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Supplemental Information

A CRISPR/Cas-Mediated Selection-free Knockin Strategy in Human Embryonic Stem Cells

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Figure S1

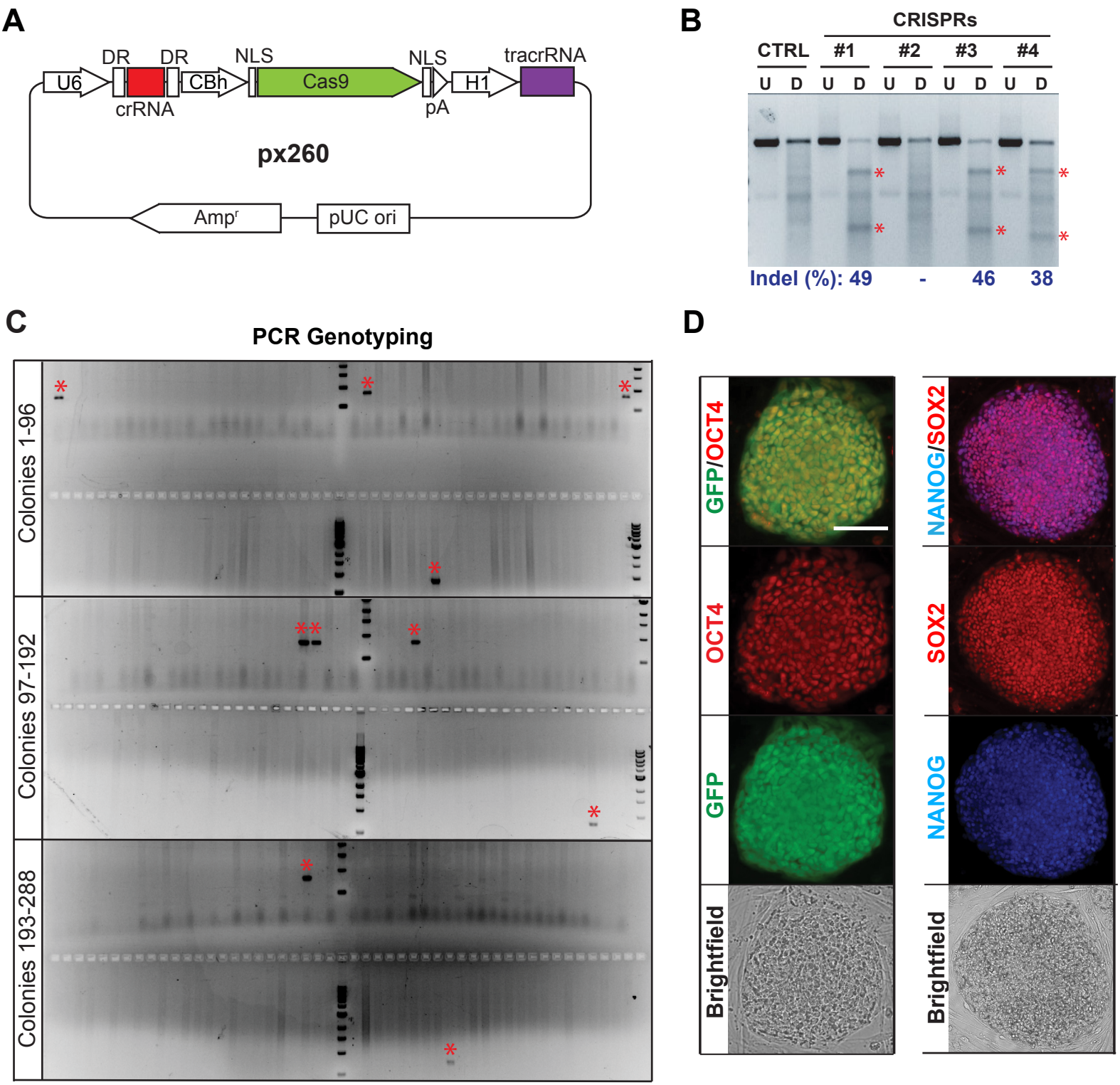

A
DR
DR
NLS
NLS
tracrRNA
U6
CBh
Cas9
H1
crRNA
pA
px260
Amp^r
pUC ori
B
CRISPRs
CTRL
#1
#2
#3
#4
U D U D U D U D U D
Indel (%): 49 - 46 38
C
PCR Genotyping
Colonies 1-96
Colonies 97-192
Colonies 193-288
D
GFP/OCT4
OCT4
GFP
Brightfield
NANOG/SOX2
SOX2
NANOG
Brightfield

Figure S2

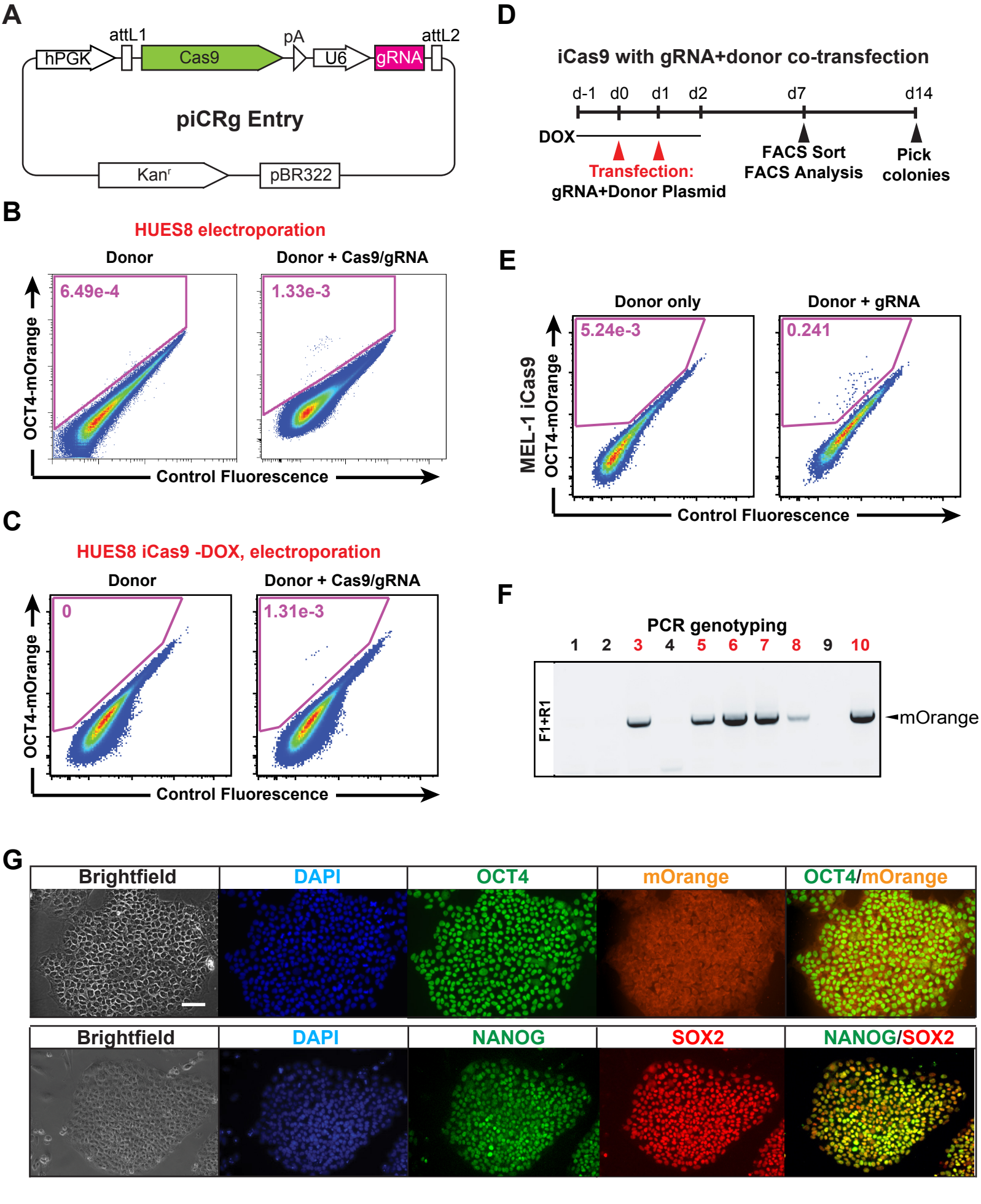
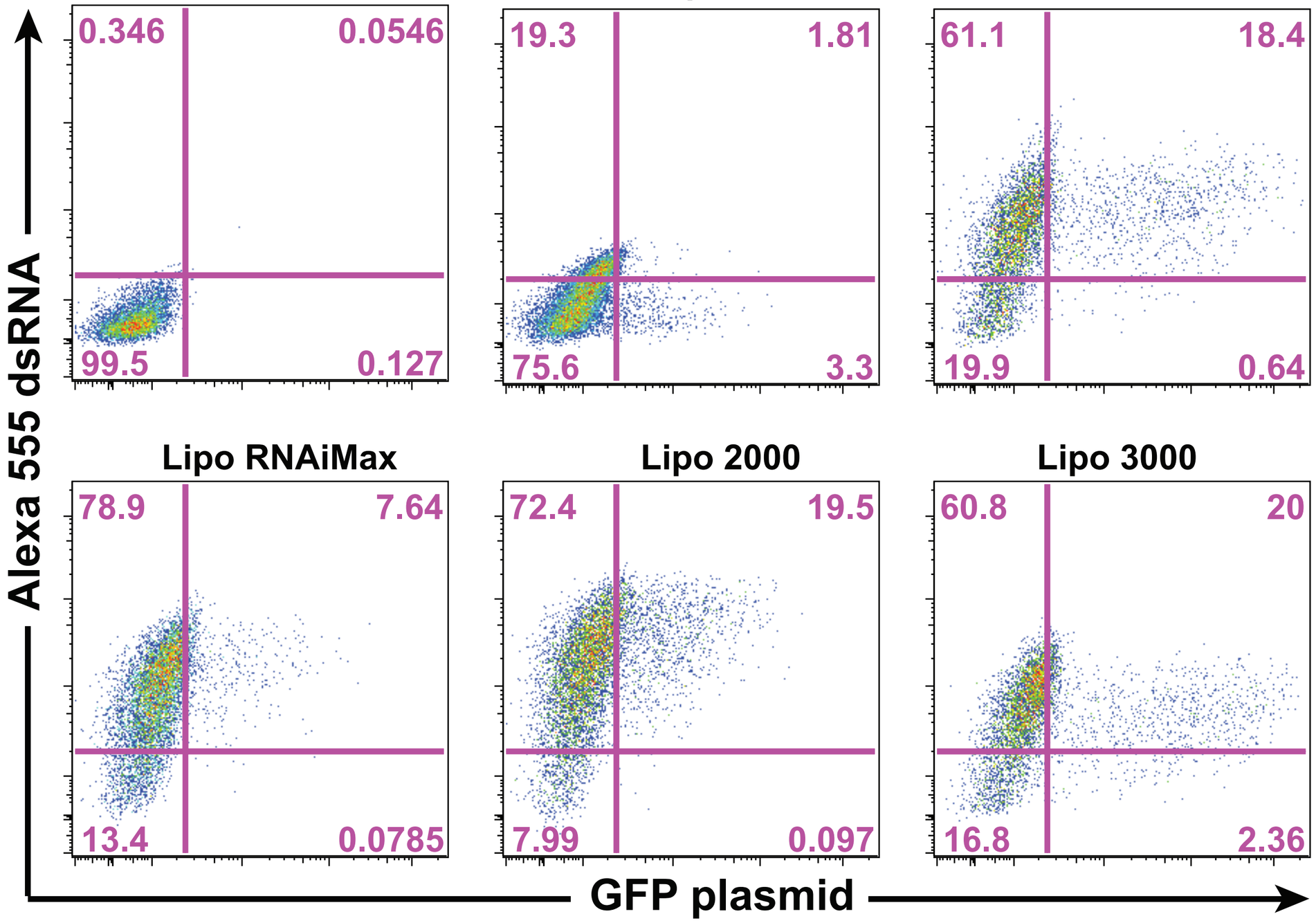

A
attL1
pA
attL2
hPGK
Cas9
U6
gRNA
piCRg Entry
Kanr
pBR322
D
iCas9 with gRNA+donor co-transfection
d-1 d0 d1 d2 d7 d14
DOX
Transfection:
gRNA+Donor Plasmid
FACS Sort
FACS Analysis
Pick
colonies
B
HUES8 electroporation
Donor
Donor + Cas9/gRNA
6.49e-4
1.33e-3
OCT4-mOrange
Control Fluorescence
E
Donor only
Donor + gRNA
5.24e-3
0.241
MEL-1 iCas9
OCT4-mOrange
Control Fluorescence
C
HUES8 iCas9 -DOX, electroporation
Donor
Donor + Cas9/gRNA
0
1.31e-3
OCT4-mOrange
Control Fluorescence
F
PCR genotyping
1 2 3 4 5 6 7 8 9 10
F1+R1
mOrange
G
Brightfield
DAPI
OCT4
mOrange
OCT4/mOrange
Brightfield
DAPI
NANOG
SOX2
NANOG/SOX2

Figure S3

A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0.0546
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19.3
1.81
75.6
3.3
61.1
18.4
19.9
0.64
Lipo RNAiMax
Lipo 2000
Lipo 3000
78.9
7.64
13.4
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7.99
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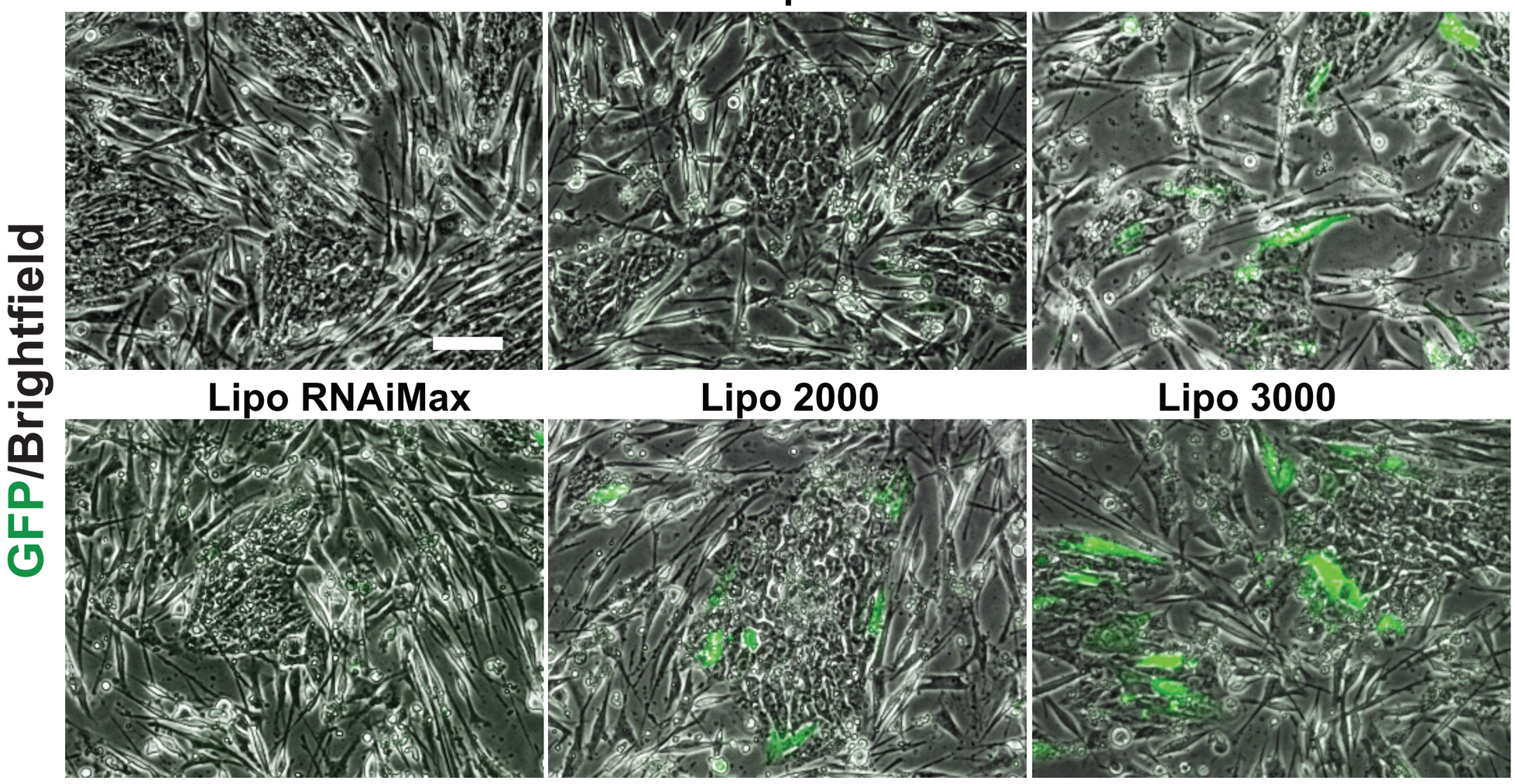
60.8
20
16.8
2.36
Alexa 555 dsRNA
GFP plasmid

B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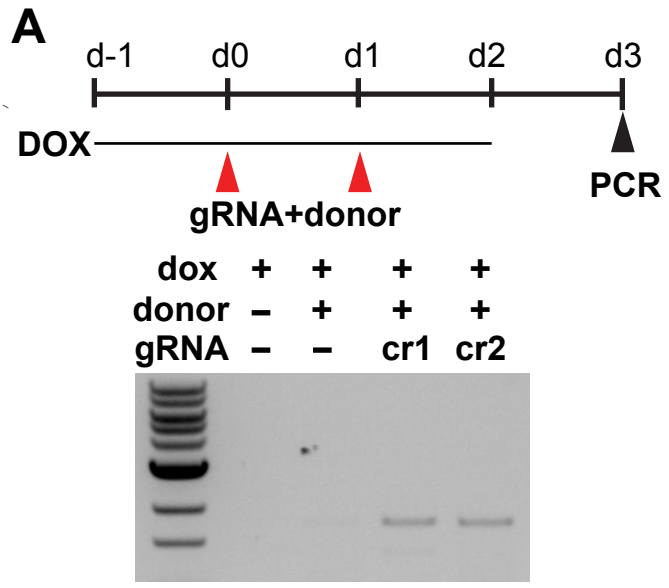
Electroporation
JetPrime
Lipo RNAiMax
Lipo 2000
Lipo 3000
GFP/Brightfield

Figure S4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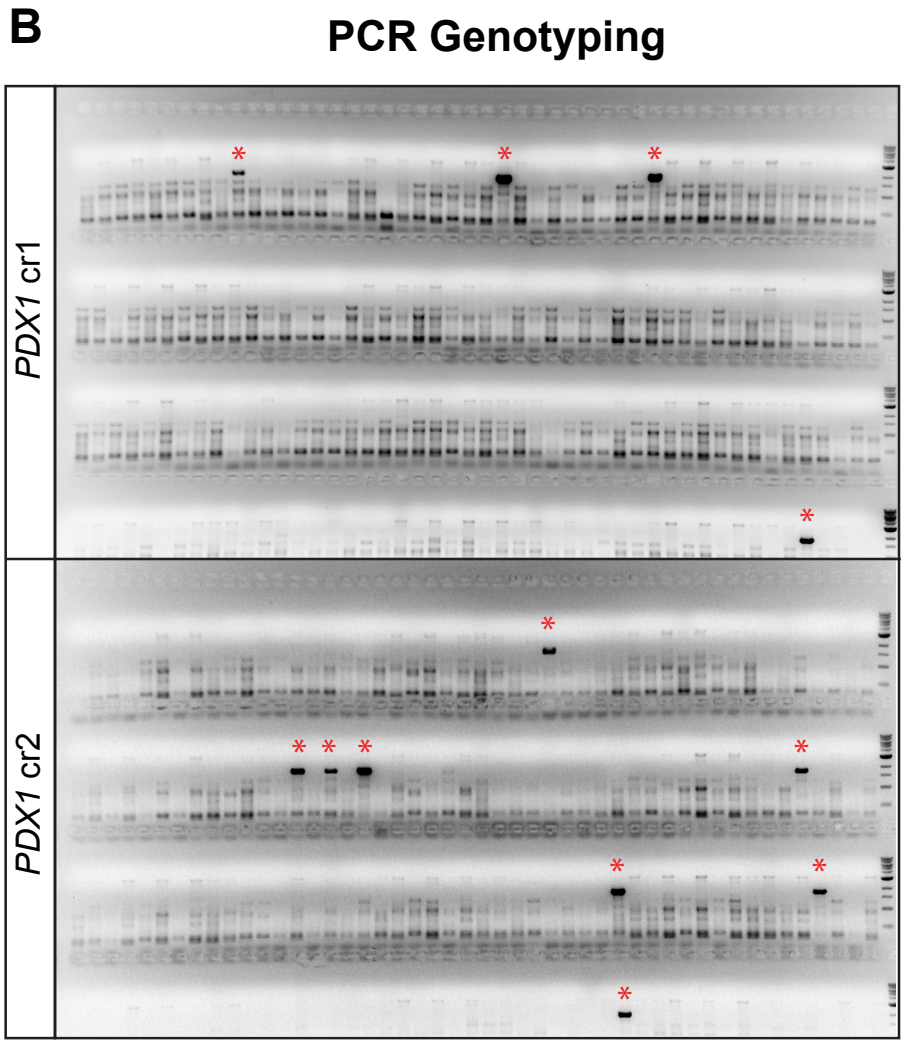
d0
d1
d2
d3
DOX
PCR
gRNA+donor
dox + + + +
donor – + + +
gRNA – – cr1 cr2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PCR Genotyping
PDX1 cr1
PDX1 cr2

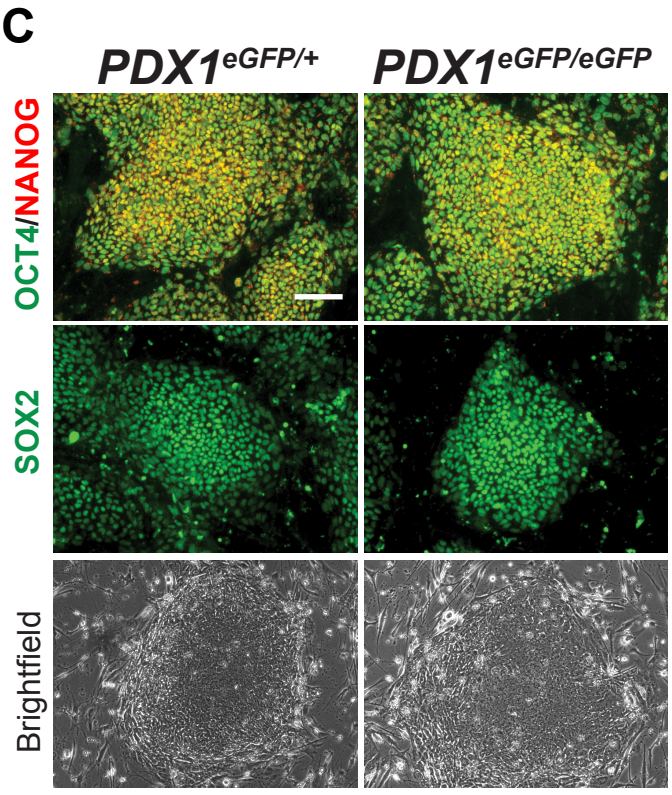
C
PDX1eGFP/+
PDX1eGFP/eGFP
OCT4/NANOG
SOX2
Brightfield

Figure S1. CRISPR-mediated targeting of the *OCT4* locus through drug selection.

(A) The vector map of the px260 plasmid for expressing Cas9 and crRNA/tracrRNA. (B) Four crRNAs were designed to target the stop codon of the *OCT4* locus. Each crRNA was cloned into the px260 vector and transfected into 293T cells. Two days after transfection genomic DNA was collected and Surveyor analysis was used to estimate the efficiency of each CRISPR in generating Indels. Asterisks indicated the cleavage products and the estimated Indel frequencies were labeled in blue. CTRL: px260 vector control; U: undigested control; D: digestion reaction with Surveyor nuclease. **(C)** PCR genotyping results showing 10 positive clones (indicated by red asterisks) identified based on the presence of a correct PCR product (811 bp). **(D)** *OCT4-eGFP* reporter hESCs were stained for pluripotency markers OCT4, NANOG and SOX2, which overlapped with GFP expression. The GFP signal was detected using a GFP antibody. Scale bar = 100 μm.

Figure S2. Targeting the *OCT4* locus without drug selection.

(A) The vector map of the piCRg Entry plasmid for expressing Cas9 and the chimeric gRNA. **(B)** FACS analysis for OCT4-mOrange expressing cells in HUES8 cells after electroporation of the OCT4-mOrange donor and the Cas9/gRNA plasmids. **(C)** FACS analysis for OCT4-mOrange expressing cells in HUES8 iCas9 cells without doxycycline treatment after electroporation of the OCT4-mOrange donor and the Cas9/gRNA plasmids. **(D)** Timeline for establishing hESC reporter lines using iCas9 hESCs. **(E)** FACS analysis for OCT4-mOrange expressing cells in MEL-1 iCas9 cells treated with doxycycline and transfected with the OCT4-mOrange plasmid and *OCT4* cr1 gRNA. **(F)** PCR genotyping for OCT4-mOrange gene targeting. Correctly targeted clones are

indicated in red. **(G)** *OCT4-mOrange* reporter hESCs were stained for pluripotency markers OCT4, NANOG and SOX2, which overlapped with mOrange expression. Scale bar = 100 μm.

Figure S3. Optimizing co-transfection of DNA and RNA into hESCs.

(A) FACS analysis of hESCs co-transfected with Alexa 555 dsRNA and a GFP-expressing plasmid (~ 10 kb) using four commonly used transfection reagents along with electroporation. Lipofectamine 3000 consistently performed better than the other conditions. Although comparable efficiencies could sometimes be achieved using JetPrime (as shown here), the outcomes were variable and appeared to relate to the amount of DNA used for transfection. **(B)** Stronger GFP expression was detected in hESCs transfected with Lipofectamine 3000, suggesting that increased copy number of GFP-expressing plasmid was transfected into the cell.

Figure S4. Generation of *PDX1-eGFP* reporter hESCs without drug selection.

(A) Population level PCR analysis of HUES8 iCas9 cells two days after transfection with gRNA and donor plasmid. **(B)** PCR genotyping of replated clonal hESCs after transfection. Correctly targeted cells are labeled with a red asterisk. **(C)** *PDX1-eGFP* reporter hESC lines displayed proper expression of pluripotency markers OCT4, NANOG and SOX2 and typical hESC morphology. The brightfield images were taken from live cells, and do not correspond to the immunofluorescence images of fixed cells.

Table S1. Off-target analysis

Sequencing analysis of potential off-target sites in *OCT4-eGFP* and *OCT4-mOrange* reporter lines

Gene	CRIPSR Target Sequence-PAM	Sequencing results in *OCT4-eGFP* reporter lines								Sequencing results in *OCT4-mOrange* reporter lines					
		1	2	4	5	6	7	9	10	3	5	6	7	8	10
***OCT4* (intended target)**	TCTCCCATGCATTCAAACTG-AGG														
DLG2	AAGCTCAGGCATTCAAACTG-TGG	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
GPHN	GCCCTCAGGCATTCAAACTG-TGG	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
IMMP2L	TAGACTTAGCATTCAAACTG-AGG	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
PEMT	GCACCCTAGCATTCAAACTG-TGG	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
POLR2J4	AAGGAGAAGCATTCAAACTG-TGG	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
POU5F1P4	TCTCCCATGCATTCAAACTG-AGG	WT	HET*	WT	HOM**	WT	HOM***	WT	WT	WT	WT	WT	WT	WT	WT
SLC33A1	CAGAAATGGCATTCAAACTG-CGG	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT

WT: Both alleles are wild-type; HET: One allele has a mutation; HOM: Both alleles have mutations;
* 6 bp insertion; ** 4 bp deletion; *** 10 bp deletion.

Sequencing analysis of potential off-target sites in *PDX1-GFP* reporter line # 9 generated using *PDX1* cr1

Gene	CRISPR Target Sequence-PAM	Sequencing results in *PDX1-GFP* reporter line #9
***PDX1* (intended target)**	GCCGCAGGAACCACGATGAG-AGG	
SUSD4	TCCTCAGGACCCACGATGGG-CAG	WT
COX7C	TCCGTAGGAGCCACTATGAG-GAG	WT
DCPS	GCCACAGGCACCACGGTGAG-GAG	WT
TRAF3IP2	GCCCCTGGGACCACGAAGAG-AGG	WT
NARF	GCCGCAGGACCCACGAGAAG-CGG	WT
ILVBL	GCAGCAGAAACCAGGAGGAG-AGG	WT
BST2	GCGGCAGGAGCCAGGACGAG-CAG	WT
SNED1	GCGGCGGGAACCCGGATGAG-GAG	WT
PSG3	CCTGCAGGAACCAGGATAAG-AGG	WT
CRAT	GGGGCAGGAACCAAGCTGAG-TGG	WT

Sequencing analysis of potential off-target sites in *PDX1-GFP* reporter line #4 generated using *PDX1* cr2

Gene	CRISPR Target Sequence-PAM	Sequencing results in *PDX1-GFP* reporter line #4
***PDX1* (intended target)**	GCCTCTCATCGTGGTTCCTG-CGG	
PSG3	TCCTCTTATCCTGGTTCCTG-CAG	WT
NUBP1	GCATCTCCCAGTGGTTCCTG-AGG	WT
SYNM	GTGTCTGACCGTGGTTCCTG-GAG	WT
HEXDC	GCCACTGCACGTGGTTCCTG-AGG	WT
PSG10P	TTCTCTTATCCTGGTTCCTG-CAG	WT
KCNQ1	TGCTCTCACCTTGGTTCCTG-GGG	WT
FBRSL1	GCCAGGCATCGGGGTTCCTG-CAG	WT
FOXJ3	ACCTGCCATCGTTGTTCCTG-GAG	WT
OR6M1	ATCTCTCTTCGTGGTTCTTG-TGG	WT
MAGEA8	ACCTCACAGCCTGGTTCCTG-GAG	WT

Table S2. Oligonucleotides used in this study

Oligonucleotides for generating Cas9/crRNA/tracrRNA or Cas9/gRNA expressing plasmids

Gene		CRISPR target sequence (5' of PAM)	Vector	Oligos used for generating the CRISPR constructs (5' to 3')
OCT4	cr1-dp	CACTCTGGGCTCTCCCATGCATTCAAACTG	px260	**F:** AAACCACTCTGGGCTCTCCCATGCATTCAAACTGGT
				R: TAAAACCAGTTTGAATGCATGGGAGAGCCCAGAGTG
	cr2-dp	TGCATTCAAACTGAGGTGCCTGCCCTTCTA	px260	**F:** AAACTGCATTCAAACTGAGGTGCCTGCCCTTCTAGT
				R: TAAAACTAGAAGGGCAGGCACCTCAGTTTGAATGCA
	cr3-dp	ATGCATTCAAACTGAGGTGCCTGCCCTTCT	px260	**F:** AAACATGCATTCAAACTGAGGTGCCTGCCCTTCTGT
				R: TAAAACAGAAGGGCAGGCACCTCAGTTTGAATGCAT
	cr4-dp	CTCTGGGCTCTCCCATGCATTCAAACTGAG	px260	**F:** AAACCTCTGGGCTCTCCCATGCATTCAAACTGAGGT
				R: TAAAACCTCAGTTTGAATGCATGGGAGAGCCCAGAG
	cr1	TCTCCCATGCATTCAAACTG	piCRg Entry	**F:** CACCGCTCCCATGCATTCAAACTG
				R: AAACCAGTTTGAATGCATGGGAGC
PDX1	cr1	GCCGCAGGAACCACGATGAG	piCRg Entry	**F:** CACCGCCGCAGGAACCACGATGAG
				R: AAACCTCATCGTGGTTCCTGCGGC
	cr2	GCCTCTCATCGTGGTTCCTG	piCRg Entry	**F:** CACCGCCTCTCATCGTGGTTCCTG
				R: AAACCAGGAACCACGATGAGAGGC

PCR Primers for generating templates for gRNA *in vitro* transcription

Gene	CRISPR target sequence (5' of PAM)	CRISPR specific forward primer (5' to 3')
OCT4	cr1 TCTCCCATGCATTCAAACTG	**F:** TAATACGACTCACTATAGGGCTCCCATGCATTCAAACTG
PDX1	cr1 GCCGCAGGAACCACGATGAG	**F:** TAATACGACTCACTATAGGGCCGCAGGAACCACGATGAG
	cr2 GCCTCTCATCGTGGTTCCTG	**F:** TAATACGACTCACTATAGGGCCTCTCATCGTGGTTCCTG

Universal reverse primer **gRNA-R:** AAAAGCACCGACTCGGTGCC

PCR Primers for donor plasmid construction

Donor plasmid		Primer sequence (5' to 3')
OCT4	Nh2AOr-F	TTCTAGCTAGCACCGGTGCCACGAACTTCTCTCTGTTAAAGCAAGCAGGAGACGTGGAAGAAAACCCCGG TCCTATGGTGAGCAAGGGCGAGGAG
	AscOr-R	CTTATGGCGCGCCTTACTTGTACAGCTCGTCCAT
PDX1-eGFP	HL-F	GTCAGTGAATTCAGGACCACTCATTGGCAGAG
	HL-R	GTCAGTCTGCAGGCTAGCTCGTGGTTCCTGCGGCCGCC
	HR-F	GTCAGTCTGCAGGGCGCGCCGAGGCAGGAGCTGCTCCTGG
	HR-R	GTCAGTGGATCCACTCACTGTATTCCACTGGC
	2A-GFP-F	CAGTGCTAGCGCCACTAACTTCTCCCTGTT
	2A-GFP-R	TCAGTCGGCGCGCCTTACTTGTACAGCTCGTCCA

PCR primers for genotyping

Gene	Primer sequence (5' to 3')
OCT4	**F:** AGTCCAAAGCTTGCCCTTGTCACC
	GFP-R: AGCTCCTCGCCCTTGCTCACC
	mOr-R: GAGGTGATGTCCAACTTGATGCCGA
PDX1	**F:** ATTTGCTGGCTCTCAGGTTG
	R: GTTCACCTTGATGCCGTTCT

PCR primers for generating Southern blot probes

Gene	Probe	Primer sequence (5' to 3')
OCT4	External	**F:** CCAGTGGGAGTCAGTGGGGCT
		R: GTCCGACTCCCAAGAGGTCACAG
	Internal Puro	**F:** TGACCGAGTACAAGCCCACGG
		R: TCGTAGAAGGGGAGGTTGC
	Internal mOrange	**F:** TGGCCATCATCAAGGAGTTCA
		R: CTTCTTCTGCATTACGGGGCCG
PDX1	External	**F:** AACACCTCTGAGGGCATTTG
		R: CGGACACTGCAGGTCAGTTA
	Internal GFP	**F:** GTTCATCTGCACCACCGG
		R: CGCGCTTCTCGTTGGGGT

Supplemental Experimental Procedures

Generation of constructs

To generate CRISPR plasmids expressing Cas9 and the crRNA/tracrRNA duplex targeting specific genomic loci, 30-bp protospacer sequences were cloned into the px260 (Addgene: 42229) as previously described (Cong et al., 2013). Briefly, vectors were digested with BbsI, treated with Antarctic Phosphatase, and gel purified. A pair of oligonucleotides containing the 30-bp protospacer sequence was annealed generating BbsI overhangs, and cloned into BbsI-digested, dephosphorylated vectors. The same procedure was also used to generate CRISPR plasmids expressing Cas9 and the chimeric gRNA with the difference that a pair of oligonucleotides containing the 20-bp protospacer sequence was cloned into the piCRg Entry plasmid (Addgene: 58904). The sequences for all oligonucleotides used for generating the CRISPR constructs are listed in Table S2.

For generation of *OCT4-eGFP* hESC reporter lines, the OCT4-2A-eGFP-PGK-Puro plasmid (Addgene: 31938) was used. The left homology arm is 697 bp and the right homology arm is 699 bp. The *OCT4-mOrange* hESC reporter lines were made using the OCT4-2A-mOrange targeting vector. To generate the OCT4-2A-mOrange targeting vector, an NheI-2AmOrange-AscI cassette was PCR amplified using the mOrange-pBAD plasmid template (Addgene: 54751) and primers Nh2AOr-F and AscOr-R. Next, the NheI-2A-mOrange-AscI PCR fragment and the OCT4-2A-eGFP-PGK-Puro plasmid were digested with NheI and AscI and ligated.

For constructing the PDX1-GFP donor plasmid, the 725-bp left (Primers: HL-F and HL-R) and 543-bp right (Primers: HR-F and HR-R) homology arms were PCR

amplified from the HUES8 genomic DNA and cloned into the pBlueScript SKII (+) plasmid to generate the pBS-PDX1 plasmid. The 2A-eGFP insert was PCR amplified from the OCT4-2A-eGFP-PGK-Puro plasmid (Addgene: 31938) using 2A-eGFP-F and 2A-eGFP-R primers, digested and cloned into the pBS-PDX1 plasmid to generate the PDX1-eGFP donor plasmid. In the donor plasmid, the 2A-eGFP sequences was fused in-frame to the last codon of *PDX1*. The sequences for all primers used for generating the donor plasmids are listed in Table S2.

Cell Culture

HUES8 (NIHhESC-09-0021) and MEL-1 (NIHhESC-11-0139) hESCs were cultured on irradiated mouse embryonic fibroblasts (iMEFs) feeder layers in DMEM/F12 medium (Life Technologies) supplemented with 20% KnockOut Serum Replacement (Life Technologies), 1X MEM Non-Essential Amino Acids (Life Technologies), 1X GlutaMAX (Life Technologies), 100U/ml Penicillin and 100 µg/ml Streptomycin (Gemini), 0.055 mM 2-mercaptoethanol (Life Technologies) and 10 ng/ml recombinant human basic FGF (EMD Millipore). Cells were incubated at 37 °C with 5% (vol/vol) CO_2, and media was changed daily. Cultures were passaged at a 1:6 - 1:12 split ratio every 4 - 6 days using 0.05% trypsin/EDTA. 5 µM Rho-associated protein kinase (ROCK) inhibitor Y-27632 (Selleck Chemicals) was added into the culture medium when passaging or thawing cells.

293T cells were maintained in DMEM supplemented with 15% fetal bovine serum, 1X GlutaMAX, 1X MEM NEAA and 1mM Sodium Pyruvate (Life Technologies).

Differentiation of hESCs

For differentiation of *OCT4* reporter lines, undifferentiated hESCs cultured on iMEF feeder layer were first adapted to the feeder-free E8 culture. Briefly, hESCs were passaged using 0.05% trypsin/EDTA and plated at a 1:3 split ratio on Matrigel (BD Biosciences) coated plates in E8 medium (Stem Cell Technologies). Cells were incubated at 37 °C with 5% (vol/vol) CO2, and media was changed daily. After 2 passages in E8 medium, the cells were differentiated: 2 days after passaging, cells were treated with 10 μM SB431542 (Sigma Aldrich) and 10 ng/mL BMP4 (R&D Systems) to initiate differentiation. After 3 days of this treatment, eGFP and mOrange fluorescence was analyzed by flow cytometry and fluorescence microscopy.

For differentiation into pancreatic lineages, undifferentiated hESC were cultured until 50-70% confluency, washed with PBS w/$Ca^{2+}Mg^{2+}$ once and treated with 2 μM BIO-acetoxime (Tocris), 100 ng/ml Activin A (R&D) in advanced RPMI (Invitrogen) for 1 day. The medium was changed to 100 ng/ml Activin A in advanced RPMI with 0.2% FBS for 2 days. On day 3, cells were treated with 50 ng/ml FGF10 (R&D) and 0.25 μM SANT1 (Sigma) in advanced RPMI with 2% FBS for 2 days. On day 5, cells were treated with 50 ng/ml FGF10, 0.25 μM SANT1, 2 μM Retinoic Acid (Sigma) and 250 nM LDN (Stemgent) in DMEM with 1% B27 (Invitrogen) for 4 days. Then on day 9, cells were treated with 1 μM Alk5 inhibitor (Axxora), 100 ng/mL Noggin (R&D system) and 1 μM DAPT (Tocris) in DMEM with 1% B27 for 4 days. After that, cells were kept in DMEM with 1% B27 for another 4 days and were examined for the expression of pancreatic cell specific markers.

In vitro transcription of gRNAs

A T7 promoter was added to gRNA templates by PCR amplification on piCRg Entry vectors using CRISPR-specific forward primers and a universal reverse primer gRNA-R (Table S2). T7-gRNA PCR products were used as templates for *in vitro* transcription using the MEGAshortscript T7 kit (Life Technologies). The resulting gRNAs were purified using the MEGAclear kit (Life Technologies), eluted in RNase-free water and stored at -80°C until use.

Electroporation

HUES8 hESCs were pre-treated with Rho-associated protein kinase (ROCK) inhibitor Y-27632 (Selleck Chemicals) 24 hours before electroporation. On the day of electroporation, hESCs were disassociated into single cells with 0.25% Trypsin/EDTA and filtered through a 40 μM cell strainer to remove cell clumps. 10 million cells were re-suspended in 800 μL cold PBS and mixed with targeting and donor plasmids (10 μg CRISPR targeting plasmid and 40 μg donor plasmid). Cells were electroporated using the Gene Pulser XCeII (Bio-Rad) at 250 V, 500 μF in a 0.4 cm Gene Pulser cuvette (Bio-Rad). Cells were recovered and re-plated on irradiated mouse embryonic fibroblast (iMEF) coated plates with ROCK inhibitor.

For the generation of *OCT4-eGFP* lines, two days after electroporation cells were treated with puromycin (0.5 μg/mL) for three days. Two weeks after electroporation individual colonies were picked and expanded for PCR genotyping, Southern blot analysis and sequencing for the establishment of reporter lines. Three of the correctly targeted clones (#1, #4 and #7) were electroporated with Cre recombinase following the same procedure described above. After electroporation with 50 μg of Cre recombinase

plasmid, the cells were recovered and replated on iMEF with ROCK inhibitor. 3 days after electroporation, GFP-expressing (GFP^+) cells were observed. GFP^+ cells were isolated by FACS and plated at a low density (2,000 cell/10cm dish) for subsequent picking and expansion of individual clones.

Transfection

HUES8 and MEL-1 iCas9 hESCs (Gonzalez et al., 2014) were treated with ROCK inhibitor and doxycycline one day before transfection. For transfection, cells were dissociated using TrypLE (Life Technologies), replated at 200,000 hESCs per well in iMEF-coated 12-well plates and transfected in suspension with gRNAs and donor plasmid. A second transfection was performed 24 hours later. Transfection of the gRNAs and donor plasmid into hESCs was performed using Lipofectamine 3000 (Life Technologies) following manufacturer's guidelines. For each targeting, gRNAs at a 10 nM final concentration and 5 μg of donor plasmid were used. Lipofectamine 3000 and gRNA + donor plasmid were diluted separately in Opti-MEM (Life Technologies), mixed together, incubated for 5 min, and added drop-wise to cultured hESCs.

Assessment of Indel mutations using the Surveyor Nuclease Assay

80-90% confluent 293T cells in 6-well dishes were transfected using JetPrime transfection reagent (Polyplus) following manufacturer's guidelines. For each well, a total of 2 μg CRISPR plasmid was transfected. After transfection the cells were incubated at 37 °C for 2 days prior to genomic DNA extraction. The Surveyor Kit (Transgenomic) was used to test the efficiency of each crRNA for producing Indels. Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's protocol. The genomic region flanking the CRISPR target site for each gene was PCR

amplified. For Surveyor analysis 16 µL of PCR product (at 25 ng/µL) was denatured and re-annealed in JumpStart buffer to a total volume of 20 µL using the following protocol: 95 °C, 5 min; 95–85 °C at −2 °C/s; 85–25 °C at −0.1 °C/s; hold at 4 °C. 10 µL of hybridized PCR products was treated with 1 µL of Surveyor Enhancer S and 1 µL of Surveyor Nuclease S at 42°C for 60 minutes. The reaction was stopped by adding 1.2 µL of Stop solution to each tube. Products were then analyzed on 2.5% agarose gel and imaged with a Gel Doc gel imaging system (Bio-Rad). Quantification was based on relative band intensities using ImageJ. Indel mutation percentage was determined by the formula: $100 \times (1 - (1 - (b + c) / (a + b + c))^{1/2})$, where *a* is the integrated intensity of the undigested PCR product, and b and c are the integrated intensities of each cleavage product.

Southern Blot Analysis

The external and internal probes were generated by PCR using the PCR DIG Probe Synthesis Kit (Roche). For the external probes we used HUES8 genomic DNA as the template. For internal probe generation we used the OCT4 2A-eGFP-PGK-Puro donor, OCT4-2A-mOrange donor and PDX1-2A-eGFP donor templates. For membrane hybridization, 5 µL of denatured DIG-labeled PCR product was added to 20 mL of hybridization buffer.

To identify correctly targeted hESC lines, genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen). 5-10 µg of genomic DNAs was digested overnight with the appropriate restriction enzymes (OCT4 external and mOrange internal: BamHI, puromycin internal: EcoRI, PDX1 external: ApaLI, eGFP internal: ApaLI) and then migrated in 1% agarose gels. The gel was denatured, neutralized, and transferred

overnight by capillarity on Hybond-N membranes (GE Healthcare) using 10x SSC transfer buffer. Hybridization with the external or the internal probe was carried out overnight at 65 °C. Probes were detected using an AP-conjugated DIG-Antibody (Roche) using CDP-Star (Roche) as a substrate for chemiluminescence as per manufacturer's instructions.

Immunofluorescence staining

For immunostaining, cells were fixed with 4% paraformaldehyde for 10 minutes, washed once with phosphate buffered saline (PBS) and permeabilized in PBST (PBS + 0.1% Triton) for 15 minutes. Blocking was done for 5 minutes at RT with blocking solution (5% donkey serum in PBST). Primary and secondary antibodies were diluted in blocking solution. Primary antibodies were incubated at RT for 1 hr. The following primary antibodies were used at a 1:100 dilution: OCT4 (mouse monoclonal, Santa Cruz sc-5279); NANOG (rabbit polyclonal, CosmobioJapan REC-RCAB0004P-F); SOX2 (goat polyclonal, Santa Cruz sc-17320), RFP (rabbit polyclonal, Life Technologies R10367), GFP (rabbit polyclonal, Life Technologies A-6455). The PDX1 antibody was used at a 1:500 dilution (goat polyclonal, R&D AF2419). After primary antibody staining the cells were washed three times with PBST and then incubated with the appropriate Molecular Probes Alexa Fluor dye conjugated secondary antibodies (Life Technologies) for 1 hr.

Reference:

Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A.*, et al.* (2013). Multiplex genome engineering using CRISPR/Cas systems. Science *339*, 819-823.