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Development of synthetic guide RNA libraries for CRISPR-mediated transcriptional activation screening for gain-of-function studies

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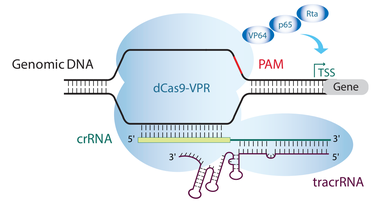
Abstract

Functional gene analysis studies have been empowered by development of CRISPR-Cas9 gene knockout tools, however the CRISPR-Cas9 system has also been adapted for inhibition or activation of gene transcription. A nuclease-deactivated Cas9 (dCas9) can be fused to various effector domains to produce an RNA-guided transcription factor for either inhibition (CRISPRi) or activation (CRISPRa) of target genes. For overexpression studies, CRISPRa holds significant advantages over traditional vector-based gene expression, because genes are upregulated from their native promoter and endogenous genomic context. The majority of CRISPRa research performed to date has utilized single guide RNA (sgRNA) expressed from a DNA vector, primarily in the context of pooled lentiviral screening approaches. Here we describe the development of CRISPRa synthetic guide RNAs for the use in arrayed screening, so that we combine this next-generation transcriptional activation method with the ability to support more complex assays in a one-gene-per well format. Considerations for arrayed activation screens will be shown. The combination of gain-of-function from CRISPRa with loss-of-function using RNAi or canonical CRISPR-Cas9 for gene knockout allows for robust characterization of gene mechanisms and pathways.

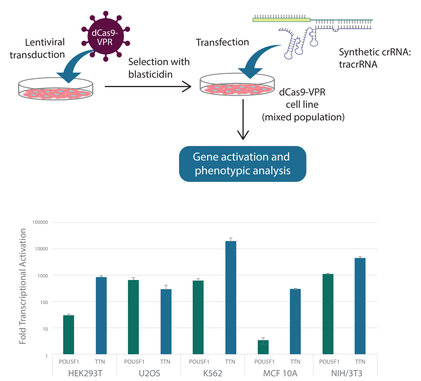
Using synthetic guide RNA with the dCas9-VPR transcriptional activation system



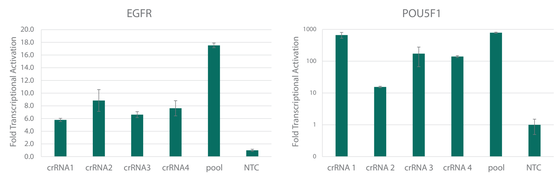
- In the VPR activation system, deactivated Cas9 is fused at the C-terminal end to three transcriptional activators, VP64, p65 and Rta (Chavez *et al.*)
- Edit-R™ CRISPRa synthetic crRNA:tracrRNA mimics the two-part guide RNA found in the native *S. pyogenes* and can be utilized with this system
- The crRNA is complementary to the gene promoter regions and induce transcription
- Designs are based on CRISPRa v2 algorithm based on machine learning (Horlbeck *et al.* 2016)

Efficient transcriptional gene activation with synthetic crRNA:tracrRNA in multiple dCas9-VPR stable cells

HEK293T, U2OS, MCF 10A, NIH/3T3 stably expressing integrated dCas9-VPR were transfected using DharmaFECT Transfection Reagents with synthetic crRNA:tracrRNA (25 nM) targeting POU5F1 and TTN. K562 cells were electroporated with synthetic crRNA:tracrRNA (400 nM) targeting POU5F1 and TTN. Cells were harvested 72 hours post-transfection and the relative gene expression was measured using RT-qPCR. The relative fold transcriptional activation for each gene was calculated with the ΔΔCq method using GAPDH as the reference gene and normalized to a non-targeting control.



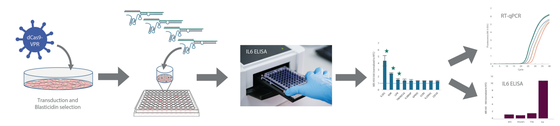
Using multiple synthetic crRNA per gene can enhance activation



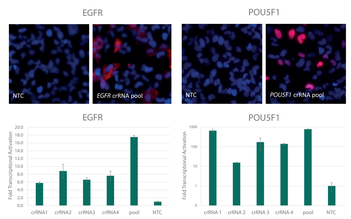
U2OS cells stably expressing integrated dCas9-VPR were transfected using DharmaFECT 4 Transfection Reagent with synthetic crRNA:tracrRNA targeting targeting EGFR and POU5F1. The pre-designed crRNAs were used either individually or pooled (to a total concentration of 25 nM). Cells were harvested 72 hours post-transfection and the relative gene expression was measured using RT-qPCR. The relative fold transcriptional activation for each gene was calculated with the ΔΔCq method using GAPDH as the housekeeping gene and normalized to a non-targeting control (NTC).

Synthetic guide RNAs are ideal for arrayed CRISPRa screening

Generate dCas9-VPR stable cells	Transfect with crRNA libraries	Phenotypic assay	Hit identification	Hit analysis

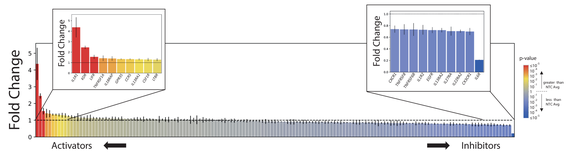


Efficient transcriptional activation on mRNA and protein levels



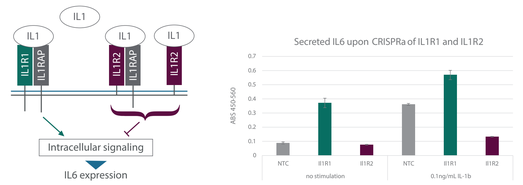
U2OS cells stably expressing dCas9-VPR were transfected with synthetic crRNA:tracrRNA (25 nM) targeting EGFR or POU5F1. Transcriptional activation was measured at 72 hours by RT-qPCR (left) or by immunofluorescence analysis (right).

Primary CRISPRa screen identifies IL6 regulators



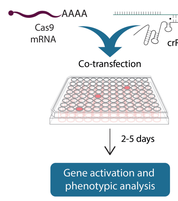
- U2OS dCas9-VPR stable cells transfected with a CRISPRa arrayed library of 153 cytokine receptors
- IL6 in supernatant was detected by ELISA assay at 72 hours
- Several positive and negative regulators of IL6 identified
- Validation of hits are in progress

IL1 signaling pathway is one of the major regulators of IL6 expression



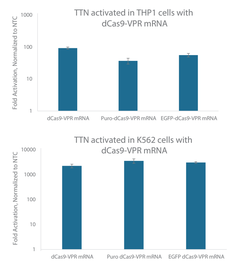
- IL1R1 is a positive regulator of IL1 signaling
- Transcriptional activation of IL1R1 by CRISPRa shows increase of secreted IL6 with or without IL1 stimulation
- IL1R2 is a negative regulator of IL1 signaling
- Transcriptional activation of IL1R2 by CRISPRa shows decrease of secreted IL6 – especially pronounced upon IL1 stimulation

Co-electroporation of dCas9-VPR mRNA and crRNA:tracrRNA results in efficient activation

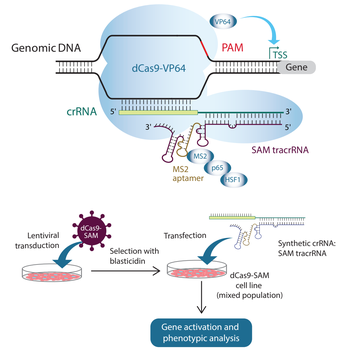


- Lentiviral transduction is not feasible for all applications
- Direct co-delivery of mRNA with synthetic crRNA:tracrRNA allows for robust activation
- Puromycin and EGFP on the mRNA can also allow for enrichment of transfected cells

THP1 and K562 cells were electroporated using the Lonza 2b system with either dCas9-VPR mRNA (5 µg, Cat #CAS12024), Puro dCas9-VPR mRNA (5 ug, Cat #CAS12026) or EGFP dCas9-VPR mRNA (5 ug, Cat #CAS12025), synthetic tracrRNA (25 nM, Cat #U-002005-05), and pooled CRISPRa crRNA targeting TTN (5 uM, Cat #P-005395-01-0005) or Non-targeting control (NTC, 25 nM, Cat #U-009500-10-05). After 48-hours cell were harvested, lysed, and total RNA was isolated. RT-qPCR was performed and activation was normalized to NTC samples for both THP1 and K562 cells, respectively.

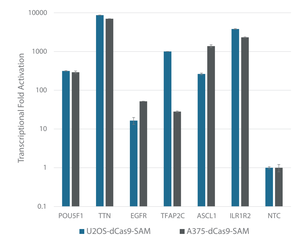


Synthetic SAM tracrRNA was developed for use with the dCas9-SAM system



- For the synergistic activation mediator (SAM activation), deactivated Cas9 is fused at the C-terminal end to VP64. The system also expresses MS2-p65-HSF1 fusion proteins (Konermann *et al.* 2015)
- This system uses an altered *S. pyogenes* guide RNA that contains an MS2 aptamer that can interact with MS2 protein
- Synthetic SAM tracrRNA has a single MS2 aptamer that can interact with the MS2 fusion protein and can be combined with Edit-R crRNA for activation

Transcriptional activation using dCas9-SAM and synthetic SAM tracrRNA



U2OS and A375 cells stably expressing dCas9-VP64 and MS2-p65-HSF1 (dCas9-SAM) were plated at 10,000 cells/well and transfected using DharmaFECT 4 (U2OS) or DharmaFECT 1 (A375) Transfection Reagent with synthetic crRNA:tracrRNA (25nM). Four pre-designed crRNAs targeting each gene were pooled (to a total concentration of 25nM). Cells were harvested 72 hours post-transfection and the relative gene expression was detected using RT-qPCR. The relative expression of each gene was calculated with the ΔΔCq method using PPIB as the housekeeping gene and normalized to a non-targeting control.

Conclusions

- Synthetic crRNA:tracrRNA can be used with dCas9-VPR for transcriptional activation
- Pooled crRNAs can enhance the transcriptional gene up-regulation activity
- Synthetic crRNAs can be successfully used for gain-of-function screening in an arrayed format enabling expansion of the phenotypic readouts to high-content and morphology-based assays
- Synthetic guide RNA can be co-delivered with dCas9-VPR mRNA for applications where a stable dCas9-VPR cell line cannot be generated
- Synthetic crRNA can also be used in the dCas9-SAM system when used in conjunction with a synthetic SAM tracrRNA
- Synthetic guide RNAs provide many advantages to expressed guide RNAs in well-by-well screens because they require fewer experimental steps, give a much more uniform delivery concentration and are less prone to off-target effects due to their transient cellular effect

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A. Chavez *et al.*, Highly efficient Cas9-mediated transcriptional programming. *Nat. Methods.* 12, 326–328 (2015)
M. A. Horlbeck *et al.*, Compact and highly active next-generation libraries for CRISPR-mediated gene repression and activation. *eLife*, 5, e19760 (2016)
S. Konermann *et al.*, Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature* 517(7536): 583–588. (2015)