CRISPR-Based Chromatin Remodeling of the Endogenous Oct4 or Sox2 Locus Enables Reprogramming to Pluripotency

Highlights

- Endogenous Oct4 and Sox2 can be targeted and activated by CRISPR activation

- Activation of endogenous Oct4 or Sox2 triggers reprogramming to pluripotency

- Oct4 promoter and enhancer are simultaneously remodeled by dCas9-SunTag-p300core

- Authentic induced pluripotent stem cells are generated with CRISPR activation

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In Brief

Ding and colleagues demonstrate that induced pluripotency can be achieved through targeted activation of endogenous Oct4 or Sox2 genes. With CRISPR activation, the promoter and enhancer are specifically remodeled, Oct4 or Sox2 is derepressed in fibroblasts, and reprogramming is triggered toward pluripotency.
CRISPR-Based Chromatin Remodeling of the Endogenous Oct4 or Sox2 Locus Enables Reprogramming to Pluripotency

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SUMMARY

Generation of induced pluripotent stem cells typically requires the ectopic expression of transcription factors to reactivate the pluripotency network. However, it remains largely unclear what remodeling events on endogenous chromatin trigger reprogramming toward induced pluripotent stem cells (iPSCs). Toward this end, we employed CRISPR activation to precisely target and remodel endogenous gene loci of Oct4 and Sox2. Interestingly, we found that single-locus targeting of Sox2 was sufficient to remodel and activate Sox2, which was followed by the induction of other pluripotent genes and establishment of the pluripotency network. Simultaneous remodeling of the Oct4 promoter and enhancer also triggered reprogramming. Authentic pluripotent cell lines were established in both cases. Finally, we showed that targeted manipulation of histone acetylation at the Oct4 gene locus could also initiate reprogramming. Our study generated authentic iPSCs with CRISPR activation through precise epigenetic remodeling of endogenous loci and shed light on how targeted chromatin remodeling triggers pluripotency induction.

INTRODUCTION

Pluripotent stem cells hold great promise for regenerative medicine. A better understanding of how endogenous chromatin remodeling leads to pluripotency induction is of significant interest. Conventionally, differentiation of somatic cells can be reprogrammed into induced pluripotent stem cells (iPSCs) by ectopic expression of Oct4, Sox2, Klf4, and c-MyC (OSKM) (Takahashi and Yamanaka, 2006). Overexpressed Oct4, Sox2, and Klf4 initially bind to and globally remodel endogenous loci across the genome (Soufi et al., 2012), ultimately leading to establishment of pluripotent regulatory circuitry.

However, it is largely unknown what precise remodeling events on endogenous chromatin trigger reprogramming toward pluripotency. First of all, whether simultaneous remodeling of a large number of pluripotency-related loci is necessary or precise remodeling of a single locus is sufficient for iPSC induction is not clear. Besides, Oct4, Sox2, and Klf4 target the distal elements of many genes required for reprogramming (Soufi et al., 2012), but how the remodeling of these distal elements would affect pluripotency induction is poorly understood. Furthermore, epigenetic remodeling is the central mechanism of cellular reprogramming (Smith et al., 2016), but it has not been determined whether iPSC induction can be initiated by epigenetic manipulation of any defined endogenous loci.

Single-cell analysis and computational modeling suggested that activation of endogenous Sox2 gene marked a deterministic event to pluripotency, likely triggering reprogramming toward iPSCs (Suganum et al., 2012). However, due to the methodological limitations, there is no direct evidence of whether pluripotency can be induced by precise remodeling of Sox2 locus.

Recently, the type II clustered regularly interspaced short palindromic repeat and Cas9 nuclease (CRISPR/Cas9) system from bacteria was repurposed as a powerful tool for genome editing in mammalian cells (Cong et al., 2013; Jinek et al., 2012; Mali et al., 2013b). A deactivated form of Cas9, dead Cas9 (dCas9), has been engineered as programmable synthetic transcription factors when fused with transactivation domains, which is termed the CRISPR activation (CRISPRa) system (Chavez et al., 2015; Gilbert et al., 2013; Konermann et al., 2015; Tanenbaum et al., 2014; Zalatan et al., 2015). This system reportedly can function as a pioneer factor to target the silenced chromatin locus with high precision and promote downstream gene transcription (Polstein et al., 2015). Moreover, Hilton and colleagues showed that dCas9-p300 core fusion protein can be used to manipulate the histone acetylation of targeted genomic sites (Hilton et al., 2015). With these features, the CRISPRa system provides an advantageous tool to precisely remodel endogenous chromatin loci for cellular reprogramming (Black et al., 2016; Chakraborty et al., 2014).

In this study, using CRISPR activation, the SunTag system, we demonstrated that precise remodeling of endogenous Oct4 or Sox2 gene locus was sufficient to induce pluripotency.
RESULTS

Activation of Endogenous Oct4 and Sox2 with dCas9-SunTag-VP64

To determine whether and how remodeling of endogenous loci initiate reprogramming toward pluripotency, we used the Sun-Tag system to precisely remodel endogenous pluripotency gene loci in mouse embryonic fibroblasts (MEFs). dCas9-Sun-Tag-VP64 was chosen for its enhanced chromatin-remodeling activity by recruiting multiple VP64 to one targeting site (Figure 1A) (Tanenbaum et al., 2014). dCas9 expression was controlled by a Tet-On promoter.

Oct4 and Sox2 loci were selected as targets because of their central roles in pluripotency induction and maintenance. Single guide RNAs (sgRNAs) were designed to target the Oct4 and Sox2 promoters, as well as the Oct4 enhancer. Besides the activation effect of sgRNAs, multiple factors were considered, regarding the genomic sequences targeted, including their proximity to but no overlapping with the binding sites of pluripotent factor and transcription machinery, histone H3K27 acetylation in pluripotent stem cells, and their potential to form promoter-enhancer loops mediated by Mediator complex (Figures S1A–S1C).

We first examined transcriptional activation of target genes with each designed Oct4 and Sox2 sgRNA delivered by lentivirus in differentiating mouse embryonic stem cells (ESCs) (Figure S1D). Mouse ESCs were first transduced with dCas9-SunTag-VP64 system and sgRNAs. Because Oct4 and Sox2 are highly expressed in ESCs, we induced ESC differentiation with 1 μM retinoic acid (RA). Meanwhile, the dCas9-SunTag-VP64 system was induced with doxycycline. Analysis of Oct4 expression showed that sgRNAs targeting a narrow promoter region close to the transcription start site (TSS), and a 200-bp region of distal enhancer can enhance the transcription (Figure S1 E). As for the Sox2 promoter, sgRNA activity showed a remarkable tendency for higher gene activation with sgRNAs closer to the TSS (Figure S1E).

Selected sgRNAs and the dCas9-SunTag-VP64 were also transduced into MEFs (Figure S1F). sgRNAs O-127 and O-71 targeting 127- and 71-bp upstream of Oct4 TSS were combined to target the promoter. Similarly, O-1965, O-2066, and O-2135 were combined to target the Oct4 enhancer; and separately, S-84, S-136, and S-148 were combined for Sox2 promoter targeting. After 4 days of dCas9 induction by doxycycline, targeting the Oct4 promoter led to about a 100-fold increase in Oct4 transcription, and targeting the enhancer resulted in modest activation (Figure S1 G). For Sox2 promoter, about 15-fold activation was detected (Figure S1G). This suggests that, guided by specific sgRNAs, dCas9-SunTag-VP64 can activate the silenced Oct4 and Sox2 in MEFs.

Figure 1. Establishment of Pluripotency Network in MEFs by Gene Activation

(A) Scheme depicting the dCas9-SunTag-VP64 function in gene activation.

(B) Scheme depicting the reprogramming procedure in OG2 MEFs.

(C) OG2 MEFs were reprogrammed to form EGFP-positive colonies. The morphology of MEFs on day 0 and reprogramming colonies on days 7 and 15 were shown (scale bar, 200 μm).

(D) Endogenous Oct4 and Sox2 transcription over 12 days.

(E) Colonies showing EGFP signal in situ and at passages 1 and 20 (scale bar, 200 μm).

(F) Nanog, Sox2, and SSEA-1 staining in the EGFP-positive colonies (scale bar, 200 μm).

(G) Pluripotent gene expression in established CRISPR iPSCs. R1 ES, R1 mouse ESCs. Data in (D) represent mean ± SD (n = 4). p values were determined by one-way ANOVA with Dunnett test. **p < 0.01. See also Figure S1.
Establishment of Pluripotency Network in MEFs by Gene Activation with dCas9-SunTag-VP64

We next sought to determine whether pluripotency network can be fully reactivated and established in MEFs. We optimized the SunTag reprogramming system in two ways. First, more gene promoters were targeted by adding the corresponding sgRNAs. Klf4, c-Myc (Takahashi and Yamanaka, 2006), Nr5a2 (Heng et al., 2010), Glis1 (Maekawa et al., 2011), and Cebpa (Di Stefano et al., 2014) were selected. For each promoter, 4–10 sgRNAs were designed and tested in differentiating ESCs (Figure S1E). 1–3 sgRNAs for each promoter were included in the previous Oct4/Sox2 sgRNA pool (Table S1). Second, a small-molecule cocktail, consisting of Parnate, Chir99021, A83-01, and Forskolin (PCAF), was added into our reprogramming medium. This chemical cocktail further increased Oct4 and Sox2 transcription by 3–4 times on day 4 (Figure S1H).

To monitor the reactivation of pluripotency network, we used OG2 MEF cells that harbor a stable Oct4-Egfp reporter and exhibit intense EGFP signal when endogenous Oct4 is actively transcribed (Szabo´ et al., 2002). After transduction of dCas9-SunTag-VP64 and the sgRNA pool (18 sgRNAs in total, Table S1), the MEF medium was changed to reprogramming medium with doxycycline. This was denoted as day 0 (Figure 1B). Since day 4, transcription of Oct4 and Sox2 became more and more robust (Figure 1D). By day 7, reprogramming clusters appeared, and after 2 weeks, EGFP-positive colonies were visible (Figure 1C). Those colonies were also positive for Nanog, Sox2, and SSEA-1 (Figure 1F).

Then, EGFP-positive colonies were expanded on feeder cells to generate CRISPR iPSC lines. Those CRISPR iPSCs formed typical mouse ES-like domed colonies with a strong EGFP signal (Figure 1E). A panel of pluripotency genes, including Oct4, Sox2, Nanog, Esrb, Nr5a2, and Utf1, was highly expressed (Figure 1G). These cells can be passaged for more than 20 passages without any sign of losing the EGFP signal or ES morphology (Figure 1E). A panel of pluripotency genes, including Oct4, Sox2, Nanog, Esrb, Nr5a2, and Utf1, was highly expressed (Figure 1G). These cells can be passaged for more than 20 passages without any sign of losing the EGFP signal or ES morphology (Figure 1E).

During OSG reprogramming, we surprisingly noticed that EGFP-positive colonies appeared when S-84 alone was used (Figure S2F), suggesting that targeting Sox2 promoter alone may be sufficient for pluripotency induction. To rule out the possibility of an off-target effect from S-84, we examined the top 10 predicted targets of S-84, and only the Sox2 gene was significantly activated (Figure S2G). Sox2 protein was also detected on day 4 (Figure 2B). Besides, we repeated the reprogramming tests with another two Sox2 sgRNAs, S-136 and S-148 (Figure 2A). These two sgRNAs individually activated endogenous Sox2 transcription (Figure 2C), and EGFP-positive colonies were obtained (Figure 2D).

Then, we examined whether the iPSCs were authentic pluripotent. Within these EGFP-positive colonies, Nanog and SSEA-1 protein was also detected, and CRISPR iPSC lines were established (Figures 2E and 2F). For line S-17, expression of key pluripotent factors was similar to that in R1 cells (Figure 2G). These cells were also karyotypically normal (Figure 2H). A more stringent assay for pluripotency was performed. S-17 cells were injected into the blastocysts of B6(Cg)-Tyrc-2J/J (B6-albino) background, and EGFP-positive cells were found in the gonadal regions of 71.4% (5 out of 7) E13.5 embryos (Figure 2I). Live-born chimeras were generated (Figure 2J), and the rate was 46.2% (6 out of 13). More importantly, the S-17 cells were also germline competent (Figure 2K). With these data, we concluded that single-locus targeting of the Sox2 promoter by one sgRNA was sufficient to reprogram MEFs into authentic pluripotent stem cells.

S-17 MEFs Are Reprogrammable with Higher Efficiency and Less Variation

With the lentiviral transduction, the reprogramming efficiency was relatively low and variable in both OG2 and 129 background MEFs (0.0%–0.013%) (Figures 2C, 3A, 3D, and 3E–C). This may be due to inefficient delivery of SunTag components and random copy numbers of the components delivered in single cells. This was reflected by the varied copy numbers of sgRNA cassette in the genomes of established iPSC lines, and one to five copies were found per cell among 12 lines (Figure 3D). To decrease the variability and enhance the efficiency, we decided to generate secondary MEFs using a CRISPR iPSC line that was derived from single colony.

S-17 iPSCs were labeled with blue fluorescence protein (BFP) and injected into B6 blastocysts, and secondary MEFs were derived from the E13.5 embryos (Figure 3A). About half of the MEFs (52.4%) were originated from the S-17 iPSCs revealed by flow cytometry (Figure 3E), and these secondary MEF cells were termed S-17 MEFs. With doxycycline, endogenous Sox2 was readily detected by both qPCR and immune-fluorescent staining, but no Sox2 was detected without doxycycline (Figures 3B and 3D). No off-target genes were dramatically elevated (Figure 3G). These data demonstrate that the SunTag system functioned properly to activate Sox2 in S-17 MEFs.

Then the S-17 MEFs were examined if they were reprogrammable. Sox2 transcription was significantly upregulated on day 4 and increased quickly to R1 mouse ES level by day 8 (Figure 3B). Following Sox2 upregulation, other core pluripotent factors, Oct4, Nanog, and Rex1, were also activated. Their transcription was detected on day 8 and elevated dramatically after that (Figure 3G). Meanwhile, morphological changes were
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observed from day 4, and EGFP-positive colonies were visible on day 7 (Figure 3E). iPSC lines could also be established (Figure 3E). With S-17 MEFs, the reprogramming efficiency (0.1%) increased by 40-fold over the lentivirus method (Figure 3F). As expected, much less variability was observed (Figure S3H).

We also tested whether more differentiated tail tip fibroblasts (TTFs) were reprogrammable. We derived S-17 TTFs from the 14-month-old chimeric mouse. In presence of doxycycline, TTFs underwent morphological changes, and EGFP-positive colonies were obtained in 2 weeks (Figure S3L). These observations show that S-17 MEFs and TTFs were reprogrammable.

Remodeling of Sox2 Promoter Triggers Reprogramming toward Pluripotency in S-17 MEFs

Without doxycycline, we could not see the activation of Sox2, and no colonies were obtained (Figures 3B and 3J). When the PCAF cocktail was removed, EGFP-positive colonies were still generated (Figure S3I), although with lower efficiency. Based on this, we concluded that endogenous Sox2 activation was the trigger for S-17 MEF reprogramming.

Then, we examined whether the reprogramming was dose dependent on Sox2 level. Sox2 was activated with a series of doxycycline concentrations (e.g., 0, 0.01, 0.1, and 1 μg/mL). We noticed that Sox2 level showed a positive correlation with the dox concentrations, and the reprogramming efficiency was clearly dependent on Sox2 level (Figure 3J).

VP64 promotes gene transcription and chromatin remodeling by recruiting multiple epigenetic modifiers (Hirai et al., 2010), so we tested how the SunTag system epigenetically remodeled the Sox2 promoter. Chromatin immunoprecipitation (ChIP) was performed with H3K27 acetylation (H3K27ac) antibody against the Sox2 promoter. As early as day 4, the H3K27ac level was already elevated 2-fold, and it further increased on days 8 and 12 (Figure 3C). This indicates that the SunTag targeting caused gradual and constant epigenetic remodeling at the Sox2 promoter. We also checked the promoters of Oct4, Nanog, and Rex1, and their H3K27ac levels increased significantly with a 4-day latency, similar to the gene transcription (Figures 3G and 3H). Interestingly, the enhancers of Oct4 showed simultaneous elevation of H3K27ac level (Figure 3F). These data suggest that activation of Sox2 facilitated following induction of other key genes for pluripotency establishment.

We then tested whether additional targeting of the Oct4 promoter in S-17 MEFs would promote the reprogramming efficiency. Transduction of O-127 led to a significant increase of Oct4 transcription, and the reprogramming efficiency was enhanced too (Figure 3K). This synergistic effect supported the idea that Oct4 and Sox2 cooperated in pluripotency induction.

We also compared the S-17 MEF reprogramming to traditional reprogramming using overexpressed factors. Unlike S-17 MEFs, the overexpressed factors failed to epigenetically remodel the Sox2 promoter on day 4 (Figure S3J), and no Sox2 transcription from the endogenous loci was effectively detected on days 4 and 12 (Figure 3L). After 3 weeks, overexpressed Oct4 or Sox2 failed to generate any colonies, and overexpression of Oct4, Sox2, and Klf4 (OSK) generated EGFP-positive colonies slightly more than S-17 MEFs with more variation between experiments (Figures 3M and S3K).

Simultaneous Remodeling of the Oct4 Promoter and Enhancer Reprograms MEFs to iPSCs

Previously, we noted that remodeling of both the Oct4 promoter and enhancer is important for pluripotency induction and that targeting the promoter alone is not sufficient for the generation of EGFP-positive colonies (Figures S2A and 2C). Given the fact that key pluripotency factors as well as p300 and the Mediator complex are enriched at the Oct4 distal enhancer in mouse ESCs (Figure 4A), we hypothesize that simultaneous remodeling of the Oct4 promoter and enhancer is required for pluripotency induction.

To test that, we used a dual-sgRNA cassette that transcribed two sgRNAs targeting different sites (Figure S4A). The O-127-2066 cassette targets the Oct4 promoter (O-127) and enhancer (O-2066) at a single-cell level. This led to simultaneous remodeling of promoter and enhancer with elevated levels of H3K27ac (Figure 4B). The gene transcription with O-127-2066 was similar to O-127 at days 4 and 8 (Figure 4C). However, after day 8, Oct4 transcription was further elevated in O-127-2066 culture. Particularly, when we replaced the cells on days 7 and 11 to allow cell expansion, the overall Oct4 expression in the population dramatically increased (Figure 4C). For O-127 and O-2066 cultures, weak Oct4 expression largely stayed unchanged after day 8 (Figure 4C). Accordingly, by day 12, EGFP-positive colonies were observed in the O-127-2066 culture, and those colonies also expressed Nanog, Sox2, and SSEA-1, indicating the acquired core pluripotency network (Figures 4E and 4F). iPSC lines could be derived from these colonies (Figure 4E). Meanwhile, no colonies were found in the O-127 or O-2066 culture (Figure 4D).

The activation of potential off-targets was checked. The top 10 predicted targets for sgRNAs O-127 and O-2066 were
Figure 3. Remodeling of Sox2 Promoter Triggers Reprogramming toward Pluripotency in S-17 MEFs

(A) Scheme showing the generation of S-17 MEFs.

(B) Sox2 activation over 12 days with or without doxycycline in S-17 MEFs.

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examined, and no dramatically activation for off-target genes was seen (Figure S4C). We also tested another two dual sgRNA cassettes O-127-1965 and O-127-2135 in parallel, and similar results were observed (Figure 4D). These data strongly supported that pluripotency was induced by simultaneous remodeling of endogenous Oct4 promoter and enhancer.

An authentic pluripotent stem cell line was also achieved. The D-9 line showed similar expression of pluripotency genes to R1 cells and a normal karyotype (Figures 4G and 4H). After injection of D-9 cells into B6-albino blastocysts, live-born chimeric mice were generated at the rate of 50% of male pups (2 out of 4) were generated at the rate of 60% of the offspring pups (6 out of 8) cells and a normal karyotype (Figures 4G and 4H). After injection of endogenous Oct4 promoter and enhancer regions (Figure 4A), p300core only has the acetyltransferase activity domain of p300 and was proved to enhance the targets’ histone acetylation (Hilton et al., 2015). So we replaced VP64 and generated a dCas9-SunTag-p300core system (Figure S4E).

Reprogramming experiments were performed with this dCas9-SunTag-p300core system. We found that p300core culture exhibited similar H3K27ac level to the VP64 counterpart at Oct4 promoter and enhancer, but only 1/30 of the Oct4 transcription was detected in p300core culture at day 5 (Figures S4F and S4G). This can be explained by p300core’s inability to recruit transcription machinery. Cultures were then passaged on days 9 and 14. Interestingly, by day 10, Oct4 levels were comparable in the VP64 and p300core conditions (Figure S4G). Accordingly, EGFP-positive colonies were produced in the p300core cultures, and iPSC lines were generated (Figures S4H and S4I). These observations indicate that the manipulation of histone acetylation with p300core led to chromatin remodeling similar to VP64, although with a noticeable latency in transcriptional activation. Together, our results show that the epigenetic remodeling of Oct4 promoter and enhancer, either through VP64 or p300core, is sufficient to trigger reprogramming toward pluripotency.

**DISCUSSION**

In this study, we reported that iPSCs were generated with CRISPRa system by targeting single genes, Oct4 or Sox2. The activation of endogenous pluripotent genes had been examined previously with CRISPRa systems, but no iPSCs were established. Several groups succeeded in activating endogenous OCT4 and SOX2 in human 293T cells by targeting the promoter, and murine cells were also tested in some cases (Cheng et al., 2013; Hilton et al., 2015; Hu et al., 2014; Mali et al., 2013a). The Feng lab systematically examined CRISPR activation effect at the mouse Oct4 promoter, and they found that sgRNAs targeting 147 to 89 bp upstream of the TSS was the most effective (Hu et al., 2014), which is similar to our finding (Figure S1E). However, they didn’t target the enhancers and only observed transient activation with the promoter, like we did with O-127 alone (Figure 4C). In our study, we designed the sgRNAs de novo and selected sgRNA target sites based on multiple parameters (Figures S1A–S1C). The SunTag system we used can be very efficient in gene activation and chromatin remodeling because as many as 24 VP64 may be recruited to the targeting sites. We observed a 100-fold increase in Oct4 activation, which was much higher that previous work (Hu et al., 2014). Besides, small molecules further enhanced the reprogramming efficiency (Figure S3H). Recently, two studies reported generation of muscle and neuron cells by activating endogenous MyoD or BAM (Brn2, Ascl1, and Myt1L) with a VP64dCas9VP64 system (Black et al., 2016; Chakraborty et al., 2014). Our work established pluripotent stem cells with CRISPRa method.

In our study, we mechanistically specified that direct remodeling of endogenous Oct4 or Sox2 is sufficient to trigger reprogramming toward pluripotency. This not only provides an alternative way for iPSC generation, but also provide insights into the molecular mechanism of pluripotency induction. The Sox2 study proved that activation of endogenous Sox2 is a critical event for pluripotency induction. This, in part, is consistent with a previous study illustrating that activation of endogenous Sox2 marked a deterministic stage to pluripotency (Buganim et al., 2012). We clearly showed that Sox2 activation was required for S-17 MEF reprogramming, the remodeling of Sox2 preceded other key pluripotent gene activation, and the reprogramming efficiency was dependent on Sox2 levels (Figures 3B, 3C, 3G–I, and 3J). Meanwhile, we also noticed that although 20% of the S-17 population activated the endogenous Sox2, only 0.1% could be

(C) The histone H3K27 acetylation levels at the Sox2 promoter on days 0, 4, 8, and 12.

(D) Detection of Sox2 expression by immunofluorescent staining in presence of doxycycline (scale bar, 100 μm).

(E) S-17 MEFs were reprogrammed to form EGFP-positive colonies, and iPSC line was established (scale bar, 200 μm)

(F) Efficiency comparison of lentiviral SunTag reprogramming and S-17 MEF reprogramming.

(G) Gene expression of Oct4, Nanog, and Rex1 over 12 days in S-17 MEF reprogramming.

(H) The histone H3K27 acetylation levels at the Oct4, Nanog, and Rex1 promoter on days 0, 4, 8, and 12.

(I) The histone H3K27 acetylation levels at the Oct4 enhancer on days 0, 4, 8, and 12.

(J) Sox2 dependency in S-17 MEF reprogramming. 4 different concentrations (0, 0.001, 0.1, and 1 μg/mL) of doxycycline were used, and the Sox2 activation (left) and reprogramming efficiency (right) were shown.

(K) The cooperativity of Oct4 and Sox2 remodeling in S-17 MEF reprogramming. Oct4 gene activation (left) and reprogramming efficiency (right) were examined.

(L) The total (left) and endogenous Sox2 (right) expression on days 4 and 12 when pluripotent genes were overexpressed (OE). Three conditions were tested, Oct4 alone, Sox2 alone and OSK (Oct4, Sox2, and Klf4), and the overexpression of mCherry (mCh) worked as control.

(M) Reprogramming efficiency comparison for reprogramming with S-17 MEFs and pluripotent gene overexpression. Data in (B), (C), (G)–(I), and (K) represent mean ± SD (n = 4). p values in (B) were determined by two-way ANOVA with Bonferroni test, p values in (C) and (G)–(I) were determined by one-way ANOVA with Dunnett test, and p values in (K) were determined by unpaired t test. *p < 0.01; **p < 0.05. See also Figure S3.
Figure 4. Simultaneous Remodeling of Oct4 Promoter and Enhancer Reprogramms MEFs to iPSCs

(A) Scheme depicting the sgRNA targeting sites for Oct4 promoter and enhancer along with the binding peaks of transcription factors (Oct4, Sox2, Nanog), histone acetyltransferase p300, and the Mediator complex, as well as the distributions of histone H3K27ac and DNase hypersensitive sites (DHS) from mouse ENCODE and previous work (Whyte et al., 2013).

(B) The histone H3K27 acetylation levels at the Oct4 enhancer and promoter on day 4. Three different sites were examined, 2.7, 1.4, and 0.2 kb upstream of the transcription start site.

(C) Endogenous Oct4 transcription in the presence of O-127, O-2066, or O-127-2066 sgRNA over 16 days.

(D) Colony numbers generated from remodeling of the Oct4 promoter (O-127), enhancer (O-2135, O-2066, O-1965), or promoter and enhancer simultaneously (O-127-2135, O-127-2066, O-127-1965). Four independent experiments are shown, no colony was observed in O-127-2135 culture of experiment 3 and O-127-2066/1965 of experiment 4.

(E) The morphology of EGFP-positive colonies in situ and the P0 iPSCs from simultaneous remodeling of Oct4 promoter and enhancer (O-127-2066) (scale bar, 200 μm).

(F) Nanog, Sox2, and Rex1 expression in the Oct4-EGFP-positive colonies (scale bar, 200 μm).

(G) Comparison of pluripotency gene expression in D-9 cell line and R1 mouse ESCs (R1 ES).

(H) Karyotyping of D-9 line.

(I–K) Characterization of the pluripotent D-9 line in vivo. The chimeric mice are generated with D-9 cells (J), and those cells contributed to the gonadal tissue represented by the cells with intensive EGFP signal (I) and gave rise to offspring (K).

Data in (B) and (C) represent mean ± SD (n = 4). p values were determined by unpaired t test. **p < 0.01. See also Figure S4.
reprogrammed into EGFP-positive colonies (Figures 3F and S3F), suggesting that sole activation of endogenous Sox2 is not determinant to pluripotent cell fate in our CRISPRa context. Further activating Oct4 enhanced the efficiency of generating EGFP-positive colonies (Figure 3K), supporting the cooperativity of multiple pluripotent locus reprogramming in pluripotency induction. In the Oct4 study, notably, we found the remodeling of enhancer region was required for pluripotency induction. We observed robust transcriptional activation from the promoter remodeling and modest gene activation from the enhancer remodeling. However, the remodeling of enhancer seems essential for further induction of Oct4 at a later stage (Figure 4C). This suggests that the Oct4 promoter functions as a fast trigger, and the enhancer is a regulator required for latent but higher Oct4 transcription. Whether enhancer remodeling in our study facilitated the establishment of promoter-enhancer loop as seen in naive mouse ESCs needs to be further investigated (Kagey et al., 2010). Another interesting point is that this enhancer is among the 231 superenhancers found specifically to pluripotent stem cells (Whyte et al., 2013). Our work provided functional evidence for superenhancer in pluripotency induction.

Meanwhile, the generation of iPSCs with dCas9-SunTag-p300core revealed that histone acetylation plays an essential role in iPSC generation. Cellular reprogramming involves dynamic epigenetic changes, but whether reprogramming can be achieved through epigenetic manipulation of defined genomic sites is not known. In pluripotent stem cells, histone H3K27 acetylation is highly enriched on both promoter and enhancer of Oct4, which provides an entry to tackle this question by manipulating only one type of epigenetic modification. In our study, iPSCs were generated through dCas9-SunTag-p300core simultaneous targeting of the promoter and enhancer. This also paved a way to change cell fate by site-specific manipulation of epigenetic modifications. Besides p300, several other epigenetic factors (i.e., Tet1, Dnmt3a, KRAB, and LSD1) have been verified as functional in epigenome editing for both activating or silencing genes (Kearns et al., 2015; Liu et al., 2016; Thakore et al., 2015). These expanding CRISPR tools give rise to more possibilities to manipulate cell fate by targeting different types of DNA and histone modifications in the future.

In summary, using one of the CRISPRa systems, the SunTag system, we demonstrated that precise remodeling of endogenous Oct4 or Sox2 gene locus is sufficient to initiate reprogramming toward pluripotency. Our study not only generated iPSCs with CRISPR activation but also shed light on mechanistic understanding of cellular reprogramming. This reprogramming strategy should also work in the generation of other cell types and in other model systems, such as human cells. Because of the differences in epigenetic landscape between cell types and different genomic sequences between human and mouse, the experimental design will be different, but the strategy is straightforward.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental Information includes four figures and two tables and can be found with this article online at https://doi.org/10.1016/j.stem.2017.12.001.

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**AUTHOR CONTRIBUTIONS**

P.L. and S.D. conceived of the study. P.L. designed the experiments and analyzed data. P.L. and S.D. wrote the manuscript, and S.D. supervised the work. M.C. established the dCas9–SunTag-p300core system. Y.L. established the dCas9–SunTag-VP64 system in mouse ESCs, and L.S.Q. supervised this part of the work.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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**REFERENCES**


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# STAR METHODS

## KEY RESOURCES TABLE

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(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Sheng Ding (sheng.ding@gladstone.ucsf.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell Culture

HEK293T/17 cells were maintained in DMEM supplemented with 10% FBS.

Mouse embryonic fibroblasts (MEFs) were prepared from the E13.5 embryos, and the tail tip fibroblasts (TTFs) were derived from a 14-month old adult mouse. MEFs and TTFs were cultured in DMEM supplemented with 10% FBS and non-essential amino acid (NEAA).

All iPSC lines and R1 mouse ES cells were maintained on feeders in KO-DMEM (Invitrogen) with 5% ES-FBS (Invitrogen) and 15% KO-serum replacement (KSR, Invitrogen), 1% GlutaMAXTM (Invitrogen), 1% nonessential amino acids (NEAA, Invitrogen), 55 µM 2-mercaptoethanol (Sigma), 10 ng/ml leukemia inhibitory factor (LIF, Stemgent), 3 µM CHIR99021, and 1 µM PD0325901. For micro-injection, iPSCs were maintained under feeder-free N2B27 condition (50% DMEM/F12, 50% Neurobasal Medium, 0.5% N2 medium, 1% B27 medium, 0.1 mM 2-mercaptoethanol, 10 ng/ml leukemia inhibitory factor, 25 µg/ml BSA, 3 µM CHIR99021, and 1 µM PD0325901). For ES cell differentiation, ES cells were cultured in MEF medium supplemented with 1 µM retinoic acid for 4 days.

Mice

OG2 Mice (B6;CBA-Tg(Pou5f1-EGFP)2Mnn/J) and B6 Albino mice (B6(Cg)Tyrc-2J/J) were from the Jackson Laboratory. CD-1 mice were from Charles River (Stock#022). OG2 mice were crossed for the derivation of OG2 MEFs at embryonic day 13.5. Super-ovulated female B6(Cg)Tyrc-2J/J mice (4 weeks old) were mated to B6(Cg)Tyrc-2J/J males for blastocyst preparation. The S-17 and D-9 chimeric mice were generated from the injection of S-17 or D-9 iPSCs into the blastocysts. The injected blastocysts were implanted into uteri of 2.5 d post-coitum pseudopregnant CD-1 female mice (5–6 weeks). B6 Albino female mice (6 weeks) were used to mate with the chimeric mice (4–8 weeks) for germline transmission tests. 129 mice (129S2/SvPasCrl) were from Charles river for the derivation of 129 MEFs. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco.

METHOD DETAILS

Plasmid Construction

For the sgRNA constructs, 72-bp oligos, including specific sgRNA sequences, were synthesized for PCR amplification with primers sgRNA-F (GTATCCCTTGGAGAACCACCT) and sgRNA-R (TGCTGTTTCCAGCTTAGCTCT). The amplified fragments were purified and used for recombination reaction according to the Gibson Assembly Cloning Kit protocol (NEB) with the pSLQ1373 construct digested with BstXI and BlpI.

For the dual-sgRNA constructs, a fragment containing the second sgRNA was amplified using primers mU6-T2H-F (ctaggatccataggccGGGTACAGTGCAGGGGAA) and mU6-T2H-R2 (atacggttatccacgcGGCCGCCTAATGGATCCT) with the single sgRNA construct as a template. This fragment was purified and used for recombination reaction with the other construct containing the first sgRNA digested by NotI. The second mU6-sgRNA cassette is downstream of the first one in the same transcription direction.

For p300core cloning, the backbone was derived from pSLQ1711-pPGK-ScFV(GCN4)-sfGFP-VP64 by digestion with SbfI-HF and Rsrl (NEB) and retrieved using gel purification kit (QIAGEN). An 83-bp SV40 nuclear localization site (NLS) with a linker was cloned and added between sgFPP and p300core with the forward primer (TACAGAGGATGAGTCCAGGCCCTAATTGAGATCC) and reverse primer (AAATCGTCTAAAGCATCTCGGCGCCGGGACGCC) and reverse primer (AAATCGTCTAAAGCATCTCGGCGCCGGGACGCC) and p300core was PCR-amplified from template pcDNA-dCas9-p300 Core (Addgene 61357) using Phusion High-Fidelity DNA Polymerase (NEB) with the forward primer (AGTGGGCGGTTCCGGCGGAGGGTACGAttttcaaaccagaagaactacgac) and reverse primer (TACAGAGGATGAGTCCAGGCCCTAATTGAGATCC) and reverse primer (TACAGAGGATGAGTCCAGGCCCTAATTGAGATCC) and p300core were assembled using Gibson assembly cloning kit (NEB).

All the constructs were sequenced for confirmation.
Lentivirus Preparation and Transduction
HEK293T/17 cells (ATCC® CRL-11268) were plated 1 day ahead to reach about 70% confluency for transfection, and VSV-G envelope expressing plasmid pMD2.G (Addgene, 12259) and psPAX2 (Addgene, 12260) were used for lentiviral packaging. Plasmids (1.8 μg) with the gene of interests were mixed with psPAX2 (1.35 μg) and pMD2.G (0.45 μg) for each well of six-well plates, and 10.8 μL FUGENE HD (Promega) was added for transfection. 5 hours later, the medium was refreshed. Supernatant containing the virus was harvested at 48 hours, passed through a 0.45-μM filter to remove the cell debris, and mixed with 1 volume of fresh medium for immediate use. For SunTag system, lentiviruses for the three components (dCas9, VP64/p300core, and Tre3G) were packaged independently and mixed when used.

For transduction, MEF cells were incubated with the lentiviral supernatant in presence of 5 μg/ml polybrene (Millipore) for 8 hours or overnight. SunTag transduction was performed in two rounds of lentiviral infection as the first round for SunTag system (dCas9, VP64/p300core, and Tre3G) and the second round for sgRNAs. Media were refreshed after each infection.

Virus Titration and sgRNA Copy Number Prediction
By detecting the blue fluorescent protein (BFP) from the sgRNA cassette, the virus titration was performed in primary MEF cells, and the multiplicity of infection (MOI) can be calculated with the Poisson distribution (Arai et al., 1999):

\[ P(k) = e^{-m} m^k / k! \]

\( m \) is the MOI, \( k \) is the virus particle number, and \( P(k) \) is the fraction of cells infected by \( k \) virus particles. For our experiments, around 70% of the MEFs were positive for BFP, and the MOI was 1.20. Similarly, the fraction of cells infected by the indicated numbers of sgRNA virus particles are as follows:

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<th>Number of virus particles</th>
<th>Fraction of Cells</th>
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</tr>
<tr>
<td>1</td>
<td>0.3614</td>
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<tr>
<td>2</td>
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<tr>
<td>3</td>
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<tr>
<td>4</td>
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</tr>
<tr>
<td>5</td>
<td>0.0062</td>
</tr>
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<td>...</td>
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MEF and TTF Derivation
E13.5 embryos were used for MEF derivation. After the embryo recovery, the head, limbs, and internal organs, especially the gonads, were removed under dissection microscope. The remaining bodies of the embryos was finely minced with two blades and digested in 0.05% Trypsin-EDTA for 15 minutes. MEF medium was then added to stop the trypsinization. Further dissociation of the tissues was performed by pipetting up and down for a few times. Cells were then collected by centrifugation and plated onto 15cm dishes for expansion (P0). MEF cells were used before P3 for all tests.

For TTF derivation, 14-month old adults were used. The tail was peeled, minced into 1mm pieces, and cultured in 6cm dish. Media was half changed every 3 days until fibroblasts migrated out of the graft pieces. Cells were then passaged and ready for use (P1).

Reprogramming and iPSC Derivation
MEF cells were seeded onto gelatin-coated plates at the density of 10,000 cells/cm² 24 hours before transduction. After transduction, cells were allowed to recover in MEF medium for 24 hours. To start reprogramming, cultures were switched to reprogramming medium (ES medium supplemented with 10 μM Parnate, 3 μM Chir99021, 1 μM A83-01, and 10 μM Forskolin) with 1 μg/ml doxycycline. This was denoted as day 0. On day 3, cultures were treated with 1 mg/ml collagenase B (Roche) for 20 minutes and then 0.05% Trypsin for 5 minutes at 37°C, replated onto new wells (30,000 cells/cm²), and further cultured until the end of reprogramming. Cultures were not further replated except where indicated in Figures 4 and S4. During the entire process, media were refreshed every other day for the first 12 days. After that, normal ES medium was used and changed every day, and EGFP-positive colonies were usually ready for iPSC derivation between days 16 and 18.

For reprogramming with S-17 MEFs or mouse tail tip fibroblasts (TTFs), MEFs or TTFs were seeded onto gelatin-coated plates at the density of 5,000 cells/cm². 24 hours later, the medium was switched to reprogramming medium with 1 μg/ml doxycycline. This was denoted as day 0. Medium was changed every other day until day 14. EGFP-positive colonies were counted for reprogramming efficiency calculation or used for iPSC line derivation.

For iPSC derivation, the reprogramming cultures were incubated with 1 mg/ml collagenase B (Roche) for 20 minutes at 37°C. Single colonies were picked up under microscope and digested in 0.05% trypsin for 5–10 minutes for single-cell suspensions. Cells were then seeded on feeders in normal ES medium, and these cells are considered as P0 iPSCs.
Off-target Prediction
The off-targets of sgRNAs were predicted by the CCTop-CRISPR/Cas9 target online predictor (Stemmer et al., 2015). For each prediction, the core sequence was set at 12 bp. The maximum mismatches of core sequence were 2 bp, and the maximum mismatch of all mismatches was 4 bp.

Quantification of sgRNA Cassette in Genome
QPCR primers were designed for the amplification of Sox2 gene and the sgRNA cassette in the genome, and the amplification of Sox2 worked to normalize the genome for each cell line. Plasmids containing the targets was used for standard curve generation. The standard curves were generated by plotting Ct values against the plasmid copy numbers of a serial of plasmid dilutions (10 pg, 1 pg, 0.1 pg, and 0.01 pg), and the copy numbers of Sox2 gene and sgRNA cassettes in around 30 ng of genomic DNA was calculated based on the standard curve. sgRNA copy numbers were then calculated by normalizing to Sox2 gene (2 copies/cell).

Quantitative RT-PCR (qPCR)
Total RNA was extracted from samples at the indicated times with the RNeasy Plus mini kit with QiaShredder (QIAGEN) and treated with DNA-free Kit (Ambion) to remove genomic DNA. RNA was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad). Quantitative PCR was performed with iQ™ SYBR Green Supermix (Bio-Rad) on the 7500 Fast Real-Time PCR System (Applied Biosystems). All reactions were done in quadruplicate. All data were statistically analyzed with Prism 7.

Flow Cytometry
Cells were trypsinized to form single cell suspension in FACS buffer (2% FBS in DPBS). The suspension was filtered through 40 μm cell strainer before it was examined by the MACSQuant VYB flow cytometer. The data was analyzed with FlowJo v10.

Immunofluorescent Staining
Cells were washed three times with DPBS and fixed with 4%PFA for 30 minutes at 4°C. Donkey serum (10% in DPBS) was used for blocking for 1 hour at 4°C. Antibodies were diluted in DPBS with 1% BSA. The following primary antibodies were used for staining: anti-Sox2 (1:1000, Millipore, AB5603), anti-Oct4 (1:1000, Santa Cruz, sc-5279), anti-Nanog (1:500, Abcam, 80892), and anti-SSEA-1 (1:200, Stemgent, 09-0095).

Chromatin Immuno-Precipitation (ChIP)
All ChIP experiments were performed with EZ-ChIP Chromatin Immunoprecipitation kit (Millipore, 17-371), following the protocol provided with the kit with modifications. Briefly, about 2×10⁶ cells were crosslinked with 0.275 mL of 37% formaldehyde to 10 mL of growth medium. 1 mL of 1.25 M glycine (10X) were added to quench unreacted formaldehyde. 0.12 mL of SDS lysis buffer was used for each sample. Genomic DNA was then sheared to a length of 100–500 bp on Covaris S2 Sonicator with optimized conditions. 1.5 μg of H3K27 acetylation antibody (Abcam, ab4729) and 15 μL of magnetic protein A/G beads (Millipore 16-663) were used for each sample. Finally, DNA fragments were eluted with 50 μL of elution buffer C, which was used for downstream qPCR.

Cell Line Karyotyping
iPS lines karyotyping was performed at Cell Line Genetics by analyzing the Giemsa binding. Detail method are as reported elsewhere.

Chimeric Mice Generation and Germline Transmission Tests
For blastocyst injection, iPSCs were cultured under N2B27 condition without feeders. On the day of injection, cells were suspended in Blastocyst Injection Medium (25 mM HEPES-buffered DMEM plus 10% FBS, pH 7.4).
Super-ovulated female B6(Cg)-Tyr<sup>C2/J</sup> mice (4 weeks old) were mated to B6(Cg)-Tyr<sup>C2/J</sup> males. Morulae (2.5 d post-coitum) were collected and cultured overnight in KSOM medium (Millipore) at 37°C in 5% CO<sub>2</sub>. The next morning, the blastocysts were ready for iPSC injection, and approximately 10–20 cells were injected for each blastocyst. Injected blastocysts were cultured in KSOM medium at 37°C in 5% CO<sub>2</sub> for 1–2 hours and then implanted into uteri of 2.5 d post-coitum pseudopregnant CD1 female mice. Chimeric mice can be identified by the mosaic coat color. The male chimeric mice are further mated with female B6(Cg)-Tyr<sup>C2/J</sup> mice, and pups with black coat color are considered as successful germline transmission.

For gonadal contribution, the injected embryos were recovered 10 days (E13.5) after implantation. The gonadal regions of each embryo are collected and visualized under microscope for EGFP signal.

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco.

QUANTIFICATION AND STATISTICAL ANALYSIS
Statistical analyses were performed in GraphPad Prism 7. Significance and the value of n were calculated with the indicated methods in each figure legend. The data are presented as the mean ± SD. *p < 0.05; **p < 0.01; ns, non-significant.