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SHORT COMMUNICATION

CRISPR-on system for the activation of the endogenous human *INS* gene

CA Giménez[1], M Ielpi[1], A Mutto[2], L Grosembacher[3], P Argibay[1] and F Pereyra-Bonnet[1]

Advances in the field of epigenetics have allowed the design of new therapeutic strategies to address complex diseases such as type 1 diabetes (T1D). Clustered regularly interspaced short palindromic repeats (CRISPR)-on is a novel and powerful RNA-guided transcriptional activator system that can turn on specific gene expression; however, it remains unclear whether this system can be widely used or whether its use will be restricted depending on cell types, methylation promoter statuses or the capacity to modulate chromatin state. Our results revealed that the CRISPR-on system fused with transcriptional activators (dCas9-VP160) activated endogenous human *INS*, which is a silenced gene with a fully methylated promoter. Similarly, we observed a synergistic effect on gene activation when multiple single guide RNAs were used, and the transcriptional activation was maintained until day 21. Regarding the epigenetic profile, the targeted promoter gene did not exhibit alteration in its methylation status but rather exhibited altered levels of H3K9ac following treatment. Importantly, we showed that dCas9-VP160 acts on patients' cells *in vitro*, particularly the fibroblasts of patients with T1D.

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INTRODUCTION

Although the genetic backgrounds of diseases tend to receive the most attention, epigenetic mechanisms, such as chromatin remodeling, DNA methylation and histone modifications, are increasingly appreciated as relevant to the pathogenesis of diabetes.[1] The field of epigenetics facing this new challenge in biomedicine is called 'epigenetic therapy' and involves the manipulation of the epigenetic regulation of endogenous genes to 'turn off' or 'turn on' transcriptional expression.[2]

In the past decade, diverse research groups have developed strategies that employ zinc fingers or transcription activator-like effectors (TALEs) to direct specific enzyme machineries to regulate single genes. However, these strategies are difficult to design and are expensive.[2] A new tool for molecular editing has emerged from a bacterial and archeaen defense system called the clustered regularly interspaced short palindromic repeats (CRISPR)-associated Cas protein system (CRISPR/cas). Recently, the use of CRISPR/Cas-derived biotechnology in eukaryotic cells has become a practical RNA-guided platform for targeting DNA loci with diverse applications.[3–5]

The CRISPR system for gene induction (CRISPR-on) is a novel and powerful RNA-guided transcriptional activation system. The CRISPR-on system consists of the inactive DNA nuclease Cas9 (dCas9) fused to activation domains and co-expressed single guide RNAs (sgRNAs) that are designed to hybridize a target sequence. Combined, these elements can generate a DNA complex that recognizes a target locus and activates a specific gene.[6–9] The possibilities of a simple tool with the ability to modulate the transcriptional state of a gene with high specificity have elicited enormous expectations in reprogramming strategies and medicine.[10–13]

We sought to determine whether the CRISPR-on system was affected by the cell type used and the fully methylated status of the target promoter. This allowed identification of the capacity of the CRISPR-on system to modulate epigenetic marks associated to gene activation. Due to the potential clinical relevance of this work, we chose the endogenous human insulin (*INS*) gene as a model.

RESULTS AND DISCUSSION

To determine whether the CRISPR-on system allowed for the activation of endogenous genes, we co-transfected NIH3T3 mouse cells with an expression plasmid consisting of dCas9 fused to 10 copies of VP16 (dCas9-VP160) and three sgRNAs targeting the endogenous *Oct4* transcription factor (T1, T2 and NT3, previously tested by Hu *et al.*).[14] The reverse transcription PCR (RT-PCR) analysis revealed that the CRISPR-on system in NIH3T3 was able to turn on *Oct4* expression (data not shown). These results confirmed that the system could be used to activate endogenous genes.

Having established that dCas9-VP160 was able to activate endogenous genes in our hands, we designed four sgRNAs that targeted the endogenous human *INS* proximal promoter and studied their abilities to activate this gene in human embryonic kidney 293T cells (HEK293T) (Figures 1a–c). The sgRNAs target sequences and their positions on the human insulin gene promoter are detailed in Table 1. The different *INS* promoter sites that were chosen indicated that the expression of a single sgRNA was sufficient to induce *INS* gene activation (Figures 2a and b). To quantify and validate these observations, we performed individual quantitative PCRs (qPCRs) for the four sgRNAs that were transfected and co-transfected in HEK293T cells. We observed synergistic activations when multiple sgRNAs were used (Figure 2c), in agreement with other reports involving the CRISPR-on system.[8–10] Collectively, these observations indicate that gene activations with the CRISPR-on system using dCas9-VP160 are multiplicatively

[1]Basic Science and Experimental Medicine Institute, University Institute of the Italian Hospital of Buenos Aires (HIBA), Buenos Aires, Argentina; [2]Laboratory of Reproductive Biotechnologies and Animal Genetic Improvement, Biotechnology Research Institute, National University of General San Martín, Buenos Aires, Argentina and [3]Endocrinology and Nuclear Medicine Service, HIBA, Buenos Aires, Argentina. Correspondence: Dr F Pereyra-Bonnet, ICBME, Hospital Italiano, Buenos Aires C1181ACH, Argentina.
E-mail: Federico.pereyra@hospitalitaliano.org.ar
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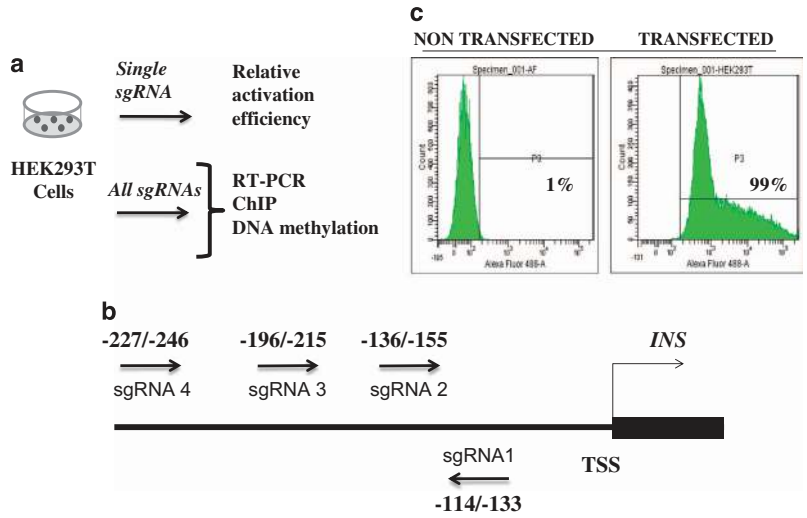

Figure 1. Research design. (**a**) Schematic diagram showing the design of the experiments for the CRISPR-on *INS* activation and epigenetic analysis. (**b**) Schematic of the human *INS* promoter region. The location of transcription start site (TSS) is indicated. The short lines indicate the targeting sites and the directions of the sgRNAs (sgRNAs 1, 2, 3 and 4). The numbers indicate the positions relative to the TSS. (**c**) FACS analyses of the non-transfected and transfected HEK293T cells with the CRISPR-on system following the delivery of the pCX-GFP expression plasmid as a transfection control (10% total plasmid).

Table 1. sgRNA target sequences and positions on the human insulin gene promoter

sgRNA name	*Target sequence (5′–3′)*	*Position relative to TSS*
sgRNA 1	GGGGCTGAGGCTGCAATTTC	−114/−133
sgRNA 2	CCAGCACCAGGGAAATGGTC	−136/−155
sgRNA 3	CTAATGACCCGCTGGTCCTG	−196/−215
sgRNA 4	AGGTCTGGCCACCGGGCCCC	−227/−246

Abbreviation: TSS, transcription start site.

increased by binding events at different sites of the proximal promoter regions. Although we observed reproducibility of the CRISPR-on activation, there were differences in gene expression between replicates (Figure 2c). This observation could be attributed to the random features of the procedure. Further investigation will be required to turn this procedure into a non-stochastic process.

To estimate the duration of the dCas9-VP160 stimulation, we performed out sequential RT-PCR analyses on a culture of HEK293T cells that were co-transfected with all of the sgRNAs. We found that the activation of the *INS* gene was sustained for at least day 21 post transfection, just before that an increase in caspase-3 messenger RNA levels (apoptosis marker) was observed by qPCR indicating the possible onset of cell death (Figure 2d; Supplementary Figure 1). This result is important regarding the potential future uses of CRISPR-on systems in biomedical research. Nevertheless, lack of insulin protein expression was observed in HEK293T cells at day 4 by immmunofluorescence (data not shown). The absence of protein in HEK293T cells could be due to either low rate of *INS* messenger RNA expression (compared with human pancreas control; Supplementary Figure 2), to post-transcriptional regulation by 5-methylcytosine of RNA[15] (Supplementary Figure 3) or to lack of specific *INS* RNA-binding proteins in this phenotype.[16] Importantly in this line, a recent work by Balboa and colleagues[13] reported not only activation of transcription but also of translation using a CRISPR-on variant, showing the reach of this technique.

Very few studies have described epigenetic statuses previous to the onset of the CRISPR-on systems. To determine whether the activity of dCas9-VP160 was affected by the full methylation status of the *INS* promoter and how this status was influenced by the application of dCas9-VP160, DNA bisulfite conversion followed by direct sequencing was employed. We were able to activate the *INS* gene even when pretreatment bisulfite analyses revealed high degrees of methylation in the *INS* promoters of the HEK293T cells (Figure 3a). This finding is consistent with one recent study in which the prior methylation statuses of the promoters of targeted genes were not found to predict successful gene activation by the CRISPR-on system and contrasts the findings related to other epigenetic editing tools based on transcription activator-like effector nuclease.[14] The methylation level in the *INS* promoter was high even in the CpG dinucleotide-binding sites targeted by our four sgRNAs (Supplementary Figure 4). These observations might be explained by the DNA–RNA recognition natures of CRISPR systems, which can be abolished by mismatches between the sgRNA and target DNA at different positions but can tolerate both methylated and non-methylated cytosines without considering these two variants as mismatches.[17] Furthermore, the *INS* promoters remained hypermethylated even after *INS* transcription was found to be significantly upregulated in the post-treatment analyses (Figure 3a). Also, analysis of DNA associated with active epigenetic marks coupling chromatin immunoprecipitation (ChIP) and bisulfite analysis did not reveal robust hypomethylation (Figure 3b).

In the same line, our results suggest that the CRISPR-on system using VP16 domains was able to increase the acetylation levels on H3K9. To quantify the targeted epigenetic activation of the markers H3K4me3 and H3K9ac on the proximal promoter mediated by dCas9-VP160, we performed ChIP with specific antibodies followed by qPCR in HEK293T cells that were co-transfected with all four sgRNAs. The qPCR analysis revealed that although H3K4me3 did not exhibit any changes, the H3K9ac marker on the human *INS* proximal promoter exhibited an increase of >2.5-fold in cells that were transfected with the CRISPR-on system (Figure 3c). H3K9ac epigenetic marks in proximal promoter regions are known to be associated with transcriptionally active genes due to their role in transcriptional initiation.[18] Previous reports have shown that the overexpression of p300 associated with the VP16 domain can enhance a CRISPR-on system (sgRNA/dCas9-VP64).[17,19] In addition, our preliminary results in co-immnunoprecipitation indicated that dCas9-VP160 interact with p300 (data not shown). In this line of work, it is widely documented that p300 catalyzes H3K27 acetylation,[20] but it has recently been reported that p300 may also intervene in H3K9 acetylation.[21,22] Further studies are needed to

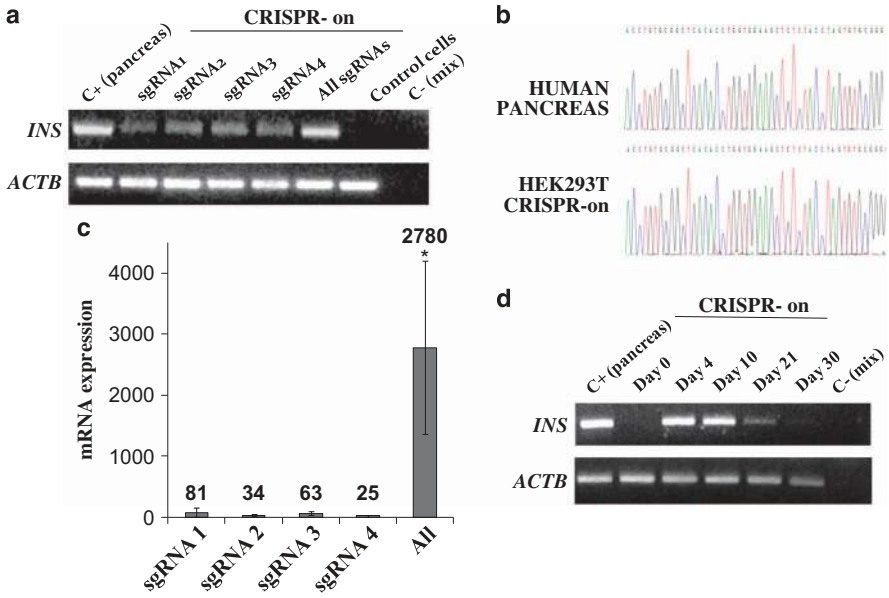

Figure 2. Activation of the endogenous *INS* gene via the CRISPR-on system using dCas9-VP160. (**a**) The four sgRNAs expression plasmids were co-transfected with dCas9-VP160 individually and in combination into HEK293T cells, and *INS* gene activation was assessed by RT-PCR. The control cells were transfected with empty sgRNA expression plasmids, and β-actin was used as the internal control (*ACTB*). The data represent the outcomes of two biological replicate assays. (**b**) To validate the *INS* product identity, electrophoretic automatic sequencing analyses were performed with the PCR products obtained in (**a**). The sequences on the electropherograms from the CRISPR-on cells and human pancreas cells (positive control) exhibited a perfect match in accordance with the results obtained by BLASTN. (**c**) *INS* messenger RNA (mRNA) expression levels in the HEK293T cells co-transfected with dCas9-VP160 and single or multiples sgRNAs were measured by qPCR. The gene expressions were normalized to the β-actin gene expression levels and are shown relative to those of the control cells. The data are shown as the means ± s.e.m. ($n = 2$ biological replicates). The treatment with the combination of sgRNAs was significantly different than all other treatments (*$P < 0.05$; ANOVA followed by Turkey's tests). (**d**) *INS* gene expression was assessed by RT-PCR on days 0 (control cells), 4, 10, 21 and 30 post transfection in the CRISPR-on systems with all of the sgRNA in the HEK293T cells.

identify specific manipulations of the CRISPR-on system to improve its accuracy in chromatin accessibility.

Finally, to determine whether dCas9-VP160 could activate *INS* gene expression in other cell types, we co-transfected all four sgRNAs into HeLa cells and skin fibroblasts obtained from two patients with type 1 diabetes in two different *in vitro* experiments. We observed gene activation in every cell type that was transfected with the CRISPR-on system via RT-PCR and no activation in the non-transfected control cells (Figure 4). Our results, particularly those involving the fibroblasts from the type 1 diabetes patients, could have particular relevance to future biotechnological and medical applications. In this work, we chose the most straightforward approach to activate the *INS* gene; however, it is clear that a deeper reprogramming process is needed to obtain 'bonafide' beta-like cells. One current possibility is the use of the CRISPR-on system to improve reprogramming strategies.[13] In addition, future studies may examine CRISPR-on system delivery *in vivo* for the conversion of alpha or ductal into beta cells via the activation of key β-cell development genes such as *INS*.[23]

In summary, we report the first four validated sgRNAs for the endogenous activation of human *INS* observed in diverse cell types, including an extensively description of the epigenetic changes underlying the CRISPR-on activation of *INS* gene expression. This work provides new clues that will enable further discoveries in this novel area.

MATERIALS AND METHODS

Ethics statement

The protocols were approved by the Institutional Ethics Committee (Res 1672). The two fibroblast donors provided written informed consent for the collection of all samples and subsequent analyses.

Cell culture and transfection of HEK293T, HeLa and human fibroblasts

HEK293T cells and HeLa cells were cultured under standard culture conditions. Skin biopsies were obtained from two type 1 diabetes patients and expanded as described previously.[24] The transfections of the HEK293T and HeLa cells were performed using Lipofectamine 2000 (Invitrogen; Carlsbad, CA, USA) using a 1:1 DNA/reagent ratio, and the transfection of the human fibroblasts was performed with Lipofectamine LTX Plus Reagent (Invitrogen) according to the manufacturer's instructions. For all cell types, the dCas9-VP160 plasmids were transfected at a mass ratio of 1:1 to either the individual sgRNA expression plasmids or the identical amount of sgRNA expression plasmid consisting of a mixture of equal amounts of the four sgRNAs. Control groups cells were transfected with the dCas9-VP160 plasmid and empty sgRNA expression plasmids at a mass ratio of 1:1.

Plasmids

The sgRNA expression plasmid (pSPgRNA) and the plasmid encoding dCas9-VP160 (pAC94-pmax-dCas9-VP160-2A-puro) were developed previously by others[8,9] and obtained from Addgene (plasmids #47108 and #48226). The oligonucleotides containing the target sequences were synthesized, hybridized, phosphorylated and cloned on the pSPgRNA plasmid using the *Bbs*I sites as described by Ran *et al*.[25] The insertion was verified by clone sequencing. The oligonucleotide sequences for cloning are provided in Supplementary Table 1.

Gene expression analysis

The human fibroblasts were sorted by fluorescence-activated cell sorting (FACS) following the delivery of the pCX-EGFP expression plasmid (transfection control plasmid). HEK293T cells were similarly

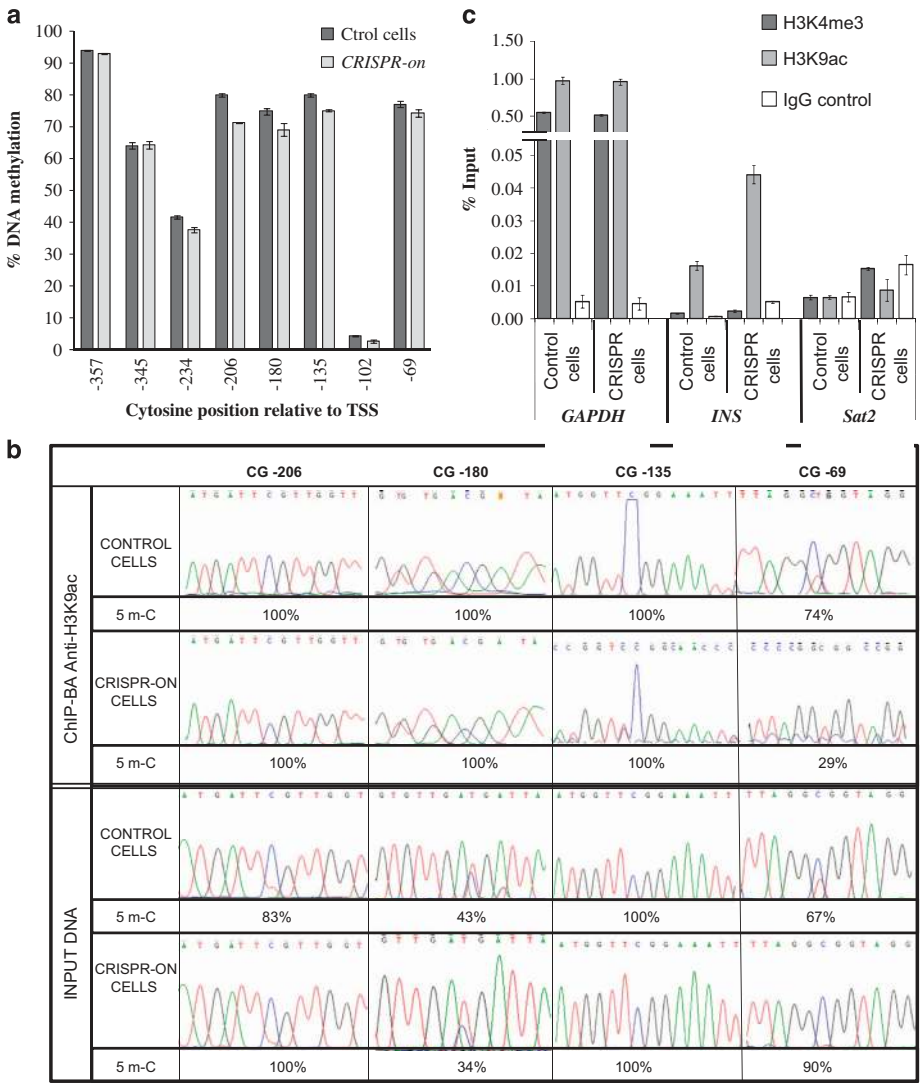

Figure 3. Epigenetic analysis of the *INS* promoter activation by the CRISPR-on system with all sgRNAs. (**a**) DNA methylation analyses of the *INS* proximal promoter in the HEK293T control and CRISPR-on-transfected cells. The methylation level following treatment with the combination of sgRNAs was not significantly different from that of the control group ($P > 0.05$; unpaired *t*-test with Welch's correction). The data are shown as the means ± s.e.m. ($n = 3$ biological replicates). (**b**) ChIP bisulfite analysis of *INS* proximal promoter. Electropherograms sequences images from a replicate of HEK293T CRISPR-on cells and control cells are shown for the input DNA (sonicated) and DNA precipitated with active epigenetic mark H3K9ac. Percentage of 5-methilcytosine (5-mC) is indicated for each dinucleotide CG. (**c**) ChIP–qPCR analyses of H3K4me3 and H3K9ac in the *INS* proximal promoter, the *GAPDH* proximal promoter (control+) and the heterochromatin repetitive sequence *Sat2* (control −) in the HEK293T control and CRISPR-on-transfected cells. IgG control antibody was used as an immunoprecipitation background control. The values were obtained using the percent input method. The data are shown as the means ± s.e.m. ($n = 5$ *INS* H3K9ac; $n = 2$ *INS* H3K4me3; $n = 2$ IgG control; $n = 3$ GADPH and Sat2).

treated without FACS. Total RNA was isolated with a MicroKit (Qiagen; Venlo, Netherlands). For the RT-PCR and qPCR, the RNA was reverse-transcribed using ImProm-II™ Reverse Transcriptase (Promega; Fitchburg, MA, USA). Specific intron-spanning primers were used in the PCR and qPCR (Supplementary Table 2). The qPCR was performed using KAPA SYBR FAST qPCR Kit Master Mix (2×) Universal (Kapa Biosystems; Wilmington, MA, USA). The reported values are the means and the s.e.m. from two biological replications ($n = 2$) with technical duplicates that were averaged for each experiment. For sequential RT-PCR analyses, HEK293T cells were co-transfected with all of the sgRNAs and keep on culture for 0, 4, 10, 21 and 30 days without cell splitting.

ChIP–qPCR analysis

A pool of cells obtained from two independent transfection assays was used as a starting material for the ChIP–qPCR analysis. HEK293T cells were collected 4 days after co-transfection of dCas9-VP160 and the four sgRNAs. A ChIP assay was performed using an OneDay ChIP kit (Qiagen) according to the manufacturer's instructions. Anti-human H3K4me3, anti-human H3K9ac and their IgG controls (Qiagen; GAH-8208,GAH-1209) were used for the immunoprecipitation. The primers used for the qPCR are listed in Supplementary Table 2. The percent input method was used to analyze the data.[26] The reported values are the means and the s.e.m. from technical replicates using the pool of cells ($n = 2$–5).

DNA methylation analysis

The genomic DNAs of the CRISPR-on HEK293T cells and control cells were purified using a MiniKit column (Qiagen) and treated using a bisulfite kit (Qiagen). The post-bisulfite promoter region of the human *INS* gene (NCBI ID 3630) was amplified by PCR using specific primers (Supplementary Table 2). The PCR products were purified and directly sequenced by Macrogen (Macrogen, Inc; Tokyo, Japan.), and the methylation levels were measured as described by Jiang

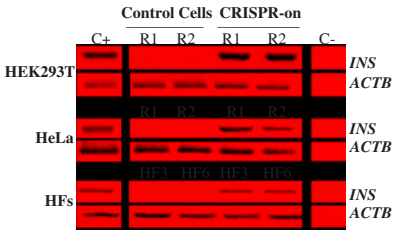

Figure 4. The CRISPR-on system using dCas9-VP160 with all sgRNAs is portable to other cell types, including skin fibroblasts from patients with TD1. RT-PCR analyses of the endogenous *INS* expressions in the HEK293T cells, HeLa cells and fibroblasts from patients with type 1 diabetes (human fibroblasts, HFs) in the control and CRISPR-on groups. β-Actin was used as the internal control (*ACTB*). For the HFs, the data represent the outcomes of two biological replicate assays. The dividing lines mark the different parts of the same gel. R1 and R2, biological replicates 1 and 2; HF3 and HF6, human fibroblasts from patient 3 and 6; C+: human pancreas cDNA; C−: control mix.

et al.[27] The reported values are the means and the s.e.s' of the mean from three biological replications ($n = 3$) with technical duplicates that were averaged for each experiment. The ChIP bisulfite analysis DNAs of the CRISPR-on HEK293T cells and control cells precipitaded with anti-H3K9ac were treated using a bisulfite kit (Qiagen) according to Matarrazo *et al.*[28] Primers used for ChIP bisulfite analysis are described in Supplementary Table 2.[29]

sgRNA design

The sgRNAs were designed using the CRISPR Design Tool (Feng Zhang Lab, MIT, Cambridge, MA, USA). The algorithm used by this program is based on a previously described specificity analysis.[17]

Statistical analysis

The statistical analyses were performed using the GraphPad Prism 5.0 software (La Jolla, CA, USA). The results were considered significant when the *P*-values was < 0.05.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

CG, PA and FPB conceived and designed the experiments. CG, MI and FPB performed the experiments. CG and FPB analyzed the data. AM and LG contributed reagents/materials/analysis tools. CG and FPB wrote the paper. FPB is the guarantor of this work and as such had full access to all of the data in the study and assumes responsibility for the integrity of the data and the accuracy of the data analysis.

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Supplementary Information accompanies this paper on Gene Therapy website (http://www.nature.com/gt)