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Supplemental Information

Nascent Induced Pluripotent Stem Cells Efficiently Generate Entirely iPSC-Derived Mice while Expressing Differentiation-Associated Genes

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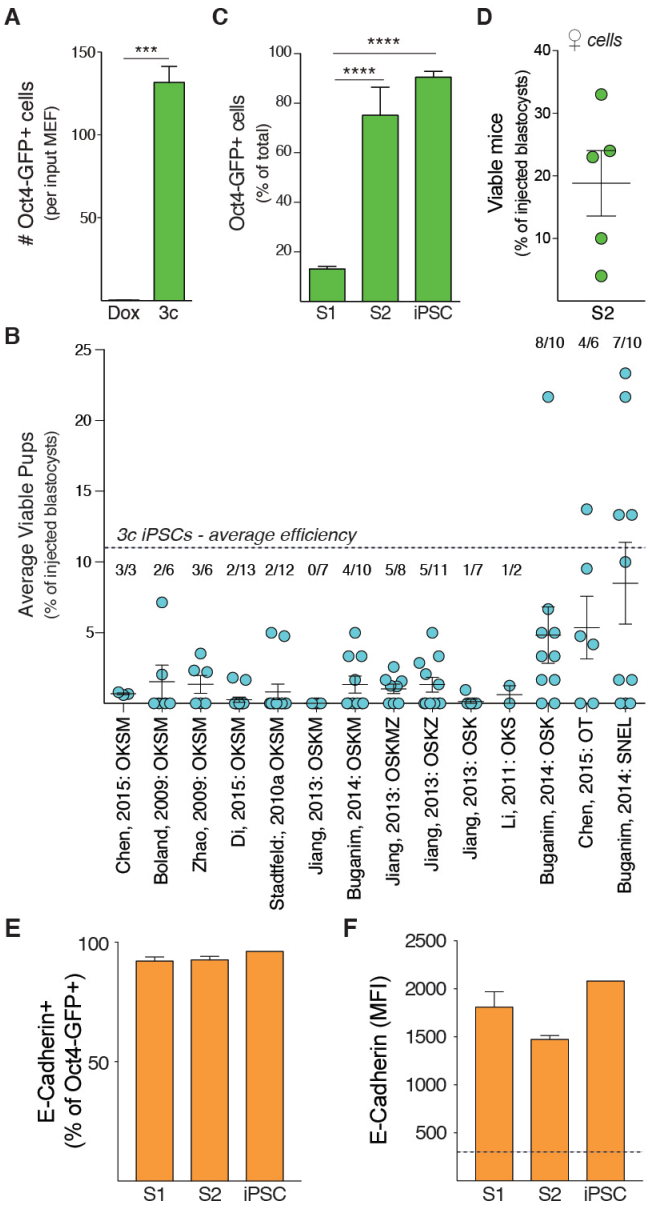

Figure S1, Related to Figure 1. (A) Number of Oct4-GFP+ cells generated per input MEF at Day 6 of reprogramming in either basal (Dox) or 3c conditions. (B) Published rates of viable mice upon 4n complementation with fibroblast-derived iPSCs. First author, year of publication and reprogramming factors used are shown. E = Esrrb, L = Lin28, N = Nanog, S = Sall4, T = Tet1, Z = Zscan4. Dotted line shows average success rate with established 3c-iPSCs. (C) Average percentage of viable cells expressing Oct4-GFP at the indicated stages of reprogramming. (D) "All-iPSC" mice obtained upon injection of S2 cells derived from female MEFs under 3c conditions. (E) Average percentage of Oct4-GFP+ cells expressing E-Cadherin, measured by flow cytometry. (F) Average mean fluorescence intensity (MFI) of E-Cadherin signal in Oct4-GFP+ cells. The dotted line indicates background fluorescence levels in unstained iPSCs. Statistical significance was determined with an unpaired t-test (A), or a one-way ANOVA with Tukey post-test (C) and (D), with *** and **** indicating $p<0.001$ and $p<0.0001$, respectively. Error bars indicated standard errors (n = 3 independent cultures per condition).

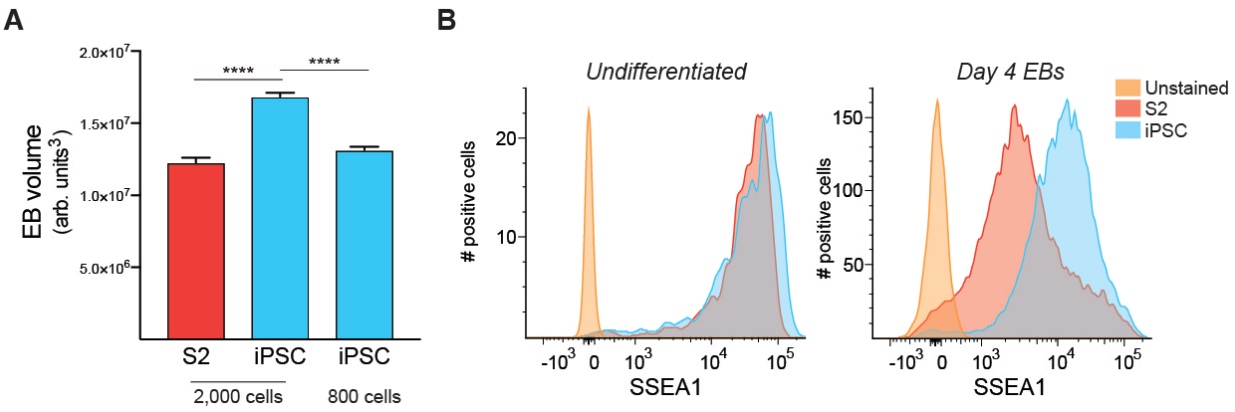

Figure S2, Related to Figure 2. (A) Average volume of 20 EBs, which were initiated with the indicated number and type of input cells. Statistical significance was determined using a one-way ANOVA with Tukey post-test, with **** indicating $p<0.0001$. EBs initiated with 2,000 S2 cells versus 800 iPSCs were not significantly different in size. Error bars indicated standard error (n = 3 independent cultures per condition). (B) Expression of the pluripotency-associated cell surface marker SSEA1 by undifferentiated cells (left) and by cells isolated from Day 4 EBs (right), measured by flow cytometry.

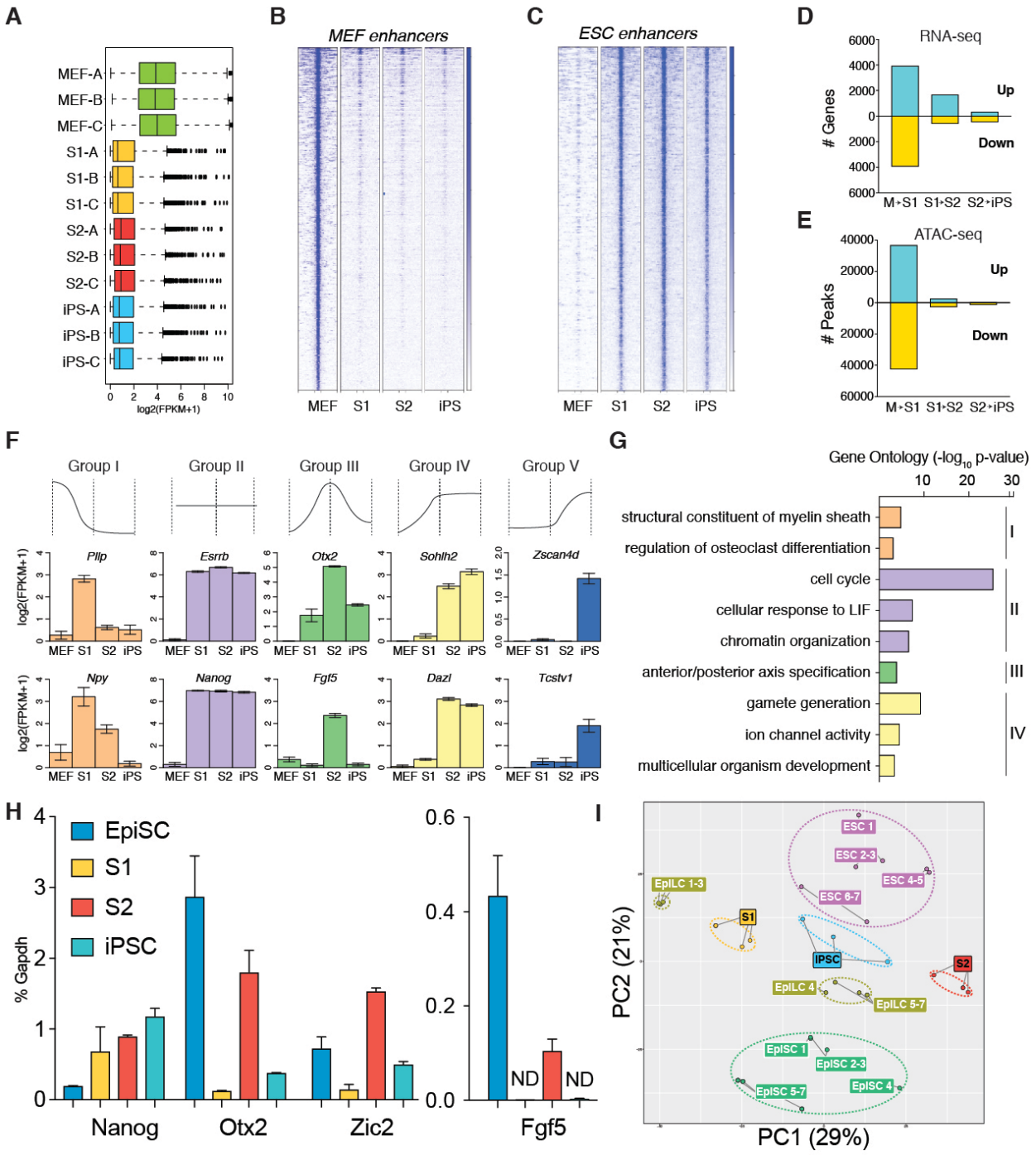

Figure S3, Related to Figure 3. (A) Box plots showing expression levels of MEF-associated genes at indicated stages of iPSC derivation (n = 3 replicates per stage), defined as 5x higher in MEFs vs established iPSCs. (B, C) ATAC-seq signal at (B) MEF-associated and (C) ESC-associated candidate enhancer elements, and the regions 2500bp upstream and downstream of these elements, at indicated stages of iPSC formation. Enhancer elements were defined by presence of MED1/MED12 and H3K27ac (see Supplemental Methods). Scale ranges from most accessible (blue) to least accessible (white). (D) Number of gene loci that change their expression levels significantly (>2-fold; adj. p<0.05) during the indicated transitions of iPSC

derivation. (E) Number of genomic regions that change their accessibility significantly (>1.5-fold; adj. p<0.1) during the indicated transitions during iPSC derivation. (F) Generic kinetics (top row) and examples of genes belonging to the five main groups defined by RNA-seq expression kinetics. Error bars indicate standard error (n = 3 replicates per stage). (G) Select, highly significant Gene Ontology (GO) terms associated with the indicated gene expression groups (see also Supplemental Table 3). (H) Expression of indicated transcripts in EpiSCs and during reprogramming. Error bars indicate standard error (n=2 per cell type or stage). ND = not detected or detected at very low levels. (I) Principal component analysis of our S1, S2 and iPS cells compared to published datasets for ESCs, EpiSCs and EpiLCs. Published datasets were referenced as follows: ESC 1 (Morikawa et al., 2016)(GSE70578), ESC 2-3 (Acampora et al., 2016)(E-MTAB-3856), ESC 4-5 (Chang et al., 2014)(GSE36290), ESC 6-7 (D'Aniello et al., 2017)(GSE84137), EpiLC 1-3 (von Meyenn et al., 2016)(GSE86903), EpiLC 4 (Buecker et al., 2014)(GSE56096), EpiLC 5-7 (Williams et al., 2016)(GSE81494), EpiSC 1 (Morikawa et al., 2016)(GSE70578), EpiSC 2-3 (Acampora et al., 2016)(E-MTAB-3856), EpiSC 4 (Sudheer et al., 2016)(E-MTAB-3784), EpiSC 5-7 (Bao et al., 2017)(GSE99491).

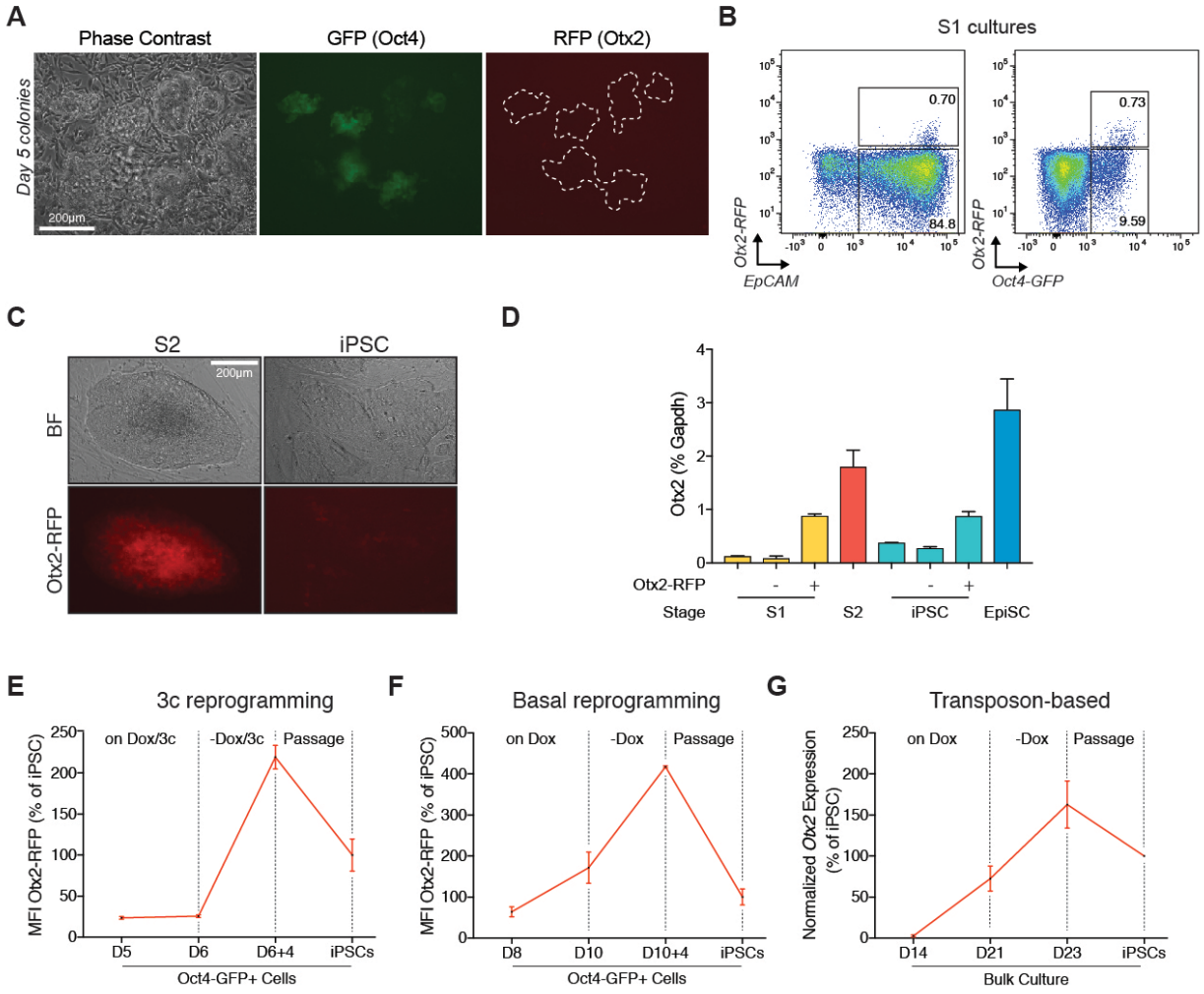

Figure S4, Related to Figure 4. (A) Representative colonies observed at Day 5 of 3c reprogramming initiated with MEFs harboring both Oct4-GFP and Otx2-RFP alleles. Note that Oct4-GFP+ cells are Otx2-RFP- at this stage. (B) Representative FACS plots of S1 cultures showing co-expression of Otx2-RFP and EpCAM (left panel) and Otx2-RFP and Oct4-GFP (right panel), respectively. (C) Representative images of Otx2-RFP fluorescence in S2 cells and established iPSCs. (D) Expression of *Otx2* in S1, S2, iPSC, and EpiSC cultures, and S1 and iPSCs sorted based on Otx2-RFP expression, measured by qPCR. (E, F) Mean fluorescent intensity (MFI) of Otx2-RFP in Oct4-GFP+ cells at indicated time points during reprogramming under (E) 3c, or (F) basal conditions, measured by flow cytometry and represented relative to the MFI of iPSCs. (G) Relative *Otx2* expression during transposon-mediated reprogramming, measured by RNA-seq on bulk cultures (Golipour et al., 2012). Note that the varying time points in E, F and G reflect the particular reprogramming kinetics (3c is faster than both basal and transposon-based reprogramming) of the different systems.

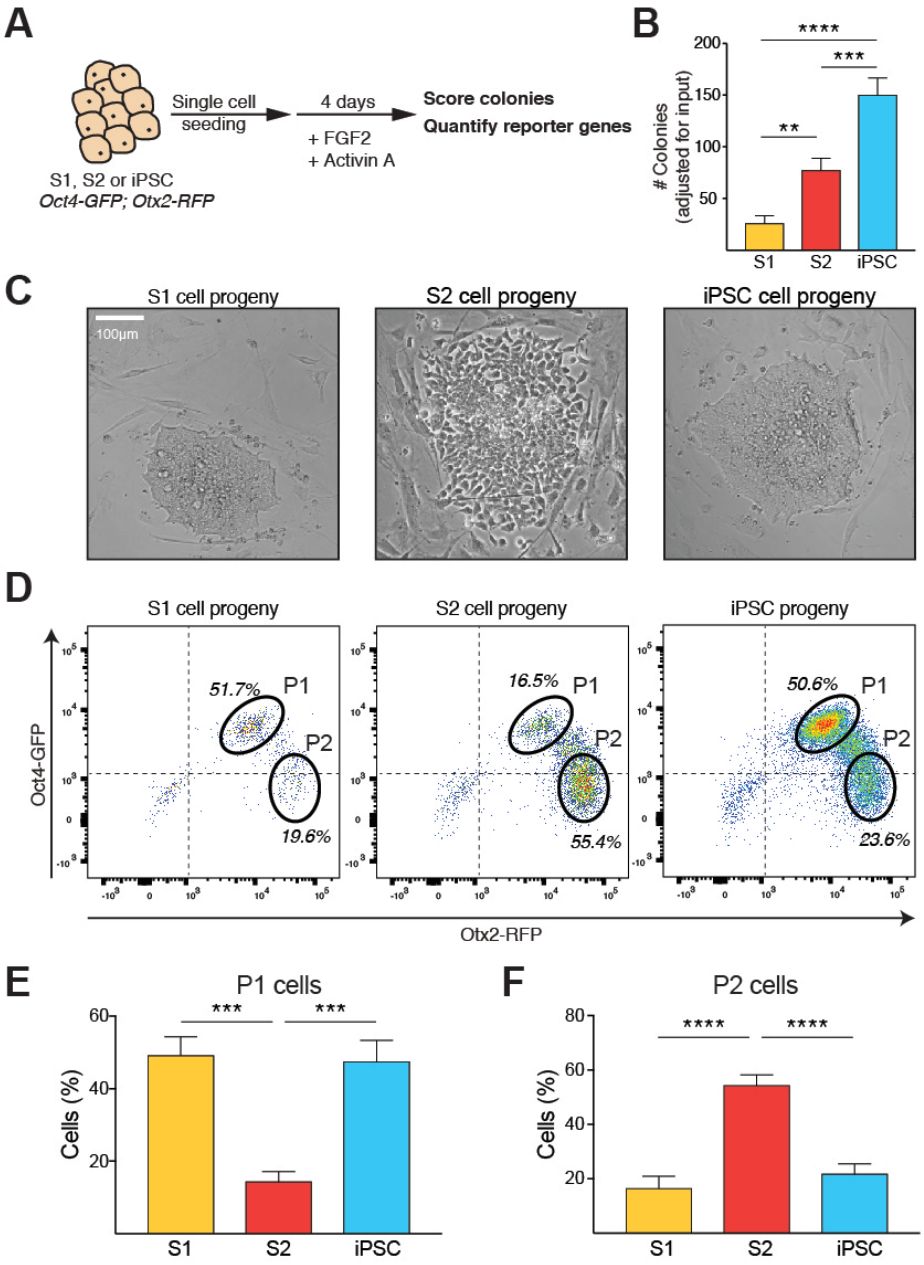

Figure S5, Related to Figure 4. (A) Experimental strategy to determine how cells at different stages of reprogramming respond to withdrawal of LIF and culture in presence of FGF2 and Activin A at clonal density. (B) Quantification of colony formation after single-cell sorting of Oct4-GFP+ cells isolated from indicated reprogramming stages treated as outlined in A. Error bars indicate standard error (n=3 per stage). (C) Representative colonies derived from Oct4-GFP+ cells isolated from indicated stages. (D) Representative flow cytometry plots and gating strategy to identify Oct4-GFP^{HIGH}Otx2-RFP^{LOW} ("P1") and Oct4-GFP^{LOW}Otx2-RFP^{HIGH} ("P2") cell populations. (E, F) Percent of P1 and P2 cells derived from indicated reprogramming stages after four days of culture, measured by flow cytometry. Error bars indicate standard error (n=3 per stage). Significance is indicated by ** ($p<0.01$), *** ($p<0.001$) and **** ($p<0.0001$) based on a one-way ANOVA with Tukey post-test.

Table S1: 4n injection data, Related to Figure 1

Fig.	Stage	Clone	Sex	Blasts.	Pups	%	Notes
1C	**iPSC (dox)**	#1	♂	20	2	**10%**	Derived without 3c
	iPSC (dox)	#1	♂	20	0	**0%**	Derived without 3c
	iPSC (dox)	#2	♂	24	0	**0%**	Derived without 3c
	iPSC (dox)	#2	♂	23	0	**0%**	Derived without 3c
	iPSC (dox)	#3	♂	20	1	**5%**	Derived without 3c
	iPSC (3c)	#1	♂	21	0	**0%**	
	iPSC (3c)	#1	♂	21	0	**0%**	
	iPSC (3c)	#2	♂	19	3	**16%**	
	iPSC (3c)	#2	♂	19	5	**26%**	
	iPSC (3c)	#3	♂	19	5	**26%**	
	iPSC (3c)	#3	♂	19	0	**0%**	
	iPSC (3c)	#4	♂	23	5	**22%**	
	iPSC (3c)	#4	♂	23	0	**0%**	
	iPSC (3c)	#5	♂	20	4	**20%**	
	iPSC (3c)	#5	♂	21	0	**0%**	
	ESC	#1	♂	20	2	**10%**	
	ESC	#1	♂	20	0	**0%**	
	ESC	#2	♂	20	0	**0%**	
	ESC	#2	♂	20	5	**25%**	
1F	**S1**	Polyclonal	♂	20	0	**0%**	
	S1	Polyclonal	♂	20	0	**0%**	
	S1	Polyclonal	♂	20	0	**0%**	
	S1	Polyclonal	♂	20	0	**0%**	
	S1	Polyclonal	♂	20	0	**0%**	
	S1	Polyclonal	♂	20	3	**15%**	
	S1	Polyclonal	♂	20	0	**0%**	
	S1	Polyclonal	♂	20	0	**0%**	
	S2	Polyclonal	♂	20	10	**50%**	
	S2	Polyclonal	♂	20	11	**55%**	
	S2	Polyclonal	♂	20	0	**0%**	
	S2	Polyclonal	♂	22	11	**50%**	
	S2	Polyclonal	♂	22	0	**0%**	
	S2	Polyclonal	♂	22	0	**0%**	
	S2	Polyclonal	♂	20	6	**30%**	
	S2	Polyclonal	♂	22	10	**45%**	
	S2	Polyclonal	♂	22	11	**50%**	

	iPSC (3c)	Polyclonal	♂	20	5	**25%**	
	iPSC (3c)	Polyclonal	♂	20	0	**0%**	
	iPSC (3c)	Polyclonal	♂	24	7	**29%**	
	iPSC (3c)	Polyclonal	♂	11	1	**9%**	
	iPSC (3c)	Polyclonal	♂	10	2	**20%**	
	iPSC (3c)	Polyclonal	♂	22	0	**0%**	
	iPSC (3c)	Polyclonal	♂	22	2	**9%**	
	iPSC (3c)	Polyclonal	♂	22	0	**0%**	
	iPSC (3c)	Polyclonal	♂	10	0	**0%**	
	iPSC (3c)	Polyclonal	♂	11	0	**0%**	
1G	**S1**	Polyclonal	♂	22	0	**0%**	Oct4-GFP+ Sorted
	S1	Polyclonal	♂	35	0	**0%**	Oct4-GFP+ Sorted
	S1	Polyclonal	♂	25	0	**0%**	Oct4-GFP+ Sorted
	S2	Polyclonal	♂	21	6	**29%**	Oct4-GFP+ Sorted
	S2	Polyclonal	♂	25	2	**8%**	Oct4-GFP+ Sorted
	S2	Polyclonal	♂	22	5	**23%**	Oct4-GFP+ Sorted
4C	**S2**	Polyclonal	♂	20	3	**15%**	Otx2-RFP+, Oct4-GFP+ Sorted
	S2	Polyclonal	♂	20	3	**15%**	Otx2-RFP+, Oct4-GFP+ Sorted
	S2	Polyclonal	♂	20	4	**20%**	Otx2-RFP+, Oct4-GFP+ Sorted
	S2	Polyclonal	♂	20	4	**20%**	Otx2-RFP+, Oct4-GFP+ Sorted
	iPSC (3c)	Polyclonal	♂	20	0	**0%**	Otx2-RFP+, Oct4-GFP+ Sorted
	iPSC (3c)	Polyclonal	♂	20	1	**5%**	Otx2-RFP+, Oct4-GFP+ Sorted
	iPSC (3c)	Polyclonal	♂	20	1	**5%**	Otx2-RFP+, Oct4-GFP+ Sorted
	iPSC (3c)	Polyclonal	♂	20	0	**0%**	Otx2-RFP+, Oct4-GFP+ Sorted
S1D	**S2**	Polyclonal	♀	21	5	**24%**	
	S2	Polyclonal	♀	18	6	**33%**	
	S2	Polyclonal	♀	23	1	**4%**	
	S2	Polyclonal	♀	22	5	**23%**	
	S2	Polyclonal	♀	20	2	**10%**	

Supplemental Experimental Procedures

Flow Cytometry

For quantification of *Oct4-GFP*, *Otx2-RFP* and cell surface marker expression, cultures were harvested by trypsin digestion and stained with DAPI or 7-aminoactinomycin D to allow dead cell exclusion. Cells were also stained as needed with biotinylated antibodies against E-cadherin (13-3249-80, eBioscience) or SSEA1 (13-8813-82, eBioscience), followed by streptavidin-APC. Samples were acquired on a LSR II Flow Cytometer (BD Biosciences), and analyzed with FlowJo software (TreeStar). FACS for purposes other than blastocyst injection was performed using FACSAria (BD Biosciences), a Beckman Coulter MoFlo (Cytomation) or a SY3200 (Sony). FACS prior to blastocyst injections are described in the main text under '4n blastocyst complementation'.

Alkaline Phosphatase Staining

Cultures were stained using the VECTOR Red Alkaline Phosphatase kit (Vector Laboratories) per the manufacturer's protocol.

Gene Groups based on RNA-seq

MEF genes were identified as higher in MEFs than in established iPSCs (fold-change > 5, adjusted p-value < 0.05). Pluripotency genes were identified as higher in at least one reprogramming stage vs MEFs (fold-change > 5, adjusted p-value < 0.05).
Gene groups (see Figures 3C and S3F) were defined based on pluripotency genes by intersection of pair-wise comparisons between stages, based on higher expression (fold-change > 2, adjusted p-value < 0.05), or similar levels (fold-change < 2 and/or an adjusted p-value > 0.05). Groups with less than 100 genes (see heatmap in Figure 3C) were not analyzed further.

- Group I: Genes expressed higher in S1 vs S2, and S1 vs iPSCs.
- Group II: Genes expressed at similar levels between S1 vs S2, and S2 vs iPSCs, and S1 vs iPSCs.
- Group III: Genes expressed at higher levels in S2 vs S1, and S2 vs iPSCs. Genes that fulfilled these criteria, but whose significant fold-change between iPSC vs S1 was larger than S2 vs iPSC, were assigned to Group IV (iPSC vs S1 minus S2 vs iPSC ≥ log(0.5), n=18/124), e.g. *Lefty2*, *Aire*.
- Group IV: Genes expressed at higher levels in S2 vs S1, and iPSC vs S1, and at similar levels between S2 vs iPSCs.
- Group V: Genes expressed at higher levels in iPSCs vs S1, and iPSCs vs S2. Genes that fulfilled these criteria, but whose significant fold-change between S2 vs S1 was larger than iPSCs vs S2, were assigned to Group IV (S2 vs S1 minus iPSCs vs S2 ≥ log(0.5), n=48/124), e.g: *Lefty1, Aurkc*.

Comparative analysis of published 4n-complementation injections

Published reports describing 4n complementation with fibroblast-derived murine iPSCs (Boland et al., 2009; Buganim et al., 2014; Chen et al., 2015; Di et al., 2015; Jiang et al., 2013; Li et al., 2011; Stadtfeld et al., 2010; Zhao et al., 2009) were mined for percentage of viable pups born per blastocyst transferred. If viable pup data was unavailable, pups born was used. If number of blastocysts transferred was unavailable, number of blastocysts injected was used.

Definition of Enhancer Regions

MED1/MED12 (Kagey et al., 2010) and H3K27ac (Liu et al., 2017) ChIP-seq reads were trimmed for adaptors (cutadapt 1.8.1) and low-quality reads (sickle 1.33), and mapped to mm10 permitting a maximum of one mismatch in seed alignment (Bowtie 2.2.5). Reads marked as positional duplicates or which overlapped with mouseENCODE black listed genomic regions (liftOver to mm10; ENCODE Project Consortium 2012) were removed. Replicates were merged, and ChIP-seq peaks (enrichment of signals over background) were called (MACS 2.1.1) at a P value threshold of 10^{-2} (H3K27ac) and 10^{-5} (MED1/MED12). Enhancer regions for either ESCs or MEFs were defined as overlap between H3K27ac and at least one of MED1 or MED12, excluding regions of H3K27ac in the other cell type (Bedtools 2.26).

Comparison to published datasets for ESCs, EpiSCs, and EpiLCs

Transcripts per million were quantified in Salmon (version 0.8.2) for the study data and selected published studies (indicated in legend for Figure S3I). Batch effect was corrected using linear regression, as previously described (Bulut-Karslioglu et al., 2016). Signature genes for S1, S2 and iPS cells (totaling 2319 genes) were identified through pairwise differentially tests in limma (version 3.6) by the thresholds of fold change 2 and p-value of 0.01. Principal component analysis was performed on these samples.

Supplemental References

Acampora, D., Omodei, D., Petrosino, G., Garofalo, A., Savarese, M., Nigro, V., Di Giovannantonio, L.G., Mercadante, V., and Simeone, A. (2016). Loss of the Otx2-Binding Site in the Nanog Promoter Affects the Integrity of Embryonic Stem Cell Subtypes and Specification of Inner Cell Mass-Derived Epiblast. Cell Rep *15*, 2651-2664.

Bao, S., Tang, W.W., Wu, B., Kim, S., Li, J., Li, L., Kobayashi, T., Lee, C., Chen, Y., Wei, M., *et al.* (2017). Derivation of hypermethylated pluripotent embryonic stem cells with high potency. Cell Res.

Boland, M.J., Hazen, J.L., Nazor, K.L., Rodriguez, A.R., Gifford, W., Martin, G., Kupriyanov, S., and Baldwin, K.K. (2009). Adult mice generated from induced pluripotent stem cells. Nature.

Buecker, C., Srinivasan, R., Wu, Z., Calo, E., Acampora, D., Faial, T., Simeone, A., Tan, M., Swigut, T., and Wysocka, J. (2014). Reorganization of enhancer patterns in transition from naive to primed pluripotency. Cell Stem Cell *14*, 838-853.

Buganim, Y., Markoulaki, S., van Wietmarschen, N., Hoke, H., Wu, T., Ganz, K., Akhtar-Zaidi, B., He, Y., Abraham, B.J., Porubsky, D., *et al.* (2014). The developmental potential of iPSCs is greatly influenced by reprogramming factor selection. Cell Stem Cell *15*, 295-309.

Bulut-Karslioglu, A., Biechele, S., Jin, H., Macrae, T.A., Hejna, M., Gertsenstein, M., Song, J.S., and Ramalho-Santos, M. (2016). Inhibition of mTOR induces a paused pluripotent state. Nature *540*, 119-123.

Chang, G., Gao, S., Hou, X., Xu, Z., Liu, Y., Kang, L., Tao, Y., Liu, W., Huang, B., Kou, X., *et al.* (2014). High-throughput sequencing reveals the disruption of methylation of imprinted gene in induced pluripotent stem cells. Cell Res *24*, 293-306.

Chen, J., Gao, Y., Huang, H., Xu, K., Chen, X., Jiang, Y., Li, H., Gao, S., Tao, Y., Wang, H., *et al.* (2015). The combination of Tet1 with Oct4 generates high-quality mouse-induced pluripotent stem cells. Stem Cells *33*, 686-698.

D'Aniello, C., Habibi, E., Cermola, F., Paris, D., Russo, F., Fiorenzano, A., Di Napoli, G., Melck, D.J., Cobellis, G., Angelini, C., *et al*. (2017). Vitamin C and l-Proline Antagonistic Effects Capture Alternative States in the Pluripotency Continuum. Stem Cell Reports *8*, 1-10.

Di, K.Q., Gao, S., Cui, L.F., Chang, G., Wu, F.J., Ren, L.K., An, L., Miao, K., Tan, K., Tao, L., *et al*. (2015). Generation of fully pluripotent female murine-induced pluripotent stem cells. Biol Reprod *92*, 123.

Golipour, A., David, L., Liu, Y., Jayakumaran, G., Hirsch, C.L., Trcka, D., and Wrana, J.L. (2012). A late transition in somatic cell reprogramming requires regulators distinct from the pluripotency network. Cell Stem Cell *11*, 769-782.

Jiang, J., Lv, W., Ye, X., Wang, L., Zhang, M., Yang, H., Okuka, M., Zhou, C., Zhang, X., Liu, L., *et al*. (2013). Zscan4 promotes genomic stability during reprogramming and dramatically improves the quality of iPS cells as demonstrated by tetraploid complementation. Cell Res *23*, 92-106.

Kagey, M.H., Newman, J.J., Bilodeau, S., Zhan, Y., Orlando, D.A., van Berkum, N.L., Ebmeier, C.C., Goossens, J., Rahl, P.B., Levine, S.S., *et al*. (2010). Mediator and cohesin connect gene expression and chromatin architecture. Nature *467*, 430-435.

Li, W., Zhao, X.Y., Wan, H.F., Zhang, Y., Liu, L., Lv, Z., Wang, X.J., Wang, L., and Zhou, Q. (2011). iPS cells generated without c-Myc have active Dlk1-Dio3 region and are capable of producing full-term mice through tetraploid complementation. Cell research *21*, 550-553.

Liu, Y., Pelham-Webb, B., Di Giammartino, D.C., Li, J., Kim, D., Kita, K., Saiz, N., Garg, V., Doane, A., Giannakakou, P., *et al*. (2017). Widespread Mitotic Bookmarking by Histone Marks and Transcription Factors in Pluripotent Stem Cells. Cell Rep *19*, 1283-1293.

Morikawa, M., Koinuma, D., Mizutani, A., Kawasaki, N., Holmborn, K., Sundqvist, A., Tsutsumi, S., Watabe, T., Aburatani, H., Heldin, C.H., *et al*. (2016). BMP Sustains Embryonic Stem Cell Self-Renewal through Distinct Functions of Different Kruppel-like Factors. Stem Cell Reports *6*, 64-73.

Stadtfeld, M., Apostolou, E., Akutsu, H., Fukuda, A., Follett, P., Natesan, S., Kono, T., Shioda, T., and Hochedlinger, K. (2010). Aberrant silencing of imprinted genes on chromosome 12qF1 in mouse induced pluripotent stem cells. Nature *465*, 175-181.

Sudheer, S., Liu, J., Marks, M., Koch, F., Anurin, A., Scholze, M., Senft, A.D., Wittler, L., Macura, K., Grote, P., *et al*. (2016). Different Concentrations of FGF Ligands, FGF2 or FGF8 Determine Distinct States of WNT-Induced Presomitic Mesoderm. Stem Cells *34*, 1790-1800.

von Meyenn, F., Berrens, R.V., Andrews, S., Santos, F., Collier, A.J., Krueger, F., Osorno, R., Dean, W., Rugg-Gunn, P.J., and Reik, W. (2016). Comparative Principles of DNA Methylation Reprogramming during Human and Mouse In Vitro Primordial Germ Cell Specification. Dev Cell *39*, 104-115.

Williams, E.O., Taylor, A.K., Bell, E.L., Lim, R., Kim, D.M., and Guarente, L. (2016). Sirtuin 1 Promotes Deacetylation of Oct4 and Maintenance of Naive Pluripotency. Cell Rep *17*, 809-820.

Zhao, X.Y., Li, W., Lv, Z., Liu, L., Tong, M., Hai, T., Hao, J., Guo, C.L., Ma, Q.W., Wang, L., *et al*. (2009). iPS cells produce viable mice through tetraploid complementation. Nature *461*, 86-90.