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Review Article

Engineered protein switches for exogenous control of gene expression

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There is an ongoing need in the synthetic biology community for novel ways to regulate gene expression. Protein switches, which sense biological inputs and respond with functional outputs, represent one way to meet this need. Despite the fact that there is already a large pool of transcription factors and signaling proteins available, the pool of existing switches lacks the substrate specificities and activities required for certain applications. Therefore, a large number of techniques have been applied to engineer switches with novel properties. Here we discuss some of these techniques by broadly organizing them into three approaches. We show how novel switches can be created through mutagenesis, domain swapping, or domain insertion. We then briefly discuss their use as biosensors and in complex genetic circuits.

Introduction

Protein switches exist across all domains of life, serving the crucial role of sensing and responding to an organism's environment [1]. Broadly, a protein switch modulates an output function, such as catalysis or binding, in response to an exogenous input, such as small molecule binding, a change in pH or temperature, or exposure to light. Transcription factors represent one common type of protein switch [2]. These proteins recognize and bind to their cognate DNA sequence and modulate transcription at an adjacent promoter. They repress transcription by sterically blocking binding or transcription of RNA polymerase and activate transcription by recruiting RNA polymerase to the promoter. The DNA affinity, and therefore the strength of their repression or activation, can be modulated by a wide variety of stimuli, but is most commonly affected by the binding of a small molecule.

Transcription factors are ubiquitously used across many fields of study [3]. While there are thousands of known transcription factors, a relatively small portion of them are actually used for research purposes. While this small pool of transcription factors is often sufficient, there are situations in which novel transcription factors are necessary. This commonly occurs when a transcription factor that responds to a specific compound of interest is required. Often transcription factors that recognize that particular compound simply do not exist. The need for novel transcription factors is also apparent when designing large, complex gene circuits. A pool of parts that recognize a wide variety of different compounds and promoters and have a large range of activities is important for the construction of circuits with the desired properties. The tools that are commonly used today are often insufficient.

Nature can act as one source of novel transcription factors. Indeed, genome mining has identified many potentially useful proteins. However, these transcription factors are often ineffective outside of their native context and their utility is therefore limited [4]. Alternatively, novel transcription factors that have customized properties can be engineered from existing transcription factors and regulatory parts [5]. Gene expression engineering has been the subject of several recent reviews [5–8]. Here, we focus on the engineering approaches to engineering novel one-component transcription factors that function as protein switches. We broadly separate them into three approaches. Novel proteins can be

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generated through mutation of a few residues, swapping of domains from existing transcription factors, and insertion of regulatory domains into transcriptional effectors. We conclude by highlighting recent examples of applications of these engineered protein switches.

Genetic switches made through mutagenesis

We start by considering protein switches that are engineered through mutagenesis of an existing transcription factor to change its binding targets or activity (Figure 1A). We use engineered switches derived from the *E. coli* lactose operon repressor protein, LacI, as prototypical examples for this section, as this protein is well studied and extensive engineering has been performed on it [9–16]. LacI binds to the *lac* operator and represses transcription at the *lac* promoter [17]. Binding of allolactose or the synthetic inducer IPTG, triggers a conformational change in LacI that causes it to release the operator and allow for transcription at the *lac* promoter.

Mutating the substrate-binding domain of transcription factors allows researchers to expand the bank of potential compounds to use as inducers. However, this strategy is typically limited to using compounds that are structurally similar to existing inducers. To generate proteins that bind structurally dissimilar inducers would require a change of the protein's binding pocket that is too drastic for current methods to access. For example, Taylor et al. [12] generated a large number of LacI mutants that could be induced by gentiobiose, fucose, lactitol, or sucralose. Another common target for mutagenesis is the residues that impart DNA binding specificity. Multiple groups have found that mutating a few key residues within the DNA recognition helix of LacI generates proteins that recognize varied three-nucleotide sequences along the *lac* promoter [9–11]. This change in sequence specificity is enough to confer a substantial degree of orthogonality between the LacI variants [16]. Beyond changing substrate and DNA binding specificity, groups have also engineered LacI variants that respond differentially to inducer [13–15]. These anti-Lac variants bind to the *lac* operator in the presence of IPTG rather than the absence. Mutations that change DNA binding specificity and inducer response are largely orthogonal [16]. That is, these mutations can be combined to generate an additional set of LacI variants that both recognize an alternate promoter sequence and display a reversed response to IPTG. While the orthogonality of the substrate-binding domain mutations has not been demonstrated, it is likely that they will also be able to be combined with other mutations to generate an even larger number of unique transcription factors.

Both rational mutagenesis and directed evolution have been used to identify mutations that alter ligand and DNA affinity [9–16]. While not essential for engineering, structural information is useful for determining where mutagenesis should occur. It is particularly important when using computational methods to predict suitable mutants or site saturation mutagenesis. However, engineering can be performed in the absence of structural information using techniques such as random mutagenesis or comprehensive codon mutagenesis. Taylor et al. [12] used multiple library construction methods to generate LacI variants that recognized alternative inducers. This allowed them to compare the efficacy of each of these methods. They used computational design, comprehensive single codon saturation mutagenesis, and random mutagenesis via error-prone PCR.

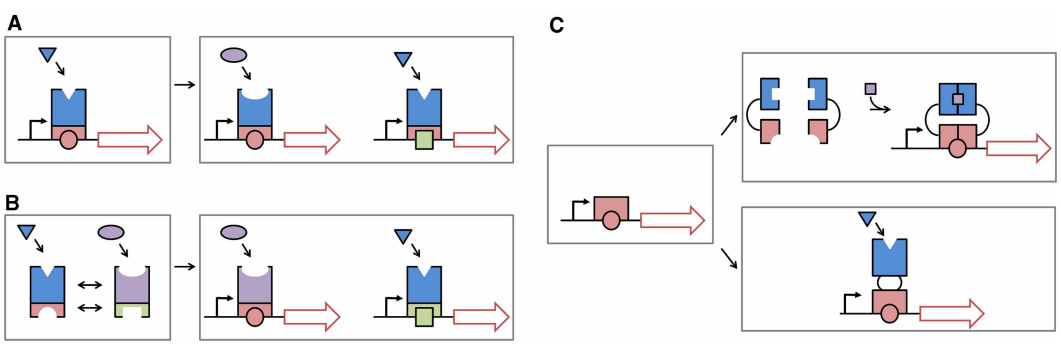

Figure 1. Protein switch approaches for generating novel transcription factors.
(**A**) Mutating a transcription factor to alter its allosteric effector or cognate DNA. (**B**) Swapping domains between homologous transcription factors to produce proteins with novel combinations of effectors and cognate DNA sequences. (**C**) Inserting regulatory domains into existing transcriptional regulators to introduce new exogenous inputs such as induced dimerization (top) or small molecule binding (bottom).

Generally, the most effective mutants had multiple mutations. These variants, which often had four or more mutations were only accessible through the computational method. One benefit of using the other two methods was that they were able to access mutations at distal regions that influence substrate binding, but are difficult to predict using computational methods. That being said, the most effective variants, ones which exhibited the largest change in gene expression upon induction and the greatest inducer specificity, were generated through multiple rounds of mutagenesis that combined these techniques.

Engineered switches with novel binding properties have also been made using other common transcription factors such as XylR [18], TetR [19], and AraC [20]. More recent work has been directed to designing proteins that respond to specific desired compounds including aromatic aldehydes such as vanillin and hydroxybenzaldehydes [21] and metalloids such as antimony [22]. Additionally, Rondon and Wilson recently performed engineering on the *E. coli* regulatory protein BetI [4], which releases its target operator in the presence of choline. This protein was previously unsuitable for use as a transcriptional regulator due to its poor response to choline. They generated a library of variants that controlled gene expression over a wide range of choline concentrations, induced gene expression up to approximately 300-fold, and were both positively and negatively regulated by choline. Similarly, Meyer et al. performed directed evolution to optimize 12 existing transcription factors, increasing their sensitivity to inducer and dynamic range, while decreasing their baseline expression in the absence of inducer and cross-reactivity to other inducers [23]. They subsequently incorporated each of these mutated transcription factors into the *E. coli* genome to generate 'Marionette' strains. In principle, these strains should allow for the independent regulation of 12 separate genes at once.

This review focuses on engineering single component transcription factor systems. However, the approaches we outline can be applied to more complex systems such as two-component systems. For example, McClune et al. [24] performed random mutagenesis on histidine kinases along with their corresponding response regulators to generate protein pairs with novel interactions that were orthogonal to most or all of the existing *E. coli* kinase systems. The large number of functional variants that they generated and the relative ease with which they generated them led the authors to conclude that the sequence space of two-component *E. coli* kinases was sparsely occupied. Further exploration of this space should yield more novel kinase variants.

Genetic switches made through domain swapping

Next, we consider novel switches that are made by swapping domains of existing switches (Figure 1B). Many switch proteins are made up of multiple, discrete domains [1]. The least complex of such proteins have one domain that recognizes an input signal and another that returns a function based on the signal it receives. Because these domains are often structurally distinct, input and output domains from separate switch proteins can be swapped to introduce novel outputs for given inputs.

We once again use engineered switches derived from LacI and related proteins from the LacI/GalR family of transcription factors as prototypical examples of this approach. Proteins in this family bind to a unique operator sequence on the DNA with a strength that is modulated by an allosteric effector [25]. They also have a conserved tertiary structure. They contain a ∼40 amino acid N-terminal helix-turn-helix (HTH) domain that recognizes operator DNA, followed by a ∼20 amino acid linker sequence, and a ∼300 amino acid C-terminal regulatory domain that is responsible for binding to the allosteric effector as well as forming multimers.

Tungtur et al. [26] performed the initial work on LacI/GalR hybrids that we consider. They swapped the C-terminal domain of LacI with the corresponding C-terminal domain of PurR. PurR, a member of the LacI/GalR family, binds to its target operator and represses transcription in the presence of purines such as adenine. The hybrid LacI-PurR protein showed a slight (two-fold) decrease in transcription at the *lac* promoter in the presence of adenine. Additionally, this protein was toxic; in certain cell lines, they were unable to produce detectable levels of protein or grow cells transformed with the plasmid containing the gene for the hybrid protein. They hypothesized that the hybrid protein interacted with other critical *E. coli* operators, limiting growth. For these reasons, they continued to generate iterative versions of this hybrid protein. It had previously been shown that residues in the linker that connects the DNA and substrate-binding domains mediate interactions between the two domains that are essential for allostery [27]. Therefore, in an attempt to identify variants with a larger change in gene expression on exposure to adenine while also potentially generating less toxic variants, Tungtur et al. [26] generated a series of LacI-PurR hybrids that contained mutated linker sequences. Some of these variants were successful and had a larger (up to 10-fold) reduction in expression at the *lac* promoter upon the addition of adenine. They were also able to generate variants that were less toxic but did not perform follow-up investigations into possible causes of this change in toxicity. Nevertheless, this represents a

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potential complicating factor in engineering other, similar, systems. Meinhardt et al. [28] expanded on this study to generate eight additional hybrid transcription factors that were comprised of the LacI DNA binding and linker domains connected to a LacI/GalR substrate-binding domain. Their goal was to generate proteins that responded to novel compounds such as, galactose, ribose, and cytidine while still recognizing the *lac* operator. These proteins showed variable responses, from over 100-fold induction to no response at all, to their corresponding effectors. Once again, the researchers were able to improve some of these proteins' responses to effector by introducing mutations to their linker regions. Recently, Dimas et al. [29] further expanded on this work to create a much larger set of hybrid proteins. They combined eight DNA binding domains with five substrate recognition domains from the LacI/GalR family to generate 35 novel transcription factors. They subsequently measured the expression of GFP under the control of these hybrid proteins and found low basil GFP expression and high (greater than 10-fold) induction levels for 22 out of the 35 proteins. They also found only a few interactions between hybrid proteins and off-target promoters, demonstrating the orthogonality of their engineered transcription factors.

Recently, other groups have performed similar work on proteins from other families, such as the TetR [30] and LuxR families [31]. These works generated hybrid proteins that recognized novel combinations of DNA operators and small molecule effectors. They also demonstrated similar design constraints as the above studies, such as the importance of the linker region and the need for iterative improvements to generate high-quality variants. This hybrid protein approach can also be used to tune the properties of existing transcription factors. For example, a hybrid LysR-type transcription factor was recently engineered, combining the DNA binding domain from the *Herbasperillum seropedicae* protein FdeR and the substrate-binding domain of the *Sinorhizobium meliloti* protein NodD1 [32]. While both proteins are flavonoid biosensors, the hybrid protein combined the *E. coli* compatible promoter of FdeR with the greater substrate specificity of NodD1. Groups have also used chimeric membrane proteins as another avenue for generating novel proteins to control gene expression [33–35]. Often, the extracellular substrate-binding domain of one membrane protein is combined with the intracellular effector domain of another. Common effector domains include kinases that activate transcription factors [33] or proteases that release transcription factors from the membrane [34,35]. This strategy provides an additional set of sensors and effectors to use and also eliminates the need for import of the substrate of interest into the cells.

Genetic switches made through regulatory domain insertion

Lastly, we consider engineered protein switches made through domain insertion, i.e. switches that are made by inserting a regulatory domain into a protein that is already capable of influencing gene expression (Figure 1C). We primarily use engineering performed on the CRISPR associated protein, Cas9, as an example of this type of work [36]. Cas9 is an endonuclease native to a variety of bacterial species and is responsible for identifying and cleaving foreign DNA. It targets DNA in a sequence-specific manner through normal Watson–Crick base-pairing interactions between the target DNA and a short guide RNA molecule that the protein forms a complex with. This protein can therefore flexibly target a wide variety of sequences simply by changing the sequence of the guide RNA. Most of our discussion centers around the nuclease-null mutant (D10A and H840A) *Streptococcus pyogenes* Cas9 protein, which is referred to as dCas9 [37]. This variant binds DNA but does not cleave. This engineered transcription factor silences gene expression when it targets select sites in the gene's coding region or promoter.

A wide variety of regulatory domains have been incorporated into Cas9 and dCas9 to confer switching behavior [38–44]. Systems that use Cas9 rather than dCas9 measure switching based on DNA cleavage or gene editing efficiency [38,39,41,43,44]. In some of these systems, switching with dCas9 has not been explicitly demonstrated [43,44]. However, results with systems that have been tested with both Cas9 and dCas9 indicate that a Cas9 variant that acts as a switch will still act as a switch when mutated to dCas9 [38,39,41]. Many of the regulatory domains that we will discuss respond to small molecules in a similar manner to existing transcription factors [38–40]. For example, Oakes et al. [38] generated a dCas9 variant that represses gene expression in the presence of 4-hydroxytamoxifen. This variant was identified by randomly inserting the ligand-binding domain of the human estrogen receptor-α [45] into the dCas9 gene and screening the library for variants that displayed 4-hydroxytamoxifen-dependent changes in expression of a reporter gene. A large number of dCas9 switches have also been created through the use of domains that dimerize in the presence of a small molecule [39,40] or light [41,42]. Researchers start by identifying a point on the protein, usually

located between two domains, to split dCas9 into two fragments. Compared with the domain swapping approach, where it is often clear where the break should be made, researchers usually need to test multiple sets of fragments split at different locations, as the fragments should have no activity on their own, but still be capable of functional assembly in the right context. Next, researchers fuse a set of heterologous dimerization domains to each of the fragments [39,41,42]. In the presence of the appropriate inducer, the domains dimerize, bringing the dCas9 fragments together into an active complex. Alternatively, dimerization domains have been attached to intact dCas9 and transcriptional activators or repressors [40]. In this case, dCas9 always binds to its target DNA, but only affects transcription when the regulatory domain is recruited through induced dimerization. Other methods of controlling dCas9 activity include inserting domains that affect its stability or its folding [43,44]. For example, fusing the FKBP12-derived destabilizing domain [46] to dCas9's N-terminal increases its susceptibility to proteasome cleavage [43]. This tag reduces Cas9 stability and lowers its activity in cells to undetectable levels. The synthetic ligand, Shield-1, binds to the destabilizing domain, stabilizing it and preventing degradation of the protein. Therefore, upon the addition of this ligand, Cas9 activity is restored. Cas9's folding can be disrupted by inserting a ligand-responsive intein domain [44,47]. When splicing is triggered through the addition of ligand, Cas9 folding and activity is restored. However, in contrast with other systems, we have discussed so far, this intein-based switch is irreversible due to the nature of the splicing reaction.

Similar strategies have been applied to other artificial transcription factors as well as natural transcription factors. For example, Slomovic and Collins inserted trans-splicing inteins [48] into a zinc finger (ZF) DNA binding domain and the mammalian transcriptional activator, VP64 [49]. When the two domains are within close proximity to one another, splicing occurs, and the ZF is joined to the transcriptional activator, leading to increased expression at a targeted promoter. The two inteins are brought together by additional ZF domains. This allows for the recognition of intracellular DNA, such as viral DNA. Additionally, Moon et al. [50] fused light-oxygen-voltage (LOV) domains to the tetracycline repressor protein, TetR [51]. This protein's switching mechanism contrasts with that of the other light-induced switches we have mentioned so far. In the presence of blue light, rather than dimerizing, these domains undergo a conformational change, altering the protein's affinity for DNA. The affinity change was minor compared with the change associated with tetracycline binding; exposure to light changed the protein's dissociation constant only by approximately two-fold. However, the conformational changes associated with the LOV domains allowed for fine tuning of TetR's activity.

Applications of engineered genetic switches

There is an ongoing need in the field of synthetic biology for new, effective protein switches for controlling gene expression [5,6,52]. Often these switches are used as biosensors [22,33]. For example, a switch that responds to a specific compound can be used to control the expression of a reporter gene such as GFP. Researchers are then able to monitor the presence of that particular compound based on fluorescence. In doing so, they have a way to screen for desirable cellular phenotypes such as the production of that compound or disease states associated with changes in that compound's concentration. For example, Rogers and Church used the acrylate-sensing protein, AcuR [53], along with two helper proteins which converted 3-hydroxypropionate (3HP) to acrylate to identify growth conditions that maximized production of 3HP [54]. Fluorescent reporter systems for identifying compound production are faster and more efficient than methods such as HPLC and mass spectrometry [55]. This allows for high-throughput screening of a large number of conditions. More recently, two groups have engineered transcription factors for more sensitive detection of natural products such as macrolides [56] and the antibiotic pamamycin [57]. Alternatively, rather than inducing expression of a reporter protein, these biosensors can be used to induce the expression of a protein effector that controls the flux of a metabolic pathway, acts as a therapeutic for treating disease, or performs some other beneficial function [5]. Recently, Bojar et al. [58] developed a set of caffeine biosensors that used the caffeine-binding single-domain antibody, aCaffVHH. Using this system, they were able to induce expression of the synthetic human glucagon-like peptide, which can be used for the treatment of type 2 diabetes [59], in diabetic mice. When the mice were fed caffeine, the researchers found significant changes in the concentration of glucose and insulin within the mice's bloodstream [58].

A diverse set of transcription factors is also useful for creating genetic circuits in which multiple inputs interact to influence one or more outputs [7]. The set of LacI/GalR chimeras created by Meinhardt et al. [28] was used in conjunction with LacI DNA binding domain mutants generated by Daber and Lewis [9] to generate

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multi-input logic gates [60]. They were able to create AND gates using two or more chimeras (up to four) that recognized the same promoter. The reporter gene under the control of this promoter was expressed only when all of the inducers were present. They could maintain an additional, orthogonal AND gate when an additional promoter that is recognized by mutant LacI variants was used. These gates were able to act independently and showed low expression in the absence of one or more inducer. Recently, in a separate study, these chimeras were used to generate 'passcode' circuits, which allowed for cell survival only in the presence of a unique combination of orthogonal inducers [61]. This circuit can be used to ensure the biocontainment of engineered cell lines. Because the inducers were not naturally occurring, any cells that escape into the natural environment will be unable to survive. Gao et al. [40] were able to generate AND and OR circuits in mammalian cells using the dCas9 variants that dimerized with transcriptional activators or repressors in the presence of a small molecule effector. Multiple circuits could be used simultaneously when two dCas9 orthologs that recognize different guide RNA sequences were employed.

Chimeric transcription factors have also recently been used as a tool to aid in natural product discovery [31]. Genome mining has revealed many biosynthetic gene clusters in bacteria. However, many of them are not expressed under normal laboratory conditions and the requirements to induce expression are unknown [62]. Mukherji et al. [31] identified a 'silent' gene cluster that was regulated by PseR, a LuxR family transcription factor with an unknown inducer. They replaced this protein's substrate-binding domain with the substrate-binding domain from another LuxR family transcription factor, LasR, which activates transcription of its target operator in the presence of 3-oxo-C10 HSL. This chimeric protein was able to activate transcription at the silent gene cluster and Mukherji et al. were able to characterize a novel natural product, which they called pseudomonal.

Perspectives

- Synthetic biologists need novel genetic parts that can produce varied responses to a wide variety of signals.
- As a result of this need, many different approaches have been developed to modify existing switches to create novel ones with desired properties. These approaches vary by the degree of changes made to the original switch.
- As these approaches continue to develop, our bank of useful tools will grow, as will our ability to quickly and easily create new switches.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contributions

S.S. drafted the review. Both authors contributed to the design, editing, and revision of the review.

Abbreviations

Cas9, CRISPR associated protein; CRISPR, clustered regularly interspaced short palindromic repeat; *E. coli*, *Escherichia coli*; GFP, green fluorescent protein; HTH, helix-turn-helix; HPLC, high-performance liquid chromatography; 3HP, 3-hydroxypropionate; IPTG, isopropyl β-D-1-thiogalactopyanoside; LOV, light-oxygen-voltage; PCR, polymerase chain reaction; ZF, zinc finger.

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