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LETTER TO THE EDITOR

Functional annotation of *cis*-regulatory elements in human cells by dCas9/sgRNA

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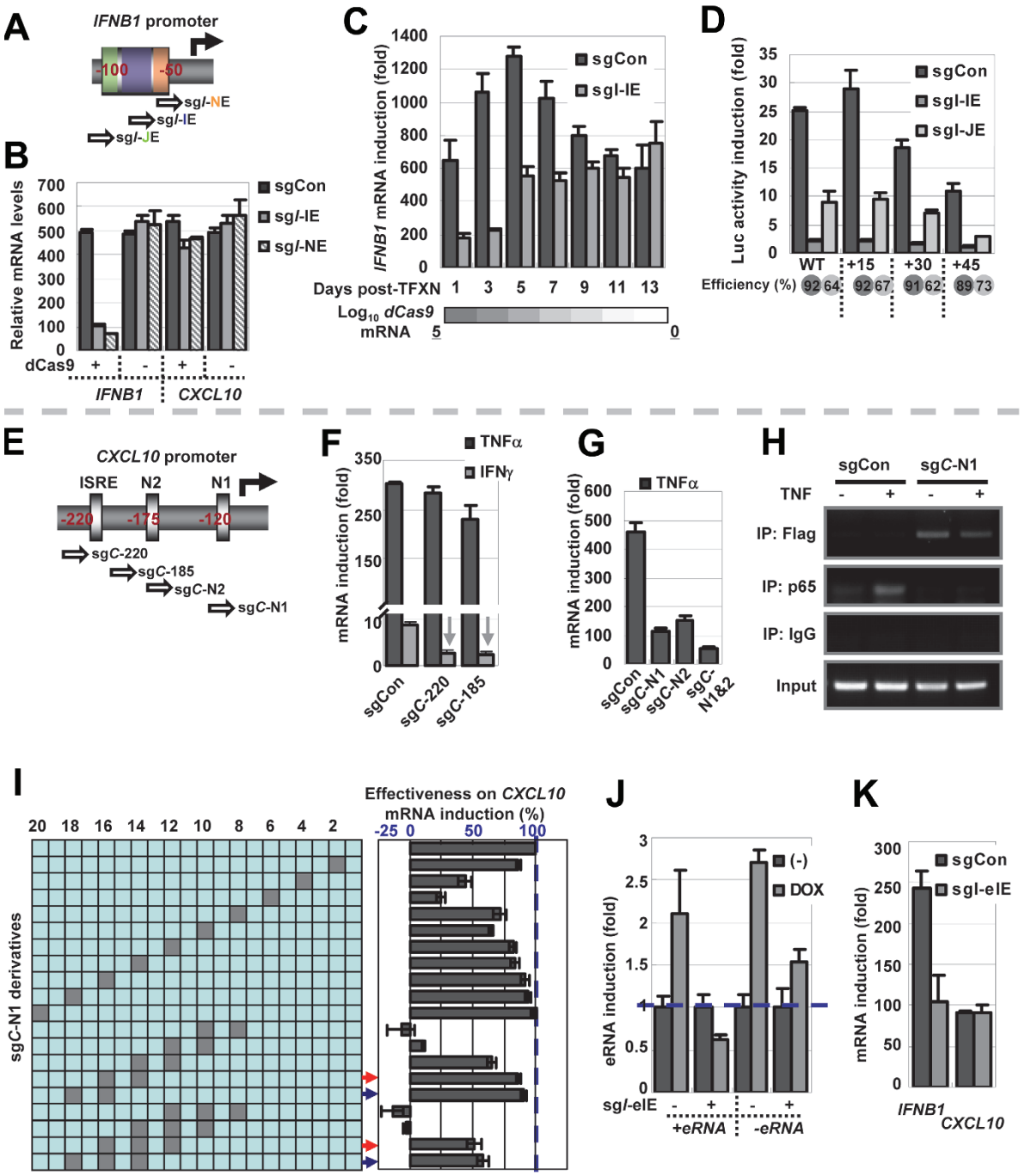
Dear Editor,

It is now believed that the human genome contains a significant portion of regulatory bases, including a great number of transcription factor (TF)-binding *cis*-elements [1], whose functions are largely unexplored due to the lack of convenient experimental methods to model element-specific loss-of-function. Based on recent technological breakthroughs adapting the bacterial type II CRISPR/Cas9 system for RNA-guided gene editing and transcriptional programming [2], we tested the application of the non-cleavage dCas9/sgRNA as a sequence-specific *cis*-element-targeting platform in human cells. Our study established a convenient, efficient and flexible approach that may pave the way for functional annotation of TF-binding *cis*-elements in higher organisms.

The sequence-specific DNA-binding property of dCas9/sgRNA was previously harnessed to map transcriptional *cis*-regulatory elements in *E. coli* and yeast [3, 4]. Although a similar approach in human cells targeting *cis*-elements in the *SV40* viral promoter of an SV-40-GFP reporter was inefficient [4], no vigorous tests against endogenous *cis*-elements have been carried out. For proof-of-principle, we first focused on several *cis*-elements in proximal promoters, which mediate strong transcriptional outputs. Transcriptional activation of *IFNB1* (gene for one of the anti-viral type I IFNs) was used as an initial test model due to the substantial understandings of the underlying mechanisms [5]. Signaling pathways triggered by virus-derived nucleic acid patterns commonly result in concurrent activation of TFs c-Jun/ATF2, IRF3/7 and NF-κB, which are in turn recruited to a tandem of closely situated *cis*-regulatory elements in the proximal promoter of *IFNB1* to cooperatively activate its transcription (Supplementary information, Figure S1A). To mimic the latter process, we introduced a tet-on expression construct for a viral RNA-responsive signaling adaptor, i.e., MAVS [5], into human embryonic kidney 293T cells. Upon deoxycyclin (DOX) administration, *IFNB1* expression was robustly induced in these cells (Supplementary information, Figure S1B and S1C). We next designed sgRNAs largely covering each (or in one case two) of the TF-binding sites within the *IFNB1* promoter (Figure 1A and Supplementary information, Figure S1A). Remarkably, these sgRNAs all effectively inhibited the DOX-induced activities of co-transfected *IFNB1* promoter reporter (Supplementary information, Figure S1D, upper panel). Similar results were obtained when the activity of an integrated *IFNB1* promoter reporter (Supplementary information, Figure S1E) or the endogenous *IFNB1* mRNA level was analyzed (Figure 1B). The inhibition of *IFNB1* mRNA induction by the sgRNAs was dCas9-dependent (Figure 1B) and accompanied by the inhibition of *MX2*, a classic example of IFNβ-stimulated genes (ISGs; Supplementary information, Figure S1F). Consistently, the culture supernatant from the *cis*-element (*IFNB1*)-targeted cells was markedly less active in increasing the protein levels of another ISG, i.e., *STAT1*, in naïve cells (Supplementary information, Figure S1G). Furthermore, the inhibitory effects by the transfected dCas9/sgRNA were dose- and time-dependent (Supplementary information, Figure S1H and Figure 1C), clearly demonstrating a tunable feature. In contrast to the drastic decrease of *IFNB1* expression, the mRNA level of a chemokine gene *CXCL10* was only slightly reduced (Figure 1B and Supplementary information, Figure S1H). Such a minor effect on a non-target gene (*CXCL10*) may possibly be attributed to autocrine regulation by type I IFN (Supplementary information, Figure S1I).

In comparison to previously described sgRNAs that enabled dCas9 to trigger strong interference with transcriptional initiation/elongation (targeting the −50 to +300 bp region relative to the transcription start site (TSS)) [3, 6], some of the sgRNAs described thus far in our study, e.g., sg*I*-IE and sg*I*-JE, were designed against the *IFNB1* promoter sequences upstream of the −50-bp position. Nevertheless, to confirm that sgRNAs could guide inhibition of *IFNB1* transcription independently of general interference, we inserted a 15-, 30- or 45-bp spacer sequence to the 5′ side of the TATA-box in the



IFNB1 luciferase reporter to move the clustered virus-responsive *cis*-elements further upstream (Figure 1D). The results demonstrated that the inclusion of none of the spacers affected the inhibitory efficiency of either sg*I*-IE or sg*I*-JE, strongly supporting that dCas9/sgRNA-mediated *cis*-element-specific blockage underlies the suppression of *IFNB1* transcription.

Since the clustered TF-binding sites within the proximal promoter of *IFNB1* function cooperatively [5], we seek other systems to examine the spatial rule, where the dCas9/sgRNA complex may selectively block a *cis*-element without affecting another one nearby. We constructed an artificial double-*cis*-element reporter with an upstream two-copy NFκB-responsive element (2×NκRE) and a downstream (following a 60-bp spacer) five-copy interferon stimulation-responsive element (5×ISRE [7]), which are respectively targeted by TNFα and IFNα (Supplementary information, Figure S2A). Two sepa-

rate sgRNAs against the 2×NκRE were both effective in abrogating TNFα-induced reporter activity. In contrast, neither of these two sgRNAs affected IFNα-induced reporter activity (5×ISRE-mediated), clearly demonstrating an example of sub-promoter, *cis*-element-level functional selectivity of dCas9/sgRNA (Supplementary information, Figure S2B). Next, to probe the nucleotide-level resolution of dCas9/sgRNA-mediated *cis*-element targeting, we focused on the endogenous promoter of *CXCL10* that harbors an array of single-copy *cis*-elements, including two proximal NκREs (denoted as N1 and N2) and an upstream ISRE, respectively activated by TNFα and IFNγ [8]. We designed several sgRNAs targeting these *cis*-regulatory elements (Figure 1E). Indeed, two sgRNAs closely flanking the *CXCL10* ISRE (sg*C*-220 and sg*C*-185) substantially inhibited IFNγ-induced *CXCL10* expression (Figure 1F). In contrast, neither of these two ISRE-oriented sgRNAs markedly affected TNFα-induced *CXCL10* expression (Figure 1F). Since the base-paired target of sg*C*-185 is only 10 bp away from the N2 element that is TNFα-responsive (Figure 1E), the latter result suggests that the dCas9/sgRNA complex exhibits a rather localized, ~40-nt *cis*-element-targeting resolution (20-nt paired bases plus 10 flanking nucleotides on either side) in live cells. Such results are in general agreement with the spatial details of CRISPR/Cas9 foot-printing data *in vitro* [9].

Since TNFα acts via both of the NκREs within the *CXCL10* promoter [8], we designed two corresponding sgRNAs (sg*C*-N1 and sg*C*-N2, Figure 1E). These two sgRNAs were both effective in inhibiting TNFα-induced *CXCL10* expression (Figure 1G). Additionally, ChIP experiments confirmed the binding of dCas9/sg*C*-N1 to the proximal promoter of *CXCL10*, accompanied by a concomitant loss of TNFα-dependent NFκB recruitment (Figure 1H), providing direct evidence that dCas9/sgRNA inhibits *cis*-element activities via a mechanism of exclusive binding. Furthermore, when sg*C*-N1 and sg*C*-N2 were applied together to simultaneously target both the N1 and N2 elements, an additive inhibitory effect on TNFα-induced *CXCL10* expression was observed (Figure 1G), consistent with the notion that dCas9/sgRNA-mediated targeting of *cis*-elements is multiplexible.

To further assess the fidelity of dCas9/sgRNA-mediated *cis*-element inhibition, we made a series of single-, double- and triple-nucleotide mutations in sg*C*-N1 and analyzed their effects on endogenous *CXCL10* mRNA induction upon TNFα treatment. For this particular target site, single-nucleotide mismatches at most of the positions (except for positions 4 and 6) were tolerated (Figure 1I). In contrast, double- or triple-nucleotide mismatches around the center of the target site abrogated the targeting activities (Figure 1I). Although sg*C*-N1 variants with double or triple mismatches near the 5′ end were still relatively effective, triple mismatches therein were apparently less tolerated than the double mismatches (see paired comparisons indicated by red and blue arrows, Figure 1I). The overall trend was reproduced using similar mutants of sg*I*-IE targeting the *IFNB1* promoter reporter, although in the latter case, a generally more stringent base-pairing requirement was observed (Supplementary information, Figure S2D). Therefore, CRISPR/

Figure 1 A dCas9/sgRNA platform for efficient and flexible targeting of TF-responsive *cis*-elements. **(A)** A schematic display of the proximal promoter of *IFNB1*. The position of the nucleotides in relation to the TSS is presented in red. The sgRNAs (named according to their overlaps with IRF-responsive element, NFκB-responsive element and Jun/ATF2-responsive element as sg*I*-IE, sg*I*-NE and sg*I*-JE, respectively) are presented as small block arrows pointing towards the PAM. **(B)** MAVS tet-on cells were transfected with the indicated sgRNAs with or without dCas9 and subjected to drug selection. Twenty-four hours later, cells were treated with DOX for another 24 h. The cDNAs were analyzed in quadruplicates via qPCR. **(C)** Cells were transfected with sgCon or sg*I*-IE in combination with dCas9, and were drug-selected for the initial 48 h. At the indicated time points following the completion of drug selection, cells were treated with DOX for 24 h before harvest. The folds of *IFNB1* mRNA induction are presented in bar graphs. The relative levels of *dCas9* mRNA (log scale) were also determined and presented in a heat-map below (created by IrfanView). A 10^5-fold decrease of *dCas9* mRNA level can be observed over two weeks. **(D)** A 15-, 30- or 45-bp spacer sequence was placed upstream of the TATA-box in the *IFNB1*-Luc reporter. MAVS tet-on cells were co-transfected with the indicated dCas9/sgRNA and each modified reporter in triplicates. The DOX-inducible reporter activity (24 h) was determined. The inhibitory efficiency (%) by either sgRNA on each reporter was calculated (numbers in shaded circles). **(E)** A schematic display of the endogenous *CXCL10* proximal promoter is presented as in **A**. The two NFκB-responsive elements are marked as N1 and N2, respectively. The sgRNAs were named either according to the overlapped *cis*-elements (i.e., sg*C*-N1 and sg*C*-N2), or to their target positions (220 bp (sg*C*-220) and 185 bp (sg*C*-185) upstream of TSS). **(F**, **G)** Cells were transfected with the indicated dCas9/sgRNA. Following 36 h of drug selection, cells were treated with TNFα or IFNγ for 12 h. The levels of *CXCL10* mRNA were measured. In **F**, notable inhibitory effects were marked with grey arrows. **(H)** ChIP was performed using antibodies against Flag (dCas9) or p65 (NFκB) in dCas9/sgRNA-transfected cells treated with or without TNFα for 3 h. Primers corresponding to the proximal promoter of *CXCL10* were used for PCR analysis. **(I)** Single-, double- or triple-nucleotide mutations were introduced into various positions in sg*C*-N1. Experiments were performed similar to **G**. The inhibitory efficiency by the WT sg*C*-N1 (inhibition of TNFα-induced expression of *CXCL10* mRNA) was set as 100%. Error bars represent normalized STDEV from results of quadruplicated qPCR reactions. **(J**, **K)** MAVS tet-on cells were transfected with sgRNA targeting the *IFNB1* enhancer IRF3 element (sg*I*-eIE) together with dCas9, drug-selected for the initial 48 h and then treated with DOX for 24 h. The levels of indicated eRNAs **(J)** and mRNAs **(K)** were analyzed.

dCas9-mediated non-cleavage *cis*-element targeting is dependent on extensive base matches between the sgRNA and target DNA. Consistently, the *cis*-element-targeting efficiencies of sg*C*-N1 variants when coexpressed with dCas9 largely correlated with their indel-inducing activities when coexpressed with Cas9 (Supplementary information, Figure S2C).

Lastly, we tested the application of the dCas9/sgRNA system on a non-promoter, TF-responsive *cis*-element. In principle, such *cis*-elements are commonly situated within accessible chromatin domains [1] permissive to dCas9/sgRNA binding [10, 11]. It came to our attention that a very recent report identified a novel upstream (~20 kb) enhancer region for human *IFNB1*, where a bi-directional eRNA transcription was also observed following viral stimulation [12]. Interestingly, the enhancer region contains an IRF3-binding site located between the bi-directional eRNAs, potentially serving as a critical element that drives eRNA transcription. To probe the physiological connections among the events of IRF3 binding, eRNA transcription and enhancer activation, we designed dCas9/sgRNA against this novel *IFNB1* enhancer IRF3-responsive element (*I*-eIE) in the MAVS tet-on cells (Supplementary information, Figure S2E). We found that the *I*-eIE-targeting dCas9/sgRNA significantly inhibited DOX-induced expression of both eRNA strands (Figure 1J). Additionally, the same dCas9/sgRNA also partially reduced DOX-induced *IFNB1*, but not *CXCL10* mRNA levels (Figure 1K), confirming the role of this enhancer-born IRF3 *cis*-element in downstream transcription of *IFNB1* [12]. Therefore, our data suggest that the dCas9/sgRNA platform is also suitable for mapping non-promoter, TF-responsive *cis*-elements at their endogenous loci.

In summary, by using a simple non-cleavage dCas9/sgRNA platform with minimal manipulation and optimization, we have consistently (11 out of 11 sgRNAs tested) achieved effective *cis*-element targeting in several promoter/enhancer models in a human cell line. Such efficiencies are remarkable, considering that no additional repressor domains [2] were fused to dCas9 and that the method was designed to directly limit the access of endogenous TFs to the targeted *cis*-elements. The latter attribute translates into a direct, *cis*-element-oriented, loss-of-function perturbation strategy that is indeed suitable for faithful functional annotation of novel genomic loci. Our data further demonstrate that in addition to high resolution and multiplexibility, dCas9/sgRNA-mediated *cis*-element mapping also features good functional fidelity, consistent with a recent report on CRISPRi analyses [6]. Moreover, dCas9/sgRNA-mediated *cis*-element targeting is intrinsically reversible and tunable, owing to the underlying non-cleavage, site-blockage mechanism. Therefore, the present study establishes a useful and flexible method that may be further exploited in relevant cell types and even in animal models to ultimately characterize the large-numbered, function- and disease-associated *cis*-regulatory elements revealed by accumulating human genomics studies [1]. Such a new approach shall make significant contributions to diverse areas of basic and applied research in biology and medicine.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)