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Supplemental Information

A Role for KLF4 in Promoting the Metabolic Shift via TCL1 during Induced Pluripotent Stem Cell Generation

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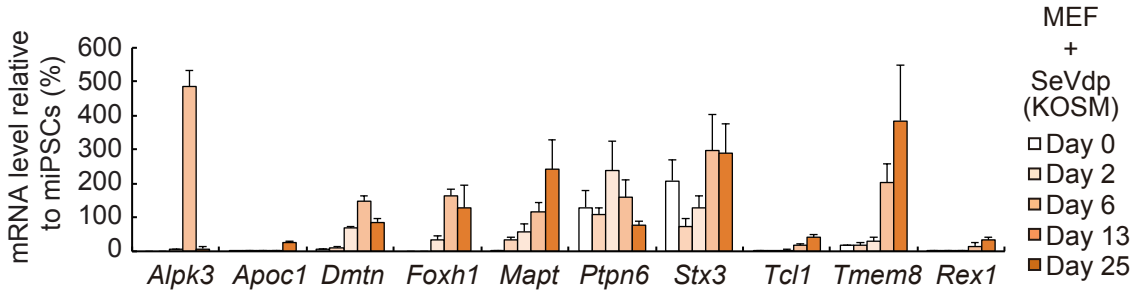
A

Gene symbol	Gene product	Accession No.
Alpk3	alpha-kinase 3	NM_054085
Apoc1	apolipoprotein C-I	NM_007469.5
Dmtn	dematin actin binding protein	NM_001252662.1
Foxh1	forkhead box H1	NM_007989.4
Mapt	microtubule-associated protein tau	NM_001038609.2
Ptpn6	protein tyrosine phosphatase, non-receptor type 6	NM_013545.3
Stx3	syntaxin 3	NM_152220.2
Tcl1	T cell lymphoma breakpoint 1	NM_009337.3
Tmem8	transmembrane protein 8	NM_021793.2
Rex1 (Zfp42)	zinc finger protein 42	NM_009556.3

B



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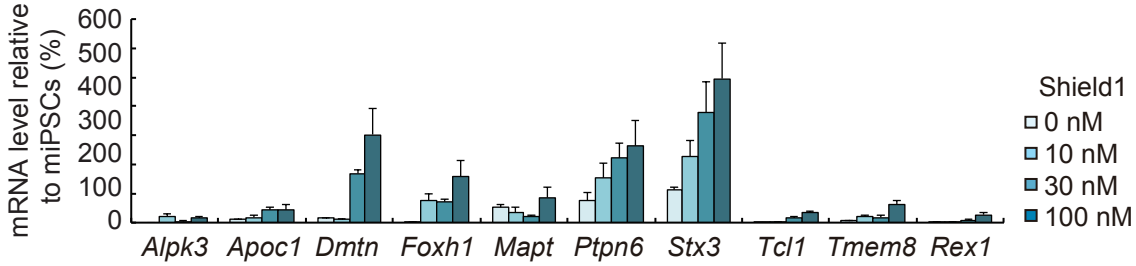
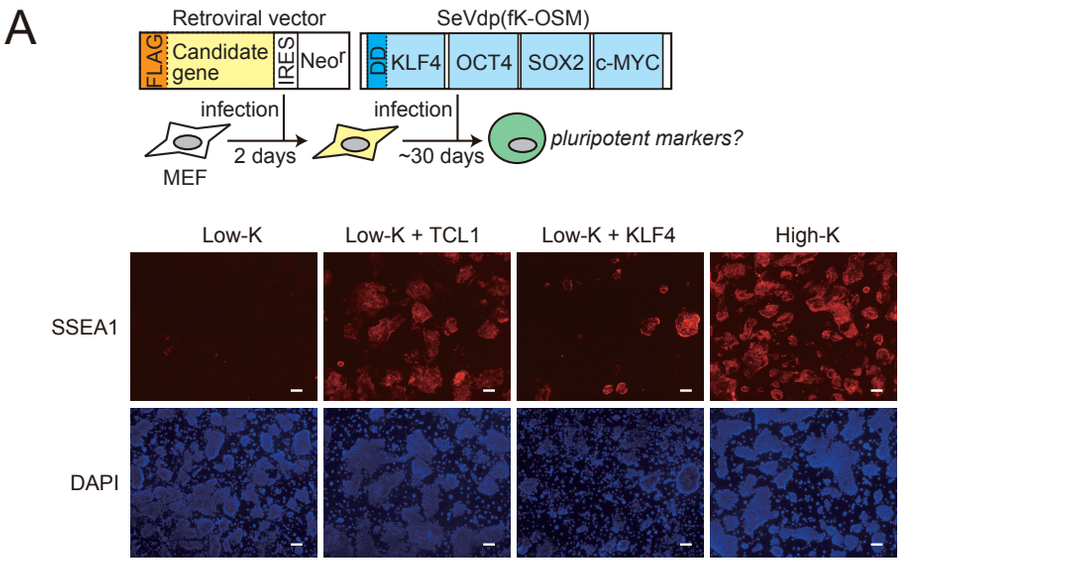


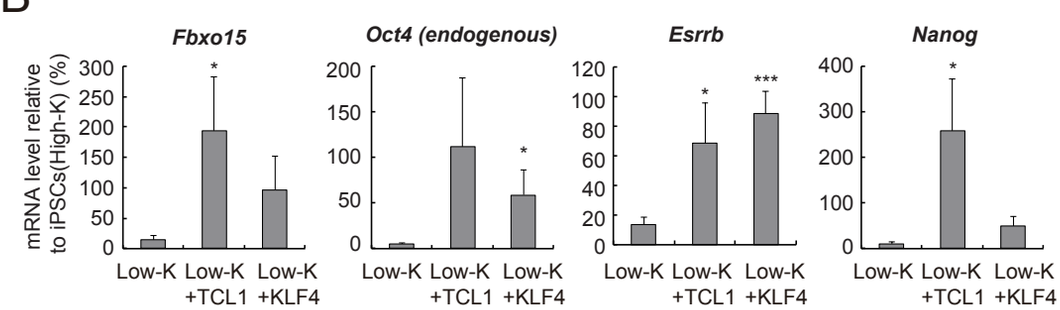
Figure S1. Expression of the candidate genes during iPSC generation (Related to Figure 1)

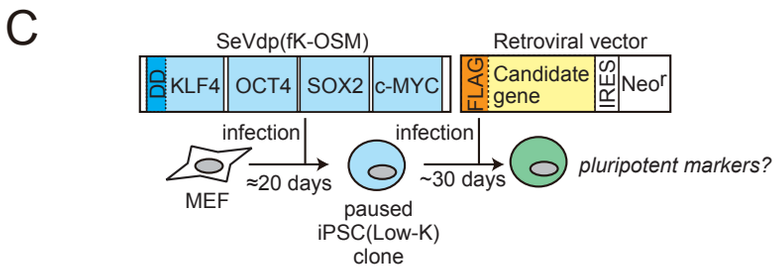
(A) List of 10 candidate genes that were selected by the screening.

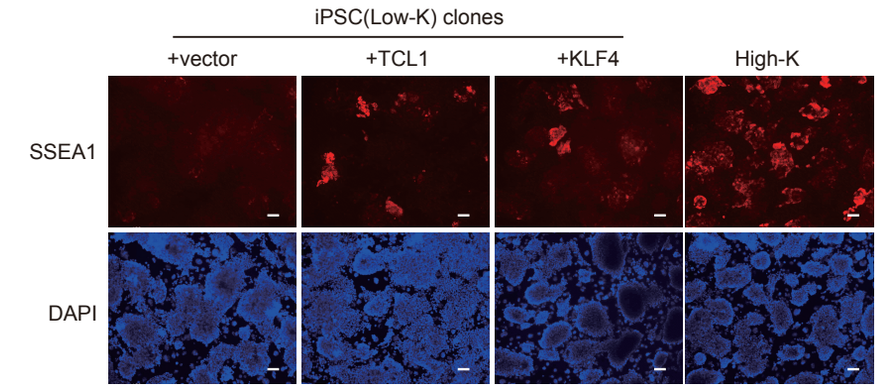
(B) Changes in the mRNA expression level of candidate genes during iPSC generation. MEFs were reprogrammed by SeVdp(KOSM) and the mRNA levels of candidate genes were determined at days 0, 2, 6, 13, and 25. Data represent means ± SEM of three independent experiments.

(C) KLF4 dose-dependent mRNA expression of candidate genes. MEFs were reprogrammed for 27 days by SeVdp (fK-OSM) with 0, 10, 30, or 100 nM of Shield1, and the mRNA levels of the candidate genes were determined. Data represent means ± SEM of three independent experiments.


A
Retroviral vector
FLAG
Candidate gene
IRES
Neor
SeVdp(fK-OSM)
DD
KLF4
OCT4
SOX2
c-MYC
infection
2 days
infection
~30 days
pluripotent markers?
MEF
Low-K
Low-K + TCL1
Low-K + KLF4
High-K
SSEA1
DAPI


B
Fbxo15
Oct4 (endogenous)
Esrrb
Nanog
mRNA level relative to iPSCs(High-K) (%)
Low-K
Low-K +TCL1
Low-K +KLF4


C
SeVdp(fK-OSM)
DD
KLF4
OCT4
SOX2
c-MYC
Retroviral vector
FLAG
Candidate gene
IRES
Neor
infection
≈20 days
infection
~30 days
pluripotent markers?
MEF
paused iPSC(Low-K) clone


iPSC(Low-K) clones
+vector
+TCL1
+KLF4
High-K
SSEA1
DAPI

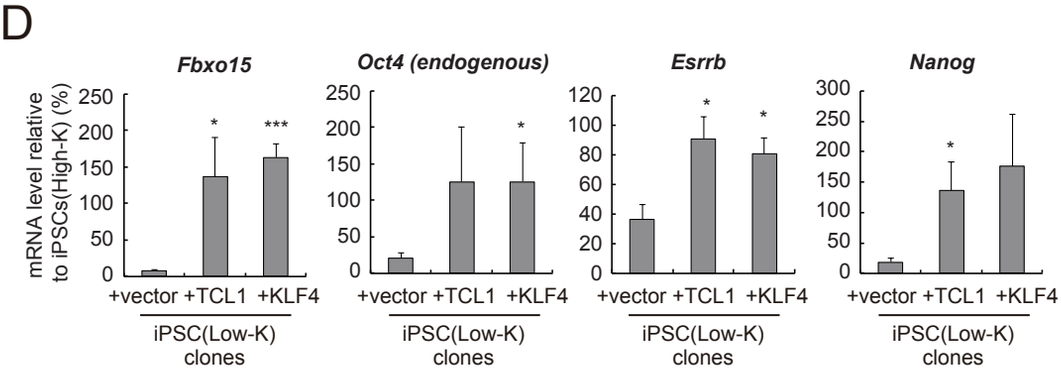

D
Fbxo15
Oct4 (endogenous)
Esrrb
Nanog
mRNA level relative to iPSCs(High-K) (%)
+vector
+TCL1
+KLF4
iPSC(Low-K) clones

Figure S2. Expression of pluripotency markers induced by TCL1 or KLF4 (Related to Figure 2)

(A) SSEA1 expression in MEFs reprogrammed by SeVdp(fK-OSM) with overexpressed TCL1 or KLF4. Two days prior to the reprogramming, MEFs were infected with a retrovirus expressing TCL1 or KLF4 and then selected with G418. Cells were immunostained with an anti-SSEA1 antibody at day 29. Scale bars, 100 μm.

(B) The mRNA levels of *Fbxo15*, endogenous *Oct4*, *Esrrb*, and *Nanog* in MEFs reprogrammed for 30 days by SeVdp (fK-OSM) with overexpressed TCL1 or KLF4. Data represent means ± SEM of three independent experiments. **P* < 0.05 versus iPSCs(Low-K).

(C) SSEA1 expression in paused iPSC(Low-K) clones that express TCL1 or KLF4. MEFs were first reprogrammed by SeVdp(fK-OSM) without Shield1 to generate paused iPSC(Low-K) clones, which were then infected with an empty retrovirus or a retrovirus expressing TCL1 or KLF4 and selected with G418. Cells were immunostained with an anti-SSEA1 antibody 27 days after the retrovirus infection. Scale bars, 100 μm.

(D) The mRNA levels of *Fbxo15*, endogenous *Oct4*, *Esrrb*, and *Nanog* in paused iPSC(Low-K) clones that express TCL1 or KLF4, which were introduced by the retrovirus 30 days prior to mRNA isolation. Data represent means ± SEM of three independent experiments. **P* < 0.05, ****P* < 0.005 versus paused iPSC(Low-K) clones infected with an empty retrovirus.

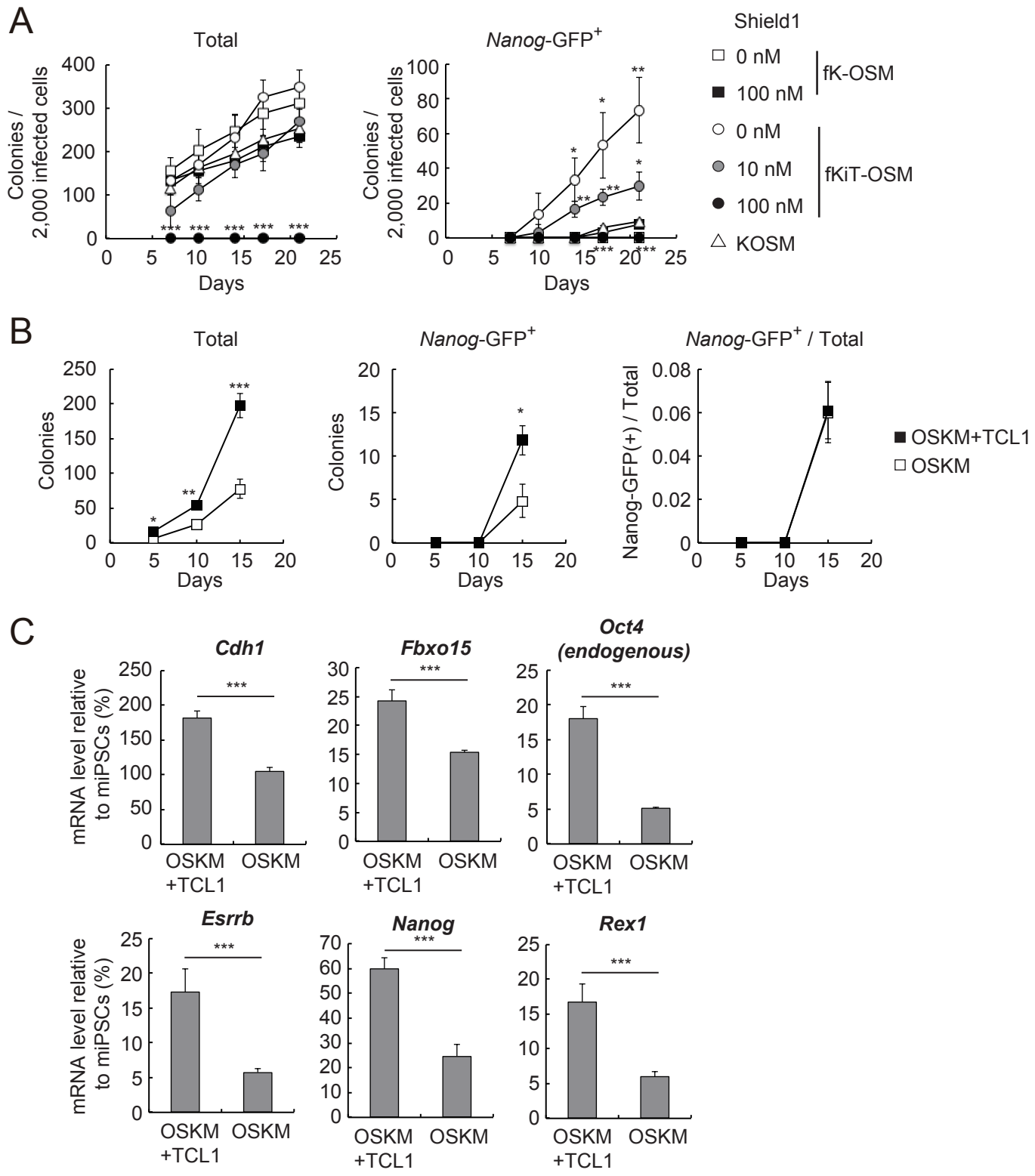


Figure S3. Enhanced efficiency of reprogramming by TCL1 (Related to Figure 3)

(A) Time course of colony formation of iPSCs generated by SeVdp(fKiT-OSM). MEFs were reprogrammed with the indicated vectors with 0, 10, or 100 nM Shield1. The numbers of total colonies and *Nanog*-GFP$^+$ colonies per 2 x 10^3 vector-infected cells were counted at indicated days. Data represent means ± SEM of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$ versus SeVdp (KOSM).

(B) Time course of colony formation of iPSCs generated by the retrovirally-expressed four Yamanaka factors with or without a TCL1-expressing retrovirus. MEFs were infected by the retroviruses expressing the four reprogramming factors with or without TCL1 (OSKM+TCL1 or OSKM, respectively) for two days, then passaged onto mitomycin C-treated SNL76/7 feeder cells, and subsequently grown in KSR medium for indicated days. The numbers of total colonies and *Nanog*-GFP$^+$ colonies were counted at indicated days. Data represent means ± SEM of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$ versus reprogramming without TCL1.

(C) Expression of pluripotency markers in retrovirus-mediated reprogramming with or without TCL1. MEFs were reprogrammed as in (B), and total RNA was extracted 30 days after the retrovirus infection. Data represent means ± SEM of three independent experiments. ***$P < 0.005$.

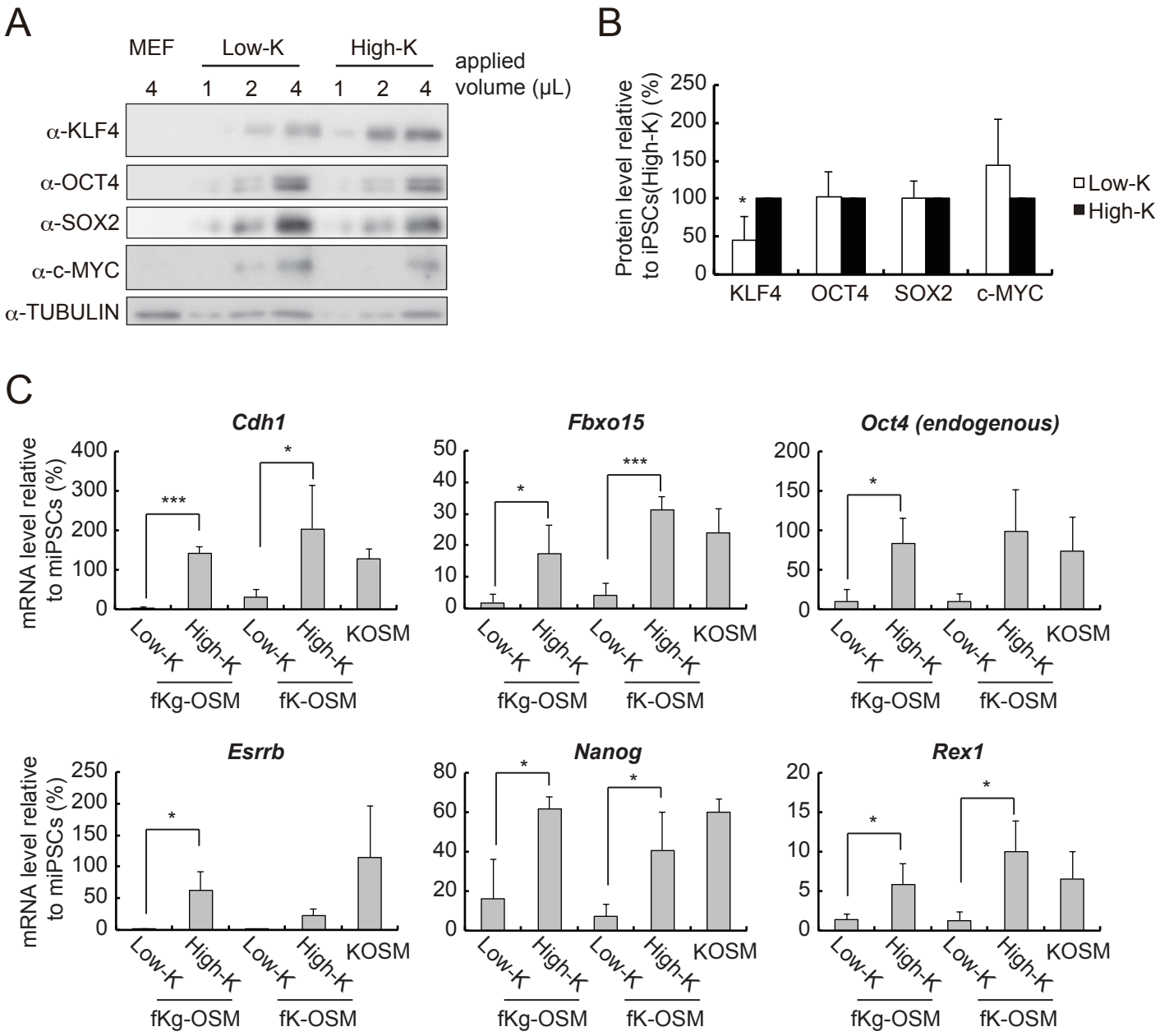


Figure S4. iPSC generation using SeVdp(fKg-OSM) (Related to Figure 4)

(A) Quantitative western blots to determine the relative protein levels of the reprogramming factors. Cell extracts were prepared from MEFs infected with SeVdp(fKg-OSM) for two days with or without 100 nM Shield1. The indicated amounts of the extracts were applied and quantified by western blotting using an antibody against each reprogramming factor. An anti-α-TUBULIN was used to normalize the sample amounts.

(B) Intensity of each band in (A) was determined using LAS4000. The protein level of each reprogramming factor in iPSCs (Low-K) is shown relative to that in iPSCs(High-K). Data represent means ± SEM of three independent experiments. $*P < 0.05$ versus iPSCs(High-K).

(C) Expression of the pluripotency marker mRNAs in the cells that were infected with the indicated vectors and then cultured for 21 days with or without Shield1. Data represent means ± SEM of three independent experiments. $*P < 0.05$, $***P < 0.005$.

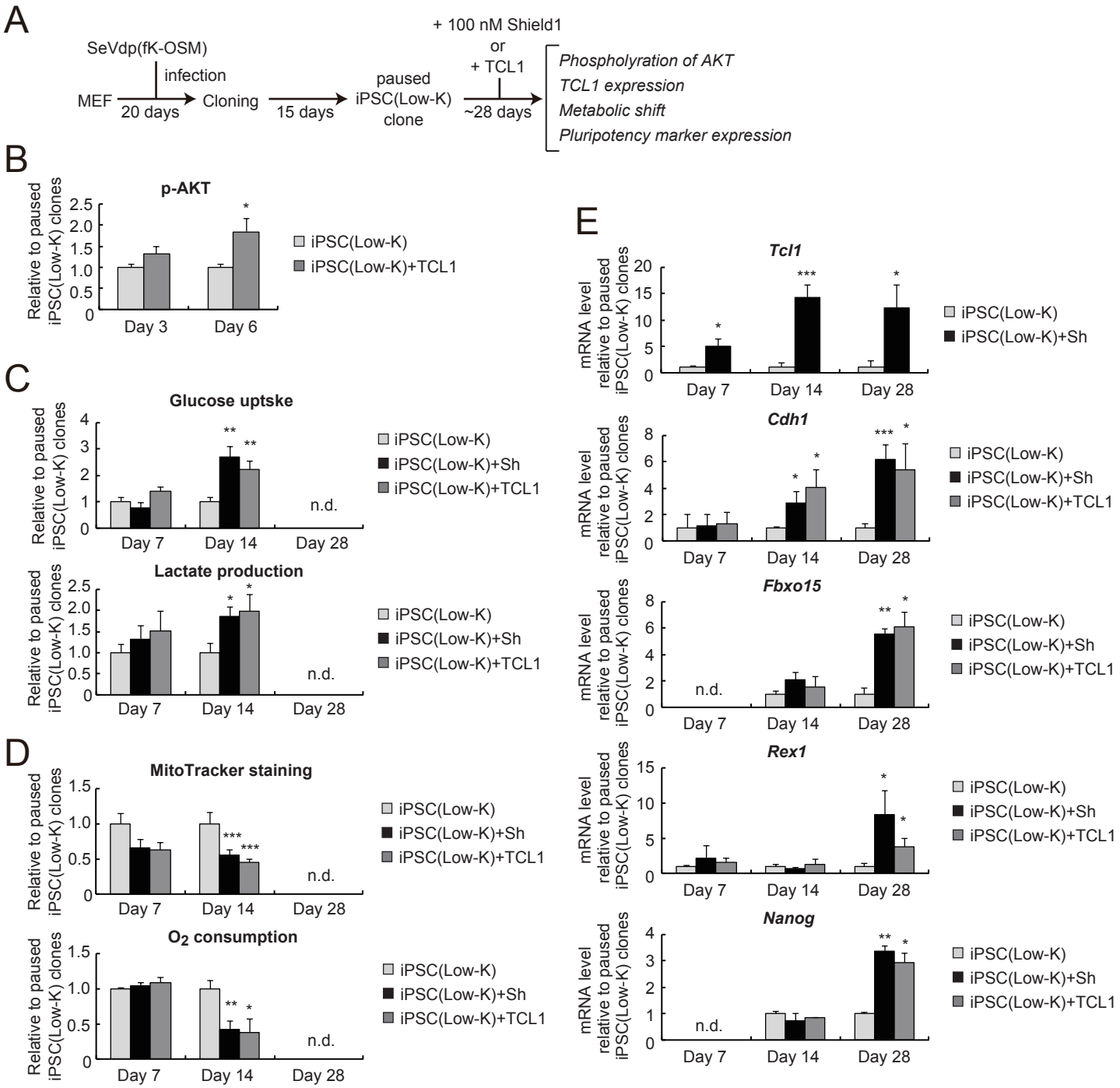


Figure S5. Time course analyses of the metabolic shift and the expression of pluripotency markers in paused iPSC (Low-K) clones which were infected with a TCL1-expressinig retrovirus or cultured with 100 nM Shield1 (Related to Figures 5, 6, and 7)

MEFs were first reprogrammed by SeVdp(fK-OSM) without Shield1 to generate paused iPSC(Low-K) clones. The clones were infected with the retrovirus expressing TCL1 and then selected by G418. Alternatively, the clones were cultured with 100 nM Shield1 (Sh) to increase the KLF4 level (A). Phosphorylation of AKT (B), glucose uptake (C), lactate production (C), mitochondrial content (D), oxygen consumption (D), *Tcl1* expression (E), and pluripotency marker expression (E) were determined at indicated days. Data represent means ± SEM of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$ versus paused iPSC(Low-K) clones. n.d.; not done.

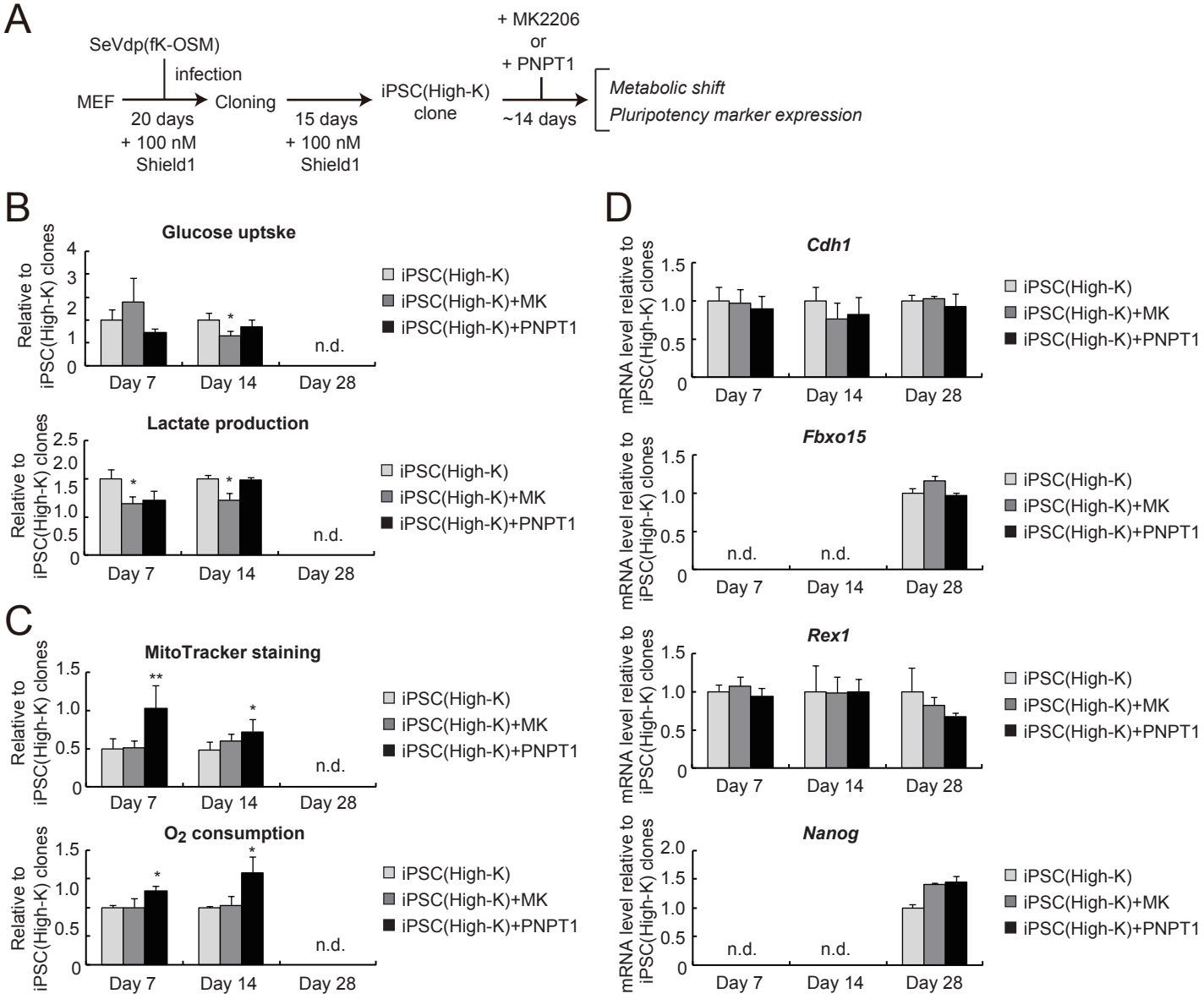


Figure S6. Time course analyses of the metabolic shift and the expression of pluripotency markers in iPSC(High-K) clones, which were infected with a PNPT1-expressinig retrovirus or cultured with MK2206 (Related to Figures 5, 6, and 7)
MEFs were first reprogrammed by SeVdp(fK-OSM) with 100 nM Shield1 to generate iPSC(High-K) clones. The clones were infected with the retrovirus expressing PNPT1 and selected with puromycin. Alternatively the clones were cultured with 1 μM MK2206 to inhibit the AKT activity (A). Glucose uptake (B), lactate production (B), mitochondrial content (C), oxygen consumption (C), and pluripotency marker expression (D) were determined at indicated days. Data represent means ± SEM of three independent experiments. *$P < 0.05$, **$P < 0.01$ versus iPSC(High-K) clones.

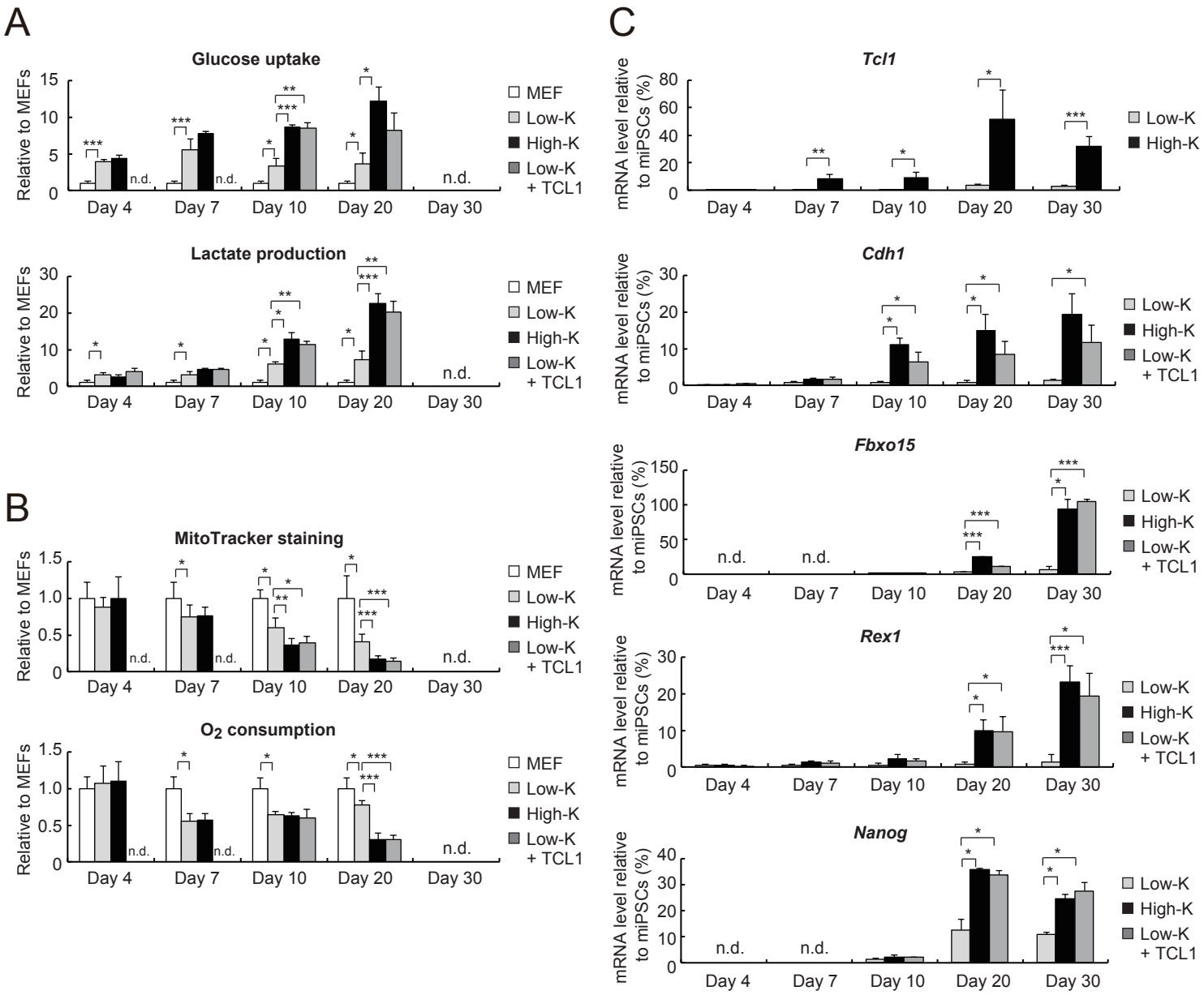


Figure S7. Time course analyses of the metabolic shift and the expression of pluripotency markers during reprogramming by SeVdp(fK-OSM) with or without a TCL1-expression retrovirus (Related to Figures 5, 6, and 7)

Two days prior to the reprogramming, MEFs were infected with the retrovirus expressing TCL1 and then selected with G418. The selected cells were reprogrammed by SeVdp(fK-OSM) without Shield1. MEFs were also reprogrammed, without prior retrovirus infection, by SeVdp(fK-OSM) with or without 100 nM Shield1. Glucose uptake (A), lactate production (A), mitochondrial contents (B), oxygen consumption (B), *Tcl1* expression (C), and pluripotent marker expression (C) were determined at indicated days. Data represent means ± SEM of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$. n.d.; not done.

Table S1. Primer sequences for quantitative RT-PCR analyses and ChIP-qPCR analyses (Related to Figures 1, 2, 3, 4, 5, 6, 7, S1, S2, S3, S4, S5, S6, and S7)

• For RT-qPCR

Gene	Sequence
Alpk3	AATGCCAAACCTTCCAGC
	GTACATCAGTCATCTTCCAGTC
Apoc1	TTTGGGAACACTTTGGAAGAC
	AACTTCTCCTTCACTTTGCC
Cdh1	ACGTCCCCCTTTACTGCTG
	TATCCGCGAGCTTGAGATG
Dmtn	TCACTGTCACCCAAATCCAC
	TGAGAGATGATTCCAAGAGTCC
Esrrb	TGGCAGGCAAGGATGACAGA
	TTTACATGAGGGCCGTGGGA
Fbxo15	CTCATCTGTCACGAAGCAGC
	AGGTCACCGCATCCAAGTAA
Foxh1	CCATGATCGCCTTGGTAATTC
	TTCCAGCCCTCATAGTCGTC
Glut1	TGGGAATCGTCGTTGGCATC
	ATGACACTGAGCAGCAGAGG
Hk2	AGAGGGTGGAGATGGAGAAC
	GGCAATGTGGTCAAACAGC
Ldha	CAAACTCAAGGGCGAGATG
	GTTCGCAGTTACACAGTAGTC
Mapt	CGCTGGGCATGTGACTCAA
	TTTCTTCTCGTCATTTCCTGTCC
Nanog	ACCTGAGCTATAAGCAGGTTAAGAC
	GTGCTGAGCCCTTCTGAATCAGAC
Oct4 (endogeneous)	CTGTTCCCGTCACTGCTCTG
	AACCCCAAAGCTCCAGGTTC
Pdk1	TGCTACTCAACCAGCACTC
	CTTTAATGACCTCCACCACG
Pkm2	TGTCTGGAGAAACAGCCAAG
	GTTCCTCGAATAGCTGCAA
Ptpn6	TGCTCAATGACCAGCCCAAG
	ACCCACAGTATAGCGTCCAC
Rex1	TTGATGGCTGCGAGAAGAG
	ACCCAGCCTGAGGACAATC
Stx3	TGAGATTGCCATTGACAACAC
	AGCTTCCTCTACATGCTCC
Tcl1	GGAGAAGCACGTGTACTTGGA
	TGACTGGGGGACATAGCTTC
Tmem8	CCCAGAAGATCCAGGTGAAG
	TCAACCACCCTCATTACCAG

• For ChIP-qPCR

Gene	Sequence
Tcl1 Enhancer	AAACACCCCCATCCACTAC
	TCTTCCTTGTTTGGGCTCC
Tcl1 Promoter	AAGGGAAGCATAGGGCAGTG
	AGACCTGACCCCATCAAACC
Tcl1 Gene body	CGACTCCATGTATTGGCAG
	ACCAGGATCACTCAAGATCAC

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Production of SeVdp vectors

The SeVdp vector genomic cDNA was constructed as described (Nishimura et al., 2011). To construct cDNA for SeVdp(fKiT-OSM), we amplified the sequence of an internal ribosome entry site (IRES) and the mouse *Tcl1* gene by PCR and inserted them after the *Klf4* gene in SeVdp(fK-OSM) cDNA using In-Fusion HD Cloning Kit (Takara Bio). We amplified the FLAG-tagged *Klf4* gene by PCR with primers, one of which containing the FLAG tag sequence. Then, the *Klf4* gene of SeVdp(fK-OSM) vector cDNA was replaced by the FLAG-tagged *Klf4* gene to construct cDNA for SeVdp(fKg-OSM).

To prepare vector-packaging cells, 2.0 x 10^5 BHK/T7/151M 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 (Thermo Fisher) 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 (Nishimura et al., 2007).

Production of retroviral vectors

We amplified sequence of the neomycin resistant gene and puromycin resistant gene by PCR from pcDNA3 plasmid (Thermo Fisher) and pMXs-U6-Puro plasmid (Cambridge bioscience), and the amplified genes were used to replace the GFP gene in pMCsΔYY1-IRES-GFP plasmid to construct pMCsΔYY1-IRES-Neo plasmid and pMCsΔYY1-IRES-Puro plasmid, respectively. The cDNAs encoding the APOC1, DMTN, FOXH1, TCL1, TMEM8, and REX1 proteins were amplified from mouse embryonic stem cell cDNA and inserted into pMCsΔYY1-IRES-Neo plasmid to construct retroviral vectors expressing each protein. The cDNAs encoding the PNPT1 proteins were amplified from MEF cDNA and inserted into pMCsΔYY1-IRES-Puro plasmid. Annealed DNA oligonucleotides (5'-gatccCGTCCTGTCGCTGATTAAAttcaagagaTTTAATCAGCGACAGGACGttttttggaaag-3' and 5'-aattctttccaaaaaaCGTCCTGTCGCTGATTAAAtctcttgaaTTTAATCAGCGACAGGACGg-3') were inserted into pMXs-U6-Puro plasmid to construct the retroviral vector expressing shRNA against the *Tcl1* gene. Retrovirus expressing OCT4, SOX2, KLF4, and c-MYC for retrovirus-mediated reprogramming were prepared from pMXs-hOCT3/4, pMXs-hSOX2, pMXs-hKLF4, and pMXs-hc-MYC (Addgene), respectively.

To prepare retrovirus stock, 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 counting neomycin- or puromycin-resistant NIH3T3 cells infected with a diluted vector suspension.

Quantitative RT-PCR

To avoid any contamination of feeder cells, reprogrammed cells, initially grown on feeder cells, were 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 (Thermo Fisher). Quantitative PCR (qPCR) analyses were performed using 7500 Fast Real-time PCR System (Applied Biosystems) with GoTaq qPCR Master Mix (Promega). As controls, we used cDNA from mESCs, EB5 (RIKEN BioResource Center) or miPSCs generated by the SeVdp vector system (Nishimura et al., 2011). The expression levels were normalized against that of TATA-box binding protein (TBP). The DNA sequences of the primers are listed in Table S1.

Immunofluorescence staining

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 anti-mouse IgG conjugated with AlexaFlour555 (1:500, A32727, Thermo Fisher) or anti-mouse IgM conjugated with AlexaFlour555 (1:500, A21426, Thermo Fisher) as secondary antibodies. 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., 2007) and anti-SSEA-1 (1:250, sc-21702, Santa Cruz).

Luciferase reporter assays

We amplified the 3.0 or 1.1 kb genomic region upstream of the *Tcl1* transcription start site by PCR using a mouse BAC clone, B6Ng01-102P12 (RIKEN BioResource Center) containing the *Tcl1* gene, as a template. The amplified 3.0 or 1.1 kb genomic region was inserted into pGL3-Basic vector (Promega) to construct pL-*Tcl1*-Luc or pS-*Tcl1*-Luc reporter plasmid, respectively. KLF4 or OCT4 cDNA, amplified by PCR, was inserted into the pcDNA3 vector (Thermo Fisher) to construct pcDNA3-KLF4 or pcDNA3-OCT4 that expresses either KLF4 or OCT4, respectively.

For luciferase reporter assays, MEFs were transfected with the indicated reporter plasmid together

with one of the expression vectors (pcDNA3, pcDNA3-KLF4, or pcDNA3-OCT4) and pRL-SV40 (Promega) using Lipofectamine LTX with Plus Reagent (Thermo Fisher). Two days after transfection, cells were collected and luciferase activities were measured by Dual-Luciferase Reporter Assay System (Promega), and the transfection efficiency was normalized by the fluorescence values from pRL-SV40.

ChIP assays

Cells were cultured without feeder cells, cross-linked for 10 min at room temperature with 0.75% formaldehyde directly added to the cell culture plates, followed by 5-min quenching with 125 mM glycine, and then washed with cold-PBS three times. Collected cells were lysed in FA lysis buffer (50 mM HEPES-KOH, pH7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, and Protease Inhibitor Cocktail (Sigma)) and sonicated to shear the chromatin. The sheared chromatin was cleared by centrifugation at 15,000 rpm for 5 min at 4˚C, and the supernatant was used as a chromatin suspension for ChIP assays.

The chromatin suspension was diluted 10 fold with Dilution buffer (16.7 mM Tris, 167 mM NaCl, 1.2 mM EDTA, 1.1% Triton X-100, 0.01% SDS). One μg of each antibody was added to the diluted chromatin, which was incubated for 16 hours at 4 ˚C and for additional 6 hours at 4 ˚C after addition of Dynabeads Protein G (Thermo Fisher). Precipitated antibody-chromatin mixture was washed twice with Wash buffer (20 mM Tris, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS), once with Final Wash buffer (20 mM Tris, 500 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS), once with LiCl Wash buffer (20 mM Tris, 500 mM LiCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS), and once with TE buffer (10 mM Tris, 1 mM EDTA). Chromatin DNA was extracted in Elution buffer (10 mM Tris, 1 mM EDTA, 1% SDS) by vortexing for 30 min at 30˚C, and the extracted sample was analyzed in qPCR.

For each ChIP assay, we employed the following primary antibodies; anti-DYKDDDDK tag (FLAG-tag) (018-22381, Wako), anti-OCT4 (ab19857, Abcam), anti-H3K4me3 (ab8580, Abcam), anti-H3K27me3 (07-449, Millipore), anti-H3K27Ac (ab4729, Abcam), and anti-H3K4me1 (ab8895 Abcam). The DNA sequences of the primers used for ChIP-qPCR are listed in Table S1.

Determination of protein expression by western blotting

After SeVdp vectors were infected to MEFs, the cells were collected two days after infection and lysed on ice in SDS-PAGE gel loading buffer (50 mM Tris-HCl (pH6.8), 2% SDS, 10% glycerol, 0.1 mg/mL Bromophenol blue, 5% 2-mercaptoethanol), followed by boiling for 5 min, and the whole cell extracts were subjected to western blot analysis. Protein expression levels were quantified and calculated as described previously (Nishimura et al., 2014). We employed the following primary antibodies; anti-KLF4,

anti-SOX2, and anti-c-MYC purified from immunized rabbit serum (Nishimura et al., 2014), Anti-OCT4 (1:5,000, ab19857, Abcam), anti-α-TUBULIN (1:10,000, ab7291, Abcam), anti-phosphorylated AKT (S473) (1:5,000, ab81283, Abcam), anti-AKT (1:5,000, ab32505, Abcam).

Statistical analysis

Student's t-tests were used to test for a statistically significant difference between data sets. A value of $P < 0.05$ was considered statistically significant.

SUPPLEMENTAL REFERENCE

Nishimura, K., Segawa, H., Goto, T., Morishita, M., Masago, A., Takahashi, H., Ohmiya, Y., Sakaguchi, T., Asada, M., Imamura, T.*, et al.* (2007). Persistent and stable gene expression by a cytoplasmic RNA replicon based on a noncytopathic variant Sendai virus. J Biol Chem *282*, 27383-27391.