Stem Cell Reports

Supplemental Information

KLF4 N-Terminal Variance Modulates Induced Reprogramming to Pluripotency

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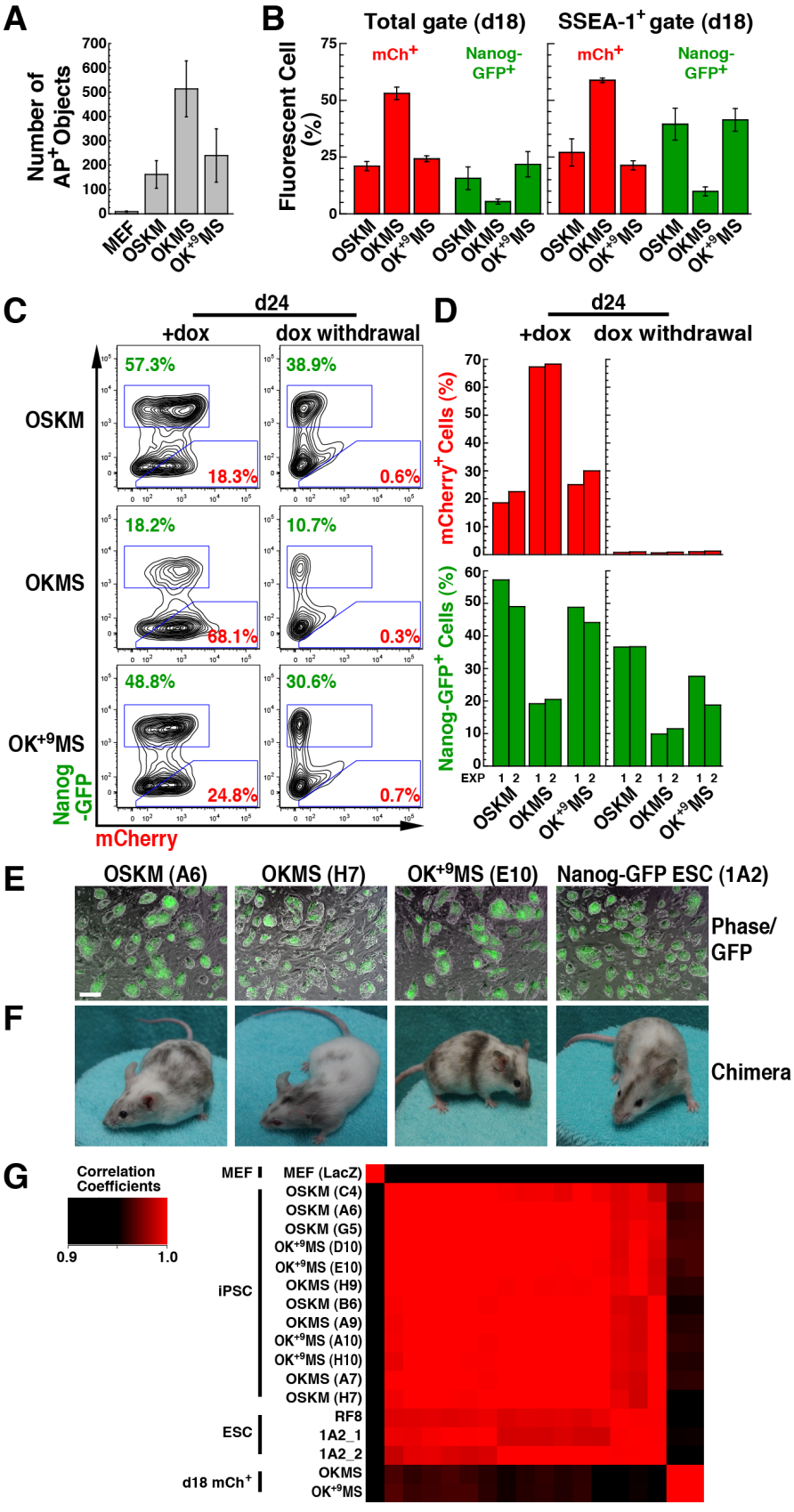

Figure S1. The Efficiencies of Dox-Independent, Nanog-GFP iPS Cell Formation by Polycistronic Vectors Differs According to the N-terminus of KLF4, Related to Figure 1

(A) Quantification of AP positive colony formation using three different polycistronic reprogramming cassettes: OSKM, OKMS, and $OK^{+9}MS$. Means ± SE for three independent experiments.

(B) Percentages of $mCherry^{+}$ and Nanog-GFP^{+} populations from FACS analysis of reprogramming cultures. The reporter-positive fractions of the Total (left) and SSEA-1^{+} (right) populations are shown. Means ± SE for three independent experiments.

(C-D) FACS analysis (d24) showing the effect of continued dox treatment, or dox withdrawal on d18 reprogramming cultures. Relative $mCherry^{+}$ and Nanog-GFP^{+} cell fractions remain essentially unchanged following extended dox treatment (compare d24 +dox to d18 levels, Figure 1C and 1F). On the other hand, $mCherry^{+}$ cells are absent after 6 days of culture in the absence of dox. An ~30% decrease in GFP cell numbers is observed for all cassettes following dox withdrawal. The data in panel C is summarized for two experiments in panel D.

(E) Phase and fluorescence microscopy images of colony morphology and Nanog-GFP expression from representative dox-independent iPS cell clones, as compared to parental Nanog-GFP ES cells (1A2). Scale bar, 200 μm.

(F) Chimeric mice derived from iPS cell clones and parental ES cells (1A2) injected into ICR (albino) host embryos.

(G) Correlation plot of microarray transcriptome analysis for mock-transfected MEFs (lacZ), dox-independent iPS cell clones (4 clones each for OSKM, OKMS, and $OK^{+9}MS$), grandparental RF8 and parental 1A2 ES cells (two independent passages), and d18 $mCherry^{+}$ cells from OKMS and $OK^{+9}MS$.

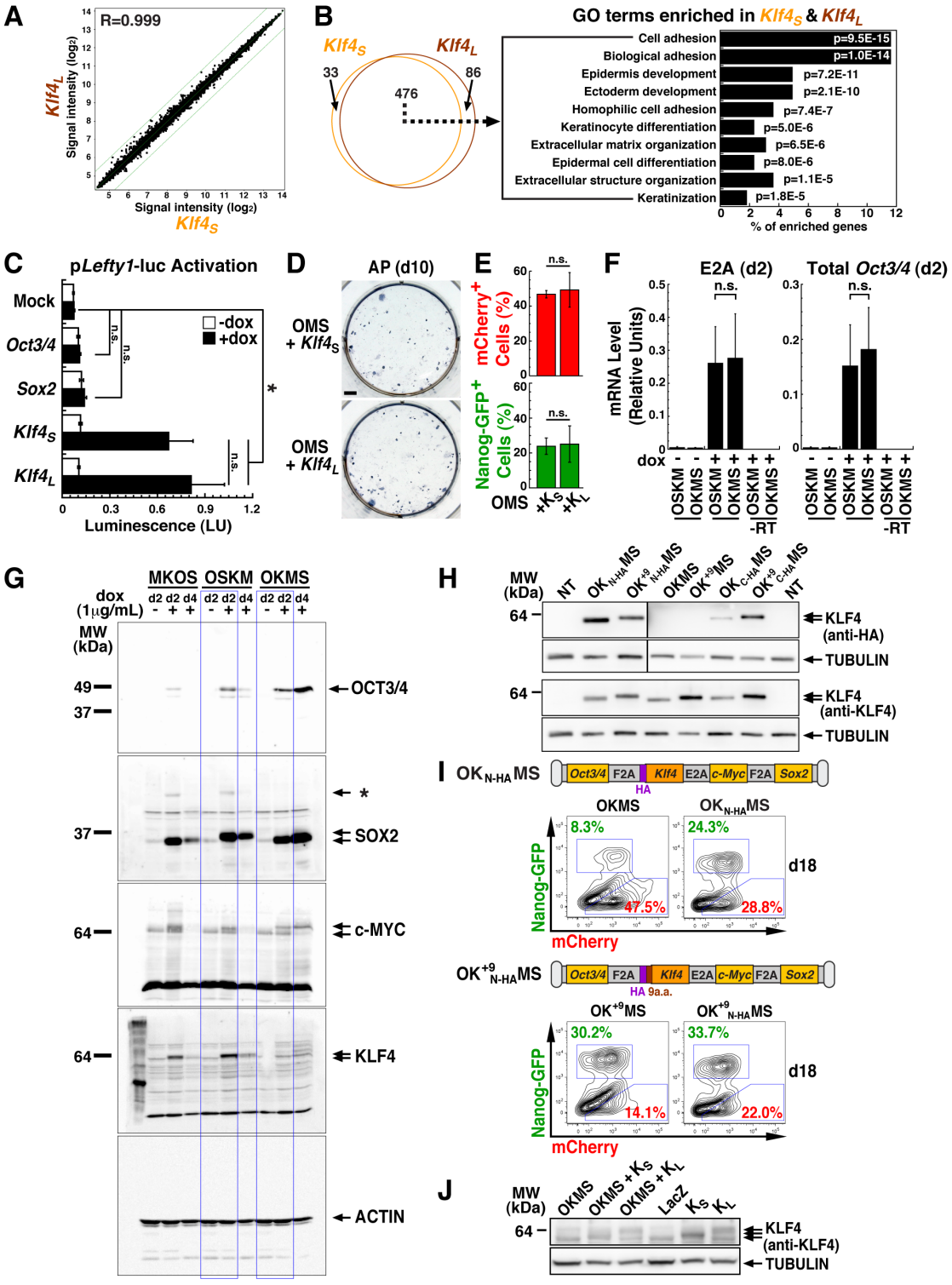

Figure S2. *Klf4* Variant-Associated Phenotypes are Linked to Protein Expression Levels, and are Not Exclusive to N-terminal Identity, Related to Figure 2

(A) Pairwise comparison of genes induced in $Klf4_S$ (x-axis) and $Klf4_L$ (y-axis) transfected MEFs on d6 of dox treatment (mCherry$^+$ fraction). Green lines indicate two-fold changes in $\log_2$ signal intensity between the two data sets. Signal intensities were normalized with the RMA algorithm (GeneSpring).

(B) Venn diagram (left) illustrating genes expressed >2-fold higher in *Klf4*$_S$ and *Klf4*$_L$ transfected cells as compared to LacZ mock-transfected MEFs. *Klf4*$_S$ and *Klf4*$_L$ show a high degree of overlap. Gene Ontology (GO) analysis of 476 shared genes, arranged by p-value and indicating the proportion of genes represented for each enriched GO term (right).

(C) Activation of a *Lefty1* promoter in HeLa cells transfected with PB-TAC expressing LacZ (Mock), *Oct3/4*, *Sox2*, or one of two *Klf4* variants, as measured by luciferase activity in the presence or absence of dox. Means ± SE for three independent experiments. n.s., not significant. *, p<0.05. Student t-test.

(D) AP staining on d10 following transfection of OMS in combination with *Klf4*$_S$ or *Klf4*$_L$. Scale bar, 4000 μm.

(E) Percentages of mCherry$^+$ and Nanog-GFP$^+$ populations in the SSEA-1$^+$ fraction from FACS analysis of OMS + *Klf4*$_S$ or *Klf4*$_L$ reprogramming cultures on d18. Means ± SE for three independent experiments. *, p<0.05. Student t-test.

(F) qRT-PCR analysis of the common E2A region and total *Oct3/4* mRNA in OSKM and OKMS transfected MEFs cultured for 2 days with (+) or without (-) dox treatment. cDNA synthesis without reverse transcriptase (-RT) was used as a negative control. All mRNA levels were normalized to *Hprt*. Means ± SE for three independent experiments. *, p<0.05. Student t-test.

(G) Western blot analysis of OCT3/4, SOX2, KLF4, and c-MYC in OSKM or OKMS transfected MEFs cultured on d2 without or d2, 4 with dox treatment. MKOS transfected MEFs were included as a reference (Kaji et al., 2009). ACTIN was used as a loading control. Note that proteins produced from positions 1, 2 and 3 in the quadracistronic vectors are C-terminally modified with residual 2A sequences, resulting in slightly higher molecular weights. Asterisk indicates presumptive mega-protein detected with the SOX2 antibody. Blue boxes indicate samples in which the mCherry positive cell numbers are most similar between OSKM and OKMS.

(H) Western blot analysis of protein levels in HEK293T cells following modification of *Klf4* in OKMS. OKMS and OK^{+9}MS are shown in lanes 4 and 5. KLF4 from OKMS and OK^{+9}MS was N- (lanes 2 and 3) or C-terminally (lanes 6 and 7) tagged with HA and probed with a HA antibody (upper panels) to exclude the possibility of differences in native antibody recognition between KLF4$_S$ and KLF4$_L$ epitopes with the KLF4 antibody (lower panels). Non-transfected (NT) cells are included as negative controls (lanes 1 and 8). TUBULIN was used as a loading control.

(I) N-terminal HA tagging of KLF4 in OKMS (OK$_{N\text{-}HA}$MS) and OK^{+9}MS (OK$^{+9}_{N\text{-}HA}$MS) cassettes. FACS analysis of mCherry and Nanog-GFP expression in the d18 SSEA-1$^+$ population (bottom).

(J) Western blot analysis of OKMS supplementation with *Klf4*$_S$ and *Klf4*$_L$ (lanes 1-3), compared to expression in MEFs (lanes 5, 6) on d2 after dox induction. Mock transfected MEFs (LacZ) are included as a control (lane 4). TUBULIN was used as a loading control.

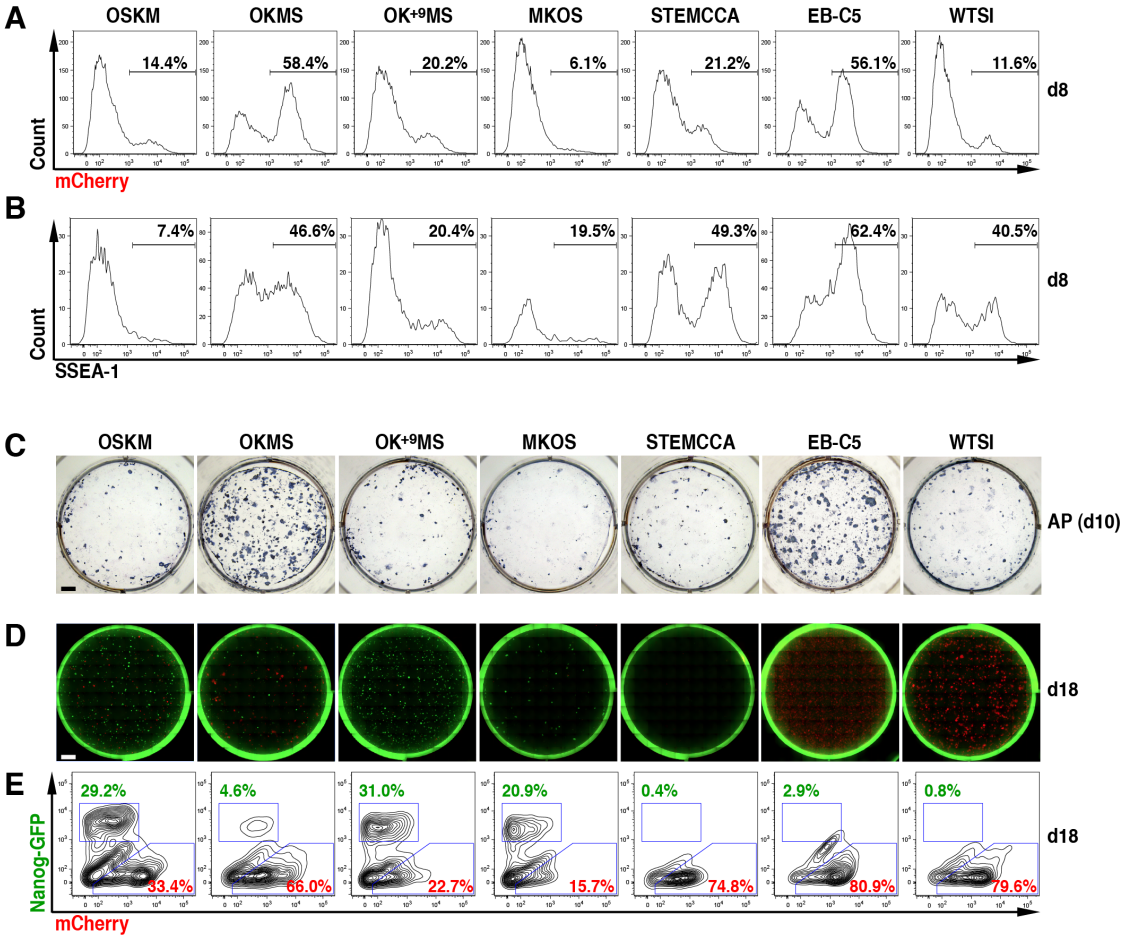

Figure S3. Comparative Evaluation of Polycistronic Vectors, Related to Table 1 and Figure 3

(A) FACS analysis of mCherry expression in OSKM, OKMS, OK^{+9}MS, MKOS, STEMCCA, EB-C5, and WTSI transfected MEFs cultured for 8 days with dox treatment.

(B) Expression of SSEA-1 in mCherry^{+} cell fractions from panel A.

(C) AP staining on d10. Scale bar, 4000 μm.

(D) Day 18 fluorescence microscopy of entire wells (composite 10 × 10 fields) for and mCherry^{+} and Nanog-GFP^{+}. Scale bar, 4000 μm.

(E) FACS analysis of mCherry expression and Nanog-GFP in the SSEA-1^{+} population of d18 cultures described in panel D. The results in panels A-E are representative of at least two to three independent experiments.

Table S1. Genes Commonly Upregulated in the *Klf4*$_L$ Cluster, Related to Figure 3

The table containing the gene list is provided as a separate Excel file.

Supplemental Experimental Procedures

Luciferase Assay

HeLa cells were seeded in DMEM containing 10% FBS, penicillin-streptomycin and L-glutamine on 96-well plate at a density of 5×10^4 cells per 0.32 cm^2 and transfected with 200 ng p*Lefty1*-luc reporter plasmid (Nakatake et al., 2006), 200 ng PB-CAG-rtTA (Woltjen et al., 2009), 200 ng PB-TAC-LacZ, -*Oct3/4*, -*Sox2*, -$Klf4_S$, or -$Klf4_L$, and 20 ng of Renilla luciferase internal control plasmid (pGL4.74[hRluc/TK], Invitrogen) using Lipofectamine 2000 (Invitrogen). *piggyBac* transposase was not included for these transient expression assays. After 24 hr, culture media was replaced with medium containing doxycycline (1 μg/mL). After additional 24 hr, transfected cells were harvested and luciferase assays were performed using a Dual-Glo® Luciferase Assay System (Promega) and luminescence was measured by a 2104 EnVision Multilabel Reader (Perkin Elmer).

Chimera Generation

A total of twelve dox-independent iPS clones generated with OSKM (C4, G5, A6, B6), OKMS (A7, H7, A9, H9), or $OK^{+9}MS$ (A10, D10, E10, H10) were grown under standard ES cell conditions. Cells were harvested as a feeder-depleted (pre-plated on non-treated dishes for 30-45 min) single cell suspension on the date of injection, and each ICR blastocyst received 8–20 iPS cells. After injection, blastocysts were cultured in KSOM and placed at 37°C until transferred to recipient females. About 20–27 injected blastocysts were transferred to each uterine horn of 2.5 day postcoitum pseudopregnant B6D2F1 female. Chimeras were maintained and documented by photography at 4-6 weeks of age. No incidence of mortality or tumor formation was noted in mice over 12 months of age. The care of the animals was in accordance with Kyoto University guidelines, and all animal experiments were approved by the CiRA Animal Experiment Committee.