Potent transcriptional activation using CRISPRa with synthetic crRNA

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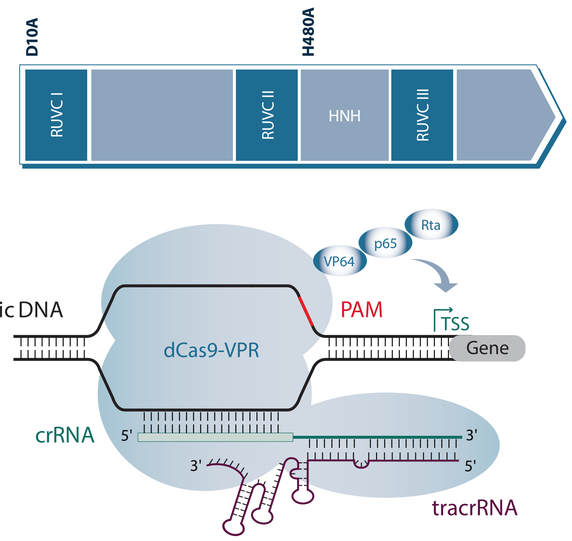
Abstract

The CRISPR-Cas system derived from *Streptococcus pyogenes* has been adapted to upregulate any gene in its endogenous context, enabling overexpression experiments without a need for exogenous overexpression plasmids. For CRISPR activation (CRISPRa), the guide RNA forms a complex with a nuclease-deactivated Cas9 (dCas9, D10A and H840A) which is in turn fused to transcriptional activators. The machinery then acts upstream of the transcription start sites to upregulate expression of a target gene. The ease of programming the CRISPRa system with small RNA guides is transformative for performing gain-of function studies as an alternative method for identification of gene functions that might be undiscovered with loss-of function methods alone.

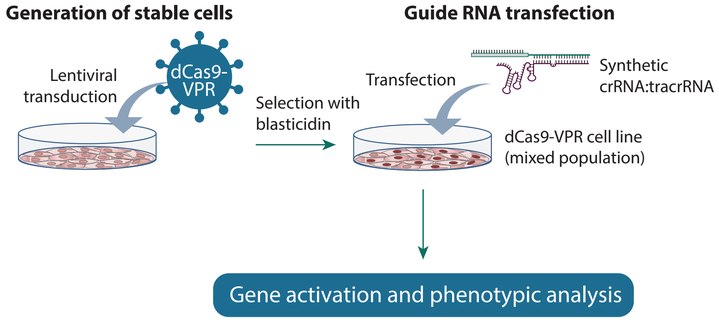
Here we demonstrate a strategy to conduct CRISPRa experiments using synthetic crRNA reagents. We examine the functionality and advantages of using the synthetic guide RNA approach for CRISPRa in multiple cell lines including pooling of crRNAs to enhance target gene activation, as well as activating multiple genes simultaneously. Additionally, we demonstrate transcriptional activation leading to a significant increase in the target's protein level, which in turn causes phenotypic effects by inhibiting or activating downstream genes. This demonstrates that CRISPRa using synthetic crRNAs could be used for low- or high-throughput studies of downstream signaling and pathway analysis. The methods presented are broadly applicable as a strategy to upregulate any gene including systematic functional gene analysis in an arrayed screening format.

Edit-R CRISPRa transcriptional activation system

CRISPRa uses deactivated or "dead" Cas9 (dCas9), a mutant variant of the Cas9 protein that is nuclease-deficient due to mutations in the RuvCI and HNH domains. The dCas9 is fused to the transcriptional activators VP64, p65, and Rta (VPR), which act upstream of the transcriptional start site (TSS) to up-regulate expression of the target gene. Chemically synthesized crRNA:tracrRNA molecules enable fast assessment of multiple target sites per gene, or for multiple genes without any cloning.

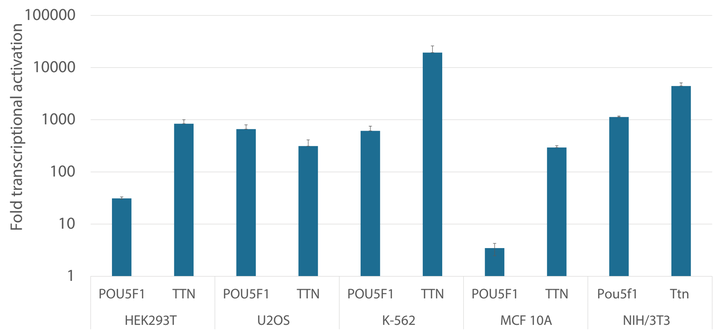


CRISPRa experimental workflow



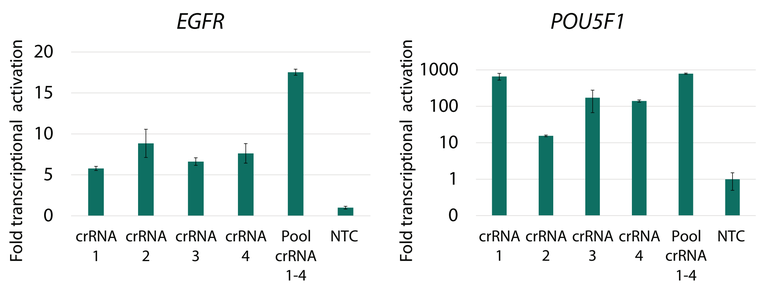
Efficient transcriptional gene activation with synthetic crRNA in dCas9-VPR stable cells

HEK293T, U2OS, MCF 10A, and NIH/3T3 cells stably expressing dCas9-VPR were transfected with synthetic crRNA:tracrRNA (25 nM) targeting *POU5F1* and *TTN* using DharmaFECT transfection reagents. K-562 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 assessed using RT-qPCR. The relative expression of each gene was calculated using the ΔΔCq method with *GAPDH* as the reference gene, and normalized to a non-targeting control. Varying levels of gene activation of *POU5F1* and *TTN* are observed in all cell lines compared to the endogenous gene expression level.



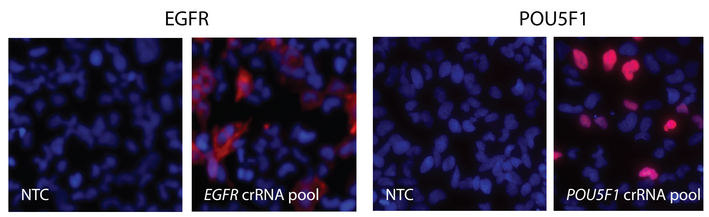
Pooling of crRNAs can enhance transcriptional activation

U2OS cells stably expressing dCas9-VPR were transfected with synthetic crRNA:tracrRNA targeting *EGFR* or *POU5F1* using DharmaFECT 4 transfection reagent. The crRNAs were used either individually or pooled (at a total concentration of 25 nM). The pooled crRNAs demonstrate gene up-regulation activity as good or better than the most active single crRNA. The ability to easily pool crRNAs is an advantage of utilizing chemically synthesized crRNA:tracrRNA.

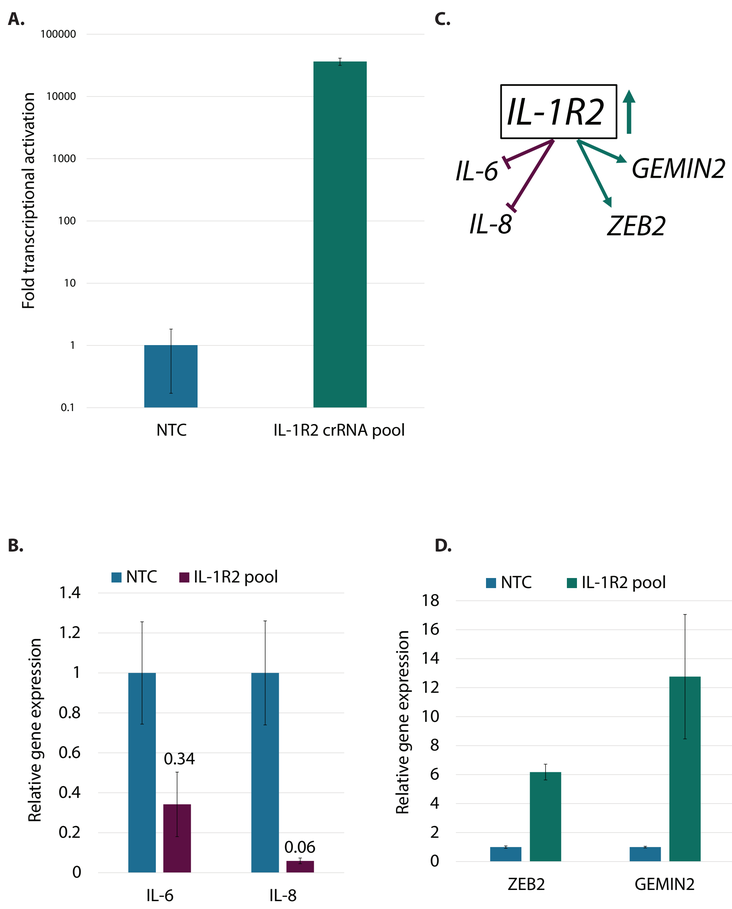


Immunofluorescence analysis indicates effective gene activation

U2OS-dCas9-VPR stable cells were transfected with a pool of four synthetic crRNAs targeting *EGFR* or *POU5F1* (25 nM total concentration) using DharmaFECT 4 transfection reagent. 72 hours post-transfection, cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. Cells were stained with Hoechst 33342, primary antibodies targeting the gene of interest, and secondary antibodies conjugated to Dylight 550.



Activation of *IL-1R2* affects expression of downstream genes



A. U2OS cells stably expressing integrated dCas9-VPR were transfected with a pool of synthetic crRNA:tracrRNA (25 nM) targeting *IL-1R2* using DharmaFECT 4 transfection reagent. Gene activation of *IL-1R2* was increased from undetectable with the NTC to more than 10,000 fold. **B.** Diagram showing predicted gene expression changes upon activation of *IL-1R2* from previous publications. IL-1R2 is an antagonist of IL1 signaling and prevents IL1-enabled IL-6 and IL-8 cytokine production (Peters *et al., Brain Behav Immun* 2013). Other studies have shown that IL1-R2, along with ZEB2 and GEMIN2 (SIP-1), have a role in regulation of cell morphology and EMT transition (Leshem *et al., PLoS ONE* 2011 & Chang *et al., Cytokine* 2009). **C.** Upon *IL-1R2* activation, we detect the predicted down-regulation of *IL-6* and *IL-8*. **D.** CRISPRa activation also leads to the predicted up-regulation of *ZEB2* and *GEMIN2 (SIP-1)*.

Conclusions

- Potent up-regulation of target genes with CRISPRa can be achieved with chemically synthesized crRNA:tracrRNA enabling gain-of-function studies.
- Advantages of synthetic crRNA compared to plasmid-based sgRNA for CRISPRa include the ease of testing multiple target sites per gene, easily pooling multiple sequences for increased gene activation effect, and activation of multiple genes at the same time.
- CRISPRa with synthetic crRNA can be effective in studying the downstream effects of target gene activation.