Simultaneous gene knockout and transcriptional activation using *S. pyogenes* VPR-Cas9 mRNA

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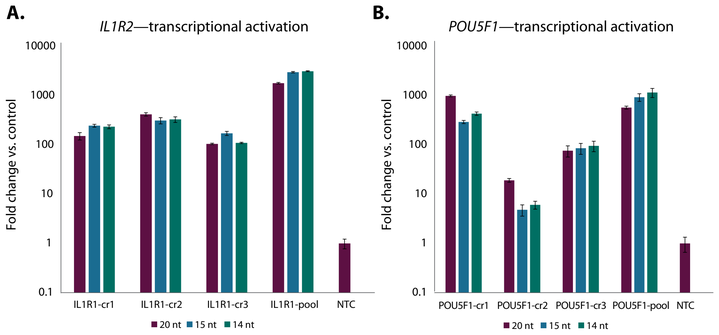
Abstract

Combinatorial genetic manipulations within the same cell populations have been essential to unraveling complex gene regulatory networks in biological systems. For example, being able to simultaneously knock out one gene while activating another gene in large scale allows for in-depth analysis of gene function in the context of signaling pathway modulation. Previously, these simultaneous gene knockout and activation experiments have been performed using two orthogonal Cas enzymes with unique guide RNA requirements—one to perform gene editing and the other for transcriptional activation.

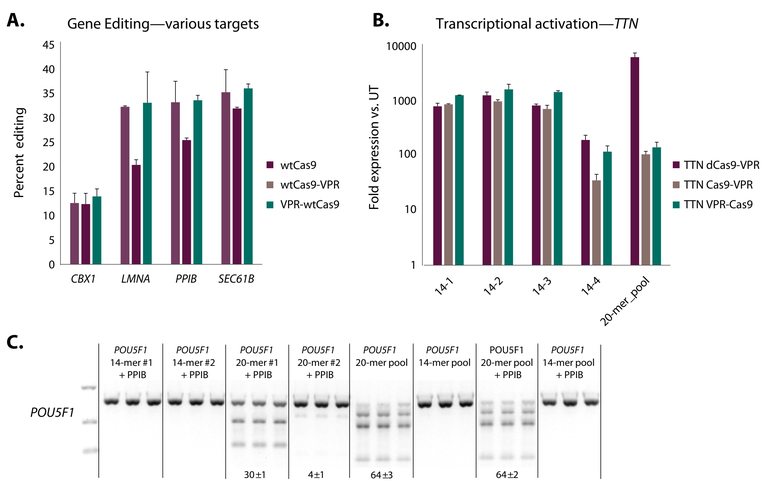
An alternative approach is to use a catalytically active Cas9 nuclease fused with transcriptional activators and different length guide RNAs: a standard guide RNA to localize to the target site in the genome and create a double-strand break and a truncated guide RNA containing a 14-nt targeting sequence which is sufficient to localize to the target site in the genome, but does not result in DNA cleavage. In our hands, both truncated and full-length guide RNAs resulted in transcriptional activation when co-delivered with deactivated Cas9 fused to the transcriptional activators VPS64, P64, and Rta (VPR) mRNA. Therefore, we set out to test if simultaneous gene knockout and transcriptional activation using wild-type *S. pyogenes* Cas9-VPR was feasible using synthetic guide RNAs and Cas9-VPR mRNA. For this purpose, we optimized the location of the transcriptional activating domain. N-terminal and C-terminal VPR positioning on active Cas9 resulted in equal transcriptional activation, but gene editing was significantly compromised by the C-terminal VPR fusion. We then showed that this optimized VPR-Cas9 mRNA was capable of simultaneous gene editing and transcriptional activation when co-transfected with 20 nt targeting synthetic guide RNA for gene editing and 14 nt targeting synthetic guide RNAs for transcriptional activation. Therefore VPR-Cas9 provides a useful single effector for both gene editing and transcriptional activation that can be programmed via synthetic guide RNA and synthetic truncated guide RNA to aid in the interrogation of complex regulatory pathways.

Synthetic-truncated guide RNAs are capable of transcriptional activation



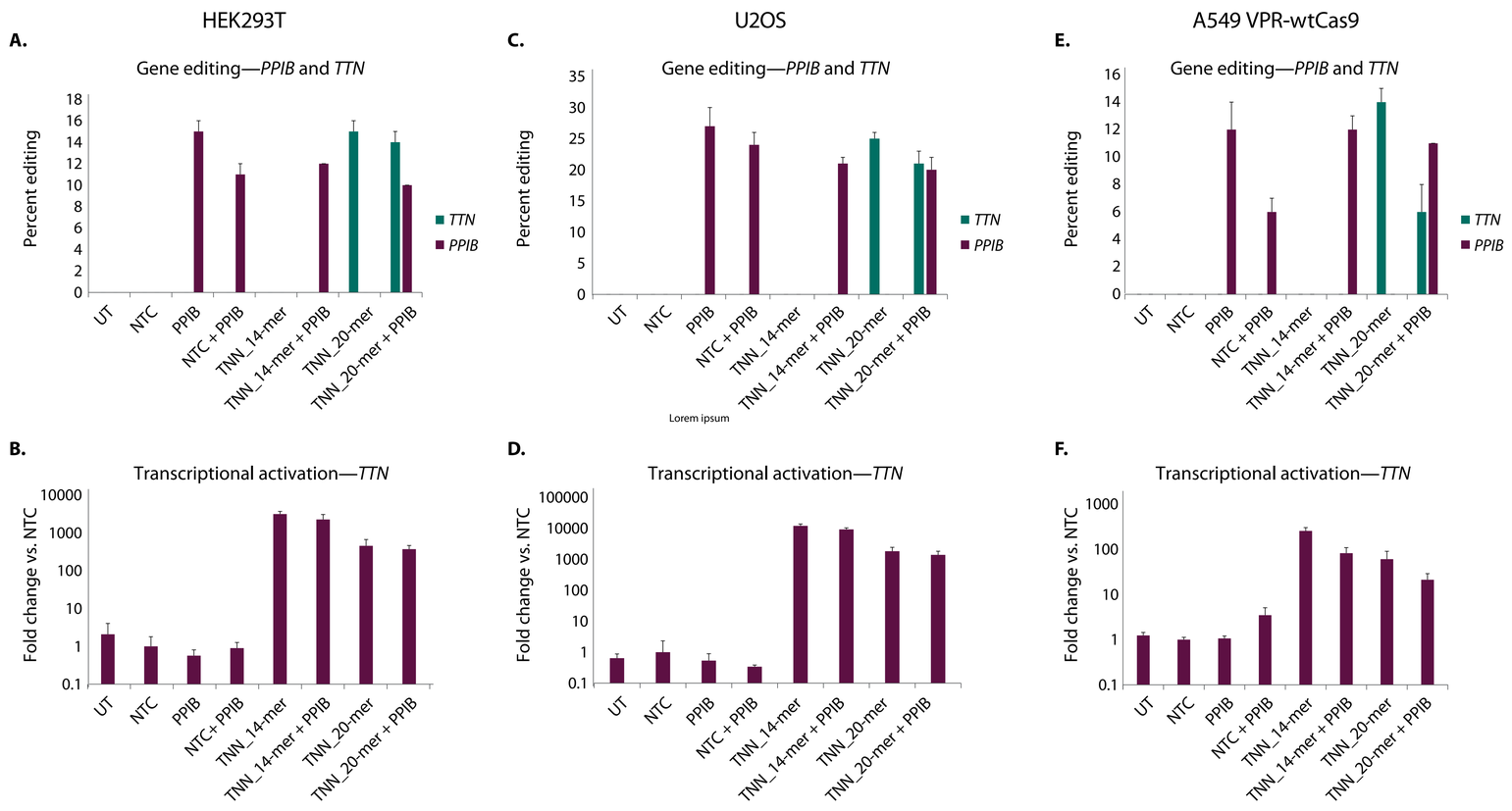
(A–B) U2OS-dCas9-VPR cells were transfected with synthetic guide RNAs with target sequences of variable lengths, and transcriptional activation was assessed at 72 hours by RT-qPCR. (NTC = non-targeting control)

Positioning VPR at the Cas9 N-terminus does not effect Cas9 function



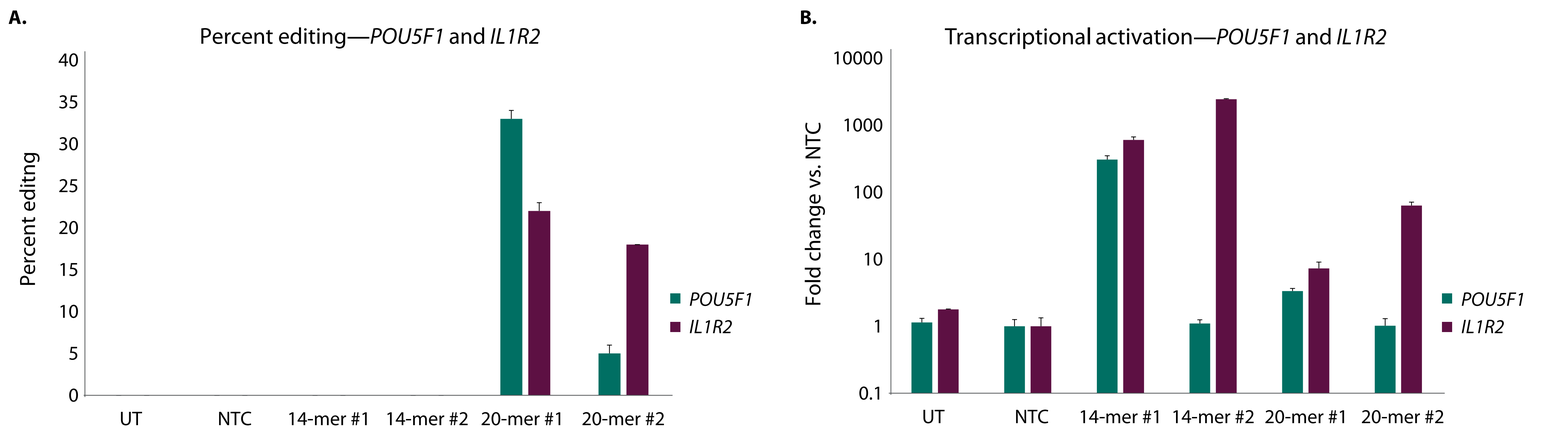
U2OS cells were co-transfected with synthetic 20-mer guide RNAs and mRNA with VPR at the N- or C-terminus of wtCas9 for assessment of **(A)** gene editing (T7EI mismatch detection assay) and **(B)** transcriptional activation (RT-qPCR) 72 hours post-transfection. **(C)** U2OS cells were co-transfected with 14-mer or 20-mer synthetic guide RNAs and VPR-wtCas9 mRNA. Gene editing was determined 72 hours post-transfection via a T7EI mismatch detection assay. (UT = untreated control)

Simultaneous gene editing and transcriptional activation with different gene targets and cell lines



HEK293T **(A–B)**, U2OS **(C–D)**, or A549-VPR-wtCas9 stable cells **(E–F)** were transfected with VPR-wtCas9 mRNA **(A–D)** and were co-transfected with synthetic guide RNAs with variable length target sequences. Cell lysates were generated 72 hours post-transfection. Gene editing was assessed by a mismatch detection assay, T7EI **(A, C, and E)** and transcriptional activation was assessed by RT-qPCR **(B, D, and F)**. (UT = untransfected control, NTC = non-targeting control)

Guide RNAs display a positive association between gene editing performance and transcriptional activation efficiencies



U2OS cells were co-transfected with VPR-wtCas9 mRNA and either *POU5F1* or *IL1R2* CRISPRa algorithm designed synthetic guide RNAs with variable length target sequences Cell lysates were generated 72 hours post-transfection and the amount of either gene editing (by T7EI mismatch detection assay) **(A)** or transcriptional activation (RT-qPCR) **(B)** was determined. (UT = untransfected control, NTC = non-targeting control)

Conclusions

- 14-mer truncated guide RNAs are capable of recruiting VPR-wtCas9 for transcriptional activation.
- N-terminal placement of VPR on Cas9 maintains maximal gene editing function when delivered as mRNA.
- 14-mer guide RNAs do not result in detectable gene editing when delivered with wtCas9 in multiple cell types.
- VPR-Cas9 provides a useful single effector for both gene editing and transcriptional activation that can be used in the interrogation of gene function in different transcriptional cellular contexts.
- Guide RNAs with higher efficiency for gene editing have a higher transcriptional activation potential.