**GENE THERAPY**

**CRISPR-mediated activation of a promoter or enhancer rescues obesity caused by haploinsufficiency**


**INTRODUCTION:** Loss-of-function mutations in one gene copy can lead to reduced amounts of protein and, consequently, human disease, a condition termed haploinsufficiency. The delivery of extra copies of the gene by way of gene therapy is a promising therapeutic strategy to increase gene dosage in such conditions. Recombinant adeno-associated virus (rAAV) provides a promising tool for delivery of transgenes in an efficient and safe way for gene therapy. However, it has some limitations, including an optimal DNA packaging constraint of 4700 base pairs and ectopic expression.

**RATIONALE:** Increasing the expression levels of the normal gene copy by directly targeting the endogenous gene regulatory elements that control it could potentially correct haploinsufficiency. CRISPR-mediated activation (CRISPRa), whereby a nuclease-deficient Cas9 (dCas9) is used to target a transcriptional activator to the gene’s regulatory element (promoter or enhancer), could be used for this purpose. Such an approach could overcome the ectopic expression and DNA packaging limitations of rAAV. Using obesity as a model, we tested in mice whether CRISPR-mediated activation of the existing normal copy of two different genes, Sim1 or Mc4r, where loss-of-function mutations that lead to haploinsufficiency are a major cause of human obesity, can rescue their obesity phenotype.

**RESULTS:** We first generated a transgenic CRISPRa system using dCas9 fused to a transcriptional activator, VP64, to test whether it can rescue the obesity phenotype in a Sim1 haploinsufficient mouse model. CRISPRa targeting of the Sim1 promoter or its hypothalamic-specific enhancer, which is 270 kilobases away from the gene, in Sim1 haploinsufficient mice increased the expression of the normal copy of Sim1. This up-regulation was sufficient to rescue the obesity phenotype of Sim1 heterozygous mice and led to significantly reduced food intake and body fat content in these mice. We assessed the off-targeting effects of CRISPRa using both RNA sequencing (RNA-seq) and Cas9 chromatin immunoprecipitation sequencing (ChIP-seq) analyses. We found CRISPRa targeting to be highly specific and without any overt changes in the expression of other genes. We also observed that Sim1 up-regulation occurred only in tissues where the regulatory element (promoter or enhancer) that was being targeted was active. Although promoter-CRISPRa–targeted mice up-regulated Sim1 in all the tissues where it is expressed, the enhancer-CRISPRa–targeted mice showed Sim1 up-regulation only in the hypothalamus.

We then delivered CRISPRa packaged into rAAV targeting the Sim1 promoter or its hypothalamic-specific enhancer using either Streptococcus pyogenes or the shorter Staphylococcus aureus CRISPRa system. We show that postnatal injection of CRISPRa-rAAV into the hypothalamus can up-regulate Sim1 expression and rescue the obesity phenotype in Sim1 haploinsufficient mice in a long-lasting manner. To further highlight the therapeutic potential of this approach to rescue other haploinsufficient genes, we targeted Mc4r, where haploinsufficiency leads to severe obesity in mice and humans. CRISPRa-rAAV targeting of the Mc4r promoter rescued the obesity phenotype of Mc4r heterozygous mice.

**CONCLUSION:** These findings show that the CRISPRa system can rescue a haploinsufficient phenotype in vivo. This CRISPR-mediated activation strategy is different from a conventional gene therapy strategy, as it uses the endogenous regulatory elements to up-regulate the existing functional gene copy. As such, it can overcome the problem of ectopic gene expression. In addition, it could be used for genes that are not amenable to conventional gene therapy because their coding sequences are longer than the rAAV packaging limit. Our results provide a framework to further develop CRISPRa as a potential tool to treat gene dosage–sensitive diseases.

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CRISPR-mediated activation of a promoter or enhancer rescues obesity caused by haploinsufficiency

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A wide range of human diseases result from haploinsufficiency, where the function of one of the two gene copies is lost. Here, we targeted the remaining functional copy of a haploinsufficient gene using CRISPR-mediated activation (CRISPRa) in Sim1 and Mc4r heterozygous mouse models to rescue their obesity phenotype. Transgenic-based CRISPRa targeting of the Sim1 promoter or its distant hypothalamic enhancer up-regulated its expression from the endogenous functional allele in a tissue-specific manner, rescuing the obesity phenotype in Sim1 heterozygous mice. To evaluate the therapeutic potential of CRISPRa, we injected CRISPRa-recombinant adeno-associated virus into the hypothalamus, which led to reversal of the obesity phenotype in Sim1 and Mc4r haploinsufficient mice. Our results suggest that endogenous gene up-regulation could be a potential strategy to treat altered gene dosage diseases.

More than 660 genes are currently estimated to cause human disease due to haploinsufficiency (table S1) (1,2), leading to a wide range of phenotypes that include cancer, neurological diseases, developmental disorders, immunological diseases, metabolic disorders, infertility, kidney disease, limb malformations, and many others (1,2). Large-scale exome sequencing analyses estimate that there could be over 3000 human genes that are haploinsufficient (3). Gene therapy in which a functional recombinant copy or copies replace the mutant gene holds great promise in addressing diseases caused by haploinsufficiency. Numerous clinical trials are being carried out for gene therapy, most of which use recombinant adeno-associated virus (rAAV) to deliver the transgene (4). rAAV is a preferred gene delivery method because of its ability to provide long-lasting gene expression of the transgene, delivering DNA without integrating into the genome, and with limited pathogenicity (5). However, current rAAV approaches tend to use promoters to drive transgenes that can lead to nondesirable ectopic expression (6,7). Another crucial limitation is that AAV has an optimal 4.7-kilobase (kb) packaging capacity (8), limiting its gene therapy use for genes longer than 3.5 kb (taking into account additional regulatory sequences needed for its stable expression). Analysis of the 660 haploinsufficiency disease-causing genes and 3320 predicted heterozygous loss-of-function (LoF) genes reveals that 135 (20%) and 730 (23%) of them, respectively, have coding sequences longer than 3.5 kb (fig. S1), rendering them unsuitable for rAAV gene therapy.

CRISPR gene editing can potentially fix haploinsufficient mutations; however, this would require that the editing strategy be customized for each mutation. Moreover, it may not be feasible to correct heterozygous LoF microdeletions. To address these challenges, we devised a strategy that could potentially treat haploinsufficiency by using CRISPR activation (CRISPRa). CRISPRa takes advantage of the RNA-guided targeting ability of CRISPR to direct a nuclease-deficient Cas9 (dCas9) fused with a transcriptional activator to regulatory element(s) of a specific gene, thus increasing its expression (9–15). Here, we tested whether this system can be used to rescue a haploinsufficient phenotype by increasing the transcription of the normal endogenous gene. As a proof-of-concept model, we chose a quantitative trait, obesity caused by haploinsufficiency of either the single-minded 1 (SIM1) or melanocortin 4 receptor (MC4R) gene.

SIM1 is a transcription factor that is expressed in the developing kidney and central nervous system and is essential for the formation of the supraoptic nuclei (SON) and paraventricular nuclei (PVN) of the hypothalamus (16). SIM1 also plays a role in the maintenance of long-term energy homeostasis by acting downstream of the leptin-melanocortin pathway (17). In humans, haploinsufficiency of SIM1 due to chromosomal aberrations results in hyperphagic obesity (18), and SIM1 coding mutations, many of which are LoF mutations, are thought to be a major cause of severe obesity in humans (19–21). Sim1 homozygous null mice die perinatally, whereas Sim1 heterozygous mice (Sim1+/-) survive, are hyperphagic, and develop early-onset obesity with increased linear growth, hyperinsulinemia, and hyperleptinemia (22). A postnatal conditional knockout of hypothalamic Sim1 leads to a similar phenotype in heterozygous mice (23), delineating an additional role for Sim1 as an important regulator of energy homeostasis in adults. Over-expression of SIM1, by using a human bacterial artificial chromosome in mice, rescues diet-induced obesity and reduced food intake (24), suggesting a potential role for SIM1 in preventing development of an obesity phenotype.

MC4R is a heterotrimetric guanine nucleotide-binding protein (G protein)–coupled receptor that is essential for the long-term regulation of energy homeostasis and other physiological processes. MC4R expression in the PVN of the hypothalamus is both necessary and sufficient for most of its effects on the regulation of body weight (25). Haploinsufficient mutations in MC4R are the most common cause of monogenic severe obesity, estimated at 2.6 to 5% of cases of early-onset and/or adult class 3 obesity (body mass index >40 kg/m²) (26–28). Mice haploinsufficient for Mc4r become obese with hyperphagia, hyperinsulinemia, and hyperglycemia (29).

We initially tested the ability of a transgenic CRISPRa system to rescue the obesity phenotype in Sim1+/− mice. CRISPRa using a single guide RNA (sgRNA) targeted to either the Sim1 promoter or its ~270-kb distant enhancer up-regulated Sim1 expression and rescued Sim1-mediated obesity in haploinsufficient animals. This transgenic approach also showed that Sim1 up-regulation occurred only in tissues where the promoter or enhancer is active, suggesting that the targeted cis-regulatory elements can determine CRISPRa tissue specificity. We also used these transgenic mice to assess the targeting specificity of CRISPRa by using RNA sequencing (RNA-seq) and chromatin immunoprecipitation sequencing (ChiP-seq), which we found to be highly specific and without any apparent off-target effects. To further show that CRISPRa could be used as a potential strategy to treat haploinsufficient phenotypes, we used rAAV-mediated delivery of CRISPRa to the hypothalamus, preventing excessive weight gain in postnatal Sim1+/− mice. To demonstrate that this strategy could be used for other haploinsufficient genes, we also targeted the Mc4r promoter by means of a similar CRISPRa-rAAV approach and reduced weight gain in Mc4r+/− mice. Our results present a potential strategy for treating haploinsufficiency and additional gene dosage–related functional abnormalities.

Results

Up-regulation of Sim1 in vitro by CRISPRa

To increase expression of the wild-type Sim1 gene, we optimized CRISPRa conditions in vitro.
**SUMMARY:**

The paper describes the use of CRISPR-A technology to rescue obesity in a mouse model. The authors used a transgenic CRISPR-A system with dCas9-VP64 and either sgRNA targeting the wild-type littermates and weighed weekly until 16 weeks of age along with Sim1+/− mice. They observed a significant reduction in body weight for the promoter and enhancer, respectively (Fig. S5). Of note, they observed slight differences in body weight trajectories between female and male mice (i.e., when compared to wild-type mice, Sim1+/− females gained weight more rapidly than males), similar to what was observed in previous Sim1 knockout studies (22, 23). Taken together, these results show that both Prm-CRISPRa and Enh-CRISPRa mice have reduced body weight due to lower food intake, which likely leads to their reduced body fat levels.

**Up-regulation of Sim1 in vivo by transgenic CRISPRa rescues obesity**

To test the ability of the CRISPRa system to rescue obesity in Sim1+/− mice, we generated knockin mouse lines using TARGATT technology (34). Using this technology, we inserted spdCas9-V64 into the mouse Hipp1 locus (a region that is known to allow robust transgene expression (35)) having three copies of attP (Hipp1P3CAG-dCas9-V64) and either sgRNA, targeting the Sim1 promoter (R26P3(Spm-Cas9-sgRNA)) or SCE2 (R26P3(spmCas9-sgRNA)), in the Rosa26 locus that has three attP sites (Fig. 1C and fig. S3). We then crossed these mice to Sim1−/− mice that develop severe obesity (22). Mice having all three alleles (Sim1+/− × Hipp1P3CAG-dCas9-V64 and R26P3(Spm-Cas9-sgRNA) or R26P3(spmCas9-sgRNA)) were weighed weekly until 10 weeks of age along with wild-type littermates and Sim1+/− and Sim1−/− × Hipp1P3CAG-dCas9-V64 mice, both of which become severely obese (negative control). Analysis of at least 10 females and 10 males per condition showed that Sim1−/− mice carrying both spdCas9-V64 and either Sim1 promoter or enhancer sgRNA had a significant reduction in body weight compared to Sim1−/− × Hipp1P3CAG-dCas9-V64 and Sim1+/− (Fig. 1, D and E, and fig. S4). Sim1+/− mice carrying spdCas9-V64 and either Sim1 promoter or enhancer sgRNA also showed a reduction in body weight compared to wild-type mice (Fig. S4). We also analyzed body fat content and food intake for all genotypes: Sim1−/− × Hipp1P3CAG-dCas9-V64 × R26P3(spmCas9-sgRNA) (PrmCRISPRa), Sim1+/− × Hipp1P3CAG-dCas9-V64 × R26P3(spmCas9-sgRNA) (Enh-CRISPRa), Sim1+/−, and wild-type mice. Both Prm-CRISPRa and Enh-CRISPRa mice showed significantly reduced body fat content and food intake compared to Sim1−/− in both females and males (Fig. S5). Of note, we observed slight differences in body weight trajectories between female and male mice (i.e., when compared to wild-type mice, Sim1+/− females gained weight more rapidly than males), similar to what was observed in previous Sim1 knockout studies (22, 23). Taken together, these results show that both Prm-CRISPRa and Enh-CRISPRa mice have reduced body weight due to lower food intake, which likely leads to their reduced body fat levels.

**CRISPRa up-regulation of Sim1 is tissue specific**

To test for Sim1 activation levels and tissue specificity in Prm-CRISPRa and Enh-CRISPRa mice, we measured its mRNA expression levels in different tissues. We selected two tissues where Sim1 is expressed, hypothalamus and kidney, and two tissues where it is not expressed, lung and liver, based on previous studies (36, 37) and our analysis of Sim1 expression in different tissues (fig. S6). We first measured spdCas9 expression and found that it was expressed in all four tissues, as expected, because we used a ubiquitous cytoplasmic antibody against Cas9 (CAG) promoter to drive its expression (Fig. 2A). By contrast, for Sim1, we observed significantly higher mRNA levels in both the hypothalamus and kidney in Prm-CRISPRa mice but only in the hypothalamus of Enh-CRISPRa mice compared to Sim1−/− mice (Fig. 2B). In Sim1+/− mice, we observed half the levels of mRNA expression when compared to wild-type mice, both in the hypothalamus and kidney.

Because we did not observe any significant differences between the obesity phenotype of Prm-CRISPRa and Enh-CRISPRa mice, we speculate that the activation of Sim1 in the hypothalamus is sufficient to rescue the Sim1−/− obesity phenotype. In tissues where Sim1 is not expressed (i.e., liver and lung), we could not detect Sim1 expression in Prm-CRISPRa or Enh-CRISPRa mice despite spdCas9 being expressed. These results imply that despite ubiquitous expression, spdCas9-V64 could only up-regulate Sim1 in tissues where its target cis-regulatory elements are active. This suggests that cis-regulatory elements could be used to define the tissue specificity of CRISPRa.

**Sim1 CRISPRa targeting is highly specific**

To check for CRISPRa off-target effects, we undertook two genome-level approaches: We analyzed the hypothalamic transcriptome (RNA-seq) of wild-type, Sim1−/−, Prm-CRISPRa, and Enh-CRISPRa mice. Three males and three females were used for each genotype (total of 24 samples; 6 biological replicates per condition). We also carried out ChiP-seq using an antibody against S. pyogenes Cas9 in the hypothalamus of Prm-CRISPRa, Enh-CRISPRa, and dCas9-VP64 (negative control) mice. A pool of four mice was used for each genotype, and two biological replicates.

In the RNA-seq analyses, we identified 24 differentially expressed genes (at a false discovery rate (FDR) of 0.1) between Sim1−/− and wild-type mice, of which 17 were up-regulated and 7 were down-regulated. For all of the 17 up-regulated and 6 of the 7 down-regulated genes, we observed fold changes that were similar to that of wild-type versus Sim1−/− for Prm-CRISPRa or Enh-CRISPRa when compared to Sim1−/− (Fig. 2, C and D, and table S3). We also observed that genes that were significantly up-regulated or down-regulated in Prm-CRISPRa versus Sim1−/− were also up-regulated or down-regulated in Enh-CRISPRa and vice versa, highlighting that the overall gene expression profile in Prm-CRISPRa and Enh-CRISPRa was similar (Fig. 2, C and D, and table S3).

Next, we carried out ChIP-seq analysis to identify off-target spdCas9-V64 binding. We found the most significant on-target enrichment at the Sim1 promoter in Prm-CRISPRa and SCE2 in Enh-CRISPRa mice (fig. S8, A and B, and table S2). In addition, we found 91 and 136 significantly affected peaks (FDR adjusted p value ≤ 10−4) in Prm-CRISPRa and Enh-CRISPRa, respectively (table S2). We then looked for predicted sequence-specific genomic off-targets due to Prm-sgRNA or Enh-sgRNA mismatches, allowing for zero to three nucleotide mismatches, using Cas-OFFFinder (38). For the promoter-targeting sgRNA, we found the one expected on-target site, and one off-target site with three nucleotide mismatches (fig. S9, S and table S2). For the enhancer-targeting sgRNA, we found the one expected on-target site and eight off-target sites with three nucleotide mismatches (fig. S9D and table S2). None of the Prm-CRISPRa or Enh-CRISPRa ChIP-seq peaks overlapped a corresponding predicted off-target site (table S2). We next analyzed the RNA-seq datasets for the expression of the neighboring genes (+500 kb upstream and downstream) near the ChIP-seq peaks and sgRNA off-target sites, including the Sim1 target-specific peaks (fig. S8, C and D, and table S3). Of the genes within 500 kb of the off-target ChIP-seq peaks or predicted off-target sites, none showed differential gene expression (Fig. 2, E and F, and fig. S8, E and F). In addition, ChIP for dCas9 followed by qPCR for Ascc3, Gprc6a, and the Sim1 promoter or SCE2 showed binding.
Fig. 1. CRISPRa Sim1 up-regulation in vitro and obesity rescue in vivo. (A) Schema of the mouse Sim1 genomic locus, showing the LacZ-driven hypothalamic expression of SCE2 (En) from 56-day-old mice. (B) CRISPRa in Neuro-2a cells targeting the Sim1 promoter (Prm-CRISPRa) or enhancer (Enh-CRISPRa). Results are expressed as mRNA fold increase normalized to Actb using the ΔΔCT method. The data are represented as means ± the lower and upper quartile, and lines represent the minimum and maximum from three independent experiments and three technical replicates. *p < 0.001; ***p < 0.0005 (ANOVA, Tukey test). (C) Schema showing the mating scheme used to generate Sim1 +/- CRISPRa mice. A CAG-dCas9-VP64 cassette was knocked into the Hipp11 (H11P3) locus, and an sgRNA targeting either the Sim1 promoter (U6-Prm-sgRNA) or SCE2 (U6-Enh-sgRNA) was knocked into the Rosa26 locus. (D) Weekly weight measurements of wild-type, Sim1 +/-, H11P3CAG-dCas9-VP64 x R26P3Sim1Pr-sgRNA (Prm-CRISPRa), and H11P3CAG-dCas9-VP64 x R26P3SCE2En-sgRNA (Enh-CRISPRa). At least 10 male and female mice were measured per genotype. Mean values ± SD are shown. p-value statistics are listed in table S5. (E and F) Photos of 26-week-old male mice for each genotype: Sim1 +/-, H11P3CAG-dCas9-VP64 x R26P3Sim1Pr-sgRNA (Prm), and wild type (WT) (E) and Sim1 +/-, H11P3CAG-dCas9-VP64 x R26P3SCE2En-sgRNA (Enh), and wild type (WT) (F). Genotype, weight, and length of each mouse are depicted below.
Fig. 2. dCas9 and Sim1 mRNA expression levels in CRISPRa transgenic mice. (A) dCas9 mRNA expression in the hypothalamus, kidney, lung, and liver from four Sim1+/- x H1P3CAG-dCas9-VP64 mice. The data are represented as means ± the lower and upper quartile, and lines represent the minimum and maximum from at least four mice with three technical replicates. (B) Sim1 mRNA expression in the hypothalamus, kidney, lung, and liver for the following genotypes: wild type, Sim1+/-, H1P3CAG-dCas9-VP64 x R26PrSim1Pr-sgRNA (Prm-CRISPRa), and H1P3CAG-dCas9-VP64 x R26PrSCE2En-sgRNA (Enh-CRISPRa). The data are represented as means ± the lower and upper quartile, and lines represent the minimum and maximum from four mice (two females and two males) and three technical replicates. All experiments were determined based on mRNA fold increase compared to wild type and normalized to Actb or Rpl38 using the ΔΔCT method or ΔCT for (A). BDL, below detectable levels. (C and D) A Michaelis-Menten plot showing differentially expressed genes in the hypothalamus between Sim1+/- and wild-type mice on the x axis and Prm-CRISPRa (C) or Enh-CRISPRa (D) versus Sim1+/- mice on the y axis. The larger circles are genes that are differentially expressed with a raw p value ≤0.05, and the outlined circles have a FDR ≤0.1. (E and F) A Michaelis-Menten plot showing differentially expressed genes in the hypothalamus that are nearby ChIP-seq peaks and predicted off-target sgRNAs between Sim1+/- and wild-type mice on the x axis and Prm-CRISPRa (E) or Enh-CRISPRa (F) versus Sim1+/- mice on the y axis. The outlined circles are genes that show differential expression with a raw p value ≤0.05, and the larger circles are genes that overlap nearby off-target sites (both Sim1 promoter and SCE2 were predicted targets even up to three mismatches).
only in the Sim1 promoter for Prm-CRISPRa and SCE2 in Enh-CRISPRa mice (fig. S7, C and D), similar to what we observed in the ChIP-seq data. Our results show that Sim1 Prm-CRISPRa and Enh-CRISPRa are highly specific, without any apparent off-target effects.

Delivery of Sim1 CRISPRa rAAV to the PVN rescues the weight gain phenotype in Sim1+/− mice

To further investigate the translational potential of this approach to rescue haploinsufficiency in adult mice, we took advantage of rAAV to deliver CRISPRa into the hypothalamus of Sim1+/− mice. We generated the following three rAAV vectors: (i) S. pyogenes dCas9-Vp64 driven by a CMV promoter (pCMV-spdCas9-Vp64); (ii) Sim1 promoter sgRNA along with mCherry (pU6-Sim1Pr-CMV-mCherry); and (iii) SCE2 sgRNA along with mCherry (pU6-SCE2-CMV-mCherry) (Fig. 3A). These vectors were packaged individually into AAV-DJ serotype (39). We first tested if the rAAV CRISPRa vectors could up-regulate Sim1 in vitro using Neuro-2a cells. We observed a four- and fivefold increase in Sim1 mRNA expression when targeting the promoter or enhancer, respectively (Fig. 3B and fig. S9).

Next, we performed stereotactic injections to deliver virus carrying pCMV-spdCas9-Vp64 and either pU6-Sim1Pr-CMV-mCherry (Prm-CRISPRa-AAV) or pU6-SCE2-CMV-mCherry (Enh-CRISPRa-AAV) into the PVN of the hypothalamus of Sim1+/− mice at 4 weeks of age, before the mice start becoming obese. As an injection-based negative control, we also injected Sim1+/− mice with pCMV-spdCas9-Vp64 virus only. We first optimized the stereotaxic injection conditions and coordinates (see Methods) and tested for the expression of mCherry from the pU6-Sim1Pr-CMV-mCherry cassette in the PVN by performing immunostaining on the hypothalami of injected mice (Fig. 3C and fig. S10). Next, we carried out stereotactic injections into the PVN of Sim1+/− mice at 4 weeks of age using S. pyogenes CRISPRa-AAV. To test whether Sim1 expression levels were increased by delivering CRISPRa-AAV to the hypothalamus of Sim1+/− mice, we measured mRNA expression levels for both dCas9 and Sim1 from 11-week-old AAV-injected mice. dCas9 was expressed in the hypothalami of all the pCMV-spdCas9-Vp64 AAV-injected mice (Fig. 3D). Sim1 up-regulation was observed in both Prm-CRISPRa-AAV- and Enh-CRISPRa-AAV-injected hypothalami, but not in mice injected with only pCMV-spdCas9-Vp64-AAV (Fig. 3E). To observe the extent of Sim1 up-regulation that could be achieved, we injected Prm-CRISPRa-AAV into the hypothalami of wild-type mice using two different titers. We observed up to 1.8-fold up-regulation with the higher viral titer (fig. S11).

Because the length of S. pyogenes dCas9-Vp64 exceeds the optimal packaging load for AAV (i.e., 4.7 kb), we generated a Staphylococcus aureus
obtaining higher titers for dCas9-VP64 virons DJ serotype of the off-targets (table S2). We then generated an AAV-DJ serotype of the S. aureus CRISPRa vectors, obtaining higher titers for dCas9-VP64 virons than S. pyogenes CRISPRa-AAVs (see Methods). We infected Neuro-2a cells with the viruses and selected a single sgRNA for the promoter or SCE2 that significantly increased Sim1 expression (Fig. 3B and fig. S12E). Next, we carried out stereotactic injections into the PVN of Sim1/− mice at 4 weeks of age using S. aureus CRISPRa-AAV. To test whether Sim1 expression levels were increased by delivering S. aureus CRISPRa-AAV to the hypothalamus of Sim1/− mice, we measured mRNA expression levels from hypothalami of 11-week-old AAV-injected mice. Compared to S. pyogenes dCas9 expression levels (Fig. 3D), we found higher expression levels of S. aureus dCas9 in the hypothalami of all pCMV-saCas9-VP64 AAV–injected mice (Fig. 3F). We also observed higher Sim1 up-regulation (Fig. 3G) compared to S. pyogenes CRISPRa (Fig. 3E).

Next, we tested whether Sim1 up-regulation by CRISPRa-AAV can lead to a reduction in body weight of Sim1 haploinsufficient mice. CRISPRa-AAV–injected Sim1/− mice were measured for body weight up to 11 weeks of age (Fig. 4A). We observed a significant weight reduction in the Prm-CRISPRa-AAV– or Enh-CRISPRa-AAV–injected mice compared to the Sim1+/− or pCMV-dCas9-VP64–injected mice both for S. pyogenes (Fig. 4B) and S. aureus (Fig. 4C). These results suggest that CRISPRa-AAV can rescue the Sim1 haploinsufficiency obesity phenotype.

Finally, we analyzed whether CRISPRa would have a long-term body weight effect on these mice. Although many of the injected mice were analyzed in the aforementioned gene expression studies, a few were maintained and showed significant weight reduction compared to the Sim1+/− or pCMV-spdCas9-VP64–injected Sim1/− mice 9 months after injection (Fig. 4, D and E). Similar results were also observed in S. aureus–injected mice (fig. S13). These results show that CRISPRa-AAV–mediated up-regulation could have a long-lasting effect on phenotype.

**Delivery of Mc4r CRISPRa rAAV to the PVN rescues the weight gain phenotype in Mc4r+/− mice**

To further investigate whether CRISPRa can rescue an additional haploinsufficient obesity model, we carried out S. aureus CRISPRa targeting of the Mc4r promoter in Mc4r+/− mice, which become obese as a result of heterozygous LoF of Mc4r (29). We first screened five sgRNAs targeting the Mc4r promoter and selected one that led to robust Mc4r up-regulation in Neuro-2a cells using both transient transfection and rAAV infections (fig. S14). We then carried out stereotactic injections into the PVN of 4-week-old Mc4r+/− mice with either pCMV-sadCas9-VP64 AAV as a negative control or pCMV-sadCas9-VP64 AAV and pU6-Mc4rPr-CMV-mCherry (Mc4rPrm-CRISPRa-AAV) (Fig. 5, A and B). We observed an increase in Mc4r expression up to 2.7-fold in Mc4rPrm-CRISPRa-AAV mice (Fig. 5C). Body weight measurements 8 weeks after injection showed a significant weight reduction in the Mc4rPrm-CRISPRa-AAV–injected mice compared to the Mc4r+/− or pCMV-sadCas9-VP64–injected Mc4r+/− mice (Fig. 5, D and E).

These results further suggest that CRISPRa can be used to rescue other haploinsufficient phenotypes.

**Discussion**

CRISPR-based gene editing is a promising therapeutic technology for correcting genetic mutations. However, it is a challenging approach for treating haploinsufficiency, limited by low homology-directed repair efficiencies (i.e., editing only a small portion of cells) and the need to customize specific guides and donor sequences for each individual mutation. In addition, it may not be a feasible therapeutic strategy for microdeletions, more than 200 of which are known to cause human disease (40), primarily because of haploinsufficiency. In this study, we used a CRISPR-mediated activation approach to tackle these hurdles and show how a haploinsufficient phenotype could be corrected by increasing the transcriptional output from the existing functional allele with CRISPRa.
Using CRISPRa targeting for either the promoter or enhancer of Sim1, we could rescue the obesity phenotype in a tissue-specific manner in mice that are haploinsufficient for Sim1. Because this approach takes advantage of the existing functional allele, it has several benefits: (i) It overcomes the need to custom-tailor CRISPR gene editing approaches for different haploinsufficiency-causing mutations in the same gene. (ii) This approach could potentially be used to target two or more genes. It could serve as a potential therapeutic strategy for microdeletions-related diseases that are caused by the heterozygous LoF of more than one gene. (iii) CRISPRa-AAV could be used to rescue haploinsufficient phenotypes caused by genes that are longer than its optimal packaging capability. (iv) Tissue specificity is a major concern for gene therapy. CRISPRa-based approaches can take advantage of cis-regulatory elements to guide tissue specificity (Fig. 6A). The availability of large-scale tissue-specific maps of gene regulatory elements could provide ample candidates for this approach. We observed distinct differences in tissue-specific activation of Sim1 based on the targeted cis-regulatory element, which can be attributed to chromatin accessibility of the locus in various tissues. Previous large-scale Cas9 and dCas9 cell culture screens have shown a targeting preference for regions with low nucleosome occupancy, thus being more amenable to dCas9 targeting. CRISPRa uses a nuclease-deficient Cas9 (dCas9) fused to a transcriptional activator and as such does not edit the genome. However, it can lead to transcriptional modulated off-target effects. To test for such effects, we carried out both RNA-seq and ChIP-seq in vitro and in vivo. We did not observe any apparent CRISPRa off-target binding that resulted in significant transcriptional changes. Additional analyses of predicted off-targeting loci due to sgRNA mismatches did not find any transcriptional changes surrounding these loci. The dCas9-VP64 fusion used in our CRISPRa system is known to activate loci that are programmed for transcription, such as promoters or enhancers (14, 42). Taken together, our results suggest that CRISPRa has high specificity.

Our dCas9-VP64 mouse and rAAV vectors can be a useful tool for targeted gene activation in vivo by delivering sgRNA(s) targeted to a specific gene in certain tissues or cell types. This approach could be used to assess gene-gene interactions or for the identification of the target gene(s) of a specific regulatory element in vivo by measuring its expression level following activation. Another potential area of study could be neuronal circuit manipulation. Discrepancies between acute and chronic neuronal circuit manipulations have been observed (43) that could potentially be addressed by rAAV-CRISPRa and transgenic-CRISPRa strategies, respectively.

Haploinsufficiency of SIM1 is associated with severe obesity (19–21) in humans and mice (22). Whether this is caused by the reduction in PVN

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**Fig. 5. CRISPRa-AAV injection in the PVN decreases weight gain in Mc4r+/− mice.** (A) Schema showing the CRISPR AAVs used for injection into Mc4r+/− mice. (B) Timeline for weight measurement post CRISPRa-AAV injection in PVN. (C and D) dCas9 (C) and Mc4r (D) mRNA expression from uninjected wild-type and Mc4r+/− mice along with pCMV-sadCas9-VP64 (dCas9-VP64)− and pCMV-sadCas9-VP64 + pMc4rPr-mCherry (Prm-CRISPRa)− injected Mc4r+/− mice. Four mice were used for each genotype with three technical replicates. The data are represented as means ± the lower and upper quartile, and lines represent the minimum and maximum. Values were determined based on mRNA fold increase compared to wild-type mice and normalized to Actb using the ΔΔCT method for Mc4r expression and relative Actb ΔΔCT log2 for dCas9 expression. (E and F) Weight gain determined over an 8-week period from Mc4r+/− female (E) or male (F) mice injected with pCMV-sadCas9-VP64 (dCas9-VP64)− or pCMV-sadCas9-VP64 + pMc4rPr-mCherry (Prm-CRISPRa) compared to uninjected wild-type littermates and Mc4r+/− mice. Means ± SD and number of mice (N) are shown for each condition. ***p < 0.0005; (ANOVA, Tukey test).
size during development that is observed in Sim1+/− mice (22) or by disturbed energy homeostasis during adulthood was largely known. The obesity phenotype observed in the postnatal conditional knockout of hypothalamic Sim1 (23) reinforced the hypothesis that Sim1 does indeed have a role in energy homeostasis later during adulthood. Our results showing phenotypic rescue in adult mice by rAAV CRISPRa further corroborate this role.

Mutations in MC4R are the most commonly found in individuals with class 3 obesity (body mass index >40 kg/m²), with an estimated 2.6 to 26% of this population having mutations in this gene are associated with an obesity phenotype (26–28). Heterozygous LoF mutations in this gene are associated with an obesity phenotype (26–28). MC4R is a promising drug target for anti-obesity drugs, and several agonists have been approved (29, 30). Mc4r haploinsufficiency in cases where this allele is not a complete LoF. The obesity phenotype observed in the postnatal conditional knockout of hypothalamic Sim1 (23) reinforced the hypothesis that Sim1 does indeed have a role in energy homeostasis later during adulthood. Our results showing phenotypic rescue in adult mice by rAAV CRISPRa further corroborate this role.

Materials and methods

**Plasmids**

The pMSCV-LTR-dCas9-Vp64-BFP vector, encoding a mammalian codon-optimized Streptococcus pyogenes dCas9 fused to two C-terminal SV40 NLSs and tagBFP along with a Vp64 domain and the U6-sgRNA-CMV-mCherry-T2A-Puro plasmids were used for cell line transfections (both kind gifts from J. S. Weissman and S. Qi). sgRNAs (table S4) were cloned into the In-Fusion HD cloning kit (Clontech) following the manufacturer’s protocol into the Bst XI and Xho I sites. Mouse knockin vectors were generated by cloning dCas9-Vp64 and U6-sgRNA-CMV-mCherry expression cassettes from the aforementioned vectors into the TARGATT (CAG + Poly A) plasmid (Applied StemCell). For AAV vectors, pDNA-dCas9-Vp64 (Addgene 47070), and U6-sgRNA-CMV-mCherry-WPREPa were cloned replacing the Ef3a-FAS-hChr2(H334R)-mCherry-WPRE-pA with that of the U6-sgRNA-CMV-mCherry-WPREa into the backbone of pAAV-EF3a-FAS-hChr2(H334R)-mCherry-WPRE-pA (Addgene 37090). S. aureus dCas9-Vp64 vector was constructed from Addgene Plasmid #68495, AAV-NLS-SaCas9-NLS-VPF by removing the RelA(p65) activation domain and Rta Activation domain using XbaI and EcoRI enzymes and introducing a stop codon after the Vp64 domain followed by bGHPolyA. We named it pAAV-CMV-sadCas9-Vp64-pA. pAAV-U6-sasgRNA-CMV-mCherry-WPREPa was cloned by replacing the CMV-sadCas9-Vp64-pA cassette in pAAV-CMV-sadCas9-Vp64-Vp64-pA backbone with that of U6-sasgRNA-CMV-mCherry-WPREPa cassette from pLTR-1120-MP17-U6-sasgRNA-mcherry (a kind gift from B. Huang at UCSF). S. aureus sgRNAs (table S4) were cloned using the In-Fusion HD cloning kit (Clontech) following the manufacturer’s protocol into the Bst XI and Xho I restriction enzyme sites.

**AAV production**

Particles of rAAV-DJ serotype, which is a chimera of type 2, 8, and 9 that was shown to achieve high expression levels in multiple tissues (39), were produced for all vectors using the Stanford Gene Vector and Virus core. The packaging load for pCMV-spCas9-Vp64 was 5.4 kb and for pU6-Sim1Pr-CMV-mCherry and pU6-SCE2-CMV-mCherry 2.5 kb. Titers were ascertained by WPRE and ITR probes to be 1.40 × 10³ viral genome/vm for pCMV-spCas9-Vp64 and around 3.30 × 10³ viral genome/vm for pU6-Sim1Pr-CMV-mCherry and 2.20 × 10³ viral genome/vm for pU6-SCE2-CMV-mCherry. The packaging load for pCMV-sadCas9-Vp64-pA was 4.3 kb and for pU6-sasgRNA-CMV-mCherry was 2.5 kb. Genetic titers for pCMV-sadCas9-Vp64-pA were 1.60 × 10¹² viral genome/ml, for pU6-sasgRNA3Sim1Pr-CMV-mCherry 1.58 × 10¹³ viral genome/ml, for pU6-sasgRNAAS3Sim1Pr-CMV-mCherry 2.0 × 10¹³ viral genome/ml, and for pU6-sasgRNA2Ms4rp-CMV-mCherry 1.0 × 10¹² viral genome/ml.

**Cell culture**

Neuroblastoma 2a cells (Neuro-2a; ATCC® CCL-121) were grown following ATCC guidelines. Plasmids were transfected into Neuro-2a cells using X-tremeGENE HP DNA transfection reagent (Roche) following the manufacturer’s protocol. AAV particles were infected into Neuro-2a cells at different multiplicity of infection (MOI) ratios (figs. S9 and S12E). Neuroblastoma 2a cells (Neuro-2a; ATCC® CCL-121) were grown following ATCC guidelines. Plasmids were transfected into Neuro-2a cells using X-tremeGENE HP DNA transfection reagent (Roche) following the manufacturer’s protocol.
harvested 48 hours after transfection and 5 days after infection to isolate RNA for qRT-PCR analysis.

**RNA isolation, RNA-seq, and quantitative reverse-transcription PCR**

RNA was isolated from cells or tissues using the RNeasy Mini Kit (Qiagen) following the manufacturer’s protocol. For mice, animals were euthanized and tissues were harvested directly into the RNA lysis buffer of the RNeasy Mini Kit. The hypothalamus was dissected using a mouse brain matrix and slicers (Zivic Instruments). Each hypothalamus was sampled the same way for each animal with 1.0-mm coronal section slice intervals. The coronal brain section of the hypothalamus was sliced with two blades 3 mm apart (Fig. S15, A to C). The hypothalamus was dissected out from this coronal section. Care was taken to orient the brain on the matrix and to align the hypothalamus each time corresponding to the same coronal section slots. For qRT-PCR, cDNA was prepared using SuperScript III First-Strand Synthesis System (Invitrogen) using the manufacturer’s protocol along with deoxyribonuclease I digestion. qPCR was performed with SsoFast EvaGreen Supermix (Bio-Rad) and primers that overlap the region that was knocked out (Sim1−/−’ primers listed in table S4), to further validate our qPCR results for Sim1. We also performed qPCR on wild-type, Sim1−/− and Sim1+/− Prm-CRISPRa-AAV−, Enh-CRISPRa-AAV−, or pCMV-dCas9-VP64-AAV−–injected mice with mice that overlap the region that was knocked out (Sim1−/−’ primers listed in table S4), obtaining similar results (Fig. S13, D to G). The results were expressed as fold-increase mRNA expression of the gene of interest normalized to either Actb or Rpl38 expression by the ΔΔCT method followed by analysis of variance (ANOVA) and Tukey test for statistical analysis. Reported values are the mean ± SEM from three independent experiments performed on different days (N = 3) with technical duplicates that were averaged for each experiment. For RNA-seq, three males and three females were used for each genotype (24 samples total; 6 biological replicates per condition). cDNA was amplified using Ovation V2 kit (NuGEN), and sequencing libraries were generated using NexteraXT kit (Illumina). RNA-seq was carried out on an Illumina HiSeq 4000. Sequence alignment was performed using STAR (32). Mappings were restricted to those that were uniquely aligned to the mouse genome (GRCm38.67). Replicates were pooled to call peaks against a background of the nontargeting VP64 ChIP using MACS2 (54). All ChIP-seq data were deposited in NCBI as Bioproject PRJNA438723.

**Mice**

Sim1−/− mice (22) on a mixed genetic background were obtained as a kind gift from J. Michaud’s lab. In these mice, a 1-kb fragment containing 750 bp of the 5′ region, the initiation codon, and the sequence coding for the basic domain (the first 17 amino acids) was replaced by a Pgk−neo cassette that was used for genotyping (see table S4 for primers) with KAPA mouse genotyping kit (KAPA Biosystems). To generate dCas9-VP64 and sgRNA mice, we used TARGATT technology (34). DNA for injection was prepared and purified as minicircles by using the TARGATT Transgenic Kit, V6 (Applied StemCell). The injection mix contained 3 ng/μl DNA and 48 ng/μl of in vitro–transcribed cDNA mRNA in microinjection TE buffer [0.1 mM EDTA, 10 mM Tris (pH 7.5)], and injections were done using standard mouse transgenic protocols (55). dCas9-VP64 was inserted into the mouse Hipp11 locus and sgRNAs into the Rosa26 locus. Mice were genotyped using the KAPA mouse genotyping kit. F0 H11P3CAG-dCas9-VP64 TARGATT knockins were assessed in injected mice with primers specific dCas9-VP64 and background of the nontargeting VP64 ChIP using MACS2 (54). All ChIP-seq data were deposited in NCBI as Bioproject PRJNA438723.

**Chromatin immunoprecipitation**

Fresh tissue was homogenized using a hand-held dounce homogenizer, cross-linked in phosphate-buffered saline (PBS) containing 1% formaldehyde for 10 min, quenched with 125 mM glycine for 5 min, and washed three times with PBS. Cross-linked tissue pellet was processed further for chromatin immunoprecipitation using the Low cell Chip Kit (Diagenode; catalog no. C01010072) following the manufacturer’s protocol. An S. pyogenes Cas9 polyclonal antibody (Diagenode; catalog no. C15310258) and an S. aureus Cas9 monoclonal antibody (Diagenode; catalog no. C15200230) were used for the pull-down. Enrichment of target regions were assessed by RT-qPCR using SsoFast EvaGreen Supermix (Bio-Rad) and primers listed in table S4. Results were expressed as %input using the ΔΔCT method. Reported values are the mean ± SEM from three independent experiments performed on different days (N = 2) with technical duplicates that were averaged for each experiment. For ChIP-seq, a pool of four mice was used for each genotype and two biological replicates. Libraries were made by using the ThruPLEX DNA-seq kit (Rubicon Genomics; catalog no. R400428) and sequencing was carried out with an Illumina HiSeq-4000. Sequencing reads were mapped to the genome using STAR (32). Mapping was restricted to reads that were uniquely assigned to the mouse genome (GRCh38.67). Replicates were pooled to call peaks against a background of the nontargeting VP64 ChIP using MACS2 (54). All ChIP-seq data were deposited in NCBI as Bioproject PRJNA438723.

**Body composition and food intake analyses**

Body composition was measured using either dual energy x-ray absorptiometry (DEXA) or Echo Magnetic Resonance Imaging (EchoMRI; Echo Medical System). For DEXA, mice were anesthetized with isoflurane and measured for bone mineral density and tissue composition (fat mass and lean mass) with the Lunar PIXImus. EchoMRI (Echo Medical System) was used to measure whole-body composition parameters such as total body fat, lean mass, body fluids, and total body water in live mice without the need for anesthesia or sedation. Food intake was measured using the Columbus Instruments Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments). Mice were housed individually and acclimatized on powdered picodiet 5058 for 3 to 4 days, and food intake measurements were done over 4 to 5 days. Three males and three females from each genotype: wild-type littersmates, Sim1−/−, Sim1+/− × H11P3CAG-dCas9-VP64, R26PsSim1Pr-sgRNA, Sim1−/− × H11P3CAG-dCas9-VP64 or R26PsC528En-sgRNA were measured for their body weights from 4 to 16 weeks of age on a weekly basis.

**Transgenic mice body weight measurements**

H11P3CAG-dCas9-VP64, R26PsSim1Pr-sgRNA and R26PsC528En-sgRNA mice were mated with FVB mice for three to five generations to assess germ-line transmission. Three independent integrants were used from each line to set up matings. H11P3CAG-dCas9-VP64−/− were mated with Sim1+/− and subsequent Sim1−/− × H11P3CAG-dCas9-VP64−/− mice were crossed with either R26PsSim1Pr-sgRNA or R26PsC528En-sgRNA to generate mice having all three unlinked alleles. Mice were maintained at Picodiet.5058 throughout the study, and at least 10 females and 10 males from all genotypes (wild type, Sim1−/−, Sim1+/− × H11P3CAG-dCas9-VP64−/− or Sim1−/− × H11P3CAG-dCas9-VP64+, R26PsSim1Pr-sgRNA or Sim1−/− × H11P3CAG-dCas9-VP64+, R26PsC528En-sgRNA) were measured for their body weights from 4 to 16 weeks of age on a weekly basis.
injections carried out in a previous study (36). A 0.5-mm hole was created in the cranium with a high-speed model 1911 Stereotactic Drill with a 0.02-inch drill bit (David Kopf Instruments). Using a 31-gauge 1-ml Hamilton microsyringe, we injected a dose of 0.5 × 10^7 vg/ml of sgRNA-AAV along with 2.5 × 10^6 vg/kg of spdCas9-VP64-titer and double the amount (1 × 10^7 vg/ml of the PVN unilaterally over a 10-min period. This performed as previously described (50) m30% sucrose in PBS for 72 hours. Brains were weighed on a weekly basis.


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Data and materials availability: All RNA-seq data were deposited in NCBI as Bioproject PRJNA438712. All ChIP-seq data were deposited in NCBI as Bioproject PRJNA438723.

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/363/6424/eaau0629/suppl/DC1

Figs. S1 to S15

Tables S1 to S5

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CRISPR-mediated activation of a promoter or enhancer rescues obesity caused by haploinsufficiency


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CRISPRa corrects haploinsufficient obesity

Loss-of-function mutation in one gene copy, termed haploinsufficiency, can lead to insufficient protein levels and result in human disease. Matharu et al. tested whether a CRISPR-based activation system (CRISPRa) could rescue a haploinsufficient phenotype by increasing the gene expression levels of the existing normal copy (see the Perspective by Montefiori and Nobrega). By delivering this system into the mouse hypothalamus using adeno-associated virus, they rescued the obesity phenotype caused by haploinsufficiency of either of two genes known to promote obesity when mutated in mice and humans. These results highlight the translational potential of the CRISPR activation system to treat haploinsufficient disease.

Science, this issue p. eaau0629; see also p. 231

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