Inducible and multiplex gene regulation using CRISPR–Cpf1-based transcription factors

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Targeted and inducible regulation of mammalian gene expression is a broadly important capability. We engineered drug-inducible catalytically inactive Cpf1 nucleases fused to transcriptional activation domains to tune the expression of endogenous genes in human cells. Leveraging the multiplex capability of the Cpf1 platform, we demonstrate both synergistic and combinatorial gene expression in human cells. Our work should enable the development of multiplex gene perturbation library screens for understanding complex cellular phenotypes.

Sequence-specific RNA-guided CRISPR–Cas nucleases are simple to program1,2; the widely used Streptococcus pyogenes Cas9 (SpCas9) can be targeted to specific DNA sequences by an associated complementary guide RNA (gRNA), provided that a protospacer-adjacent motif (PAM) of the form NGG is also present. Catalytically inactive SpCas9 (dSpCas9) has been fused to transcriptional activation or repression domains to alter the expression of individual genes or to perform genome-wide library screens in mammalian cells3. Both small-molecule- and light-inducible dSpCas9-based fusions have been developed4–6, which has enabled researchers to regulate the activity of this gene regulatory platform. Recently described CRISPR–Cpf1 nucleases offer additional capabilities beyond those of SpCas9—including shorter length CRISPR RNAs (crRNAs) for guiding Cpf1 to targets, the ability to target T-rich PAMs7,8, and RNase processing of multiple crRNAs from a single transcript by RNase activity of Cpf1 (refs. 9,10). However, to our knowledge, ‘dead’ Cpf1 (dCpf1)-based gene regulators have thus far only been shown to repress gene expression in bacteria11,12 and plants (Arabidopsis)13.

Based on preliminary experiments suggesting its higher activity in a GFP reporter gene experiment (Supplementary Note 1 and Supplementary Fig. 1), we fused dCpf1 from Lachnospiraceae bacterium (dLbCpf1) to the strong synthetic VPR activator (herpes simplex virus-derived VP16 activator, the human NF-KB p65 activation domain, and the Epstein–Barr-virus-derived R transactivator (Rta))14. We targeted these dLbCpf1–VPR fusions to the promoters of three different endogenous genes that are either epigenetically silenced according to ENCODE data (HBB) or expressed at low levels (AR and NPY1R) in HEK293 cells by designing three crRNAs for each promoter located at various distances within 1 kb upstream of the transcription start site (TSS). At least one crRNA for each of the three target genes achieved robust transcriptional activation, as assayed by quantitative reverse transcription PCR (RT-qPCR); whereas a dLbCpf1–p65 fusion alone showed little or no transcriptional activation of the target gene promoter (Fig. 1a), which confirmed previous observations with dSpCas9 (ref. 14). We also tested a larger series of 32 crRNAs positioned within 1 kb upstream or 500 bp downstream of the TSSs of two additional endogenous genes, CD5 and CD22, which encode cell surface proteins. Most of the 32 crRNAs tested could significantly (P < 0.05) activate the target gene promoter when positioned between ~600 bp upstream and ~400 bp downstream of the TSSs (Supplementary Fig. 2), and this was consistent with results obtained using dSpCas9 activators15. The levels of activation observed with dLbCpf1-based activators are comparable to what might be observed in naturally occurring biological systems and are similar to what has been previously reported for analogous dCas9-based activators16.

To construct drug-regulated versions of dCpf1–VPR, we used DmrA and DmrC domains, which only interact in the presence of a rapamycin analog known as the A/C heterodimerizer4,17. We created split dLbCpf1 activators consisting of two fusions: dLbCpf1 fused to a DmrA domain and a DmrC domain fused to VPR or p65 (Fig. 1b); this made activation dependent on the A/C drug. Although single crRNAs failed to reveal inducible activation of the HBB, AR, or NPY1R genes (Fig. 1c), dLbCpf1 fusions harboring two, three, or four tandem copies of the DmrA domain in the presence of DmrC–VPR and the A/C heterodimerizer drug increased gene activation at two of the three endogenous gene promoters (HBB and NPY1R; Fig. 1c). The degree of activation observed correlated with the number of DmrA domains, with maximum levels reaching approximately half of those observed with direct dLbCpf1–VPR fusions (Fig. 1c). We found that using a DmrC–p65 fusion with dLbCpf1–DmrA fusions led to drug-dependent transcriptional upregulation from all three target gene promoters (Fig. 1c)—an unexpected result given the lack of activation observed with dLbCpf1–p65 direct fusions and these same crRNAs (Fig. 1a).

Compared with Cas9, one major advantage of Cpf1 is its ability to encode two or more crRNAs in a multiplex single transcript (MST), and subsequent processing of individual crRNAs by the
RNase-processing activity of Cpf1 (refs. 9 and 10) (Fig. 2) potentially simplifies the implementation of synergistic and multiplex gene activation. We tested the use of MST crRNAs for synergistic activation by encoding sets of the three crRNAs targeting the promoters of HBB, AR, or NPY1R genes on single transcripts (Fig. 2a). With dLbCpf1–VPR, we observed synergistic activation for HBB and AR but not for NPY1R (Supplementary Fig. 3). We also failed to observe synergistic activation of any of the three genes with dLbCpf1–p65 with either MST crRNAs or pooled single crRNAs (Supplementary Fig. 3). We also observed drug-dependent synergy with dLbCpf1–DmrA(×4) (dLbCpf1 fused to four copies of DmrA) and DmrC–VPR at the HBB and AR promoters but again not at the NPY1R promoter (even though synergy was observed with pooled single crRNAs introduced together) (Fig. 2b). A similar pattern of results for these three gene promoters was observed with dLbCpf1–DmrA(×4) and DmrC–p65 (Fig. 2b), and higher activation was observed on the HBB and AR genes relative to comparable experiments performed with DmrC–VPR (Fig. 2b). For the HBB gene, this higher activation was not seen with the single crRNA that worked the best among the three single crRNAs tested (Fig. 1c). Although the reason for this difference is not known, it is possible that it relates to the number and configuration of transcriptional activation domains recruited to the promoter.

We also tested the use of MST crRNAs for multiplex activation of different gene promoters in the same cells (Fig. 2c). We found that MST crRNAs could be used together with (i) dLbCpf1–VPR, (ii) dLbCpf1–DmrA(×4) and DmrC–VPR fusions, or (iii) dLbCpf1–DmrA(×4) and DmrC–p65 fusions to simultaneously activate the endogenous HBB, AR, and NPY1R gene promoters in HEK293 cells (Fig. 2d). The activities of MST crRNAs were generally comparable relative to single individually expressed crRNAs, and more than half of the gene/activator configurations we examined showed no statistically significant difference (P < 0.05) (Fig. 2d). For the cases where a difference was observed, the MST crRNAs generally showed only ~2-fold lower activation compared to single individually expressed crRNAs, and the absolute level of fold activation was still quite high, ranging from ~9-fold to ~40-fold. Attempts to further increase the activities of MST crRNAs did not yield substantial improvements (Supplementary Note 1 and Supplementary Fig. 4). To extend our findings to another human cell line, we also tested the direct VPR activator fusions and drug-regulated VPR and p65 activators in human U2OS cells, and we targeted the same genes (HBB, AR, and NPY1R) and more than half of the gene/activator configurations we examined showed no statistically significant difference (P < 0.05) (Fig. 2c). For the cases where a difference was observed, the MST crRNAs generally showed only ~2-fold lower activation compared to single individually expressed crRNAs, and the absolute level of fold activation was still quite high, ranging from ~9-fold to ~40-fold. Attempts to further increase the activities of MST crRNAs did not yield substantial improvements (Supplementary Note 1 and Supplementary Fig. 4). To extend our findings to another human cell line, we also tested the direct VPR activator fusions and drug-regulated VPR and p65 activators in human U2OS cells, and we targeted the same genes (HBB, AR, and NPY1R) and more than half of the gene/activator configurations we examined showed no statistically significant difference (P < 0.05) (Fig. 2d). For the cases where a difference was observed, the MST crRNAs generally showed only ~2-fold lower activation compared to single individually expressed crRNAs, and the absolute level of fold activation was still quite high, ranging from ~9-fold to ~40-fold. Attempts to further increase the activities of MST crRNAs did not yield substantial improvements (Supplementary Note 1 and Supplementary Fig. 4). To extend our findings to another human cell line, we also tested the direct VPR activator fusions and drug-regulated VPR and p65 activators in human U2OS cells, and we targeted the same genes (HBB, AR, and NPY1R) and more than half of the gene/activator configurations we examined showed no statistically significant difference (P < 0.05) (Fig. 2d). For the cases where a difference was observed, the MST crRNAs generally showed only ~2-fold lower activation compared to single individually expressed crRNAs, and the absolute level of fold activation was still quite high, ranging from ~9-fold to ~40-fold. Attempts to further increase the activities of MST crRNAs did not yield substantial improvements (Supplementary Note 1 and Supplementary Fig. 4). To extend our findings to another human cell line, we also tested the direct VPR activator fusions and drug-regulated VPR and p65 activators in human U2OS cells, and we targeted the same genes (HBB, AR, and NPY1R) and more than half of the gene/activator configurations we examined showed no statistically significant difference (P < 0.05) (Fig. 2d). For the cases where a difference was observed, the MST crRNAs generally showed only ~2-fold lower activation compared to single individually expressed crRNAs, and the absolute level of fold activation was still quite high, ranging from ~9-fold to ~40-fold. Attempts to further increase the activities of MST crRNAs did not yield substantial improvements (Supplementary Note 1 and Supplementary Fig. 4). To extend our findings to another human cell line, we also tested the direct VPR activator fusions and drug-regulated VPR and p65 activators in human U2OS cells, and we targeted the same genes (HBB, AR, and NPY1R) and more than half of the gene/activator configurations we examined showed no statistically significant difference (P < 0.05) (Fig. 2d). For the cases where a difference was observed, the MST crRNAs generally showed only ~2-fold lower activation compared to single individually expressed crRNAs, and the absolute level of fold activation was still quite high, ranging from ~9-fold to ~40-fold. Attempts to further increase the activities of MST crRNAs did not yield substantial improvements (Supplementary Note 1 and Supplementary Fig. 4).
In addition, we assessed the kinetics of activator effects on the addition and withdrawal of A/C heterodimerizer. We found that maximum activation of the HBB and AR genes was observed at 25–35 h after drug addition (Supplementary Fig. 6a), and return of activated gene expression to baseline occurred at 35–45 h after drug withdrawal (Supplementary Fig. 6b). We envision that drug inducibility could be easily extended to other orthologs, and we have successfully used these same strategies to regulate and tune dSpCas9-based activators (Supplementary Fig. 7), as have others who published similar drug-regulated dCas9-based activators while this work was in progress.

To quantify Cpf1’s ability to complement the targeting range of SpCas9, we performed an informatics analysis of sequences located between 600 bp upstream and 400 bp downstream of 32,696 TSSs in the human genome for sites with TTTV, TYCV, and TATV PAMs (see Online Methods). We identified ‘desert’ regions of ≥20 bp within these sequences in which no SpCas9 target sites could be found and summed these for each TSS. We then determined, for each TSS, what percentage of these summed desert regions could be targeted only by wild-type Cpf1 (Supplementary Fig. 8a) and the two Cpf1 PAM recognition variants with wild-type Cpf1 (Supplementary Fig. 8b). Wild-type Cpf1 permits the targeting of 66% of the sum of all desert regions, while the two PAM variants together with wild-type Cpf1 permit the targeting of 88% of these regions. As might be expected, the targeting capability increases as the size of the desert increases (Supplementary Fig. 8a,b).

Overall, our findings should enable and encourage the use of dCpf1-based gene regulatory proteins for performing both focused and genome-wide gene-perturbation screens in mammalian cells. More broadly, our results should also motivate researchers to engineer dCpf1-based direct and drug-regulated fusions.
to other heterologous regulatory domains (e.g., p300, DNMT, TET1) as has previously been done with dSpCas9 (refs. 18–20). Successful development of such fusions could enable recruitment of two different effector domains in a controlled manner using dCas9 and dCpf1 scaffolds targeted to the same promoter.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

Y.E.T., B.P.K., J.K.N., J.Y.H., J.S.W., and J.K.J. conceived of and designed experiments. Y.E.T., B.P.K., J.K.N., J.Y.H., J.E.H., and J.G. performed experiments. Y.E.T., B.P.K., J.K.N., J.Y.H., J.S.W., and J.K.J. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Plasmids and oligonucleotides. A diagram of constructs and a list of plasmids and related sequences used in this study are found in Supplemental Note 2; LbCpf1 target sites, multiplex crRNA sequences, and SpCas9 target sites are found Supplemental Table 1. Plasmids dLbCpf1-p65 (JG1202) and dLbCpf1-VPR (JG1211) were constructed by cloning p65 and VPR domains into dLbCpf1 (D832A) (MMW1578) using BstZ17I and NotI sites through isothermal assembly. VPR was amplified from dsSpCas9-VPR, which was a gift from G. Church (Harvard Medical School, Boston, Massachusetts; Addgene plasmid 63798)14. Plasmids encoding dLbCpf1-DmrA(×1), dLbCpf1-DmrA(×2), dLbCpf1-DmrA(×3), and dLbCpf1-DmrA(×4) (JG674, JG676, JG693, and YET1000, respectively) were generated by subcloning dLbCpf1(D832A) into AgeI- and Xhol-digested constructs that have different numbers of DmrA domains (BPK1019, BPK1033, BPK1140, and BPK1177 for dSpCas9-DmrA(×1) dSpCas9-DmrA(×2), dSpCas9-DmrA(×3), and dSpCas9-DmrA(×4), respectively) using isothermal assembly. Plasmids encoding dSpCas9(D10A/H840A) effector fusions to VP64, p65, or DmrA were cloned via isothermal assembly (for a complete list of plasmids, please see Supplemental Note 2).

The DmrC entry vector was digested with NruI, and p65 or VPR were inserted via isothermal assembly to generate DmrC-p65 (BPK1169) and DmrC-VPR (MMW948). Single LbCpf1 crRNA expression plasmids were constructed by ligating annealed oligo duplexes into BsmBI-digested BP3082 (Addgene 78742)21. MST LbCpf1 crRNA plasmids were assembled by annealing, phosphorylating, and ligating three pairs of oligonucleotides into BsmBI- and HindIII-digested BP3082. Sequences for all oligo pairs are listed in Supplemental Table 1. The NF-KB p65 domain encoded in JG1202, BPK1169, BPK1160, and BPK1163 encompasses amino acids 285 to 551 of the native protein; while domain encoded in constructs containing VPR encompass amino acids 428 to 546.

Human cell culture and transfection. HEK293 cells (Invitrogen) and HEK293T cells (ATCC) were grown at 37 °C, in 5% CO2 in Dulbecco’s Modified Eagle Medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin and streptomycin. U2OS cells (obtained from T. CATHOMEN, University of Freiburg) were grown at 37 °C in 5% CO2, in advanced DMEM supplemented with 10% heat-inactivated FBS, 2 mM GlutaMax, and 1% penicillin and streptomycin. Media supernatant was analyzed biweekly for the presence of mycoplasma. For the GFP-activation assays shown in Supplemental Figure 1, 500 ng of plasmid DNA expressing dCas9-VPR-BFP (JKNp44), dAsCasP1-VPR-BFP (JKNp49), or dLbCpf1-VPR-BFP (JKNp47) and 500 ng of crRNA plasmid were cotransfected into HEK293T cells that stably encode GFP under a Tet-On 3G doxycycline-inducible promoter (Takara Clontech). Both the dLbCpf1 and dAsCasP1 protein-coding sequences were codon optimized for expression in human cells. Median GFP fluorescence was measured 3 d post-transfection using a LSR II Flow Cytometer (BD), and values were normalized to HEK293T-GFP cells with no plasmids transfected. For LbCpf1 direct-fusion experiments on endogenous genes, 750 ng of dLbCpf1-p65 or dLbCpf1-VPR plasmids with 250 ng of LbCpf1 crRNA plasmids were cotransfected using 3 µl of TransIT-LT1 Transfection Reagent (Mirus, cat. no. MIR2300) into HEK293 cells in a 12-well plate. For experiments with dLbCpf1 fusions to DmrA domains, 400 ng of dLbCpf1-DmrA fusions plasmids, 200 ng of DmrC-p65 or DmrC-VPR plasmids, and 400 ng of LbCpf1 crRNA plasmids were cotransfected using 3 µl of LT1 into HEK293 cells in a 12-well plate. A complete media containing 500 µM A/C heterodimerizer (Takara Clontech) was used at the time of transfection. For LbCpf1 experiments in U2OS cells, the same amount of plasmids used in HEK293 cells was cotransfected by nucleofection using the DN-100 program on a Lonza 4-D Nucleofector with the s.e.m. Cell Line Kit (Lonza). For the experiments shown in Supplemental Figure 6, 1,000 ng of dLbCpf1-DmrA fusions plasmids, 500 ng of DmrC-p65 plasmid, and 1,000 ng of LbCpf1 MST crRNA plasmids targeting HBB promoter or AR promoter were cotransfected using 7.5 µl of LT1 into HEK293 cells in a six-well plate. At 34 h post-transfection, cells were split into 24-well plates to harvest at different time points.

Quantitative reverse-transcription polymerase chain reaction. Total RNA was extracted from the transfected cells 72 h post-transfection using the NucleoSpin RNA Plus (Clontech, cat. no. 740984.250), and 250 ng of purified RNA was used for cdNA synthesis using High-Capacity RNA-cDNA kit (ThermoFisher, cat. no. 4387406). cdNA was diluted 1:20, and 3 µl of cdNA was used for quantitative PCR (qPCR). qPCR reaction samples were prepared using cdNA, SYBR (ThermoFisher, cat. no. 4385612), and primers detecting each target transcript. Primer sequences are listed in Supplemental Table 2. qPCR was performed using Roche LightCycler480 with the following cycling protocols (Supplemental Table 2). When (Ct) values were over 35, we considered them to be 35, because (Ct) values fluctuate for very weakly expressed transcripts. Samples that were transfected with LbCpf1 crRNA backbone plasmid (BP3082) were used as negative controls; and the levels of fold activation over negative controls were normalized to the expression of HPTRI.

Flow cytometry. Cells were washed with cell-staining buffer (BioLegends, cat. no. 420201) after 72 h post-transfection and incubated with CD22 (BioLegends, cat. no. 363511) or CD5 antibody (BioLegends, cat. no. 364008) for 15 min; this was followed by two washes with cell-staining buffer. PE/Cy-7 for CD5-positive cells and BV421 for CD22-positive cells were measured by a LSR Fortessa X-20 flow cytometer (BD).

VEGFA ELISA. HEK293 cells were seeded in 24-well plates roughly 20 h prior to transfection using Lipofectamine 3000 (Thermo Fisher Scientific). Unless otherwise indicated, 250 ng of dCas9-DmrA plasmid was cotransfected with 250 ng of sgRNA plasmid (single VEGFA site 3 sgRNA or pooled VEGFA sites 1, 2, and 3 sgRNAs2) and 125 ng of DmrC-effecter plasmid. A complete media exchange was performed approximately 20 h post-transfection, with the exchanged media containing 500 µM A/C heterodimerizer (Takara Clontech), unless otherwise noted. Approximately 42 h post-transfection, supernatant media were removed and clarified before analysis using a Human VEGF Quantikine ELISA kit (R&D Systems). Optical density of stopped ELISA reactions was determined using a Model 860 Microplate reader (Bio-Rad).

Western blot. HEK293T cells were transfected in six-well plates with 3 µg of plasmids encoding VPR-fused dCas9, dAsCpf1,
and dLbCpf1 and 3 µg of guide-RNA-encoding plasmid. Cells were harvested 48 h post-transfection with PBS and lysed in RIPA buffer (Thermo Fisher Scientific) supplemented with protease inhibitors (Sigma-Aldrich, cat. no. 11867431001). Soluble lysates were quantified by BCA protein assay (Thermo Scientific), and 40 µg of each sample was loaded into a 4–12% Bis–Tris protein gel (Thermo Scientific). HA-tagged VPR fusions were detected with rat anti-HA antibody (Sigma-Aldrich, cat. no. 11867431001). GAPDH was detected with rabbit anti-GAPDH antibody (Cell Signaling Technology, cat. no. 2118S). Blots were imaged by LI-COR Odyssey CLx.

**Computational analysis of Cpf1-targetable sites in the human genome.** Cpf1-targeting range was characterized by analyzing sequences −600 bp and +400 bp relative to the TSS of 32,696 genes in the human genome (GRCh37/hg19). To assess the complementarity between the targeting ranges of Cpf1 and SpCas9, ‘desert’ regions of ≥20 bp that are not targetable by SpCas9 (20 bp spacer + NGG PAM) were enumerated and summed for each gene. These desert regions were then analyzed to quantify the proportion that could be targetable by wild-type Cpf1 alone or with the two Cpf1 PAM recognition variants (TYCV and TATV) together with wild-type Cpf1. The desert region sums for each gene were binned into the following groups to assess the distributions: 20–100 bp, 101–200 bp, 201–300 bp, 301–400 bp, 401–500 bp, and 501–1,000 bp. Codes for the analysis performed are available from the corresponding author upon reasonable request.

**Data availability statement.** The data that support the findings of this study are available from the corresponding author upon reasonable request.

**A Life Sciences Reporting Summary** is available.

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