Transgenic mice for in vivo epigenome editing with CRISPR-based systems

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CRISPR-Cas9 technologies have dramatically increased the ease of targeting DNA sequences in the genomes of living systems. The fusion of chromatin-modifying domains to nuclease-deactivated Cas9 (dCas9) has enabled targeted epigenome editing in both cultured cells and animal models. However, delivering large dCas9 fusion proteins to target cells and tissues is an obstacle to the widespread adoption of these tools for in vivo studies. Here, we describe the generation and characterization of two conditional transgenic mouse lines for epigenome editing, Rosa26:LSL-dCas9-p300 for gene activation and Rosa26:LSL-dCas9-KRAB for gene repression. By targeting the guide RNAs to transcriptional start sites or distal enhancer elements, we demonstrate regulation of target genes and corresponding changes to epigenetic states and downstream phenotypes in the brain and liver in vivo, and in T cells and fibroblasts ex vivo. These mouse lines are convenient and valuable tools for facile, temporally controlled, and tissue-restricted epigenome editing and manipulation of gene expression in vivo.

Epigenome editing with CRISPR-Cas9 systems has become a widespread approach for investigating fundamental aspects of biological processes through the targeted regulation of genes and the non-coding genome. Fusion of the nuclease-deactivated Cas9 (dCas9) to transcriptional activator or repressor domains enables targeted manipulation of gene expression. Initial work to characterize dCas9 fused to the tetramer of the VP16 acidic activation peptide (dCas9VP64) laid the framework for the development of a wide range of next-generation transcriptional activators, including SAM, VPR, p300core, Tet1CD and others 8–9. In addition, dCas9 fusions that can function through direct or indirect chromatin modification using domains and enzymes such as KRAB, MECP2 and DNMT3a (refs. 2,10). In particular, we and others have shown that dCas9p300 and dCas9KRAB can be targeted to enhancers or to positions near transcriptional start sites (TSSs), leading to targeted histone acetylation or methylation and subsequent changes in gene expression 11,12. These epigenome-modifying dCas9 fusion proteins have been used for studies of gene regulation 12–15, directed cell differentiation 16–20, therapeutic gene modulation 21,22, and high-throughput screening of putative gene regulatory elements 13–20.

Although the majority of studies using CRISPR-based epigenome editing tools have focused on ex vivo cell culture systems, in which delivery challenges are readily addressable, there are several examples of the powerful utility of targeted gene activation and repression in vivo. The smaller dCas9 from Staphylococcus aureus 27 was incorporated into a dCas9KRAB fusion protein and delivered with a guide RNA (gRNA) expression cassette via adeno-associated virus (AAV) for targeted gene repression in the mouse liver. Short ‘dead’ gRNAs were used to activate gene expression in the liver muscle of the Cas9 transgenic mouse using AAV co-delivery of these gRNAs with an MPH activator module 28. A dCas9-SunTag transgenic mouse has been used to activate gene expression in the midbrain 29 and liver 11. Another study used a constitutively expressed dCas9KRAB knock-in to activate expression of the Sim1 gene and reverse obesity resulting from Sim1 haploinsufficiency when crossed to mice transgenic for gRNA expression cassettes 30. Although most of the previous studies have focused on gene activation, one recent study generated a tetracycline-inducible dCas9KRAB mouse line. Hematopoietic stem cells from this line were then engineered ex vivo to assess the impact of five transcription factors on hematopoietic lineage determination after transplantation into a host animal 31. Plasmids encoding either dCas9VP64 or dCas9KRAB have also been transfected into the mouse brain to investigate the role of epigenetic regulation in addiction behaviors 31. These experiments highlight the potential of CRISPR-based epigenome editing in studies of gene regulation. Accordingly, we sought to generate widely applicable transgenic mouse lines to readily perform temporally controlled and tissue-restricted epigenome editing for both gene activation and repression with the simple addition of the gRNA. There are several potential advantages to using Cre-inducible expression of dCas9-based epigenome editors from

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transgenic mouse lines compared with viral delivery. First, several epigenome editors, such as dCas9 fusions to the acetyltransferase p300, are too large for viral vectors. Second, a single genomic copy ensures a more uniform expression level between cells and animals. Third, the use of a genetically encoded Cre recombinase adds tissue or cell type specificity when a corresponding promoter that can be packaged into viral vectors is unavailable. Fourth, it is possible to genetically encode both the gRNA and the Cre recombinase to alter gene expression or program epigenetic states in cell types that are not readily transduced by viral vectors. And last, the transgenic expression of dCas9-based epigenome editors may overcome challenges associated with immune system recognition of the bacterial Cas9 protein14–27. Here, we characterize and demonstrate the utility of two mouse lines, Rosa26-LSL-dCas9-p300core (dCas9p300) and Rosa26-LSL-dCas9-KRAB (dCas9KRAB), for in vivo epigenome editing and modulation of gene expression.

Results
Generation and characterization of the mouse lines. We generated dCas9 epigenome editor mice by inserting a Cre-inducible cassette into the Rosa26 locus using traditional homologous recombination in mouse embryonic stem cells (Fig. 1a). The inserted transgene consists of a CAG promoter followed by aloxP-stop-loxP (LSL) cassette, followed by the complementary DNA encoding the dCas9 fusion protein containing a FLAG epitope. This allows for inducible expression of the dCas9 fusion protein in response to Cre recombinase activity that results in removal of the stop signal between theloxP sites. To evaluate the inducibility of the dCas9p300 and dCas9KRAB lines, we treated the mice with AAV9-CMV.Cre and found Cre-dependent expression in the spleen, skeletal muscle, liver, pancreas and heart (Supplementary Figs. 1 and 9b).

Targeted gene activation in the liver. To demonstrate activation of gene expression with dCas9p300 in cells harboring a single genomic insertion of the dCas9p300 expression cassette, we isolated primary fibroblasts from the gastrocnemius and tibialis anterior hind limb muscles of the dCas9p300 mouse to test gRNAs in cell culture. We tested four gRNAs targeting the TSS of Pdx1 (pancreatic and duodenal homeobox 1) (Supplementary Fig. 2a). Pdx1 was originally selected as a target due to its ability to induce liver cells to develop into a pancreatic β-cell-like phenotype, including glucose-responsive insulin expression13. Cells were co-transduced with lentiviral vectors encoding Cre and a single gRNA expression cassette and cultured for 4 d, at which point RNA was isolated for gene expression analysis using quantitative polymerase chain reaction with reverse transcription (RT–qPCR). Two of the four gRNAs significantly increased Pdx1 messenger RNA levels compared with a control gRNA targeting Myod. For all future experiments, we continued with gRNA no. 4 given that it showed the greatest activation of Pdx1 mRNA expression, at an ~150-fold increase relative to controls (Supplementary Fig. 2b).

This Pdx1-targeting gRNA was cloned into an AAV vector containing a ubiquitous CBh promoter driving Cre (AAV9-CBh.Cre-Pdx1.gRNA), which we injected into 8-week-old mice, from which we then collected liver tissue 2 weeks later to assess Pdx1 mRNA expression (Fig. 1b). The Pdx1 mRNA levels were increased several thousandfold compared with treatment with a corresponding AAV vector containing a non-targeting control gRNA (Fig. 1c). We confirmed an increase in PDX1 protein levels in tissue sections of the livers treated with the Pdx1-targeting gRNA compared with those treated with the non-targeting gRNA control as measured with immunofluorescence staining (Fig. 1d). In the animals treated with Pdx1 gRNA, 7.0% of nuclei were PDX1 positive, compared with 0.03% of cells in livers treated with the control gRNA (Fig. 1e). However, we did not detect an increase in insulin mRNA using RT–qPCR or protein using immunostaining in any treated animals. This result is supported by previous studies showing that hyperactive PDX1-VP16 overexpression is necessary to achieve robust insulin induction28.

After demonstrating robust target gene activation, we characterized the specificity of gene regulation in the dCas9p300 mice. Although the p300 acetyltransferase has many well-characterized targets involved in gene regulation, previous studies showed deposition of acetylation of lysine 27 on histone subunit 3 (H3K27ac) in regions of dCas9p300 binding that is concomitant with an increase in target gene mRNA levels14,15,18,24. To assess the genome-wide specificity of both dCas9 binding and targeted acetylation, we performed chromatin immunoprecipitation and sequencing (ChIP-seq) using antibodies against dCas9 and H3K27ac. Significant levels of specific binding of dCas9p300 to the targeted region of Pdx1 (Fig. 1f, g and Supplementary Fig. 3) and increased H3K27ac in the surrounding regions were readily detectable (Fig. 1f and Supplementary Fig. 4). Genome-wide comparisons showed that the only significantly different dCas9 binding site between treatment with the Pdx1-targeting gRNA and that with the non-targeting gRNA controls was at the Pdx1 gRNA target site (Fig. 1b). Genome-wide, H3K27ac levels were not significantly different between the Pdx1-targeting gRNA and non-targeting gRNA control treatments (Supplementary Fig. 5a). However, we observed a twofold increase in H3K27ac at the Pdx1 gene target binding site as compared with the non-targeting control gRNA (Fig. 1i). Comparing the mice injected with AAV encoding Cre and the Pdx1-targeted gRNA with the control mice injected with saline, we found widespread differential H3K27ac levels, which appears to be attributable to gRNA-independent

**Fig. 1 | AAV-based gRNA and Cre recombinase delivery to Rosa26-LSL-dCas9p300 mice activates Pdx1 gene expression and catalyzes targeted histone acetylation.** a, Schematic diagram of the dCas9p300 knock-in locus. BGH, bovine growth hormone. b, Schematic diagram of the experiment for in vivo Pdx1 activation. vc, vector genomes. c, Pdx1 mRNA quantification 2 weeks after injection in liver tissue lysates isolated from mice injected with PBS, or AAV9 encoding Cre and either a control non-targeting or Pdx1-targeting gRNA (n = 4 per group, Kruskal–Wallis one-way ANOVA with Dunnett’s post-hoc test, *P = 0.0132). d, PDX1 immunostaining of liver tissue sections at 14 d after injection of mice treated with control and Pdx1-targeted gRNAs. Scale bars, 50 μm. e, Quantification of PDX1-positive nuclei in control gRNA- and Pdx1 gRNA-treated animals (70% versus 0.03%, *P = 0.019, Student’s t-test, n = 3 animals, with 3 images counted per animal). f, Representative browser tracks of dCas9 and H2K27ac ChIP-seq data from treated livers at 2 weeks after treatment. chr-5 (mm10), chromosome 5 (Genome Reference Consortium Mouse Build 38). g, dCas9 ChIP-seq quantification of sequencing counts (CPM, counts per million) in the gRNA target region of Pdx1 in samples from mice treated with Pdx1-targeted gRNA, control gRNA or PBS (n = 4, one-way ANOVA with Dunnett’s post-hoc test, P = 0.059). h, RNA-seq and Pdx1 ChIP-seq analyses showing the relationship between changes in gene expression and occupancy of dCas9p300 genome-wide (n = 4, FDR < 0.05). i, H3K27ac ChIP-seq quantification of sequencing counts in a 1kb window centered on the gRNA target site near the TSS of Pdx1 in samples from mice treated with the Pdx1-targeted gRNA, control gRNA or PBS (n = 4, two-tailed Student’s t-test, P = 0.07). j, RNA-seq and H3K27ac ChIP-seq analysis showing the relationship between changes in gene expression and genome-wide H3K27 acetylation for samples from mice treated with the Pdx1-targeted gRNA and control gRNA (n = 4, FDR < 0.05). Cebpb, CCAAT enhancer-binding protein-β. DEG, differentially expressed gene (orange dot); Diff. ChIP, differentially enriched ChIP-seq signal (blue dot); DEG and Diff. ChIP, differentially expressed gene and ChIP-seq enrichment (red dot). All bar-plot error bars represent standard deviation, and all box plots are drawn from the 25th to 75th percentile with the horizontal bar at the mean and the whiskers extending to the minima and maxima.
consequences of overexpression of the constitutively active p300 acetyltransferase catalytic domain (Supplementary Fig. 5d).

To assess the specificity of changes in gene expression downstream of targeted histone acetylation, we performed RNA sequencing (RNA-seq) on mRNA collected from treated mouse livers. When compared with treatment with Cre and a non-targeting gRNA, the Pdx1-targeted gRNA led to increases in Pdx1 expression that were the greatest change in gene expression transcriptome-wide (Fig. 1h,j and Supplementary Figs. 5g and 6a). This is consistent with previous levels of specificity reported for dCas9p300 in cultured cells3. However, when comparing Pdx1-targeting gRNA or control non-targeting gRNA with saline-injected controls, we observed widespread gRNA-independent changes in gene expression as a result of dCas9p300 expression (Supplementary Fig. 6b,c). These results support gRNA-mediated specific epigenome editing and changes to target gene expression in vivo in this dCas9p300 mouse, but also underscore the need for proper controls to account for gRNA-independent effects when overexpressing constitutively active catalytic domains of epigenome editors.

**Targeted gene activation in the brain.** To test gene activation in differentiated neurons with a single genomic insertion of the dCas9p300
Fig. 2 | Epigenomic enhancement of Fos in vivo increases excitability in CA1 neurons. a, Contralateral AAV injection strategy for comparison of targeting and non-targeting control gRNAs. b, Immunofluorescence imaging of neurons in the dentate gyrus region of the hippocampus after transduction with AAV containing LacZ control gRNA or Fos Enh2 gRNA and stimulation with novel objects. AAV-gRNA-positive neurons, green; FOS-positive neurons, red. Scale bars, 20 µm. c, Quantification of those FOS-positive neurons in b. The lines connect measurements from the two sides of the same mouse, and the counts are of FOS-positive cells in tissue slices from n = 6 paired ROIs per condition from 3 animals. *P = 0.002 (Student’s paired two-sided t-test). d, Representative current-clamp traces from acute hippocampal slices. e, Action potential stimulus–response curves for neurons expressing Fos Enh2 gRNA compared with neurons expressing the LacZ control gRNA. Current, F(10,240) = 31.12 (P < 0.0001); virus, F(1,24) = 1.05 (P = 0.32); current × virus interaction, F(10,240) = 4.64 (P < 0.0001). The difference between Fos Enh2 gRNA (n = 14) and the LacZ control gRNA (n = 12) at 350 pA (P = 0.016), at 400 pA (P = 0.028) and at 500 pA (P = 0.026) was significant (two-way repeated measures ANOVA with post-hoc Fisher’s LSD test; *P < 0.05, the error band represents the s.e.m.). f–i, The rheobase (P = 0.023) (f) and the latency to first spike (P = 0.018) (g) were significantly lower for the neurons expressing Fos Enh2 gRNA compared with the LacZ controls, but the input resistance (P = 0.092) (h) and the membrane time constant (P = 0.091) (i) were not significantly different. LacZ control gRNA, n = 12; Fos Enh2 gRNA, n = 14; each from 2 animals (two-tailed Student’s t-test; horizontal bars represent the mean; error bars show the s.e.m.).
expression cassette, we first sought to regulate gene expression in cultured primary neurons. Previously, we showed that gRNA-mediated recruitment of dCas9<sup>9,794</sup> over-expressed from a lentiviral vector is sufficient to drive expression of the mature neuronal NMDA receptor subunit Grin2c in developing cerebellar granule neurons (CGNs)<sup>31</sup>. Here, to determine whether the Cre-inducible dCas9<sup>9,100</sup> transgene is similarly effective for activating gene expression, we cultured CGNs heterozygous for the dCas9<sup>9,100</sup> transgene and then either induced dCas9<sup>9,100</sup> expression by lentiviral delivery of Cre or over-expressed dCas9<sup>9,794</sup> from a lentiviral vector for comparison. Recruitment of dCas9<sup>9,100</sup> to the Grin2c promoter activated Grin2c expression to a similar degree to that by dCas9 VP64 recruitment (Supplementary Fig. 7a). This activation was specific for Grin2c and was not due to accelerated neuronal maturation because the expression of another developmentally upregulated gene, Wnt7a, was not different in any of the conditions ( Supplementary Fig. 7b). In addition, these data suggest that the dCas9<sup>9,100</sup> transgene is at least as effective as lentiviral dCas9<sup>9,794</sup> for inducing expression of developmental genes in cultured primary neurons.

To determine the ability of transgenic dCas9<sup>9,100</sup> to regulate gene expression in neurons in vivo, we measured the induction of neuronal activity-dependent genes in the hippocampus, a brain region that is important for spatial learning and memory. Fos transcription is rapidly and robustly induced by the physiological changes in neuronal firing that follow sensory experience. The Fos gene is flanked by five enhancer elements that regulate its stimulus-dependent transcription. We previously showed that expression of dCas9<sup>9,794</sup> from a transfected plasmid and its recruitment to enhancer 2 (Enh2) is sufficient to increase Fos mRNA and protein expression levels in cultured neurons<sup>11</sup>. To recruit dCas9<sup>9,100</sup> to Enh2 in vivo, we delivered an AAV vector encoding a gRNA expression cassette, Cre recombinase, under the control of the neuron-specific Syn1 promoter, and green fluorescent protein (GFP) to track transduced cells, by stereotactic injection into the dorsal hippocampus of dCas9<sup>9,100</sup> heterozygous mice (Fig. 2a and Supplementary Fig. 8a). For each mouse, one side of the brain was injected with a gRNA targeting Fos Enh2 and the other side was injected with a control non-targeting gRNA against LacZ as an in-animal control. Both sides of the hippocampus showed similar GFP expression, and immunofluorescence staining showed robust detection of Cas9 in neurons, confirming that the Cre virus was inducing conditional dCas9<sup>10,100</sup> expression (Supplementary Fig. 8b). We allowed one cohort of the mice to explore a set of novel objects in the open field, which is a stimulus that induces expression of Fos in the hippocampus<sup>41</sup>. As expected, immunostaining for FOS protein in the dentate gyrus region of the hippocampus showed very few cells expressing a high level of induced FOS. However, in each brain there was a significantly greater number of high FOS-positive cells on the side expressing the Fos Enh2-targeting gRNA than the side expressing the LacZ control gRNA (Fig. 2b,c). Importantly, quantification of FOS protein levels across all of the transduced cells showed that the distribution of FOS was significantly increased in the cells expressing the Fos Enh2-targeting gRNA compared with those expressing the LacZ-targeting gRNA (Supplementary Fig. 8c), consistent with dCas9<sup>9,100</sup>-dependent regulation of Fos gene expression in a cell-autonomous manner.

A key application of transgenic dCas9<sup>9,100</sup> mice in the regulation of neuronal gene expression in vivo is the ability to determine the consequences of epigenome editing on the function of physiologically relevant neuronal circuits in the intact brain. To determine whether dCas9<sup>9,100</sup>-driven increases in FOS protein levels were sufficient to change neuronal physiology, we cut acute slices of hippocampus from a second cohort of dCas9<sup>9,100</sup> mice and performed current-clamp recordings from virally transduced CA1 neurons (Supplementary Fig. 8d,e). Neurons expressing either the Fos Enh2 gRNA or the LacZ gRNA fired action potentials in response to progressive current steps (Fig. 2d). However, the action potential stimulus–response curves were significantly different between the two treatments. The maximum firing rate was reduced for the neurons expressing the Fos Enh2 gRNA compared with neurons expressing the LacZ control gRNA (Fig. 2e). Moreover, the rheobase, that is, the lowest input current at which neurons begin to fire spikes, and the initial spike latency were both significantly reduced in neurons expressing the Fos Enh2 gRNA compared with the LacZ gRNA controls (Fig. 2f,g). These data indicate increased excitability of the Fos Enh2-edited neurons. Importantly, the input resistance and the membrane time constants were unchanged between the conditions (Fig. 2h,i). This suggests that the differences in action potential firing were due to changes in active, rather than passive, membrane properties. Taken together, these data show that dCas9<sup>9,100</sup>-mediated epigenome editing of a single Fos enhancer in the adult brain in vivo induces changes in FOS protein levels that are sufficient to modulate neuronal physiology.

Targeted gene repression in the liver. For characterization of the dCas9<sup>9,100</sup> mice, we chose to target Pcsk9 given that loss of PCSK9 protein is known to reduce serum levels of low-density lipoprotein (LDL) cholesterol<sup>41</sup>. In a previous study, AAV co-delivery of the smaller dCas9 from S. aureus fused to the KRAB domain and a Pcsk9-targeted gRNA repressed Pcsk9 in the liver, resulting in reduced serum LDL cholesterol levels<sup>32</sup>. We delivered AAV9
encoding Cre and an S. pyogenes gRNA targeting the same sequence in the Pcsk9 promoter by tail vein injection to 8-week-old dCas9KRAB mice (Fig. 3a), and compared them with control mice injected with saline or a corresponding vector containing a non-targeting control gRNA (Fig. 3b). At 8 weeks after injection, we collected the mouse livers and assessed the Pcsk9 mRNA levels. We found that Pcsk9 mRNA levels were reduced by ~70% when dCas9KRAB was targeted to the promoter of Pcsk9 compared with the non-targeting gRNA and saline controls (Fig. 3c). We also measured serum PCSK9 levels at 4 weeks after injection and found an ~90% reduction in samples treated with the Pcsk9-targeted gRNA as compared with the controls (Fig. 3d). Additionally, serum was collected every 2 weeks during the 8 week experiment to assess LDL cholesterol levels (Fig. 3b). There was an ~45% reduction in serum LDL cholesterol levels at each time point from 2 to 8 weeks (Fig. 3e). Upon Pcsk9 repression, LDL receptor protein levels increase due to lack of receptor degradation43, which was confirmed by western blot in three of the four Pcsk9-targeted animals (Supplementary Fig. 9a).

To assess the specificity of gene regulation by dCas9KRAB in these transgenic mice, we performed RNA-seq and ChIP-seq on liver tissue samples from dCas9KRAB heterozygous mice injected with AAV encoding Cre and the Pcsk9-targeted gRNA, AAV encoding Cre and a corresponding vector containing a non-targeting control gRNA (Fig. 3b). At 8 weeks after injection, we collected the mouse livers and assessed the Pcsk9 mRNA levels. We found that Pcsk9 mRNA levels were reduced by ~70% when dCas9KRAB was targeted to the promoter of Pcsk9 compared with the non-targeting gRNA and saline controls (Fig. 3c). We also measured serum PCSK9 levels at 4 weeks after injection and found an ~90% reduction in samples treated with the Pcsk9-targeted gRNA as compared with the controls (Fig. 3d). Additionally, serum was collected every 2 weeks during the 8 week experiment to assess LDL cholesterol levels (Fig. 3b). There was an ~45% reduction in serum LDL cholesterol levels at each time point from 2 to 8 weeks (Fig. 3e). Upon Pcsk9 repression, LDL receptor protein levels increase due to lack of receptor degradation43, which was confirmed by western blot in three of the four Pcsk9-targeted animals (Supplementary Fig. 9a).

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the control non-targeting gRNA, or saline controls. For RNA-seq, we performed comparisons of treatment with the Pcsk9-targeted gRNA with both the control gRNA and the saline controls. In both cases, Pcsk9 was one of the most strongly downregulated genes, with only 15 and 6 other genes, respectively, that were significantly changed transcriptome-wide (Benjamini–Hochberg false discovery rate (FDR) < 0.01) (Supplementary Fig. 6d–f). ChIP-seq was performed using an anti-FLAG antibody for dCas9 binding specificity and a histone 3 lysine 9 trimethylation (H3K9me3) antibody to detect histone methylation as a result of dCas9KRAB-mediated recruitment of methyltransferases (Fig. 3f–j and Supplementary Figs. 10 and 11). Specific and significantly enriched dCas9 binding to the gRNA target site upstream of Pcsk9 was readily detected (Fig. 3f,g). Additionally, significant increases in H3K9me3 deposition in the target region of the Pcsk9-targeted gRNA were evident compared with the non-targeting control gRNA or saline controls (Fig. 3f,i). The dCas9KRAB binding was also highly specific genome-wide, given that the Pcsk9 gRNA target site was one of only two significantly different peaks between samples treated with the Pcsk9 gRNA and the control gRNA in the dCas9 ChIP (Fig. 3h) and the only significantly different peak in the H3K9me3 ChIP (Fig. 3j). Treatment with dCas9p300, we performed ChIP-seq for H3K27ac in Th0 cells (Fig. 4h and Supplementary Figs. 13). To further demonstrate the versatility of the dCas9p300 and the control gRNA, we performed ChIP-seq for H3K27ac in Th0 cells, we found that relative to Th0 cells treated with the control non-targeting gRNA, was the only differentially expressed gene in the Th0 cells, we describe the generation and characterization of Cre-inducible targeted epigenome editing to function as Treg cells, we used an in vitro suppression assay to test their ability to limit the proliferation of activated CD4+ T cells in co-culture. The cells treated with Foxp3-targeting gRNA showed enhanced suppression relative to untreated controls or cells treated with gRNA controls, and had a suppressive activity similar to that of the positive control induced (i)Treg cells generated in vitro using established protocols (Fig. 4d,c). To verify that the suppression activity was mediated by the gRNA-treated cells, we performed a suppression assay using serial dilutions of the number of Foxp3-induced cells in co-culture and found a dose-dependent suppressive effect (Supplementary Fig. 12f,g). To demonstrate gene repression in another cell type from the dCas9KRAB mice ex vivo, we isolated naïve CD4+ T cells from the lymph nodes and spleen of Cd4:Cre/dCas9KRAB mice and generated FOX3-positive iTreg cells in vitro via T-cell antigen receptor activation and treatment with IL-2 and transforming growth factor (TGF)-β1. The iTreg cells were transduced with retrovirus containing either the Foxp3-targeting or non-targeting gRNAs. We achieved an ~70% reduction in CD4+ Foxp3 mRNA levels in cells with dCas9KRAB-targeted to the Foxp3 promoter as compared with untreated iTreg cells or those treated with the control gRNA (Fig. 4i). In addition, we assessed FOXP3 protein levels by immunostaining and found a significant reduction in the percentage of FOXP3-positive cells when transduced with the Foxp3-targeting gRNA as compared with controls (Fig. 4j,k). These results confirm our ability to repress genes in multiple tissues and cell types of the dCas9KRAB mouse line both in vivo and ex vivo.

Discussion

Perturbation of gene expression has long been a powerful tool for uncovering the functions of genes. Recent technological advances have enabled not only the study of gene function, but also the mechanism by which the genes themselves are regulated. Using dCas9 fused to epigenome modifiers has proven to be a productive strategy to uncover unknown gene function, to map regions of the non-coding genome and to validate potential therapeutic applications using CRISPR-based epigenome editors in vivo. Here, we describe the generation and characterization of Cre-inducible dCas9 and dCas9KRAB transgenic mouse lines for targeted activation or repression of promoters and non-coding regulatory elements in vivo or in primary cells ex vivo, including in the liver, T cells, fibroblasts and neurons. We demonstrate induction of dCas9 epigenome editor expression using viral or transgenic Cre delivery combined with viral gRNA delivery. The targeted gene activation or repression induced changes in both mRNA transcript levels and protein changes that elicit downstream phenotypes. Concomitant with expression changes, we show targeted deposition of histone 

RNA-seq and ChIP-seq analyses highlight the specificity and efficacy of targeting dCas9KRAB to one location of the genome (Fig. 4h). However, when we compared RNA-seq and ChIP-seq in Th0 cells treated with the Foxp3 gRNA from mice with or without Cd4:Cre, we observed numerous differences in gene expression and H3K27 acetylation (Supplementary Fig. 5h). When comparing RNA-seq data from Cre-negative and Cre-positive cells, the same set of genes was found to be differentially expressed regardless of whether the cells were treated with Foxp3-targeting or control gRNA, further supporting a gene-expression-independent effect of active dCas9KRAB expression on the baseline epigenetic state in this mouse strain (Supplementary Fig. 13). This effect is specific to the dCas9KRAB mice and is not observed in dCas9KRAB mice when comparing liver samples from mice treated with either Pcsk9-targeting gRNA or saline (Supplementary Fig. 5f,i). These results illustrate the need for experimental controls that include an active dCas9KRAB for comparisons with an accurate baseline epigenetic state.

To test the capacity of FOX3-positive Th0 cells generated by targeted epigenome editing to function as Treg cells, we used an in vitro suppression assay to test their ability to limit the proliferation of activated CD4+ T cells in co-culture. The cells treated with Foxp3-targeting gRNA showed enhanced suppression relative to untreated controls or cells treated with gRNA controls, and had a suppressive activity similar to that of the positive control induced (i)Treg cells generated in vitro using established protocols (Fig. 4d,c). To verify that the suppression activity was mediated by the gRNA-treated cells, we performed a suppression assay using serial dilutions of the number of Foxp3-induced cells in co-culture and found a dose-dependent suppressive effect (Supplementary Fig. 12f,g).

To demonstrate gene repression in another cell type from the dCas9KRAB mice ex vivo, we isolated naïve CD4+ T cells from the lymph nodes and spleen of Cd4:Cre/dCas9KRAB mice and generated FOX3-positive iTreg cells in vitro via T-cell antigen receptor activation and treatment with IL-2 and transforming growth factor (TGF)-β1. The iTreg cells were transduced with retrovirus containing either the Foxp3-targeting or non-targeting gRNAs. We achieved an ~70% reduction in FOX3 mRNA levels in cells with dCas9KRAB-targeted to the Foxp3 promoter as compared with untreated iTreg cells or those treated with the control gRNA (Fig. 4i). In addition, we assessed FOXP3 protein levels by immunostaining and found a significant reduction in the percentage of FOX3-positive cells when transduced with the Foxp3-targeting gRNA as compared with controls (Fig. 4j,k). These results confirm our ability to repress genes in multiple tissues and cell types of the dCas9KRAB mouse line both in vivo and ex vivo.
Fig. 4 | Epigenome editing in T cells for activation and repression of Foxp3. a. Flow cytometry analysis of FOXP3-eGFP expression in CD4+ T cells purified from dCas9KRX:CD4-Cre;Foxp3-eGFP mice cultured in vitro under Th0 polarization conditions (IL-2) after transduction with retrovirus encoding either the Foxp3 gRNA or the control gRNA. Cells cultured in iTreg cell polarization conditions (IL-2, TGF-β1) were included as a positive control for Foxp3 expression. b. The percentage of Thy1.1-positive cells that were FOXP3-eGFP positive (P < 0.0001; one-way ANOVA with Dunnett’s post-hoc test; iTreg cells, n = 3; control gRNA and Foxp3 gRNA, n = 4). c. RT-qPCR measurement of Foxp3 mRNA levels in Th0 cells with no virus compared with cells treated with Foxp3-targeting gRNA or with control non-targeting gRNA (P = 0.0190, one-way ANOVA with Dunnnett’s post-hoc test, n = 3 per condition). d. Flow cytometry histograms showing proliferation of Cell Trace Violet (CTV)-labeled CD4+ FOXP3-eGFP–conventional T cells (Tconv) after 72 h of in vitro co-culture with aCD3/ aCD28 dynabeads and either FOXP3-eGFP* iTreg cells (n = 3), Th0 cells (n = 2), FACs-purified Thy1.1+ FOXP3-eGFP* cells treated with Foxp3 gRNA (n = 3) or Thy1.1+ cells treated with control non-targeting gRNA (n = 3). Unstimulated T cells served as a no-activation control. e. Suppressive capacity of iTreg cells summarized as the division index of Tconv (P < 0.0001, one-way ANOVA with Tukey’s post-hoc test). f. Browser track of H3K27ac ChIP-seq read counts at the Foxp3 locus in transduced Th0 cells from a Cd4:Cre-positive or Cd4:Cre-negative Rosa26:LSL-dCas9KRX mouse. g. H3K27ac ChIP-seq read counts per million in the MACS2–called peak that intersects the Foxp3 gRNA target site for each genotype and gRNA treatment (Foxp3 gRNA-treated Cd4:Cre-positive Th0 cells, control gRNA-treated Cd4:Cre-positive Th0 cells, and Foxp3 gRNA-treated Cd4:Cre-negative Th0 cells, n = 3 per condition). h. Scatter plot showing log2(fold-change) of gene expression and H3K27ac enrichment when comparing read counts from Cd4:Cre-positive Rosa26:LSL-dCas9KRX Th0 cells treated with Foxp3 gRNA to control gRNA (FDR < 0.01). DEG, differentially expressed gene (orange dot); Diff. ChIP, differentially enriched ChIP-seq signal (blue dot); DEG and Diff. ChIP, differentially expressed gene and ChIP-seq enrichment (red dot). i. Flow cytometry analysis of FOXP3 expression in CD4+ T cells purified from dCas9KRX mice cultured in vitro under iTreg cell polarization conditions and transduced with retrovirus encoding the indicated gRNAs. j. The percentage of Thy1.1-positive cells that were FOXP3-eGFP–positive for each gRNA treatment (P < 0.0001, one-way ANOVA with Dunnnett’s post-hoc test, n = 3 per condition). k. RT-qPCR measurement of Foxp3 mRNA levels in iTreg cells treated with no virus, control gRNA or Foxp3-targeting gRNA (P < 0.0135, one-way ANOVA with Dunnett’s post-hoc test, n = 3 per condition). All bar-plot error bars represent the standard deviation, and all boxplots are drawn from the 25th to 75th percentile with the horizontal bar at the mean and the whiskers extending to the minima and maxima.
marks subsequent to the recruitment of dCas9-based epigenetic effectors at both promoters and enhancers.

An unbiased, comprehensive, genome-wide analysis of epigenome editing specificity using ChIP-seq and RNA-seq showed both a high level of precision in DNA targeting and interesting differences between the dCas9RE100 and dCas9KRAB editors. The dCas9RE100 editor was highly precise and robust when compared with a control non-targeting gRNA. However, when compared with cells in which dCas9RE100 was not induced, there were widespread changes to H3K27ac and gene expression. Interestingly, these changes did not appear to result in any adverse phenotype. Given that the off-target changes were similar between the targeting and non-targeting gRNA in T cells, we interpret the changes to be gRNA independent and related to the expression of the constitutively active core catalytic domain of the p300 acetyltransferase that may alter the genome-wide epigenetic baseline of these cells. This is consistent with previous observations that dCas9 fused to catalytic enzymes such as DNA methyltransferases can exhibit gRNA-independent changes, and underscores the importance of using proper gRNA controls to determine the consequences of modulating gene expression through epigenome editing. In contrast to dCas9RE100, which has inherent non-specific catalytic activity, dCas9KRAB serves as a scaffold for the recruitment of other enzymatic histone modifiers. Accordingly, target changes to gene expression and epigenetic state were highly specific when compared with the non-targeting gRNA or saline controls, which was consistent with the high level of specificity of DNA targeting and histone modifications.

Following the development of CRISPR-Cas9 for genome editing in human cells,3-5, a Cre-inducible Cas9 nucleas transgenic mouse was quickly generated and widely distributed for in vivo studies of gene function and the genetics of disease. This mouse line has been essential to numerous important breakthroughs including in vivo disease dissection of gene function in studies of gene regulatory networks, and in vivo genetic screens. We expect these new dCas9 epigenome editor transgenic mice to be similarly powerful for enabling in vivo studies of endogenous gene knockdown, activation and perturbation of epigenetic states. Accordingly, the mice have been deposited for distribution via The Jackson Laboratory (stock numbers 033065 (dCas9RE100) and 033066 (dCas9KRAB)).

Online content
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Methods

Generation of Rosa26:LSL-dCas9-p300 and Rosa26:LSL-dCas9-KRAB transgenic lines. All experiments involving animals were conducted with strict adherence to the guidelines for the care and use of laboratory animals of the National Institutes of Health. All experiments were approved by the Institutional Animal Care and Use Committee at Duke University.

To generate mouse lines for conditional expression of these dCas9 fusion proteins, we used a modified pA9 targeting vector. The pA9 vector targets the Rosa26 locus and contains the 5′ Rosa homology arm, a CAG promoter, a loxP-flanked triple polyadenylation (pa) signal stop cassette (LSS), a codon-optimized dCas9-p300 cassette or dCas9-KRAB (ref. 12) with either a 1X FLAG or 3X FLAG, respectively, a woodchuck hepatitis post-transcriptional regulatory element (WPRE), a bovine growth hormone pa, a phosphoglycerate kinase (PGK)-Neo-pαA selection cassette, and the 3′ homology arm. This modified pA9 targeting vector was electroporated into hybrid G4 B6N/129S6 ES cells and, targeting for the ROSA locus was confirmed on PCR and sequencing. Positive clones were expanded and injected into the 8-cell morulae of ICR mice. Chimeric mice were then mated to establish the transgenic line. The mice are genotyped using a forward primer, GCACGGCTCTGTTCACATAC, and a reverse primer, AAGTGCTCCTCTAGTGTTAT. The PCR conditions used for genotyping are 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 57°C for 45 s, 72°C for 2 min 72°C and 2 min 30 s, with a 12°C hold. The expected product size for the WT band is 235 bp, and that for the knock-in is 162 bp. All mice used in the experiments were dCas9p300 or dCas9p300 heterozygous animals.

The new mouse lines are available from The Jackson Laboratory, with the stock numbers of 033065 for Rosa26-LSL-dCas9-p300 and 033066 for Rosa26-LSL-dCas9-KRAB.

All mice were housed in pathogen-free barrier conditions with the exception of those mice used for stereotaxic injections. The mice were kept in ventilated racks at a temperature of 22°C ± 1°C and a humidity of 30–70%, with food and water ad libitum. The mice used for the liver- and brain-targeting experiments had a 12–12h light–dark cycle, whereas the mice used for T-cell experiments had a 14–10h light–dark cycle. All mice were between 8 and 12 weeks old and were not selected based on sex, with the exception of those adult male mice used only for stereotaxic injection experiments.

Isolation and culture of primary dCas9p300 cells. Fibroblasts were isolated from the gastrocnemius and tibialis anterior of the hind limbs of 6-week-old dCas9p300 heterozygous animals using a modified version of a protocol by Springer et al.13. Instead of proceeding with myoblast isolation, fibroblasts were isolated through selective trypsinization of the myoblasts out of the culture using 0.25% trypsin. Fibroblasts were then grown in DMEM supplemented with 10% fetal bovine serum (FBS).

The CGNs from male and female postnatal d7 dCas9p300 heterozygous pups were cultured following our published protocols12. In brief, the cerebellar cortex was removed and dissociated with papain, the granule neuron progenitors were purified by centrifugation through a Percoll gradient, and neurons were plated on poly-d-lysine-coated plates in neurobasal media with B27 supplements (Invitrogen), 1% FBS and penicillin–streptomycin. On d 1 of in vitro culture, the cell culture medium was removed and dissociated with papain, the granule neuron progenitors were cultured following our published protocols15. In brief, the cerebellar cortex was removed and dissociated with papain, the granule neuron progenitors were purified by centrifugation through a Percoll gradient, and neurons were plated on poly-d-lysine-coated plates in neurobasal media with B27 supplements (Invitrogen), 1% FBS and penicillin–streptomycin. On d 1 of in vitro culture, the cell culture medium was removed and dissociated with papain, the granule neuron progenitors were cultured following our published protocols15. In brief, the cerebellar cortex was removed and dissociated with papain, the granule neuron progenitors were purified by centrifugation through a Percoll gradient, and neurons were plated on poly-d-lysine-coated plates in neurobasal media with B27 supplements (Invitrogen), 1% FBS and penicillin–streptomycin. On d 1 of in vitro culture, the cell culture medium was removed and dissociated with papain, the granule neuron progenitors were cultured following our published protocols15. In brief, the cerebellar cortex was removed and dissociated with papain, the granule neuron progenitors were purified by centrifugation through a Percoll gradient, and neurons were plated on poly-d-lysine-coated plates in neurobasal media with B27 supplements (Invitrogen), 1% FBS and penicillin–streptomycin. On d 1 of in vitro culture, the cell culture medium was removed and dissociated with papain, the granule neuron progenitors were cultured following our published protocols15.

AAV cloning and production. An AAV2/1 vector containing a CMV promoter (site 2-2 and site 2-3 from Frank et al.15) or a hepatitis B virus enhancer tended to cross-react with the scRNA-seq data. The scRNA-seq data were filtered to remove genes with low or no expression, and to retain genes with 2 or more counts in two or more samples. Filtered counts were then normalized using the DESeq function, which uses estimated size factors to account for library size as well as gene and global dispersion. To find significant differentially expressed genes, nbinomWaldTest was used to test the coefficients in the fitted negative binomial generalized linear model (GLM) using the previously calculated size factors and dispersion estimates. Genes with a Benjamini– Hochberg FDR < 0.05 were considered significant (unless otherwise indicated).

log(fold-change) values were shrunk towards zero using the adaptive shrinkage estimator from the astatR package16. log(fold-change) was calculated using the ratio of read counts of samples treated with the enhancer and the control sample.

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acquired with a Zeiss LSM 880 microscope. Sections were then washed and incubated with a specific secondary (10 mM citric acid, 0.05% Tween-20, pH 6.0) for 25 min at 95 °C, and were then 4% paraformaldehyde, and 12-

For PDX1 staining, livers were fixed overnight with GFP was used to identify CA1 neurons for recording.

For ChIP-seq analysis. For CGNs, chromatin immunoprecipitation was performed following the protocol of EZ-ChIP (Millipore, cat. no. 17-371). In brief, cells were lysed using SDS Lysis Buffer and sonicated for 1.5h (Diagenode Bioruptor) at 4°C on the high setting with a 30 s on–off cycle. A total of 20µl DNA dsDNA, Protein G (Thermo Fisher Scientific; cat. no. 10003D), 0.1% Tween (0.1% Tween in PBS for 1 h. The primary antibody (Anti-PDX1-Antibody, Abcam, cat. no. ab14727, 1:100) was incubated overnight at 4 °C in blocking reagent. Sections were then washed and incubated with a specific secondary antibody coupled to Alexa Fluor 488 (Goat anti-Rabbit IgG, Life Technologies, cat. no. A27034, 1:200) and 4,6-diamidino-2-phenylindole. Confocal images were acquired using a Zeiss LSM 880 microscope. For Fos staining in hippocampal sections, after 3 weeks following viral infection the mice were placed in an open field and allowed to explore three novel objects for a period of 2 h. Mice were then perfused with 4% paraformaldehyde and the brains were coronally sectioned on a freezing microtome for immunostaining. The primary antibodies used were rabbit anti–c-Fos (Calbiochem, cat. no. PC38, 1:1,000) or rabbit anti-Ca9 (EnCor Biotechnology, cat. no. RPICA-CAS9-Sp, 1:1,000). These were detected using anti-rabbit Cy3 (1:500). Z-stack images through the dentate gyrus were obtained using a Leica SP8 upright confocal with a x40 objective plus additional digital zoom. For each animal, the hemisphere infected with grNA targeting Fos Enh2 was compared with the control hemisphere expressing the control LaCZ grNA. To count high Fos-expressing cells, z-stacks were converted to sum projections and thresholded using ImageJ. These counts were compared using a paired t-test. To determine whether there were changes in Fos intensity across the population of GFP-positive cells, regions of interest (ROIs) were created for each GFP-positive cell in a given region while blind to Fos expression. The Bonferroni adjusted p-value of the intensity of the Fos channel was then measured for each of these cells, to create a distribution of Fos intensities. The Fos signal for each cell was normalized to the average control hemisphere Fos value for a given animal. The resulting distributions were then compared using a Kolmogorov–Smirnov test.

Hippocampal slice electrophysiological recordings. Three weeks after viral injections into dCas9/GrNA heterozygous mice, the mice were anesthetized with Isoflurane and transcardially perfused with 4°C 0.1% Tween in PBS for 1 h. Following ChIP, ChIP-seq libraries were prepared using a Kapa HyperPrep Kit (Kapa Biosystems) and sequenced on an Illumina HiSeq4000 or 25bp paired-end reads on an Illumina NextSeq 500. For analysis, adapter sequences were removed from the raw reads using Trimmomatic v0.32 (ref. 1). Reads were aligned to the UCSC Genome Browser (http://genome.ucsc.edu) human genome (hg19). bamCoverage (v3.0.1, ref. 2) ignoring duplicates, extending reads to 200 bp and applying reads per kilobase per million mapped reads (RPKM) normalization. Using the sequenced input controls, binding regions were identified using the calpeak function in MACS2 v2.1.2/060309 (ref. 3). All MACS2 peaks were annotated with the TSS of the nearest gene using Gencode vM19 basic annotation data. For the differential binding analysis, first, a union peak set was computed by merging individual peak calls using `macs2 callpeak -a peaksDisjoint -o peaksDisjoint` and then `macs2 callpeak -a peaksDisjoint -o peaksDisjoint --extsize 200 --selfpeak --selfpeak –smoothstep 2000 --smoothstep 2000 --smoothstep 2000`. The three peaks in peaks were estimated using featureCounts from the subread package v1.4.6-p4 (ref. 4). The difference in binding was assessed with DESeq2 v1.22.0 (ref. 5) using nbinomWaldTest to test coefficients in the fitted negative binomial GLM. Data were visualized using the pandas v0.23.3 and seaborn v0.9.0 packages in Python 2.7.11 or R v3.5.1.

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Novel object exploration and hippocampal expression of Fos. AAV vectors containing GFP and grNA targeting either LaCZ (control) or Fos Enh2 were injected into either hemisphere of the dorsal hippocampus of adult male dCas9/GrNA mice such that each mouse had one control hemisphere and one experimental hemisphere. Briefer, AAV (2 x 3 µl) was injected into the dorsal hippocampus of adult male dCas9/GrNA mice such that each mouse had one control hemisphere and one experimental hemisphere, using a bilateral injection protocol (AP: –2.3; ML: +/–1.8; DV: –2.3; ML, –2.3; AP, –0.1). Two weeks following AAV injection, mice were placed in an open field and allowed to explore novel objects for a period of 2 h. Mice were then perfused with 4% paraformaldehyde and the brains were coronally sectioned on a freezing microtome for immunostaining. The primary antibodies used were rabbit anti–c-Fos (Calbiochem, cat. no. PC38, 1:1,000) and rabbit anti-Ca9 (EnCor
Biotechnology, cat. no. RPCA-C9SH-SP, 1:1,100). These were detected using anti-rabbit Cy3 (1:500). Z-stack images through the dentate gyrus were obtained using a Leica SP8 upright confocal with a x40 objective plus additional digital zoom. For each animal, the hemisphere injected with gRNA targeting Foxp3 was compared with the control hemisphere expressing the control Lacz gRNA. To count high Foxp3-expressing cells, z-stacks were converted to sum projections and thresholded using ImageJ v1.46 (National Institutes of Health). These counts were compared using a paired t-tests. To determine whether there were changes in Foxp3 intensity across the population of GFP-positive cells, ROIs were created for each GFP-positive cell in a given region while blind to Foxp3 expression. The fluorescence intensity of the Foxp3 channel was then measured for each of these cells, to create a distribution of Fox intensities. The Fox signal for each cell was normalized to the average control hemisphere Fox value for a given animal. The resulting distributions were then compared using a Kolmogorov–Smirnoff test.

Purification of CD4 T cells for in vitro cultures. Rosa26-LSL-dCas9-p300 or Rosa26-LSL-dCas9-KRAB mice were backcrossed with a B6-Foxp3-eGFP mouse line (Jax stock no. 006772) for six generations and then crossed with the B6-Cd4:Cre mouse line (Jax stock no. 022071) to generate heterozygous Cd4Cre/dCas9-KRAB or Cd4Cre/dCas9-p300/Foxp3-eGFP experimental mice. Spleens and lymph nodes were collected, dissociated and processed with ammonium chloride potassium (ACK) lysis buffer and passed through the Magnisort Mouse CD4 T cell Enrichment Kit (Thermo Fisher, cat. no. 8804-6824-74) and sorted using a Leica SP8 upright confocal with a 40 objective plus additional digital zoom. FOXP3 protein staining and flow cytometry analysis. For dCas9-KRAB-based experiments, in vitro cultured, transduced T cells were stained with anti-Thy-1.1-PerCP-Cy5.5 (eBioscience, cat. no. 45-0900-82, 1:300) and Fixable Viability Dye eFluor780 (1:1,000) prior to fixation and permeabilization with Foxp3 Transcription Factor Staining Buffer Kit (eBioscience, cat. no. 00-5523-00) following the manufacturer’s protocol. Cells were stained intracellularly with anti-Foxp3-PE (eBioscience, cat. no. 12-5773-82, 1:200) and data were collected with a BD FACSCanto II cytometer using FACS DIVA v8.0.3. All flow cytometry was analyzed using FlowJo v10.6.2

Statistics and reproducibility. No statistical method was used to predetermine the sample size. Sample sizes were chosen to be consistent with other published reports of dCas9-based activators and repressors used in vivo [49,50]. In each of these three studies, n = 3 or 4 was used in the majority of assays that measured changes in gene expression. For high-throughput sequencing-based assays, sample sizes were chosen to be consistent with ENCODE standards [61–63]. All statistical analysis was conducted using Prism v6 or v9 (GraphPad), with the exception of Supplementary Fig. 2, which was analyzed using R v3.5.1. Sequencing data were excluded only if they failed to pass pre-existing criteria for technical quality. RT-qPCR data were excluded only if our control for RNA collection and sample processing showed an abnormally low yield, defined as more than 2 s.d. from the mean of the sample set. Electrophysiology data were excluded only if the measurement of basal membrane potentials suggested that the cell was dying. Mice for all experiments were selected randomly with respect to parent of origin, age and sex, with the exception of mice for phototoxic injection, for which only adult male mice were used. Sample collection and analysis for liver-targeted experiments were performed blind. Animals were injected, numbered and housed mixed in cages, and samples were collected based on animal number only. Sample collection and analysis for T-cell, neuron and brain experiments were not performed blind.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Raw sequencing files are available from the NCBI Gene Expression Omnibus via SuperSeries accession GSE146848. Source data are provided with this paper.

Code availability
Data processing and analysis code is made available through Zenodo [80] and on GitHub (https://github.com/RoddyLab/gemberling-et-al-NMETH-A42509C).

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Author contributions
M.P.G., K.S., E.R., K.R.T.-E., F.L., A.K., V.C., M.F.H., I.C.B., C.A.W. and J.C.B. conducted experiments and analyzed data. H.D., D.C.R. and L.L. assisted with the mouse experiments. A.B. and K.S. performed ChIP-Seq and RNA-seq analysis. M.P.G., K.S., A.E.W. and C.A.G. wrote portions of the paper. I.B.H. provided critical reagents. V.J.M. and A.A. produced AAV9 for the mouse experiments. M.C., K.D.P., T.E.R., A.E.W. and C.A.G. provided guidance on the experimental design and interpretation of results. All authors edited the text.

Competing interests
C.A.G., I.B.H. and T.E.R. have filed patent applications related to CRISPR technologies for genome engineering. C.A.G. is an advisor to Tune Therapeutics, Sarepta Therapeutics, Levo Therapeutics and Iveric Bio, and a co-founder of Tune Therapeutics, Element Genomics and Locus Biosciences. A.A. is a co-founder of and advisor to StrideBio and TorqueBio. T.E.R. is a co-founder of Element Genomics. M.P.G. is a co-founder and employee of Tune Therapeutics. All other authors have no competing interests.

Additional information
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Correspondence and requests for materials should be addressed to C.A.G.

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ChIP and RNA-seq data sets generated during this study are available on GEO in both raw and processed forms (GSE146848). Figures 1, 3, and 4, and supplemental figures 3, 4, 5, 6, 10, 11, and 13. Other data can be made available on request.

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Sample size: No statistical method was used to predetermine samples size. For CRISPR-based assays, sample sizes were chosen to be in line with other published reports of Cas9 and dCas9 based activators and repressors used in vivo. 25, 26. In each of these three studies, n=3 or 4 were used in the majority of assays measuring changes in gene expression. For high-throughput sequencing based assays, sample sizes were chosen to be in line with ENCODE standards 27, 28.

Data exclusions: Data are only excluded for failing to pass pre-existing criteria for technical quality. RT-PCR data were excluded only if our control for RNA harvesting and sample processing showed abnormally low yield, defined as more than 2 standard deviations from the mean of the sample set. Electrophysiology data were excluded only if the measurement of basal membrane properties suggested that the cell was dying.

Replication: All experiments have been replicated successfully in at least at least two independent biological replicates.

Randomization: Mice for all experiments were selected randomly with respect to parent of origin, age (between 8-12 weeks old) and sex with the exception of mice for stereotaxic injections for which only adult male animals were used.

Blinding: Sample collection and analysis for PDX1 and PCSK9 experiments were performed blind. Animals were injected, numbered, and housed mixed in cages and samples were collected based on animal number only. Sample collection and analysis for T cell and neuron experiments were not performed blind.

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Methods

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☐   ChIP-seq
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Antibodies

| Antibodies used | Supplier | Product Cat# | Lot# | Clone Name | Dilution Website |
|-----------------|---------|-------------|-----|-----------|------------------|
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| ebioscience Anti-Mouse CD3e Functional Grade Purified 16-0331-86 209296 145-2C11 1:4000 | www.thermos Fisher.com/antibody/product/CD3e-Antibody-clone-145-2C11-MonoClonal/16-0331-82 |
| ebioscience Anti-Mouse IFN-gamma Functional Grade Purified 16-7311-85 2269799 XMG1.2 1:500 | www.thermos Fisher.com/antibody/product/IFN-gamma-Antibody-clone-XMG1.2-MonoClonal/16-7311-81 |
| ebioscience Anti-Mouse IL-4 Functional Grade Purified 16-7041-85 2197861 11811 1:500 | www.thermos Fisher.com/antibody/product/IL-4-Antibody-clone-11811-MonoClonal/16-7041-81 |

Validation

All antibodies used were validated with negative control samples included in all analyses. Websites are listed following each antibody below:

- Cell Signaling Anti-Gapdh | www.cellsignal.com/products/primary-antibodies/gapdh-14c10-rabbit-mab/2118 |
- Abcam Anti-1D1R | www.abcam.com/id/receptor-antibody-ep1553y-ab52818.html |
- EMD Millipore Anti-Actin | www.emdmillipore.com/us/en/product/Anti-Actin-Antibody-clone-C4,Mm_NF-MAB1501 |
- Sigma Anti-Rabbit HRP | www.sigmaaldrich.com/catalog/product/sigma/a6154?lang=en&region=US |
- Santa Cruz Anti-Mouse HRP | www.scbt.com/p/goat-anti-mouse-igg-3hrs |
- Biotin Goat anti-mouse | 680 20253 18C1127 1:5000 | www.biotium.com/product/goat-anti-mouse-iggl |
- Life Technologies Goat Anti-Rabbit IgG AlexaFluor 488 | www.thermos Fisher.com/antibody/product/Goat-anti-Rabbit-IgG-H,-L-Secondary-Antibody-Recombinant-Polyclonal/A27034 |
- MP Biomedical anti-Hamster IgG | www.mpbio.com/us/0856984-goat-affinity-purified-antibody-to-hamster-igg-whole-molecule |
- Abcam Pdx1 | www.abcam.com/pdx1-antibody-ab47267.html |
- EnCor Biotechnology Anti-Cas9 | www.encorbio.com/products/mca-3f9/ |
- EnCor Biotechnology Rabbit anti-Cas9 | www.encorbio.com/product/rpca-cas9-sp/ |
- Calbiochem Anti-c-Fos | www.labome.com/product/EMD-Millipore/PC38-100U.html |
Eukaryotic cell lines

Policy information about cell lines

**Cell line source(s)**

PLAT-E cells were from Dan Littman (NYU), HEK293T cells were from ATCC (CRL-11268)

**Authentication**

PLAT-E cells were not authenticated in our lab and taken in good faith, HEK293T cells were authenticated by ATCC. Both of these cell lines were not used to collect data but only produce Retro and Lentivirus for downstream applications.

**Mycoplasma contamination**

Cell lines were not tested for mycoplasma contamination

**Commonly misidentified lines**

See ICTAC register

Name any commonly misidentified cell lines used in the study and provide a rationale for their use.

Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

**Laboratory animals**

Mouse Line Crossbreeding Information

Rosa26-LSL-dCas9:p300 C57BL/6N (Jax Stock No: 033066) [129S6/SvEvTac x C57BL/6N] N57+N6

Rosa26-LSL-dCas9 KRAB C57BL/6N (Jax Stock No: 033066) [129S6/SvEvTac x C57BL/6N] N37+N6

CD4Cre C57BL/6J (Jax Stock No: 022071) [C57BL/6/J x DBA/2J] N13+F5

B6-Foxp3-eGFP C57BL/6J (Jax Stock No: 006772) [129S1/Svl] N57+N3F6

CD4-Cre Rosa26-LSL-dCas9 p300 Foxp3-eGFP C57BL/6J N/A (C57BL/6/J x DBA/2J) x (129S1/Svl) x (129S6/SvEvTac x C57BL/6N) N6

CD4-Cre Rosa26-LSL-dCas9 KRAB Foxp3-eGFP C57BL/6J N/A (C57BL/6/J x DBA/2J) x (129S1/Svl) x (129S6/SvEvTac x C57BL/6N) N6

dCas9-effector mice were generated by electroporting a modified pA9 targeting vector into hybrid G4 B6N/129S6 ES cells to target transgenic insertion into the ROSA locus. Positive clones were expanded and injected into the 8-cell morulae of ICR mice. Chimeric mice were then mated to establish the transgenic line.

For T cell experiments, the resulting Rosa26-LSL-dCas9:p300 or Rosa26-LSL-dCas9 KRAB mice above were backcrossed with a B6-Foxp3EGFP mouse line [Jax Stock #006772] for at least 6 generations and then crossed with B6-CD4-Cre [Jax Stock #022071] to generate either CD4-Cre/dCas9-KRAB+/Foxp3-eGFP+ or CD4-Cre/dCas9-p300+/Foxp3-eGFP+.

All mice were used between 8-12 weeks old and not selected based on specific sex, with the exception of those adult males used only for stereotaxic injection experiments.
Wild animals

No wild animals were used in these studies.

Field-collected samples

There were no field-collected samples used in this study.

Ethics oversight

All experiments involving animals were conducted with strict adherence to the guidelines for the care and use of laboratory animals of the National Institute of Health (NIH). All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Duke University.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

ChIP-seq

Data deposition

☑ Confirm that both raw and final processed data have been deposited in a public database such as GEO.
☑ Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

Data access links

May remain private before publication.

GSE146848 - https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE146848
Reviewer Token: acpiaqenjnuvcfd

Files in database submission

mmLiver_p300.Cas9.PBS.rep10
mmLiver_p300.Cas9.PBS.rep11
mmLiver_p300.Cas9.PBS.rep12
mmLiver_p300.Cas9.PBS.rep4
mmLiver_p300.Cas9.controlRNA.rep1
mmLiver_p300.Cas9.controlRNA.rep2
mmLiver_p300.Cas9.controlRNA.rep3
mmLiver_p300.Cas9.controlRNA.rep5
mmLiver_p300.Cas9.Pdx1.rep6
mmLiver_p300.Cas9.Pdx1.rep7
mmLiver_p300.Cas9.Pdx1.rep8
mmLiver_p300.Cas9.Pdx1.rep9
mmLiver_p300.input.PBS.rep10
mmLiver_p300.input.PBS.rep11
mmLiver_p300.input.PBS.rep12
mmLiver_p300.input.PBS.rep4
mmLiver_p300.input.controlRNA.rep1
mmLiver_p300.input.controlRNA.rep2
mmLiver_p300.input.controlRNA.rep3
mmLiver_p300.input.controlRNA.rep5
mmLiver_p300.input.Pdx1.rep6
mmLiver_p300.input.Pdx1.rep7
mmLiver_p300.input.Pdx1.rep8
mmLiver_p300.input.Pdx1.rep9
mmLiver_p300.K27ac.PBS.rep10
mmLiver_p300.K27ac.PBS.rep11
mmLiver_p300.K27ac.PBS.rep12
mmLiver_p300.K27ac.PBS.rep4
mmLiver_p300.K27ac.controlRNA.rep1
mmLiver_p300.K27ac.controlRNA.rep2
mmLiver_p300.K27ac.controlRNA.rep3
mmLiver_p300.K27ac.controlRNA.rep5
mmLiver_p300.K27ac.Pdx1.rep6
mmLiver_p300.K27ac.Pdx1.rep7
mmLiver_p300.K27ac.Pdx1.rep8
mmLiver_p300.K27ac.Pdx1.rep9
mmLiver_KRAB.flag.PBS.rep12
mmLiver_KRAB.flag.PBS.rep1
mmLiver_KRAB.flag.PBS.rep2
mmLiver_KRAB.flag.PBS.rep4
mmLiver_KRAB.flag.controlRNA.rep10
mmLiver_KRAB.flag.controlRNA.rep11
mmLiver_KRAB.flag.controlRNA.rep8
mmLiver_KRAB.flag.controlRNA.rep9
mmLiver_KRAB.flag.Pcsk9.rep3
mmLiver_KRAB.flag.Pcsk9.rep5
mmLiver_KRAB.flag.Pcsk9.rep6
mmLiver_KRAB.flag.Pcsk9.rep7
mmLiver_KRAB.input.PBS.rep12
mmLiver_KRAB.input.PBS.rep1
mmLiver_KRAB.input.PBS.rep2
mmLiver_KRAB.input.PBS.rep4
mmLiver_KRAB.input.controlRNA.rep10
Methodology

Replicates: All replicates refer to individual mice either dCas9-p300 and dCas9-KRAB. We collected 4 mice (biological replicates) per treatment, with an average Spearman correlation coefficient ranging from 70-95%.

Sequencing depth: Across experiments, on average 39M reads were sequenced per replicate (CI 13.4M-71.5M). From those, 30.4M unique mapped reads were recorded on average (CI 9.5M-56.9M). All libraries were sequenced in an Illumina HiSeq4000 or NextSeq platform, and the reads were configured as S15E (51bp single-end) or S2PE (25bp paired-end) respectively.

Antibodies: Cas9 (Diagenode C15200229-100), Flag-M2 (Sigma, F1804), H3K27ac (Abcam, ab4729), or H3K9me3 (Abcam, ab8898)

Peak calling parameters: Peak calling was performed using MACS2 (see version number in methods), using the appropriate input controls (--control parameter) and the additional following parameters: --no-model --gmm --extsize <SPP_FRAG_LENGTH_ESTIMATION>. The fragment length estimation is calculated using the run_spp R script provided by Anshul Kundaje. See Software for more details.

Data quality: We ran strand cross-correlation analyses for all our samples to ensure the associated values of Relative and Normalized Strand Cross-correlation were within the acceptable ranges. Additionally, we computed the Non-redundant Read Fraction (NRF) and the Fraction of Reads in Peaks (FRIP) which showed values within range. For ChIP-seq samples of Cas9 and FLAG, some of these metrics didn’t seem to apply given the extremely low expected number of peaks. For those, we relied on Spearman correlation analysis for 10kb bins across the mouse genome.

Software: We used the CWL pipeline from the GGR project to process these samples, which has been described in a previous publication [PMID: 30097539]. All the code is publicly available and can be found in github: https://github.com/Duke-GCB/GGR-cwl/tree/master/v1.0

Flow Cytometry

Plots

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.
### Methodology

**Sample preparation**
Spleens and lymphnodes were harvested, dissociated, and processed with ammonium chloride potassium (ACK) lysis buffer and enriched with a Magnisort mouse CD4 T cell enrichment kit (eBioscience cat# 8804-6821-74)

**Instrument**
FACS was performed using either a Beckman Culture Astiros or or SONY SH800S; Flow cytometry analysis was performed using either a BD FACSCanto II cytometer or SONY SH800S in analyzer mode.

**Software**
BD FACSDiva software was used to collect samples from the Beckman Culture Astiros cell sorter and BD FACSCanto II cytometer. The SH800S software was used to collect samples from the SONY SH800S cell sorter and analyzer. Flowjo v10 was used to analyze the .fcs files. Prism6 or Prism9 and Illustrator 2020 was used to prepare figures for publication

**Cell population abundance**
all naive T cells were collected in a single sort with a final purity of ≥98% from the Beckman Culture Astiros and ≥95% from the SONY SH800S

**Gating strategy**
- Gating strategy for
  1. naive T cell sorts: Lymphocytes/Single Cells/Live cells/CD4+/CD25+/CD62L+/CD44lo
  2. CRISPR activation and interference experiments: Lymphocytes/Single Cells/Live cells/THY1.1+/FOXP3+
  3. Suppression Assays: Lymphocytes/Single Cells/Live cells/CeIl Trace Violet+ (CTV+)

- Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.