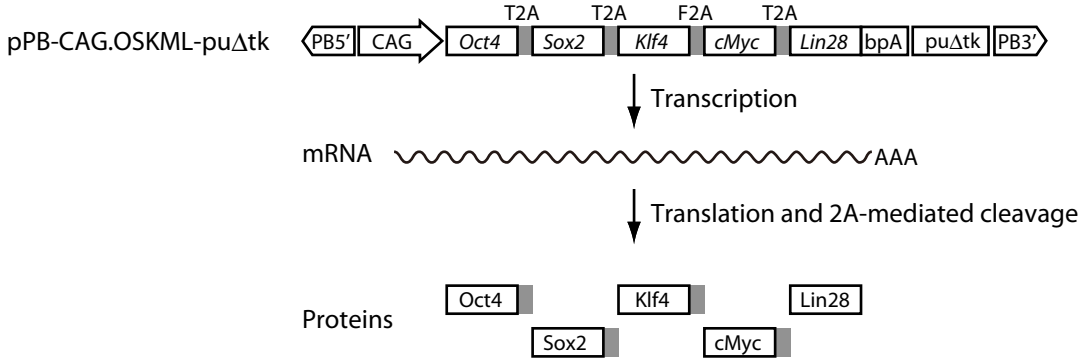
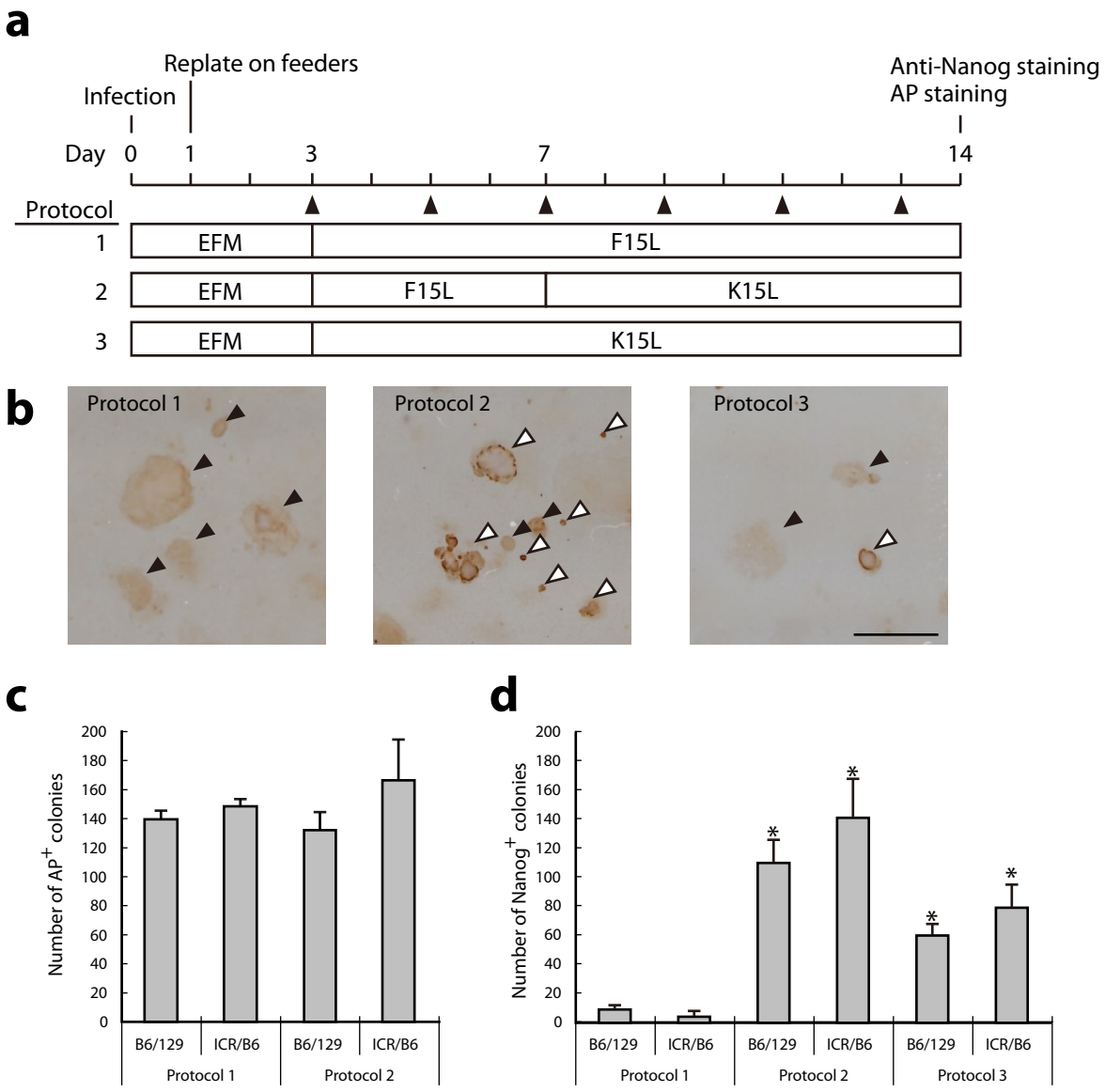
Supplementary Figure 1



Supplementary Figure 1. 2A peptide-mediated polycistronic expression.
Five genes liked by 2A peptide are transcribed into a single mRNA molecule. Polypepetides are co-translationally cleaved and release their N-terminal peptides fused with residual 2A peptide. Thus the molecular weights increase 2.5 kDa except C-terminal protein.

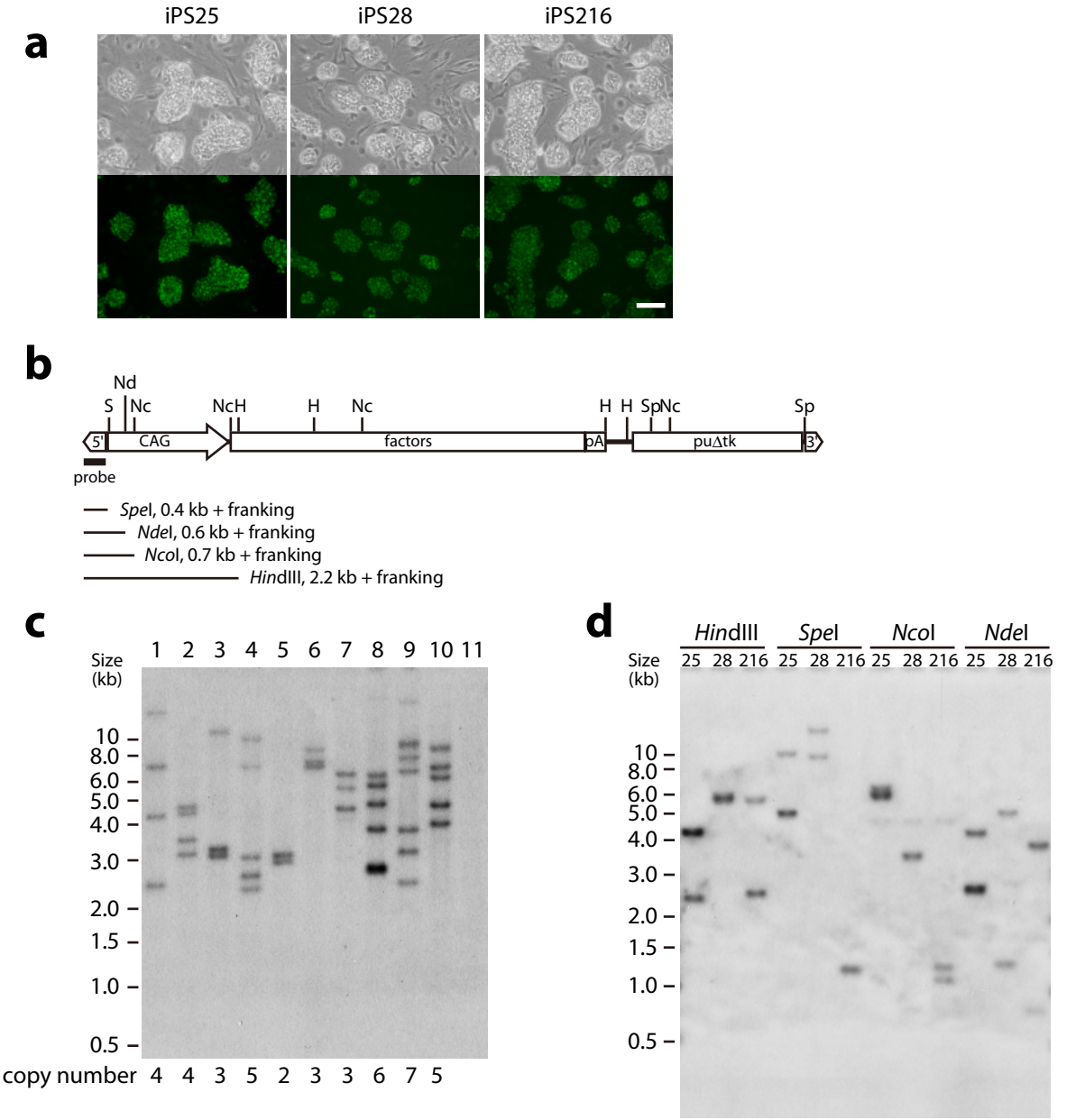
Supplementary Figure 2



Supplementary Figure 2. Enhancement of reprogramming by combination of serum-based and serum-free media.

(**a**) Time schedules of medium change from serum-based medium to serum-free medium. On day 0, MEFs were infected by a mixture of retroviruses carrying the four factors individually. Next day, infected MEFs were replated onto feeders. Cells were cultured with ESC medium between day 3 to day 14. In protocol 1 and 3, serum-based and serum-free medium was used for the entire period, respectively. In protocol 2, serum-based medium was used until day 7 and then switched to the serum-free medium. Triangles, medium changes; EFM, embryonic fibroblast medium; F15L, serum-based ESC medium; K15L, serum-free ESC medium. (**b**) Immunochemistry of iPSC colonies. On day 14, colonies were fixed and immunostained with anti-Nanog followed by chromogenic detection with DAB. Open and closed arrowheads, positive and negative for staining, respectively. Due to overdevelopment, even negative colonies show light brown. (**c, d**) The numbers of AP-positive and Nanog-positive colonies, respectively. Data are shown as mean ± s.d. (n=3). *, p<0.05 (Student' s t test compared to protocol 1). Scale bar, 5mm

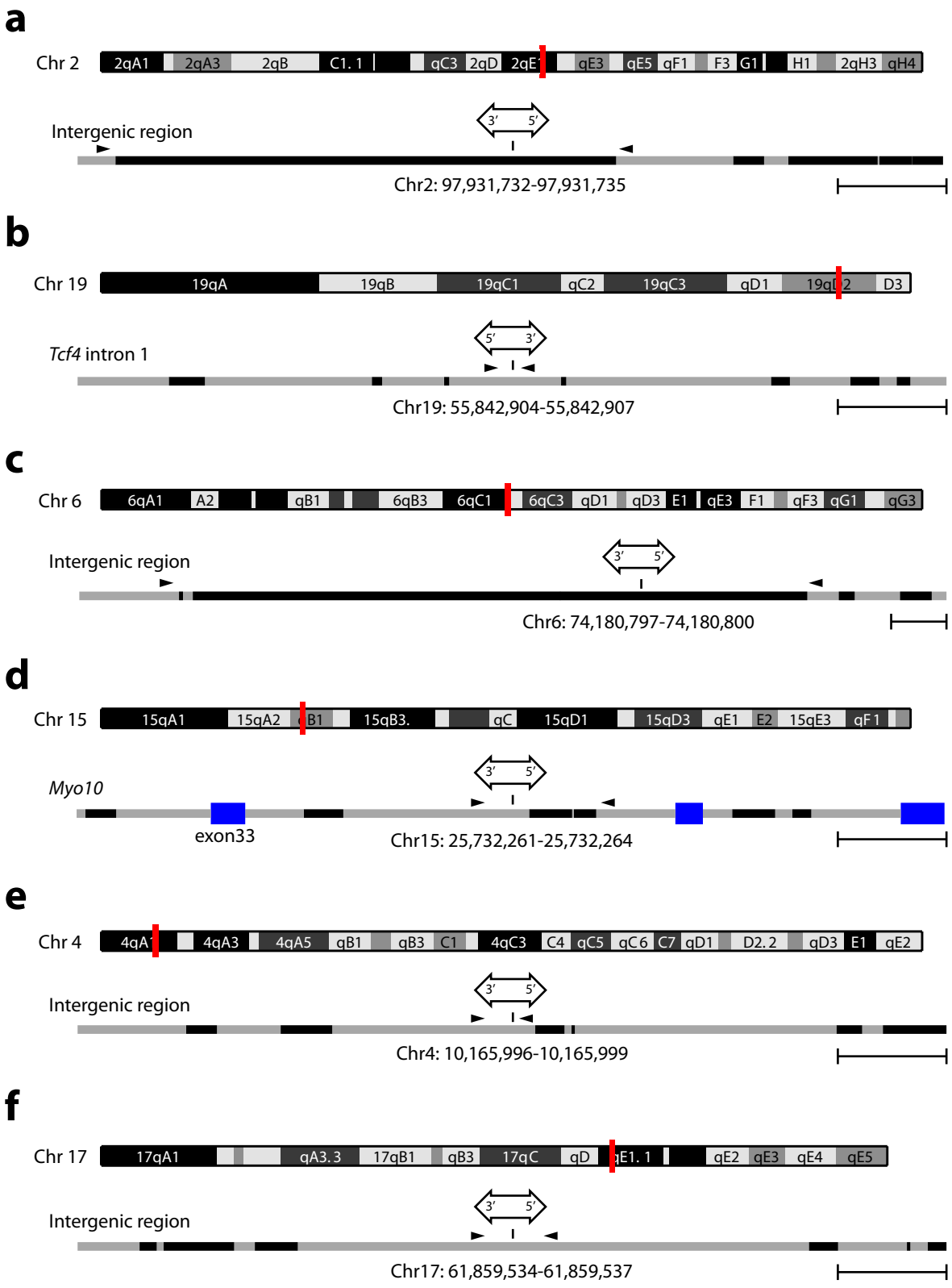
Supplementary Figure 3



Supplementary Figure 3. Characterization of primary iPSCs reprogrammed by the *piggyBac* transposon system.

(**a**), Immunostaining of Nanog in primary iPSC lines. They are morphologically indistinguishable from ESCs and express endogenous Nanog uniformly. Top, phase contrast image; bottom, Nanog immunostaining. (**b**) Restriction enzyme map of the reprogramming transposon vector. A thick line represents a probe for Southern blot analysis. (**c**) A representative result of Southern blot analysis of primary iPSCs. Genomic DNAs were digested with *Hin*dIII and hybridized with the 5' terminal repeat of the *piggyBac* transposon. Lane 1-7, primary iPSCs generated by transfection of 2.0 μg of OSKM* transposon and 2.0 μg of *piggyBac* transposase-expressing plasmid. Lane 8-10, primary iPSCs generated by transfection of 4 factors carried by transposon individually together with the *piggyBac* transposase-expressing plasmid. Lane 11, *piggyBac* transposon-free mouse genomic DNA as a negative control. The copy numbers of integrated transposon were shown under each lane. (**d**) Detailed Southern blot analysis of 3 primary iPSC clones with 2-copy integration. Genomic DNAs were digested with different restriction enzymes and hybridized with the 5' terminal repeat of the *piggyBac* transposon. In all lanes, there were no more than 3 bands, indicating that these clones had 2 transposon copies. Scale bar, 100 μm

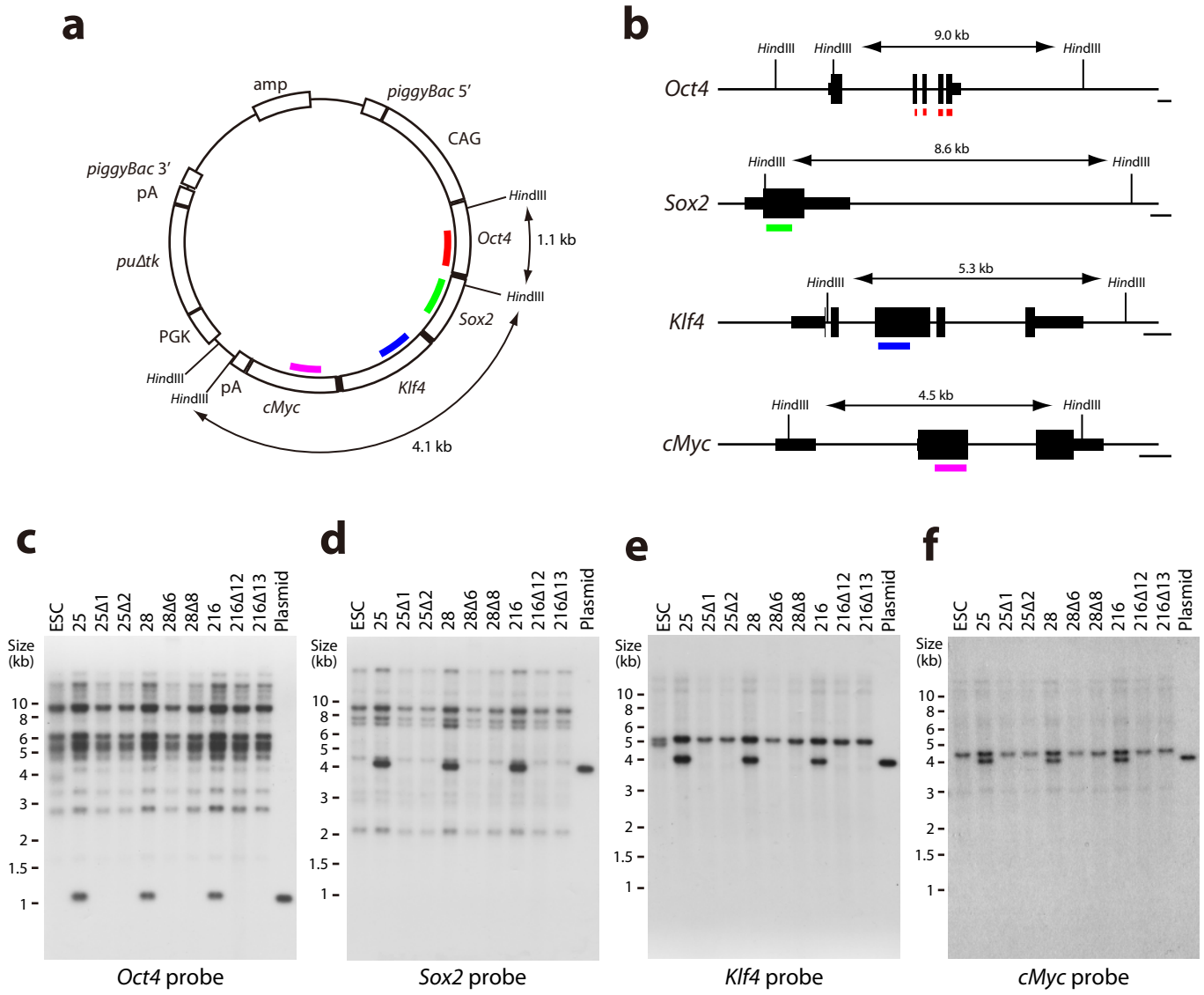
Supplementary Figure 4



Supplementary Figure 4. Transposon integration sites of 3 selected primary iPSC lines.

(**a, b**) Integration site 1 and 2 of iPS25. (**c, d**) Integration site 1 and 2 of iPS28. (**e, f**) Integration site 1 and 2 of iPS216. Gray and black lines show non-repetitive and repetitive sequences, respectively. Blue box, exon; Arrowheads, integration site-specific primers used in **Figure 3c-e**. The numbers under each line indicate positions of TTAA sequences, which are the target site of the *piggyBac* transposon (based on UCSC July 2007 assembly). Scale bar, 500 bp

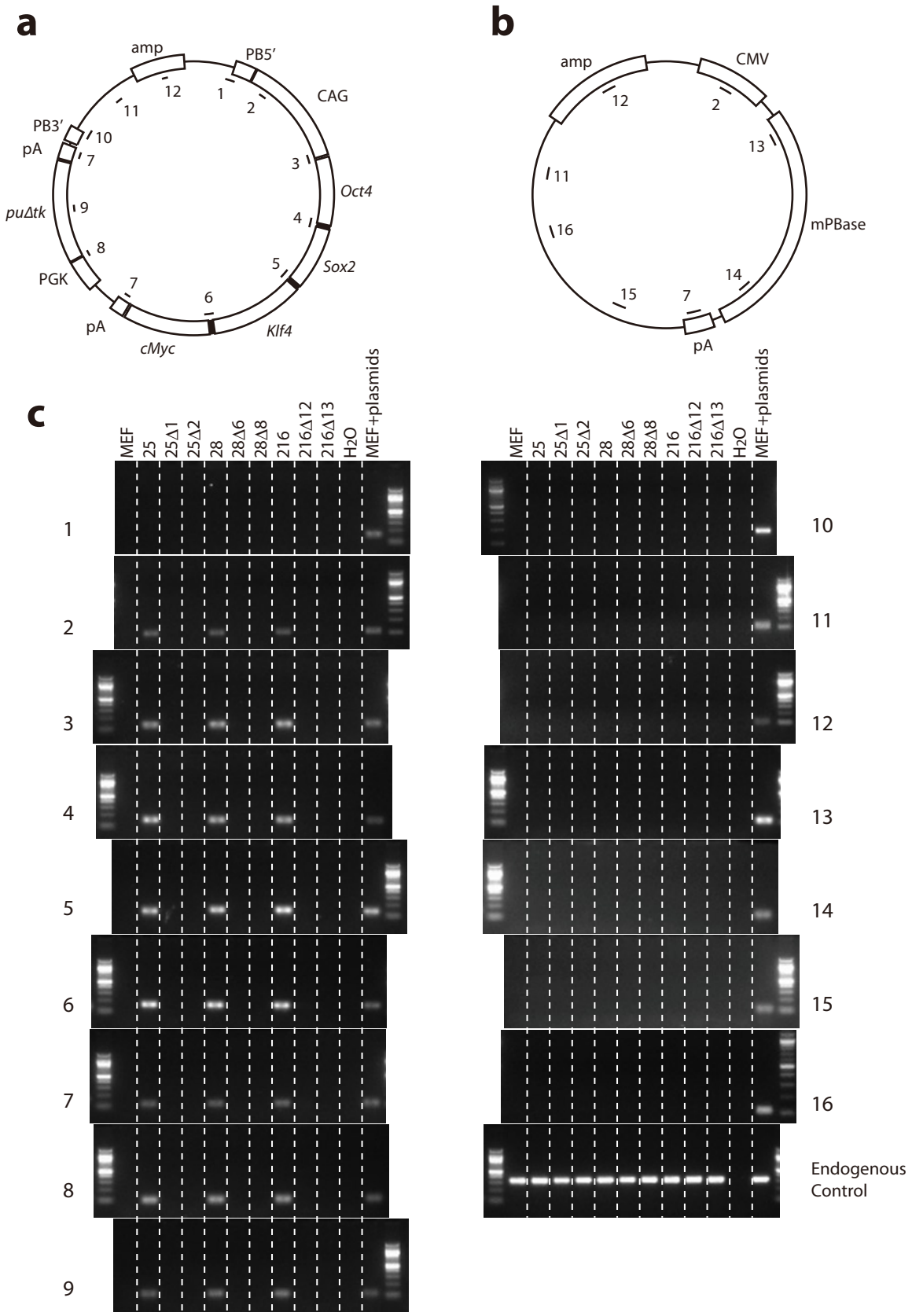
Supplementary Figure 5



Supplementary Figure 5. Southern blot analyses on integration-free iPSC genomes using probes against different parts of the transgene.

(**a**) Positions of probes (thick lines) used for Southern blot analyses. (**b**) Restriction maps of the endogenous loci of the 4 genes. (**c-f**) Southern blot analyses. Genomic DNAs were digested by *Hind*III and hybridized with probes shown in (**a**). Transgenes were detected in primary iPSC lines (iPS25, 28, and 216), but not in integration-free iPSC lines (iPS25Δ1, 25Δ2, 28Δ6, 28Δ8, 216Δ12, and 216Δ13), further proving transposon removal together with Southern blot analysis using the 5′ terminal repeat probe shown in **Figure 3b**. Note that the control ESC line used has a B6129F1 background. In Oct4 and Klf4, polymorphisms can be seen. Scale bar, 500 bp

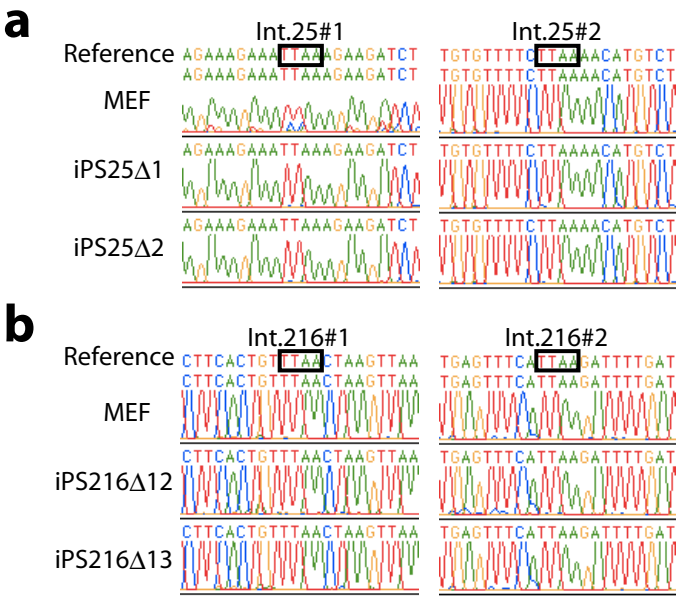
Supplementary Figure 6



Supplementary Figure 6. PCR analysis of vector integration in integration-free iPSCs.

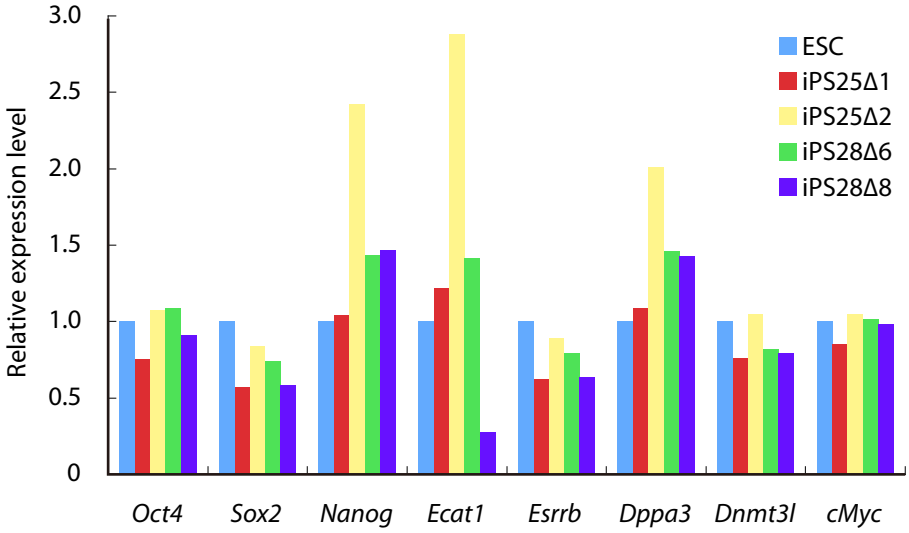
(**a, b**) Positions of PCR amplicons in the reprogramming transposon and the *piggyBac* transposase expression vectors, respectively. (**c**) PCR results indicating that there is no evidence of random integration derived from both transpson and transposase expression vectors. As a positive control, both plasmids were spiked into MEF genomic DNA at levels designed to simulate single-copy integration per genome.

Supplementary Figure 7



Supplementary Figure 7. Footprint analysis by sequencing.
(**a**) Integration sites of iPS25-derived lines. (**b**) Integration site of iPS216-derived lines. Reference sequence is the C57Bl/6J genome. Sequences in transposon-free iPSC lines were identical to corresponding sequences of MEFs, indicating the perfect repair after transposon excision. TTAA target sites are boxed.

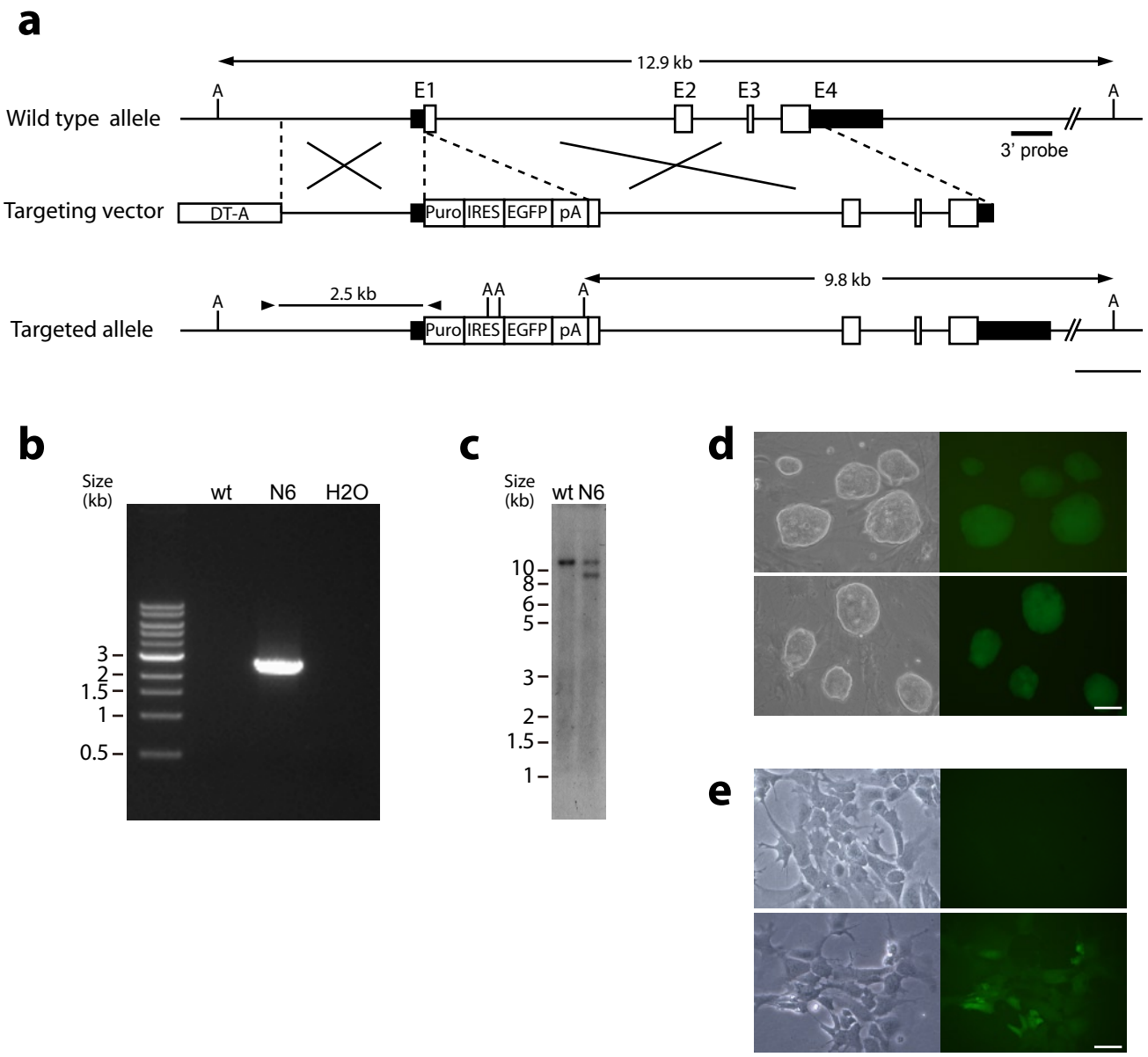
Supplementary Figure 8



Supplementary Figure 8. Quantitative RT-PCR analyses of ESC-specific genes in integration-free iPSCs.

Expression levels of ESC-specific genes were normalized to *Gapdh*. Expression levels in iPSCs were compared to levels in ESCs.

Supplementary Figure 9



Supplementary Figure 9. Targeting a puro-IRES-eGFP cassette into the *Nanog* locus.
(**a**) Schematic representation of gene targeting. A puro-IRES-eGFP cassette was knocked-in upstream of the translation initiation codon. Arrowheads, a primer pair to detect homologous recombination. A, *Afl*III sites. (**b, c**) Homologous recombination was verified by PCR across the 5′ homologous arm (**b**) and by Southern blot analysis using a 3′ external probe (**c**). (**d**) GFP expression in self-renewing iPSCs. Top, knock-in cell line, N6, derived from iPS25Δ1. Bottom, iPS25Δ1-derived cell line, U6, expressing GFP from a ubiquitous promoter. (**e**) Differentiation induced by all-*trans*-retinoic acid. At day 3, GFP expression was erased in the line N6 (top), whereas GFP was continuously expressed in the line U6 (bottom), indicating normal regulation of the *Nanog* promoter in cured iPSCs. Scale bars, 1 kb (**a**), 50 μm (**d,e**)

Supplementary Table 1. Summary of blastocyst injections

Cell line	Embryos trasnferred	Chimerism at e10.5 [3]	Contribution to gonad at e12.5 [4]	Live pups	GFP chimerism
25Δ1-U6[1]	12	7/8	n/a	n/a	n/a
	12	n/a	n/a	2	0
	10	n/a	n/a	2	1[5]
	12	n/a	n/a	0	0[6]
25Δ1-N6[2]	10	n/a	1/7	n/a	n/a
	10	n/a	n/a	5	1[6]
	10	n/a	n/a	4	1[6]

[1] iPS25Δ1-derived line with ubiquitous expression of eGFP
[2] iPS25Δ1-derived line in which eGFP has been knocked-in into the *Nanog* locus
[3] Number of GFP-positive chimeric embryos / total embryos
[4] Number of chimeric embryo with GFP-positve gonad / total embryos
[5] Genotyped by GFP primers and observation under the fluorescent stereomicroscope
[6] Genotyped by GFP primers
n/a, Not applicable

Supplementary Table 2. Oligonucleotides used in this study

F2A-linked 4-factor vector construction

Oct4-B-U1	CGGGATCCACCATGGCTGGACACCTGGCTTC
Oct4-F2A-S	ACGCGTCGACGGGCCCTGGGTTGGACTCCACGTCTCCCGCCAACTTGAGAAGGTC AAAATTCAACAGCTGTTTTCCGCTGCCGTTTGAATGCATGGGAGAGC
Sox2-X	CTCGAGATGTATAACATGATGGAGAC
Sox2-F2A-S	GTCGACGGGCCCTGGGTTGGACTCCACGTCTCCCGCCAACTTGAGAAGGTCAAAA TTCAACAGCTGTTTTCCGCTGCCCATGTGCGACAGGGGCAGTG
Klf4-X	CTCGAGATGGCTGTCAGCGACGCTCT
Klf4-F2A-S	GTCGACGGGCCCTGGGTTGGACTCCACGTCTCCCGCCAACTTGAGAAGGTCAAAA TTCAACAGCTGTTTTCCGCTGCCAAAGTGCCTCTTCATGTGTA
Myc-X	CTCGAGATGCCCCTCAACGTGAACTT
Myc-S	GTCGACTCATGCACCAGAGTTTCGAAGCT

Oct4-T2A-Sox2 modification

OctT2ASox	CCAATGCATTCAAACGGCGGCGGGTCCGGAGGAGAGGGCAGAGGAAGTCTTCTAA CATGCGGTGACGTGGAGGAGAATCCTGGCCCAATGTATAACATGATGGAGACG
OtS-seqL	GCCCTGCTGCGAGTAGGACATGCTG

Sox2-T2A-Klf4 modification

StK-U1	GCAACGGCAGCTACAGCATGATGCAG
StK-U2	CAGAGGAAGTCTTCTAACATGCGGTGACGTGGAGGAGAATCCTGGCCCAATGGCTG TCAGCGACGCTCTGC
StK-L1	CAGGAGGTCGTTGAACTCCTCGGTCTC
StK-L2	ACCGCATGTTAGAAGACTTCCTCTGCCCTCTCCTCCGGACCCGCCGCCCATGTGCG ACAGGGGCAGTGTGCCG

Lin28 cloning

Lin28	U1	CGGGATCCACCATGGGCTCGGTGTCCAACCAGCAG
	L1	GGAATTCTCAATTCTGGGCTTCTGGGAGCAG

Linking Lin28 to the 4-factor cassette

MtL	U1	TCTAACATGCGGTGACGTGGAGGAGAATCCTGGCCCAATGGGCTCGGTGTCCAAC
	L1	ACTGATCGATCTCGAGTCAATTCTGGGCTTCTGGGAG

pCAG-EBNXN construction

EBNXNlinker	U	AATTCAGATCTGCGGCCGCCCGAGATGCA
	L	TCTCGAGGCGGCCGCAGATCTG

pPB-UbC construction

UbC	U	CTAGCTAGCCTCGAGGGCCTCCGCGCCGGGTTTTGGCGCC
	L	GGAATTCATGGATCCGTCTAACAAAAAAGCCAAAAACGGC
bpA	U	GGAATTCCGACTGTGCCTTCTAGTTGCCAG
	L	GCGTCGACCCATAGAGCCCACCGCATCCCCAGC

Sex typing

Sry	F	TGGTGTGGTCCCGTGGTGAGAGGCACAAG
	R	TAGCCCTCCGATGAGGCTGATATTTATAG

Splinkerrette-PCR

Spl-top [1]	CGAATCGTAACCGTTCGTACGAGAATTCGTACGAGAATCGCTGTCCTCTCCAACGAG CCAAGG
Spl-sau [1]	GATCCCTTGGCTCGTTTTTTTTTGCAAAAA
Spl-blunt	CCTTGGCTCGTTTTTTTTTGCAAAAA
Spl-CG	CGCCTTGGCTCGTTTTTTTTTGCAAAAA

Supplementary Table 2. (*continued*)

Spl-P1 [1]	CGAATCGTAACCGTTCGTACGAGAA
Spl-P2 [1]	TCGTACGAGAATCGCTGTCCTCTC
PB5-P1	AAGCGGCGACTGAGATGTCCTAAATG
PB5-P2	GCGACGGATTCGCGCTATTTAGAAAG
PB3-P1	AAACCTCGATATACAGACCGATAAAACAC
PB3-P2	CGTCAATTTTACGCATGATTATCTTTAAC

Detection of transposon removal

Transposon 1 (Sox2-Klf4 junction)	F	GCAACGGCAGCTACAGCATGATGCAG
	R	CAGGAGGTCGTTGAACTCCTCGGTCTC
Transposon 2 (PGK promoter)	F	GCGTCGACCGACTGTGCCTTCTAGTTGCCAGCC
	R	GTTGGCGCCTACCGGTGGATGTGGAATGTG
Endogenous control	F	GGGGCCTTCTGGGGGTAAAGTTCAGAACAC
	R	TGGCTGCCTGAGGGCAAGAGGGAAAGAATC

Southern blot probes to detect vector integration

Oct4	F	AAGGAGCTAGAACAGTTTGCCAAGC
	R	GGAAAAGGGACTGAGTAGAGTGTGG
Sox2	F	GCGACCGGCGGCAACCAGAAGAACA
	R	GGGTGCCCTGCTGCGAGTAGGACAT
Klf4	F	CCGTCTGGGCTTCCTTTGCTAACAC
	R	ACGGTGGCCACAGACCTGGAGAGTG
cMyc	F	GACGATGACGGCGGCGGTGGCAACT
	R	GTCGCTGCTGGTGGTGGGCGGTGTC

Detection of random integration of the plasmids

1	F	ACGACGTTGTAAAACGACGGCCAGT
	R	GCGACGGATTCGCGCTATTTAGAAAG
2 [2]	F	TATCATATGCCAAGTACGC
	R	TAGATGTACTGCCAAGTAGGAA
3	F	AGAGCCTCTGCTAACCATGTTCATGCCTTC
	R	GAAGTCTGAAGCCAGGTGTCCAGCC
4	F	CCCTCTGTTCCCGTCACTGCTCTG
	R	GCTTCAGCTCCGTCTCCATCATGT
5	F	CCATTAACGGCACACTGCCCCTGT
	R	AGGACGGGAGCAGAGCGTCGCTGA
6	F	CCACCTTGCCTTACACATGAAGAG
	R	GAGTCGTAGTCGAGGTCATAGTTC
7	F	TGCCACTCCCACTGTCCTTTCCTA
	R	CCAATCCTCCCCCTTGCTGTCCTG
8	F	CGTCTGCCGCGCTGTTCTCCTCTT
	R	GTGGCGAGGCGCACCGTGGGCTTG
9	F	CAGGTGCTGGGGGCTTCCGAGACA
	R	TATCTGGGCGCTTGTCATTACCAC
10	F	CGTCAATTTTACGCATGATTATCTTTAAC
	R	ACACAGGAAACAGCTATGACCATGATTACG
11 [2]	F	GCCTTATCCGGTAACTATCGT
	R	GCACCGCCTACATACCTC
12 [2]	F	CCGATCGTTGTCAGAAGTAAGTTG
	R	TCACAGAAAAGCATCTTACGGA
13	F	GACGACGTGCAGTCCGACACCGAG
	R	CTGCCGGGCTGCTCGATCACGTTC
14	F	ACAGCCACAACGTGAGCAGCAAGG
	R	GGGGGCCTCCAGCCTCTTCCTCAT
15	F	TTCTGTGGAATGTGTGTCAGTTAG
	R	GGGGACTTTCCACACCTGGTTGCT

Supplementary Table 2. (*continued*)

16	F	CGGGGAGAGGCGGTTTGCGTATTG
	R	GTATTACCGCCTTTGAGTGAGCTG
Endogenous control	F	GGGGCCTTCTGGGGGTAAAGTTCAGAACAC
	R	TGGCTGCCTGAGGGCAAGAGGGAAAGAATC

Transposon excision analysis

iPS25-site 1	L	TCTGGCCTATTTTAGAGCATAATAC
	R	AAGAAAGCATGGCCTTCATGGAGCTG
iPS25-site 2	L	AGGAAGCCGCAGTTACACGCCACAG
	R	CCAAGGAAGGAGACCACCTCATCAC
iPS28-site 1	L	ATAGAATAGAGGGACACTGAATATC
	R	CAGGCCAGTGGATCACTGTAACCTAC
iPS28-site 2	L	TGCCAGCCTCCAGCGAGTGTTGTTG
	R	TGCCATCAATCGTGGGATCTCCAAG
iPS216-site 1	L	ATGCTGGCTGTGCTTGTTCTATGTC
	R	TGAGTAACCCCAGGTAATTCTGATTC
iPS216-site 2	L	AAGAATTCATTTGCTTGGGACTCTG
	R	ACTGCACAGAAGTTCCCCTACTGGT

RT-PCR analysis

Oct4	F	CCTGGAATCGGACCAGGCTCAGAGGTATTG
	R	ATTGTTGTCGGCTTCCTCCACCCACTTCTC
Sox2	F	AGCTACGCGCACATGAACGGCTGGAGCAAC
	R	TGGAGCTGGCCTCGGACTTGACCACAGAGC
Nanog	F	CCACAGTTTGCCTAGTTCTGAGGAAGCATC
	R	TACTCCACTGGTGCTGAGCCCTTCTGAATC
Ecat1	F	CGTGGAACCTCGGCTACTGGAAATC
	R	GCCGCCATACGACGACGCTCAACTC
Esrrb	F	AACCTGCCGATTTCCCCACCTGCTA
	R	GGCTCATCTGGTCCCCAAGTGTCAG
Dppa3	F	GAGGACGCTTTGGATGATACAGACG
	R	CAACAAAGTGCGGACCCTTCTCTTG
Dnmt3l	F	CCCTCTTCCTGTATGATGATGATGG
	R	CCTCTGCAGCAGTCCACTCCGTGAG
cMyc	F	GCCGCGTCCGAGTGCATTGACCCCTCAGTG
	R	TTGGCCCTCTTGGCAGCTGGATAGTCCTTC
Actb	F	CAGGGTGTGATGGTGGGAATGGGTCAGAAG
	R	TACGTACATGGCTGGGGTGTTGAAGGTCTC
Gapdh	F	CCTTCCGTGTTCCTACCCCCAATG
	R	GGAGACAACCTGGTCCTCAGTGTA

Bisulphite sequencing

Oct4 [3]	F	GGTTTTTTAGAGGATGGTTGAGTG
	R	TCCAACCCTACTAACCCATCACC
Nanog [3]	F	GATTTTGTAGGTGGGATTAATTGTGAATTT
	R	ACCAAAAAAAACCCACACTCATATCAATATA

Detection for Nanog-puro-IRES-GFP targeting

Nanog-puro	F	TAAACAGTGGGTCTGAAGCCTTCGAGGGAG
	R	GCGAGGCGCACCGTGGGCTTGTACTCGGTC
Nanog 3' probe	F	CCCCAGCTGCAGCTTTCAGAGGCTTT
	R	TGTTTTACTCATGTGCATGGGCTGG

Chimera genotyping

GFP [4]	F	CTTGATGCCGTTCTTCTGCTTGTCG
	R	CACCCTCGTGACCACCCTGACCTAC

Supplementary Table 2. (*continued*)

References
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2, Okita *et al*. Scinece 322, 949-953 (2008)
3, Takahashi, K. & Yamanaka, S. Cell 126, 663-676 (2006)
4, Horie *et al*. PNAS 98, 9191-9196 (2001)