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LETTER TO THE EDITOR

Regulation of transcriptionally active genes via the catalytically inactive Cas9 in *C. elegans* and *D. rerio*

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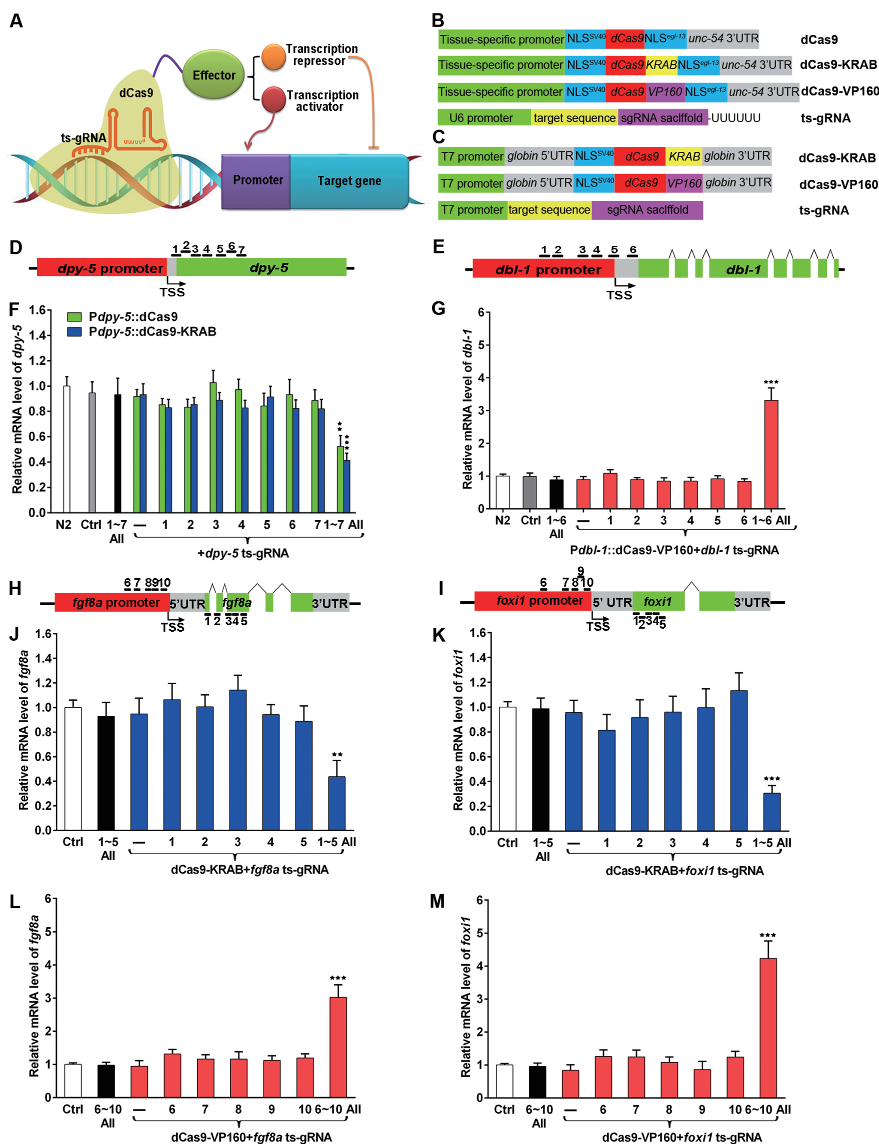
Dear Editor,

CRISPR interference (CRISPRi) is a recently developed tool used to study single guide RNA (gRNA)-mediated sequence-specific repression of transcription in both prokaryotic and eukaryotic cells [1-5]. Transcription initiation and elongation of a gene can be interfered by the presence of gRNA:DNA hetero-duplex/dCas9 (a catalytically inactive form of Cas9) complex in its promoter and exons. If Krüppel associated box (KRAB) domain is fused to dCas9, repression of target gene is more efficient. Likewise, fusion of a transcription activator such as VP16 (CRISPR-on) can increase target gene expression through three to four gene-specific gRNAs (up to seven) that recognize the proximal promoter of a target gene in cultured cells and *in vivo* [2]. We applied both CRISPRi and CRISPR-on tools in worm and zebrafish, and demonstrated here that our dCas9 fusion systems modify gene expression at or near their endogenous expression location(s) through target-specific gRNAs (ts-gRNAs).

We fused dCas9 to the KRAB domain (repressor) or the VP160 domain containing 10 tandem VP16 units (activator; Figure 1A). In *C. elegans*, dCas9 constructs were driven by tissue-specific promoters while ts-gRNAs were under control of a *U6*-type RNA polymerase III promoter (Figure 1B). Different combinations of the DNA constructs were injected into adult hermaphrodite gonads to generate transgenic worms. For zebrafish experiments, dCas9-KRAB and dCas9-VP160 mRNAs and ts-gRNAs were synthesized *in vitro* and injected into one-cell stage embryos (Figure 1C), and the resulted embryos were analyzed by real-time RT-PCR and *in situ* hybridization. Flanking sequences, including the nuclear localization sequences (NLSs) and UTRs, were indicated in Figure 1B and 1C or described previously [6].

Decrease in *dpy-5* expression levels produces short body and dumpy phenotype, known as Dpy. Dbl-1 is a TGF-β family member expressed primarily in neurons and regulates *lon-1* to adjust body length. When *dbl-1* is overexpressed, worm body length is elongated (Lon phenotype). We targeted *dpy-5* by selecting seven ts-gRNAs that recognize its non-template DNA strand of coding region spanning about 400 bp downstream of the transcription start site of *dpy-5* (Figure 1D). Transgenic worms carrying P*dpy-5*::dCas9 or P*dpy-5*::dCas9-KRAB and one of seven *dpy-5*ts-gRNAs did not exhibit obvious changes in *dpy-5* expression and body length (Figure 1F and Supplementary information, Table S1a). When all seven *dpy-5*ts-gRNAs(1-7) were included, suppression of *dpy-5* expression and Dpy phenotype occurred (Figure 1F and Supplementary information, Figure S1aA-S1aC and S1aE). It appeared that P*dpy-5*::dCas9-KRAB and *dpy-5*ts-gRNAs(1-7) combination showed slightly stronger effect on *dpy-5* expression than that of P*dpy-5*::dCas9 and *dpy-5*ts-gRNAs(1-7) combination, although both combinations resulted in the Dpy phenotype (Figure 1F and Supplementary information, Figure S1aA-S1aC, S1aE and Table S1a). We also found that combined with dCas9-KRAB, at least five ts-gRNAs were required to knockdown *dpy-5* (Supplementary information, Figure S1bA and S1bC). These results suggest that recruiting multiple dCas9/*dpy-5*ts-gRNA complexes to exonic region of *dpy-5* may account for a synergistic suppression. To directly visualize tissue-specific gene suppression, we generated P*dpy-30*::NLS^{SV40}-GFP-NLS$^{egl\text{-}13}$; P*dpy-30*::NLS^{SV40}-mCherry-NLS$^{egl\text{-}13}$ transgenic worms expressing GFP and mCherry in all cells (Supplementary information, Figure S1cA-S1cD). When dCas9-KRAB driven by an intestine-specific promoter P*ges-1* and five *gfp*ts-gRNAs driven by P*U6* promoter were introduced into double-transgenic animals, GFP was largely diminished in intestine nuclei while mCherry signal remained in most animals examined (92.7%, $n = 83$; Supplementary information, Figure S1cE-S1cH), suggesting that tissue- and gene-specific knockdown is achievable with our dCas9 fusion system. Using P*dpy-7*::GFP worms that only express GFP in the hypodermis, we found that only dCas9-KRAB driven by the hypodermis-specific *dpy-7* promoter, not that by the intestine-specific P*ges-1*, reduced GFP expression in hypodermal cells (Supplementary information, Figure S1d), indicating that dCas9 functions in a cell-autonomous manner.

To test whether *dbl-1*ts-gRNAs and dCas9-VP160 combination could activate *dbl-1* expression, we gener-



ated transgenic worms carrying dCas9-VP160 and one of six *dbl-1*ts-gRNAs or all of them (Figure 1E). We observed a significant elevation of *dbl-1* expression and the Lon phenotype (Supplementary information, Figure S1aA, S1aD, S1aF and Table S1b) only when dCas9-VP160 and all six *dbl-1*ts-gRNAs (1-6) were present (Figure 1G and Supplementary information, Figure S1bB and S1bD). In addition, using transgenic worms carrying a copy of integrated *pud-2.2*-promoter-driven GFP (P*pud-2.2*::GFP) that expresses GFP strongly and uniformly in the intestine and weakly in the hypodermis, we were able to directly monitor changes of GFP in these two tissues. P*pud-2.2*::GFP worms carrying P*ges-1*::dCas9 or P*ges-1*::dCas9-KRAB and all five *gfp*ts-gRNAs (1-5) showed suppressed *gfp* expression (Supplementary information, Figure S1e). P*pud-2.2*::GFP worms carrying P*ges-1*::dCas9-VP160 and all five *pud-2.2*ts-gRNAs (6-10) that recognize *pud-2.2* promoter showed significantly enhanced GFP expression mainly in the intestine (Supplementary information, Figure S1e and S1fC-S1fH). When hsp-16.2 (a ubiquitously expressed gene) promoter was used to drive dCas9-VP160 in the presence of all five *pud-2.2*ts-gRNAs (6-10) in P*pud-2.2*::GFP worms, an enhanced GFP expression was also mainly observed in the intestine and epidermis upon heat shock (Supplementary information, Figure S1g), suggesting that ts-gRNAs and dCas9-VP160 tend to modify gene expression in place(s) where the gene is transcriptionally active.

In zebrafish, we targeted *fgf8a* and *foxi1*, both of which are required for the induction of the otic placode [7-8]. We designed five *fgf8a*ts-gRNAs and five *foxi1*ts-gRNAs to suppress *fgf8a* and *foxi1* expression, respectively. These

Figure 1 The dCas9 fusion systems can be used to suppress or activate gene expression in both worm and zebrafish embryos. **(A)** dCas9 is fused to an effector and guided by ts-gRNAs to suppress or activate target genes. **(B)** Constructs of dCas9 fusion systems used in *C. elegans*. ts-gRNA is driven by *U6* promoter. All dCas9 constructs contain NLSs flanking dCas9-coding region that is driven by *dpy-5* or *dbl-1* promoter. **(C)** Constructs of dCas9 system used in zebrafish. All dCas9 constructs contain NLSs attached to dCas9-coding region. ts-gRNA and dCas9 mRNA were *in vitro* synthesized by T7 RNA polymerase and the RNAs were injected in zebrafish experiments. **(D)** *dpy-5* locus of *C. elegans*, where TSS (an arrow), *dpy-5* ORF (green bar), *dpy-5* promoter (red bar) and seven *dpy-5*ts-gRNA targeting sites (1-7, short black lines) are indicated. All seven ts-gRNAs target the non-template DNA strand. **(E)** *dbl-1* locus of *C. elegans*. Among the six *dbl-1*ts-gRNAs (1-6, short black lines), ts-gRNAs 1, 2, 3, 4 and 6 target the template strand while ts-gRNA5 targets the non-template strand of *dbl-1*. Spaces in the green bar represent seven *dbl-1* introns. **(F)** qRT-PCR reveals requirement of multiple ts-gRNAs to suppress *dpy-5* expression. N2, wild-type worms (white bar); Ctrl, P*col-10*::mCherry worms (grey bar); 1-7All, *pRF-4*[rol-6(su1006)] worms carrying all seven ts-gRNA plasmids (black bar). The ts-gRNA plasmids expressing none, a single gRNA (one of seven target sequences) or all seven gRNAs (1-7All) were co-expressed with P*dpy-5*::dCas9 or P*dpy-5*::dCas9-KRAB in N2 worms. **(G)** qRT-PCR reveals requirement of multiple ts-gRNAs to activate *dbl-1* expression. 1-6All, *pRF-4*[rol-6(su1006)] worms carrying all six ts-gRNA plasmids (black bar). Different combinations of ts-gRNA and P*dbl-1*::dCas9-VP160 plasmids as indicated were co-expressed in N2 worms. **(H)** The zebrafish *fgf8a* locus. *fgf8a*ts-gRNAs 1-6 target the non-template DNA strand and *fgf8a*ts-gRNA 7-10 targets the template DNA strand. **(I)** The zebrafish *foxi1* locus, *foxi1*ts-gRNAs 1-5, 7 and 8 target the non-template DNA strand while *foxi1*ts-gRNAs 6, 9 and 10 target the template DNA strand. **(J**, **K)** qRT-PCR reveals requirement of multiple ts-gRNAs in suppressing *fgf8a* **(J)** or *foxi1* **(K)** expression. Ctrl, uninjected embryos (white bar); 1-5All, injecting all five ts-gRNAs only (black bar); (−), injecting RNA transcribed from empty gRNA vector (without target sequence) in the presence of dCas9-KRAB mRNA. **(L**, **M)** qRT-PCR reveals requirement of multiple ts-gRNAs in activating *fgf8a* **(J)** or *foxi1* **(K)** expression. 6-10All, injecting all five ts-gRNAs only (black bar); (−), injecting RNA transcribed from empty gRNA vector (without target sequence in the presence of dCas9-VP160 mRNA. For worm experiments **(F**, **G)**, total RNA was isolated from synchronized young adult transgenic worms of each group and pooled together for qRT-PCR analysis. For zebrafish experiments **(J**-**M)**, RNAs are injected into wild-type zebrafish embryos at the 1-cell stage. A total of 30 embryos for each group were collected at 11 hpf and their total RNAs were isolated and pooled together for qRT-PCR analysis. In all qRT-PCR experiments, data shown represent means ± SEM of three independent experiments normalized to wild-type/control animals (N2 in **F** and **G**; uninjected embryos in **J**-**M**). $^{**}P < 0.01$; $^{***}P < 0.001$ (unpaired Student's *t*-test).

ts-gRNAs recognize the non-template strand of *fgf8a* or *foxi1* coding region (Figure 1H and 1I). When all five *fgf8a*ts-gRNAs were co-injected with dCas9-KRAB mRNA into one-cell stage wild-type fertilized eggs, the resulted embryos showed reduced *fgf8a* expression at 11 hpf (Figure 1J and Supplementary information, Figure S1hC) and smaller otic vesicle at 32 hpf (Supplementary information, Figure S1iA-S1iB). Similarly, we observed that *foxi1* expression and otic vesicle size were reduced when dCas9-KRAB mRNA and all five *foxi1*ts-gRNAs (1-5) were injected (Figure 1K, Supplementary information, Figure S1hD, S1iA and S1iC). If dCas9-VP160 mRNA and *fgf8a*ts-gRNAs (6-10) or *foxi1*ts-gRNAs (6-10) were co-injected, an elevated expression of *fgf8a* or *foxi1* was observed (Figure 1L and 1M and Supplementary information, Figure S1hE and S1hF), leading to enlarged otic vesicles (Supplementary information, Figure S1iD-S1iF), and enhanced *fgf8a* or *foxi1* expression appeared to be near their endogenous expression locations (Supplementary information, Figure S1j). We also detected slightly changed expression of *pea3* and *pax8*, downstream genes of Fgf8a and Foxi1, respectively, by *in situ* hybridization (Supplementary information, Figure S1k). Thus, our dCas9 fusion system works in both worm and zebrafish to adjust endogenous gene expression in a tissue-specific fashion. Presumably, the gene specificity is determined by an array of ts-gRNAs spreading across a short range at the target gene locus.

Zebrafish and *C. elegans* are well-established model animals with many loss-of-function and gain-of-function tools to regulate gene expression. To compare the efficiency of our dCas9 fusion systems with that of other widely used tools, we used *dpy-5* RNAi to knockdown *dpy-5* expression, and found that the loss-of-function efficiency was higher in *dpy-5* RNAi worms than that of worms expressing P*dpy-5*::dCas9/dCas9-KRAB and *dpy-5*ts-gRNAs (1-7; Supplementary information, Figure S1lA). This result was further confirmed by measuring worm body lengths (Supplementary information, Figure S1lC and Table S1c). We also measured *dbl-1* expression levels and body lengths of worms overexpressing dCas9-VP160 and *dbl-1*ts-gRNAs (1-6), and worms expressing P*dbl-1*::Dbl-1. Although *dbl-1* expression level was higher in P*dbl-1*::Dbl-1 transgenic worms than that of worms expressing dCas9-VP160 and *dbl-1*ts-gRNAs (1-6), the body lengths were comparable (Supplementary information, Figure S1lB and S1lD). In zebrafish experiments, *pea3* and *pax8* expression levels in embryos co-expressing *fgf8a* or *foxi1* were comparable to those injected with dCas9-VP160 mRNA and ts-gRNAs (Supplementary information, Figure S1m). Injecting mRNA directly into zebrafish fertilized eggs swamped all embryonic cells with abundant *fgf8a* or *foxi1* mRNA (data not shown). However, in embryos injected with dCas9-VP160 mRNA and ts-gRNAs, *fgf8a* or *foxi1* mRNA was only observed in and near the original expression places (Supplementary information, Figure S1j). It therefore appeared that enhanced *pea3* or *pax8* expression in latter experiment groups might be more physiologically relevant (due to the on-site overexpression of *fgf8a* or *foxi1*) than that of

conventional mRNA injection. On the other hand, the reduction of *pea3* and *pax8* expression level was moderate, compared to that of using *fgf8a* or *foxi1* morpholino antisense oligo (MO; Supplementary information, Figure S1m). However, MO-mediated knockdown has been frequently criticized to cause somewhat too severe phenotypes that could not match the corresponding mutants due to its toxicity, off-target effects, or some unknown reasons [9]. Therefore, the modulation of endogenous gene expression by our dCas9 fusion systems may be used to reveal *in vivo* gene functions more appropriately.

In cultured cells, transcriptionally quiescent genes could be activated by dCas9-effector and their ts-gRNAs, contrary to our results. We believe that different genome modification(s) in culture cells, i.e., epigenetic states (open chromatin state, CpG methylation, and so on), may account for the discrepancy [10-11]. The strand-specific repression by ts-gRNAs of a gene was explained to be the consequence of steric inhibition of RNA polymerase II activity in prokaryotes [5]. At a transcriptionally active gene locus, the transcriptional machinery constantly unwinds the promoter and proximal exons to recurrently produce transcripts. The active state of a gene thus likely provides more chances for dCas9/ts-gRNAs to gain access to their target DNA sequences. In fact, using multiple ts-gRNAs to adjust the endogenous gene expression level or to conduct large-scale genetic screens in mammalian cells was also thought to better reduce the off-target effects [2, 4, 10, 12-14]. However, different from previous findings that Cas9 and a single ts-gRNA are enough to induce target gene knockdown in many cases [5], we found that an individual ts-gRNA and dCas9 or its derivatives could barely change target gene expression, which may potentially compromise the simple/easy application of our fusion systems. Nevertheless, with multiple dCas9-gRNA:DNA complexes formed simultaneously in a close range on target gene locus, the possible instability of a single dCas9/ts-gRNA:DNA complex is no longer a limiting factor.

Unlike worm experiments, in which DNA constructs of dCas9 or its derivatives and multiple ts-gRNAs (Supplementary information, Table S1d) were injected, we used *in vitro*-synthesized RNAs to modulate the expression of target genes in zebrafish (Supplementary information, Figure S1h and Table S1e). Due to the stability issue of synthetic RNAs *in vivo*, injected RNAs may at best modulate actively expressed target genes during early embryogenesis. The transient suppression or activation of target genes therefore led to a mild morphological phenotype(s) (Supplementary information, Figure S1i). To achieve long-lasting and spatiotemporally controllable effects, transgenic fish carrying ubiquitous and/or inducible dCas9-KRAB, dCas9-VP160 and multiple gene-specific ts-gRNAs are desirable. To avoid injecting too many ts-gRNA-encoding constructs at a time, a plasmid containing tandem ts-gRNA sequences that could automatically release individual ts-gRNAs when transcribed *in vivo* would be an ideal option [15]. Despite the lack of yet-to-be-developed amendment, we have demonstrated that our dCas9 fusion systems are a noteworthy alternative to modulate endogenous gene expression in whole organisms. In theory, ts-gRNA-guided dCas9 fusion proteins can be used to target and perturb noncoding elements such as enhancers, silencers, insulators, introns, and so on, and with libraries of ts-gRNAs that cover whole genomes, high-throughput analyses to interrogate gene functions and genetic networks will be readily conducted in both animals.

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References

1 Bikard D, Jiang W, Samai P, *et al*. *Nucleic Acids Res* 2013; **41**:7429-7437.
2 Cheng AW, Wang H, Yang H, *et al*. *Cell Res* 2013; **23**:1163-1171.
3 Gilbert LA, Larson MH, Morsut L, *et al*. *Cell* 2013; **154**:442-451.
4 Perez-Pinera P, Kocak DD, Vockley CM, *et al*. *Nat Methods* 2013; **10**:973-976.
5 Qi LS, Larson MH, Gilbert LA, *et al*. *Cell* 2013; **152**:1173-1183.
6 Liu P, Long L, Xiong K, *et al*. *Cell Res* 2014; **24**:886-889.
7 Phillips BT, Bolding K, Riley BB. *Dev Biol* 2001; **235**:351-365.
8 Nissen RM, Yan J, Amsterdam A, *et al*. *Development* 2003; **130**:2543-2554.
9 Schulte-Merker S, Stainier DYR. *Development* 2014; **141**:3103-3104.
10 Kuscu C, Arslan S, Singh R, *et al*. *Nat Biotechnol* 2014; **32**:677-683.
11 Wu X, Scott DA, Kriz AJ, *et al*. *Nat Biotechnol* 2014; **32**:670-676.
12 Koike-Yusa H, Li Y, Tan EP, *et al*. *Nat Biotechnol* 2014; **32**:267–273.
13 Mali P, Esvelt KM, Church GM. *Nat Methods* 2013; **10**:957-963.
14 Wang T, Wei JJ, Sabatini DM, *et al*. *Science* 2014; **343**:80–84.
15 Nissim L, Perli SD, Fridkin A, *et al*. *Mol Cell* 2014; **54**:698-710.

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