Competitive dCas9 binding as a mechanism for transcriptional control

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Abstract

Catalytically dead Cas9 (dCas9) is a programmable transcription factor that can be targeted to promoters through the design of small guide RNAs (sgRNAs), where it can function as an activator or repressor. Natural promoters use overlapping binding sites as a mechanism for signal integration, where the binding of one can block, displace, or augment the activity of the other. Here, we implemented this strategy in Escherichia coli using pairs of sgRNAs designed to repress and then derepress transcription through competitive binding. When designed to target a promoter, this led to 27-fold repression and complete derepression. This system was also capable of ratiometric input comparison over two orders of magnitude. Additionally, we used this mechanism for promoter sequence-independent control by adopting it for elongation control, achieving 8-fold repression and 4-fold derepression. This work demonstrates a new genetic control mechanism that could be used to build analog circuit or implement cis-regulatory logic on CRISPRi-targeted native genes.

Keywords analog circuit; CRISPRi; ratio sensing; synthetic biology

Subject Categories Biotechnology & Synthetic Biology; Chromatin, Transcription & Genomics

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Introduction

Regulatory networks integrate environmental and cellular signals to ensure genes are expressed under the correct conditions. Integration can occur at individual promoters through the arrangement of DNA operators to which regulatory proteins bind and either recruit or interfere with transcriptional machinery (van Hijum et al., 2009; Weingarten-Gabbay & Segal, 2014). Within a promoter, the binding of one regulator can also affect the binding of others through positive or negative interactions, for example, through DNA looping or overlapping operators. This collectively generates “cis-regulatory logic”, which dictates the conditions for gene expression based on the combination of regulators that are active (Shen-Ort et al., 2002; Shen-Ort et al., 2002; Aerts et al., 2003; Buchler et al., 2003; Hermsen et al., 2006; Mayo et al., 2006; Kaplan et al., 2008; Gertz et al., 2009; van Hijum et al., 2009; Schulthess et al., 2015). For synthetic genetic circuits, the placement of multiple operators within a promoter has been used to implement combinatorial logic, for example, to create an X AND (NOT Y) gate for an edge detector by placing an operator for an activator (X) and repressor (Y) within a promoter (Cox et al., 2007; Murphy et al., 2007; Ellis et al., 2009; Tabor et al., 2009; Sharon et al., 2012; Bacchus et al., 2013; Mogno et al., 2013; Perez-Pinera et al., 2013; Rantasalo et al., 2018; Monteiro et al., 2020; Yu et al., 2021). As a design principle, combining operators is useful for “compressing” large logic operations to reduce the resource burden (Rondon et al., 2019; Groseclose et al., 2020). It is challenging to insert an operator into a promoter because the change in sequence can affect the promoter strength, an effect that can be mitigated with insulators and computational predictions (Brewster et al., 2012; Stanton et al., 2014; Zong et al., 2017, 2018; Hoque et al., 2021; preprint: Poole et al., 2021). Prokaryotic promoters are small, thus making it difficult to fit multiple operators and it is difficult or impossible to design operators that bind to multiple regulatory proteins.

dCas9 can be programmed to bind to different DNA sequences by changing the targeting sequence of its bound sgRNA (Bikard et al., 2013; Esvelt et al., 2013; Qi et al., 2013; Jiang & Doudna, 2017). DNA binding requires 5–10 bp sgRNA-DNA complementarity adjacent to an NGG PAM sequence (Boyle et al., 2017), although dCas9 can be engineered to reduce the PAM sequence requirements (Klein-stiver et al., 2015; Hu et al., 2018; Nishimasu et al., 2018; Walton et al., 2020; Collias & Beisel, 2021). When bound to DNA, dCas9 covers 30 bp and melts the strands to form a bubble that results in very slow unbinding rates (Sternberg et al., 2014; Jones et al., 2017). Thus, it can function as a repressor by sterically blocking the binding of RNA polymerase (RNAP) to a promoter or by blocking its progression through a gene (Qi et al., 2013). When targeting constitutive E. coli σ70 promoters, repression is strongest when the sgRNA is targeted within the −10 to −35 promoter core and does not depend on which strand is targeted (Bikard et al., 2013; Qi et al., 2013; Nielsen & Voigt, 2014). Targeting dCas9 downstream of a promoter represses transcriptional elongation, with much stronger repression observed when the sgRNA is targeted to the non-template strand (Bikard et al., 2013; Qi et al., 2013). dCas9 can also serve as an activator by fusing it to an activating domain that recruits RNAP (Bikard et al., 2013; Zalatan et al., 2015; Dong et al., 2018; Ho et al., 2020; Kiattisewee et al., 2021). There are often tight
spacing requirements for activation, particularly for prokaryotic promoters. Lastly, dCas9 can also be directed to bind to the operators of protein repressors, activators, or enhancers, thus blocking their impact on expression (preprint: Shur & Murray, 2017; Shariati et al., 2019; Liu et al., 2021).

An advantage of using dCas9 is that its regulatory effect can be directed to a promoter without having to insert an operator sequence. This has been used to control the regulation of native genes, for example, controlling enzymes at branch points to redirect flux through a metabolic pathway (Kim et al., 2016; Moser et al., 2018; Tian et al., 2019; Hawkins et al., 2020; Wu et al., 2020b; van Gestel et al., 2021). It also simplifies the integration of multiple signals at a single promoter by designing sgRNAs that target it to different positions. For instance, NOR gates have been constructed using two sgRNAs that target different positions in the output promoter, either of which leads to repression (Lebar & Jerala, 2016; Gander et al., 2017). When multiple sgRNAs target overlapping regions, this leads to mutually exclusive binding (Qi et al., 2013). Tan and co-workers harnessed this effect to control the strength and noise of an E. coli constitutive promoter by co-transcribing different ratios of two sgRNAs that direct dCas9 (fused to an activating domain) to overlapping positions that either recruit or block RNAP (Wu et al, 2020a).

Once expressed, the regulatory effects of dCas9 only end when the protein or sgRNA degrade or are diluted by cell division. Several approaches have been taken to control the activity of either dCas9 or the sgRNAs after they are expressed. One is to express anti-CRISPR proteins derived from phage genomes that bind to and inactivate dCas9 (Bubeck et al., 2018; Nakamura et al., 2019). This leads to the complete inactivation of dCas9, thus eliminating its ability to implement any sgRNA-mediated regulation in the cell. Different sets of genes can be controlled by expressing orthogonal dCas9 variants, each of which binds a different set of sgRNAs (Gao et al, 2016; Kim et al, 2019). These can be changed dynamically from being repressors to activators by expressing the corresponding domains as separate proteins that bind to dCas9 using modular protein–protein interaction domains (Gao et al, 2016). Another approach is to design RNA to bind to and augment the activity of a specific sgRNA. Antisense RNAs will inactivate sgRNAs by targeting them for degradation via the native bacterial Hfq system (Lee et al., 2016). The sgRNA can also be designed to fold into an inactive hairpin, thus requiring the co-expression of toehold RNAs to unfold and bind to dCas9 (Oesinghaus & Simmel, 2019; Siu & Chen, 2019; Hochrein et al., 2021). Both of these techniques require modifying the sgRNA to have additional sequences such that it can be bound by the modulating RNA.

While there are many natural examples of different repressors binding to the same operator or a repressor displacing an activator, to our knowledge, there are no examples of a repressor’s action being negated by a second protein binding to an overlapping operator. To this end, we have developed a mechanism for sgRNA-specific derepression through competitive dCas9 binding to overlapping regions. Transcription is blocked when a repressing sgRNA (sgR) directs dCas9 to the first position and a second derepressing sgRNA (sgD) directs dCas9 to a mutually exclusive second position that does not impact transcription (Fig 1A). We find that the two regions are competitive so long as they are within 14 bp and the PAM sequences are between the target sites. The repression/derepression switch is implemented in two ways (Fig 1B). The first is to design the sgR to overlap the −35 σ70-binding region of promoter and the sgD to bind just upstream. The second approach is to design repression/derepression sites within a gene by exploiting the strand dependence of RNAP elongation inhibition by dCas9. These results demonstrate a new mechanism to control the activity of dCas9-directed regulation that could be used for efficient genetic circuit design to integrate signals in a promoter or to derepress subsets of native genes to subregulate genome-encoded functions.

Results

Promoter control through repression/derepression

dCas9 can repress a promoter by sterically blocking the binding of RNAP (Figs 1B and 2A) (Bikard et al., 2013; Qi et al., 2013). An E. coli constitutive promoter can be repressed by targeting the repressing sgRNA (sgR) to overlap the −10 or −35 σ70-binding sites (Bikard et al., 2013; Qi et al., 2013; Nielsen & Voigt, 2014). Our design for derepression is based on using a second sgRNA (sgD) to recruit dCas9 to an upstream site that blocks its ability to bind to the repressing position. Importantly, sgD cannot interfere with RNAP binding or else it will also lead to repression. To quantify the

![Figure 1. The mechanisms of dCas9 repression and derepression.](image-url)
impact of dCas9:sgRNA binding on mRNA transcription rates, we defined a parameter $\alpha$ as the ratio of maximal mRNA production rates (completely unbound DNA) to the production rate when all DNA is bound by dCas9:sgRNA (saturated DNA). The ideal location to target a repression/derepression sgRNA pair would correspond to a large $\alpha$ for sgR ($\alpha >> 1$) and $\alpha \rightarrow 1$ for sgD, indicating strong repression by sgR and no repression by sgD.

A system was designed to evaluate how targeting sgR and sgD to different positions in the promoter impacts the effectiveness of repression and derepression. Two inducible promoters were used to independently transcribe sgR (IPTG-inducible Ptet) and sgD (Vanilllic acid-inducible Pvan) encoded on a p15A plasmid. The targeted constitutive promoter (PC1) was built based on the −35 to +1 core of Pcaten (Zhang & Voigt, 2018) to which a randomly generated 100-bp sequence was added upstream (Materials and Methods). Positions in PC1 were selected to be targeted for sgR/sgD by exploiting NGG PAM sites in the promoter sequence (Fig 2B). The sgR and sgD sequences were designed based on the same scaffold (Zhang & Voigt, 2018), with mutations the 20-bp spacer sequence corresponding to the target region of the promoter (Appendix Table S2). To measure promoter activity, PC1 was placed upstream of a ribozyme (Lou et al, 2012) and gene encoding red fluorescent protein (RFP). From the same plasmid, dCas9 was expressed using the aTc-inducible Ptet promoter. A concentration of 1.25 nM aTc was used for all experiments and leads to approximately 500 dCas9 molecules per cell during exponential growth (Zhang & Voigt, 2018).

Experiments were performed to measure the promoter activity that results when dCas9 is targeted to different positions. The plasmids were transformed into E. coli and cultures were grown in defined media (EZ Rich) and induced with aTc and either IPTG or Vanilllic acid (Van) for 5.5 h (Materials and Methods). Fluorescence was then measured using flow cytometry. First, we compared the repression obtained by targeting the −10 (sgRP1) or −35 (sgRP2) positions of the promoter (Fig 2B). Upon maximum sgR expression (1 mM IPTG), both sgRs are able to repress the promoter by ~30-fold (Fig 2C). This result is consistent with previously observed fold-repressions at these locations (Nielsen & Voigt, 2014; Zhang & Voigt, 2018).

A model was derived to capture the repression of a promoter by dCas9:sgR,

$$ P + S_R K_R \rightleftharpoons P_R $$

(1)

where $P$ and $P_R$ are the concentrations of promoters in the unbound and bound state, $S_R$ is the concentration of dCas9:sgR, and $K_R$ is the association constant. The production of mRNA transcripts $m$ from the promoter is described by

$$ \frac{dm}{dt} = \beta_m \left( \frac{1 + \alpha^{-1} K_R S_R}{1 + K_R S_R} \right) + \beta - \delta_m m, $$

(2)

where $\beta_m$ is the maximum transcription rate, $\beta$ is the leaky transcription rate, and $\delta_m$ is the degradation rate. Solving for steady-state yields

$$ m = \left( \frac{\beta_m}{\delta_m} \right) \frac{1 + \alpha^{-1} K_R S_R}{1 + K_R S_R} + \frac{\beta}{\delta_m}. $$

(3)

This equation can be further converted to the activity of the output promoter PC1,

$$ y = (y_{\text{max}} - y_{\text{min}}) \left( \frac{1 + \alpha^{-1} K_R S_R}{1 + K_R S_R} \right) + y_{\text{min}} $$

(4)

where $y$ is in arbitrary units (au) of RFP fluorescence and $y_{\text{max}}/y_{\text{min}}$ are the maximum/minimum measured values. $K_R$ is the strength of the promoter driving the expression of sgR, measured as au of RFP fluorescence and $K_R$ is rescaled to be in the same units. This assumes that the binding of sgR to dCas9 to form $S_R$ is in the linear (unsaturated) regime. Equation 4 was fit to the sgRP1 and sgRP2 induction curves (Fig 2C), and the parameters were extracted (Table 1).

We then designed experiments to determine the constraints of targeting dCas9:sgD to the promoter without evoking repression (Fig 2D). Seven positions were selected between −28 and −66 regions of the promoter, including orientations that target both strands (Fig 2B). The promoter activity was measured for each position when sgD is expressed (100 µM Van) and unexpressed (0 µM Van), and these data were used to estimate $\alpha$ as the ratio of these values (Fig 2D and E). We observed that $\alpha$ continuously decreases as the sgD targets regions farther from the promoter core, indicating weaker repressive abilities (Fig 2E). When sgD targets regions upstream of −60, negligible repression was observed.

It has been previously shown that dCas9:sgRNAs will compete for binding when their target regions overlap (Qi et al, 2013; Wu et al, 2020a). However, the distance constraints we measured showed that it would not be possible to obtain repression/derepression by targeting overlapping regions. Even if we used the upper-bound on TSS distance for the repressing sgRNA (30 bp) and the lower-bound on distance for the derepressing sgRNA (60 bp), the sgRNA target sequences would still have to be 10 bp away from each other. We hypothesized that while we could not utilize sgRNA target overlap, we may still be able to harness steric hindrance effects from overlap of the dCas9 protein footprint on DNA. To investigate this, we examined a crystal structure of the dCas9:sgRNA-DNA complex (Nishimasu et al, 2014), which shows that dCas9 has an overhang of ~9 bp from the PAM-proximal binding side, but only a ~1 bp overhang on the PAM-distal side of the sgRNA target. This indicated that we might be able to obtain competitive dCas9 binding without overlapping the sgRNA binding regions if we oriented two sgRNAs with their PAM sequences facing each other.

To test this hypothesis, we redesigned the system to express sgRP2 from a choline (Chol)-inducible promoter and sgDP2 from a Van-inducible promoter (Fig 2F). These promoters were chosen because they are not predicted to append disruptive 5’ sequences onto the sgRNAs (Qi et al, 2013; Meyer et al, 2019). In accordance with the crystal structure estimation of dCas9’s DNA footprint, we designed sgDP5 to be 14 bp from sgRP2. To bind sgDP5 at this distance, we had to introduce a 3-bp mutation from −60 to −62 of PC1, to create a PAM site for sgDP5, resulting in PC2. We screened for derepression by fully inducing sgRP2 with 9 mM Chol and titrating the expression of sgDP5 from 0 to 100 µM Van. Derepression occurred in a graded manner as more sgRNA is expressed, ultimately returning the promoter activity to its unpressed state (Fig 2G).

We then determined the promoter response when different amounts of sgR and sgD are expressed. This response can be viewed as a cis-regulatory logic operation (Mayo et al, 2006), where the
Figure 2.
signals from these regulators are integrated by the promoter. To obtain this function, 60 combinations of sgR<sub>p2</sub> and sgD<sub>p5</sub> induction levels were measured. These data are shown in Fig 2H as circles, where their colors are the output values of the circuit and each data point is positioned at the respective promoter activity values for sgR and sgD (error bars for each measurement are provided in Appendix Fig S4). Output from the promoter increased with sgD induction and decreased with sgR expression. It has been shown that the co-expression of multiple sgRNAs titrates a shared dCas9 resource away from the co-expressed sgRNAs (Zhang & Voigt, 2018; Huang et al., 2021). This could complicate the interpretation of the derepression data, where the expression of sgD could deplete the dCas9 pool, thus indirectly reducing the concentration of dCas9:sgR in the cell. To test for this effect, we performed a control experiment where we maximally expressed a sgR and then titrated in either a non-targeting sgRNA (sgN) (does not bind to the promoter or genome) or an off-target sgRNA (sgO) (binds to an inert region of the plasmid) (Appendix Fig S2). Neither of these sgRNAs showed derepression, indicating that the derepression we observed was not due to dCas9 titration.

Our model was then expanded to include derepression. Equation 1 can be modified to include the competitive reaction of dCas9:sgD (S<sub>D</sub>) binding to the promoter to form P<sub>D</sub>,

$$P_D + S_R \overset{K_D}{\rightarrow} P + S_R + S_D \overset{K_D}{\rightarrow} P + S_D$$

where K<sub>D</sub> is the association constant between S<sub>D</sub> and P. The production of mRNA transcripts m from the promoter is described by

$$\frac{dm}{dt} = \beta_m \frac{1 + \alpha_R^{-1}K_RS_R + \alpha_D^{-1}K_DS_D}{1 + K_RS_R + K_DS_D} + \beta - \delta_mm$$

### Table 1. Model parameters.

| Mechanism    | sgRNAs<sup>a</sup> | Parameters | \(Y_{\text{min}}\) | \(Y_{\text{max}}\) | \(K_S\) | \(K_D\) | \(\alpha_R\) | \(\alpha_D\) |
|--------------|---------------------|------------|----------------|----------------|--------|--------|-------------|-------------|
| Promoter     | sgR<sub>k1</sub>    | 1.2        | 7,700          | 0.10           |        |        |             |             |
| Promoter     | sgR<sub>p2</sub>    | 16         | 6,800          | 0.043          |        |        |             |             |
| Promoter     | sgR<sub>p2</sub> + sgD<sub>p5</sub> | 16        | 6,800          | 0.043          | 0.063  | 120    |             |             |
| Elongation   | sgR<sub>p2</sub>    | 37         | 4,200          | 0.019          |        |        |             |             |
| Elongation   | sgR<sub>p2</sub> + sgD<sub>p5</sub> | 37        | 4,200          | 0.019          | 0.0077 | 27     |             |             |

<sup>a</sup> Single sgRNA experiments were fit to Equation 4 and dual-sgRNA experiments were fit to Equation 8.
where $\beta_{\text{RO}}/\alpha_R$ and $\beta_{\text{DO}}/\alpha_D$ are the maximum transcription rates when either $S_R$ or $S_D$ are bound to the promoter. Solving for steady-state yields

$$m = \left(\frac{\beta_m}{\delta_m}\right) \frac{1 + \frac{1}{\kappa_R} K_R S_R + \frac{1}{\kappa_D} K_D S_D}{1 + \kappa_R S_R + K_D S_D} + \frac{\beta}{\delta_m} \tag{7}$$

As described for Equation 4, this can be further converted to the activity of the output promoter $P_{CG}$,

$$y = \left(y_{\text{max}} - y_{\text{min}}\right) \frac{1 + \frac{1}{\kappa_R} K_R S_R + \frac{1}{\kappa_D} K_D S_D}{1 + \kappa_R S_R + K_D S_D} + y_{\text{min}} \tag{8}$$

This equation was then fit to the 2-dimensional response of the promoter to the expression of sgR and sgD (Fig 2H). This fit was performed while keeping the parameters previously fit to Equation 4 for sgR$_2$ ($\kappa_R$, $\alpha_R$, $y_{\text{min}}$, and $y_{\text{max}}$) constant. The fit is shown as the heatmap coloration in Fig 2H, the parameters for which are provided in Table 1. The newly fit association constant $\kappa_D$ for sgD is similar to $\kappa_R$ indicating that sgR$_2$ and sgD$_{ps}$ have similar apparent binding strengths. Additionally, the observed $\alpha_D$ of 1 is consistent with sgD$_{ps}$ having little repressive effect on its own.

**Dynamics of derepression**

Qi et al (2013) showed that, in exponentially growing E. coli, the dynamics of sgRNA-based repression of RFP was identical to the cellular growth rate. This is consistent with complete repression of protein expression and first-order degradation of the protein due to dilution by cell division (Del Vecchio & Murray, 2015; Potvin-Trottier et al, 2016). However, when the authors removed the repressing sgRNA, RFP fluorescence increased with a doubling time slower than the cellular growth rate.

We monitored the dynamics of repression and derepression for the promoter repression/derepression sgRNA pair sgR$_2$ + sgD$_{ps}$ over the course of 8 h (Materials and Methods). Starting with uninduced cells in exponential phase, we diluted cells into repressing conditions (10 mM Chol, 1.25 nM aTc). Consistent with previous work, RFP fluorescence reduced at the rate of cellular division (Fig 3). After 6 h, RFP expression was 100-fold lower than the initial uninduced condition. We then washed the cells and diluted them into fresh media inducing only the derepressing sgRNA, sgD$_{ps}$ (1.25 nM aTc and 100 µM Van). This led to 47-fold derepression within 2 h.

**Control of transcriptional elongation through repression/derepression**

dCas9 can block transcription when it is directed to bind internally to a gene by physically interfering with the progression of RNAP (Fig 4A). However, being located in a gene complicates the design of a derepressing sgRNA position, which must bind to disrupt the repressing sgRNA without itself blocking elongation. To this end, we exploited the strand dependence of dCas9-based elongation repression, where it has been found that RNAP elongation is more likely to terminate when it collides with the PAM-proximal side of a dCas9-DNA complex (Bikard et al, 2013; Qi et al, 2013; Vigouroux et al, 2018). Similar to the promoter repression/derepression mechanism, these constraints on sgR and sgD repressive effects can be captured empirically with the parameter $\alpha$, which is the maximum fold-repression of mRNA production rates under saturating conditions.

A genetic system was constructed to test this design. dCas9 produces stronger repression when it is directed to the 5’-end of the gene (Bikard et al, 2013; Qi et al, 2013), so we selected a position at +232 to be targeted by a repressing sgRNA (sgRO$_1$) (Fig 4C). This location was chosen because it has two adjacent PAM sites that could be used for targeting sgD, one of which has the same 14 bp spacing as was found to be optimal when derepressing a promoter (sgDO$_2$). sgRO$_1$ was placed under the control of a Chol-inducible $P_{\text{Tac}}$ promoter. It has been observed that elongation repression can be weaker with stronger promoters, presumably due to dislodgement of dCas9 by RNAP (Vigouroux et al, 2018). To evaluate this effect, we used an IPTG-inducible promoter ($P_{\text{Rf}}$) to drive the transcription of the rfp reporter. When the promoter is strong (1 mM IPTG), we observed an 8-fold repression of RFP upon the maximum induction of sgRO$_1$ (10 mM Chol) (Fig 4D). While we initially developed the models in Equations 4 and 9 for the promoter repression/derepression mechanism, these models can be generalized to the elongation repression/derepression mechanism if the promoter states $P$, $P_{\text{Rf}}$, and $P_{\text{Tac}}$ are instead considered to be general DNA states. Therefore, the data for sgRO$_1$ were fit to Equation 4 (Table 1). The $\alpha$ for sgRO$_1$ is 4-fold lower than that from promoter repression (Table 1), indicating a limitation of elongation repressive control at this location. The dependence of repression on the $P_{\text{Tac}}$ activity was then measured (Fig 4E). Repression by sgRO$_1$ initially increased with promoter strength, but then levels off at 30-fold repression (Fig 4E). Previous work showed that elongation repression is promoter-strength invariant for saturating levels of dCas9 and for strong promoters (Vigouroux et al, 2018).

Two sgDs were designed to target positions up- (sgDO$_2$) and down-stream (sgDO$_3$) of the sgRO$_1$ position (Fig 4C). These were...
placed under the control of a Van-inducible promoter, as before. We initially attempted to calculate $\alpha$ for these derepressing sgRNAs; however, induction of sgDO2 resulted in higher, rather than lower, RFP expression. This effect was likely due to leaky sgRO1 expression of sgRO1 from PBetI which is derepressed when sgDO2 is induced (Fig 4F). Nonetheless, we tested the derepression capabilities of sgDO1 and sgDO2 (Fig 4G). To do this, sgRO1 was maximally expressed (10 mM Chol) with the strongest induction of the rfp reporter (1 mM IPTG). Maximum expression of sgDO2 (100 $\mu$M Van) showed 4-fold derepression (Fig 4G). This derepression was confirmed to not be a
The interaction between RNAP and dCas9:sgR and dCas9:sgD is shown. Transcription is "ON" when full-length transcripts are produced. The dashed lines indicate non-ideal repression by sgD.

When dCas9 is bound to the non-template strand, the PAM is proximal to RNAP, and dCas9 tends to stay bound after collision. When dCas9 is bound to the template strand, the PAM is distal to RNAP collision and RNAP can continue to elongate.

Repressing and derepressing sgRNA binding sites tested relative to rfp.

Repression of transcriptional elongation by sgR. The circuit is encoded on plasmid pDAA056, which also includes sgD (uninduced in these experiments). The curve was fit to Equation 4, yielding the parameters shown in Table 1. The x-axis is converted from the concentration of inducer (Chol) to promoter activity as described in the Materials and Methods. Inducer concentrations: 0, 9.77, 19.5, 39.1, 78.1, 156, 313, 625, 1250, 2500, 5000, 10000 µM Chol.

The dependence of sgR repression and sgD derepression on the strength of the promoter controlling the repressed gene (P

A

The regulatory logic for the co-expression of sgR and sgD was then determined (Fig 4H). Similar to promoter repression/derepression, the elongation mechanism exhibited increased expression with sgD induction and decreasing expression with sgR induction (error bars for each measurement are provided in Appendix Fig S4). Equation 8 was then fit to the 60-point sampling of the system, with xE corresponding to P

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activation and xD corresponding to P

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activation values. The fit parameters imply that the derepressing sgRNA association constant κD is 2.5-fold lower than the repressing sgRNA association constant κE (Table 1).

The dependence of derepression on the P

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promoter strength was then measured by varying IPTG (Fig 4E). Similar to the promoter strength test for relative repression, we normalized the fluorescence to that of the P

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only plasmid (pDAA040). For each level of P

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induction, the relative expression during derepression was calculated as the ratio of the RFP fluorescence from the P

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only plasmid to the RFP fluorescence from the circuit plasmid with maximal sgR01 and sgD02 induction (10 mM Chol and 100 µM Van). From this experiment, we observed that derepression is invariant over a wider range of transcription rates than that observed for repression.

Ratiometric performance of the repression/derepression

Ratiometric signal processing describes a circuit that responds to the relative value of two inputs, as opposed to their absolute magnitude. Naturally occurring ratiometric responses have been observed in ATP/ADP management (Atkinson, 1968), X versus autosomal chromosome levels (Madd & Herman, 1979), circadian clock determination (Li et al, 2016), cancer cell clinical resistance (Raisova et al, 2001), and sugar source utilization in yeast (Escarleante-Chong et al, 2015). To examine the ratiometric performance of our repression/derepression mechanisms, we looked at the circuit outputs relative to the ratio of promoter activities producing sgD and sgR (P

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/ P

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). From this, we observed that the promoter repression/derepression circuit effectively responded to over 2 orders of magnitude of sgD/sgR ratios with a 50-fold dynamic range (Fig 5A). In contrast, the elongation mechanism had a smaller overall dynamic range (10-fold) and the circuit did not perform predictably across all ratios (Fig 5B).

Discussion

This work introduces a new mode of regulatory control, where the binding of a repressor is displaced by the binding of a nearby derepressor. This motif, where both repressor and derepressor bind to the same operator, has not been observed in natural systems. This may be due to the difficulty of designing a common operator that binds to multiple proteins and a derepressing protein that blocks the repressor, but not RNAP. The programmability of CRISPRi makes the design of multiple sgRNAs binding nearby sites almost trivial and the size of dCas9, and its melting of DNA, simplifies its use to sterically inhibit the binding or elongation of RNAP. Note that the off-rate of dCas9 binding DNA is very slow, effectively irreversible, until it is displaced by DNAP or RNAP (Sternberg et al, 2014; Jones et al, 2017; Vigouroux et al, 2018). In our repression/derepression mechanism, this makes it unlikely that dCas9:sgD can displace dCas9:sgR when it is already bound. Rather, it depends on which binds first, with DNAP replication (and to a lesser-extent RNAP derepression).
elongation) “resetting” the DNA state at the rate of plasmid replication (cellular growth rate) and RNAP elongation (promoter strength) (Jones et al., 2017; Vigouroux et al., 2018). Thus, the kinetics of the system reaching steady state are expected to be dependent on growth phase and may impact their use in circuits with components with faster dynamics (Mishra et al., 2014; Takahashi et al., 2015; Ali Al-Radhawi et al., 2019; Westbrook et al., 2019).

Repression/derepression could be applied to design synthetic circuits that perform new signal integration functions. As simple cis-regulatory logic, the operation performed by these circuits is A NIMPLY B where sgD (B) overrides the regulation imposed by sgR (A) (Figs 2G and 3G). If instead the displaced regulator were an activator (based on dCas9 recruiting RNAP (Dong et al., 2018; Fontana et al., 2020a; Ho et al., 2020; Villegas Kcam et al., 2021)), this logic would be A NIMPLY B.

However, this digital combinatorial logic does not capture the potential for new signal integration performed by these mechanisms. The signal integration follows a graded, rather than switch-like transition (Figs 2H and 3H), and this can be used to build useful analog functions. The circuit based on the repression/derepression of a promoter (sgR2 and sgD5) is capable of responding to transcriptional input ratios over two orders of magnitude (Fig 5A). Ratiometric signal processing is common in natural regulatory systems and is usually achieved by a motif where two species compete for a third component and only one of the bound species results in the output (Atkinson, 1968; Madl & Herman, 1979; Raisova et al., 2001; Berg et al., 2009; Daniel et al., 2013; Escalante-Chong et al., 2015; Li et al., 2016; Antebi et al., 2017; Cherry & Qian, 2018; Lopez et al., 2018; Liu et al., 2019). For example, this competition can occur at a DNA binding site where an activating transcription factor competes with an inactive or repressive transcription factor (Perli & Lu, 2017; Zeng et al., 2019). Ratiometric circuits can be used to determine which of two continuously variable signals is larger. The sgR2/sgD5 circuit in log-space is performing a (sgD – sgR) calculation, which if connected to a cooperative switch-like output could act as a single neuron in a neural network (Li et al., 2021).

Another consideration is the transfer of this repression/derepression mechanism for transcriptional control in organisms other than E. coli. Repression of transcriptional elongation via dCas9 binding has been shown to work in a variety of different bacterial species (Peters et al., 2016, 2019; Rock et al., 2017), so we expect the elongation repression/derepression mechanism to be applicable to other species. Transferring this mechanism to eukaryotes is more difficult because dCas9 is only capable of modest elongation repression in eukaryotic systems (Gilbert et al., 2013; Qi et al., 2013). Repression in eukaryotic systems is often accomplished through the fusion of chromatin-remodeling proteins to dCas9, which are capable of robust and reversible repression of transcription (Gilbert et al., 2013, 2014; Zalatan et al., 2015; Mandegar et al., 2016). Derepressing these systems could be accomplished by targeting a dCas9 lacking the fused repression domains to the same DNA location. This approach would require two different dCas9 species, which could be accomplished using dCas9 orthologs (Esvelt et al., 2013; Kim et al., 2019; Gasiunas et al., 2020) or by encoding repression domain recruitment within the sgRNA scaffold (Zalatan et al., 2015).

Various approaches have been developed to place native genes under synthetic regulatory control through the insertion of synthetic
promoters (e.g., that respond to T7 RNAP) or other regulatory motifs (Warner et al., 2010; Wang et al., 2012; Na et al., 2013). CRISPRi has been rapidly adopted because it can exert regulatory control without having to mutate the target; however, it only imparts a single on/off signal. To this end, depression/derepression can be used to inactivate the effects of CRISPRi in the control of native genes (Nielsen & Voigt, 2014; Weinberg et al., 2017; Moser et al., 2018; Henningsen et al., 2020). For example, CRISPRi/a has been used to dynamically repress or activate enzymes to control carbon flux in metabolic engineering applications (Moser et al., 2018; Peng et al., 2018; Lu et al., 2019; Tian et al., 2019; Fontana et al., 2020b). Derepression allows for the disruption of a subset of the genes being influenced by an activating or repressing sgRNA; in effect, this would introduce cis-regulatory logic into the native genes without needing to insert operators. In this work, we had to deal with the challenge of identifying or designing PAM sites so that the sgk and sgD binding sites are appropriately positioned to satisfy the distance constraints. This restricts their utility in the control of genome-encoded genes, where the likelihood of PAM sites being appropriately positioned in a promoter is small or restricts where the regulation can occur within a gene. This is likely why the effect that we observed for the blockage and release of elongation is small. However, recent efforts to engineer dCas9 to not require a PAM site have made progress (Kleinstiver et al., 2015; Hu et al., 2018; Nishimasu et al., 2018; Walton et al., 2020; Collias & Beisel, 2021). Collectively, these advances will allow the programming of cis-regulatory logic to be applied to any gene in the genome without having to insert or modify genomic DNA.

Materials and Methods

Strains, plasmids, media, and chemicals

*Escherichia coli* NEB 10-beta (C30191, New England BioLabs, Ipswich, MA, USA) was used for all routine cloning. All genetic circuit measurements were done using *E. coli* K-12 MG1655 * [F- λ- ilvG- rfb-50 rph-1 Δ araCBAD Δ(lacI)] (Blattner et al., 1997; Nielsen & Voigt, 2014). Cells were grown in in MOPS EZ Rich Defined Medium (Teknova, M2105) with 0.2% glucose (Teknova, G0520). Kanamycin (50 µg/ml, GoldBio, K-120-5) was used to maintain plasmids. Chemiluminescent inducers used the following: vanillic acid (Van) (Millipore Sigma, 94770); isopropyl β-d-1-thiogalactopyranoside (IPTG) (GoldBio, I2481C); anhydrotetracycline (aTc) (Millipore Sigma, 37919); and choline chloride (Chol) (Millipore Sigma, C7017). DNA oligos and genes were ordered from Integrated DNA Technologies (Coralville, IA) and Twist Biosciences (San Francisco, California). All plasmids were constructed from the parental pDAA038 backbone (Appendix Table S3) using TypeIIS assembly to insert circuit components between BsaI sites. A table of genetic parts and full plasmid sequences are provided in Appendix Table S2 and S3. Key plasmid maps are shown in Appendix Fig S5.

Computational methods

The random 100-bp sequence within P_Ct was generated using the online Random DNA Sequence Generator (http://www.faculty.ucr.edu/~mmaduro/random.htm) with the GC content set to 50%. Non-linear fitting was completed with the Python scipy.optimize.curve_fit() function.

Induction assays

All growth was performed in 96-well V-bottom plates (Roskilde, Denmark, #249952) at 1,000 rpm in a microplate shaker (ELMI, #DTS-4). The day before, individual colonies were inoculated into 150 µl EZ Rich media and Kan for overnight (16 h) growth at 37°C. The next day, cultures were diluted 200-fold by adding 0.75 µl of overnight culture into 150 µl of EZ Rich media and Kan. After 2-h growth, cultures were diluted 1,000-fold into inducing conditions by adding 6 µl of culture into 198 µl fresh media and Kan and then 5 µl of that dilution into media with inducers and Kan. To induce dCas9, aTc was added to a final concentration of 1.25 nM in all experiments. Growth was performed for 5.5 h, after which samples were prepared for flow cytometry.

Flow cytometry analysis

Fluorescence characterization was performed using a BD LSR Fortessa flow cytometer with the HTS attachment (BD, Franklin Lakes, NJ). Samples were prepared by aliquoting 40 µl of cell culture into 160 µl of PBS containing 200 µg/ml Kan. All samples were run in standard mode at a flow rate of 2 µl/s. RFP fluorescence measurements were made using the green (561 nm) laser, and all data were derived from the PE-Texas Red-A channel (PMT voltage of 700 V). The FSC and SSC voltages were 650 and 270 V, respectively. At least 10,000 events were collected for each sample, and the Cytoflow Python package (https://github.com/cytobio/cytoflow) was used for analysis, including gating. The geometric mean fluorescence is calculated for all cytometry distributions.

Promoter input calculations

The following procedure was followed to convert inducer concentrations (e.g., [IPTG]) to the activities of the inducible promoter (e.g., P_Tac) reported in RFP fluorescence (au). This approach has been described previously (Nielsen et al., 2016; Zhang & Voigt, 2018). Using a separate plasmid based on pDAA038, the response function of the inducible system was measured separately by measuring RFP fluorescence using cytometry. The RFP fluorescence values in au for the inducer concentrations were then plotted as “Promoter Activity”. The response functions used are shown in Appendix Fig S1.

Repression/derepression dynamics

All growth was performed at 37°C and 350 rpm in an Innova44 (Eppendorf, NY, USA) with a 1-inch throw. The day before the experiment, a starter culture was initiated by inoculating a colony into 2 ml EZ-rich media with Kan in a 15-ml test tube. The next day, this culture was diluted to an OD600 of 0.025 into 40 ml of fresh EZ-rich media with Kan. This was allowed to grow for 2 h in a 250-ml Erlenmeyer flask to bring the cells to OD600 = 0.5. At this point, a time point was taken for t = 0 and cells were diluted to OD600 = 0.00035 in an Erlenmeyer flask using fresh media with 10 mM Choline and 1.25 mM aTc. After 2 h of growth, 30-min time points were taken. At each time point, 1 ml of culture was removed, spun
down (1 min at 16,000 g), and then resuspended in 300 µl of PBS and 2 mg/ml Kan and analyzed by cytometry. To transfer into derepressing conditions, 28 ml of culture was washed twice by centrifugation at 12°C at 4,300 g for 5 min. Then, the cultures were diluted to OD600 = 0.035 in 40 ml of media with 100 µM Van, 10 mM Choline, and 1.25 nM aTc.

Data availability

This study includes no data deposited in external repositories.

Expanded View for this article is available online.

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Author contributions

DAA and CAV conceived the study and designed the experiments. DAA performed the experiments and analyzed the data. DAA and CAV wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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