

CRISPR Activation Enhances *In Vitro* Potency of AAV Vectors Driven by Tissue-Specific Promoters

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Validation of gene transfer vectors containing tissue-specific promoters in cell-based functional assays poses a formidable challenge for gene therapy product development. Here, we describe a novel approach based on CRISPR/dCas9 transcriptional activation to achieve robust transgene expression from transgene cassettes containing tissue or cell type-specific promoters after infection with AAV vectors in cell-based systems. Guide RNA sequences targeting two promoters that are highly active within mammalian photoreceptors were screened in a novel promoter activation assay. Using this screen, we generated and characterized stable cell lines that co-express dCas9.VPR and top-performing guide RNA candidates. These cells exhibit potent activation of proviral plasmids after transfection or after infection with AAV vectors delivering transgene cassettes carrying photoreceptor-specific promoters. In addition, we interrogated mechanisms to optimize this platform through the addition of multiple guide RNA sequences and co-expression of the universal adeno-associated virus receptor (AAVR). Collectively, this investigation identifies a rapid and broadly applicable strategy to enhance *in vitro* expression and to evaluate potency of AAV vectors that rely upon cell or tissue-specific regulatory elements.

INTRODUCTION

Viral vectors occupy a unique space in drug development, as these are highly sophisticated biological particles composed of protein and nucleic acid components. Their structural and genomic complexity presents a variety of hurdles in the design and implementation of functional or potency assays for specific disease targets. A major challenge concerns the selection of tissue or cell-specific promoters to control vector transgene expression.¹ These regulatory elements provide specificity to the cell targets of interest *in vivo* but can display poor activity when applied to cell-based systems.^{2,3} In the context of retinal gene transfer, several promoter sequences enable selective and stable gene expression patterns in various cell types.^{4–8} Adeno-associated virus (AAV) vectors containing such promoters demonstrate robust safety and efficacy profiles in pre-clinical models of inherited retinal degeneration, underscoring their translational potential.^{8–11} To interrogate the function of vectors driven by photoreceptor-specific promoters, several reports have interrogated transgene activity in the 661W cell line, which is derived

from a mouse retinal tumor that expresses many functional markers indicative of a cone photoreceptor origin.^{2,12,13} Although 661W cells may be suitable for functional testing of vectors regulated by ubiquitous promoters and some retina-specific promoters, these cells do not display sufficient levels of transgene expression for all photoreceptor promoters and downstream potency assessment.²

Recent developments surrounding genome engineering technologies such as CRISPR/Cas9 provide a powerful means to create simple, rapid, and precise modifications to genomic DNA sequences.^{14–16} In addition to gene editing, more recently described systems have employed catalytically inactive variants of Cas9 (dCas9) tethered to effector domains for enabling selective regulation of transcriptional activity.¹⁷ Inactivation of Cas9 catalytic activity is achieved by mutating two amino acids critical to endonuclease function.¹⁸ Although these mutations successfully ablate nuclease activity, Cas9 still retains all functions with respect to DNA binding and recognition. Based on this core platform, numerous approaches have been described to achieve various modalities of gene regulation, including activation, repression, or epigenetic remodeling.^{18–21} Chavez et al.²² characterized a synthetic transactivation complex, termed VPR, which combines activities of the VP64, p65, and Rta activation domains. VPR was shown to stimulate higher levels of gene expression compared to dCas9 fused to the canonical VP64 transactivation domain. We hypothesized that such an approach could provide an effective means to activate cell type-specific promoters from AAV expression cassettes in cell-based functional assays.

Here, we applied CRISPR/dCas9 transcriptional reprogramming to augment *in vitro* expression of AAV vectors driven by photoreceptor promoters. Specifically, we explored the *in vitro* activation of the human rhodopsin kinase (GRK1) and cone arrestin (CAR) promoters (Supplemental Materials). We identified high-quality guide RNA sequences

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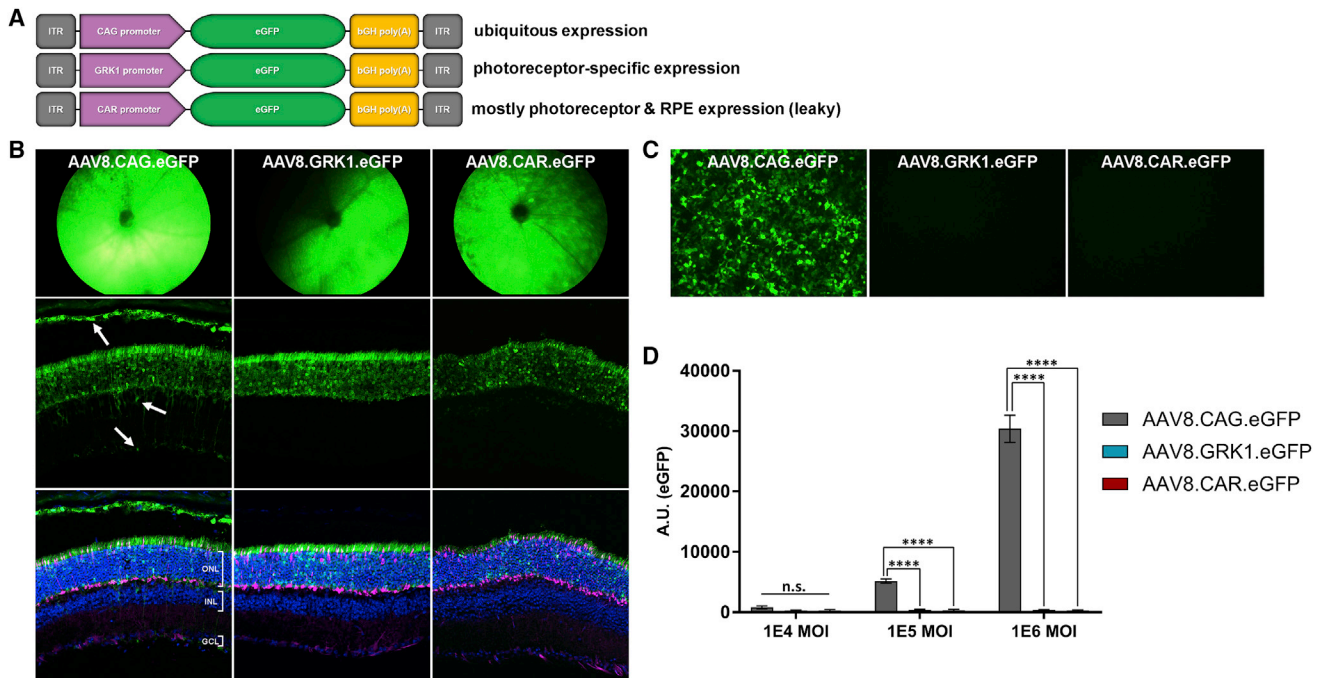


Figure 1. Differential Expression of Photoreceptor Promoters in the Mouse Retina *In Vivo* and *In Vitro*

(A) Outline of AAV reporter cassettes driven by ubiquitous or photoreceptor promoters. (B) Representative images of wild-type mouse retina following subretinal injection of AAV8 reporter vectors. The top row shows *in vivo* fundus images. Middle and bottom rows depict immunohistochemistry of retinal cross sections displaying reporter vector expression (green). Cross sections are stained with antibodies directed against cone arrestin, to mark the photoreceptor layer, and DAPI, to designate cell nuclei. White arrows indicate off-target AAV8 reporter expression driven by the CAG promoter. (C) Representative micrographs of 84-31 cells transduced with different AAV8 vectors at 1E6 MOI. (D) AAV8 reporter expression quantified after treatment with three different MOIs 48 h after transduction. Data represent the mean \pm SEM ($n = 3$). **** $p < 0.0001$.

that effectively activate these promoters by using a fluorescent reporter assay. Next, we generated a variety of cell lines that stably co-express candidate guide RNAs and dCas9.VPR. These cells display robust activation of transgene expression regulated by these photoreceptor promoters following plasmid transfection or AAV vector transduction. Finally, we identified strategies to optimize this platform with the addition of multiple guide RNAs (multiplexing) targeting a single promoter of interest and co-expression of the universal AAV receptor (AAVR).

RESULTS

Rhodopsin K and Cone Arrestin Promoters Display Robust Activity in Photoreceptors but Poor Transgene Expression in Cell-Based Systems

We generated AAV8 reporter vectors containing eGFP cDNA driven by the cytomegalovirus enhancer and chicken beta actin (CAG) hybrid promoter, GRK1 promoter, or human CAR promoter (Figure 1A). Subretinal administration of vectors demonstrates robust activity *in vivo*, as assessed by fundoscopic analysis and immunohistochemistry of retinal sections (Figure 1B). Vectors regulated by the ubiquitous CAG promoter display expression in multiple retinal cell types in the outer and inner retinal layers. AAV8.GRK1.eGFP activity was restricted specifically to photoreceptors, whereas AAV8.CAR.eGFP vectors displayed leaky expression within photoreceptors and the retinal pigment epithelium (RPE). AAV transduction with these

photoreceptor promoters *in vitro* reveals exceptionally poor activity when compared to CAG driven vectors, as demonstrated by fluorescence intensity and microscopy in cell lines (Figures 1C and 1D) or human induced pluripotent stem cell (hiPSC) models (Figure S1). These results underscore the major discrepancy in vector activity *in vivo* versus cell-based systems when transgene expression is regulated by a tissue-specific promoter. Moreover, this presents a major limitation in the design and implementation of cell-based functional and potency assays for gene therapy vectors driven by these regulatory elements.

In Vitro Stimulation of Photoreceptor Promoters with CRISPR Transcriptional Activation

We hypothesized that recently characterized dCas9-based transcriptional activators would allow stimulation of photoreceptor promoters in the context of an *in vitro* system. We screened 20 unique guide RNAs targeting the GRK1 (Table S1) or CAR promoter (Table S2) sequences using a fluorescence-based reporter assay. Guide RNAs were cloned into expression plasmids encoding eGFP driven by either the GRK1 or CAR promoters (Figure 2A). These constructs were transfected into 84-31 cells that stably express dCas9 fused to the VPR transactivation complex (84-31.VPR). Fluorescence readings 48 h after transfection revealed differential effects upon reporter expression driven by photoreceptor promoters (Figures 2B and 2C). Most guide sequences provided a modest increase in reporter expression

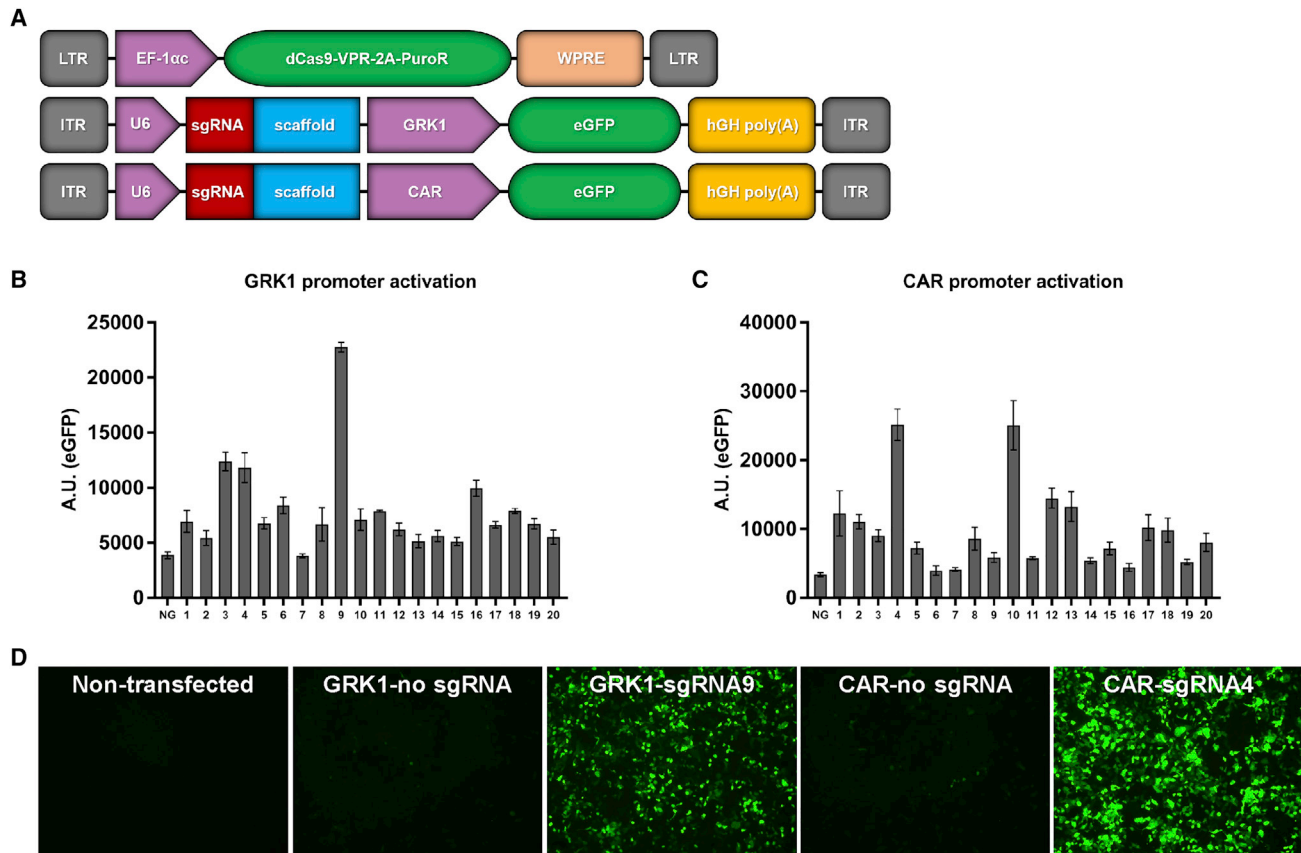


Figure 2. *In vitro* Stimulation of Photoreceptor Promoters with CRISPR-dCas9 Transcriptional Activation

(A) Outline of lentiviral vector cassette used to generate 84-31-VPR cells (top), GRK1 sgRNA-reporter construct (middle), and CAR sgRNA-reporter construct (bottom). (B) GRK1 promoter activation screen. (C) CAR promoter activation screen. The x axes denote the activity of 20 unique sgRNAs targeting each promoter. NG, no-guide control. Data are represented as the mean \pm SEM. (D) Representative micrographs of 84-31-VPR cells transfected with empty guide-reporter or sgRNA-reporter plasmids.

compared to cells transfected with plasmids harboring an empty sgRNA cassette (no guide [NG]). Guide RNA candidate 9 demonstrated the most potent stimulation of GRK1-driven reporter expression, whereas sgRNA candidates 4 and 10 stimulated similar and robust levels of CAR promoter-driven reporter activity (Figures 2B and 2C). Likewise, fluorescence microscopy revealed robust induction of reporter protein expression in 84-31.VPR cells transfected with these top-performing single-guide RNA (sgRNA)-reporter plasmids compared with empty guide control plasmids (Figure 2D).

CRISPR Activation Stimulates Tissue-Specific Promoter Expression from AAV Vectors

We generated a panel of stable cell lines that co-express dCas9.VPR and a top-performing sgRNA candidate targeting the GRK1 or CAR promoter from a single lentiviral expression cassette (Figure 3A). Transfection of AAV proviral plasmids carrying the eGFP cDNA driven by photoreceptor promoters results in substantially higher levels of gene expression compared to that seen in control cell lines (Figure S2). Similarly, transduction with AAV vectors containing the same transgene cassette resulted in robust activation of transgene expression, specif-

ically within cell lines co-expressing the guide RNA and dCas9-VPR (Figures 3B and 3C). Moreover, photoreceptor promoter expression was shown to increase in a dose-dependent manner, by using two different AAV capsids: AAV8 and AAV7m8 (Figure 3C). To determine whether this approach could be broadly applied to other cell lines, we examined vector expression in the ARPE-19 cell line, which displays morphological and biochemical features of RPE cells. As predicted, transduction of ARPE-19 cells, selected to stably co-express dCas9.VPR and sgRNA 9 targeting the GRK1 promoter (84-31.GRK1.9) resulted in enhanced levels of reporter expression, compared with the control cells (Figure 3D). Collectively, these results demonstrate the potent and potential broad applicability for activating tissue-specific promoters after infection with the appropriate AAV vectors in diverse cell lines.

Multiplexed Promoter Targeting Amplifies AAV Transgene Expression

Prior reports suggest that co-expression of multiple guide RNA sequences (multiplexing) targeting a single promoter region may vastly improve dCas9-mediated gene activation.^{14,19} Likewise, we hypothesized that inclusion of an additional guide RNA could enhance the

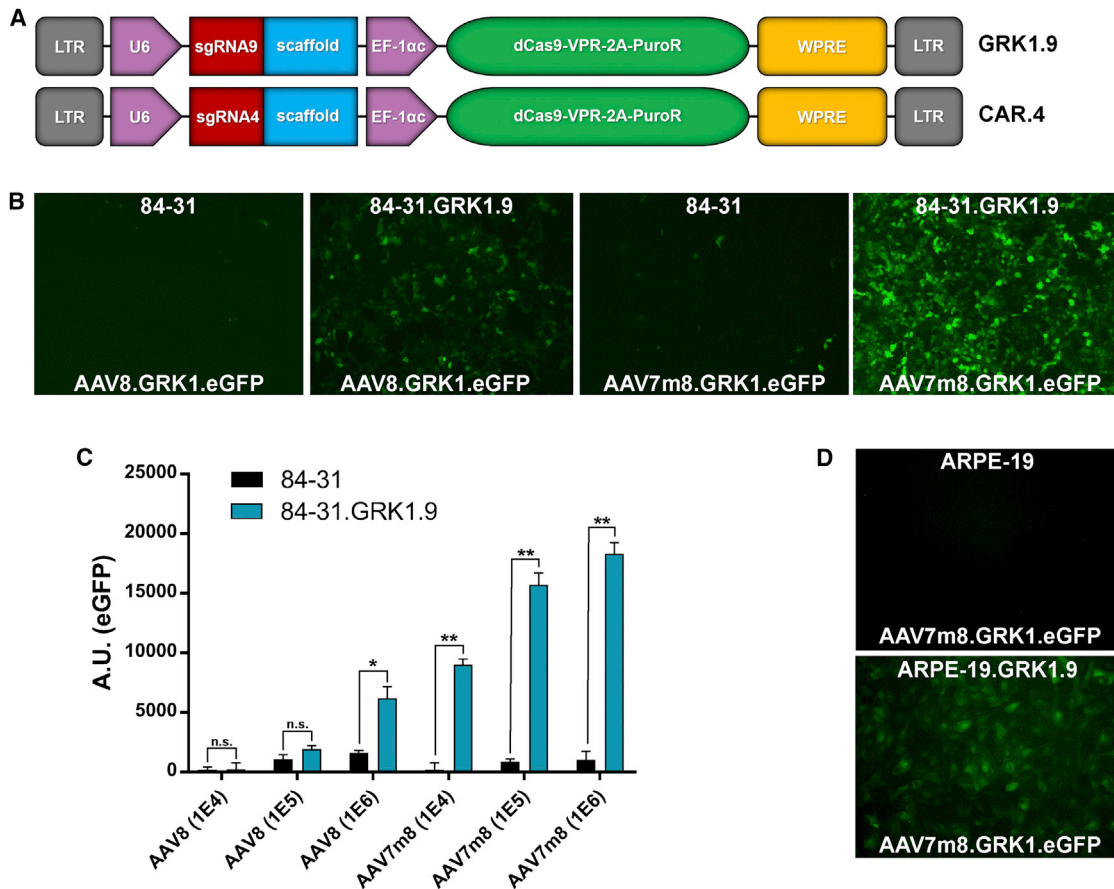


Figure 3. CRISPR Activation Stimulates Tissue-Specific Transgene Expression from AAV Vectors

(A) Outline of lentiviral constructs used to generate cell lines that co-express dCas9.VPR and sgRNAs targeting the GKR1 (top) or CAR (bottom) promoter sequences. (B) Micrographs of wild-type or cells containing the GRK1.9 promoter activation system following treatment with AAV8 or AAV7m8 vectors. (C) Quantification of fluorescence intensity in wild type or cells containing the GKR1.9 promoter activation system following AAV8 or AAV7m8 transduction at three MOIs. (D) AAV7m8 activity in ARPE-19 cells with or without the GRK1.9 promoter activation system. Data are represented as the mean \pm SEM (n = 2). *p < 0.05, **p < 0.01.

activation of photoreceptor promoters from AAV expression cassettes. We engineered a lentiviral vector encoding dCas9.VPR and the two top-performing guide RNA sequences targeting the CAR promoter (Figure 4A). We generated a stable cell line (84-31.CAR.4.10) expressing these components and selected positively transduced clones by puromycin incubation. Upon transduction with AAV8.CAR.eGFP, these cells displayed greater levels of reporter gene expression, as assayed by microscopy, fluorescence intensity readings, and gene expression analysis, compared with the control cell lines (Figures 4B and 4D). Similar to previous findings, the degree of reporter gene expression increased in a dose-dependent manner (Figure 4C), suggesting the importance of AAV genome copies as a rate-limiting determinant of promoter activation.

AAVR Augmentation Mediates a Synergistic Effect upon AAV Promoter Activation

Recent studies identified and characterized a “universal” transmembrane receptor for AAV infection denoted AAVR.^{23,24} We generated

84-31 cells that stably expressed AAVR with a lentiviral vector (Figure 5A). Transduction of 84-31 cells that stably overexpress AAVR (84-31.AAVR) displayed differential effects upon reporter gene expression, compared with control cells (Figures 5B–5C). Surprisingly, transduction with many serotypes was inhibited compared with 84-31 control cells, suggesting a discrepancy between AAVR activity and capsid or cell line-dependent properties. Importantly, the transduction efficiency of clinically relevant capsids, such as AAV8, was enhanced with AAVR supplementation. Based on these findings, we hypothesized that enhanced vector uptake through the AAVR pathway would provide additional substrate for the CRISPR activation components and subsequently improve transgene expression. 84-31 and 84-31.CAR.4.10 cells were similarly transduced with a lentiviral vector driving AAVR overexpression, and positive clones were selected with blasticidin. Transduction of 84-31-CAR.4.10.AAVR cells with AAV8.CAR.eGFP demonstrates superior transgene expression compared with control cell lines, as detected by microscopy and RT-qPCR (Figures 5D–5E). Similar effects were observed following

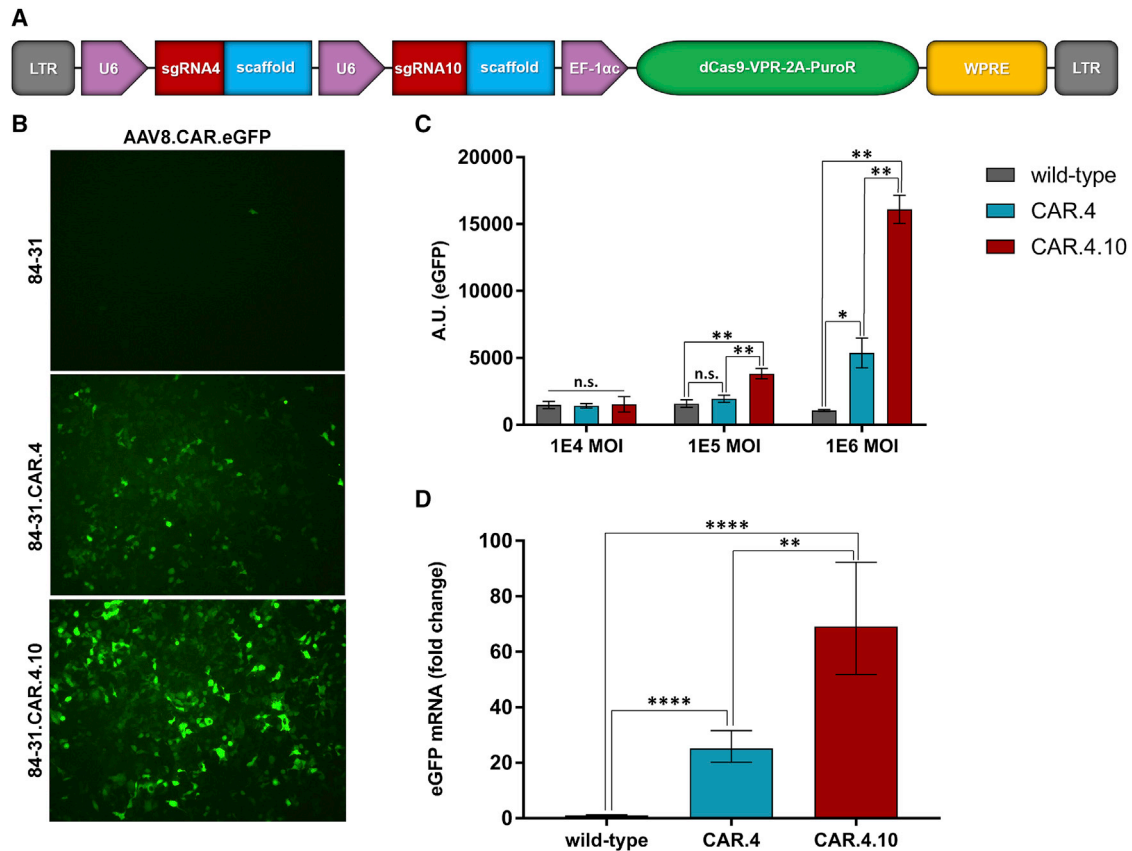


Figure 4. Multiplexed Targeting of a Single Promoter Enhances AAV Expression

(A) Lentiviral cassette co-expressing dCas9-VPR with sgRNA4 and sgRNA10 targeting the CAR promoter. Lentiviral vectors were used to generate the 84-31.CAR.4.10 cell line. (B) Representative micrographs of different cell lines transduced with AAV8.CAR.eGFP at 1E6 MOI. (C) Quantification of fluorescence intensity in different cell lines following transduction with AAV8.CAR.eGFP at different MOIs ($n = 4$). Data represent the mean \pm SEM. * $p < 0.05$, ** $p < 0.01$. (D) Quantification of eGFP gene expression in cell lines following transduction with AAV8.CAR.eGFP at 1E6 MOI ($n = 3$). Data represent the mean \pm SD. ** $p < 0.01$, **** $p < 0.0001$.

AAV8.GRK1.eGFP transduction in 8431.GRK1.9 cells harboring the AAVR expression cassette (Figure S3). Collectively, these results highlight the synergistic value of enhancing AAV promoter activity by co-expressing AAVR and CRISPR activation components. Furthermore, they once again suggest vector genome copies as the limiting factor in achieving substantial tissue-specific promoter activation from AAV transgene cassettes.

DISCUSSION

The clinical success and recent U.S. Food and Drug Administration (FDA) approval of Luxturna provides a framework for the development of gene therapies for other forms of inherited retinal degeneration.^{25,26} As viral vectors become increasingly sophisticated in capsid design and genomic composition,²⁷ new challenges will arise in the development of cell-based assays to interrogate vector function and clinical potency. Various pharmacological reagents have been shown to successfully enhance vector transduction of numerous cell types.^{28,29} Although these tools are valuable for increasing overall levels of gene transfer efficiency, these reagents are likely to provide

minimal advantages if the transgene is controlled by a tissue or cell-specific regulatory element that displays insufficient or undetectable activity within the target cell line. This raises an enormous challenge in designing vectors for retinal disease targets characterized by photoreceptor degeneration, as cell lines representative of pure photoreceptors are unavailable³⁰ and culturing photoreceptors *ex vivo* is hampered by swift kinetics of photoreceptor degeneration after a few days in culture, among other technical challenges.³¹ Moreover, currently established protocols for differentiation of human iPSCs into photoreceptors are limited by extensive culture timelines met with low yields of mature photoreceptors.³² We previously alluded to the use of the 661W cell line as a possible avenue to functionally evaluate vectors driven by retinal promoters *in vitro*. However, we were unable to detect transgene expression regulated by the GRK1 promoter following transduction with AAV8 or AAV7m8 vectors. Conversely, we did observe expression from this promoter in the 84-31.GRK1.9 cell line when compared side-by-side with the 661W cells (Figure S4). It may be possible to bypass these obstacles with several different approaches. Prior investigations allude to the use

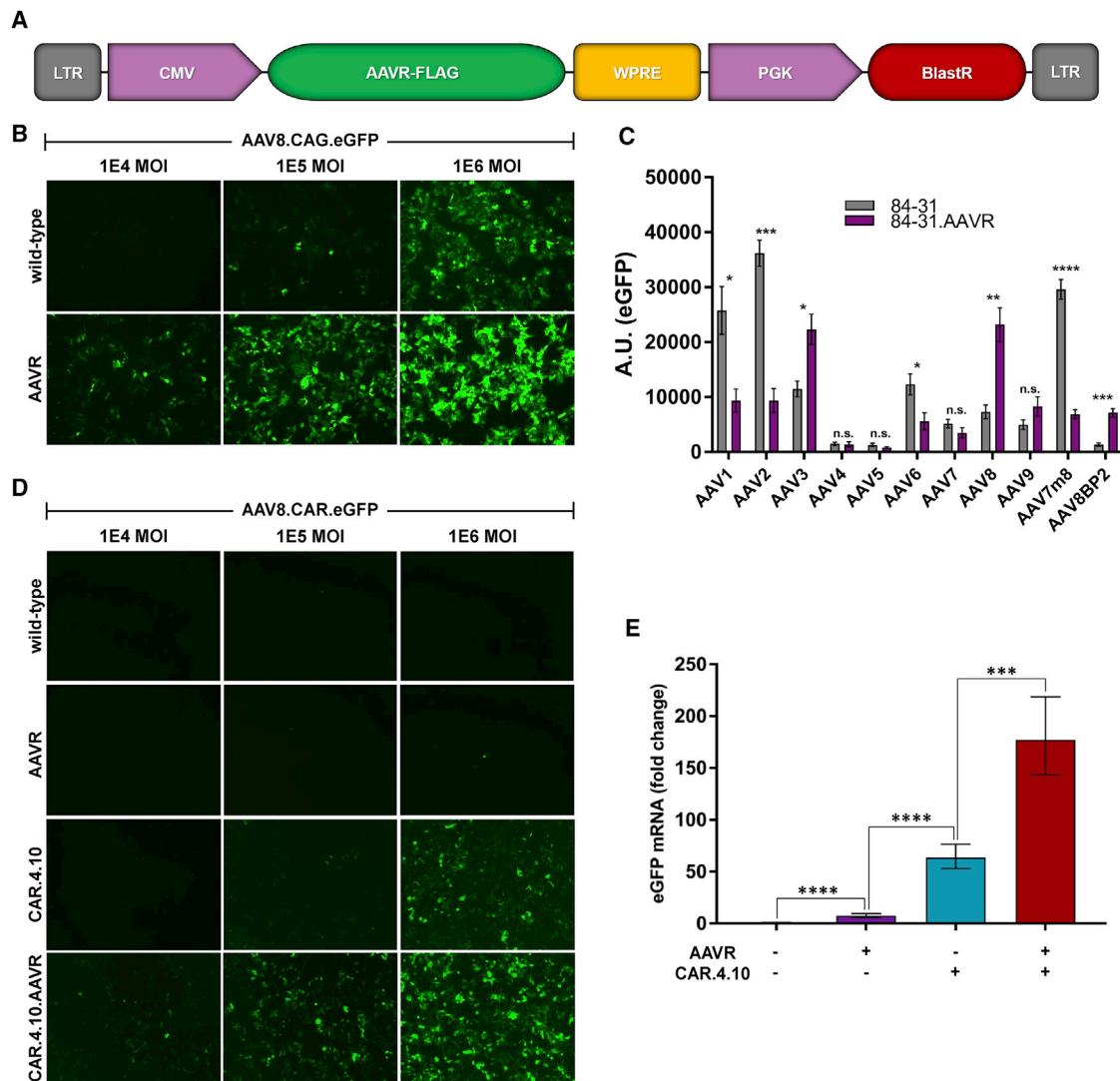


Figure 5. AAVR Co-expression Mediates a Synergistic Effect upon Promoter Activity

(A) Outline of lentiviral vector used to generate AAVR overexpression cell lines. (B) Representative micrographs displaying the effect of AAVR overexpression in 84-31 cells following AAV8.CAG.eGFP transduction at three different MOIs. (C) Quantification of eGFP fluorescence intensity in wild-type 84-31 and 84-31.AAVR cells with a panel of AAV serotypes at 5E5 MOI. Data represent mean intensity \pm SEM (n = 4). (D) Representative micrographs of different cell lines transduced with AAV8.CAR.eGFP at different MOIs. (E) Quantification of eGFP expression with qRT-PCR following transduction with AAV8.CAR.eGFP at 1E6 MOI. Data represent means \pm SD (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

of pharmacological reagents to stimulate endogenous promoter activity as a possible avenue to improve *in vitro* activity of cell-specific promoters. For example, Li et al.³³ identified a region of the cone arrestin promoter sequence that is responsive to retinoic acid. However, effects on transgene expression are likely to be transient, because of the limited half-life of the compound. In addition, it is unclear what downstream effects drug activity may exert upon cell biology. It may also be possible to amplify transgenic promoter activity by overexpressing transcription factors identified to act upon the DNA sequence of interest. However, transcription factors typically stimulate large networks of associated genetic elements, thereby imposing

a non-specific and diluted response. Similar to pharmacological induction, it is unclear how constitutive expression of a transcription factor will affect underlying cell biology and other downstream parameters of a functional assay. The present approach using CRISPR activation provides a highly potent, stable, and precise platform to stimulate cell-specific promoters from AAV vectors.

Our promoter activation assay allowed us to screen guide RNA activity by reconstituting the CRISPR activation system following transfection of guide-reporter plasmids into a cell line harboring endogenous expression of dCas9.VPR. Using this approach, we successfully

identified guide RNA candidates that displayed robust stimulation of our promoter targets. Although our specific goal was to activate photoreceptor-associated regulatory elements, it should be recognized that this assay may be universally applicable and adapted to accommodate screening of guide RNA sequences targeting other cell or tissue-specific promoter sequences for use in AAV expression cassettes. Cell lines engineered to stably co-express dCas9-VPR and guide candidates provided sufficient stimulation of tissue-specific promoters following proviral plasmid transfection or AAV transduction within these cells. Furthermore, we demonstrated the broad utility of these systems by targeting the GRK1 promoter using different vector capsids and across different cell lines.

Addition of a second sgRNA targeting the same promoter amplified AAV transgene expression compared to single-guide-containing cells and the 84-31 control cell line. Similar to previous findings from the single-guide systems, CAR-driven transgene activation from AAV8 vectors increased in a dose-dependent manner in the multiplexed cell line. It may be possible to generate multiplexed CRISPR activation systems that express sgRNAs targeting more than one specific promoter, thereby allowing the creation of a universal cellular model to interrogate the potency of gene transfer vectors regulated by different cell-specific regulatory elements.

Conventional understanding of AAV binding and entry involves the use of one or more proteoglycans, such as heparin sulfate proteoglycan (HSPG) in the case of AAV2, as a primary receptor combined with a secondary protein receptor.³⁴ Most recently, AAVR was identified as a key host receptor responsible for the attachment and entry of numerous AAV capsids.^{23,24,35} Combining CRISPR activation with AAVR overexpression provided a highly effective means of improving vector performance and thereby increasing levels of cell type-specific promoter transgene expression. Specifically, AAVR overexpression dramatically enhanced AAV8 uptake in cell lines harboring both the GRK1 or CAR promoter activation systems. We observed differential effects upon capsid uptake with AAVR overexpression using a panel of 11 distinct serotypes, suggesting capsid- or cell-type-dependent effects on viral entry. For example, AAVR overexpression within 84-31 cells greatly reduced expression of vectors, such as AAV2 and AAV7m8, compared with the control cell line. However, transduction with AAV2 showed a trending increase in transgene expression, and AAV7m8 transduction was greatly enhanced in ARPE-19 cells overexpressing AAVR (Figure S5). Although AAVR is certainly a major determinant of AAV entry, other distinct mechanisms likely contribute to the differential effects upon vector transduction observed between distinct cell lines, including the expression and distribution of capsid-specific receptors upon the cellular surface.^{34,36} It is also possible that AAVR overexpression perturbs such mechanisms in a cell-line-dependent fashion by inhibiting critical steps in vector transduction. Downstream investigations will be necessary to delineate these cell-type-dependent mechanisms of AAV entry.

Beyond functional testing of gene therapy vectors, the present approach may be useful for proof-of-concept studies in cellular

models of disease and potency assay development. This is particularly relevant in the context of inherited retinal degenerations where animal models do not always present an appreciable ocular phenotype that reflects the human course of disease.^{37,38} Because of these challenges associated with *in vivo* modeling, various studies have explored modeling of inherited retinal degenerations with iPSC-derived patient cells differentiated toward a specific retinal cell lineage. For example, Duong et al.³⁷ demonstrated rescue of protein trafficking, prenylation, and phagocytosis following AAV-mediated gene replacement in iPSC-derived RPE cells isolated from patients with choroideremia. Generation of patient-derived cellular models and application of CRISPR-activation systems such as those described here may allow rescue of cellular phenotypes and development of *in vitro* potency assays for gene therapy vectors regulated by tissue-specific promoters.

Collectively, our study outlines a broadly applicable approach for engineering cell lines to express AAV vectors regulated by tissue-specific promoters. Although our data demonstrate the utility of this approach in the context of retina-specific regulatory elements, this strategy may be applied broadly to other promoter sequences that display specific activity within a tissue or cell population of interest. Furthermore, continued identification and engineering of Cas proteins with broader PAM recognition features and enhanced DNA binding specificity will allow the continued improvement of such systems. Finally, these systems will be crucial for the downstream development of not only AAV-based gene therapy products, but also other viral and non-viral vector systems.

MATERIALS AND METHODS

Plasmid Generation

The pAAV-U6-sgRNA-CMV-eGFP-KASH plasmid was kindly supplied by Hetian Lei (85451; Addgene, Cambridge, MA, USA). The eGFP-KASH coding sequence was removed by digestion with AgeI-HF and EcoRI-HIF (NEB, Ipswich, MA, USA) and the wild-type eGFP sequence was amplified and restored between these sites. In addition, the CMV promoter cassette was removed by digestion with NheI-HF and SpeI-HF (NEB) and replaced with the GRK1 or CAR promoter sequences. Guide RNA sequences targeting either promoter were cloned between the SapI sites between the U6 promoter and scaffold. The pLenti-EF1 α -dCas9-VPR-2A-Puro plasmid was a kind gift from Kristen Brennand (99373; Addgene). pLentiCRISPRv2 was kindly provided by Feng Zhang (52961; Addgene). Top-performing candidate guide RNAs were cloned between the BsmBI (NEB) sites of pLentiCRISPRv2. Afterward, the SpCas9-2A sequence was removed by digestion with XbaI-HF and BamHI-HF (NEB). The dCas9-VPR-2A sequence was amplified and cloned into the XbaI/BamHI digested pLentiCRISPRv2 with the In-Fusion HD cloning system (Clontech, Mountain View, CA, USA) to create lentiviral expression plasmids that co-express an sgRNA and dCas9-VPR-2A-Puro. The multiplexed CAR activation lentiviral expression plasmid was generated by digestion of the single-guide CAR expression plasmid with NheI-HF (NEB), PCR

amplification of the guide expression cassette containing sgRNA candidate 10, targeting the CAR promoter, and ligation with the In-Fusion HD cloning system. A plasmid encoding AAVR-FLAG was a kind gift from Jan Carette (Stanford University). The AAVR-FLAG sequence was amplified and cloned into a lentiviral expression vector containing a downstream blasticidin resistance cassette. All plasmids were propagated in One Shot Stbl3 competent cells (Invitrogen, Carlsbad, CA, USA).

Cell Culture

HEK293T and ARPE-19 cells were supplied by ATCC (Manassas, VA, USA). HEK293T cells were cultured in DMEM-GlutaMax (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco). ARPE-19 cells were maintained in DMEM:nutrient mixture F-12 (DMEM/F12; Gibco) and supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin. 84-31 cells were provided by James M. Wilson (University of Pennsylvania) and were cultured in DMEM-GlutaMax supplemented with 10% FBS and 1% penicillin-streptomycin. hiPSCs were maintained in Stemflex medium (Invitrogen) on Matrigel-coated (3–4 $\mu\text{g}/\text{cm}^2$; Corning, Corning, NY, USA) dishes. hiPSC lines were maintained at 37°C with 5% CO₂ and 5% O₂. All other cell lines were grown at 37°C with 5% CO₂. Cell lines stably transduced with lentiviral vectors were selected and maintained in medium containing 1 $\mu\text{g}/\text{mL}$ puromycin (Sigma-Aldrich, St. Louis, MO, USA) or 10 $\mu\text{g}/\text{mL}$ blasticidin-S (Invitrogen).

Guide RNA Selection

Candidate guide RNA sequences were identified with web-based design tools, including CRISPR Design (<http://zlab.bio/guide-design-resources>) and CRISPR-ERA (<http://crispr-era.stanford.edu>).

Lentiviral Vector Production

HEK293T cells (2×10^6) were plated in 10 cm dishes. On the following day, cells were transfected with 6 μg of the appropriate lentiviral expression plasmid, 4 μg psPAX2 (supplied by Didier Trono; 12260; Addgene), and 2 μg pMDG.2 (supplied by Didier Trono; 12259; Addgene) combined with 16 μL Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. Cells were refed with fresh medium the following day. Medium containing lentiviral particles was harvested 48 and 72 h after transfection, pooled, and stored at -80°C until use.

AAV Vector Production

AAV vectors were generated by using previously described methods³⁹ and purified with CsCl gradient ultracentrifugation by the CAROT research vector core at the University of Pennsylvania.

In Vitro AAV Transduction and Fluorescence Intensity Measurements

Cells were plated (5×10^4) into individual wells of 96-well black-bottom dishes. Afterward, the cells were immediately transduced with AAV vectors at the MOIs indicated. eGFP fluorescence was quantified at 48 h after transduction on the Tecan Infinite M200 Pro plate

reader (485 nm excitation and 535 nm emission). Data represent mean arbitrary units (AUs) in eGFP fluorescence between three technical replicates per experiment. Data from each treatment group were normalized to the mean of three non-transduced control wells. Experiments were performed at least three times.

Promoter Activation Screen

84-31.VPR cells (5×10^4) were plated into individual wells of 96-well black-bottom dishes. The following day, cells were transfected with 200 ng sgRNA-reporter plasmid and 1 μL Lipofectamine 2000 (Invitrogen). eGFP fluorescence was quantified at 48 h after transfection on the Tecan Infinite M200 Pro plate reader (485 nm excitation and 535 nm emission). Data represent the mean AUs of eGFP fluorescence between three technical replicates. The screens were performed a total of three times ($n = 3$), and the data from each treatment were normalized to the mean of three non-transfected control wells.

In Vivo Studies

C57BL/6 mice were obtained from the Jackson Laboratory. Animals were housed and maintained at the University of Pennsylvania under Animal Care and Use Committee protocol 805890. Subretinal injections were performed as previously described.⁴⁰ Each retina received 1×10^9 vector genomes in a total volume of 1 μL .

Immunohistochemistry

Eyes were enucleated, fixed, and processed as frozen retinal sections, as described previously.⁴⁰ Sections were incubated in blocking buffer consisting of 10% normal goat serum (Cell Signaling Technology, Danvers, MA, USA) and 2% Triton X-100 (Sigma) and PBS (Corning, Corning, NY, USA) for 1 h at room temperature. Next, the sections were incubated in blocking solution containing rabbit anti-cone arrestin (1:400; Millipore, Burlington, MA, USA) overnight in a humidified chamber at room temperature. They were washed three times in PBS and incubated in secondary antibody solution consisting of blocking buffer and Cy5-labeled goat anti-rabbit IgG (1:400; KPL) for 1 h at room temperature. Sections were washed in PBS three times and mounted on slides with Fluoromount-G (Southern Biotech, Birmingham, AL, USA) containing DAPI.

RNA Isolation and qRT-PCR

RNA was isolated using the Nucleospin RNA kit (Macherey-Nagel, Bethlehem, PA, USA). First-strand cDNA synthesis was performed using 1,000 ng total RNA with the SuperScript III First-Strand Synthesis system, according to the manufacturer's protocol. Real-time PCR was performed with the Applied Biosystems 7500 Fast system using the Power SYBR green PCR Master Mix (Invitrogen). The following primer sequences were used: 5' CCACTCCTCCACCTTT GAC 3' (human *GAPDH* forward), 5' ACCCTGTTGCTGTAGCCA 3' (human *GAPDH* reverse), 5' CGACAACCACTACCTGAGCA 3' (*eGFP* forward), and 5' CTTGTACAGCTCGTCCATGC 3' (*eGFP* reverse). Relative gene expression was quantified with the $\Delta\Delta\text{C}_T$ method and normalized to *GAPDH*.

Statistics

Statistical analyses of differences between two groups were performed using the unpaired Student's *t* test. Comparisons of three or more groups with a one-way ANOVA with Tukey's honest significant difference (HSD) post hoc test. *p* < 0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.omtm.2019.03.004>.

AUTHOR CONTRIBUTIONS

D.S.M. conceived the study, designed and generated reagents, performed experiments, analyzed data, and wrote the manuscript. K.C.P., T.E.P., A.M., T.T.D., and J.A.M. performed experiments and collected data. S.Z. generated rAAV vectors. J.B. supervised the study. All authors edited and approved the finalized manuscript.

CONFLICTS OF INTEREST

J.B. is a scientific (non-equity-holding) founder of Spark Therapeutics and is a founder of GenSight Biologics and Perch Therapeutics. These companies were not involved in any aspect of the study.

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