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Conditional Control of Protein Function

Review

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Deciphering the myriad ways in which proteins interact with each other to give rise to complex behaviors that define living systems is a significant challenge. Using perturbations of DNA, genetic analyses have provided many insights into the functions of proteins encoded by specific genes. However, it can be difficult to study essential genes using these approaches, and many biological processes occur on a fast timescale that precludes study using genetic methods. For these reasons and others, it is often desirable to target proteins directly rather than the genes that encode them. Over the past 20 years, several methods to regulate protein function have been developed. In this review, we discuss the genesis and use of these methods, with particular emphasis on the elements of specificity, speed, and reversibility.

Introduction

Geneticists have taken advantage of naturally occurring as well as engineered mutations to perturb genes and the proteins that they encode. The knowledge harvested from a century of these studies is truly impressive. Unbiased genetic approaches (i.e., forward genetic screens) have been incredibly useful for discovering genes that, when mutated, give rise to identifiable phenotypes. These correlations have provided many insights into the functions of the proteins involved. The central dogma of biology (DNA makes RNA makes protein) lies at the heart of the genetic approach, so that mutations affecting a specific gene are faithfully transmitted to the protein product.

Genetic techniques can also be applied in a more targeted fashion to interrogate specific proteins of interest. This reverse genetic approach involves mutation of a particular gene of interest, followed by examination of the system to evaluate the role of the candidate protein in the process of interest. The mouse is the most complex vertebrate in which genetic techniques are tractable. In mice, reverse genetic studies often take the form of a knockout strategy, whereby homologous recombination is used to inactivate a gene of interest in embryonic stem cells. Mice derived from this procedure are heterozygous, possessing one wild-type allele and one disrupted allele of the gene of interest. Subsequent breeding of the heterozygotes can provide homozygous knockout (KO) mice possessing two disrupted alleles. KO mice have proven to be powerful experimental systems for studying certain genes; however, significant experimental limitations exist.

Disruption of a gene that is essential for development often leads to an embryonic lethal phenotype, which obviously limits the use of this approach in cases where a less drastic phenotype is the intended subject of study [1]. Another issue that can be even more difficult to control for is the possibility that, during development, a KO mouse will exhibit either cellular or molecular compensation for the disrupted gene [2]. Partial or complete compensation for the missing activity can preclude definitive interpretation of these experiments.

Nature tends to be parsimonious, and many proteins perform more than one function. It can be difficult to tease apart these different roles using basic genetic approaches. Specificity is crucial for interrogating a biological system, but methods to rapidly and reversibly perturb a system can be equally important, especially for essential genes. In order to circumvent embryonic lethality, methods to conditionally regulate gene function have been developed. In microorganisms, temperature-sensitive mutants that conditionally regulate protein function have been extremely useful [3]. The protein product is active and the organism is viable at the permissive temperature; however, moving the organism to the nonpermissive temperature inactivates the protein and allows observation of the phenotype. Although powerful for microorganisms, this approach does not translate well to vertebrates.

A variety of other methods to conditionally regulate gene function have been developed for use in vertebrates. Systems in which transcription of the gene of interest is under the control of a small-molecule-dependent promoter (e.g., tetracycline) have been used with considerable success in both cultured cells and whole organisms [4]. In theory, this strategy allows transcription to be turned on or off to control protein levels. At the operational level, the intrinsic stability of the protein limits the reversibility of this approach, and this feature is common to all transcriptional switches. Alternatively, the Cre recombinase can be used to disrupt specific gene sequences targeted with loxP sites [5]. In a mouse, when the Cre recombinase is driven by a tissue-specific promoter, tissue-specific disruption of a gene of interest is possible. Unfortunately, this approach is not reversible, and in some targets, the Cre-mediated disruption of target genes can require days or weeks in mice [6].

Targeting DNA sequences provides exquisite specificity; however, there is considerable room for improvement in terms of speed and reversibility. Posttranscriptionally, at least two techniques can be used to perturb mRNAs of interest. RNA interference shows immense promise and is currently the subject of intense study [7, 8]. Additionally, RNA regulatory elements that bind to small molecules (i.e., riboswitches) may also prove useful for perturbing specific genes [9]. These transcriptional and posttranscriptional strategies have been recently reviewed by Buskirk and Liu [10], and we will focus instead on techniques that directly perturb protein function.

Given their long and distinguished history as therapeutic agents, cell-permeable small molecules may be the

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best candidates to conditionally regulate protein function. Stuart Schreiber has articulated the similarities and differences between traditional genetic approaches and chemical genetic strategies for investigations of biology [11]. The use of diversity-oriented synthesis as an engine of discovery might lead to an inhibitory (and activating) ligand for every protein of interest. The day may come when one can rapidly and reversibly inhibit a specific protein without the need for any molecular biological intervention, and Nature has provided us with several small molecules that prove the principle. It may only be a question of time. How long will it take to assemble this collection of reagents? Furthermore, what metrics will be used to evaluate specificity? Even with an efficient discovery process, validating the specificity of chemical genetic tools presents a formidable challenge.

As an alternative to the "one ligand-one protein strategy" described above, one might build upon one or more known protein-ligand interactions to regulate the activity of a specific protein of interest. Properly engineered, genetic fusion of a gene of interest to a sequence encoding a ligand binding domain would produce a chimeric protein, the function of which could be regulated by a cell-permeable ligand. Ideally, this strategy would be general, so that many different proteins, either individually or as ensembles, could be conditionally perturbed using a single protein-ligand system. This "one ligand-many proteins" approach would require the use of molecular biology to fuse a ligand binding domain to the protein of interest. However, this investment (i.e., creating transgenic or knockin cell lines or mice) returns a significant dividend. Targeting the DNA ensures the specificity of this approach, and the use of cell-permeable ligands to perturb function provides the elements of speed and reversibility.

Properly implemented, a relatively small number of cell-permeable ligands could be evaluated, and their intrinsic biological activities could be cataloged with some confidence. In ideal cases, these cell-permeable molecules would be biologically silent, so that off-target interactions, if they exist, would not perturb cellular processes. Biological effects would result solely from interactions with the cognate ligand binding domains. In practice, investigators may have to control for the intrinsic activity of the perturbing ligands. Additionally, the requisite molecular biological interventions should be minimal to avoid altering the function of the protein of interest. A single genetic fusion to a protein of interest might be expected to be less perturbing than two or more artificial receptors that require posttranslational modification and specific subcellular localization.

The development of strategies to directly control the function of specific proteins began in the late 1980s, and this review focuses on those systems. Not all of the strategies allow conditional control, and not all of the conditional systems utilize small molecules as the regulatory agents. We will attempt to describe the conceptual background of each strategy to illuminate the different points of intervention. We will also try to point out specific advantages and disadvantages of each approach. An ideal experimental system to conditionally regulate protein function would be useful for any protein of interest, predictable in its behavior and easy to implement, rapid, reversible, and specific.

Hormone-Regulated Protein Function

Nuclear hormone receptors form a class of ligand-activated proteins that serve as transcription switches for a large number of genes involved in cellular development, differentiation, and regulation [12]. These receptors bind to hormones such as estrogen, dexamethasone, and progesterone, although the majority of family members do not have known ligands. Nuclear hormone receptors are comprised of several conserved regions, including a DNA binding domain and a hormone binding domain, with the hormone binding domain serving an inhibitory role in gene expression. Although cellular localization seems to be hormone dependent for some hormone receptors, with cytoplasmic localization in the absence of hormone, and nuclear localization in its presence, several hormone receptors have been shown to exhibit nuclear localization even in the absence of hormone. The most likely scenario is that the unliganded receptor establishes an equilibrium distribution between the cytoplasm and the nucleus, with transcriptional activation occurring only in the presence of hormone [13].

In an attempt to decouple the functions of various domains of the nuclear hormone receptor, Yamamoto and coworkers discovered that hormone binding domains are able to control the activity of heterologous transcription factors [14]. Initially, the authors excised the DNA binding domain from the glucocorticoid receptor (GR) and replaced it with the LexA DNA binding domain. Strikingly, hormone regulation of the protein proved to be modular, as dexamethasone regulated genes under control of the LexA promoter. Additional studies showed that the hormone binding domain alone was able to exert control over exogenous elements [15]. When the hormone binding domain of the GR was fused to E1A, an adenovirus transcription factor, the chimera showed hormone-dependent activity. In the absence of hormone, the promoter controlled by E1A was strongly repressed, with activation observed only upon addition of hormone (Figure 1A).

While these studies showed hormone-dependent activation of a GR-E1A fusion protein, they also showed that this hormonal regulation was not due entirely to subcellular localization, as the fusion protein showed some nuclear localization even in the absence of hormone [15]. Interestingly, these studies also showed that the ability of the hormone binding domain to repress activity of the heterologous protein decreased as the length of the peptide linker between the protein of interest and the hormone binding domain increased, with the length of the peptide sequence to some extent indicating a spatial separation of the two domains. It was postulated at this point and later shown that repression of E1A activity might be due in part to association of the unliganded fusion protein with the chaperone Hsp90 complex [16]. This finding offered an explanation for the distance dependence observed. Hsp90 associates with the unliganded hormone binding domain, and Hsp90's ability to sterically interfere with the function of the protein of interest might be expected to decrease with increased spatial separation.

The role of the Hsp90 complex in regulating HBD fusions is further supported by studies of a fusion between the Myc oncogene and the estrogen receptor HBD (Myc-ER) [17]. In the presence or absence of ligand

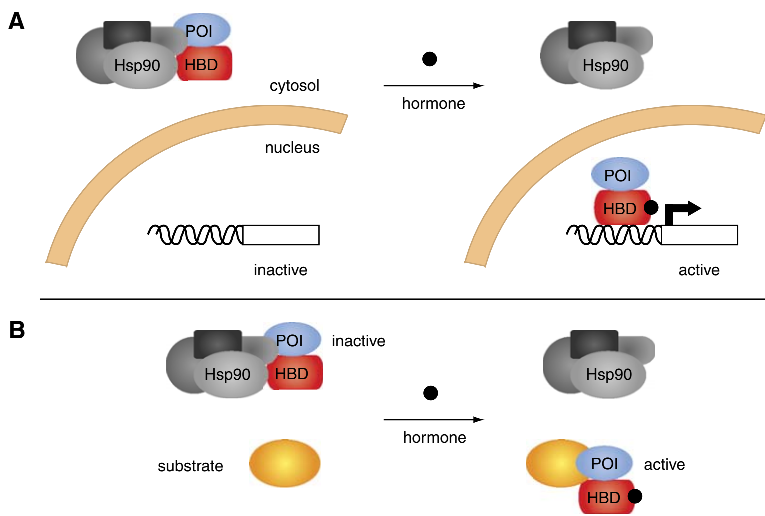

Figure 1. Hormone Binding Domains Control the Activity of Exogenous Elements in a Hormone-Dependent Fashion: Depiction of Two Possible Scenarios

(A) A hormone binding domain (HBD) fused to a transcription factor confers hormone-dependent activity on a promoter. Nuclear localization signals on the HBD are obscured by the Hsp90 complex, rendering the cytoplasmic transcription factor inactive. Hormone binding causes an allosteric change in the HBD that leads to dissociation of the Hsp90 complex and subsequent translocation into the nucleus, where the transcription factor regulates gene expression.

(B) Fusion of an HBD to a cytosolic protein of interest (POI) confers ligand-dependence on its activity. Association of the HBD fusion with the Hsp90 complex precludes protein-protein interactions between the POI and its relevant partners. Hormone binding causes an allosteric change in the HBD that leads to the dissociation of the Hsp90 complex, allowing the POI to interact with its relevant substrate or binding partner.

See text for complete details.

the Myc-ER fusion exhibits nuclear localization due to signals on the Myc protein, however nuclear localization alone is not sufficient for activity. The Myc-ER fusion protein shows activity only in the presence of estrogen, suggesting that the Hsp90 complex plays a significant role in suppressing Myc function. Thus, regulation of the hormone binding domains can be regarded as a combination of subcellular localization effects as well as inhibitory steric effects due to complexation with Hsp90.

The involvement of Hsp90 in protein regulation reveals one of the shortcomings of using hormone binding domains to control protein activity. While cytoplasmic events have been controlled in this manner, the ability of the Hsp90-associated hormone binding domain to repress activity of the protein of interest is limited to proteins involved in macromolecular interactions, such as protein-DNA binding or protein-protein interactions (Figure 1B). Indeed, enzymes, such as Ura3p, DHFR, and galactokinase, which utilize small-molecule substrates, could not be regulated by this method [18, 19].

Despite this limitation, conditional control of protein function using hormone binding domains has been quite successful. Activation of the protein of interest is rapid and reversible, because activity is small-molecule dependent. A variety of hormone binding domains, including but not limited to the androgen, estrogen, glucocorticoid, and progesterone receptors, can be used; thus, several proteins could in theory be regulated independently and specifically in the same system. Despite the fact that these steroid ligands often possess intrinsic biological activity, this approach has been shown to work across many cell lines and transgenic organisms, and has allowed for conditional regulation of dozens of transcription factors and kinases [19].

Stability-Based Degradation

The N-End Rule and Protein Stability

In 1986, Varshavsky and coworkers discovered that the half-life of a protein depends, in part, upon the identity of its N-terminal residue [20]. Chimeric proteins comprised of an N-terminal ubiquitin domain (Ub; 76 residues) followed by β-galactosidase were rapidly processed in yeast by a deubiquitylating protease to yield free ubiquitin plus the remainder of the fusion protein. The stability of the liberated β-galactosidase became a function of the newly revealed N-terminal residue, the identity of which could be controlled via site-directed mutagenesis. It was found that the N-terminal residues Met, Ser, Ala, Thr, Val, and Gly led to stable proteins ($t_{1/2}$ > 20 hr), whereas the N-terminal residues Phe, Leu, Asp, Lys, and Arg dramatically reduced the half-life of the protein ($t_{1/2}$ ~2–3 min) in yeast. When Varshavsky and coworkers attempted to test the generality of this phenomenon in yeast by preparing chimeric proteins with dihydrofolate reductase (i.e., Ub-X-DHFR), they noted that cellular lifetimes of the X-DHFR proteins were similar, regardless of the N-terminal residue [21]. This led to the realization that the identity of the N-terminal amino acid is important, but not sufficient, to confer instability to the protein of interest.

Varshavsky's initial results were obtained using a fusion protein that contained a 45 residue linker between the ubiquitin and β-galactosidase domains [20]. This linker sequence was derived from an internal segment of the *lac* repressor (lacI), and is not found in wild-type β-galactosidase. Their second-generation studies using DHFR lacked this peptide sequence [21]. When the lacI linker was inserted between ubiquitin and DHFR (i.e., Ub-X-lacI-DHFR), the processed proteins showed N-terminal-dependent stabilities similar to those observed for the X-β-galactosidase proteins. A Lys-Arg-Lys sequence found in the lacI linker is essential for its ability to confer instability. It was shown that degradation of these proteins requires recognition by an E3 ubiquitin ligase, as well as accessible lysines as sites of ubiquitylation. The two lysines present in the lacI sequence likely serve this purpose, thus increasing the rate of degradation for those proteins recognized by the E3 ligase. The polyubiquitylated proteins are then targeted for degradation by the proteasome.

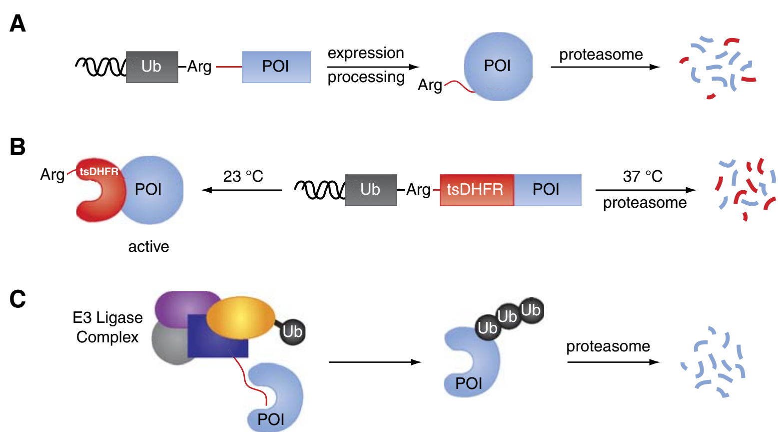

Figure 2. Posttranslational Control of Protein Stability

(A) A destabilizing N-terminal amino acid confers instability to a POI. Ub-Arg-POI is cotranslationally processed to reveal a destabilizing residue at the N terminus of the POI, targeting it for ubiquitylation and degradation.

(B) Temperature-sensitive DHFR fusions provide temporal and reversible control over the function of a POI. The processed Arg-DHFR-POI fusion is functional at the permissive temperature, but is targeted for ubiquitylation and degradation at the nonpermissive temperature.

(C) Localization of a POI is sufficient for degradation. A peptide ligand that binds to a POI is covalently fused to the E3 ligase complex. Association of the POI with its peptide ligand brings the protein in close proximity to the E3 ligase, leading to its ubiquitylation and degradation.

Following this work, Szostak and coworkers postulated that the lacI peptide fragment might be sufficient to confer instability to any number of different proteins [22, 23]. To this end, a Ub-lacI fusion was designed as a portable cassette that could be placed at the N terminus of a protein of interest expressed in yeast. Szostak and coworkers designed chimeric proteins in which one cassette revealed a stabilizing Met residue upon processing, whereas a second cassette revealed a destabilizing Arg residue. As expected, the processed protein containing an N-terminal Met retained activity, and the processed protein possessing an N-terminal Arg showed no activity due to rapid degradation (Figure 2A).

Conditional N-End Rule

Szostak and coworkers were the first to use a portable "degron" to control the stability of a heterologous protein, but the approach did not offer conditional control of protein function and, in practice, is similar to that of a genetic KO. Varshavsky and coworkers expanded this approach to offer temporal control and reversibility [24]. A DHFR mutant, in which a destabilizing Arg replaced the wild-type N-terminal Val, was used to develop a temperature-sensitive (*ts*) allele. Genetic selection in yeast was used to identify a mutant protein that was stable and active at the permissive temperature (23ºC), whereas increasing the temperature to 37ºC resulted in protein degradation. The Arg-DHFR^{ts} was then fused to Cdc28, an essential component of the cell cycle oscillator. The Arg-DHFR^{ts}-Cdc28 fusion protein is long-lived and active at 23ºC, but phenocopies a *ts*-Cdc28 allele at 37ºC (Figure 2B). These experiments demonstrate the potential for engineering new *ts* alleles for any protein of interest as long as the resulting Arg-DHFR^{ts} fusion protein is functional. To date, this method has been used to probe the function of multiple proteins, including a genome-wide study of essential genes of unknown function in fission yeast [25].

Degradation by Association

Both the Szostak and Varshavsky approaches require that the protein of interest be targeted for degradation by the proteasome through ubiquitylation. This goal is achieved by fusing the protein of interest to an unstable protein or peptide fragment that is recognized by one or more of the enzymes involved in protein recognition and proteasomal degradation. A more direct approach to induce degradation was taken independently by Howley and coworkers, as well as Gosink and Vierstra. Both groups targeted proteins of interest directly to enzymes known to be involved in proteasome-mediated protein degradation. Gosink and Vierstra created a chimeric protein comprised of a functional E2 domain fused to a peptide sequence that served as a ligand for a protein of interest [26]. Colocalization of the protein of interest with the E2 ubiquitin-conjugating enzyme was sufficient for monoubiquitylation under cell-free in vitro conditions. Howley and coworkers used a similar approach, in which the ligand for the protein of interest was fused to a subunit of an E3 ligase complex (Figure 2C) [27]. These studies were successful in targeting an otherwise stable protein for degradation in cultured mammalian cells, with the expected phenotype observed upon degradation of the target protein. This strategy of taking advantage of protein-protein interactions to noncovalently associate a degron with a target protein has been applied for proteasome-mediated degradation using both ubiquitin-dependent [28–30] and ubiquitin-independent [31] mechanisms.

Taken together, these studies involving both N-end rule-mediated degradation and localization-based degradation laid the foundation for the development of general methods for regulating protein function at the posttranslational level. These methods differ from traditional small-molecule control of protein function and traditional temperature-sensitive alleles in that they attempt to offer a general solution for targeting a large number of proteins. Szostak and coworkers controlled the posttranslational stability, and therefore function, of their target protein through genetic incorporation of a degradation signal; however, their approach offers no temporal control, and is neither tunable nor reversible. Temperature-sensitive alleles, such as those developed by Varshavsky and coworkers, are typically fast-acting alleles, with responses observed on the order of minutes, but their use is limited to systems where transition from permissive temperatures to nonpermissive temperatures is

feasible and facile (i.e., microorganisms or cultured cells). Also, the response observed using *ts* alleles is not tunable, and it is often unclear that the observed results are due to inactivation of the protein of interest, as the increase in temperature can often cause changes in the activity of off-target proteins (i.e., induction of the heat-shock proteins). Although Gosink and Vierstra, along with Howley and coworkers, offered a more direct approach for targeting a protein of interest to the proteasome, the methods inherently lack conditional control.

Small Molecules as Regulators of Protein Stability

In an effort to enhance the temporal control and generality of their DHFR *ts* allele, Varshavsky and coworkers engineered a DHFR protein, the stability of which was regulated by the high-affinity ligand, methotrexate (MTX, K_d < 1 nM) [32, 33]. Ubiquitin-DHFR fusions containing the lacI linker were prepared, which, after ubiquitin processing, revealed a destabilizing Arg at the N terminus of DHFR. In the absence of MTX, Arg-DHFR levels were considerably reduced, whereas the presence of MTX inhibited Arg-DHFR degradation. Although this high-affinity DHFR ligand inhibited Arg-DHFR degradation, it did not inhibit recognition of the protein by an E3 ligase, and therefore did not inhibit polyubiquitylation of the protein.

In this scenario, it is likely that the polyubiquitylated Arg-DHFR is targeted to the proteasome, but cannot be degraded, as the stable protein-ligand complex precludes protein unfolding. This may create a new problem, in that the proteasome may be continually occupied with a protein that it cannot degrade. This situation is likely to inhibit degradation of other cellular proteins, thereby disrupting the regulatory function of the ubiquitin-proteasome system [34]. An additional difficulty in implementing this approach in cultured mammalian cells and vertebrates is the use of MTX as the small-molecule regulator. As discussed, it is desirable that the regulatory small molecule chosen be biologically silent, but MTX is a potent inhibitor of DHFR and nucleic acid biosynthesis.

Although the MTX-DHFR system was the first to attempt to achieve small-molecule-dependent protein stabilization, it was not the first example of using a binding interaction between an engineered protein and a cell-permeable small molecule to control a cellular process. In the early 1990's, researchers discovered that the natural product FK506 possessed the ability to heterodimerize two proteins, FKBP12 (hereafter FKBP) and calcineurin [35]. In an example of research benefiting from Nature's design, a semisynthetic small molecule, FK1012, was engineered to conditionally dimerize two FKBP domains and any chimeras thereof [36]. This strategy offered a general method for conditional control of processes that require protein complex formation, and has been variously used as a transcription switch [37], a cell-death switch [38], and a control for an artificial signaling system [39]. Among the advantages to using the FK1012 system are the dose-dependent response and reversibility provided by small-molecule control, as well as FK1012's lack of intrinsic biological activity. FK1012's target, FKBP, is highly abundant, yet nonessential.

One disadvantage to the FK1012 system is the difficulty in controlling association of two different FKBP chimeras, where three dimerization products are possible. For example, if protein A and protein B were individually fused to FKBP domains, the productive heterodimerization of proteins A and B would occur only in the presence of FK1012. However, nonproductive homodimerization of two A-FKBP fusions, as well as that of two B-FKBP fusions, would be impossible to prevent. Rapamycin, a natural product that simultaneously binds to FKBP and a small domain of the TOR protein, known as FRB, supplied a tool with which selective protein heterodimerization could be achieved. Proteins of interest can be expressed as fusions to either FKBP or FRB, and their association can be induced with rapamycin [40]. Many methods to regulate cellular events have taken advantage of the FKBP•rapamycin•FRB ternary complex [41–47]. The system is attractive because rapamycin binds very tightly to both proteins [48], and because FKBP is a highly abundant yet nonessential protein that can be found in most tissue types. However, rapamycin's intrinsic ability to inhibit the TOR protein is a constant source of cellular perturbation. In rapamycin-based technologies, not only does rapamycin bind to FRB and associated fusions, but it also binds to and inhibits TOR, leading to arrest of growth and proliferation in many cell types [40]. In mice, rapamycin is teratogenic to developing embryos, precluding its use in such studies [49].

In order to bypass these difficulties, rapamycin analogs have been synthesized that contain large substituents at the FRB binding interface, resulting in a greatly reduced affinity of these "bumped" rapamycin variants for FRB. A triple mutant of FRB (called FRB*) with a compensatory "hole" restores binding specificity for C20-methallyl-rapamycin (MaRap) [50]. Initially, Crabtree and coworkers fused FRB* to GSK-3β with the goal of using the MaRap to conditionally export GSK-3β from the nucleus [51]. The GSK-3β-FRB* fusion was knocked in to mice, allowing expression of the engineered protein under the control of its endogenous regulatory promoter. Interestingly, the GSK-3β-FRB* fusion protein showed lower expression levels than the otherwise identical fusion to wild-type FRB. Furthermore, mice homozygous for the GSK-3β-FRB* allele phenocopied the conventional GSK-3β KO, which is embryonic-lethal. These observations suggest that the three mutations necessary to restore MaRap binding were detrimental to FRB* stability, and that this instability was conferred to GSK-3β. The instability of these FRB* fusions could be reversed by recruiting endogenous FKBP to the GSK-3β-FRB* fusion protein using either rapamycin or MaRap (Figure 3A). Finally, the kinase activity of the GSK-3β-FRB* fusion protein was restored due to inducible stabilization by MaRap, both in cultured cells derived from knockin mice as well as in mouse embryos themselves [51].

Fusion of a protein of interest to FRB* may be a general method with which to control protein function, as FRB* has been shown to confer instability to a variety of proteins ([51] and J.E. Gestwicki et al., submitted). The method offers predictable, conditional, reversible, and dose-dependent destabilization of a protein of interest; however, the current drug of choice, MaRap, is

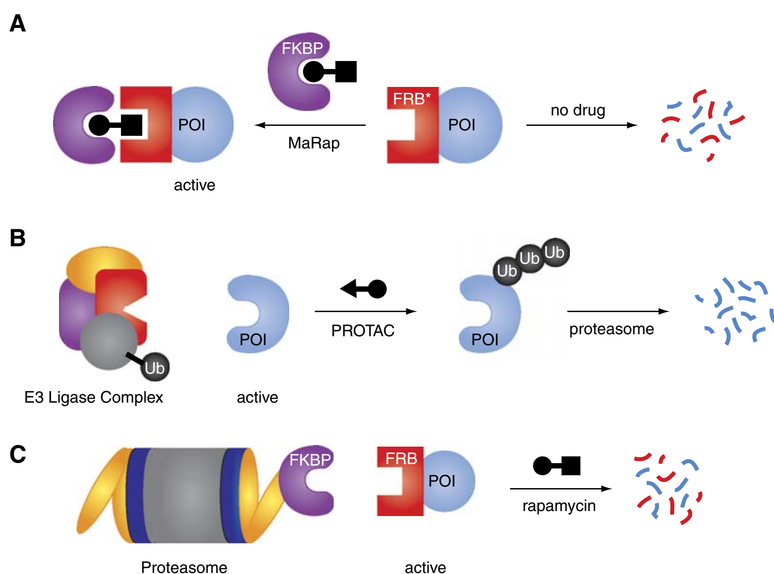

Figure 3. Small-Molecule-Dependent Protein Stability

(A) Methallyl-rapamycin (MaRap) provides rapid, reversible, and dose-dependent regulation of a POI. Instability of the FRB* mutant is conferred to a POI, leading to proteasomal degradation of the fusion protein. Formation of the FKBP•MaRap•FRB* ternary complex stabilizes FRB*, in turn stabilizing the POI.

(B) PROTACS provide rapid, reversible, and dose-dependent control of function of a POI. A PROTAC comprised of an E3 ligase ligand conjugated to a ligand for a known POI causes colocalization of the E3 ligase complex and the POI. This colocalization is sufficient for ubiquitylation and subsequent degradation of the POI.

(C) Rapamycin allows for rapid, reversible, and dose-dependent control of protein function. FKBP is fused to a noncatalytic subunit of the proteasome, and FRB is fused to a POI. Formation of the FKBP•rapamycin•FRB ternary complex causes colocalization of the POI to the proteasome, which is sufficient for degradation of the presumably unubiquitylated POI.

expensive, difficult to formulate, and exhibits poor pharmacokinetic properties in vivo. Identification of new protein/ligand pairs will be necessary in order to make in vivo studies feasible [52; this issue of *Chemistry & Biology*]. Identification of multiple protein/ligand pairs might even permit orthogonal control of multiple protein targets ([52] and J.E. Gestwicki et al., submitted).

Engineering Small-Molecule Specificity

When analyzing data from studies involving small-molecule perturbation of protein function, the question of specificity is of the utmost importance. Often, a drug will effectively regulate the function of a protein of interest, but proteomic analysis will show that it also affects at least one, if not many, off-target proteins [53–55]. Shokat and coworkers wished to use a small molecule to control the function of a specific protein kinase, a task that has been particularly difficult due to the size and homology of the protein kinase family. KOs and traditional conditional alleles have been used to study some kinases, but these approaches have been unsuccessful for kinases essential for development, or because the kinase-related events of interest occur on a timescale faster than control of the conditional allele.

To address these issues, Shokat and coworkers developed a method by which individual kinases could be targeted using a small molecule as either a specific substrate or inhibitor for a specific kinase isoform [56, 57]. The approach relies on modifying the active site of the protein of interest in such a way that the kinase maintains function, but can be differentiated from its family members. Typically, this approach involves the replacement of a large conserved residue in the ATP binding site with a small glycine or alanine, thus creating a “hole” in the binding pocket. A known kinase inhibitor possessing activity against many enzymes can then be chemically modified with a large substituent, or “bump,” that specifically complements the mutation introduced in the protein. The large substituent prevents the inhibitor from binding to wild-type kinase family members. This approach has been used successfully to probe the functional roles of several ATPases and GTPases, both in cell culture and in mice [58, 59]. The large number of sequences within these protein families, coupled with sequence conservation among the members, allows this approach to be used in a general fashion to interrogate many proteins using straightforward molecular biology manipulations.

Degradation by Small-Molecule-Induced Association

In a conceptual extension of the previously discussed work targeting proteins of interest to E2 and E3 enzymes, Crews and Deshaies have collaboratively developed an approach that uses a bifunctional molecule to mediate degradation of a protein of interest [60–62]. The bifunctional molecules, termed proteolysis targeting chimeric molecules (PROTACS), are heterodimeric molecules comprised of a targeting ligand tethered to a peptide. The targeting ligand binds to a protein of interest, and the peptide sequence is a known ligand for an E3 ligase complex. Studies show that PROTACS induce colocalization of the protein of interest to the E3 ligase complex, and that formation of this ternary complex is sufficient for ubiquitylation and subsequent degradation of the target protein (Figure 3B). Initially, these studies were performed using *Xenopus* extracts, as the PROTACS are not cell permeable [60]. Addition of a poly-D-arginine peptide sequence allowed the PROTACS to enter cells, and this technique has been shown to be effective in cultured cells [61].

Church and coworkers recently reported a complementary approach, in which proteasomal degradation is used to control activity of a protein of interest [63]. Rather than targeting the protein of interest for ubiquitylation followed by degradation, the protein of interest is targeted directly to the proteasome. Church and coworkers fused FKBP to various noncatalytic subunits

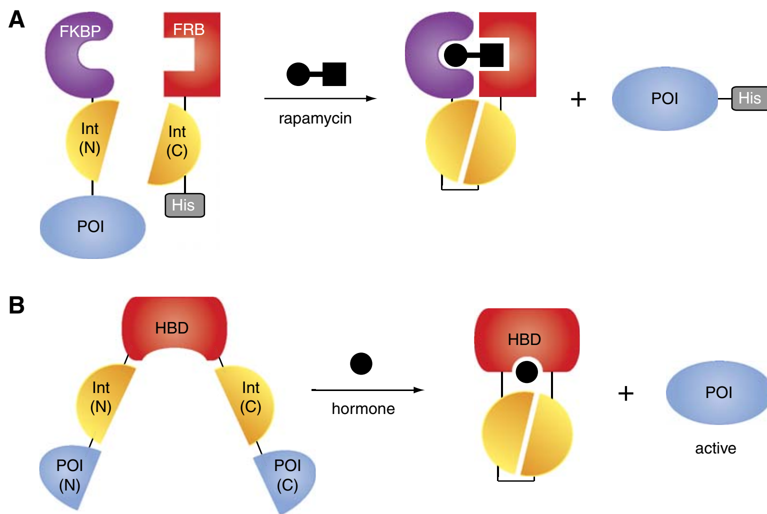

Figure 4. Small-Molecule-Dependent Protein Splicing

(A) Rapamycin provides rapid, reversible, and dose-dependent control of protein splicing. An intein is divided into two fragments (IntN and IntC) that are fused to FKBP and FRB at the endo (nonsplicing) positions. Two peptide or protein sequences of interest are placed at the splicing positions. Addition of rapamycin to form the FKBP•rapamycin•FRB ternary complex brings the two intein fragments into close proximity, which is sufficient to facilitate splicing and covalent ligation of the peptide sequences at the extein positions.

(B) Estrogen allows for rapid, reversible, and dose-dependent control of protein splicing. Both the POI and an intein are split into two fragments, with the intein fragments separated by HBD. Addition of estrogen causes an allosteric change in the hormone binding domain, bringing the intein fragments into close proximity. This colocalization allows for intein splicing and functional reconstitution of the POI.

of the proteasome, and FRB was fused to a protein of interest. These two chimeric proteins were then expressed in yeast. In the absence of rapamycin, no interaction between the two proteins is observed, and the protein of interest is stably expressed. Target protein levels decrease when rapamycin is added, demonstrating that localization to the proteasome is sufficient for degradation (Figure 3C). This approach could be used to achieve rapid and reversible control of stability and function for a wide variety of proteins, provided that the fusion protein maintains its native activity.

While both of these methods target proteins of interest for degradation, they differ in several fundamental ways. PROTACS mediate degradation of the protein of interest through ubiquitylation by involving both the E2 and E3 complexes and the proteasome. Church and coworkers bypassed the ubiquitylation machinery by localizing the presumably unubiquitylated protein of interest directly to the proteasome. Crews and Deshaies point out that one strength of PROTACS is the fact that molecular biology manipulations are unnecessary. A known ligand for the protein of interest can simply be covalently linked to a known E3 ligase substrate. In the absence of a known ligand, some form of ligand discovery would be required to design and synthesize the necessary bifunctional molecule, which means that generality is limited to proteins with known high-affinity ligands. In contrast, Church and coworkers view their approach as a modular method to conditionally control function of a large number of proteins through fusion to FRB. This is certainly true to the extent that investigators can use yeast as their model organism; however, this approach would require significant experimental investment for use in vertebrates, and rapamycin's inhibitory effects on TOR would need to be addressed.

Fragment Complementation and Conditional Protein Splicing

Recently, fragment complementation has been developed as an alternative to the yeast two-hybrid system [64]. The yeast two-hybrid system is typically engineered as a transcriptional switch that requires protein-protein interactions to occur in the nucleus [65]. Fragment complementation allows direct detection of protein-protein interactions in a wider variety of cellular environments. For example, β-galactosidase can be split into two noncatalytic fragments, and each fragment can be fused to a protein of interest. When properly crafted, β-galactosidase enzymatic activity will be reconstituted only when the two proteins of interest show a reasonable affinity for one another [66]. Similar systems have been developed employing ubiquitin [67], β-lactamase [68], DHFR [69], GFP [70], and adenylate cyclase [71] fragments as sensors for protein-protein interactions. Along these lines, several groups have developed additional technologies with which to control protein function at the posttranslational level.

Muir and coworkers have developed a method of conditional protein splicing to control protein structure in vitro and in mammalian cell culture [72]. During protein splicing, an intervening sequence, termed an intein, autocatalytically excises itself out of a precursor polypeptide, with concomitant linkage of the two flanking sequences through a peptide bond. When properly designed, the presence of an intein renders the precursor polypeptide inactive, and function is restored upon protein splicing. Muir and coworkers fused two domains of interest to the N and C termini of a *cis*-splicing intein. They then divided the intein into N- and C-terminal domains. At this site of division (the nonsplicing positions), they fused FKBP to one half of the intein and FRB to the other half, creating two separate polypeptides that can be colocalized with rapamycin (Figure 4A). The FKBP and FRB domains heterodimerize in the presence of rapamycin, and, in turn, the intein is reconstituted due to close proximity of the two domains. The reconstituted intein is then able to self-splice, covalently linking the two domains of interest.

Studies have successfully linked a target protein to an epitope tag in cell culture, but they have not as yet reconstituted a functional protein of interest. This method has also been used to conditionally regulate protein

Table 1. Conditional Control of Protein Function

Method	Conditional	Reversible	General
Hormone-dependent protein function	Yes	Yes	Limited to targets involved in macromolecular interactions
Degron cassette based upon the N-end rule	No	No	Yes
ts-DHFR cassette	Yes	Yes	Limited to cultured cells or microorganisms that can be subjected to temperature shifts
E2/E3 localization-based degradation	No	No	Yes
Small-Molecule-Dependent Protein Stability			
FKBP-MaRap-FRB*	Yes	Yes	Shown to be general in mice and primary cell lines
PROTACS	Yes	Yes	Requires a known ligand for the protein of interest
Degradation due to localization to the proteasome	Yes	Yes	Limited to yeast due to its use of rapamycin
Small-Molecule-Dependent Protein Splicing			
Rapamycin-dependent intein splicing	Yes	Yes	Depends on properties of the engineered protein
Hormone-dependent intein splicing	Yes	Yes	Depends on properties of the engineered protein

kinase A when rapamycin-mediated intein splicing is used to excise an autoinhibitory peptide [73]. One drawback to this technique might be the difficulty in predicting appropriate cleavage sites and the necessary fusion orientation for the protein of interest such that the protein is active when reconstituted through splicing. The technique might also be limited by a lack of reversibility and the fact that some fragments may be unstable or unable to fold outside the context of the complete protein.

Liu and coworkers took a different approach to achieve small-molecule dependence [74]. The authors wished to exert small-molecule control over the typically autocatalytic process of intein splicing. To realize this goal, an intein must acquire the ability to bind to a small molecule, and this binding event must translate into a signal that initiates splicing (Figure 4B). Liu and coworkers used molecular evolution to achieve these goals. Small-molecule dependence was conferred upon intein splicing by inserting an estrogen binding domain (ER) into the middle of an intein. However, simple insertion of a ligand binding domain did not result in ligand-dependent splicing.

Error-prone PCR was used to generate a library of point-mutated intein(N)-ER-intein(C) genes, which resulted in mutants that showed splicing activity both in the presence and absence of ligand. These clones were subjected to several additional rounds of selection and screening to identify an intein that showed strong and specific ligand-dependent activity. The generality of this method was demonstrated by inserting the evolved intein(N)-ER-intein(C) regulatory element into four different proteins, and all four displayed small-molecule-dependent intein excision to reconstitute protein function.

Conclusions

Many factors contribute to the power of genetics as an approach for studying living systems, but two particularly stand out in the context of this review. First, DNA perturbations are faithfully transmitted to the proteins that they encode. This fact typically ensures a high degree of specificity between a particular mutation and the observed phenotype. Second, genetic approaches usually involve the study of proteins in their natural environments. Proteins, either wild-type or mutated, are expressed and observed in the context of a living cell or organism. The native environment is a single cell for microorganisms, such as bacteria and yeast. This native environment is more heterogeneous for multicellular organisms, such as worms, flies, and mice. Regardless, the proteins under investigation are typically expressed in the usual quantities and are allowed to interact with their usual partners (i.e., cofactors or other proteins). New methods to directly interrogate protein function should try to achieve the same goal: perturbations of specific proteins in their natural environments.

In a sense, large-scale genome sequencing efforts have provided life scientists with parts lists for various organisms of interest. One of our current challenges, and it is likely to be a challenge for some time, is to learn how all of these parts interact with each other to give rise to the complex systems that govern life. If the successes of genetics have been an inspiration, then one way of approaching this formidable challenge would be to perturb the system of interest and monitor the results of one or more perturbations. However, a detailed understanding of these systems will require new tools for interrogation. We will need to invent new ways to specifically perturb proteins, and these methods will ideally allow perturbation of function that is rapid and reversible.

In the near term, several of the modular approaches discussed in this review appear to be attractive techniques for regulating the function of specific proteins (Table 1). The use of a small molecule to regulate either protein stability or protein splicing provides a reasonably rapid and specific degree of control over protein function. An essential protein could be expressed through key developmental milestones, and could be reversibly and conditionally inactivated at later time points. A significant investment of effort is required to implement these approaches in the least disruptive manner. However, the specificity that is achieved by targeting the endogenous genetic locus makes these approaches

attractive, especially in the context of the complex mixture of potential targets that is the cellular environment.

When one takes a longer view of the challenge of interrogating the roles of specific proteins, a disadvantage appears when considering these modular approaches. Many proteins play more than one role in cells, and methods that control protein existence do not provide an obvious mechanism to probe more than one function. The case of one member of the phosphoinositide 3-kinase (PI3K) family is illustrative.

Several groups independently showed that KO mice lacking PI3Kγ were apparently healthy but had reduced immune responses when challenged with inflammatory stimuli [75–77]. This finding would suggest that specific inhibitors of PI3Kγ might be powerful anti-inflammatory drugs. However, as time progressed and the mice were monitored further, it became apparent that the PI3Kγ KO mice had a cardiac defect characterized by increased contractility [78]. This observation would understandably reduce the attractiveness of PI3Kγ as a potential drug target. More recently, Hirsch and coworkers engineered knockin mice in which the endogenous PI3Kγ allele was replaced with a mutated version that is catalytically inactive [79]. These kinase-dead knockin mice retain their immunological defects, but do not display the increased cardiac contractility observed in the PI3Kγ KO mice. Additional investigations into the molecular mechanisms of these two phenotypes showed that PI3Kγ played distinct, yet critical, roles in two separable processes.

PI3Kγ interacts with PDE3B to regulate cAMP levels in the heart to control contractility, and this role is independent of its kinase activity. However, the lipid kinase activity of PI3Kγ is required to generate phosphatidylinositol-3,4,5-trisphosphate, which leads to activation of Erk and MAP kinase family members. Thus, the kinase activity PI3Kγ is directly responsible for its role in inflammatory responses. It is not clear how any one of the conditional strategies for regulating protein function covered in this review would have revealed these separable functions of PI3Kγ. Conditional inactivation of PI3Kγ function would be expected to give rise to both the immunological phenotype and the cardiac phenotype. It would not be immediately obvious if these two phenotypes were (or were not) mechanistically intertwined. On the other hand, a cell-permeable inhibitor that was specific for PI3Kγ would be expected to reveal the immunological phenotype, but not the cardiac phenotype, even after prolonged administration.

In the case of PI3Kγ, more than one approach was required to identify and illuminate two distinct functions of this protein. Perturbing the functions of specific proteins will become increasingly important as life scientists seek to understand how networks of interacting proteins give rise to complex biological behavior. There is plenty of room for success, and multiple approaches will be required to deconvolute the individual contributions of different proteins in complex systems.

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