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Creating Small-Molecule-Dependent Switches to Modulate Biological Functions

Review

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Biological small-molecule-dependent switches sense external chemical signals and transduce them into appropriate internal signals and cellular responses. Artificial molecular switches that control the function of any protein of interest using a small molecule are powerful tools for studying biology because they enable cellular responses to be controlled by inputs chosen by researcher. Furthermore, these switches can combine the generality of genetic regulation with the reversibility and temporal control afforded by small molecules. Three approaches to creating molecular switches include altering a natural switch to recognize new exogenous ligands, engineering novel allosteric responses to ligand binding, or enforcing protein localization with chemical dimerizers. Here, we discuss the development of small-molecule-dependent switches that control in a general fashion transcriptional activation, translational initiation, and protein activity posttranslationally.

Introduction

A defining characteristic of life is the ability to sense changes in the environment and react appropriately. Cells must respond to a dizzying array of stimuli, including nutrient gradients, chemical signals, temperature, mechanical stress, light, and sound. Nature has accordingly evolved complex systems that monitor the environment and rapidly alter gene expression and protein activity in response to changing conditions.

Regulation of the *E. coli lac* operon, elucidated in 1961 by Jacob and Monod [1], was the first well-characterized example of a genetic response to a chemical signal. Fluctuations in the amount of available nutrients require that bacteria rapidly switch between metabolizing different substrates, but the synthesis of high levels of nonessential enzymes is energetically costly. In the absence of lactose, *E. coli* cells repress *lacZYA* expression such that there are fewer than five copies of the LacZ protein per cell; 10 min after induction with lactose, there can be ~5000 copies per cell. This regulation is accomplished by the repressor LacI, which binds to the DNA sequence (the "operator") upstream of the *lacZYA* genes and blocks RNA polymerase from initiating transcription. Upon binding the inducer allolactose, LacI undergoes a conformational change that lowers its affinity for the operator DNA, dissociating the repressor-DNA complex and permitting transcription and translation of *lacZYA*.

Specialized sensing molecules such as LacI act as molecular switches and lie at the heart of biological responses to stimuli. They play the crucial role of recognizing an external signal and transducing it into a relevant functional output. In many cases, secondary switches relay cellular signals in a cascade of signal transduction events mediated by protein phosphorylation. This review, however, focuses on small-molecule inputs and will use the term "molecular switch" to refer to biological macromolecules (RNA or protein) that effect *small-molecule-dependent changes* in the expression level or activity of a protein. Furthermore, we will focus on switches that display *generality*: the ability to regulate expression or protein function in a manner that does not depend on any specific property of the target protein.

In addition to their central role in biology, molecular switches have proven to be important tools for analyzing biological systems. In this context, switches activated by small molecules offer significant advantages over switches regulated by other inputs. Many small molecules diffuse freely across cell membranes and can be added to a culture of cells or injected into an organism, enabling precise temporal and spatial control. In contrast to classical genetic approaches that introduce changes at the DNA level, chemical effectors can act reversibly, rapidly, and yield a dose-dependent response in which modulating the concentration of the effector can result in a proportional change in activity.

The major challenge of using small molecules alone to perturb protein function is the need to synthesize and identify a different small-molecule effector for every protein of interest. In addition, some molecules that bind a target with high affinity can also bind other proteins in vivo, complicating the interpretation of these studies. If nontarget proteins with modest affinity for a small molecule are more abundant or accessible than the desired target, side effects and loss of ligand potency can result. While researchers screening combinatorial libraries of small molecules have succeeded in targeting numerous proteins of interest, the discovery of a specific chemical inhibitor for every protein in a cell remains an outstanding challenge.

In contrast, genetic approaches offer both the generality to target any gene of interest and the specificity for one and only one gene. These desirable features arise from the precision of genetic techniques that alter the DNA sequence encoding a protein of interest. Balancing these advantages, however, are the difficulties of genetic manipulation in higher organisms, the fact that alterations at the genomic level are nonreversible, and the potential cellular lethality of genetic changes to essential genes. Although the use of conditional temperature-dependent alleles overcomes some of these difficulties, the mechanism of temperature dependence is often uncertain, and temperature shifts themselves can exert confounding effects on a biological system [2].

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Artificial RNA or protein molecular switches triggered by small molecules can combine some of the advantages of genetic and chemical approaches. Small-molecule control of the switch offers temporal and spatial precision. Moreover, since the switch is expressed in vivo and acts only on the gene of interest, downstream functions can be regulated with high specificity. These advantages, however, typically come at the expense of slower kinetics than approaches that rely solely on small molecules, and genetic intervention is still required. Because natural switches do not possess the generality and independence from cellular systems that are necessary for their application, artificial molecular switches must be created to exploit the above advantages.

Essential Features of Molecular Switches

As defined above, molecular switches must bind at least two molecules: the small-molecule effector and the downstream target. The LacI protein, for example, binds to the small-molecule inducer isopropyl-β-D-thiogalactopyranoside (IPTG) with a ~1 μM dissociation constant and to the DNA of the operator site with K_d = 10^{-13} M. Not only must these interactions be of high affinity, but they must also be highly specific: LacI binds the inducer allolactose but does not bind other sugars differing only in the stereochemical configuration at a single carbon atom. Likewise, LacI recognizes the ten base pair *lac* operator sequence amidst a vast excess of noncognate genomic DNA.

Jacques Monod emphasized a key property of molecular switches: *they bind molecules that have no necessary chemical relation to one another* [3]. The functional relationship between the two binding sites is provided by the switch itself. One approach to engineering artificial molecular switches is therefore to redesign ligand-receptor interfaces so that downstream function is controlled by small-molecule inputs chosen by the researcher (Figure 1). Analogs of natural small-molecule ligands can avoid interference with other cellular processes. Even greater control over a system can be gained by engineering an orthogonal receptor that responds only to the exogenous ligand. Together, a synthetic ligand and altered receptor constitute an *orthogonal pair*.

In addition to binding a small molecule tightly and specifically, a molecular switch must also convert the binding of the allosteric effector into an altered functional output. A current model of protein structure, based on NMR studies of folding dynamics, postulates a collection of conformational states in which some regions of the protein are disordered due to local unfolding [4]. According to this model, the binding of a ligand at a distal site may bias the conformational equilibrium toward a structure in which the active site is more active or less active. In the case of LacI, IPTG binding favors a conformation that is incapable of DNA binding, lowering affinity for the operator by three orders of magnitude.

The work of Freire and others has shown that the two binding sites of allosteric proteins typically differ in that regulatory regions are of high flexibility, whereas active sites usually maintain high stability in order to bind and orient reactants productively [5]. Theoretical work suggests that a "pathway" of energetically coupled residues must exist between the two sites for the energy of binding to an allosteric regulator to affect catalysis [6]. A molecular switch must therefore possess finely tuned thermodynamic properties that link small-molecule binding with a change in protein function. Another approach to engineering molecular switches that will be discussed below is to change the connectivity of the two binding sites in a switch. Alteration of the allosteric conformational shift of a switch can result in different responses to a given input; for example, the effect of ligand binding can shift from repression to activation of a protein's function (Figure 1).

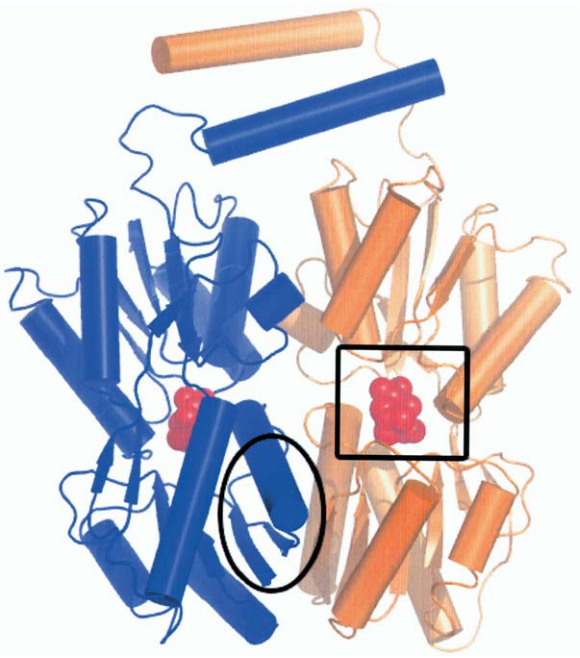

Figure 1. Two Strategies for Engineering Molecular Switches

The structure of the LacI dimer bound to the inducer IPTG is shown [69]. One approach to creating switches is altering ligand binding specificity to create proteins that respond to nonnatural inputs (boxed). A second approach is to alter the allosteric response to ligand binding; the oval highlights a region on the LacI structure between the IPTG binding site and DNA binding domains (not shown). Mutations in this region have been identified genetically that reverse LacI response upon ligand binding [70].

Molecular switches can exert control over three stages of gene expression: transcription, translation, and protein function. Transcriptional regulation offers the potential to effect the widest change in protein levels. Translational control with small molecules was discovered only recently, but appears to have intermediate properties of speed and breadth of regulation. Direct regulation of protein function (posttranslational control) gives the most temporal precision, as ligand binding is transduced directly into altered protein function. Below, we discuss examples of engineering small-molecule-dependent genetic switches that operate at each of these three levels.

Regulation of Transcription

Most organisms regulate gene expression primarily through altering messenger RNA levels. An important

characteristic of transcriptional regulation is that the regulatory signal is amplified. Binding of a transcription factor to one or several copies of the gene can generate many mRNAs and thousands of active protein molecules. Of the three levels of regulation, transcriptional control typically provides the widest range of response. The price paid for this range is a relatively slow response time; although the *E. coli lac* operon responds to transcriptional activation in less than 10 min, some mammalian genes require ~24 hr for the level of active protein to plateau.

According to the recruitment model of Ptashne and Gann [7], transcription is regulated by sequence-specific DNA binding proteins that localize components of transcriptional machinery to a gene. The majority of eukaryotic transcription factors are modular in the sense that the DNA binding and activation activities are located in separate and distinct domains. We will discuss efforts to engineer DNA binding domains to have ligand-dependent DNA binding activity, then efforts to alter the specificity of natural allosteric activators, and, lastly, approaches to engineering the interaction between the DNA binding and activation domains themselves.

Creating Small-Molecule-Dependent DNA Binding Domains

Several researchers have developed directed-evolution strategies for creating DNA binding proteins that bind to arbitrarily chosen DNA sequences [8–11]. Zinc-finger domains each bind to three base pairs of DNA and can be fused together to recognize longer sites. Using phage-displayed zinc finger libraries and in vitro DNA binding selections, Barbas and coworkers [12] evolved zinc fingers that bind to each individual GNN codon. The fusion of DNA binding domains with nuclear receptor ligand binding domains (LBDs) makes transcriptional activation dependent on steroid hormones. The combination of three-finger Zif268 proteins evolved using phage display, the VP16 activation domain, and the estrogen receptor LBD created transcriptional switches that were activated in cells ~500-fold upon ligand binding [13]. These studies demonstrated that the zinc-finger technology can be used to target promoters in a ligand-regulated manner.

Barbas, Schultz, and coworkers engineered a natural zinc-finger protein, Zif268, to bind to DNA only in the presence of a small-molecule ligand [14]. His125 and Phe116, which participate in zinc binding and hydrophobic packing, were mutated to Ala and Gly, respectively (Figure 2). The resulting cavity around the zinc ion abolished transcriptional activation. Screening 250 heterocyclic molecules for their ability to activate a luciferase gene identified 2-(4′-quinoline)-benzimidazole (**1**) as a small molecule that increases gene activity in this system by 18-fold. This small molecule likely fills the hydrophobic cavity and provides another coordinating aromatic amine ligand for the zinc ion. The authors show that ligand binding increases the affinity of the mutant protein for its DNA recognition site in vitro by 16-fold. This strategy of generating cavities in a protein and then screening small molecules to fill the cavity is similar to the "bump-hole" approach that has been used to alter ligand binding specificity [15, 16]. In this case, however, the presence of the ligand stabilizes the protein structure and restores DNA binding activity.

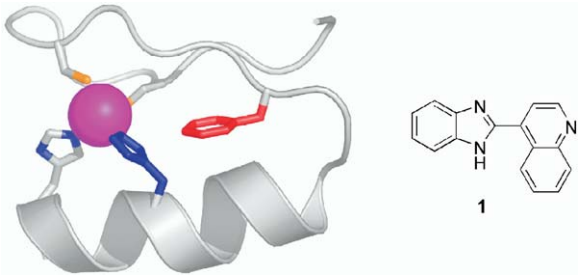

Figure 2. Engineering a Ligand-Dependent DNA Binding Protein

Phe116 (red) was mutated to Gly and His125 (blue) to Ala in the zinc-finger domain from Zif268 [14]. The small molecule **1** was isolated from a screen and found to enhance DNA binding of the doubly mutated Zif268 by 16-fold.

An impressive set of experiments by Hillen and coworkers on the tetracycline repressor (TetR) represents a second example of engineered ligand-dependent DNA binding. TetR represses expression of the tetracycline resistance protein TetA, which pumps tetracycline out of the cell [17]. Overexpression of TetA is toxic to bacteria, and TetR has evolved very high affinity for its operator site ($K_d = 10^{-14}$ M) to prevent TetA expression. Because TetR must bind tetracycline and allow expression of the resistance protein before the antibiotic reaches levels that inhibit translation, TetR has evolved tight tetracycline binding ($K_d = 10^{-9}$ M). Upon binding tetracycline, the repressor loses affinity for the operator.

Researchers attempting to develop orthogonal regulatory systems in eukaryotic cells have taken advantage of TetR's remarkable properties. Fusion of an activation domain to TetR created a hybrid transcription factor regulated by tetracycline in mammalian cells [18]. This tetracycline transcriptional activator (tTA) binds DNA and activates transcription only in the absence of tetracycline; removal of the ligand from the media was shown to increase expression of a luciferase reporter gene by five orders of magnitude. This system has been used in many contexts ranging in complexity from yeast cells to mice (Figure 3) [19].

One practical drawback of the tTA system is that tetracycline must be present to keep gene expression repressed, and ligand removal yields slower responses than ligand addition. To address these issues, Hillen and coworkers used a directed evolution approach to create TetR mutants that only bind the operator in the *presence* of tetracycline or its close analog doxycycline [20]. The researchers developed a selection in *E. coli* in which the functional DNA binding of TetR leads to survival and LacZ activity through the repression of a repressor [21]. Selection for reversed TetR function yielded proteins that remain bound to DNA in the presence of a nontoxic tetracycline analog. In the wild-type TetR, ligand binding induces β-turn formation at the end of helix 6, resulting in a 3 Å translation of the N-terminal helices and preventing interaction with the major groove of DNA. Mutations at positions 101 and 102 map to residues in this critical β turn and prevent ligand binding from reducing DNA binding affinity [22]. Not only must these mutations be compatible with the DNA-bound conformation, but they also must alter the conforma-

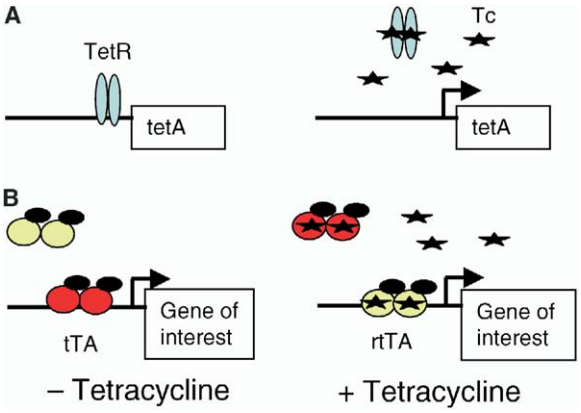

Figure 3. Tetracycline-Controlled Gene Regulation

(A) The wild-type TetR protein binds to its operator site and represses expression of the *tetA* gene. Upon tetracycline (Tc) binding, the receptor dissociates from DNA and *tetA* is expressed.

(B) TetR fused to the VP16 activation domain (tTA, red) activates eukaryotic gene expression in the absence but not in the presence of tetracycline. The reverse TetR activator (rtTA, yellow) evolved by Hillen [20] activates transcription only in the presence of tetracycline.

tional equilibrium to ensure that ligand binding is *required* to assume that conformation. In a follow-up study, the authors show that even single mutations in TetR can convey the reverse phenotype [23]. The directed evolution approach was successful despite the heavily disfavored thermodynamics of the starting materials: unliganded wild-type TetR has a 10^9-fold higher affinity for DNA than the liganded receptor. This experiment is an example of the evolution of a novel cooperative linkage between two binding sites (one for tetracycline and one for DNA) that yields a switch with altered functionality.

Fusion of the VP16 activation domain to this novel DNA binding domain generated the reverse tetracycline transcriptional activator (rtTA), which activates transcription only when induced by the addition of tetracycline analogs (Figure 3). In some applications, it was noted that the rtTA system exhibited a low-level background activity in the absence of doxycycline and low sensitivity to induction. To address these problems, Hillen and coworkers set up a second selection for reversed TetR function, this time based on transcriptional activation in *S. cerevisiae* [24]. One clone exhibited 10-fold lower unliganded background and a second showed 10-fold higher response to doxycycline induction than the original rtTA. These studies collectively imply the existence of a surprisingly large number of solutions in sequence space to the functional reversal of TetR. In addition, they highlight the power of molecular evolution approaches to generate and optimize protein conformational changes induced upon ligand binding.

Creating Orthogonal Transcriptional Activation Domains

In contrast to these studies that alter DNA binding domains, the majority of efforts to create small-molecule-dependent transcriptional switches alter the ligand specificity of natural allosteric activation domains. This approach has been highly successful with the nuclear hormone receptor family, generating orthogonal receptor-ligand pairs that have been used extensively to control eukaryotic gene expression.

The fact that small-molecule derivatives of NR ligands can be readily accessed by organic synthesis makes these natural allosteric transcriptional switches attractive targets for engineering. Apart from their use in regulating transcription, it has been shown that fusion of steroid receptor ligand binding domains to several classes of proteins renders the target protein hormone dependent [25]. Fusion to recombinases and kinases involved with cellular signaling (such as Src or Raf) inhibits macromolecular association of the target protein and its substrates. This inhibition arises from steric occlusion by the Hsp90 chaperone complex bound to unliganded LBDs [25]. Hormone binding induces a conformational shift, dissociation of the chaperone complex, and activation of target protein function.

One of the most widely used orthogonal mutants of a steroid receptor LBD is the Gly521Arg mutant of the estrogen receptor [known as ER(T)] that is induced with 4-hydroxytamoxifen (4-OHT) but does not respond to the natural hormone β-estradiol (Figure 4C) [26]. This mutation in the human ER-LBD was characterized during a rational site-directed mutagenesis study by Parker and coworkers aimed at elucidating the determinants of ER dimerization and ligand binding [27, 28]. Further analysis showed that the Gly521Arg mutation reduces affinity of the receptor for β-estradiol by more than four orders of magnitude yet reduces the affinity for 4-OHT by only 10-fold [29]. Analysis of the β-estradiol-bound [30] and 4-OHT-bound [31] crystal structures of the ER-LBD reveals how this altered specificity may be achieved (Figure 4). The side chain of residue 521 protrudes into the ligand binding pocket, and the greater steric bulk of an arginine side chain reduces the cavity size. 4-OHT may be less affected by this change than β-estradiol, which binds farther into the cavity.

Several groups have reported structure-based rational design strategies that remodel hydrophobic interfaces or alter charges in the active site of the retinoic acid receptor or estrogen receptor (for a review see [32] and [33]). In general, remodeling of the binding site shape has been more successful than attempts to reconfigure hydrogen-bonding patterns or ionic interactions. As nuclear hormone receptors are further characterized at the biochemical and structural levels, design strategies to alter the ligand specificity of natural allosteric transcription factors may prove increasingly fruitful. The use of structural information to inform directed evolution approaches has proven to be a particularly powerful strategy in altering the ligand specificity of the retinoic X receptor [34].

The above examples altered the specificity of natural activation domains. In contrast, we recently reported the creation de novo of an artificial allosteric activation domain made of RNA [35]. Using a genetic selection in yeast, we tethered random RNA sequences to a promoter and identified a short RNA sequence that activates transcription with potency rivaling some of the strongest natural protein activation domains [36]. Mutagenesis and secondary structure prediction identified crucial sequence and structural elements. Guided by this information, we designed an insertion site to ac-

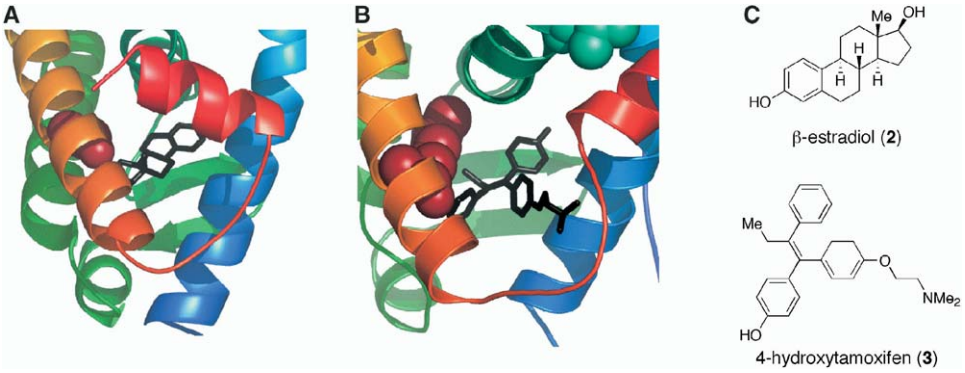

Figure 4. Estradiol and 4-OHT Binding Modes

The structure of the estrogen receptor bound to β-estradiol (A) shows the ligand bound deeply in a hydrophobic cleft [30]. Modeling of the mutation Gly521Arg (red spheres) in the 4-OHT-bound structure (B) shows how the Arg side chain partially fills the ligand binding pocket and shows the shallower binding of 4-OHT [31]. The structures of the ligands are depicted in (C).

commodate a short RNA sequence previously selected to bind the small-molecule dye tetramethylrosamine (TMR) [37]. Screening and selection of a library of linker sequences optimized a conformational shift in the RNA activator, such that transcription is 10-fold higher in the presence of TMR [35]. Although the resulting allosteric RNA-based activators are less responsive to ligand than typical protein transcriptional factors, these studies demonstrate the strengths of RNA evolution and design methods to create an allosteric linkage between a ligand binding and functional domain.

Regulation of Domain Interaction

Rather than engineer individual domains of transcription factors to be regulated by nonnatural small molecules, the highly successful "chemical inducer of dimerization" (CID) strategy pioneered by Schreiber and Clackson creates molecular switches by taking advantage of the modular nature of eukaryotic transcription factors [38]. According to the simple recruitment model of transcription described above, there is no strict requirement for transcription factor domains to be covalently fused. Association of the domains through protein-protein interactions, for example, is sufficient to restore robust transcriptional activation.

Two separate protein components comprise the original CID strategy: a DNA binding domain (DBD) and an activation domain (AD), each fused to a small molecule binding domain [38, 39]. In the presence of an appropriate dimeric small molecule (a "dimerizer"), both fusion proteins bind the small molecule, recruiting the activation domain to the promoter of interest (Figure 5). The most widely used chemical dimerizer system is based on the small molecule binding domains FKBP and FRAP, which bind to the small molecule rapamycin. Gilman and coworkers fused the FRB (FKBP and rapamycin binding) domain of FRAP to the transcriptional activator from NF-κB to create one hybrid protein and fused a zinc-finger DNA binding domain to FKBP to serve as the second [40]. Transcriptional induction of $\geq$10,000-fold in the presence of rapamycin has been achieved using this system in mammalian cells [40].

The nature of the components of the CID strategy offer benefits compared with the previously described approaches to allosteric transcription factors. Small molecules that resemble rapamycin can be synthesized that interfere with dimerization by competition for one of the drug binding domains, providing a rapid way to shut a signal off. In addition, this approach offers very low background signal in the absence of dimerizer, because the split transcription factor domains and the drug binding domains have no instrinsic affinity for each other. A third advantage is the modularity of the system. Since signaling depends only on induced proximity, each of the domains can be substituted to achieve different DNA binding, activation, or repression functions. This modularity raises the possibility of using several orthogonal dimerizer systems in the cell to regulate separate genes of interest simultaneously [38].

Translational Control

Recent work by Breaker and coworkers has led to the discovery of RNA-mediated small-molecule regulation of translation. Termed "riboswitches," these natural translational switches are found in the 5′-untranslated region (5′-UTR) of mRNAs of many prokaryotes [41]. They consist of two domains: an aptamer region that

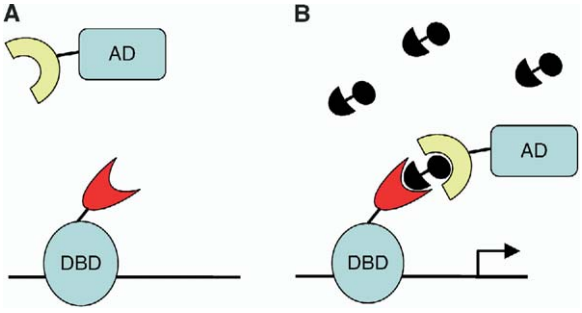

Figure 5. Chemical Inducer of Dimerization Strategy to Control Transcription

(A) In the absence of inducer, the split DNA binding and activation domains of a transcription factor do not interact.

(B) Upon addition of a chemical dimerizer, the small-molecule binding domains (red and yellow) colocalize the DNA binding domain and activation domain, resulting in transcription of a gene of interest.

binds a specific metabolite and an expression platform that interacts with regulatory elements in the mRNA, such as the Shine-Dalgarno sequence. The aptamer domains are usually 70–170 nucleotides long and bind their cognate ligands with low nanomolar to low micromolar dissociation constants. In one well-characterized riboswitch [42], the binding of thiamine pyrophosphate (TPP) blocks translational initiation by up to 16-fold in several genes involved with thiamine biosynthesis in *E. coli*. Riboswitches responsive to lysine, the nucleobases, coenzyme B_{12}, S-adenosylmethionine, and flavins have been characterized [41]. The scope of this regulation is surprising: over 70 genes in *B. subtilis* (approximately 2% of the genome) are under the control of riboswitches. The presence and conservation of riboswitches in many species of bacteria suggest that they may be primitive genetic regulatory elements that have not been replaced by protein-based machinery.

Researchers have shown that the principles of translational control seen in many natural riboswitches can be applied to the creation of artificial genetic switches. (Indeed, natural riboswitches were discovered after researchers had created similar switches in the laboratory; see the work of Green below.) The generation of RNA sequences that bind molecules not involved in cellular metabolism relies on in vitro evolution techniques. Over the last 15 years, it has been shown that RNAs can be isolated from large random libraries to bind many disparate small-molecule and macromolecule targets [43]. These RNAs, known as aptamers, can be inserted into the 5′-UTR of a gene of interest in such a way as to regulate transcriptional initiation. This step in gene expression varies in mechanism between prokaryotes and eukaryotes; two distinct approaches have therefore been developed.

In prokaryotes, occlusion of the ribosome binding site prevents translational initiation. It is thought that some natural riboswitches form RNA secondary structures that prevent ribosome binding. As ligand binding typically stabilizes these structures, most riboswitches act as repressors of translation in the presence of ligand. In a recent study, Hillen and coworkers designed an artificial riboswitch that acts as an activator, increasing translation 8-fold upon the addition of theophylline [44]. This study made use of an RNA aptamer for theophylline [45] and a structural bridge previously evolved by Soukup and Breaker [46]. Upon ligand binding, a conformational change occurs and the double-stranded structural bridge slips by one base (Figure 6). Guided by secondary structure prediction programs, Hillen designed an inhibiting structure that frees one extra base near the Shine-Dalgarno sequence upon theophylline binding, allowing initiation of translation. This example differs from most riboswitches in that the sense of regulation is reversed; addition of ligand activates, rather than represses, gene expression.

In contrast to the simpler mechanism of prokaryotic translational initiation, the eukaryotic ribosomal machinery recognizes the cap structure at the 5′ end of mRNA and scans until it forms the initiation complex at the first AUG codon. This scanning process is known to be inhibited by the binding of natural proteins (such as the iron response protein acting on the ferritin mRNA) [47]. In 1998, Green and coworkers reported the selection of aptamers to a small-molecule dye and the insertion of two such sequences into the 5′-UTR of a β-galactosidase reporter gene. Gene expression in mammalian cells decreased 10-fold in the presence of the ligand [48]. This pioneering study showed that RNA aptamers evolved in vitro can bind their ligands tightly and specifically in vivo (Figure 6) and that small-molecule binding can structure the 5′-UTR in such a way as to prohibit eukaryotic translational initiation, probably through the inhibition of ribosome scanning.

A similar mechanism of action led to inhibition of translation in *S. cerevisiae* in work reported by Wilson and coworkers [49]. The researchers inserted an aptamer previously selected in vitro to bind the small molecule tetramethylrosamine (TMR) into the 5′-UTR of the *CLB2* cyclin gene. TMR inhibited Clb2 translation in this system 10-fold. A phenotype of cell-cycle slowing and elongated cellular morphology was also observed, confirming that this methodology can alter natural biological function and serve as a genetic tool [49]. The use of aptamers to inhibit translational initiation is somewhat limited by the fact that repression greater than 10-fold has not yet been reported.

Rather than blocking the initiation of translation, Mulligan and coworkers have developed a strategy to destroy mRNA in a ligand-dependent manner through the use of self-cleaving ribozymes [50]. Screening a number of natural and engineered ribozymes for activity in vivo followed by optimization identified a mutant *Schistosoma monsoni* hammerhead ribozyme that is active in mammalian cells. The difficulty in finding a ribozyme that functions in living cells underscores an advantage of developing genetic switches in vivo: namely, that the resulting switch naturally functions in the desired context.

When inserted into a reporter mRNA immediately upstream of translational start, the optimized ribozyme self-cleaves before translation can occur, resulting in degradation of the mRNA and loss of gene expression. Mulligan and coworkers screened ~50,000 small molecules with in vivo assays to find a small-molecule ligand that inhibited ribozyme cleavage. The nucleoside analog toyocamycin inhibited mRNA cleavage and restored gene expression roughly 250-fold (for comparison, an inactive ribozyme restored expression 3000-fold). Insertion of a ribozyme-luciferase vector into a mouse model showed 40- to 190-fold induction of luciferase expression in the retina upon toyocamyin addition [50]. Unpublished experiments reveal that toyocamycin may function by incorporation into the mRNA, rather than direct binding and inhibition of ribozyme function (R. Mulligan, personal communication). When combined with evolution-based technologies that allow the creation of small-molecule-dependent ribozyme function [51] and other RNA engineering tools [52], it may be possible to engineer similar ribozymes to respond to small-molecule inducers of choice.

In summary, recent studies have shown that natural small-molecule binding aptamers can act as riboswitches to inhibit translation [41] or, when combined with ribozymes, can induce RNA cleavage [53] in bacteria. Several reports have shown that evolved aptamers can be inserted into the 5′-UTR of eukaryotic mRNAs to block ribosome scanning and initiation, or into prokaryotic

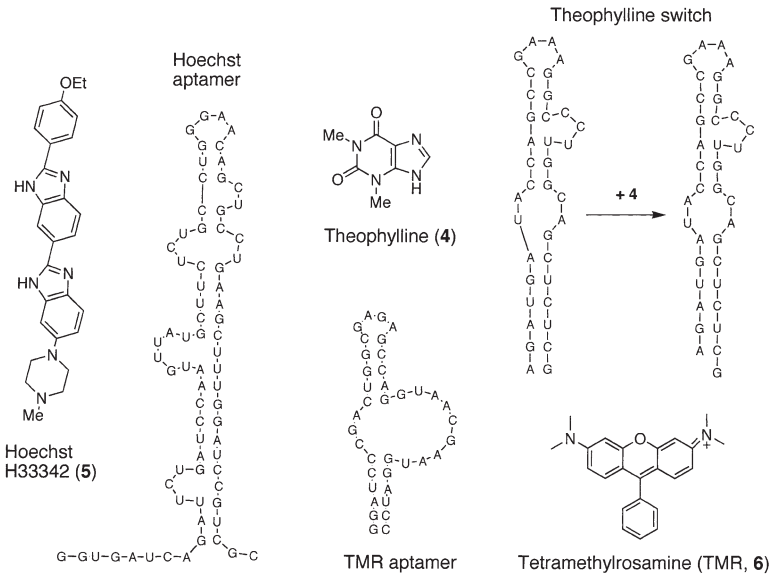

Figure 6. Structure of Aptamers and Ligands Used in Engineering Translational Control

mRNAs to block ribosome binding, though the breadth of regulation is somewhat limited. Ligand-dependent ribozymes have also been shown to destroy mRNA and effect regulation of gene expression in mammalian cells at levels that rival transcriptional control elements. These strategies do not require specialized promoters or separate regulatory proteins and greatly increase the number of inducers that can be used. Although the development of RNA-based translational switches is still in its infancy, this approach may yield faster responses than transcriptional regulation, especially in eukaryotes.

Direct Regulation of Protein Function

Posttranslational regulation of protein function can provide high temporal precision and is frequently used in natural systems to rapidly modulate protein activities. While binding of a small-molecule inhibitor to a protein target can exert an immediate effect, this direct regulation of protein function suffers from a lack of generality. Whereas a single genetic switch can regulate transcription or translation of an arbitrary gene of interest, small molecule-protein interactions are usually specific to a single protein target. The synthesis and identification of potent and selective inhibitors or activators for a target protein remain significant challenges despite improvements in synthesis and screening methods. Two strategies have recently emerged to generate genetic switches that combine generality with the rapid kinetics and dose dependence that are characteristic of small-molecule control of protein function.

Regulation of Protein Localization and Degradation

The first strategy has already been previewed above and consists of controlling protein localization with chemical inducers of dimerization. The colocalization of catalytic activities and substrates through protein-protein interactions is one of the major mechanisms of enforcing specificity in signaling pathways. In the early 1990s, two natural small molecules, FK506 and rapamycin, were found to dimerize proteins in vivo. FK506 binds to a small monomeric protein known as FKBP and also binds to and inhibits calcineurin. Rapamycin also pairs with FKBP, using a chemical substructure similar to that of FK506, but complexes and inhibits a protein known as FRAP (FKBP-rapamycin associated protein) [16]. It was soon recognized by Schreiber, Crabtree, and coworkers that these natural systems could be generalized into a method to control biological function with small-molecule dimerizers of proteins.

The first protein function artificially regulated using chemical dimerizers was signaling by the T cell antigen receptor (TCR) [54]. The signaling domain of the TCR was fused with FKBP to confer the ability to bind FK506 analogs. A semisynthetic FK506 dimer was prepared that binds FKBP but not calcineurin. The addition of the FK506 dimer oligomerized the signaling domain-FKBP fusions, activating a reporter gene downstream of the signaling pathway. The chemical dimerization strategy developed by Schreiber, Crabtree, and coworkers induces a protein-protein interaction. Chemical inducers of dimerization have proven to be effective for controlling the function of membrane receptors, protein kinases, and death domains, as well as transcription factors as described in the first section (for a list of dimerizer references, see http://www.ariad.com/regulationkits/reg_ref1.html). If the logic of induced proximity can control a protein function of interest, this strategy can be used to create small-molecule-dependent switches that act rapidly and with tunable potency.

One shortcoming of the use of the natural immunophilin ligands is that their binding proteins are endogenously expressed at high levels in many cells. Clackson and coworkers attempted to solve this specificity problem (for a review see [16]) through the creation of an orthogonal dimerizer-FKBP pair using the "bump-hole" approach. The addition of an ethyl group "bump" effectively destroyed the ability of a known synthetic ligand to bind to wild-type FKBP [55]. Analysis of the cocrystal structure and modeling studies pre-

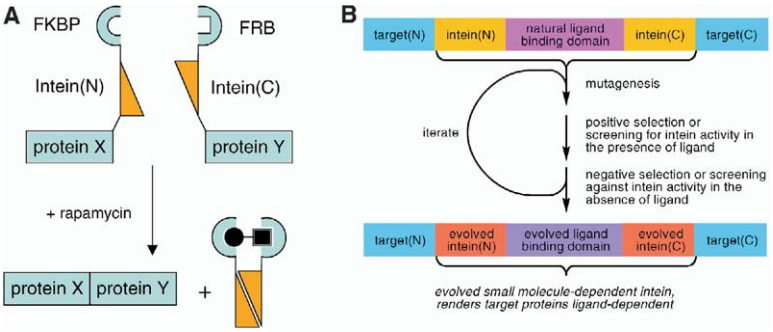

Figure 7. Small-Molecule-Dependent Protein Splicing Strategies

dicted that mutation of Phe36 of FKBP to a smaller residue (creating a "hole") would accommodate the larger ligand. The synthetic ligand AP1903 binds to the FKBP Phe36Val mutant with affinity comparable to that of wild-type FK506 for wild-type FKBP, but with 1000-fold specificity for the FKBP mutant [55].

The synthetic dimerizers described above are both homodimers, precluding their use in predictably forcing the interaction of two different FKBP-fusion proteins. The natural heterodimerizer rapamycin has good pharmacokinetic properties and binds both FKBP and the FRB domain of FRAP. Schreiber and coworkers reported the synthesis of a bumped rapamycin analog [56] which interacts only with a mutant FRB that was identified through mutagenesis and selection; this ligand-receptor pair is fully orthogonal to cellular systems.

In the course of their work to develop the orthogonal FKBP described above, Clackson and coworkers identified a mutation (Phe36Met) that reverses the ligand dependence of FKBP dimerization. This mutant FKBP forms discrete dimers that dissociate upon ligand binding [57]. Such a switch can shut off activities that are induced by proximity or turn on activities that are repressed by oligomerization. These "conditional aggregation domains" act rapidly; in one example, large aggregates of GFP were created and dissolved in vivo within minutes. In a further study, multimers of the Phe36Met FKBP mutant were used to aggregate insulin in the endoplasmic reticulum, preventing secretion [58]. Rapid and transient secretion was observed following treatment with the ligand in a mouse model.

A promising new application of the dimerization strategy provides small-molecule-mediated control of protein degradation. Rather than creating small molecules that inhibit protein function, Crews and coworkers have developed chemical inducers of dimerization that recruit the proteosome to a protein target, in effect generating a "chemical knockout" [59, 60]. Proteins are marked for degradation upon the covalent attachment of ubiquitin by a class of proteins known as the E3 ligases. Using a dimerization strategy, Crews and coworkers effectively redirected the substrate specificity of these enzymes and made the process ligand dependent.

The dimerizer ligands (termed proteolysis targeting chimeric molecules, or "PROTACS") consist of a small-molecule-based ligand for the protein target covalently linked to a short amino acid sequence recognized by an endogenous E3 ligase. The dimerizer is also attached to a poly-D-arginine tag at the C terminus to enhance cell permeability [61]. In one example, the Phe36Val mutant of FKBP described above was fused genetically to the green fluorescent protein (GFP) in HeLa cells [59]. A dimerizer consisting of an FKBP ligand similar to AP1903 and the peptide-based E3 targeting element induced a dramatic loss of fluorescence due to degradation of the GFP-FKBP protein. Fusion of a target of interest to a ligand binding protein such as FKBP may make this approach general for any arbitrary target. Proteins with natural small molecule binding sites, such as steroid receptors, can be targeted without any genetic manipulation simply by synthesizing dimerizers containing their natural ligands [59]. Although the generality, level of regulation, and detailed kinetics of the PROTACS approach are not yet known, this novel application of the dimerization system may allow small-molecule control over the important regulatory step of protein degradation.

Small-Molecule-Dependent Protein Splicing

The second strategy for engineering ligand-dependent switches that act posttranslationally is based on the natural process of protein splicing. Inteins are self-splicing protein elements, analogous to introns in RNA, which catalyze their excision from within a larger protein context (called the N- and C-exteins) [62]. Insertion of an intein into a protein target of interest typically renders the target protein inactive until splicing occurs. Ligand-dependent inteins represent attractive molecular switches because protein splicing occurs in almost any protein context and is rapid compared with transcription and translation. Natural inteins are not regulated by small-molecule ligands, however; splicing begins to take place immediately upon translation.

Two distinct approaches to the creation of ligand-dependent protein-splicing elements have been reported. In a phenomenon known as *trans*-splicing, an intein can be split into inactive N- and C-terminal halves; when combined, these halves can reconstitute an active intein structure fully capable of splicing [63]. Taking advantage of the dimerization strategy described above, Mootz and Muir developed a *trans*-splicing system in which the two intein fragments are brought together by the addition of rapamycin (Figure 7) [64]. The *S. cerevisiae* VMA intein was split such that the halves had very low affinity for one another. The N-terminal intein was fused to MBP (as an extein) and

FKBP; the C-terminal intein fragment was fused to FRB and a His_6 tag. Binding of rapamycin to FKBP and FRB colocalizes the intein fragments, which then fold together correctly and splice, yielding an MBP-His_6 product. The authors show that splicing occurs in vitro only in the presence of rapamycin and that splicing is rapid, with 50% splicing after roughly 60 min.

In a further study, Muir and coworkers reported successful conditional *trans*-splicing in mammalian cells [65]. Formation of the MBP-His_6 product occurred in vivo solely in the presence of rapamycin, with product visible after only 10 min. Splicing was shown to be tunable over a wide dynamic range depending on the amount of ligand added. The authors also showed that the splicing reaction could be stopped by addition of a competitor ligand that binds FKBP but does not recruit FRB. It should be noted that one characteristic of intein-based switches is that the splicing reaction is irreversible; once the intein is excised and the target protein is formed, it will be present until naturally degraded. In contrast, the original CID system can be shut off rapidly by addition of a competitive ligand because only protein localization and not synthesis and degradation is being regulated.

A second approach to the creation of ligand-dependent inteins relies on allosteric control of intein splicing rather than localization of split-intein halves. Since inteins do not naturally bind small molecules, we inserted the estrogen receptor ligand binding domain (LBD) between the halves of the intein, creating an intein-ER fusion that is incapable of splicing [66]. Using genetic selections in *S. cerevisiae*, we evolved intein-ER mutants capable of splicing. The ligand dependence of the resulting intein mutants was greatly improved by screening yeast cells expressing mutant intein-ER libraries inserted into GFP (Figure 7). Such "negative screening" against protein function in the absence of ligand is an important directed evolution tool when a high degree of ligand dependence is desirable.

After three rounds of mutagenesis, selection, and screening, we identified intein-ER mutants that exhibit excellent ligand dependence in yeast cells, only splicing in the presence of 4-OHT. The evolved switch acts at a posttranslational level, and spliced product appears within 30 min of exposure to ligand. When 4-OHT concentrations are varied across a wide range, the evolved intein exhibits a graded dose dependence. Insertion of one evolved intein into four different protein contexts (KanR, GFP, the endogenous yeast Ade2 protein, and LacZ) rendered each of these protein targets inactive until the inducer was added, demonstrating the generality of this switch. While early in the stages of development and application, both of these small-molecule-dependent intein strategies may serve as powerful and general tools to control protein function directly with small molecules.

Conclusions

Research in the area of small-molecule-dependent genetic switches has produced a number of mechanistic insights and powerful tools for use in genetics, cell biology, and gene therapy. Three common strategies have emerged for creating artificial molecular switches that control biological function in response to exogenous small-molecule ligands. The first strategy modifies the ligand binding specificity of a natural switch to recognize a nonnatural ligand. This strategy requires the synthesis of analogs of the small-molecule effector and engineering or evolution of its protein partner to create an orthogonal ligand-receptor pair. In addition to nuclear receptor ligand binding domains [67] and immunophilins [56], the tetracycline repressor (TetR) has been altered using directed evolution approaches to recognize new ligands [68]. The introduction of screens or selections against binding of the natural ligand may further enhance the ability of evolutionary approaches to create truly altered, rather than broadened, specificity.

The second strategy for creating small-molecule-dependent switches is to regulate protein activity through the colocalization of modular functional domains. Chemical inducers of dimerization were shown to activate transcription through induced proximity of DNA binding and activation domains [38, 39]. The dimerizer strategy is also capable of direct regulation of protein activity, for example, activating signaling pathways through the colocalization of kinases and their substrates [54] or targeting the proteosome machinery to a protein target [59]. When this approach is applied to fragments of a target protein fused to *trans*-splicing systems, a small molecule can trigger the covalent assembly of the intact protein together with the concommitant loss of the dimerization and intein domains [65].

The third strategy is to engineer allosteric regulation, manipulating a conformational change induced by ligand binding to create a novel downstream protein or RNA function. The evolution of a reversed allosteric response in the TetR protein [20] and the engineering of ligand binding sites in a DNA binding domain [14] are examples of engineered allosteric regulation of protein function with small molecules. Similarly, small-molecule aptamers evolved in vitro can be inserted into the 5′-UTR of mRNAs to effect a conformational shift in the RNA upon ligand binding that regulates translational initiation in prokaryotes [44] and eukaryotes [48]. Our studies combining functional and ligand binding domains, in both RNA-based transcription factors [35] and inteins [66], underscore the ability of directed evolution to create strong linkages between unrelated domains. We anticipate that directed evolution approaches will play an increasingly important role in creating small-molecule-dependent genetic switches for the study and control of biological systems.

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