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Tailoring the CRISPR system to transactivate coagulation gene promoters in normal and mutated contexts

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ABSTRACT

Engineered transcription factors (TF) have expanded our ability to modulate gene expression and hold great promise as bio-therapeutics. The first-generation TF, based on Zinc Fingers or Transcription-Activator-like Effectors (TALE), required complex and time-consuming assembly protocols, and were indeed replaced in recent years by the CRISPR activation (CRISPRa) technology. Here, with coagulation *F7/F8* gene promoters as models, we exploited a CRISPRa system based on deactivated (d)Cas9, fused with a transcriptional activator (VPR), which is driven to its target by a single guide (sg)RNA.

Reporter gene assays in hepatoma cells identified a sgRNA ($sgRNA_{F7.5}$) triggering a ~35-fold increase in the activity of *F7* promoter, either wild-type, or defective due to the c.-61T > G mutation. The effect was higher (~15-fold) than that of an engineered TALE-TF (TF4) targeting the same promoter region. Noticeably, when challenged on the endogenous *F7* gene, the dCas9-VPR/$sgRNA_{F7.5}$ combination was more efficient (~6.5-fold) in promoting factor VII (FVII) protein secretion/activity than TF4 (~3.8-fold). The approach was translated to the promoter of *F8*, whose reduced expression causes hemophilia A. Reporter gene assays in hepatic and endothelial cells identified sgRNAs that, respectively, appreciably increased *F8* promoter activity ($sgRNA_{F8.1}$, ~8-fold and 3-fold; $sgRNA_{F8.2}$, ~19-fold and 2-fold) with synergistic effects (~38-fold and 2.7-fold). Since modest increases in *F7/F8* expression would ameliorate patients' phenotype, the CRISPRa-mediated transactivation extent might approach the low therapeutic threshold.

Through this pioneer study we demonstrated that the CRISPRa system is easily tailorable to increase expression, or rescue disease-causing mutations, of different promoters, with potential intriguing implications for human disease models.

1. Introduction

In the last decade the modulation of gene transcription has attracted attention for therapeutic purposes in human disease, either by gene activation/repression or modification of the chromatin architecture. So far the most used approaches have been based on the manipulation of Zinc-fingers (ZFs) [1] or Transcription activator-like effectors (TALEs) [2] fused to transcriptional regulators.

ZFs typically occur in tandem arrays, and many transcription factors have three or more fingers working together to recognize specific targets on DNA. The ZFs activators have been successfully exploited to enhance endogenous expression of different human genes, including the erythropoietin [3], ERBB2 [4] and EGF2 [5] genes.

TALE proteins have a complex DNA-binding domain, which is composed of a variable number of tandem repeats differentiated only for residues at positions 12–13 of each monomer (repeat variable di-residue, RVD). The RVD dictates the specificity of recognition of the target DNA sequence [6,7]. TALE-based transcription activators (TALE-TF) have been exploited to drive the expression of different genes such as Oct4 [8,9], Frataxin [2] and for cellular reprogramming [10].

However, despite their efficacy, the exploitation of ZFs and TALE tools requires a tedious and time-consuming protocols.

In this context, the advent of the technology based on CRISPR, so far extensively exploited for genome editing [11], has opened new perspectives. The CRISPR activation (CRISPRa) system is composed of a deactivated CRISPR-associated protein 9 (dCas9), obtained by means of

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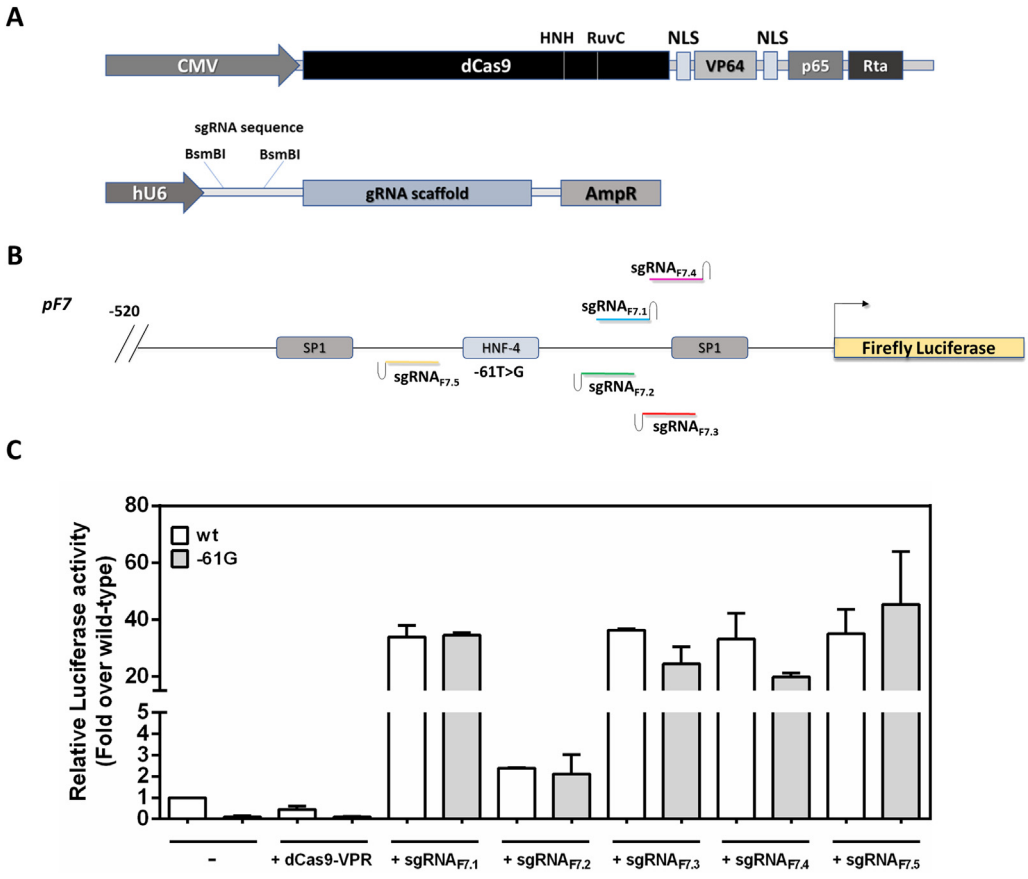

Fig. 1. Activity of the CRISPRa approach on *F7* promoter in reporter assays.

A) Schematic representation of the pSP-dCas9-VPR (upper scheme) and sgRNA backbone (MLM3636, lower scheme). dCas9-VPR is composed of a deactivated (d)Cas9 from *Streptococcus pyogenes* (HNH and RuvC inactivated domains) fused with a VPR domain, which consists of three different transcription activators (VP64, p65 and Rta) recruiting the transcriptional machinery. The single guide (sg)RNAs are under the control of the human U6 promoter. The scaffold sequence is necessary for the recruitment of Cas9.

B) Representation of the reporter p*F7* constructs, which include the proximal *F7* promoter region (520 bp) driving the expression of the firefly luciferase. The localization of the five sgRNAs (sgRNAs $_{F7.1–5}$) is reported above/below the scheme. The mutated HNF4 binding sequence (−61 T > G) is also indicated.

C) Transactivation activity of the designed sgRNAs on the wild-type and mutant -61G promoters. Histograms report the fold increase of the luciferase activity over the pF7wt. The results are expressed as mean ± standard deviation from three independent experiments conducted in HepG2 cells.

two missense mutations in the RuvC and HNH domains, fused with a transcriptional activator, and a single guide RNA (sgRNA) [12]. Since the first usage of this technology many steps have been done using different kinds of activators. First-generation activators were directly fused to the dCas9, while second-generation consist of activators arranged in a scaffold (SAM, SunTag) [13,14], exerting a synergistic effect, or of a tripartite activator composed by VP64, p65 and Rta (named as VPR) [15].

These features of the CRISPRa system, easily driven to the target by appropriately designed sgRNAs, makes it easy to be designed and realized, as well as highly versatile. However, a very few studies have been performed on the manipulation of gene expression by CRISPRa [16], particularly on the transactivation of defective promoters or of poorly expressed genes, which prevents the assessment and comparison with the previous approaches. In this field, we have recently demonstrated that a properly designed TALE-TF was able to rescue *F7* promoter activity affected by mutations lowering coagulation factor VII (FVII) expression and associated with severe deficiency [17].

Here, by exploiting the CRISPRa system in the model of coagulation, we modulated the expression of normal and mutated *F7* promoter. Noticeably, we extended the application to *F8*, with a higher degree of complexity and associated with the most frequent bleeding disorder hemophilia A, and which is poorly expressed. We demonstrated that the CRISPRa is easily tailorable on both promoter models and can guarantee a stronger effect than that of TALE TF.

2. Materials and methods

2.1. Expression vectors

The pF7$_{wt}$ and the pF7$_{-61G}$ vectors were obtained as previously described [17].

To create the pF8 reporter construct (pF8), the *F8* promoter region (1175 bp) was PCR amplified with primers $^{5'}$AAACTCGAGAAATAAATGAATAAATGCCA$^{3'}$ and $^{5'}$AAAAAGCTTCATGACTTATTGCTACAAAT$^{3'}$ and cloned upstream of the firefly luciferase sequence into the pGL3 Basic Vector (Promega, Madison, WI, USA) [17].

The sgRNAs were designed with the ZiFit web tool (http://zifit.partners.org/ZiFiT/) by entering the 92 bp of the proximal region of the *F7* promoter or 333 bp of the *F8* promoter, and scanning for the *Streptococcus pyogenes* PAM sequence (NGG) both in sense and antisense strands. Potential off-targets were predicted through the Cas OFFinder tool (http://www.rgenome.net). To generate sgRNAs expression cassette, the pMLM3636 plasmid was digested with *BsmBI* and cloned with a pair of annealed oligonucleotides (Supplementary Table 1). pSP-dCas9-VPR was a gift from George Church (Addgene plasmid #63798) and pMLM3636 was a gift from Keith Joung (Addgene plasmid #43860).

2.2. Transfection and evaluation of luciferase and FVII expression

The human hepatoma HepG2 and Huh-7 cells were transfected in 12-well plates as described [17]. For reporter gene assays, cells were transfected with 1 μg of pF7/pF8 plasmids and i) 0.5 μg of pdCas9-VPR and 0.25 μg of pMLM3636 or ii) 0.5 μg of pTF4. To normalize for transfection efficiency, cells were co-transfected with 100 ng of the pRluc control plasmid (Promega) expressing the Renilla luciferase. To evaluate the endogenous FVII expression, cells were transfected in 6-well plates with 1 μg of pdCas9-VPR and 0.5 μg of pMLM3636 or 1 μg of pTF4.

Forty-eight hours post-transfection, luciferase activity was assessed on cell lysates by the Dual-Luciferase assay (Promega) according to the manufacturer's protocol. FVII-containing medium was collected to measure FVII secreted protein and activity levels by ELISA and fluorogenic assays monitoring the generation of activated factor X (FXa), respectively [18].

2.3. Data analysis

The specific parameter lag time in functional assays was obtained by extrapolating the first derivative of relative fluorescence units (RFU) as a function of time (seconds) [19]. Statistical differences were evaluated by the *t*-test.

3. Results and discussion

To evaluate the CRISPRa transactivation potential and to provide with an informative comparison with the TALE-TF approach, we have chosen as model the *F7* gene promoter either wild-type or bearing the -61 T > G mutation, remarkably affecting the promoter activity and leading to severe FVII deficiency [20,21].

To this purpose, we chose a second-generation dCas9 fused with the tripartite transcription activator VPR (Fig. 1A), which has been demonstrated to be highly efficient [15,22].

3.1. Screening and identification of sgRNAs driving F7 promoter transactivation in cultured cells

Computational analysis led us to design a panel of five sgRNAs targeting the *F7* promoter in the region from −89 to +1, in particular that falling between the HNF4 binding motif and the transcription start site (sgRNAs $_{F7.1–4}$) (Fig. 1B). An additional sgRNA (sgRNA$_{F7.5}$) was designed to bind the sequence between SP1 and HNF4 binding sites, a region overlapping with that recognized by the most active TALE-TF (TF4) previously identified by us [17].

Reporter gene assays in HepG2 cells transfected with dCas9-VPR and the sgRNA-expressing vector demonstrated that all sgRNAs induced an increase of transcriptional activity ranging from 2-fold (sgRNA$_{F7.2}$) to over 40-fold with the other sgRNAs in the wild-type context (Fig. 1C). In the mutated -61 T > G context the CRISPRa system resulted in a promoter activity overlapping with that of the wild-type promoter in the same experimental conditions. As a matter of fact, as compared to untreated cells, the luciferase activity in the presence of the sgRNA$_{F7.5}$ was increased over 45-fold.

Altogether these studies demonstrated the transactivation activity of the CRISPRa approach on defective *F7* promoter and identified the sgRNA$_{F7.5}$ as the most active.

3.2. Comparison between CRISPRa and TALE-TF transactivation activities in reporter gene assays

The observation that the selected sgRNA$_{F7.5}$ targets the region between the SP1 and HNF4 binding sites, as the most active TF4 (Fig. 2A) that we previously characterized [17], prompted us to directly compare their activity in the same experimental set-up. As shown in Fig. 2B, the co-expression of dCas9-VPR with the sgRNA$_{F7.5}$ increased *F7* promoter activity, either wild-type or mutated, of ~30-fold whereas the effect of the TF4 was approximately ~18-fold, taken the activity of the untreated cells expressing the wild-type construct as 1. Noticeably, the dCas9-based approach resulted in a transactivation efficiency significantly higher than that promoted by TF4 in both wild-type ($p = 0.0424$) and mutated ($p = 0.0123$) *F7* contexts. This experimental evidence could be explained by the accessibility of the whole region of the *F7* promoter to natural transcription factors, as suggested by analysis of ChIPseq data (ENCODE database, https://www.encodeproject.org/), which points toward favorable interactions of both sgRNA$_{F7.5}$ and TF4 to the sequence between the SP1 and HNF4 motifs (Supplementary Fig. 1).

Although it is difficult to assess the molar amount of the different molecular components acting in the two systems, these data point toward an increased transactivation capacity of the CRISPRa approach. This finding might be in part attributable to the structural differences between CRISPRa and TALE-TF, the former exploiting the VPR-fused dCas9 [15] that is known to be more efficient than the dCas9 fused with the VP64 single domain typical of TALE-TF.

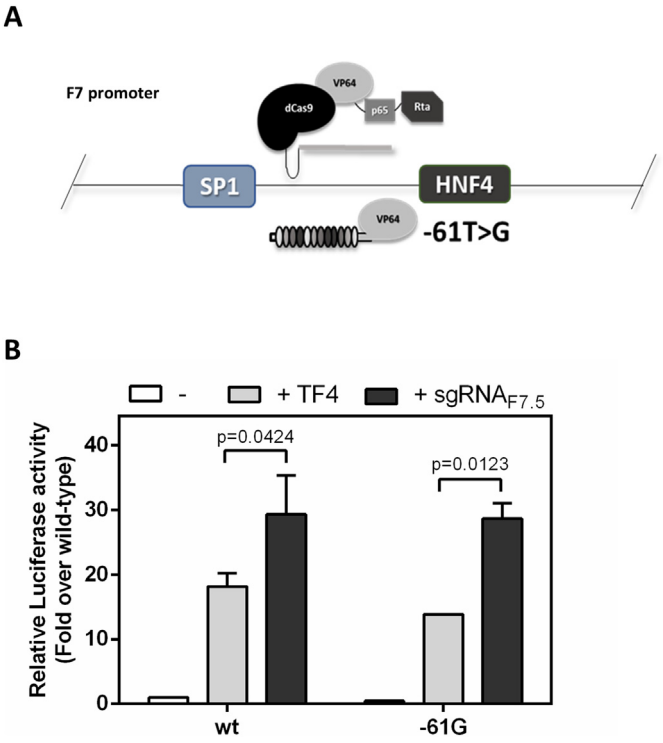

Fig. 2. Comparison of transactivation activity between sgRNA$_{F7.5}$ and TF4.
A) Schematic representation of the recognized sequences by sgRNA$_{F7.5}$ and TF4 in the region between SP1 and HNF4 transcription binding sites.
B) Co-expression in HepG2 cells of the reporter plasmids pF7wt and pF7-61G alone or in the presence of TF4 and dCas9/sgRNA$_{F7.5}$. Histograms report the fold increase of relative luciferase activity over that of the pF7wt alone. Results are expressed as mean ± standard deviation from three independent experiments.

3.3. Comparison of CRISPRa and TF4 activity on endogenous FVII protein expression in human hepatoma cells

To assess the transactivation capacity in the chromatin context we challenged the dCas9-VPR/sgRNA$_{F7.5}$ complex and TF4 on the endogenous *F7* gene expression in HepG2 cells. We therefore assessed their impact on secreted FVII protein levels, and particularly on the activity through very sensitive fluorogenic functional assays, optimized to detect very low activity levels, measuring the ability of FVII to activate its physiologic substrate factor X [23]. As shown in Fig. 3A, supplementation of FVII-deficient plasma with medium from HepG2 cells transfected with the dCas9-VPR/sgRNA$_{F7.5}$ combination resulted in an appreciable shortening of lag times (from 1400 s to 867 s) as compared with medium from untransfected cells, to indicate an increased FVII activity. Noticeably, the impact on lag times was stronger than that of medium from cells transfected with TF4 (1050 s). When extrapolated by comparison with serial dilutions of pooled normal plasma, the activity upon treatment with dCas9-VPR/sgRNA$_{F7.5}$ or TF4 resulted to be increased of 6.5 ± 0.49 or 3.8 ± 0.53 fold over the negative control, respectively (Fig. 3B). It is worth noting that the lag time, a key time parameter indicating when the generation of FXa begins, is dictated by FVII activity levels [24], thus being a clear hallmark of FVII-dependent improvement of the activity measured in plasma.

These data in cellular models further support the greater transactivation potential of the CRISPRa approach toward the TALE-TF.

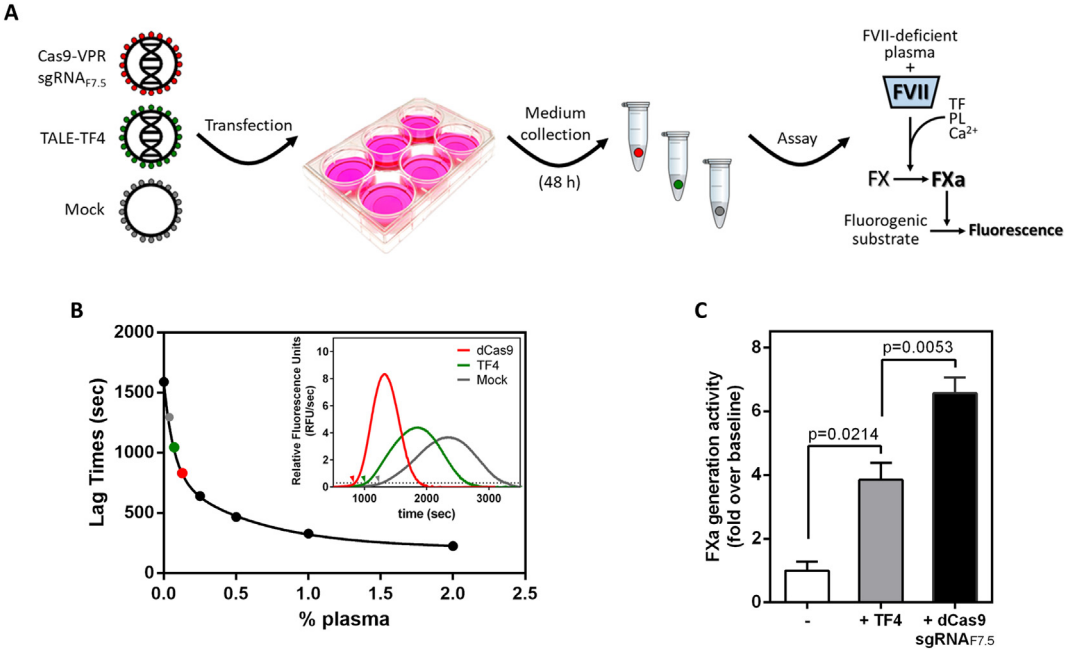

Fig. 3. Transactivation activity on the endogenous *F7* gene in HepG2 cells.

Activated FX (FXa) generation activity in FVII-deficient plasma supplemented with medium from HepG2 cells before (−, untreated) or after transactivation with either TF4 or dCas9/sgRNA$_{F7.5}$.

A) Schematic representation of the workflow from transient transfection to activity assays performed to assess the FXa generation activity of FVII-containing medium after transactivation.

TF, tissue factor; PL, phospholipids; FX, factor X, FXa, activated factor X.

B) Representative calibration curve prepared with serial dilutions (indicated as %) of pooled normal plasma showing the shortening of lag times as a function of increasing FVII protein amounts secreted in medium after dCas9/sgRNA$_{F7.5}$- or TF4-mediated transactivation of endogenous *F7*.

Inset. Activity curves obtained by supplementation of FVII-deficient plasma with FVII-containing or mock medium. Colored arrows indicate the time points used to extrapolate lag times.

C) Histograms representing the fold increase after transactivation of endogenous *F7* expression with TF4 or dCas9/sgRNA$_{F7.5}$ relatively to untransfected cells (−). Results are reported as fold increase over endogenous FVII from untreated cells (mean ± standard deviation from three independent experiments).

3.4. Tailoring the CRISPRa approach for coagulation F8 promoter

To further challenge the CRISPRa system, and assess its versatility, we tested it on the *F8* gene promoter driving the expression of coagulation factor VIII (FVIII), a key cofactor whose reduced levels lead to hemophilia A [25]. Although the frequency of promoter mutations in hemophilia A is relatively lower (0.2–0.3%;http://www.factorviii-db.org/) [26,27] than in hemophilia B (~2.5%) [28], the *F8* gene as model exemplifies the potential of the CRISPRa-mediated approach due to the higher complexity of its promoter (Fig. 4A) as compared with that of *F7* (Fig. 2A).

We therefore designed a panel of five sgRNAs to cover a region of ~300 bp upstream of the transcription start site. Moreover, we designed a single sgRNA (sgRNA$_{F8.2}$) to partially target the proximal C/EBPβ binding motif (Fig. 4A), known to play a crucial role on *F8* gene expression [29].

In reporter assays in which the expression of the firefly luciferase was driven by the whole *F8* proximal promoter region (1175 bp) [30] we detected a very low *F8* promoter activity (~2 fold) as compared with the negative control (pBasic) (Fig. 4B, inset), which is consistent with the weakness of the *F8* promoter. The screening for transactivation activity with the dCas9-VPR and the different sgRNAs revealed a variable increase of luciferase levels ranging from 4- (sgRNA$_{F8.4}$) to 20-fold with the sgRNA$_{F8.2}$ (Fig. 4B), which partially targets the C/EBPβ binding motif.

Prompted by previous studies demonstrating that two or more sgRNAs can magnify the transactivation effect [11,12] we tested the sgRNA$_{F8}$ variants in combination. As shown in Fig. 4C, each combination led to an improved activity over the single sgRNA$_{F8}$, with the highest effect (~40 fold) obtained with the combination of sgRNAs$_{F8.2}$, the most active when challenged alone, with sgRNA$_{F8.1}$.

To strengthen this finding we challenged the CRISPRa approach in endothelial EA.hy926 cells, which better resembles the physiological context for FVIII. In this setting, among all the gRNAs tested, the sgRNA$_{F8.1}$ (3.2-fold) and sgRNA$_{F8.2}$ (2-fold) showed the highest transactivation activity of *F8* promoter. In particular, when used in combination, the two sgRNAs resulted in the most significant fold change (2.7) difference ($p < 0.0001$) in comparison with the dCas-VPR alone (Fig. 5), as we observed in hepatic cells. The differential effect of sgRNA$_{F8.1}$ and sgRNA$_{F8.2}$ in comparison with that observed in hepatic cells could be attributable to the very low transfection efficiency of EA.hy926 cells [31].

Overall, these data, albeit on the artificial context of a reporter construct, demonstrate that the CRISPRa can be tailored on *F8* gene and identified, in two different cell models, the sgRNA$_{F8.1}$ and sgRNA$_{F8.2}$, alone or in combination, as those being able to recruit the dCas9-VPR component and remarkably increase *F8* promoter activity. It is worth to note that the CRISPRa system could be extended also to those missense mutations exerting a combined impact on splicing and protein biosynthesis/activity [32], which would benefit from enhanced promoter activation.

4. Conclusions

Overall, in this pioneer study using *F7* and *F8* as paradigmatic models for coagulation genes, we demonstrated that the CRISPRa system is highly versatile and can be adapted to gene promoters with different degree of complexity to increase physiologic expression or to

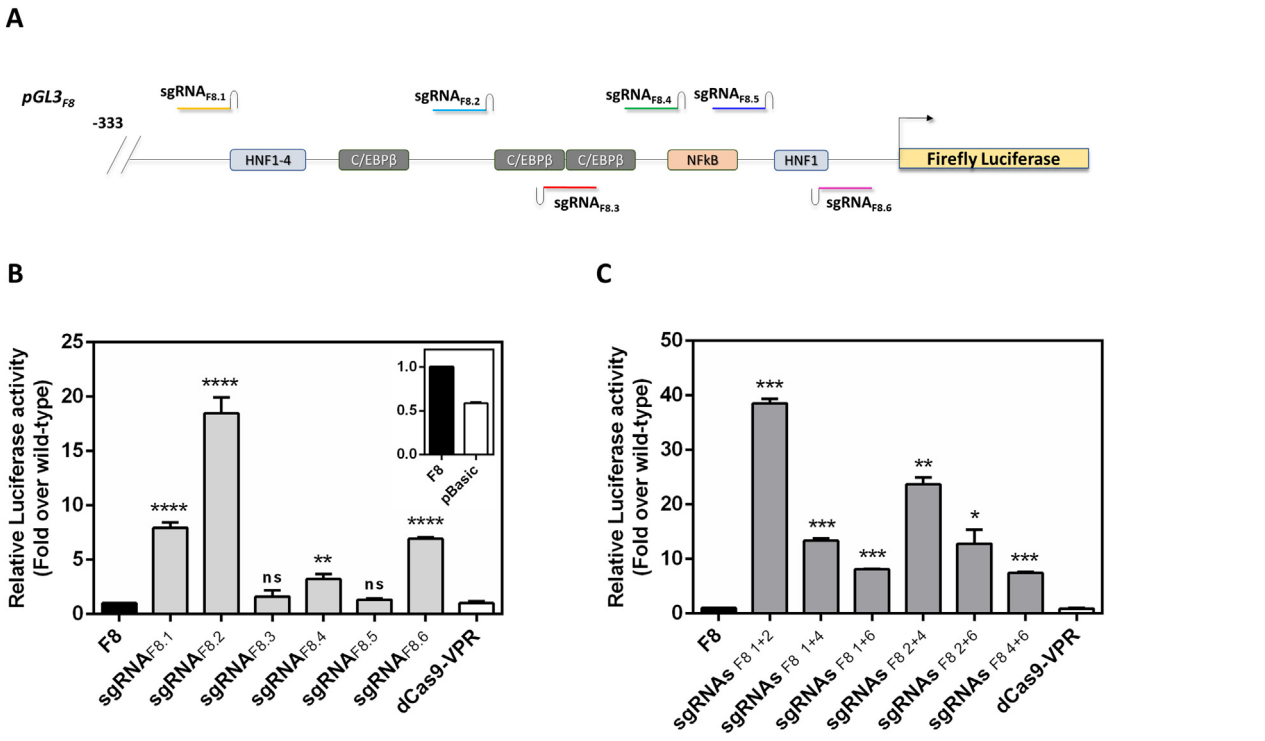

Fig. 4. Tailoring of the CRISPRa system on *F8* promoter transactivation.
A) Schematic representation of the proximal *F8* promoter region cloned upstream of the luciferase-encoding sequence. Designed sgRNAs are shown above/below the predicted target region.
B,C) Promoter activity of the *F8* reporter vector after transfection with single (B) or combinations (C) of sgRNAs targeting the region shown in A.
Histograms report the fold increase of relative luciferase activity over that of F8 alone. The dCas9-VPR, devoid of the DNA-binding component, and pBasic (B, inset), were used as negative controls.
Results are expressed as mean ± standard deviation from three independent experiments.
*, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$; ****, $p < 0.0001$; ns, not significant.

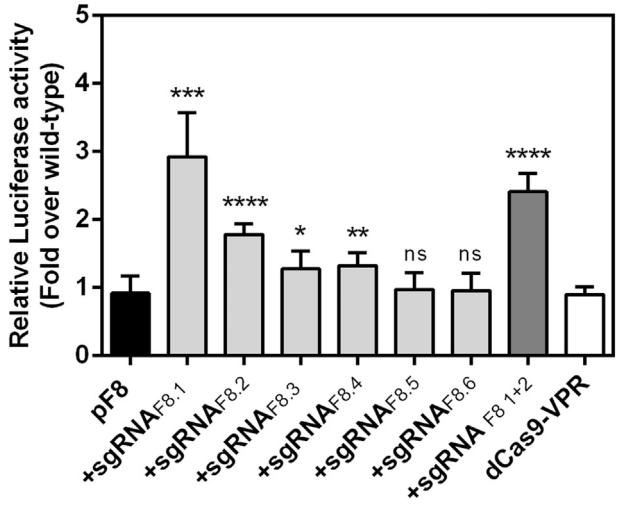

Fig. 5. *F8* promoter transactivation in endothelial EA.hy926 cells.
Promoter activity of the *F8* reporter vector after transfection with sgRNAs, targeting the region shown in Fig. 4A, represented as fold increase of relative luciferase activity over that of *F8* alone.
Results are expressed as mean ± standard deviation from at least three independent experiments.
*, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$; ****, $p < 0.0001$; ns, not significant.

rescue promoter mutations associated with human disease. However, the very low expression profile of FVIII in hepatic cells and the poor transfection efficiency of endothelial cells hampered the proper evaluation of the impact on secreted FVIII levels. In this view, although we are aware of the limit of our study in the use of hepatic cells, physiological for FVII but not for FVIII, the observed transactivation should be considered in light of the capacity of the CRISPRa system to act on both *F7* and *F8* promoters. Noticeably, the extent of the improvements obtained through the identified sgRNAs, if cautiously translated into patients, might approach the low therapeutic threshold for FVII deficiency and hemophilia A [33,34], in which, at variance from the majority of human disorders, even a modest increase in FVII or FVIII levels would ameliorate the clinical phenotype.

Notwithstanding, these data encourage further studies aimed at assessing the therapeutic potential and the safety profile of this approach, which could be extended to several disease genes.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbagrm.2019.04.002.

Author contributions

S.P. conceived the study, performed reporter gene assays as well as transactivation studies on the endogenous *F7* promoter; F.Z. created the reporter construct for *F8* promoter and performed screening experiments for transactivation activity; E.B. designed sgRNAs and the cloning strategies; A.F. supervised S.P. for reporter assays in endothelial cells; M.B. carefully revised the manuscript; M.P., A.B. and F.B. conceived the study, analyzed and interpreted data and wrote the manuscript. All authors revised and approved the final version of the manuscript.

Transparency document

The Transparency document associated this article can be found, in online version.

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Conflicts of interest

The authors state that they have no conflicts of interest.

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