Supplemental Information

CRISPR Interference Efficiently Induces Specific and Reversible Gene Silencing in Human iPSCs

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Figure S1

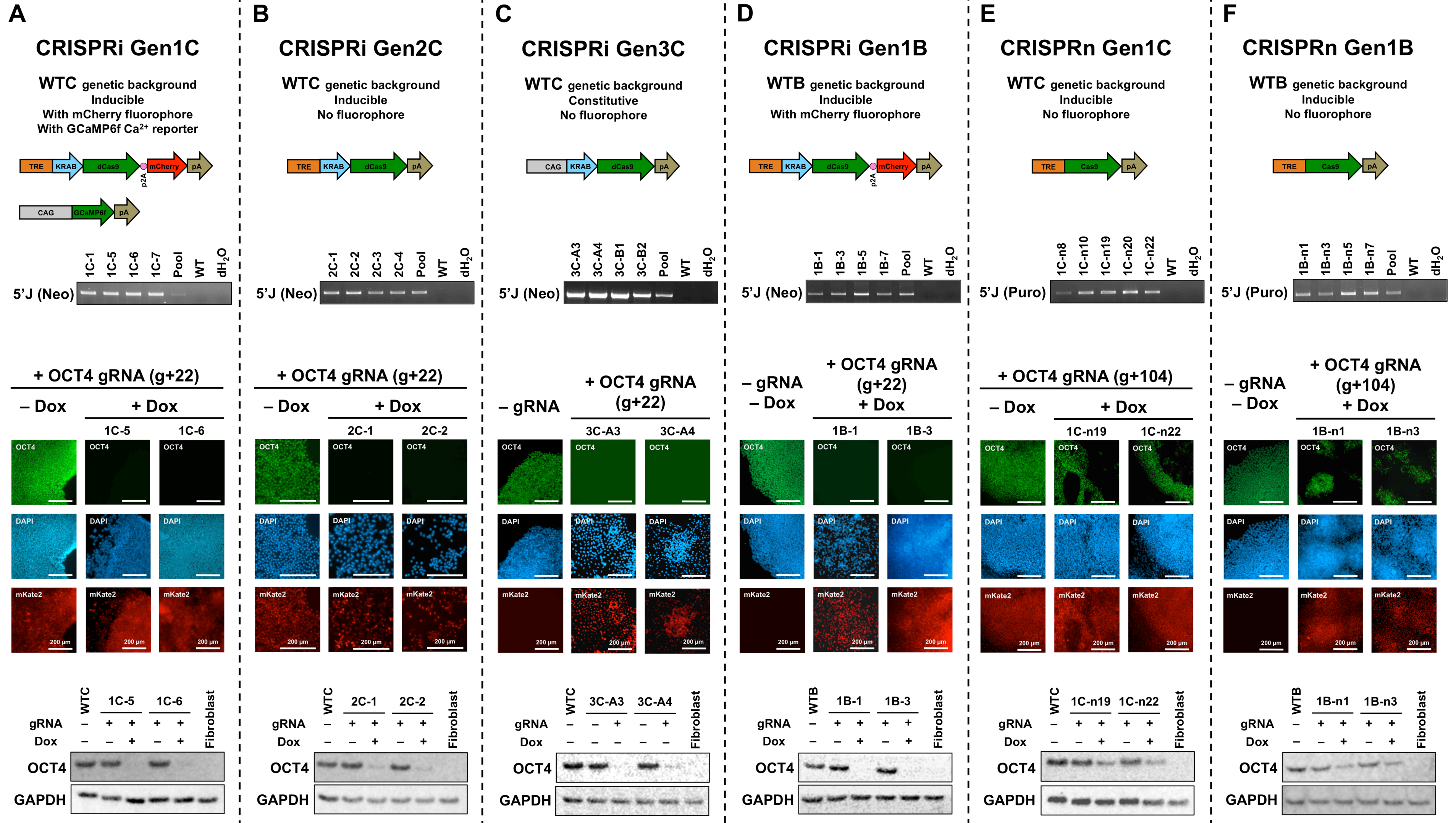


Figure S1. Derivation and validation of CRISPRi and CRISPRn iPSCs. Related to Figure 1

(A-F) Schematic diagrams show CRISPRi and CRISPRn targeting constructs used in two different iPSC genetic backgrounds (WTC and WTB). Multiple clones from each targeting event were isolated. A subset of putative clones and a pooled population from each condition were analyzed using junction PCR and confirmed on-target integration of the cassette into the AAVS1 locus. Two putative clones from each condition were initially tested in polyclonal populations containing an OCT4-specific gRNA. Samples were either cultured in the presence or absence of doxycycline (2 µM) for 7 days and analyzed using immunocytochemistry. Nuclei were counterstained with DAPI. Clones were also analyzed using western blot with an antibody against OCT4, and GAPDH was used as a loading control. Scale bars = 200 µm.

Figure S2

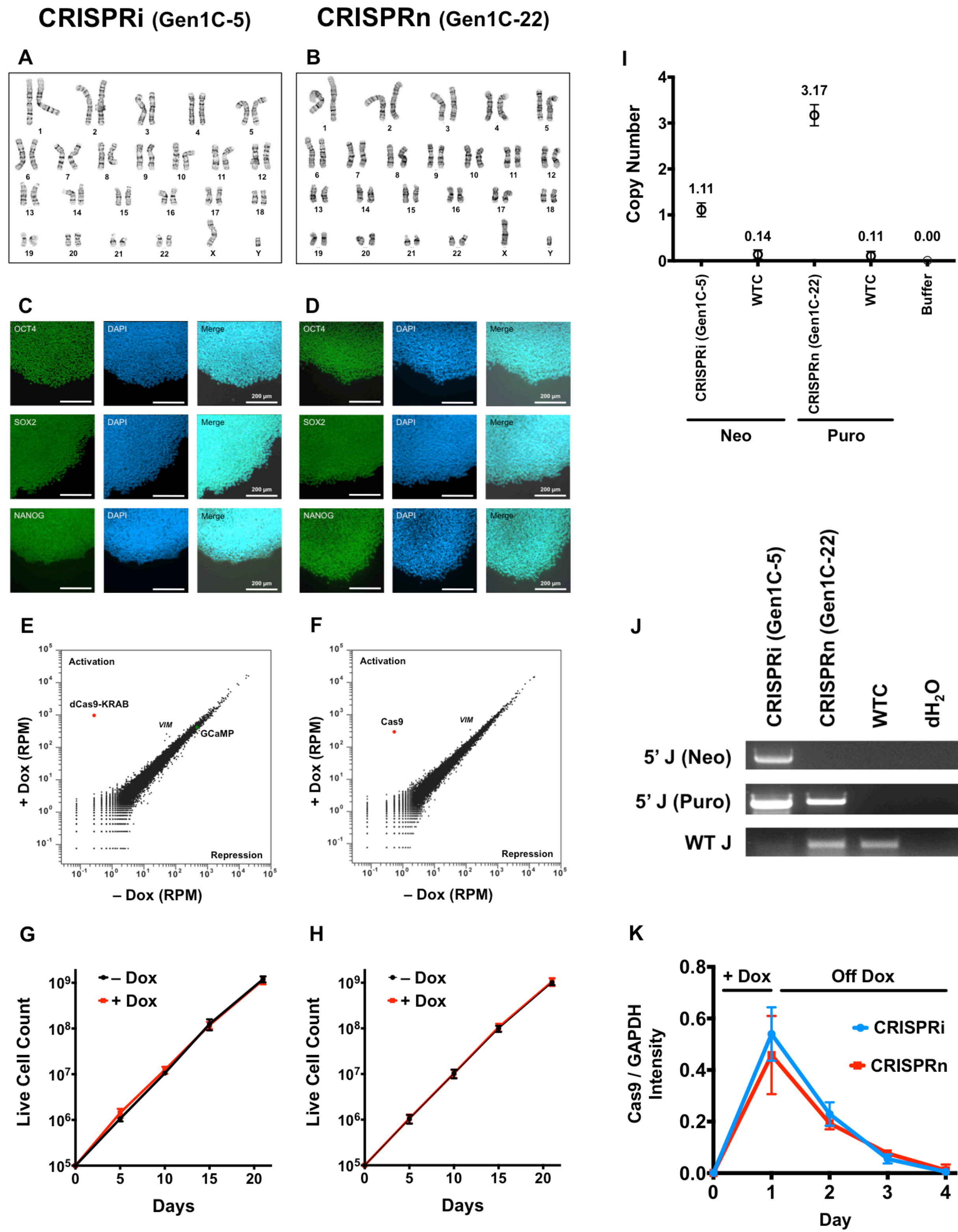


Figure S2. Characterization and doxycycline response of lead CRISPRi and CRISPRn iPSCs. Related to Figure 1.

(A, B) Karyotyping of lead CRISPRi (Gen1C 5) and CRISPRn (Gen1C 22) iPSC clones showed both lines are normal. Autosomal and sex chromosomes are annotated. (C, D) Immunostaining of CRISPRi and CRISPRn lines with pluripotency markers OCT4, SOX2, and NANOG (all in green), respectively. Nuclei were counterstained with DAPI. All cells expressed the pluripotency markers, indicating that they maintained their pluripotency after genetic modification. RNA-sequencing RPM (reads per million) are plotted for (E) CRISPRi and (F) CRISPRn iPSC, before and after 7 days of doxycycline treatment (2 μM). Expression profiles show robust induction of dCas9-KRAB and Cas9 with few off-target changes. Data is representative of two independent biological replicates. (G, H) CRISPRi and CRISPRn iPSCs were cultured with doxycycline for 3 weeks (4 passages). There were no adverse effects of dCas9-KRAB or Cas9 expression on the proliferative potential of iPSCs. (I) Droplet digital PCR (ddPCR) was used to identify the total number of transgene integration events for CRISPRi and CRISPRn clones with Neomycin- and Puromycin-specific probes, respectively. (J) Junction PCR confirmed on-target integration into the AAVS1 locus of the CRISPRi and CRISPRn clones. The CRISPRi clone also contains the GCaMP expression cassette at the other AAVS1 allele. (K) Intensity analysis of dCas9-KRAB and Cas9 was performed on two independent western blots normalizing the dCas9-KRAB and Cas9 signal intensity to GAPDH using ImageJ. Both the CRISPRi and CRISPRn clones have similar induction profiles. Error bars, SD. Scale bars = 200 μm.

Figure S3

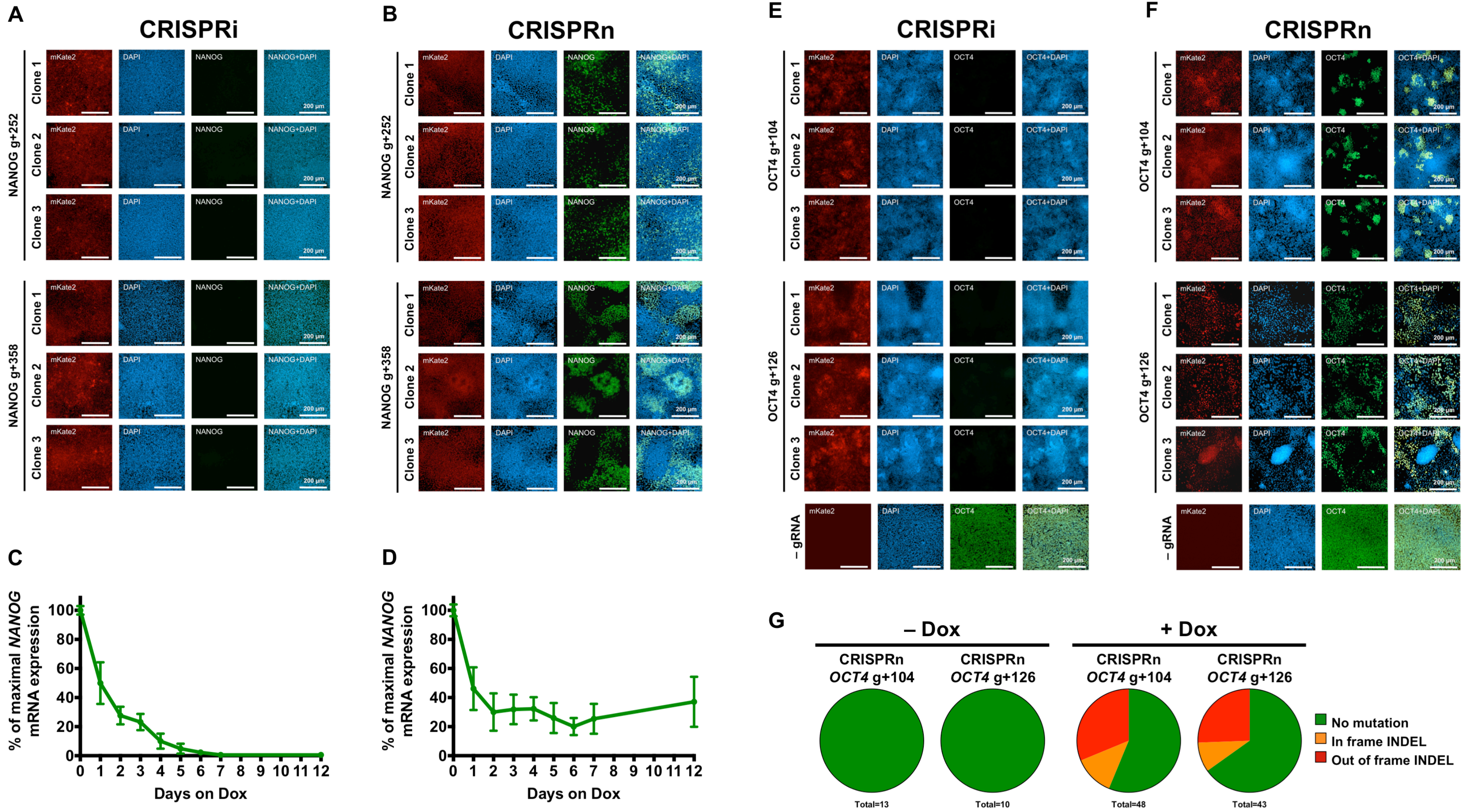
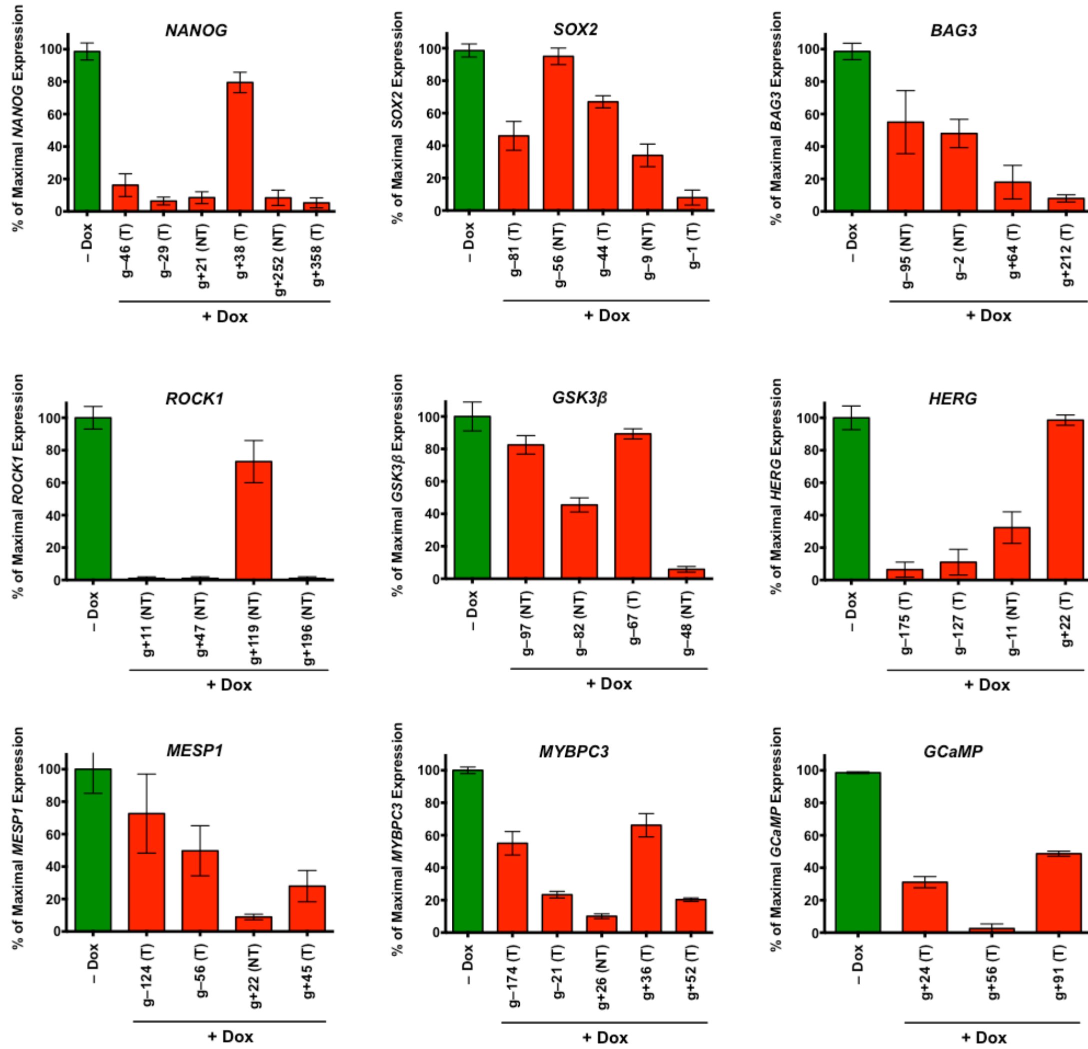
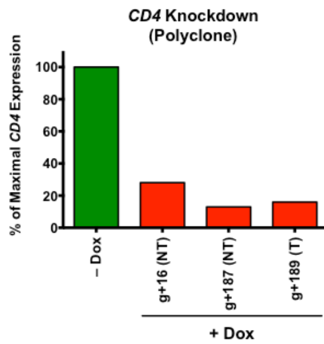


Figure S3. Comparison of efficiency of CRISPRi knockdown and CRISPRn knockout. Related to Figure 2.

Immunostaining of three independently-derived (A) CRISPRi and (B) CRISPRn colonies, containing different gRNAs (g+252 and g+358) against the first exon of *NANOG*. All CRISPRi gRNA-containing colonies showed complete knockdown of the target gene, while virtually all CRISPRn colonies showed variegated pattern of *NANOG* knockout. The mKate2 signal highlights the integration of the gRNA-expressing vector in all the cells within each clone. Nuclei were counterstained with DAPI. (C) qPCR analysis of CRISPRi cells show gradual loss of NANOG mRNA levels post initiation of knockdown. (D) For CRISPRn, mRNA levels rapidly drop within 2–3 days of knockout induction, however, remain stable thereafter. Immunostaining of three independently-derived stable (E) CRISPRi and (F) CRISPRn colonies containing different gRNAs (g+104 and g+126) against the first exon of *OCT4*. Using CRISPRi, *OCT4* expression was completely lost by 7 days post-doxycycline induction. While using CRISPRn, *OCT4* showed a variegated pattern of knockout and was expressed in 20-30% of the cells 7 days post-doxycycline induction. The mKate2 signal shows the presence of gRNA-expressing vector in all the cells. Nuclei were counterstained with DAPI. (G) Stable CRISPRn clones containing *OCT4* gRNA+104 and gRNA+126 were subjected to continuous doxycycline treatment for 14 days. Genomic DNA was extracted from non-doxycycline- and continuously doxycycline-treated cells and subjected to DNA sequencing. Even after 14 days of continuous doxycycline treatment, 55–65% of sequenced alleles contained no mutation and 30–40% of mutated alleles were in-frame INDELs. Red, out-of-frame INDELs; orange, in-frame INDELs; green, non-mutated alleles. The total number of sequenced colonies is listed below each pie graph. Error bars, SD. Scale bars = 200 μm.

Figure S4





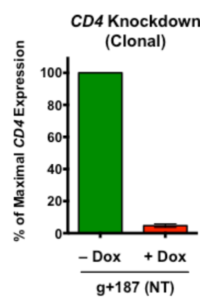


Figure S4. gRNA knockdown efficiency. Related to Figure 3.
Three to five gRNAs were designed and tested in polyclonal (A) iPSC and (B) CEM cell populations. In iPSCs, knockdown efficiency was tested using qPCR (except for GCaMP knockdown efficiency which was measured using flow cytometry). For CEM cells, the knockdown efficiency was measured using flow cytometry. The binding location of each gRNA is indicated relative to the TSS of the gene of interest and whether it targets the template (T) or non-template (NT) strand. (C) Three independent CEM clonal lines containing CD4 g+187 were isolated and assayed for CRISPRi knockdown. Each data point is an average of 2–4 technical replicates. Error bars, SD.

Figure S5

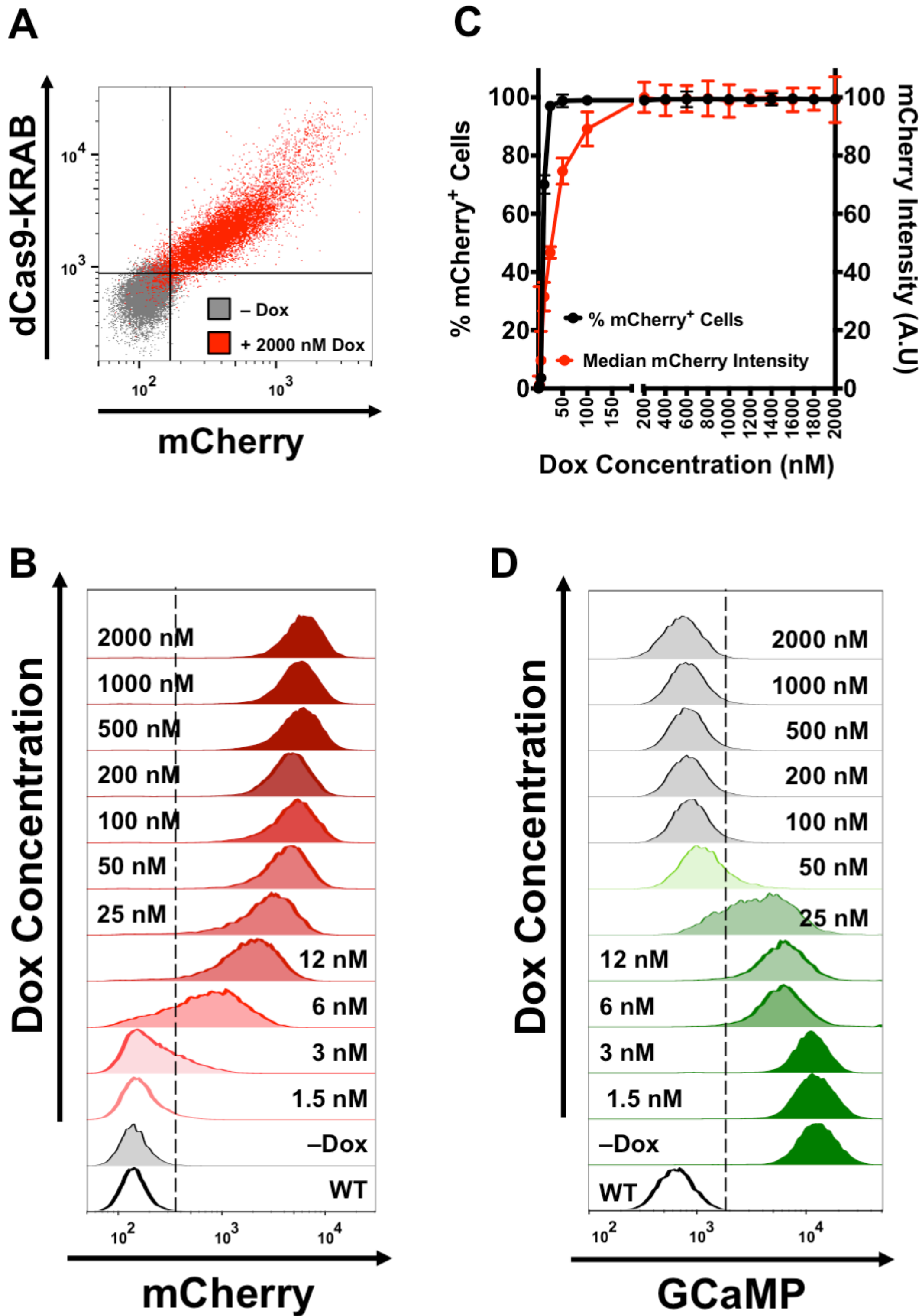
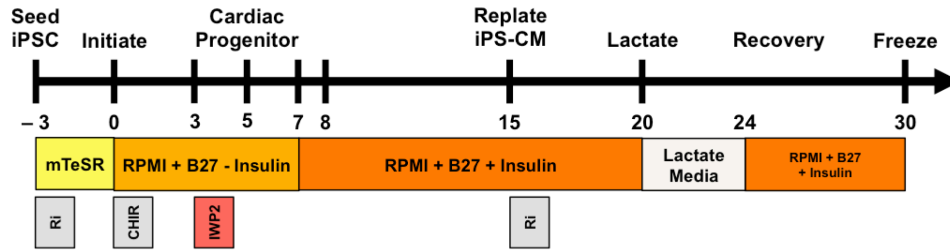
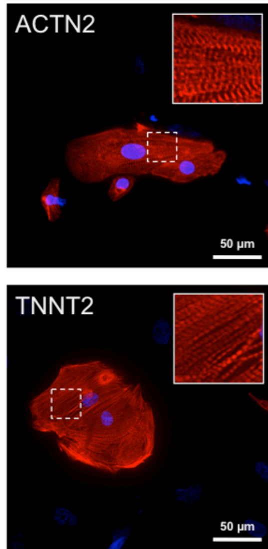


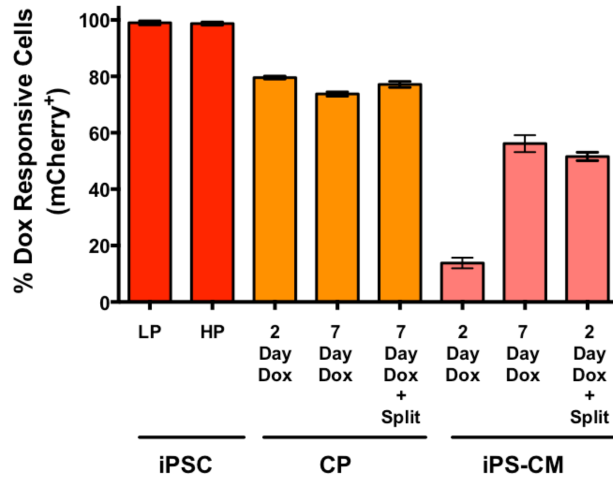
Figure S5. Tuning CRISPRi knockdown by titrating doxycycline concentration. Related to Figure 4.

(A) Flow cytometry scatterplot of CRISPRi iPSCs treated with 2 μM of doxycycline shows dCas9-KRAB and mCherry are expressed at proportional levels (translated from a bicystronic transcript). (B) mCherry expression was measured using flow cytometery to test the response of the CRISPRi cells to various doses of doxycycline. (C) The percentage of mCherry-positive cells and mCherry fluorescent intensity as measured by the median fluorescent intensity) were plotted at different doxycycline concentrations. As a single copy, the TetO promoter behaves similar to a binary switch and only within a narrow range of doxycycline concentration, the expression can be robustly tuned. (D) By titrating the dose of doxycycline, GCaMP expression levels could be tuned within a narrow range (3-12 nM). Flow cytometry plots representative of three biological replicates. Error bars, SD.

Figure S6







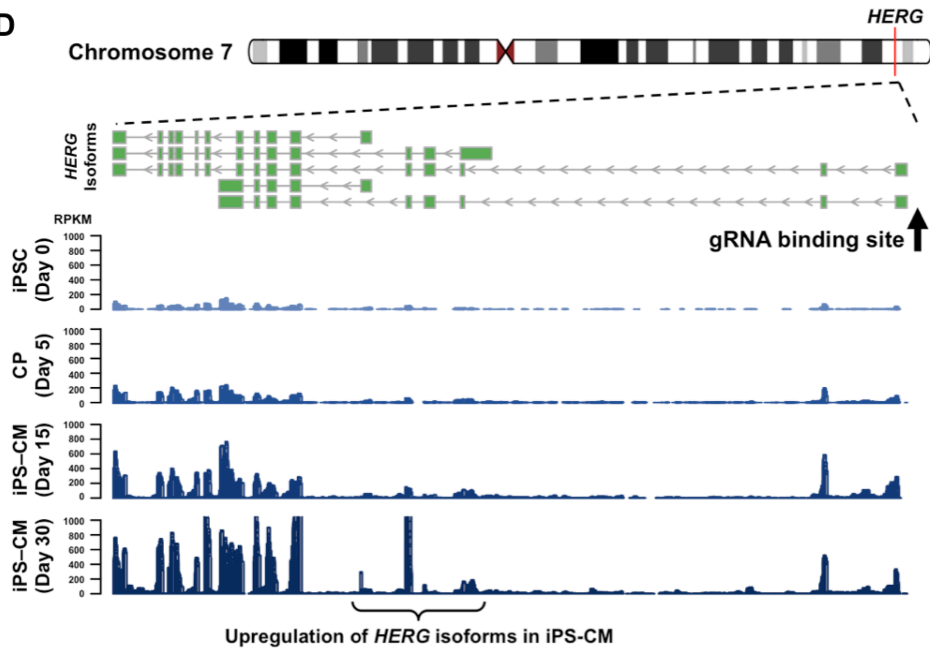
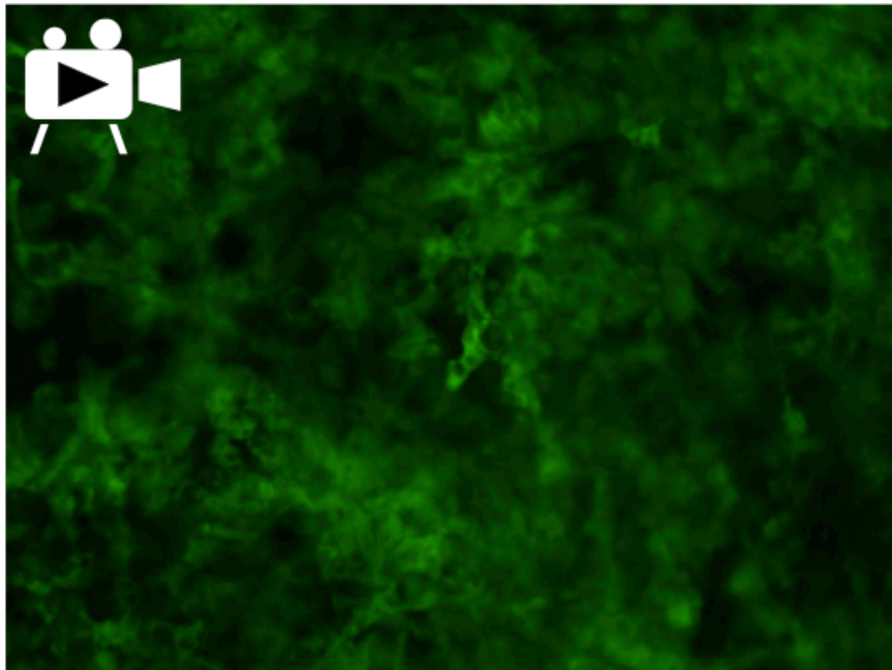


Figure S6. Differentiation, purification and doxycycline response of iPS-CMs. Related to Figure 6.

(A) Schematic diagram of the modified WNT-differentiation protocol for iPS-derived cardiomyocyte (iPS-CM) differentiation, lactate purification, and cryopreservation. (B) Lactate-purified iPS-CMs were stained with sarcomeric-specific markers ACTN2 and TNNT2. (C) Flow cytometry was used to measure doxycycline responsiveness (using mCherry expression) of low-passage (LP) and high-passage (HP; 3 months in culture) iPSC, cardiac progenitors (CP; day 5) and iPS-derived cardiomyocyte (iPS-CM; day 15) CRISPRi cells. Both low and high passage iPSC fully respond to doxycycline. 75-80% of cardiac progenitors cells respond to doxycycline. Less than 20% of iPS-CM responded to doxycycline after 2 days of treatment, while increasing the duration of doxycycline treatment to 7 days increases the percentage of responsive cells. Furthermore, only 2 days of doxycycline treatment with splitting the cells enabled more than 50% of the cells to respond. This indicates that the differentiated cells are prone to silencing at the TetO promoter. (D) RNA-Seq reads from the *HERG* transcript isoforms during iPS-CM differentiation. An arrow indicates the gRNA binding location, which targets the major transcript expressed in iPSCs. During cardiac differentiation, activation of the other isoforms (indicated by a bracket) is observed. Error bars, SD. Scale bars = 50 μm.



Video S1. Video of CRISPRi iPS-CMs under the GFP channel showing the calcium waves caused by the GCaMP fluorescent signal. Related to Figure 6.

Supplemental Experimental Procedures

iPSC Culture

Episomal reprogramming (Okita et al., 2011) was used to reprogram dermal fibroblasts from a healthy male and female individual to wild-type C (WTC) and wild-type B (WTB) human iPSC, respectively. The committee on Human Research at the University of California, San Francisco (UCSF) approved the iPSC-research protocol (#10-02521).

CRISPRi, CRISPRn, and GCaMP6f AAVS1 Knock-In Vectors

To generate iPSC lines with inducible CRISPR interference (CRISPRi) and CRISPR nuclease (CRISPRn), a single tetracycline-inducible vector was constructed to contain both the reverse tetracycline-controlled transcriptional activator (rtTA) and the tetracycline-response element (TRE3G). A strong constitutive promoter (CAG) transcribes rtTA and is oriented in the opposite direction of TRE3G element to ensure no leaky expression of the transgene without doxycycline. The TetO promoter transcribes dCas9-KRAB-P2A-mCherry (CRISPRi Gen1), dCas9-KRAB (CRISPRi Gen2) or Cas9 (CRISPRn Gen1). In the non-inducible CRISPRi clones (CRISPRi Gen3) dCas9-KRAB is expressed from the constitutive CAG promoter. In all CRISPRi lines, the KRAB domain is fused at the N-terminus of dCas9 (Gilbert et al., 2014). All CRISPRi and CRISPRn targeting vectors contained left- and right-homology arms (~800 bp) that flank the genomic-cut site in the AAVS1 locus. The vectors also contained a splice-acceptor (SA) site followed by the open reading frame (ORF) of a promoterless T2A-neomycin or T2A-puromycin resistance gene cassette, respectively (Figures 1A and 1B). The GCaMP6f knock-in vector that is specific for AAVS1 contains ~800 bp left and right of the AAVS1-homology arms, an SA site followed by the ORF of a promoterless T2A-puromycin resistance gene, and the GCaMP6f ORF driven off the CAG promoter (Figure 4).

Description of vectors

Vector Name **(Size)**	**Description & Addgene ID**	**Promoter** **Transgene of interest**	**Promoter** **Mammalian Resistance Gene**	**Bacterial Resistance Gene**
AAVS1 TALEN F (8,345 bp)	Homodimeric AAVS1 TALEN right pair	CMV RVD-FOK1	SV40 *Neo*^{R}	*Amp* and *Kan*
AAVS1 TALEN R (8,345 bp)	Homodimeric AAVS1 TALEN left pair	CMV RVD-FOK1	SV40 *Neo*^{R}	*Amp* and *Kan*
pAAVS1-NDi-CRISPRi (Gen1) (13,834 bp)	Dox-inducible CRISPR interference (CRISPRi) knock in construct into the AAVS1 locus with mCherry marker	TetO (TRE3G) dCas9-KRAB P2A mCherry	Endogenous AAV *Neo*^{R}	*Amp*

	(Gen1 CRISPRi vector)	(KRAB domain is fused at the N-terminus of dCas9)		
pAAVS1-NDi-CRISPRi (Gen2) (13,069 bp)	Dox-inducible CRISPR interference (CRISPRi) knock in construct into the AAVS1 locus (Gen2 CRISPRi vector)	TetO (TRE3G) dCas9-KRAB (KRAB domain is fused at the N-terminus of dCas9)	Endogenous AAV *Neo*^{R}	*Amp*
pAAVS1-NC-CRISPRi (Gen3) (11,437 bp)	Constitutive CRISPR interference (CRISPRi) knock in construct into the AAVS1 locus (Gen3 CRISPRi vector) Addgene ID: 73499	CAG dCas9-KRAB (KRAB domain is fused at the N-terminus of dCas9)	Endogenous AAV *Neo*^{R}	*Amp*
pAAVS1-PDi-CRISPRn (12,658 bp)	Dox-inducible CRISPR nuclease (CRISPRn) knock in construct into the AAVS1 locus	TetO (TRE3G) spCas9	Endogenous AAV *Puro*^{R} *(Pac)*	*Amp*
pgRNA-CKB (9,596 bp)	Guide RNA expression vector (with mKate2) Addgene ID: 73501	CAG NLS-mKate2-T2A-Bsd Mouse U6 gRNA	CAG *Bsd*^{R} *(Bsr)*	*Amp*
pgRNA-CGB (9,617 bp)	Guide RNA expression vector (with GFP) Addgene ID: 73502	CAG NLS-GFP-T2A-Bsd Mouse U6 gRNA	CAG *Bsd*^{R} (Bsr)	*Amp*
pAAVS1-PC-GCaMP6f (8,007 bp)	GCaMP knock in construct into the AAVS1 locus Addgene ID: 73503	CAG GCaMP6f	Endogenous AAV Puro^{R} (Pac)	*Amp*

gRNA Design and Cloning into the gRNA-Expression Vector

If empirical data were available based on RNAseq or cDNA sequences from iPSC and iPS-CM, those were given priority over the NCBI database. For CRISPRn, up to three gRNAs were designed to target within the first common exon of the gene of interest with a minimal number of off-target sites in the genome. MIT CRISPR design (http://crispr.mit.edu) was used to design and predict the number of off-target events in the genome. When possible, the location of the gRNA-binding site was scattered along the chosen stretch of DNA and targeted to both the template (T) and non-template (NT) strands while

choosing the highest-ranking gRNAs with the least number of predicted off-target events in the genome.

The parental gRNA-expression vector (pgRNA-CKB) contained a "16nt" sequence (GGAGACGGACGTCTCC) with two BsmBI restriction sites for cloning the gRNA oligos. This vector served as the "scrambled" gRNA sequence. The gRNA-expression vector expressed only a single gRNA of interest from a mouse U6 promoter and contained a reporter and selection cassette with nuclear-localized mKate2-T2A-Blasticidin, driven off the CAG promoter. For cloning each gRNA into the expression vector (pgRNA-CKB), one forward oligo was designed with its reverse complement and ordered from Integrated DNA Technologies (IDT). In addition, a 4-nt overhang “TTGG” was added to the 5’ end of the forward primer and a 4-nt overhang of “AAAC” was added to the 5’ end of the reverse primer (see below for examples of gRNA oligos). Each forward and reverse oligo (100 µM) was placed in the same reaction, phosphorylated using T4 PNK (NEB), and annealed by first heating to 95ºC and then slowly ramping down to 25ºC at 5ºC per min. The pgRNA-CKB vector was digested with BsmBI (NEB), treated with FastAP (Life Technologies), and run on a 1% (w/v) agarose (Sigma) gel. The ~9.6 kb linear DNA fragment was extracted using the QIAquick Gel Extraction Kit (Qiagen). The linearized vector (50–100 ng) and diluted phospho-annealed oligos (1 µl of 1:100) were ligated overnight at room temperature with T4 DNA ligase (NEB). The ligated product was transformed into Turbo competent E. coli (NEB). Sequencing primers gRNA Seq F (5’-GAGATCCAGTTTGGTTAGTACCGGG-3’) and gRNA Seq R (5’-ATGCATGGCGGTAATACGG TTAT-3’) were used to confirm the ligation of the correct gRNA.

gRNA oligo sequences are listed as below. gRNA naming is based on the binding coordinates relative to the transcription start site (TSS) of the gene of interest, and whether they target the template (T) and non-template (NT) strand. A negative coordinate indicates a binding location upstream of the TSS and a positive coordinate indication a binding location downstream of the TSS. The most commonly used gRNAs for efficient knockdown are indicated with a bold text and column outline. Forward and reverse primers for cloning into the pgRNA-CKB gRNA-expression vector are listed from 5’ to 3’. The 4-nt overhang sequences on the forward and reverse primers (highlighted in red) are used to clone phospho-annealed products into the pgRNA-CKB vector after BsmBI digest. The gRNA protospacer sequence is in black and designated as $(N)_{20}$ and the constant-tracer sequence is in blue.

5’–NNNNNNNNNNNNNNNNNNNNGTTTAAGAGCTATGCTGGAAACAGCATAGCAAGTTTAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTT –3’

gRNA Name (Targeting Strand)	**Oligo Sequences** **5’ – Forward Primer – 3’** **5’ – Reverse Primer – 3’**	Notes
OCT4 g–142 (T)	TTGGGGGGCGCCAGTTGTGTCTCC AAACGGAGACACAACTGGCGCCCC	

OCT4 g–105 (NT)	TTGGGGCGAAGGATGTTTGCCTAA AAACTTAGGCAAACATCCTTCGCC	
OCT4 g–7 (T)	TTGGAAGGCTAGTGGGTGGGACTG AAACCAGTCCCACCCACTAGCCTT	
OCT4 g–4 (T)	TTGGGCTAGTGGGTGGGACTGGGG AAACCCCCAGTCCCACCCACTAGC	Because the PAM-binding site contains an SNP at the *OCT4* locus, this gRNA binds to only one *OCT4* allele and knocks down *OCT4* by ~40%.
***OCT4* g+22 (NT)**	TTGGGGTGAAATGAGGGCTTGCGA AAACTCGCAAGCCCTCATTTCACC	*OCT4* g+22 is the most commonly used gRNA for efficient *OCT4* knockdown.
OCT4 g+42 (T)	TTGGTCGCAAGCCCTCATTTCACC AAACGGTGAAATGAGGGCTTGCGA	This gRNA does not knock down *OCT4*.
OCT4 g+56 (T)	TTGGTTCACCAGGCCCCCGGCTTG AAACCAAGCCGGGGGCCTGGTGAA	
OCT4 g+104 (NT)	TTGGACCACCTGGAGGGGGCGAGA AAACTCTCGCCCCCTCCAGGTGGT	This gRNA was used for both CRISPRi and CRISPRn
OCT4 g+126 (T)	TTGGTCGCCCCCTCCAGGTGGTGG AAACCCACCACCTGGAGGGGGCGA	This gRNA was used for both CRISPRi and CRISPRn
OCT4 g+701 (T)	TTGGCGAAGAGACAACTGCCGGTG AAACCACCGGCAGTTGTCTCTTCG	This gRNA does not knock down *OCT4*.
OCT4 g+1305 (NT)	TTGGGCTTACACTTGTCGCCTTGA AAACTCAAGGCGACAAGTGTAAGC	This gRNA does not knock down *OCT4*.
OCT4 g+2390 (NT)	TTGGGGAGTGCACTGGCGCGATCT AAACAGATCGCGCCAGTGCACTCC	This gRNA does not knock down *OCT4*.
OCT4 g+3410 (NT)	TTGGGTCTGTAAATCCTAGCACTT AAACAAGTGCTAGGATTTACAGAC	This gRNA does not knock down *OCT4*.
OCT4 g+4580 (T)	TTGGGTAGGTTCTTGAATCCCGAA AAACTTCGGGATTCAAGAACCTAC	This gRNA does not knock down *OCT4*.
OCT4 g+5632 (T)	TTGGCACCTCGCTTTCCCTAGCTC AAACGAGCTAGGGAAAGCGAGGTG	This gRNA does not knock down *OCT4*.
NANOG g–46 (T)	TTGGTCACAAGGGTGGGTCAGTAG AAACCTACTGACCCACCCTTGTGA	
NANOG g–29 (T)	TTGGTAGGGGGTGTGCCCGCCAGG AAACCCTGGCGGGCACACCCCCTA	
***NANOG* g+21 (NT)**	TTGGCCAGCAGAACGTTAAAATCC AAACGGATTTTAACGTTCTGCTGG	*NANOG* g+21 is the most commonly used gRNA for efficient *NANOG* knockdown.
NANOG g+38 (T)	TTGGCCAGGATTTTAACGTTCTGC AAACGCAGAACGTTAAAATCCTGG	
NANOG g+252 (NT)	TTGGCAGTCGGATGCTTCAAAGCA AAACTGCTTTGAAGCATCCGACTG	This gRNA was used for both CRISPRi and CRISPRn.
NANOG g+358 (T)	TTGGTTCTGCTGAGATGCCTCACA AAACTGTGAGGCATCTCAGCAGAA	This gRNA was used for both CRISPRi and CRISPRn.
SOX2 g–81 (T)	TTGGTCATGCAAAACCCGGCAGCG AAACCGCTGCCGGGTTTTGCATGA	
SOX2 g–56 (NT)	TTGGAGCGACCAATCAGCGCGCGG AAACCCGCGCGCTGATTGGTCGCT	
SOX2 g–44 (T)	TTGGAGGAGCCGCCGCGCGCTGAT AAACATCAGCGCGCGGCGGCTCCT	
SOX2 g–9 (NT)	TTGGGACAACCATCCATGTGACGG AAACCCGTCACATGGATGGTTGTC	
***SOX2* g–1 (T)**	TTGGCCCTGACAGCCCCCGTCACA AAACTGTGACGGGGGCTGTCAGGG	*SOX2* g–1 is the most commonly used gRNA for efficient *SOX2* knockdown.
BAG3 g–95 (NT)	TTGGTTCCGACTCGTGCGCGTGCC AAACGGCACGCGCACGAGTCGGAA	
BAG3 g–2 (NT)	TTGGGTCATCGGCTATAATCGCGG	

	AAACCCGCGATTATAGCCGATGAC	
BAG3 g+64 (T)	TTGGCGGGCCGCGGCCAACTTCTC AAACGAGAAGTTGGCCGCGGCCCG	
***BAG3* g+212 (T)**	TTGGTTCATAAAGGTGCCCGGCGC AAACGCGCCGGGCACCTTTATGAA	*BAG3* g+212 is the most commonly used gRNA for efficient *BAG3* knockdown.
***ROCK1* g+11 (NT)**	TTGGCGGGGCGCGGACGCTCGGAA AAACTTCCGAGCGTCCGCGCCCCG	*ROCK1* g+11 is the most commonly used gRNA for efficient *ROCK1* knockdown.
ROCK1 g+47 (NT)	TTGGCAAACAAACGGAGACCGCCG AAACCGGCGGTCTCCGTTTGTTTG	
ROCK1 g+119 (NT)	TTGGAGTCGCGGCGGCGAATGCCT AAACAGGCATTCGCCGCCGCGACT	
ROCK1 g+196 (NT)	TTGGAGACGATAGTTGGGTCCCGG AAACCCGGGACCCAACTATCGTCT	
GSK3β g–97 (NT)	TTGGGGATCCGGCGGGCTGACGGC AAACGCCGTCAGCCCGCCGGATCC	
GSK3β g–82 (NT)	TTGGCTCCGGCAAGCCGCGGGATC AAACGATCCCGCGGCTTGCCGGAG	
GSK3β g–67 (T)	TTGGCGCCGGATCCCGCGGCTTGC AAACGCAAGCCGCGGGATCCGGCG	
***GSK3β* g–48 (NT)**	TTGGGGGTGGCTCGGAGATGCGAC AAACGTCGCATCTCCGAGCCACCC	*GSK3β* g–48 is the most commonly used gRNA for efficient *GSK3β* knockdown.
***HERG* g–175 (T)**	TTGGTTCTGGGCGCGCGAGTCCCA AAACTGGGACTCGCGCGCCCAGAA	*HERG* g–175 is the most commonly used gRNA for efficient *HERG* knockdown in iPSC and iPS-CM.
HERG g–127 (T)	TTGGCGTTGGGGGAGCACTCGGCG AAACCGCCGAGTGCTCCCCCAACG	
HERG g–11 (NT)	TTGGTAATGCGGCGCGCGCCCCTC AAACGAGGGGCGCGCGCCGCATTA	
HERG g+22 (T)	TTGGCGCATTAACCCTTCCGCGGC AAACGCCGCGGAAGGGTTAATGCG	
MESP1 g–124 (T)	TTGGTGGGTCGGGCGCCCAAGCGA AAACTCGCTTGGGCGCCCGACCCA	
MESP1 g–56 (T)	TTGGCCCCCCGCCGTGGATTCAAA AAACTTTGAATCCACGGCGGGGGG	
***MESP1* g+22 (NT)**	TTGGGCCGCTTTATGCCGAGCCCG AAACCGGGCTCGGCATAAAGCGGC	*MESP1* g+22 is the most commonly used gRNA for efficient *MESP1* knockdown.
MESP1 g+45 (T)	TTGGGCTCGGCATAAAGCGGCCGC AAACGCGGCCGCTTTATGCCGAGC	
MYBPC3 g–174 (T)	TTGGAATTGTGCTGCGGGGGGTGA AAACTCACCCCCCGCAGCACAATT	
MYBPC3 g–21 (T)	TTGGGGGAGGTCCCCATATATAGT AAACACTATATATGGGGACCTCCC	
***MYBPC3* g+26 (NT)**	TTGGCGTCACACCAGGCACGAAGC AAACGCTTCGTGCCTGGTGTGACG	*MYBPC3* g+26 is the most commonly used gRNA for efficient *MYBPC3* knockdown.
MYBPC3 g+36 (T)	TTGGACCTGTGCCTGCTTCGTGCC AAACGGCACGAAGCAGGCACAGGT	
MYBPC3 g+52 (T)	TTGGTGCCTGGTGTGACGTCTCTC AAACGAGAGACGTCACACCAGGCA	
GCaMP g+24 (T)	TTGGTTGACTCATCACGTCGTAAG AAACCTTACGACGTGATGAGTCAA	gRNAs target the template strand of GCaMP6f open reading frame. Unlike guides targeting endogenous loci, the coordinates of the GCaMP guides are based on the translation start site (starting
***GCaMP* g+56 (T)**	TTGGGGTCACGCAGTCAGAGCTAT AAACATAGCTCTGACTGCGTGACC	

GCaMP g+91 (T)	TTGGACTCGAGAACGTCTATATCA AAACTGATATAGACGTTCTCGAGT	from ATG). GCaMP g+56 was the most efficient guide at knockdown and was used for the reversibility and RNA-Seq experiments.
CD4 g+16 (NT)	TTGGGCTCCTCCACACCCTAGGCC GTTTAAGAGC TTAGCTCTTAAACGGCCTAGGGTG TGGAGGAGCCCAACAAG	gRNA oligo sequences targeting near the *CD4* TSS targeting either the template (T) or non-template (NT) strand. *CD4* gRNA oligos were annealed and cloned into the pSLQ1371 lentiviral expression vector using BstXI and BlpI (Gilbert et al., 2014). CD4 g+187 (NT) was the most efficient gRNA at *CD4* knockdown.
CD4 g+187 (NT)	TTGGAGTCTGACCACCTTACCTCT GTTTAAGAGC TTAGCTCTTAAACAGAGGTAAGGT GGTCAGACTCCAACAAG	
CD4 g+189 (T)	TTGGCAAGAAAGACGCAAGCCCAG GTTTAAGAGC TTAGCTCTTAAACCTGGGCTTGCG TCTTTCTTGCCAACAAG	

Genomic DNA Preparation from Cells
Genomic DNA was extracted from ~10^5 cells with the DNeasy Blood & Tissue Kit (Qiagen). DNA samples were eluted in dH_2O, and sample concentrations were normalized to 100 ng/µl.

Genotyping Junction PCR
100 ng of genomic DNA were used in a 25 µl of PCR reaction mix using Phusion High-Fidelity DNA Polymerase (NEB). Standard PCR conditions were used: 62ºC annealing temperature and 30 seconds of extension at 72ºC per 1 kb of product. Primers used for genotyping PCR amplification are listed below.

Genotyping PCR Primers

Primer	Primer sequence (5' – 3')	Notes
WT AAV F	CGGTTAATGTGGCTCTGGTT	Amplifies the WT AAVS1 junction spanning the TALEN cut site. Expected PCR product size = 254 bp
WT AAV R	AGGATCCTCTCTGGCTCCAT	
AAV 5'J F	CTGCCGTCTCTCTCCTGAGT	Amplifies the 5' integration junction of knock-in vectors into the AAVS1 locus Depending on the antibiotic resistance of the knock-in vector either the Neo J R or the Puro J R primer should be used For Puro junction: Expected PCR product size = 1068 bp For Neo junction: Expected PCR product size = 1258 bp
Neo J R	CTCGTCCTGCAGTTCATTCA	
Puro J R	GTGGGCTTGTACTCGGTCAT	

TOPO TA Cloning and Sequencing

Genomic DNA was extracted from CRISPRi and CRISPRn clones containing *OCT4* and *NANOG* gRNA before and after doxycycline treatment. The region spanning the first exon of *OCT4* and *NANOG* was amplified using amplification primers listed below using Phusion High-Fidelity DNA Polymerase (NEB). PCR products were cloned into TOPO-TA cloning vector (Life Technologies) and transformed into Turbo competent *E. coli* (NEB) according to manufacturer's instructions. For each condition, individual colonies (13–48) were picked and plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen) and sequenced using the T7 primer.

OCT4 and _NANOG_ Amplification Primers

Primer	Primer sequence (5' – 3')	Notes
OCT4 Seq F	TCCACCCATCCAGGGGGCGG	For genomic DNA amplification around the first exon of *OCT4* to identify mutations Expected PCR product size = 580 bp
OCT4 Seq R	CATGACCACCTCCCCACACC	
NANOG Seq F	CTTTTCCTTCTGGAGGTCCTAT	For genomic DNA amplification around the first exon of *NANOG* to identify mutations Expected PCR product size = 400 bp
NANOG Seq R	GGATTAGTTGATAATAACACTTCTTTA	

Copy Number Assay using Droplet Digital PCR

50 ng of genomic DNA from each sample was digested with 2.5 U of HaeIII (NEB) in 1x CutSmart buffer in a total volume of 20 µl. Samples were incubated at 37ºC for 1 h and then heat inactivated at 65ºC for 20 min. 5 µM of each forward and reverse primer and 18 µM Taqman MGB (FAM) probe for Neomycin or Puromycin-resistance genes (kindly provided by Jen Berman and Samantha Cooper at Bio-Rad) were mixed in dH_2O. ddPCR reactions took place in a total volume of 25 µl containing 2 µl of digested DNA, 12.5 µl of 2x ddPCR Supermix for Probes (Bio-Rad), 1.25 µl of the premixed (FAM) primers/probe mixture, 1.25 µl of 20X (HEX) RPP30 reference primers/probe premix (Bio-Rad) and 10 µl dH_2O. Droplet generation was performed according to the manufacturer's instructions on a QX100 Droplet Generator (Bio-Rad). The ddPCR thermocycling conditions were: step 1, 95ºC 10 min; step 2, 94ºC 30 s; step 3, 58ºC (pre-optimized) 1 min; repeat steps 2 and 3 39 times; step 4, 98ºC 10 min. The PCR amplified droplets were analyzed on the QX100 droplet reader (Bio-Rad) with the chosen setting "CNV2" (for 2 copies). The copy number was analyzed using Quantasoft software (Bio-Rad).

Primers and Probes for ddPCR Assay

Primer / Probe	Primer / Probe sequence (5' – 3')	Notes
ddPCR-NeoF Primer	CATGGCTGATGCAATGCG	Optimal annealing temp = 58ºC Expected Amplicon size = 68 bp
ddPCR-NeoR Primer	TCGCTTGGTGGTCGAATG	

ddPCR-Neo Probe	CGCTTGATCCGGCTACCTGCC	
ddPCR-PuroF Primer	GTCACCGAGCTGCAAGAA	Optimal annealing temp = 58ºC Expected Amplicon size = 57 bp
ddPCR-PuroR Primer	CACCTTGCCGATGTCGAG	
ddPCR-Puro Probe	CTCTTCCTCACGCGCGTCGG	

Karyotyping
Samples were sent to Cell Line Genetics for karyotypic analyses, where 20 metaphases were analyzed using G-band karyotyping.

Immunocytochemistry
Cells were fixed in 4% (v/v) paraformaldehyde (Affymetrix) in PBS for 15 min and permeabilized in 0.1% (v/v) Triton X-100 (Sigma) for 15 min. Cells were blocked in 5% (w/v) bovine serum albumin (BSA) (Sigma) with 0.1% (v/v) Triton X-100 in PBS for 60 min. Cells were incubated overnight at 4ºC with primary antibodies diluted in 5% (w/v) BSA and 0.1% (v/v) Triton X-100 in PBS. Then, cells were washed three times in PBS for 15 min each. Cells were then incubated for 1 h at room temperature with secondary antibodies diluted in 5% (w/v) BSA and 0.1% (v/v) Triton X-100 in PBS. Then, cells were washed three times in PBS for 15 min each. Finally, cell nuclei were counterstained using VECTASHIELD mounting medium with DAPI (Vector Laboratories). Images were taken under a Zeiss Axio Observer microscope and processed using ZEN 2012 software version 8.0. Table below contains a list of the primary and secondary antibodies and their appropriate dilution.

Flow Cytometry
iPSCs and iPS-CMs were singularized with accutase. Cells were washed twice with PBS and fixed in 4% (v/v) paraformaldehyde (Affymetrix) in PBS for 10 min. Cells were then pelleted and washed with chilled (4ºC) FACS buffer consisting of 0.5% BSA (w/v) and 2 mM EDTA in PBS. Next, the samples were incubated in primary antibody for 30 min, followed by three washes in PBS. Finally, the samples were incubated in the appropriate secondary antibody for 30 min, followed by washes in PBS. Table below lists the antibodies used for these experiments. All experiments that measure GCaMP or mCherry intensity were performed on live cells without fixation, immediately after harvesting in PBS. For each sample, 20,000 events were captured on the MACSQuant VYB flow cytometer and analysed with FlowJo X 10.0.7r2.

Western blots
Cell pellets were collected, washed with PBS and resuspended in RIPA lysis buffer (150 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate, 50 mM Tris-HCl, 0.1% SDS, 2% TritonX-100, pH 8.0) containing a protease inhibitor cocktail (Roche). Samples were incubated for 30 min on ice and sonicated for 10 sec. 20 µg of each lysate were loaded per lane of a NuPAGE 4–12% Bis-Tris

polyacrylamide gel (Life Technologies). The gel was transferred onto a Nitrocellulose iBlot gel transfer stack using the iBlot gel transfer device (Life Technologies). The membrane was blocked in Odyssey Blocking Buffer (PBS) (LI-COR) for 1 h. Membranes were probed with the appropriate primary and secondary antibodies listed in the Table below. All primary antibodies were incubated overnight at 4ºC while secondary antibodies were left at room temperature for 1 h. Blots were imaged using the Odyssey Fc imaging system (LI-COR). Quantification of band intensities was performed using imageJ.

Primary and Secondary Antibodies

Type	Antibody	Application	Dilution	Species	Manufacturer and Catalog Number
Primary	Anti-OCT4	Immunocytochemistry	1:200	Mouse monoclonal	Santa Cruz Biotechnology sc-5279
		Western blot	1:1000		
		Flow cytometry	1:50		
	Anti-NANOG	Immunocytochemistry	1:200	Mouse monoclonal	Millipore MABD24
		Western blot	1:1000		
	Anti-SOX2	Immunocytochemistry	1:200	Rabbit polyclonal	Abcam ab59776
	Anti-BAG3	Immunocytochemistry	1:2000	Rabbit polyclonal	Abcam ab47124
	Anti-MYBPC3	Immunocytochemistry	1:200	Rabbit polyclonal	Abcam ab110832
		Western blot	1:1000		
	Anti-ACTN2	Immunocytochemistry	1:500	Mouse monoclonal	Sigma A7732
		Western blot	1:1000		
	Anti-TNNT2	Flow cytometry	1:100	Mouse monoclonal	Thermo scientific MS-295-P1
	Anti-GAPDH	Western blot	1:1000	Rabbit polyclonal	Abcam ab9485
	Anti-FLAG	Flow cytometry	1:100	Mouse monoclonal	Sigma F3165
	Anti-Cas9	Immunocytochemistry	1:200	Mouse monoclonal	Diagenode C15200203
		Western blot	1:1000		
		Flow cytometry	1:100		
	Anti-CD4 APC-Conjugated	Flow cytometry	1:100	Mouse monoclonal	BD 555349
Secondary	Goat anti-Mouse IgG (H+L), Alexa Fluor 647 conjugate	Immunocytochemistry	1:500	Goat anti-mouse IgG (H+L)	Life Technologies A-21236
	Chicken anti-Rabbit IgG (H+L), Alexa Fluor 647 conjugate	Immunocytochemistry	1:500	Chicken anti-rabbit IgG (H+L)	Life Technologies A-21443

	Goat anti-Mouse IgG (H+L), Alexa Fluor 488 conjugate	Immunocytochemistry	1:500	Goat anti-mouse IgG (H+L)	Life Technologies A-11001
	IRDye® 800CW Donkey anti-Rabbit IgG (H + L)	Western blot	1:2500	Donkey anti-rabbit IgG (H + L)	Li-COR 926-32213
	IRDye® 680LT Donkey anti-Mouse IgG (H + L)	Western blot	1:2500	Donkey anti-mouse IgG (H + L)	Li-COR 926-68022

CRISPRi and CRISPRn Knockdown and Knockout Assays in iPSCs
Initial rounds of knockdown or knockout screening with different gRNAs per gene were performed in polyclonal populations (greater than ~90% positive for mKate2). Unless specified, in all knockdown and knockout assays, iPSCs were cultured in mTeSR supplemented with doxycycline (2 μM; Sigma) for 7 continuous days before analysis. All corresponding negative controls (minus doxycycline) were maintained in mTeSR for 7 days. After identifying the most efficient gRNAs by TaqMan qPCR, immunocytochemistry, or flow cytometry, polyclonal populations of cells carrying the most efficient gRNA were subcloned by serial dilution. Subsequent analysis and assays were performed on clonal populations to obtain clean knockdowns or knockouts.

CRISPRi Knockdown Assays in Cardiac Mesoderm and iPS-CM
For CRISPRi knockdown in cardiac progenitor cells, stable iPSCs containing gRNA were differentiated towards the cardiac lineage with the WNT-differentiation protocol (described below). Half of the samples were treated with doxycycline (2 μM) from day 0 of differentiation (Figure S5A). Samples were harvested on day 4 of differentiation and analyzed using Taqman qPCR. For CRISPRi knockdown in iPS-CM, stable polyclonal iPSCs containing gRNA were differentiated into iPS-CM. On day 5 of differentiation, cells were enzymatically dissociated and replated onto Matrigel-coated plates at a density of $2.5x10^4$ cells/cm^2. Half of the cells were treated with media supplemented with doxycycline (2 μM) and the other half was treated with media only. Doxycycline was maintained throughout the differentiation process (either day 15 for non-lactate-treated cells or day-35 for lactate-treated cells) until cells were harvested.

RNA Extraction and TaqMan qPCR Analysis
RNA was extracted from approximately 10^5 cells with TRIzol reagent (Life Technologies) and cleaned up with the PureLink RNA Kit (Life Technologies) according to manufacturer's instructions. Samples were then treated with DNaseI (Life Technologies) for 30 min at 37ºC. Then, 1 μg of total RNA was reverse-transcribed into first-strand cDNA using SuperScript III (Life Technologies) with random hexamers, following the manufacturer's instructions. Real-Time qPCR reactions were performed in TaqMan Universal PCR Master Mix (Life Technologies) with the TaqMan probes listed in the table below. Quantification of gene expression was carried out with probes against the target gene and normalized against three ubiquitously expressed endogenous controls *18S*,

GAPDH, and *UBC* for iPSCs and cardiac mesoderm cells. For iPS-CM, qPCR results were validated with three independent biological replicates to minimize batch-to-batch variability in the timing of cardiac-specific marker expression. In addition to three housekeeping genes (18S, *GAPDH*, and *UBC*), two cardiac-specific markers (*TNNT2* and *MYH6*) were used to normalize expression of target genes. Relative expression of the gene of interest was normalized against endogenous or cardiac-specific genes using the difference in threshold-cycle (C_T) values between the gene of interest and endogenous control by the $2^{-\Delta\Delta C_T}$ method (Schmittgen and Livak, 2008).

TaqMan qPCR Probes

Gene Probe	Gene ID	Exon Boundary	Amplicon Length (bp)	Marker
OCT4	Hs00742896_s1	1–1	65	Pluripotency
NANOG	Hs02387400_g1	1–2	109	Pluripotency
SOX2	Hs01053049_s1	1–1	91	Pluripotency
ROCK1	Hs01127699_m1	1–2	79	Kinase
GSK3β	Hs01047719_m1	1–2	65	Kinase
BAG3	Hs00188713_m1	1–2	83	Co-chaperone protein
HERG	Hs00542479_g1	6–7	67	K^+ Ion channel
PAX6	Hs01088112_m1	4–5	55	Neuronal marker
T	Hs00610080_m1	8–9	132	Mesoderm marker
MESP1	Hs00251489_m1	1–2	80	Cardiac mesoderm marker
MYBPC3	Hs00165232_m1	12–13	56	Cardiac sarcomeric protein
TNNT2	Hs00165960_m1	10–11	89	Cardiac sarcomeric protein
MYH6	Hs01101425_m1	20–21	67	Cardiac sarcomeric protein
UBC	Hs00824723_m1	1–2	71	Housekeeping
18S	Hs99999901_s1	1–1	187	Housekeeping
GAPDH	Hs02758991_g1	6–7	93	Housekeeping

iPS-CM Differentiation

iPSCs were differentiated into iPS-CM using the WNT modulation-differentiation method (Lian et al., 2012) (Figure S5A). Briefly, iPSCs were seeded at 1.25–2.5×10^4 cells/cm^2 onto cell-culture plates coated with 80 μg/μl growth factor-reduced Matrigel (BD Biosciences) in mTeSR supplemented with 10 μM Y-27632 (Selleckchem) for 24 h (day –3). mTeSR medium was changed daily for the next 2 days. On day 0, iPSCs were treated with 12 μM CHIR99021 (CHIR) (Tocris) in RPMI/B27 without insulin (Life Technologies) for exactly 24 h. On day 1, the culture medium was replaced with fresh RPMI/B27 without insulin and maintained for 48 h. On day 3, cells were treated with 5 μM IWP2 (Tocris) in RPMI/B27 without insulin and maintained for 48 h. On day 5, fresh RPMI/B27 without insulin was added to the cells, and on day 7, the medium was switched to RPMI/B27 with insulin. Afterward, fresh RPMI/B27 with insulin was added to the

cells every 3 days. Functional iPS-CM appeared in culture between days 8 and 10 post-CHIR treatment.

Lactate Purification of iPS-CM

iPS-CM were purified via a modified version of the lactate metabolic-selection method (Tohyama et al., 2013). Briefly, 1 M lactate-stock solution was prepared in 1 M HEPES buffer (Sigma) with sodium L-lactate powder (Sigma). Glucose-free DMEM (Life Technologies) supplemented with 4 mM lactate solution, 1X Glutamax, and 1X non-essential amino acids (Life Technologies) was prepared as the selection medium. On day 15 post-CHIR treatment, iPS-CMs were split 1:2 onto Matrigel-coated 10-cm dishes in RPMI/B27 (Life Technologies) supplemented with insulin and Y-27632 (10 μM). Replated cells were allowed to recover for 5 days in RPMI/B27 supplemented with insulin before selection. Cells were then washed with PBS and incubated in lactate-selection medium, changed every other day for 4 days. Then, the medium was replaced with RPMI/B27 supplemented with insulin. Cells were allowed to recover for 3 days before being harvested.

Calcium-Transient Analysis of iPS-CMs

Lactate-purified iPS-CMs were replated onto Matrigel-coated plates and allowed to recover for 10 days before phenotypic analysis and immunostaining. Calcium transients in iPS-CMs were measured using the GCaMP signal to indicate intensity changes in GFP-flourescence. Videos were recorded with a Zeiss Axio Observer microscope, processed with ZEN 2012 software version 8.0, and analyzed with ImageJ.

Electrophysiology

Contracting iPS-CM were dissociated with trypsin (0.25%) and replated onto Matrigel-coated coverslips. After reconfirming visible beating, coverslips were placed in a superfusion bath (Warner, RC26-GLP) on a Nikon TiS inverted microscope equipped with a microfluorometer (IonOptix LLC). Superfusion solutions were warmed to 30°C with a superfusion system and heated perfusion pencil (ValveLink, AutoMate Scientific). Small clusters (five or fewer cells) of spontaneously contracting iPS-CMs were selected for study, with one cell under amphotericin B-perforated patch clamp (Spencer et al., 2014). Briefly, patch electrodes of approximately 2–4 MΩ (WPI) were tip-filled by dipping (20 s) them in an intracellular solution containing: KCl (120 mM), NaHEPES (20 mM), MgATP (10 mN), K_2EGTA (5 mM), $MgCl_2$ (2 mM), and adjusted to pH 7.1 with KOH. This solution was then back filled with the same solution, including amphotericin B (240 μg/ml). Coverslips were superfused at a constant flow (Warner, DN series) with modified Tyrode's extracellular solution containing: NaCl (137 mM), NaHEPES (10 mM), dextrose (10 mM), KCl (5 mM), $CaCl_2$ (2 mM), and $MgCl_2$ (1 mM), set to pH 7.4 with NaOH. Spontaneous action potentials (APs) were recorded in current clamp mode with zero applied current, and Ca^{2+} signals were low-pass filtered at 2 kHz and digitized at 5 kHz for 30 s per data file.

Supplemental References

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