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Orthogonal modular gene repression in E. coli using engineered CRISPR/Cas9

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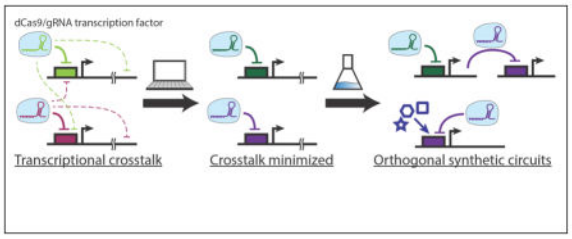
Abstract

The progress in development of synthetic gene circuits has been hindered by the limited repertoire of available transcription factors. Recently, it has been greatly expanded using the CRISPR/Cas9 system. However, this system is limited by its imperfect DNA sequence specificity, leading to potential crosstalk with host genome or circuit components. Furthermore, CRISPR/Cas9-mediated gene regulation is context dependent, affecting the modularity of Cas9 based transcription factors.

In this paper we address the problems of specificity and modularity by developing a computational approach for selecting Cas9/gRNA transcription factor/promoter pairs that are maximally orthogonal to each other as well as to the host genome and synthetic circuit components. We validate the method by designing and experimentally testing four orthogonal promoter/repressor pairs in the context of a strong promoter P_L from phage lambda. We demonstrate that these promoters can be interfaced by constructing a double and a triple inverter circuits. To address the problem of modularity we propose and experimentally validate a scheme to predictably incorporate orthogonal CRISPR/Cas9 regulation into a large class of natural promoters.

Graphical Abstract



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Supporting information

Supporting-information.pdf: Figures S1–S3, Table S1; SI-sequences.zip: plasmid maps and the list of pre-screened sequences; Compute-algorithm-code.tgz: Computer programs to generate and screen orthogonal gRNA sequences.

Keywords

CRISPR/Cas9; orthogonal repressors; synthetic gene circuit engineering

1 Introduction

Recent advances in synthetic biology have led to development of a number of gene circuits with applications in medical diagnostics, disease treatment, and bioproduction (1–4). Despite a number of successes, the complexity of synthetic circuits has been limited by the availability of a large repertoire of transcription factors required to implement and control complex cellular behavior (5, 6). To enable robust rational design of such circuits they should be built from modular orthogonal components. This ensures that the circuit parts can be predictably and independently connected, exhibiting minimal crosstalk with each other as well as with the circuits of the host cell (7).

Recently it was demonstrated that CRISPR/Cas9 system can be used to engineer synthetic transcription factors that target any sequence of interest on DNA (8–12). This system consists of Cas9 protein and a short chimeric RNA called guide RNA (gRNA). The gRNA determines the DNA binding specificity of Cas9/gRNA ribonucleoprotein complex by direct complementarity of a 20-nucleotide guide sequence in gRNA with the target DNA (9). Both repressors and activators of transcription in various organisms have been constructed using a mutant of Cas9 protein lacking its wild-type nuclease activity (dCas9) (10). In prokaryotes repressors of transcription have been constructed by guiding dCas9/gRNA complexes to interfere with transcription initiation or elongation (10, 12). In eukaryotes both transcriptional repressors and activators have been implemented by guiding dCas9 fusions with corresponding transcriptional repressor or activator domains to the promoters of interest (13–15).

DNA binding site sequence orthogonality is one of the major requirements in design of libraries of transcription factors. However, CRISPR/Cas9 system has a noticeable limitation in this respect. dCas9/gRNA binding can tolerate multiple mismatches between the 20-mer guide sequence of gRNA and the target DNA binding site with the change in the binding efficiency dependent on the position of the mismatch (9, 10, 12, 16). Ideally, one would like to design a collection of gRNA sequences that guide dCas9 only to their respective target DNA binding sites while at the same time minimizing undesired mutual crosstalk as well as off-target interactions with the host and circuit DNA (17, 18). Specifically, off-target binding sites on the genome may sequester dCas9/gRNA complexes or even disrupt gene activity required for proper functioning of the host (12). Minimal off-target interaction with the circuit components is similarly important, and several algorithms have been proposed to achieve this (16, 18, 19). A systematic approach to satisfy these two requirements is especially important when designing large libraries of orthogonal circuit components where off-target effects may otherwise happen by chance.

To be effective, synthetic genetic circuits should be modular, i.e. the parts that constitute them should work predictably and interchangeably independent of their context. However, CRISPR/Cas9-mediated gene regulation strongly depends on its binding position within the

target gene (10, 12). Therefore, CRISPR/Cas9 binding sites should be carefully chosen and consistently positioned within the target gene. Furthermore, to predictably integrate the native regulation of the target gene with the orthogonal regulation by CRISPR/Cas9, the location of dCas9/gRNA binding sites needs to be chosen in such a way that it does not interfere with binding of the native transcription factors yet allows strong regulation by CRISPR/Cas9.

In this paper we describe an approach to construct modular and orthogonal CRISPR/Cas9 regulatable promoters based on both constitutive as well as natively regulatable promoters in *E. coli*. First, to design a set of gRNA sequences that have minimal off-target binding to the host genome as well as many popular plasmid backbones, we develop a computer algorithm that generates gRNA sequences taking into account DNA binding sequence specificity of dCas9/gRNA complex. Second, we experimentally validate the performance of the method by constructing and testing four orthogonally repressible promoters based on a strong promoter P_L from phage lambda (20). We demonstrate that these orthogonal promoter-repressor pairs can be interfaced to form more complex circuits by constructing and testing a double and a triple inverters. Finally, we describe a modular design of inducible hybrid promoters in *E. coli* which are capable of being simultaneously coregulated by their native transcription factors and orthogonal dCas9/gRNA repressors. We validate this approach by constructing and testing three hybrid promoters based on widely used inducible promoters $P_{LlacO-1}$, P_{BAD}, and P_{luxI} (21–23).

2 Results

We conducted our initial computational design and experimental testing of orthogonal CRISPR/Cas9 repressors in the context of a promoter P_L from phage lambda – a strong well-characterized promoter widely used in synthetic biology (Figure 1A). This promoter is constitutively active in the absence of lambda repressor protein. We chose to install dCas9/gRNA DNA-binding sequence just upstream of the −35 box of the promoter (Figure 1A). Sequence changes in this position are less likely to disturb the unrepressed promoter activity even though dCas9/gRNA repressor binding in this position may result in weaker repression compared to binding between the −35 and −10 promoter boxes or just downstream of the −10 box (10, 24).

To design orthogonal CRISPR/Cas9 repressors that are strongly specific to their DNA target we created a computer algorithm described briefly below (for the details see Methods). The method consists in selection of guide gRNA sequences maximally orthogonal to the host and circuit DNA, followed by selection of the most mutually orthogonal dCas9/gRNA transcription factor/promoter pairs. Specifically, we start with generation of a random library of 20-nucleotide guide gRNA sequences. Sequences containing GG and CC dinucleotides are excluded, since these dinucleotides constitute functional protospacer adjacent motif (PAM) sites of *S. pyogenes* Cas9 (see below). Next, a stringent selection of the generated gRNA sequences is performed based on their similarity to the DNA sequence of the host genome and the sequences of the plasmids that encode the synthetic circuit. The target DNA binding site of *S. pyogenes* Cas9/gRNA complex is determined by direct complementarity with a 20-nucleotide long guide sequence at 5′ terminus of the gRNA followed by 5′-NGG

trinucleotide protospacer adjacent motif (PAM). The complementarity of a 10–12 nt "seed" sequence at the 3′ end of the 20 nt gRNA guide sequence with the target DNA is particularly important for the specificity of DNA recognition by Cas9/gRNA complex (9, 25–27). Correspondingly, the 8–10 nucleotides at the 5′ of the gRNA guide sequence are significantly less sensitive to the mismatches with the target DNA. Specifically, up to 8 mismatches at the 5′ end of the guide RNA can be tolerated by CRISPR/Cas9 repressors in *E. coli* (12). In our initial experiments, we found that two mismatches between the "seed" gRNA sequence and the target DNA site could be tolerated in the context of P_L promoter, while four mismatches almost completely disrupted dCas9/gRNA binding to this DNA site (Figure S1 of the Supporting Information). Therefore, in order to capture the position-dependent mismatch sensitivity of dCas9/gRNA DNA binding, we characterize its affinity to each potential binding site using a coarse-grained weighted Hamming distance where the mismatches within the "seed" sequence are added with the constant weight of 1, while the mismatches in the 5′ gRNA sequence are added with a progressively lower weight with increasing distance from the "seed" sequence (see Methods and Figure S2 of the Supporting Information).

Based on the mismatch sensitivity data discussed above we select the candidate gRNA guide sequences with a minimum weighted Hamming distance of at least 5 for every possible position in *E. coli* MG1655 genome (Figure 1B). Due to the large size of the genome, this selection results in only a small fraction of sequences being retained (approximately $7{\cdot}10^4$ out of $1.4{\cdot}10^{10}$). The gRNA guide sequences screened in this step were chosen from a similar selection step with a minimum weighted Hamming distance of at least 6.3 against the sequences of the circuit plasmids. At this step we also screened the gRNA sequences against other synthetic biology parts using the same score of 6.3, so that these parts can be incorporated into future circuits with the designed dCas9/gRNA repressors. In particular, the candidate guide gRNA sequences were screened against plasmid replication origins (p15A, ColE1, CloDF13, RSF1030), antibiotic resistance genes (coding resistance to ampicillin, kanamycin, gentamicin, zeocin, and chloramphenicol), promoters ($P_{LlacO\text{-}1}$, $P_{LtetO\text{-}1}$, P_{lacIQ}), protein coding genes (superfolder GFP, mCherry, maltose binding protein, glutathione S-transferase, and TEV protease) (see Supporting Information for the list of sequences). The minimum score of 6.3 was chosen in this step to retain roughly 10% of sequences after the screen. Finally, we applied the most stringent selection against the sequence of P_L promoter and the sequences in its immediate vicinity. Only the candidate gRNA guide sequences with minimum weighted Hamming distance of 9 were chosen (about 9% of the screened sequences). As a result 633 gRNA guide sequences satisfying all the above criteria were selected. These sequences were then sorted by their mutual orthogonality (see Methods). The mismatch scores of the 20 best sequences are shown in Figure 1C,D.

Four best sequences were selected for experimental validation (Figure 1E and Table S1 of the Supporting Information). gRNAs were expressed under control of a strong anhydrotetracycline (aTc) inducible promoter $P_{LtetO\text{-}1}$ (21). dCas9 was produced as an N-terminal maltose binding protein fusion (MBP-dCas9), which was cleaved off *in situ* by co-expressed tobacco etch virus (TEV) protease (28). Both dCas9 and TEV protease were expressed from a strong isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible promoter $P_{LlacO\text{-}1}$. Simultaneous induction of dCas9, TEV protease, and gRNA with both inducers

results in repression of the respective target promoter (Figure S3 of the Supporting Information). For each promoter/repressor pair the repression range was defined as a ratio of sfGFP reporter (29) fluorescence with the corresponding gRNA not induced or fully induced by aTc. All four synthetic promoters could be repressed to similar extent (about 14 fold) by their corresponding gRNAs and showed no apparent crosstalk between different gRNA/promoter pairs (Figure 1E).

We combined two and three of the designed orthogonal promoter/repressor pairs to form double and triple inverter circuits, respectively (Figure 2). Here, the aTc inducible input promoter $P_{LtetO-1}$ produces the first gRNA in the cascade, which in turn represses the promoter producing the next orthogonal gRNA and so on. The output promoter produces sfGFP as described above. In both cases we measured the ratio of sfGFP fluorescence of the last promoter in the cascade in fully aTc induced versus uninduced state (referred to as induction range below). As a control we used a circuit with the output promoter designed to be orthogonal to all the gRNAs within the circuits. Both double and triple inverters were functional with the corresponding induction range of approximately 9 and 5, while the controls essentially did not respond to aTc, as expected.

It is important to be able to predictably integrate functionality of natural regulatable promoters with orthogonal CRISPR/Cas9 control. A large number of prokaryotic promoters have their native regulatory sequences upstream of −35 box. Therefore the upstream position of the Cas9/gRNA binding site in the promoters described above may be limiting. We explored the possibility of installing Cas9/gRNA binding site just downstream of the transcription start site (Figure 3A). Since the sequence of the first few nucleotides downstream of the transcription start site of a promoter may have an effect on the transcription rate, we chose to keep the sequence at the positions +1 ..+3 unchanged with respect to the wild-type P_L promoter. Since the sense strand nucleotide at +3 position of the P_L promoter is a cytosine and therefore can be a part of a functional PAM site on the antisense strand, we installed Cas9/gRNA binding site flanked with the PAM sites at positions +3 .. +28 of P_L promoter driving expression of superfolder GFP (Figure 3A). As a result, in this system dCas9/gRNA was able to repress a P_L promoter using gRNA both complementary to the sense and antisense DNA strands in this position (Figure 3B).

In order to interface CRISPR/Cas9 circuits based on promoters using this design they should be able to transcribe functional gRNA. gRNAs expressed from the promoters described above will have 28 nucleotides added 5′ to their DNA targeting sequence. It has been shown previously that gRNAs can tolerate extra 5′ sequences which are not complementary to the target DNA (10). We tested whether such gRNAs can efficiently guide dCas9 to the targeted DNA in the context of the promoters described here. We found that adding 28 nucleotides to the 5′ end of a gRNA lowers dCas9/gRNA repression activity by approximately 1.3 fold (Figure 3C). As a result, the hybrid promoters described here can be layered to construct more complex circuits.

To demonstrate that this approach can be straightforwardly applied to add orthogonal dCas9/gRNA repression functionality to native regulatable promoters we designed and tested hybrid $P_{LlacO-1}$, P_{BAD}, and P_{luxI} promoters (Figure 4A). As before, we kept the +1 .. +3

sequence of the native promoters unchanged. Since the nucleotide at the +3 position of both $P_{LlacO-1}$ and P_{BAD} promoters is a cytosine we were able to test repression of the corresponding hybrid promoters using gRNA complementary to both sense and antisense strands of the installed dCas9/gRNA binding site. However, since the native P_{luxI} promoter has an adenine at the +3 position, we only tested repression of the hybrid P_{luxI} promoter using gRNA complementary to the antisense DNA strand. As in the previous case, gRNA was produced from an aTc inducible $P_{LtetO-1}$ promoter, however MBP-dCas9 and TEV protease were constitutively expressed from P_L promoter. The three hybrid promoters were fully activated with their respective inducers (IPTG, L-arabinose, and N-(3-oxo-hexanoyl)-L-homoserine lactone (3-oxo-C6-HSL)) and well repressed upon gRNA induction with aTc (Figure 4B).

3 Discussion

In this paper we described a novel strategy to create modular orthogonal CRISPR/Cas9-regulatable promoters. Currently available computational CRISPR/Cas9 gRNA selection algorithms focus on selection of gRNAs against existing genomic targets and therefore testing a specific set of user specified guide sequences for off-target binding with the host genome (reviewed in ref. (18)). Alternatively, for a small number of dCas9/gRNA transcription factors, manual design and selection of the best candidates using BLAST have been employed (11). Our automated computational approach allows one to systematically design gRNA sequences orthogonal to a given genome, circuit DNA sequences, and to each other within the context of a specific promoter. To validate this approach, we computationally designed a set of 20 mutually orthogonal gRNA sequences that minimize potential parasitic interactions with *E. coli* genome and the sequences of various widely used synthetic biology parts. We experimentally tested four of these sequences in the context of a strong promoter P_L from phage lambda in *E. coli* and used them to create two layered circuits – a double and a triple inverters.

Using these repressors, we designed a set of hybrid promoters which allow to combine native regulation of many prokaryotic promoters with orthogonal CRISPR/Cas9 regulation. We demonstrated the applicability of this approach by creating three hybrid promoters based on widely used promoters $P_{LlacO-1}$, P_{BAD}, and P_{luxI}. Our assays showed that they retain their native activation by IPTG, L-arabinose, and 3-oxo-C6-HSL correspondingly while gaining repression by dCas9/gRNA. This approach can be straightforwardly extended to a large class of native prokaryotic promoters lacking regulatory sequences downstream of the transcription start site. One of the potential drawbacks of installing dCas9/gRNA binding sequence downstream of the transcription start site is that it is transcribed into the resulting RNA. This in turn may interfere with its activity, although not strongly, as shown in Figure 3C. This problem could be circumvented by cleaving the CRISPR/Cas9 binding sequence from the downstream RNA by inserting a known RNAse cleavage site, e.g. that of Csy4 RNAse (30, 31). We believe the hybrid promoter design described here will be useful in creating complex synthetic circuits such as toggle switches and oscillators that can be regulated either externally or by the host itself. Such promoters could add an independent layer of regulation by CRISPR/Cas9 “wires” which will help engineer novel synthetic circuits with expanded functionality.

We have implemented the pre-screening portion of the computational design algorithm as a separate program which allows scoring any gRNA guiding sequence (for example, any of the 20 orthogonal gRNA sequences described in the paper) against an arbitrary DNA sequence (for example, the sequences of synthetic parts not used in the pre-screening in this paper) (see Supporting Information). We hope that this program will facilitate adapting the orthogonal repressors described in this paper to new synthetic circuits.

E. coli is a major workhorse of synthetic biology that is widely used to prototype and build novel scientifically, medically, and industrially important synthetic circuits. It is also closely related to human pathogen and potential synthetic biology therapeutic agent *Salmonella typhimurium* (32). Therefore, we believe that the overall approach and the specific CRISPR/Cas9 regulatable orthogonal promoters described in this paper will be useful for a wide community of synthetic biologists.

4 Methods

Computational design of orthogonal repressors

The purpose of this algorithm is to make sets of gRNA sequences that will cause dCas9/gRNA complexes to interact minimally with the genome and with each other (orthogonality), and will therefore be very specific to their target sequences. *S. pyogenes* dCas9/gRNA complexes recognize 20 bp DNA sequence followed by a NGG protospacer adjacent motif (PAM) site (where N is any nucleotide). Thus we generate a set of sequences in the space of 23-dimensional vectors $\boldsymbol{S} = [S_1, \ldots, S_{23}]$, where $S_i \in \{G,C, A, T\}$. To quantify the mismatch between a particular search sequence $\boldsymbol{S}^s=[S_1^s, \ldots, S_{23}^s]$ and a particular target sequence $\boldsymbol{S}^t=[S_1^t, \ldots, S_{23}^t]$ we introduce two 23-dimensional vectors, $\boldsymbol{m}$ and $\boldsymbol{w}$. The components m_i of the binary mismatch vector $\boldsymbol{m} = [m_1, \ldots, m_{23}]$ are zero if $S_i^s=S_i^t$ and 1 otherwise. The components of the constant vector $\boldsymbol{w} = [w_1, \ldots, w_{23}]$ are defined as follows. The first ten 5′ nucleotides are assigned increasing mismatch weight $w_i = i/10$, for $i = 1, \ldots, 10$. The next 10 nucleotides (the "seed" sequence) are assigned a constant weight, $w_i = 1$ where $i = 11, \ldots, 20$. The nucleotide at position 21 does not influence binding and is thus is given a zero weight, $w_{21} = 0$. The GG nucleotides at recognition positions 22 and 23 are very important for binding, so we assign them a large weight, $w_{22,23} = 2$ (see Figure S2 of the Supporting Information).

The weighted Hamming distance, δ, between two sequences is defined as the scalar product of the mismatch vector, $\boldsymbol{m}$, with the weight vector, $\boldsymbol{w}$, such that $\sum_{i=1}^{23} w_i m_i$. The minimum weighted Hamming distance, δ_{min}, is found by scanning the search sequence along all possible binding positions within the target DNA sequence and its reverse complement, and taking the minimum δ.

We use δ_{min} as the measure to find candidate gRNA sequences that lead to minimal interaction of the corresponding dCas9/gRNA repressors with the host DNA and circuit DNA sequences. The full algorithm consists of four main steps:

1. **Random sequence generation:** Random sequences of 20 nucleotides, [S_1, …, S_{20}], are generated from a uniform distribution. All sequences containing GG or CC dinucleotides are discarded in order to avoid potential PAM sites within the designed sequences, and therefore decrease the likelihood of non-specific binding. Then the NGG sequence is appended at positions 21, 22, 23 to complete the search sequence vector ***S***.

2. **Pre-screen:** The generated candidate sequences are pre-screened for incompatibility with three important sets of sequences in the cell:

    1. λP_L target promoter region (0.1 kbp upstream and 0.15 kbp downstream; see Supporting Information),

    2. the inserted plasmids and other circuit parts (around 22.4 kbp total; see Supporting Information),

    3. *E. coli* strain MG1655 genomic DNA (4.6Mbp; NCBI Reference Sequence: NC 000913.2).

    Mutual mismatch score thresholds for filtering out low-scoring sequences at each prescreen substep are set at: 5.0, 6.3, and 9.0 respectively. The reasoning behind the choice of these thresholds is discussed in the Results section.

3. **Mutual screen:** The 633 prescreened sequences are now screened against each other in the context of λP_L promoter to find their pairwise minimal Hamming distance $\delta_{min}(p, q)$, where $p, q \in [1, \ldots, 633]$ are indices of the prescreen sequences. Overlap flanks of 22 base pairs around each targeted 20-mer are also considered in the estimation of $\delta_{min}(p, q)$. The resultant matrix $\delta_{min}(p, q)$ (asymmetric because of the inclusion of the constant overlap flank sequences) is symmetrized, by defining $\delta_s(p, q) = \delta_s(q, p) = min(\delta_{min}(p, q), \delta_{min}(q, p))$.

4. **Orthogonal set selection:** The matrix of mutual screen scores $\delta_s(p, q)$ is analyzed to find the sequences which are the most mutually orthogonal. This begins by finding the pair of sequences with the highest $\delta_s(p, q)$ and putting them at the top of the orthogonal set. A sequence is then found whose minimum $\delta_s(p, q)$ between itself and each sequence in the orthogonal set is the largest of all the sequences. This sequence is then added to the orthogonal set and the process is repeated to grow the set to a desired size.

This algorithm was implemented as a custom program written in Fortran and Matlab (see Supporting Information).

Experimental methods

The plasmids used in this study were constructed by a combination of conventional restriction-ligation cloning, circular polymerase extension cloning (CPEC), Gibson assembly, efficient mutagenesis independent of ligation (EMILI), or inverse PCR mutagenesis (33–37). The sequences of the resulting plasmids are given in Supporting Information. The relevant parts of the plasmids were verified by Sanger sequencing.

The random gRNA targeting sequence (5′-GTGATGGGTCCAGAATGAAC) used in the mismatch sensitivity assay (Figure S1 of Supporting Information) has been generated using a random sequence generator with equal probabilities of each of the four nucleobases at each position. This specific gRNA sequence has at least 8.1 weighted mismatches (as defined by the algorithm described in the paper) within the DNA sequence spanning the nucleotides −85 to +803 with respect to the transcription start site of the reporter promoter (the promoter through the entire GFP coding sequence).

The mismatch sensitivity assay was performed in our lab standard strain, Keio collection derived *E. coli* K-12 JS006 (Δ*lacI* Δ*araC* Kan_S) (38). This strain naturally lacks tetracycline repressor *tetR*, therefore both $P_{LlacO-1}$ and $P_{LtetO-1}$ promoters are constitutively active in this strain. To allow regulation of these promoters with IPTG and aTc respectively, all promoter repression and circuit activity experiments were conducted in a closely related strain *E. coli* K-12 MG1655Z1 (*lacI*$^+$ *tetR*$^+$ Sp^R) (21, 39).

For the promoter activity assays overnight cultures were diluted 1:100 in LB-Lennox media (5 g/L sodium chloride) with the standard amount of antibiotics and grown for approximately 16–20 hrs at +37°C with vigorous shaking. Fluorescence was measured at excitation wavelength = 485 nm, emission wavelength = 520 nm using NovoStar or Tecan Infinite 200 Pro platereaders. Final fluorescence measurements were normalized by the optical density of cell culture and background corrected. Experimental errors were calculated as the average deviation of two or more independent measurements.

Promoter titration assay (Figure S3 of Supporting Information) was performed at the same conditions using two biological replicates. In IPTG titration experiments, the aTc concentration was kept constant at 100 ng/ml and IPTG concentrations were $1/3^n$ mM, where $n \in [0, \ldots, 8]$. In aTc titration experiments, the IPTG concentration was 0.3 mM and aTc concentrations were $200/1.8^n$ ng/ml, where $n \in [0, \ldots, 8]$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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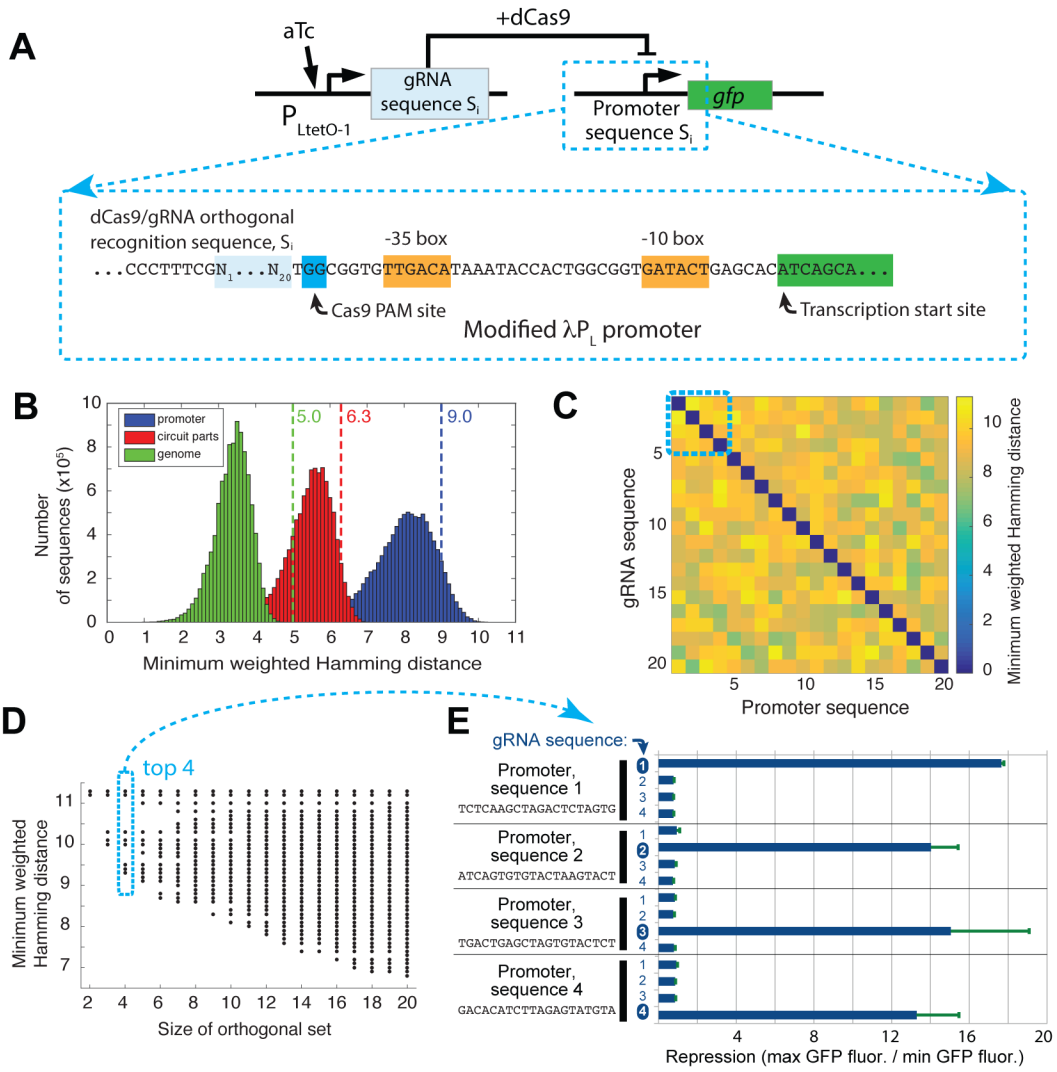

Figure 1.

Computational design and experimental validation of orthogonal CRISPR/Cas9 repressor/promoter pairs. (A) dCas9/gRNA binding site was installed upstream of −35 box of λP_L promoter driving expression of green fluorescent protein gene; gRNAs were expressed from anhydrotetracycline (aTc) inducible promoter. (B) Computed distributions of minimum weighted Hamming distances of candidate gRNA sequences against the sequence of *E. coli* genome (green), widely used synthetic circuit parts (red), the output promoter and the sequences in its immediate vicinity (blue). Only the candidate gRNA sequences with minimum weighted Hamming distances above the indicated thresholds were selected. (C) The matrix of 20 most orthogonal gRNA/promoter sequences selected by our algorithm. (D) Distribution of scores as a function of size of the orthogonal set. (E) Experimental validation of the best four computationally designed gRNA/promoter pairs. Repression strength was defined as the ratio of the fluorescence of the reporter GFP with the gRNA promoter $P_{LtetO-1}$ not induced versus fully induced with saturating amounts of aTc.

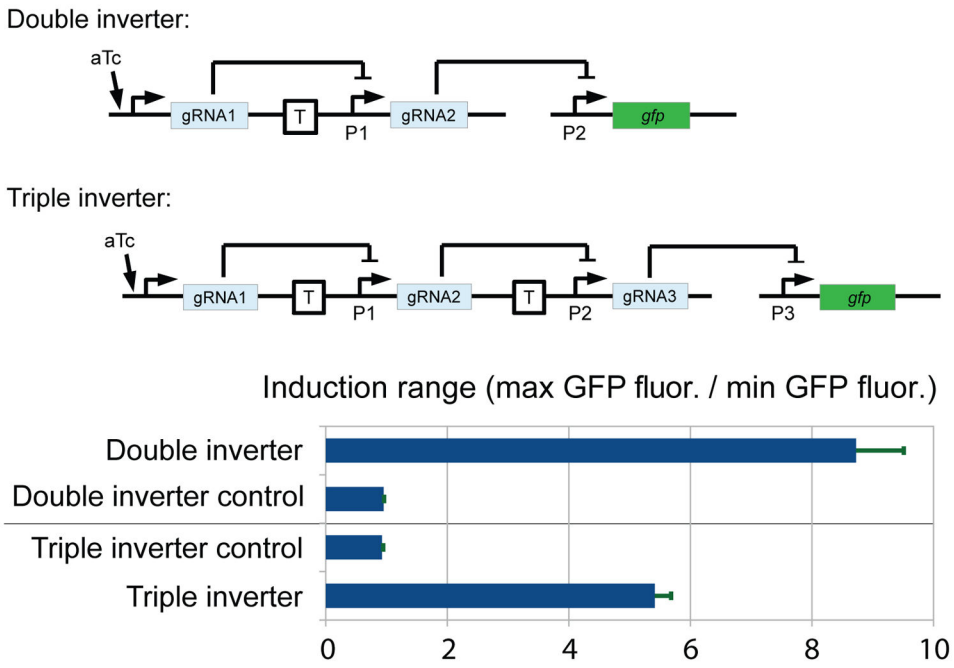

Figure 2.

Double and triple inverter circuits based on the designed orthogonal gRNA/promoter pairs. The circuits were constructed by connecting correspondingly two or three orthogonal promoters in series; the first gRNA is inducible with anhydrotetracycline (aTc). Induction range was defined as the ratio of the maximum to minimum reporter GFP fluorescence achievable in the circuit upon induction with aTc.

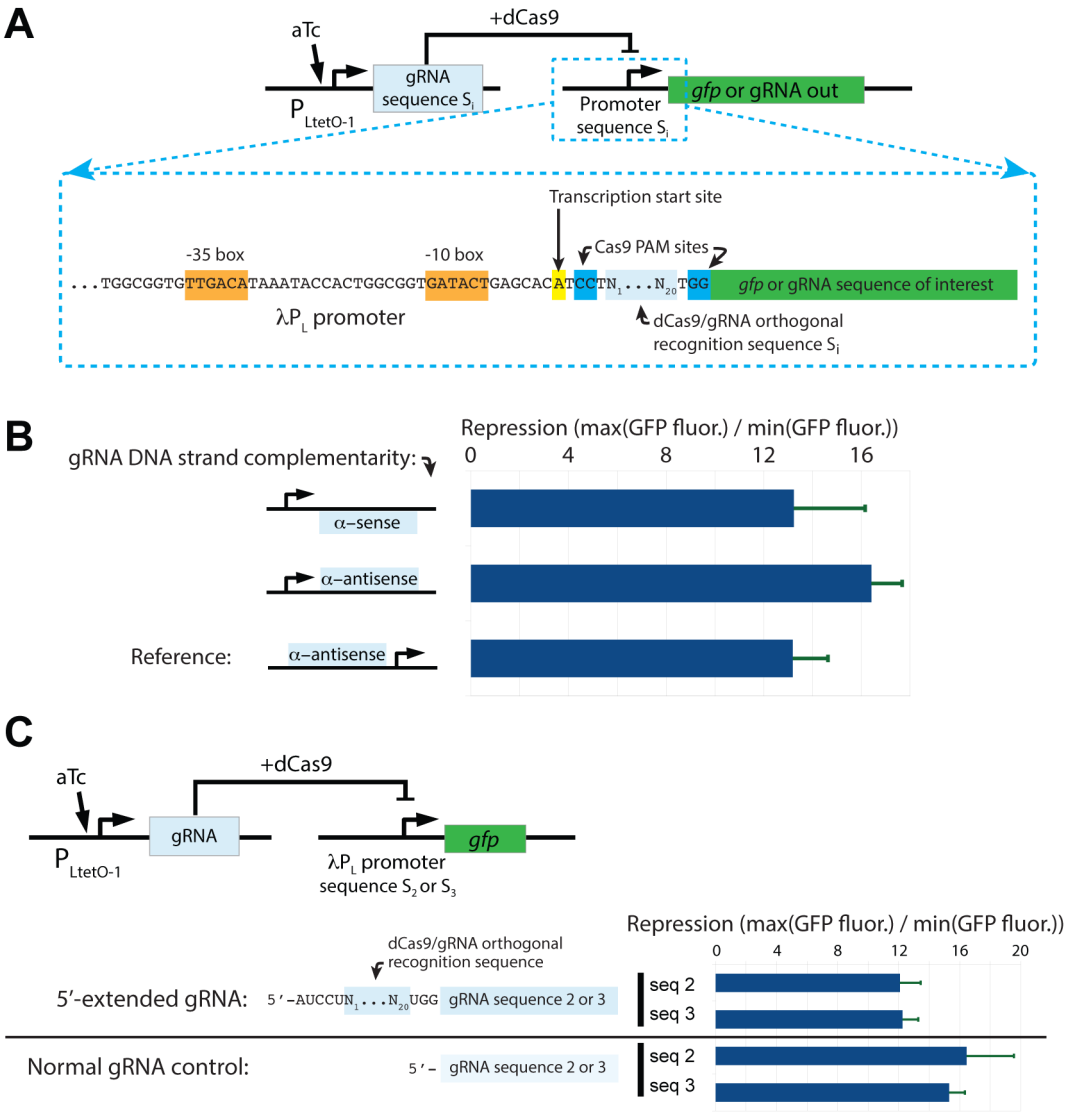

Figure 3.

Orthogonal promoters with CRISPR/Cas9 site downstream of the transcription start site. (A) dCas9/gRNA binding site was installed just downstream of the transcription start site of λP_L promoter. Two *S. pyogenes* Cas9 PAM sites were installed flanking the sequence so that dCas9/gRNA repressors can be recruited to both sense and antisense DNA strands in this position. (B) Targeting dCas9 to both sense and anti-sense DNA strands in this position results in promoter repression as strong as or stronger than repression just upstream of −35 box of the promoter. (C) gRNAs with extra 28 nucleotides 5′ of the guide sequence retain their dCas9 targeting functionality.

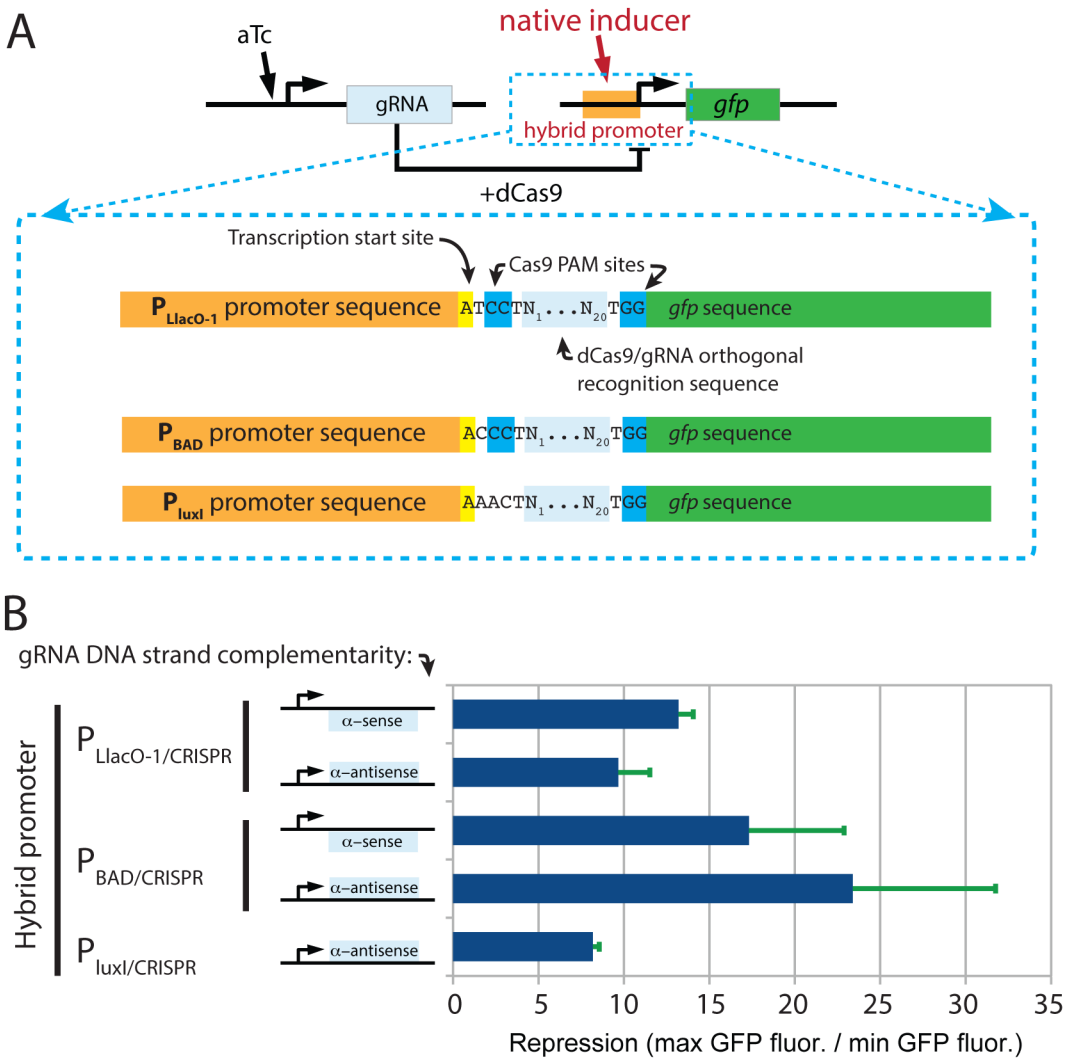

Figure 4.

Inducible hybrid CRISPR/Cas9-regulatable promoters. (A) dCas9/gRNA binding site was installed just downstream of the transcription start site of inducible promoters $P_{LlacO-1}$, P_{BAD}, P_{luxI}. (B) Hybrid promoter performance. All hybrid promoters were induced with saturating amounts of corresponding inducers (isopropyl β-D-1-thiogalactopyranoside, L-arabinose, and N-(3-oxo-hexanoyl)-L-homoserine lactone). Repression strength was defined as the ratio of the fluorescence of the reporter GFP with the gRNA promoter $P_{LtetO-1}$ not induced versus fully induced with saturating amounts of aTc.