SeVdp(KO-fSM) or SeVdp(KOS-fM). The DNA fragment coding for CL1 or PEST was amplified from pST-blue-T7-GFP-CL/PEST and inserted into the C-terminus of the KLF4 gene to construct SeVdp(Kcp-OSM) or SeVdp(Kp-OSM) cDNA. We removed the KLF4 gene expression cassette from SeVdp(KOSM) cDNA to create SeVdp(OSM) cDNA.

To prepare vector-packaging cells, 2.0 x 10^5 BHK/T7/151M(SE) cells in a 6-well plate were transfected with each vector cDNA (2 μg) and the expression vector plasmids for SeV proteins (NP, P/C, M, F, HN, L) (1 μg each) using Lipofectamine LTX with Plus Reagent (Life Technologies) and cultured at 32 ˚C for 6 days. Then, the packaging cells were further transfected with the expression vector plasmids for SeV M, F and HN proteins and cultured at 32 ˚C for additional 4 days to rescue the SeVdp vector, which was released into the culture supernatant. The supernatant was filtered through a 0.45 μm cellulose acetate filter and stored at -80 ˚C until use. Titers of the SeVdp vectors were determined by examining NIH3T3 cells infected with a diluted vector suspension by immunostaining using an anti-SeV NP antibody.

Production of retroviral vector

The cDNAs encoding the OCT4, SOX2, KLF4 and c-MYC proteins were amplified from mouse embryonic stem cell cDNA and inserted into pMXs (Addgene) to construct the retroviral vector expressing each protein. PLAT-E cells were transfected with each vector using

Lipofectamine LTX with Plus Reagent. Viral supernatant was collected 2 days after transfection and filtered through a 0.45 μm cellulose acetate filter and stored at -80 ˚C until use. Titers of the retroviral vectors were determined by examining NIH3T3 cells infected with a diluted vector suspension by immunostaining using an antibody against OCT4 (1:1,000, ab19857, Abcam), SOX2 (1:250, sc-17320, Santa Cruz), KLF4 (1:250, sc-20691, Santa Cruz) or c-MYC (1:250, sc-764, Santa Cruz).

Generation of chimeric mice

Chimeric mice were generated by a conventional method in which SeVdp(fK-OSM)-free iPSCs (Sh100-36 and Sh100-43) were microinjected into blastocysts (3.5 days post-coitum) of C57BL/6J mice, followed by transferring to the uterus of foster mothers (Joyner, 1993). Genomic DNAs were extracted from the resultant chimeric mouse tails by HotSHOT method (Truett et al., 2000), and used for genotyping the mice by PCR using primers; 5'-TGG GAT CCC TAT GCT ACT CCG TCG AAG TTC-3' and 5'-CTA GGC AAA CTG TGG GGA CCA GGA AGA C-3' to detect the *Nanog*-GFP construct derived from the iPSCs as described in http://www2.brc.riken.jp/animal/pdf/02290_PCR.pdf.

Immunofluorescence and alkaline phosphatase staining

For immunostaining, cells were fixed with 3.7% formaldehyde in phosphate buffered saline (PBS). After permeablization in 0.1% Triton X-100/PBS, cells were stained with primary antibodies followed by secondary antibodies (AlexaFlour, Life Technologies). Nuclei were counterstained with DAPI using VECTASHIELD Mounting Medium with DAPI (Vector). We employed the following primary antibodies; anti-SeV NP (1:1,000) (Nishimura et al., 2011), anti-SSEA-1 (1:250, sc-21702, Santa Cruz), anti-β-CATENIN (1:250, 610153, BD Biosciences) and anti-SSEA-4 (1:1,000, MAB4304, Millipore). Detection of alkaline phosphatase was carried out using VECTOR Red Alkaline Phosphatase Substrate Kit (Vector) according to manufacturer's instructions.

Quantitative RT-PCR

To avoid any contamination of feeder cells, reprogrammed cells were grown initially on feeder cells and then passaged under a feeder-free condition before RNA extraction. Total RNA was extracted using ISOGEN (Nippon Gene), and reverse transcription was performed using Superscript III First-Strand Synthesis System (Life Technologies). Quantitative PCR (qPCR)

analyses were performed in technical triplicates using 7500 Fast Real-time PCR System (Applied Biosystems) with GoTaq qPCR Master Mix (Promega). The expression levels were normalized against that of TATA-box binding protein (TBP). In case of human iPSCs, we collected the reprogrammed cells together with feeder cells but used primers specific for human genes for qPCR analyses. All primer sequences are listed in Table S2.

Microarray analysis

Total RNA was purified by ReliaPrep RNA Cell Miniprep System (Promega), and then reverse-transcribed and labeled using Illumina TotalPrep RNA Amplification Kit (Life Technologies). Arrays (MouseRef-8 v2.0 Expression BeadChip Kit), processed according to manufacture's instructions, were scanned with BeadArray Reader (Illumina). Each scanned image was analyzed with GenomeStudio software (Illumina). All of the data analyses were done by GeneSpring 12.5 software (Agilent). The data was filtered to remove low-confidence measurements and normalized globally per array such that 75 percentile of the signal intensity was set at 1.0. Differentially expressed genes were identified with a student's t-test ($P < 0.05$) for multiple comparisons. Hierarchical clustering was performed using the Ward's method. Pathway analysis was performed by referring to WikiPathway.

Statistical analysis

The statistical analyses were performed using StatView-J 5.0 software. Student's t-tests were used to test for statistically significant difference between data sets.

SUPPLEMENTAL REFERENCES

Joyner, A.L. (1993). Gene targeting : a practical approach (Oxford, IRL Press at Oxford University Press).

Truett, G.E., Heeger, P., Mynatt, R.L., Truett, A.A., Walker, J.A., and Warman, M.L. (2000). Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). Biotechniques *29*, 52, 54.