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RESEARCH REPORT

TECHNIQUES AND RESOURCES

Cas9 effector-mediated regulation of transcription and differentiation in human pluripotent stem cells

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ABSTRACT

The identification of the *trans*-acting factors and *cis*-regulatory modules that are involved in human pluripotent stem cell (hPSC) maintenance and differentiation is necessary to dissect the operating regulatory networks in these processes and thereby identify nodes where signal input will direct desired cell fate decisions *in vitro* or *in vivo*. To deconvolute these networks, we established a method to influence the differentiation state of hPSCs with a CRISPR-associated catalytically inactive dCas9 fused to an effector domain. In human embryonic stem cells, we find that the dCas9 effectors can exert positive or negative regulation on the expression of developmentally relevant genes, which can influence cell differentiation status when impinging on a key node in the regulatory network that governs the cell state. This system provides a platform for the interrogation of the underlying regulators governing specific differentiation decisions, which can then be employed to direct cellular differentiation down desired pathways.

KEY WORDS: CRISPR, Cas9, Differentiation, Gene activation, Pluripotent stem cell, Transcriptional repression

INTRODUCTION

Human pluripotent stem cells (hPSCs) offer a unique avenue to study normal as well as defective cellular differentiation and function *in vitro*, and have great potential to advance understanding and treatment of diseases. However, for many cell types of interest (e.g. mature pancreatic β-cells and thymic epithelial cells), our inability to guide hPSCs towards the desired mature and functional cell types through the application of exogenous signaling molecules precludes utilization of this *in vitro* system in many areas. Exogenous delivery of transcription factors provides an alternative method to influence cell identity in hPSCs and to elucidate regulatory networks underlying these cell fate decisions. Recently, an RNA-guided adaptive immune system that is widespread in bacteria and archaea (Wiedenheft et al., 2012) has been adapted for targeted DNA cleavage or gene regulation in prokaryotic and eukaryotic genomes (Charpentier and Doudna, 2013). Applied to hPSCs, such a system could provide a platform for the systematic and high-throughput identification of factors relevant to stem cell differentiation.

CRISPR (clustered regularly interspaced short palindromic repeat) RNA sequences and CRISPR-associated (Cas) genes generate catalytic protein-RNA complexes that utilize the incorporated RNA to generate sequence-specific double-strand breaks at a complementary DNA sequence (Bhaya et al., 2011). The Cas9 nuclease from *Streptococcus pyogenes* (hereafter, Cas9) can be guided to specific sites in the human genome through base-pair complementation between a 20-nucleotide guide region of an engineered single-guide RNA (sgRNA) and a genomic target sequence (Mali et al., 2013b; Cho et al., 2013; Cong et al., 2013; Jinek et al., 2013). A catalytically inactive programmable RNA-dependent DNA-binding protein (dCas9) can be generated by mutating the endonuclease domains within Cas9 (Qi et al., 2013), which can modulate transcription in bacteria or eukaryotes either directly (Qi et al., 2013; Bikard et al., 2013) or through an incorporated effector domain (Gilbert et al., 2013; Mali et al., 2013a; Konermann et al., 2013; Maeder et al., 2013; Perez-Pinera et al., 2013). However, the ability of a dCas9-effector (dCas9-E) system to influence the differentiation status of stem cells has not been addressed. Here, we demonstrate the ability of the CRISPR effector (CRISPRe) system to modulate gene expression in human embryonic stem cells (hESCs), using either CRISPR interference (CRISPRi) or CRISPR activation (CRISPRa). We describe the application of CRISPRe to influence directly the differentiation status of hESCs, providing a platform for interrogating transcriptional regulatory networks *in vitro* that underpin hPSC differentiation decisions.

RESULTS AND DISCUSSION

We created a lentiviral delivery-based dCas9-E/CRISPRe transcription effector system for application in hESCs by generating a human codon-optimized, catalytically inactive version of Cas9 (dCas9) (Jinek et al., 2012; Qi et al., 2013), which is fused to either a VP16 tetramer activation domain (VP64) or a Krüppel-associated box (KRAB) repressor domain (supplementary material Figs S1, S2). Following lentiviral infection of hESCs, we confirmed constitutive or doxycycline-inducible expression of dCas9-E under the control of EF1α or tetracycline-responsive (TRE) promoters, respectively (supplementary material Fig. S2). A separate U6 promoter-based lentiviral delivery system for sgRNA expression was generated to program dCas9-E for specific genomic targets (Jinek et al., 2012).

We first tested whether the dCas9-VP64 system could be employed to activate a developmentally relevant gene in hESCs. *SOX17* is a gene linked to differentiation of definitive endoderm (Kanai-Azuma et al., 2002) that is repressed in hESCs and exhibits a classical bivalent H3K4me3 activation and an H3K27me3

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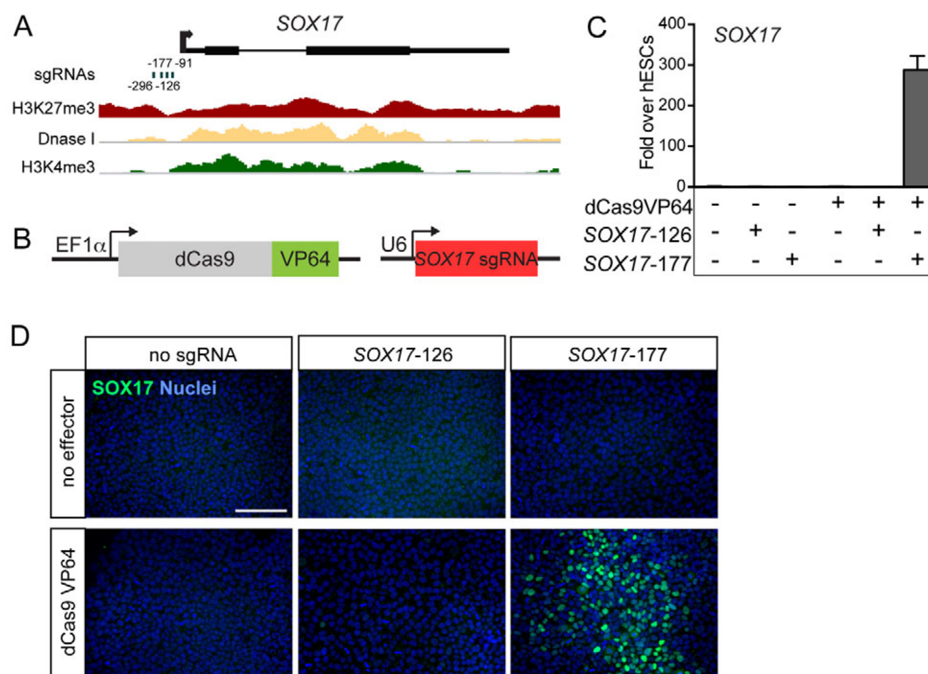

Fig. 1. CRISPRa can upregulate expression of a developmentally relevant transcription factor in hESCs. (A) Genomic view of the *SOX17* locus, showing the sgRNA targets, and key epigenetic marks indicating the active (H3K4me3) or repressed (H3K27me3) status, and overall accessibility (Dnase I) of the gene and its surrounding genomic area. (B) Schematic of the constitutive EF1α-regulated dCas9-VP64 and *SOX17* sgRNA constructs. (C) Quantitative gene expression analysis of EF1α-regulated dCas9-VP64 cells transduced with *SOX17* sgRNAs. Data are expressed as fold over hESCs ± s.d. (*n*=3) (D) Immunofluorescence analysis of SOX17 in EF1α-regulated dCas9-VP64 and control cells 6 days after transduction with sgRNAs. Scale bar: 100 µm.

repression epigenetic mark of a poised gene (Rada-Iglesias et al., 2011) (Fig. 1A). This regulator offers a unique opportunity to address whether we could activate a differentiation marker in a poised state. To assess the ability of dCas9-VP64 to upregulate expression of *SOX17* in hESCs, we expressed dCas9-VP64 in hESCs under control of the ubiquitously expressed EF1α promoter and designed two sgRNAs to target regions upstream of the *SOX17* transcriptional start site (TSS) (Fig. 1A,B). Neither expression of the dCas9-VP64 variant alone nor the presence of *SOX17*-specific sgRNAs alone led to significant increases in *SOX17* expression levels. Likewise, delivery of dCas9-VP64 in conjunction with the *SOX17*-126 sgRNA had no detectable effect. By contrast, co-delivery of dCas9-VP64 and *SOX17*-177 sgRNA increased expression of *SOX17* by 287(±35)-fold (Fig. 1C). This increase in gene expression was sufficient to allow the accumulation of SOX17 protein in the treated hESC cultures based on immunofluorescence analysis (Fig. 1D). Thus, despite the presence of repressive epigenetic marks, the CRISPRa system can drive expression of developmentally relevant genes in hESCs with one sgRNA.

To ascertain the number of hESCs responding to the CRISPRa system, we delivered *SOX17*-177 sgRNA or a control sgRNA into TRE-regulated dCas9-VP64 cells (supplementary material Fig. S3A). In this system, selection of the cells with neomycin and puromycin enables the combined enrichment of TRE-regulated dCas9-VP64 and each sgRNA. Combined selection was followed by 6 days of doxycycline treatment, resulting in 25.4% of cells with antibody-detectable SOX17 expression compared with 0% of control cells (supplementary material Fig. S3B,C). This variability might be due to variations in the expression levels of CRISRPa components due to the location of viral integration or subsequent silencing of the viral constructs. Variation in the underlying epigenetic state of each cell might also contribute to its overall responsiveness to the CRISPRa system.

The ability of an sgRNA to mediate dCas9-VP64-based changes in gene expression in hESCs is consistent with studies of similar dCas9 activators in transformed human cell lines (Cheng et al., 2013; Mali et al., 2013a; Maeder et al., 2013; Gilbert et al., 2013; Perez-Pinera et al., 2013). However, in these studies co-expression of multiple sgRNAs is typically required for efficient gene activation (Cheng et al., 2013; Gilbert et al., 2013; Mali et al., 2013a Perez-Pinera et al., 2013). To determine whether a combination of sgRNAs would further increase SOX17 expression through CRISPRa in hESCs, we transduced cells with *SOX17*-177, *SOX17*-126, *SOX17*-296 or *SOX17*-91 sgRNAs individually or in combination. A combination of all *SOX17* sgRNAs increased SOX17 protein-specific immunoreactivity in a subset of cells compared with individual sgRNAs (supplementary material Fig. S3B,D). Quantification of expression levels in the SOX17+ populations revealed a statistically significant increase (P<0.01) in the mean expression level of SOX17 when multiple sgRNAs were employed, consistent with an additive effect between different sgRNAs targeting the same promoter (supplementary material Fig. S3E). We conclude that, although a specific sgRNA suffices to increase gene expression in hESCs, multiple sgRNAs can further improve expression levels of a target gene.

Although the imposed activity of specific transcription factors has proven effective in the programming of cell fate decisions (reviewed by Ladewig et al., 2013), the destabilization of transcription factors that govern cell identity can also be used to change cell differentiation status (e.g. Cobaleda et al., 2007; Hay et al., 2004). To address whether our dCas9-E system can be harnessed for this purpose in hESCs, we targeted the pluripotency network by combining the dCas9-KRAB transcriptional repressor with *OCT4*-targeting sgRNAs. Two isoforms of *OCT4* (also known as *POU5F1*) are expressed in hESCs; isoform A is important for maintaining pluripotency whereas isoform B does not activate transcription of *OCT4*-dependent promoters (Lee et al., 2006). We designed sgRNAs to target the TSS of either *OCT4* isoform A or isoform B (Fig. 2A,B). Six days after co-expression of dCas9-KRAB and individual sgRNAs, we observed a more flattened morphology of cultures that contained *OCT4A*-specific sgRNAs compared with hESCs that received *OCT4B*-specific sgRNAs (Fig. 2C; supplementary material Fig. S4A). In addition, a large number of OCT4-negative cells were present in the cultures that received

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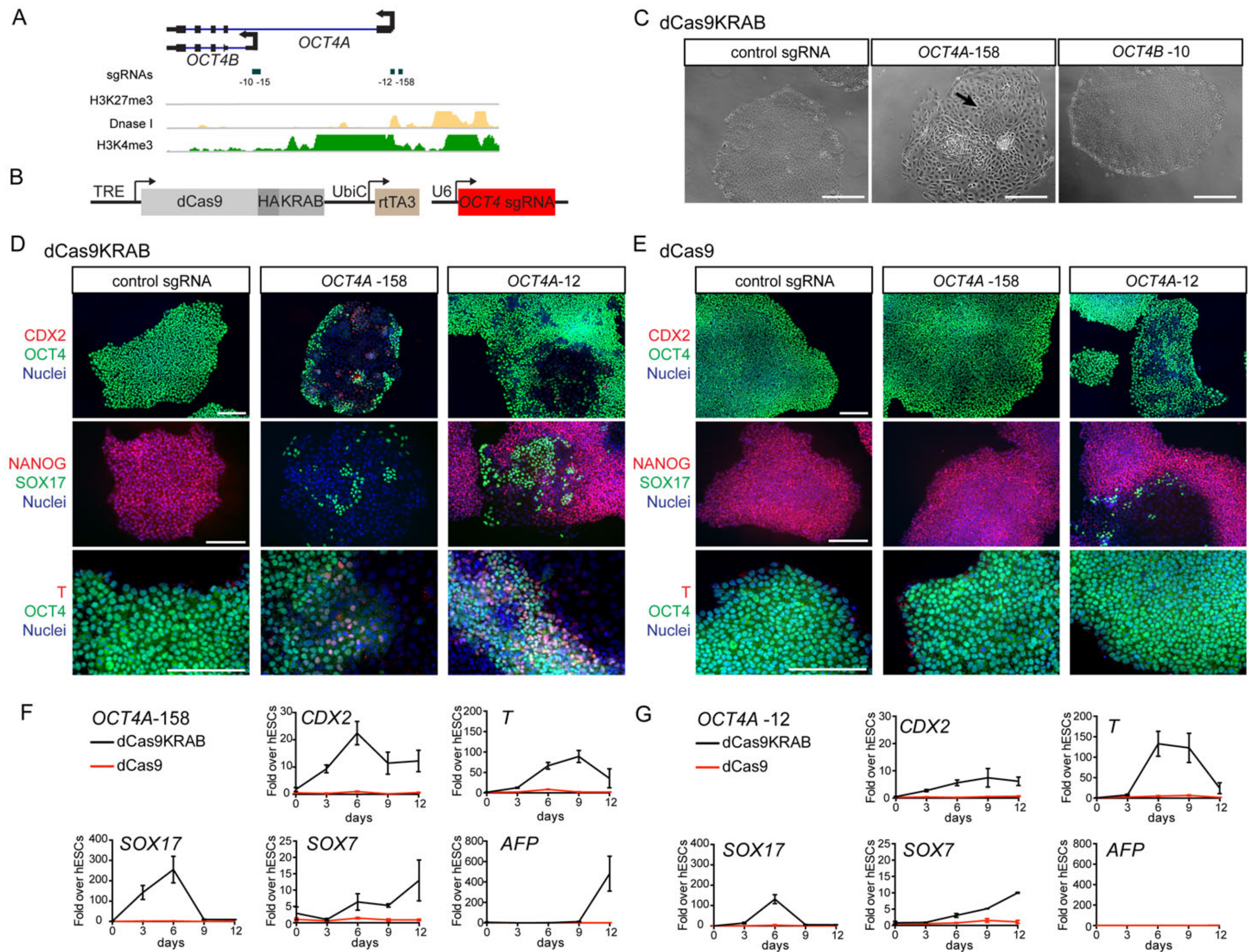

Fig. 2. Repression of the pluripotency network by CRISPRi-mediated downregulation of *OCT4A* in hESCs leads to differentiation into different cell lineages. (A) Genomic view of the *OCT4* locus indicating *OCT4* isoforms A and B. sgRNA targets, and key epigenetic marks indicating the active (H3K4me3) or repressed (H3K27me3) status, and overall accessibility (Dnase I) of the gene and its surrounding genomic area are indicated. (B) Schematic of the inducible TRE-regulated dCas9-KRAB and *OCT4* sgRNA constructs. (C) Phase-contrast images of cells expressing unrelated (control), *OCT4A* or *OCT4B* sgRNA in TRE-regulated dCas9-KRAB cells after 6 days of doxycycline treatment. Arrow indicates morphological changes. (D,E) Immunofluorescence analysis of TRE-regulated dCas9-KRAB cells (D) or TRE-regulated dCas9 cells (F) expressing unrelated (control) or *OCT4A* sgRNA after 6 days of doxycycline treatment for CDX2, OCT4A, NANOG, SOX17 and T. (F) Quantitative gene expression analysis of differentiation markers *CDX2*, *T*, *SOX17*, *SOX7* and *AFP* in *OCT4A*-158 sgRNA expressing TRE-regulated dCas9-KRAB or TRE-regulated dCas9 cells over 12 days of doxycycline treatment. Data are expressed as fold over hESCs ± s.d. (G) Quantitative gene expression analysis of differentiation markers *CDX2*, *T*, *SOX17*, *SOX7* and *AFP* in *OCT4A*-12 sgRNA expressing TRE-regulated dCas9-KRAB or TRE-regulated dCas9 cells over 12 days of doxycycline treatment. Data are expressed as fold over hESCs ± s.d. (*n*=3). Scale bars: 200 μm.

OCT4A-specific sgRNAs (supplementary material Fig. S4B), indicating the desired silencing of this locus.

To address whether dCas9-KRAB mediated repression of a pluripotency-associated gene is sufficient to influence hESC differentiation status, we analyzed the dCas9-KRAB cultures at day 6 after sgRNA delivery for expression of the pluripotency factor NANOG, as well as for differentiation markers for trophectoderm (CDX2), endoderm (SOX17) and mesendoderm/mesoderm (T). Whereas virtually every cell in the dCas9-KRAB cultures with a control sgRNA expressed both OCT4 and NANOG, large areas of OCT4 and NANOG-negative cells were observed with *OCT4A*-specific sgRNAs (Fig. 2D; supplementary material Fig. S4C). The expression of the dCas9-KRAB variant with a control sgRNA did not influence OCT4 expression or colony morphology, suggesting that the dCas9-E/CRISPRe components are not negatively influencing self-renewal independent of their gene-specific targeting function. We conclude that the dCas9-E/CRISPRe system is able to influence hESC differentiation with one sgRNA.

Recently, it was reported that a CRISPRi system can be implemented by steric interference with RNA Polymerase II transcriptional initiation or elongation via dCas9 binding (Qi et al., 2013; Gilbert et al., 2013). To address whether the observed effects of dCas9-KRAB on hESCs in our system were due to an obstruction mechanism rather than KRAB-mediated repression, we tested the impact of a dCas9 variant lacking the KRAB effector domain on *OCT4* expression. We did not observe any morphological changes in hESC cultures co-expressing dCas9 and the *OCT4A* sgRNA targeting the −158 region upstream of the TSS (supplementary

material Fig. S4D). Interestingly, we found rare cells with morphological changes in the cultures co-expressing dCas9 and the *OCT4A* sgRNA proximal to the TSS (*OCT4A*-12; supplementary material Fig. S4D). To address whether the morphological changes were accompanied by a change in expression of pluripotency genes or differentiation-associated factors, we analyzed the cultures for the presence of OCT4 and NANOG, or CDX2, SOX17 and T, respectively. In concordance with the subtle morphological changes, we observed patches of OCT4 and NANOG downregulation in hESC dCas9 cultures containing the *OCT4A*-12 sgRNA, but not in cultures containing the *OCT4A*-158 sgRNA (Fig. 2E). In addition, SOX17 antibody-reactive cells were detected within the NANOG-downregulated regions of the dCas9/*OCT4A*-12 sgRNA-treated cultures. Overall, the impact of the effector-less dCas9 on differentiation-associated genes is attenuated relative to dCas9-KRAB. Thus, some degree of repression associated with the dCas9-KRAB/*OCT4A*-12 sgRNA combination is likely to be due to direct interference of dCas9 with the function of RNA Polymerase II, whereas the repression associated with the dCas9-KRAB/*OCT4A*-158 sgRNA combination is dependent on the KRAB effector domain.

To address whether the effector-independent dCas9 might impose a delayed effect on differentiation relative to the dCas9-KRAB effector, we analyzed the appearance of differentiation-associated transcripts in a time course for both versions of dCas9 (Fig. 2F,G). No significant increases in *CDX2*, *T*, *SOX17*, *SOX7* or *AFP* transcripts were detected in dCas9/*OCT4A* sgRNA-treated cultures over the course of 12 days. By contrast, dCas9-KRAB/*OCT4A-158* sgRNA-treated cultures showed increases in *CDX2*, *SOX17* and *T* starting at day 3, in *SOX7* at day 6 and in *AFP* at day 12, consistent with the differentiation of a fraction of the treated cells down various developmental pathways. Cells treated with dCas9-KRAB/*OCT4A-12* sgRNA displayed similar expression of differentiation markers (Fig. 2G).

This proof-of-principle study demonstrates the potency of dCas9-E/CRISPRe for the activation or repression of key transcription factors in hPSCs that can have dramatic effects on gene expression and differentiation status. We suggest that this approach will have wide applicability in altering gene expression to modulate cell fate decisions in various stem cell populations. These experiments could be performed in a directed manner, or using library-based lentiviral approaches similar to those employed with shRNA libraries (Moffat et al., 2006; Kagey et al., 2010). We envisage that this system will be instrumental in dissecting regulatory networks in hPSC derivatives and thereby improving our understanding of their contribution to development or disease.

MATERIALS AND METHODS

sgRNA *in silico* design

Candidate sgRNAs were identified by searching for $G(N)_{20}GG$ motifs 300 bases upstream and 100 bases downstream of the TSS that conform with the nucleotide requirements for U6 Pol III transcription and the spCas9 PAM recognition element (NGG) (Jinek et al., 2012; Mali et al., 2013b). Bowtie2 was used to map candidate targets to the human genome (build GRCh37) (Langmead and Salzberg, 2012) with sensitive parameters (--local -f -k 10 --very-sensitive-local -L 9 -N 1) to detect potential off-target sites (supplementary material Table S1). All our sgRNAs had no other genomic matches at the alignment stringency used.

Plasmid design and construction

The human codon-optimized, nuclease-deficient Cas9 [dCas9; D10A, H840A (Jinek et al., 2012; Qi et al., 2013)] was generated by gene synthesis (GenScript). The KRAB repressor domain [residues 1-75 from ZFN10 (*Homo sapiens*) (Cong et al., 2012), Addgene 42945] was subcloned to the 3′-end of the dCas9-NLS-3xHA to generate the dCas9-KRAB lentiviral expression construct. The VP64 activation domain [from Addgene 32188 (Zhang et al., 2011)] was subcloned to the 3′-end of the dCas9-NLS-3xHA to generate the dCas9-VP64 lentiviral expression construct. The sgRNA expression lentiviral vector is based on the pLKO.1 plasmid with an oligonucleotide cloning site containing two *Bfu*AI sites for inserting guide sequences via 4-bp 5′ overhangs (ACCG and AAAC) into the sgRNA sequence (Jinek et al., 2012) (supplementary material Fig. S1).

Virus production

HEK293T/17 cells were maintained in Dulbecco's Modified Eagle Medium (Gibco, 11965) supplemented with 10% fetal bovine serum (Valley Biomedical, BS3033) and Glutamax (Gibco, 03505). HEK293T/17 cells were split and plated at 1.3×10^5 cells/cm^2. The following day, packaging plasmids and dCas9-, dCas9-E- or sgRNA-coding plasmids were transfected using TransIT-293 transfection reagent (Mirus, 2700) in Opti-MEM (Gibco, 31985) according to the manufacturer's instructions. Virus was harvested 48 hours after transfection.

Human embryonic stem cell culture

H1 cell lines were maintained on Matrigel (BD Biosciences, 354277) in mTeSR1 (Stem Cell Technologies, 05850). TRE-regulated dCas9 or dCas9-E lines were supplemented with 100 ng/ml geneticin (Gibco, 10131) and EF1α-regulated dCas9-E lines with 1 µg/ml puromycin (Sigma-Aldrich). Cells were fed daily and split every 3-4 days with TrypLE Express (Gibco, 12604) in the presence of 10 µM Y-27632 (Selleck Chemicals, S1049).

Generation of stable dCas9/dCas9-E cell lines and co-expression with sgRNAs

H1 cells were washed with PBS and singularized with TrypLE Express. Cells were incubated with TRE-regulated or EF1α-regulated dCas9 or dCas9-E lentivirus on low attachment plates. After 3 hours, cells were plated onto Matrigel-coated plates with 10 µM Y-27632. From 48 hours after transduction, EF1α-regulated dCas9-E transduced cells were treated with 1 µg/ml puromycin and TRE-regulated dCas9 or dCas9-E transduced cells with 100 ng/ml geneticin to select and maintain stable cell lines. For experiments utilizing sgRNAs, the appropriate stable dCas9 or dCas9-E cell lines were incubated with sgRNA lentiviruses as above and plated at 1.25×10^4 cells/cm^2. Forty-eight hours after transduction, TRE-regulated dCas9 or dCas9-E sgRNA transduced cells were treated with 1 µg/ml puromycin to select for cells expressing the sgRNA and 2 µg/ml doxycycline (Sigma-Aldrich) to induce expression of dCas9 or dCas9-E (day 0). An sgRNA targeting the CAG (CMV-IE, chicken actin, rabbit beta globin) promoter was used as an off-target control.

Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde for 30 minutes at room temperature then blocked for 45 minutes with 5% donkey serum (Jackson ImmunoResearch, 017-000-121) in PBST [PBS + 0.2% Triton X-100 (Sigma-Aldrich)]. Cells were incubated with primary antibodies in blocking buffer (supplementary material Table S2) for 3 hours at room temperature, then washed three times with PBST. Cells were incubated with Alexa-Fluor-conjugated secondary antibodies (Invitrogen, 1:300) for 2 hours at room temperature and washed three times with PBST. Nuclei were stained with Hoechst (Invitrogen, H3570). Phase contrast images were acquired on Nikon Eclipse TS100 and fluorescent images on Nikon Eclipse Ti microscopes. Cells were quantified using NIS-Elements Analysis Software. Ten random fields at 20× magnification were counted (3400-5000 cells identified through Hoechst staining) and the mean fluorescence intensity of SOX17 signal in each cell calculated based on a threshold set using the CAG-sgRNA control.

Quantitative PCR analysis

RNA was isolated using Trizol Reagent (Invitrogen, 15596-018) according to the manufacturer's instructions. Total RNA (2 µg for *SOX17* analysis, or 250 ng for *OCT4* analysis) was reverse-transcribed using SuperScript III

First-Strand Synthesis System (Invitrogen, 18080-051). cDNA (30 ng for *SOX17* analysis, or 3.75 ng for *OCT4* analysis) was utilized in qPCR reactions using specific primers listed in supplementary material Table S3 in iTAQ Universal SYBR Green Supermix (Bio-Rad, 172-5124) or by the following TaqMan assays: *ACTB* (Hs01060665_g1), *SOX17* (Hs00751752_s1). Relative gene expression was calculated using the ΔΔCT method; all genes were normalized to *ACTB*.

Epigenetic data analysis

Fig. 1A and Fig. 2A were generated from the Integrative Genome Viewer (IGV) (Robinson et al., 2011) using the publicly available ENCODE epigenetic sequence data for the human H1 cell line.

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Competing interests

The authors declare no competing financial interests.

Author contributions

R.M. and S.A.W. developed the concepts and approach, performed experiments and data analysis. N.A.K., R.M.J.G. and M.S.E. developed the approach, performed experiments and data analysis. M.G. developed the sgRNA target analysis approach. All authors prepared and edited the manuscript prior to submission.

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Supplementary material

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