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Chemical Biology Strategies for Posttranslational Control of Protein Function

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A common strategy to understand a biological system is to selectively perturb it and observe its response. Although technologies now exist to manipulate cellular systems at the genetic and transcript level, the direct manipulation of functions at the protein level can offer significant advantages in precision, speed, and reversibility. Combining the specificity of genetic manipulation and the spatiotemporal resolution of light- and small molecule-based approaches now allows exquisite control over biological systems to subtly perturb a system of interest in vitro and in vivo. Conditional perturbation mechanisms may be broadly characterized by change in intracellular localization, intramolecular activation, or degradation of a protein-of-interest. Here we review recent advances in technologies for conditional regulation of protein function and suggest further areas of potential development.

Mechanistic understanding of cell biology and physiology is driven in part by the tools available to manipulate the system and to assay the phenotypic response. The study of a particular process requires specialized approaches to enable dissection of components, connectivity, and, ultimately, causation. A wide variety of molecular techniques are now available to examine processes at different length scales and levels of biological complexity. Understanding the mechanistic underpinnings requires the ability to selectively alter specific structural or functional elements in order to gauge their phenotypic consequences. Successive technological advances allow interrogation of the system under study with greater precision while minimizing off-target perturbation. Wide adoption of a technique also requires it to be robust, low cost, and relatively easy to use. Although there have been many advances in the ability to observe cells and especially intact organisms (Dean and Palmer, 2014; Sinha et al., 2013), here we focus on the control of individual genes in order to understand their function in specific contexts.

One of the fundamental ways to deduce the role of a gene is to increase or decrease its function and observe the response of the system. Current strategies can target each step in the conversion of a gene into its functional product, mimicking natural control processes of the central dogma (Figure 1). Generally speaking, genetic techniques targeting DNA are robust and specific but have been difficult to implement, slow, and poorly reversible. Site-specific genomic editing by homologous recombination is robust in certain model organisms but until recently has been hampered by very low efficiency in mammalian systems. Homologous recombination or loss-of-function mutations can be stimulated using site-specific double-strand breaks using zinc-finger nucleases, transcription activator-like effector nucleases (TALENs), or clustered regulatory interspaced short palindromic repeat (CRISPR)-Cas9 (Cheng and Alper, 2014). Control over RNA stability through RNAi is faster and easier to implement than traditional DNA manipulation techniques, though it is limited by the efficiency of knockdown and the possibility of off-target effects (Milstein et al., 2013). Each of these also acts indirectly on the functional molecule. Directly targeting proteins via small-molecule inhibitors or activators is fast, conditional, and simple if a perturbant is available but may be limited by specificity and exhibit off-target effects (Fabian et al., 2005; Lounkine et al., 2012). Each of these technologies is generalizable to the study of many or all genes but may work better or worse for particular genes, in different organisms or cell types. For example, studying essential genes requires conditional techniques in order to propagate cells and allow organismal development.

Experimental Considerations for Choice in Perturbation Technique

The choice of experimental strategy (or combination of strategies) is typically a trade-off between precision requirements and ease of use (summarized in Tables S1 and S2). A perturbation strategy has a number of desirable attributes. Ideally, the technique should be: (1) specific, (2) robust, (3) conditional, (4) efficient, (5) reversible, (6) tunable, (7) rapid, (8) orthogonal, (9) spatially localized, (10) simple, and (11) low cost. From a pragmatic standpoint, technologies requiring several genetic manipulations, specialized microscopes, or custom synthesized small-molecule reagents increase the barrier to entry and limit both the adoption of new technologies and their use to low-throughput modes.

From a scientific standpoint, the perturbation should be specific; that is, the intervention should minimize unwanted side effects, especially unforeseen ones that are difficult to control for. This is especially a concern for RNAi, in which validation using independent small interfering RNAs (siRNAs) targeting the same gene is required, or for poorly characterized small molecules, especially those that must be used at high concentrations, which may bind to proteins other than the desired target. The perturbation strategy should also be robust; that is, it should be readily usable without requiring extensive validation and optimization and additionally should yield consistent and reproducible results. Conditional strategies allow a trigger to enact the modification of interest, which can be required when studying essential genes or when the modification is desired in only a subset of an organism's cells.

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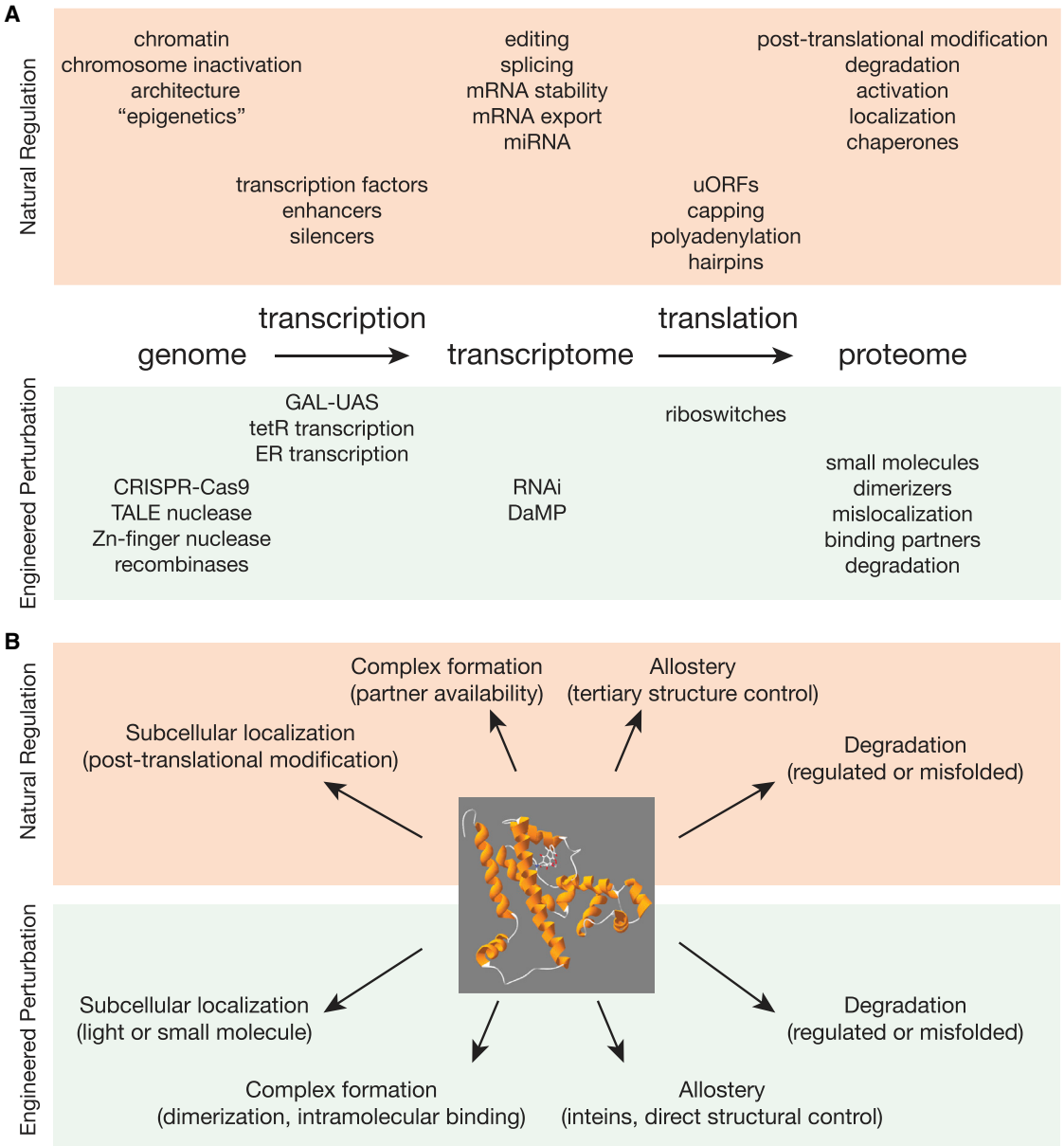

Figure 1. Comparison of Naturally Occurring and Synthetic Engineered Control Processes
(A) The central dogma of molecular biology governs the transcription of DNA (genome) into mRNA (transcriptome), which is then translated into proteins. Every step is regulated by processes, some of which are listed here above the relevant step in the flow of information from DNA into proteins. Engineered experimental perturbation strategies mimic various natural regulatory steps, some of which are shown here.
(B) Protein activity is regulated intracellularly by a variety of processes, including its availability in a particular subcompartment, whether it is part of a complex, its tertiary structure and dynamics, and its abundance. Each of these strategies is exploited experimentally for conditional posttranslational control of protein function.

Efficient control over gene expression should minimize effects from residual amounts of remaining active protein from inefficient knockdown in RNAi, leaky expression from transcriptional control strategies, or incorrectly localized or undegraded protein. Alternately, being able to reversibly and tunably regulate a protein-of-interest allows facile study of whether the observed phenotype is specific to the gene being studied and opens up quantitative studies. Rapidity must be measured relative to the speed of the process being studied but at minimum should avoid conflating adaptive responses to a protein being depleted, overexpressed, or activated. In particular, different technologies for posttranslational control may act by turning on (e.g., inteins) or turning off (destabilization domains) protein activity and rely on natural, slow mechanisms in the other direction; care should be taken to choose a technique when observing a biological phenomenon requiring a gain of function, for which rapidly increasing protein activity is desirable, or a loss-of-function, for which rapidly decreasing a protein's activity may be required.

It may at times also be desirable to manipulate several genes simultaneously; these should be orthogonal to one another in addition to the biological system. Ideally, the technology would allow temporal and spatial precision within the physiological context.

A well-characterized small molecule has many of the attributes listed above. Small molecules act rapidly in comparison with genetic perturbation tools, can tunably modulate protein activity, and can reversibly bind a protein target. However, their specificity is not guaranteed: many small molecules are known to interact with more than one protein target (Gaulton et al., 2012). Additionally, the vast majority of genes and proteins do not have high-affinity small-molecule binding partners.

Combining genetic and chemical perturbation (often called "chemical genetics") allows researchers to potentially take advantage of the specificity of genetic techniques with the speed and reversibility of small-molecule-based approaches (Banaszynski and Wandless, 2006). Typically, a genetic fusion is made between an effector molecule and a small-molecule-dependent control module and expressed exogenously on either a wild-type or mutant loss-of-function background. These control elements may act directly on the protein-of-interest, indirectly to control gene expression, and so on. Recent advances in genomic targeting through TALENs or CRISPR-Cas9 have made tagging of endogenous alleles possible, though it still requires weeks to months of effort (Dean and Palmer, 2014; Gaj et al., 2013). This allows the control of a protein-of-interest without competition from endogenous proteins. Chemical genetic strategies using bio-orthogonal ligands, small molecules that are minimally perturbing to the biological system being studied, should be used whenever possible in order to minimize off-target effects; however, expediency, availability, and familiarity often compel researchers to use rapamycin-based dimerizer systems or other small-molecule ligands that are known to not be biologically silent (Edwards and Wandless, 2007).

Several situations would also demand spatial regulation of protein function; localization of a ubiquitously expressed protein could affect its function, the protein-of-interest may be active only in a certain subcellular compartment or, on a longer length scale, genes in subcompartments of tissues could be controlled. Within metazoans, cell-specific activation can be accomplished through tissue-specific promoters or activators, but specificity is dictated by the underlying biological specificity of the chosen promoter or enhancer (Kistner et al., 1996). An alternative would be to control spatial activity with light. Use of light rather than a small molecule as the control element offers a number of advantages. Although perfusion setups can limit the diffusion of small molecules, light-mediated activity is not limited as such (Taylor et al., 2010). Activation and inactivation kinetics of light-mediated approaches can be fast and do not require media changes. On the other hand, spatially targeting a light beam often requires specialized microscopes and software (Wu et al., 2009). Unless spatial regulation is strictly required, small-molecule approaches still offer advantages in not requiring continuous application of the light, which may cause phototoxicity and local heating and limit throughput.

Chemical genetic approaches, including those regulated by light, can act directly on gene expression by targeting the protein-of-interest or indirectly by altering the gene or transcript. Small-molecule-dependent transcription, through either fusion of the tetracycline or estrogen receptor ligand binding domain to sequence specific transactivator domains, has been widely adopted for conditional regulation (Banaszynski and Wandless, 2006; Kistner et al., 1996). Although significant improvements to lowering transcription in the absence of activating drug have been made, transcriptional approaches still face the fundamental limitations in waiting for the natural clearance of the protein-of-interest from the cell and, practically, in the lack of predictably tunable gene expression. Here, we focus on posttranslational control of protein function to overcome these limitations, because the protein-of-interest is targeted directly. The historical and foundational groundwork for these technologies is covered in earlier reviews (Banaszynski and Wandless, 2006); recent advances and applications are emphasized here.

Strategies for posttranslational control of protein function can be classified by their modes of action (Figure 1B). First, because the biological activity of a protein-of-interest is often limited to a specific subcompartment of the cell, altering its localization can control its activity. Small molecules or light can be used to change the location of the protein within the cell, in order to either activate it or inactivate it. This is usually accomplished by dimerization of the protein-of-interest with other proteins known to be localized to the desired cellular compartment. Conditional (mis) localization is often very fast, but its use is limited to those proteins that have compartment-specific activity. Second, a protein-of-interest can be activated or inhibited directly without changing its physical abundance in the cell. This can be accomplished through a variety of both intermolecular and intramolecular mechanisms, discussed below. Last, because a protein cannot exert its activity if it does not exist, several approaches to reduce a protein's function by altering its intracellular stability are discussed.

Conditional Dimerization: (Mis)localization

One of the most commonly used strategies to conditionally regulate protein activity involves promoting the association of any two proteins-of-interest. Over the past two decades, small molecules have emerged as effective means by which to engineer such protein-protein interactions. These compounds, commonly referred to as chemical inducers of dimerization (CIDs), have the ability to simultaneously bind two protein domains, thereby inducing their proximity. As illustrated in the following discussion, CIDs have been used to mediate protein activity in one of three ways: by promoting transcriptional activity, by recruiting target proteins to specific cellular compartments, or by facilitating protein aggregation and disaggregation (Figure 2).

The first example of a naturally occurring CID was described in 1991, when the immunosuppressant drug FK506 was reported to inhibit T cell receptor-mediated signaling by simultaneously binding FK506-binding protein (FKBP12) and calcineurin (Liu et al., 1991). On the basis of these seminal findings, a synthetic dimer of FK506 (named FK1012) was prepared, which was able to dimerize FKBP12 (Spencer et al., 1993). Fusion of the FKBP12 domain to the ζ chain of the T cell receptor resulted in chimeras that exhibited FK1012-dependent signal transduction in cultured cells. Notably, FK1012-induced signaling was found to be rapid, dose dependent, and reversible. In addition, the synthetic ligand lacked its parent monomer's intrinsic biological

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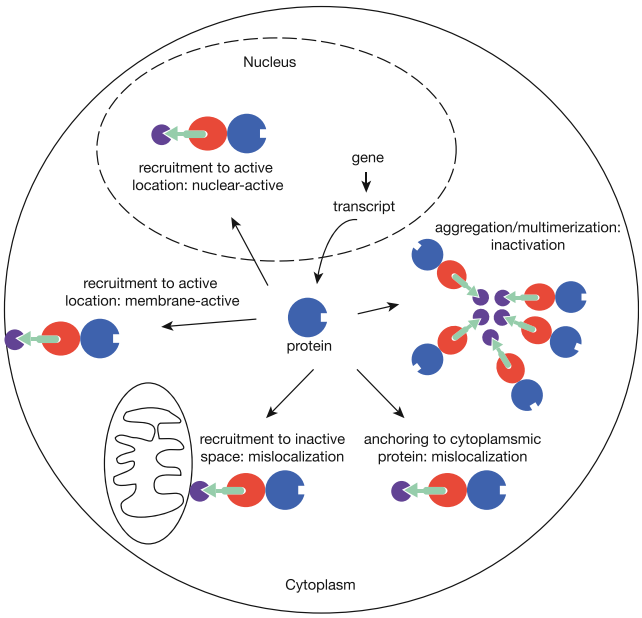

Figure 2. Subcellular Localization as a Strategy to Control Protein Function

Upon translation in the cytoplasm, a protein's activity can be controlled by the availability of its substrates. CIDs have been used both to trigger protein activity by recruitment to its site of action, such as at the plasma membrane or in the nucleus, and to be inactivated by its aggregation or sequestration in a subcellular compartment where it cannot act.

activity. This and other homodimerization systems have been used to stimulate the activity of transcription factors (Ho et al., 1996), induce Fas-mediated apoptosis (Belshaw et al., 1996a), and activate the Raf1-kinase signaling cascade (Farrar et al., 1996).

Although the FK1012 system was the first to chemically induce the proximity of engineered proteins, its utility is most suitable for proteins whose function is dependent on homodimerization. In 1996, several bivalent molecules capable of selectively dimerizing two different proteins were reported (Belshaw et al., 1996b; Licitra and Liu, 1996; Rivera et al., 1996). Among these, rapamycin represents the most thoroughly studied chemical dimerizer. This macrolide natural product mediates the interaction between FKBP12 and the FRB domain of FKBP-rapamycin associated protein (mTOR) (Brown et al., 1994). Expression of two proteins-of-interest as their FKBP and FRB fusions results in their rapamycin-inducible association. Rapamycin facilitates FKBP-FRB complexation on the order of minutes, has low nanomolar affinity for both protein domains, and exhibits good pharmacokinetics (Banaszynski et al., 2005). However, FKBP and FRB fusion proteins compete with endogenous FKBP and mTOR for rapamycin binding, leading to nonproductive interactions. Moreover, the binding and inhibition of mTOR leads to cell-cycle arrest, further complicating in vivo studies, though rapamycin resistant strains or cell lines may be used to mitigate these effects (Haruki et al., 2008). In order to mitigate its off-target effects, rapamycin was derivatized to display a "bump" at its FRB-binding interface (Liberles et al., 1997). Site-directed mutagenesis of the FRB domain delivered a triple mutant, FRB*, bearing a compensatory "hole" that selectively binds the new rapamycin analog (MaRap). Although this modified dimerization system does not possess immunosuppressive activity, MaRap is unstable, is difficult to prepare, and exhibits poor pharmacokinetic properties, precluding in vivo applications (Stankunas et al., 2003).

The "bump and hole" strategy has also been applied to develop FKBP mutants that have high affinity for other biologically silent rapamycin mimics. Interestingly, introduction of a single point mutation (F36M) in the FKBP active site affords a variant (FKBP*) that spontaneously undergoes multimerization in the absence of its cognate ligand, AP21998 (Rollins et al., 2000). The FKBP* aggregate could be readily dissociated upon addition of the small molecule, providing a peculiar example of a reverse dimerization system. Fusion of tandem copies of FKBP* to a protein-of-interest generates a protein target that self-associates and displays AP21998-dependent disaggregation. This tool was first applied to conditionally control protein secretion: when insulin is properly tagged with a signal peptide, an FKBP* tetramer, and a furin protease cleavage site, the chimeric protein undergoes aggregation in the endoplasmic reticulum and becomes too large to be properly exported (Rivera et al., 2000). Exposure to AP21998 releases monomeric insulin-FKBP* fusions and allows for their export via the secretory pathway, during which the furin protease cleaves the FKBP* domain to release free insulin. More recently, Arnold and colleagues exploited the FKBP* conditional secretion system to monitor the trafficking of cargo from the endoplasmic reticulum to the Golgi and subsequently the plasma membrane in neurons (Al-Bassam et al., 2012). This "pulse-chase" system allowed them to differentiate modes of axonal and dendritic vesicle transport and discriminate between competing transport models.

The more recent characterization of new protein-ligand pairs has led to the development of other CID systems. For example, Cornish and coworkers used a dexamethasone-methotrexate (Dex-Mtx) conjugate to induce the interaction between glucocorticoid receptor (GR) and dihydrofolate reductase (DHFR) (Lin et al., 2000). Coexpression of the yeast DNA-binding domain of LexA and the transcriptional activation domain of B42 as their DHFR and GR fusions, respectively, stimulated transcription of the lacZ reporter gene in the presence of Dex-Mtx. Unfortunately, Mtx is a promiscuous inhibitor of DHFR, which limits its broader utility. An improved system that uses a trimethoprim-SLF dimerizer was developed, a bivalent ligand that triggers the interaction between *E. coli* DHFR-FKBP12 fusions (Czlapinski et al., 2008). Importantly, this compound was not found to bind endogenous protein targets and has been used to conditionally activate a mammalian fucosyltransferase.

Despite its cytotoxic properties, rapamycin continues to be the most widely implemented CID. To control the activity of essential proteins in yeast, two studies used a strategy by which nuclear proteins can be conditionally sequestered to the cytoplasm, where they can no longer perform their cellular functions (Geda et al., 2008; Robinson et al., 2010). In practice, this technique achieves rapamycin-mediated mislocalization by genetically tagging a nuclear protein and a cytoplasmic anchor protein with FRB and FKBP, respectively. To function properly, a suitable anchor was envisioned to be highly abundant protein domain that traffics in and out of the nucleus, so that it transiently encounters the target. The ribosomal subunit RPL13A ultimately

served as the best anchor: FKBP-RPL13A fusions were found to shuttle more than 40 essential nuclear proteins to the cytoplasm in a rapamycin-dependent manner, leading to loss-of-function mutant phenotypes. Although this method has proved useful in rapidly generating conditional knockouts, it also presents several limitations. First, these studies necessitated the use of rapamycin-resistant strain of yeast to circumvent inhibition of TOR. More important, it remains unclear how the current system can be employed to mislocalize and regulate cytoplasmic proteins; in principle, targeting a cytoplasmic protein to the nucleus or plasma membrane may inactivate it, though this must be tested on a case-by-case basis. Toward this goal, a recent study demonstrated that an FKBP-tethered adaptor protein-1 complex undergoes rapamycin-mediated localization to mitochondria when coexpressed with FRB fused to a mitochondrial targeting sequence. Robinson and colleagues were able to achieve fast (approximately minutes) reduction in the activity of several proteins in the endocytosis pathway. Importantly, the rapidity of the "knock-sideways" system allowed the researchers to observe a phenotype distinct from siRNA-mediated knockdown of the same protein, an effect they attributed to compensatory responses from slow knockdown (Robinson et al., 2010). Alternatively, sequestration does not necessarily require targeting to a subcellular compartment at all. Heo and colleagues recently showed that simply multimerizing and clustering a protein through is sufficient to reduce its activity without limiting its subcellular localization (Lee et al., 2014).

Despite their general utility, the CIDs discussed thus far are not without limitations. Many of these small molecules are expensive and require multistep syntheses, which can restrict their accessibility. In addition, their application in live animals is often impeded by their promiscuous binding profiles and/or affinity for endogenous proteins, interactions that often lead to cytotoxic effects. Moreover, given the nanomolar affinity between each of these ligand-protein pairs, the reversibility of their association is typically contingent upon the addition of a second, high-affinity ligand to displace the bivalent molecule. For example, rapamycin-mediated FKBP-FRB dimerization can often be reversed by the addition of exogenous FK506, which competitively binds the FKBP active site. In some instances, rapamycin-induced proximity is irreversible (Haruki et al., 2008).

To address these challenges, researchers have exploited small-molecule-mediated signaling pathways that have recently been characterized in plants. One such pathway involves abscisic acid (ABA), a hormone that stimulates several components of plant development. Mechanistically, ABA inhibits type 2C protein phosphatases (PP2Cs) by mediating their association with the pyrabactin resistance (PYR)/PYR1-like (PYL) family of protein receptors (Cutler et al., 2010). In 2011, Crabtree and coworkers designed PYL and PP2C domains that, when individually fused to proteins-of-interest, can reconstitute Gal4 transcriptional activity, localize protein targets to the nucleus or cytoplasm, and induce extracellular signal-regulated kinase phosphorylation in mammalian cells upon exposure to ABA (Liang et al., 2011). This plant-specific hormone is inexpensive, does not bind endogenous mammalian proteins, and was found to be nontoxic to cultured cells and mice. However, high micromolar concentrations of ABA are necessary to induce PYL/PP2C proximity. Nevertheless, this interaction is more readily reversible than the corresponding ternary interaction mediated by rapamycin: upon ABA washout, protein activity was reduced to basal levels within 24 hr.

Notably, the addition of plant-based dimerizers to the standing collection of CIDs presents an interesting opportunity to regulate the activity of multiple protein signaling pathways. Specifically, use of a plant dimerizer in concert with a rapamycin-inducible system could allow for the simultaneous and orthogonal control of two different proteins. The net result would be a synthetic logic gate, wherein defined cellular output results from each of the small-molecule inputs. Inoue and coworkers examined the feasibility of such a dual-proximity system by first engineering a CID on the basis of a different plant hormone, gibberellin (GA_3) (Miyamoto et al., 2012). To exert its effects, GA_3 facilitates the interaction between gibberellin-insensitive dwarf1 (GID1) and gibberellin insensitive (GAI) (Hirano et al., 2008). After optimization of gibberellin (GA_3-AM) and the GID1-GAI domains, the authors constructed intracellular AND and OR logic gates using changes in cell morphology as the phenotypic output signal. For the OR gate, FKBP, GID1, and YFP were fused to Tiam1, a guanine nucleotide exchange factor that activates Rac1 and results in membrane ruffling when it is recruited to the plasma membrane. Coexpression of the Tiam1 fusion and the membrane-targeted Lyn-CFP-FRB-GAI in mammalian cells led to significant membrane ruffling only the presence of GA_3-AM, rapamycin, or both chemical inputs. Importantly, both small molecules can facilitate Tiam1 membrane recruitment on the order of seconds to minutes. Unfortunately, the dissociation kinetics for both systems remain relatively slow. Moving forward, orthogonal CIDs that exhibit fast on and off kinetics will greatly improve the use of logic gates in live cells.

Mislocalization by Light-Mediated Dimerization

The current palette of chemical dimerization systems has allowed researchers to tunably and reversibly regulate a number of protein-protein interactions on the order of minutes to hours. In contrast, protein signaling pathways in living cells typically occur on a second timescale, a time frame that is difficult to recapitulate using small molecules. Additionally, specific subcellular localization of a CID cannot always be achieved, a property that further limits their potential applications. In order to significantly improve the spatiotemporal precision of these tools, recent efforts have turned to the use of light to control the association of two proteins-of-interest. In particular, several studies have taken advantage of naturally occurring photosensitive protein domains that undergo a dimerization event when exposed to a specific wavelength of light. An additional advantage with light-controlled systems is that they do not typically require the addition of an exogenous ligand, mitigating the potential for off-target or toxic effects.

The most commonly used photoswitchable domains take advantage of light-sensitive signaling proteins identified in the flowering plant *Arabidopsis thaliana*. One such protein is exemplified by phytochrome B (PhyB): upon irradiation with red light (650 nm), PhyB undergoes a conformation change and binds phytochrome interaction factor 3 (PIF3), resulting in transcriptional activation (Ni et al., 1999). Exposure of this complex to 750 nm light reverses this interaction, such that PhyB no longer has significant binding affinity for PIF3. To determine the ability

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of this photoswitchable system to stimulate Rac activation, mammalian cells were cotransfected with constructs encoding a plasma membrane-anchored PhyB and a PIF3-tagged Tiam1 (Levskaya et al., 2009). Irradiation of these cells at 650 nm results in membrane recruitment and activation of Tiam1 within seconds, leading to lamellipodia formation. Interestingly, focusing a red laser at precise cellular locations stimulated localized protrusions that could be patterned at will, providing a handle by which to precisely control cell morphology. The PhyB-PIF interaction was recently adapted to develop a photoswitchable anchoring system in yeast (Yang et al., 2013). By fusing PhyB to different organelle-targeting sequences, a PIF3-tagged protein target could be conditionally recruited to eight distinct subcellular locations, including endosomes, peroxisomes, the nucleus, and the spindle pole body. This strategy is conceptually similar to the rapamycin-based anchoring system discussed above but offers faster association kinetics and is readily reversible. Notably, the spatiotemporal precision imparted by this approach allowed the authors to dissect the multifunctional role of Clb2, a mitotic cyclin found to be critical for proper nuclear fission and spindle pole body disassembly. A caveat to this strategy is that it requires the addition of phycocyanobilin, a ligand that mediates the PhyB allosteric transition.

To eliminate the need for exogenous cofactors, a second pair of photosensitive dimerizers was established. In this case, the blue light-dependent interaction between the *A. thaliana* FKF1 protein and GIGANTEA (GI) (Sawa et al., 2007) was harnessed to conditionally activate Rac signaling and activate the Gal4 transcription factor (Yazawa et al., 2009). In contrast to the PhyB-PIF system, photoexcitation of FKF1 triggers a conformational change controlled by flavin mononucleotide, a cofactor that is naturally produced by mammalian cells. Unfortunately, FKF1 and GI fusion proteins exhibit blue light-induced association on the timescale of minutes and required more than 1.5 hr to dissociate once illumination is ceased. Tucker and coworkers recently implemented a new pair of photosensitive domains with improved on/off kinetics on the basis of cryptochrome 2 (CRY2) and cryptochrome-interacting basic-helix-loop-helix 1 (CIB1) (Kennedy et al., 2010). When optimized in mammalian cells, photo-stimulated CRY2-CIB1 association was observed to occur within 300 μs, but the reverse process took minutes to complete. This represents the first system that achieves dimerization on a subsecond timescale. Ideally, the association and dissociation kinetics of the dimerization domains would occur on similar timescales; however, no such systems have been reported to date.

An indirect approach to light-induced proximity involves genetically incorporating a photosensory domain into a peptide, such that the peptide's affinity for its cognate protein is rendered dependent on light. Fusion of the modified peptide and its binding partner to proteins-of-interest affords targets that then exhibit light-dependent association. Two studies independently created photoswitchable peptides on the basis of the light, oxygen, or voltage (LOV2) domain of *Avena sativa* phototropin1 (Strickland et al., 2012; Lungu et al., 2012). LOV2 comprises a PAS domain that bears a C-terminal α helix (Jα), which remains docked to the PAS fold in the dark state. Photoexcitation with blue light facilitates Jα unfolding and dissociation from the LOV core. Vinculin-binding (Lungu et al., 2012) and PDZ-binding (Strickland et al., 2012) peptides were engineered into the Jα helix, such that the peptide was caged in the dark state and accessible to its binding partner in the lit state. LOV2-based technologies benefit from the fact that the LOV2-Jα interaction is biophysically well characterized, allowing a more predictable modulation of this dimerizer's dynamic range.

Taken collectively, the intensive research behind conditional dimerization has culminated in the development of tools that operate at the second timescale, localize targets with high spatial resolution, and are biologically orthogonal. Whether small molecule or light based, many of these approaches are still in their infancy, and each presents its own drawbacks. For example, light-mediated dimerization systems often display limited localization differences in the lit versus dark state; from published data, the CRY2 system appears to have the best signal-to-noise ratio (Kennedy et al., 2010), but smaller fold differences may nonetheless be useful in interrogating biological systems, as Yang et al. (2013) demonstrated in elucidating the spatiotemporal role of Clb2 activity in the cell cycle. It will be interesting to take note of their future applications in elucidating the mechanisms of complex signaling pathways. Within this context, the combination of small-molecule- and light-based approaches to simultaneously mediate more than one protein-protein interaction may facilitate the construction of artificial signaling networks. Although rapamycin-based approaches continue to serve as the dimerization modules of choice, in-depth studies of more recently developed proximity tools—such as improvements in their on/off kinetics and more thorough biophysical characterization of the dimerization domains—should facilitate their widespread use.

Activation of Preproteins

Rather than controlling protein activity by localization to a specific cellular compartment, an alternative approach relies on splitting a protein-of-interest into two inactive fragments. When properly engineered, these fragments can be induced to associate, thereby restoring the target's structure and cellular function. This process, also known as fragment complementation, was first combined with chemical dimerization tools in 2002 (Mootz and Muir, 2002) (Figure 3). In this study, Muir and coworkers developed a conditional protein splicing system based on inteins, protein domains that catalytically excise themselves from a polypeptide chain and simultaneously ligate their two flanking sequences. Specifically, the *Saccharomyces cerevisiae* VMA intein was split into N- and C-terminal halves: the former was fused between FKBP and maltose-binding protein (MBP) and the latter between FRB and a His tag. Rapamycin-induced dimerization of these fusions induced the proximity of the intein domains, which restored intein splicing activity and led to the formation of a His-tagged MBP.

This exploratory study illustrated that intein activity could be manipulated to conditionally tag a protein with a small peptide. In order to control the activity of a split protein, Liu and coworkers took a directed evolution approach to design a conceptually similar intein splicing system (Buskirk et al., 2004). In this case, the estrogen receptor-binding domain (ER) was initially inserted into an intein to give an intein$_N$-ER-intein$_C$ fusion that lacked splicing activity. A library of intein candidates was prepared by error-prone PCR, then cloned between the

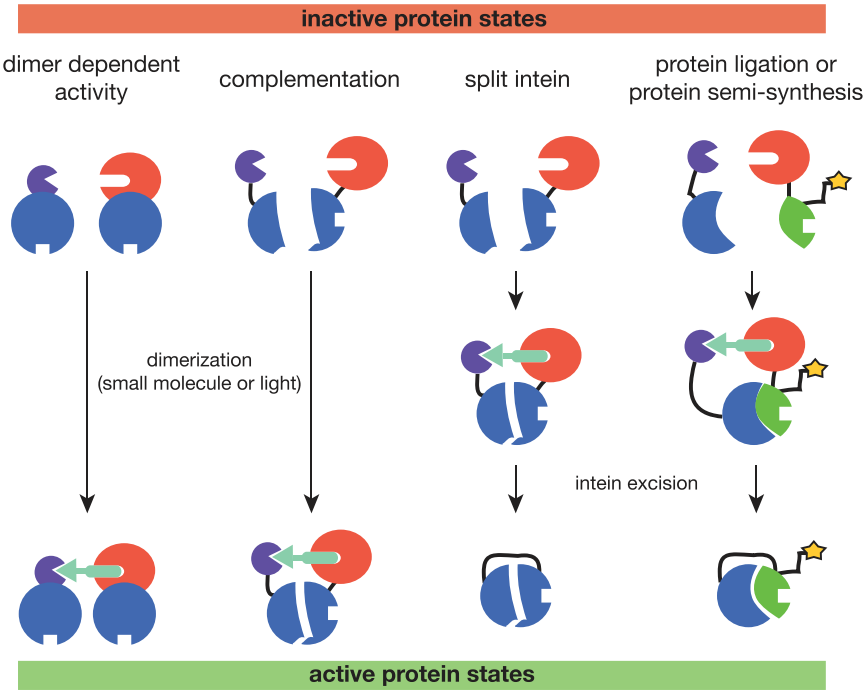

Figure 3. Conditional Dimerization Rescues Protein Function
(A) Proteins that require dimerization to function (e.g., transcription factors, some enzymes) can be activated directly by hetero- or homodimerization.
(B) Regulated protein fragment complementation. The protein's primary sequence is split into two pieces, each of which is fused to a dimerization domain. Dimerization allows reconstitution of the holoprotein.
(C) Split inteins. Self-excising intein protein domains can be fused with the split protein fragments to allow complementation of the protein-of-interest. Upon intermolecular excision and ligation of the intein, the extein fragments are fused to form a single active polypeptide with no intervening dimerization domains.
(D) Expressed protein ligation. Similar to split inteins, except that one of the fragments is synthetically derived. This can be especially useful to produce functionalized proteins or proteins with homogeneous stoichiometric posttranslational modifications.

N- and C-terminal portions of the KanR gene. Mutants that exhibited ligand-dependent splicing were identified by their ability to confer Geneticin resistance in the presence of 4-hydroxytamoxifen (4-HT), a high-affinity ER ligand. Additional rounds of selection delivered an evolved intein that could restore the activity of four different proteins in a 4-HT-dependent fashion. Although originally devised in yeast, improved mutants allowed this 4-HT-dependent intein splicing to also be used in mammalian cells (Peck et al., 2011). Alternatively, split inteins' function can be controlled via dimerization of the two halves using small-molecule rapamycin-FKBP-FRB (Schwartz et al., 2007), light-based PhyB dimerization (Tyszkiewicz and Muir, 2008), or protein-based coiled-coil-based dimerization (Selgrade et al., 2013) systems.

A major drawback of intein-based fragment complementation systems is their lack of reversibility. Intein splicing is an inherently irreversible process, and it is difficult to imagine how one might alter this mode of reactivity to inactivate the extein product. One solution to this problem involves fusing each of a protein's fragments directly to a pair of dimerization domains, such that the activity of the engineered protein is dependent solely on ligand-induced proximity. On a more general level, these approaches require researchers to judiciously determine how to best separate their protein target into two halves without creating disordered polypeptides that are not metabolically stable. Consequently, such studies often require a systematic screen of various N- and C-terminal fragment pairs, which can become laborious and requires optimization on a case-by-case basis. Depending on the protein-of-interest, it may prove difficult or impossible to split the target in such a way that maintains its structural and functional integrity.

Intein mechanisms have also been exploited to produce caged semisynthetic proteins by expressed protein ligation (Muir et al., 1998). Again, a protein-of-interest is judiciously split into an expressed fragment and a shorter peptide containing important functional residues. Conditional protein activity can be achieved by functionalizing key residues in the synthetic peptide with light-dissociable 2-nitrobenzyl leaving groups followed by incorporation of this peptide into the full-length protein by expressed protein ligation. This strategy allowed, for example, the Imperiali group to produce caged myosin protein with excellent spatial and temporal control profiles (Goguen et al., 2011). This approach, however, currently requires microinjection of the semisynthetic protein that has been produced in vitro, and the general availability of these reagents may be limited. In the future, direct protein transfection with supercharged proteins may be possible (McNaughton et al., 2009).

In attempting to develop a general fragment complementation system, Ghosh and coworkers designed kinases that could tolerate small loop insertions in their catalytic domain without compromising their catalytic activity (Camacho-Soto et al., 2014). Insertions were deliberately incorporated into regions of the catalytic domain of several tyrosine kinases that shared little sequence homology, suggesting that these regions were not essential for activity. The insertion then served as a flexible handle through which a kinase could be fragmented into two domains and fused to FKBP and FRB. This strategy was used to develop split versions of Lyn, Fak, and Src kinases, and the AGC kinase PKA, all of which exhibit rapamycin-inducible activation.

Engineered Proteins for Orthogonal Small-Molecule Control

As has been discussed, small-molecule inhibitors and activators offer fast, tunable control over proteins-of-interest when available. An alternative to finding a specific small molecule for each protein-of-interest is using a functionalized substrate analog approach coupled with mutation in the protein-of-interest in order to accept the functionalized analog. This strategy has been used in a variety of contexts, including for guanosine

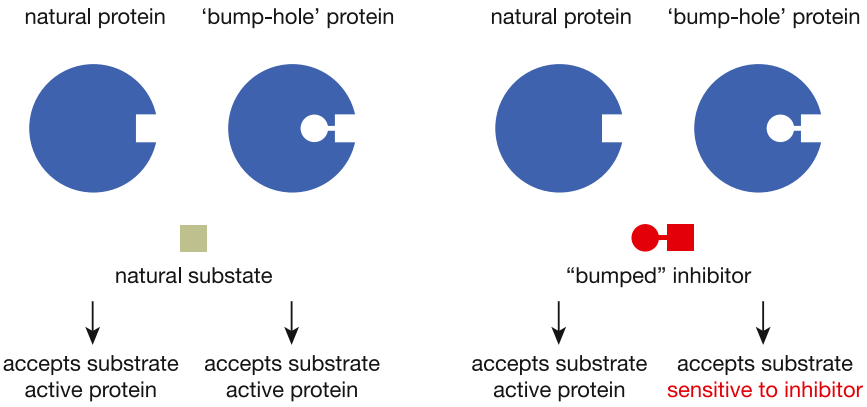

Figure 4. The "Bump-Hole" Strategy
The protein-of-interest is mutated to create a cavity so that it will accept both the natural substrate and a larger substrate analog. The substrate analog will bind only to the engineered mutant protein. The substrate analog can either be an inhibitor, to selectively inhibit the mutant protein, or it can add a novel functionality, so the enzyme's target is unnaturally modified, which can then be exploited for purification of its targets.

triphosphate (GTP)-binding proteins, seven-pass transmembrane proteins, and perhaps most generally for protein kinases (Bishop et al., 2000a, 2000b) (Figure 4). The so-called bump-hole strategy, pioneered by the Shokat group, uses a "bumped" substrate analog possessing bulky side-chain substituents that have greatly reduced binding to wild-type kinases but bind with high affinity to kinases with a mutation in the gatekeeper residues to produce a complementary "hole." When expressed in cells, the mutant kinase can be specifically inhibited by the addition of the bumped inhibitor.

Importantly, the bump-hole strategy seems readily generalizable to an arbitrary protein kinase of interest whereby the compensatory mutation can be identified using homology searching. In cases in which the hole-forming mutation is destabilizing, additional compensatory stabilizing mutations can be found (Zhang et al., 2005). In addition to its use as a control element, mutated kinases can also accept derivatized substrates to selectively tag their target proteins. Using this approach, Shokat, Morgan, and coworkers were able to identify targets of the *S. cerevisiae* Cdk1 homolog Cdc28 (Ubersax et al., 2003). Because of the ease of homologous recombination and haploid propagation in yeast, it has been possible to express these mutated kinases without competition from its endogenous wild-type counterpart. In mammalian cells, the use of the bump-hole kinases as control elements has been limited largely to those with dominant effects, such as in the oncogenes v-Src and Fyn kinases (Bishop et al., 1999). The advent of facile genome-editing techniques in metazoans should now allow a fuller characterization of human kinases using the bump-hole strategy.

The intuitively simple bump-hole strategy has also been expanded to other classes of enzymes. Luo and colleagues adapted the bump-hole strategy for protein (histone) methyltransferases (Luo, 2012). Protein methylation, like phosphorylation, can play an important part in a protein's posttranslational regulation. Epigenetic control through histone methylation in particular is an important mode of gene expression regulation. Luo and colleagues make use of S-adenosyl-L-methionine analogs in conjunction with mutating conserved gatekeeper tyrosine residues in the EuHMT1 and EuHMT2 methyltransferases to identify their targets (Islam et al., 2013). This strategy should be readily adaptable to control methyltransferase activity as appropriate.

Engineered Allosteric Control over Protein Function

Naturally occurring allosteric control of enzyme function drives functional regulation of primary metabolism and many other cellular processes (Gunasekaran et al., 2004). Small-molecule or protein binding distal to the active site causes either a conformational or dynamical change in the host protein, altering its activity. In engineering allosteric regulation, researchers have typically used one of two strategies (Figure 5). First, two protein domains are fused together such that the folding of a control domain affects the output of the second domain. Key factors are the site of attachment and the length and flexibility linkers used. Sites of attachment can be semiempirically determined or computationally predicted. Second, an intramolecular binding event can be switched to control the gross topology of the protein. The first approach requires sophisticated structural knowledge and optimization but is in general more compact, whereas the second strategy is often more modular in nature. Modular assembly of several domains can even be used to control input-output behavior of designed proteins.

An extreme case of designed allosteric regulation is the Loh group's mutually exclusive folding strategy (Radley et al., 2003). From known structures, a protein domain is genetically inserted into a loop on the surface of the host protein. The relative thermodynamic stability of the inserted domain determines which of the two domains can fold: the loop can be pushed apart if the inserted domain is more stable, disrupting the structure of the host protein, or if the stability of the inserted domain is less stable than the host protein, it will be unfolded. The relative thermodynamic stability of the two domains can then be manipulated by the addition of small- or large-molecule-binding partners for each domain to create a bifunctional switchable protein unit (Ha et al., 2006). Although mutually exclusive folding has been used to engineer several switchable proteins, it has been used only in vitro; its practical utility to control the function of an arbitrary protein-of-interest remains to be established.

Engineered allosteric regulation can also affect a protein's activity without gross structural alteration. The focal adhesion kinase G-loop governs the position of the incoming ATP; insertion of an FKBP domain in a loop distal to the G-loop is thought to increase the flexibility of the G-loop, interfering with the proper catalytic activity of the kinase. Hahn and colleagues showed that rapamycin mediated dimerization with an FRB domain decreases the flexibility of this loop, restoring proper activity of Fak (Karginov et al., 2010). Because of the high degree of conservation of kinases, this strategy may be generalizable without extensive engineering.

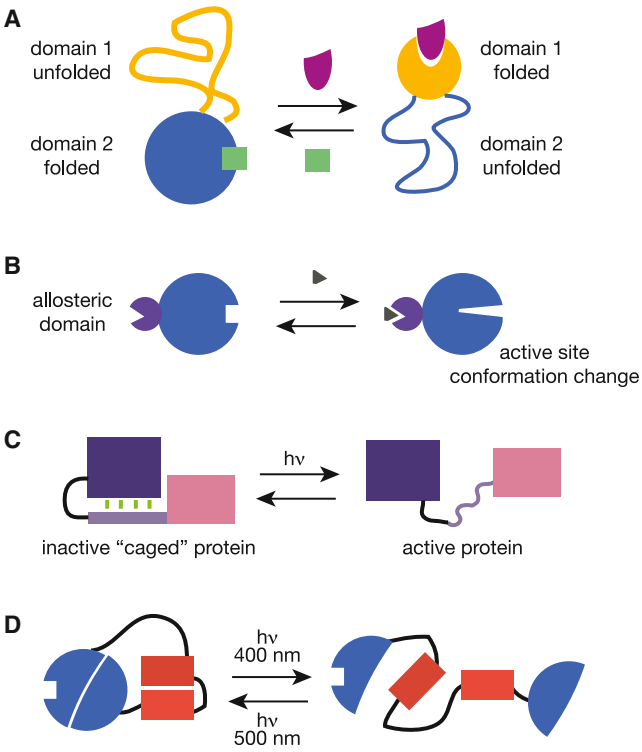

Figure 5. Allosteric Control of Protein Function

(A) Mutually exclusive folding. One protein domain is inserted into an exterior loop of another protein, creating mechanical stress on its structure. The inserted domain can become unfolded, relieving this stress, allowing the host protein to fold properly. Thermodynamically controlling the stability of each of the domains by the addition of ligands for either the host or inserted domain can control the relative stability of the two domains.

(B) "Classic" allostery. Binding at a site distal to the active site causes a conformational or dynamical change affecting the enzyme's active site.

(C) Light-driven reversible intramolecular binding. Protein activity is sequestered by the close association of a Lov2 domain with the protein-of-interest. Flexibility introduced by unfolding of the J-α helix allows protein activity. Reversion is not controlled but relies on dark-state relaxation.

(D) Similar to (C), except the photoconvertible protein Dronpa allows direct reversibility sequential application by violet (400 nm) or cyan (500 nm) light.

In addition to its use as conditional dimerizer, the LOV2 domain is often used as a modular control unit in allosteric control strategies because of its small size and well-characterized conformational change upon illumination with blue light. Sosnick and coworkers fused the Lov2 domain to the *Escherichia coli* trp repressor at 12 different insertion points (Strickland et al., 2008). One of these exhibited light-dependent DNA binding, though no functional studies were performed. The Hahn group also used a Lov2 domain to control the function of several small Rho-family GTPases (Wu et al., 2009). They found a Lov2-constitutively active Rac1 fusion (photoactive-Rac1 [PA-Rac1]) that exhibited light-dependent binding to effector PAK. Again, optimizing attachment points was key to finding light-dependent activity of the fusion protein. Structural analysis and comparison with Cdc42 allowed the construction of a Lov2-fusion photoactivatable analog, PA-Cdc42. The fast kinetics and robust reversibility of PA-Rac1 allowed the Hahn group to tease apart the hierarchy of signaling events in actin-dependent membrane remodeling.

Optimization of insertion site and linker lengths have dramatic effects on the success of allosteric regulation strategies but are often empirically determined (Cutler et al., 2009). As an alternative to this, Ranganathan and coworkers used statistical coupling analysis (SCA) to identify sectors connected to the surface residues as targets for allosteric modulation (Lee et al., 2008). SCA uses coevolution of amino acids within a protein to identify functionally relevant "sectors" rather than structurally defined protein "domains." Fusing *E. coli* DHFR and Lov2 domain at these predicted sites produced a fusion protein whose enzymatic activity could be modulated by 2-fold, leading to measurable differences in growth rate of an auxotrophic *E. coli* strain (Reynolds et al., 2011).

As an alternative to using Lov2 as the allosteric control domain, the Lin lab recently showed that a Lys145Asn mutant of the photoconvertible protein Dronpa undergoes reversible oligomerization/dissociation upon illumination cyan or violet light (Zhou et al., 2012). Inserting a tethered Dronpa 145N into the Cdc42 GEF Dbl homology domain or HCV NS3-4A protease rendered them light activatable. The kinetics of Dronpa 145N-based control are somewhat slower than Lov2, but may be easier to generalize and benefit from intrinsic fluorescence.

Protein Stability Control

Of the various methods for posttranslational control over protein function, controlling its intracellular stability is most similar to traditional genetic and transcription control methods. The protein-of-interest cannot be active if it is not there. Most conditional protein degradation technologies take advantage of the ubiquitin proteasome system (UPS) through intervention at several points in the pathway (Figure 6). The UPS is a mechanism for the regulated proteolysis of intracellular proteins. Proteins are targeted for degradation if they are damaged or their activity interferes with other cellular processes (Figure 6). Specific recognition molecules tag the protein to be degraded by the conjugation to ubiquitin. The addition of several ubiquitin molecules is typically sufficient to target the protein to the proteasome, where it is unfolded and processively degraded by the proteasome. Proteins can be targeted for degradation using the cells' natural ubiquitylation machinery (N-end rule, destabilizing domains, HaloTag-Hyt13) (Chu et al., 2008; Dohmen et al., 1994; Iwamoto et al., 2010; Neklesa et al., 2011), via recruitment to specific ubiquitin E3 ligases (PROTACs, LIDs, deGradFP, AID, ubiquibodies) (Bonger et al., 2011, 2014; Caussinus et al., 2011; Nishimura et al., 2009; Portnoff et al., 2014; Sakamoto et al., 2001), or can bypass the ubiquitylation step altogether by small-molecule-mediated direct recruitment to the proteasome (dimerizers, degrons) (Janse et al., 2004; Renicke et al., 2013). Again, chemical genetic protein stability control technologies are more easily generalizable than those that rely solely on small-molecule-mediated degradation but do not affect endogenous pools of the protein-of-interest.

Historically, temperature-sensitive alleles of a particular gene in yeast have been isolated that create conditional alleles of gene of interest, but a new mutant needed to be found for each gene being studied. In order to generalize this approach to an arbitrary gene-of-interest, Dohmen and Varshavsky created a tripartate fusion consisting of an N-end rule substrate, a known temperature sensitive allele of (mouse) DHFR, and the

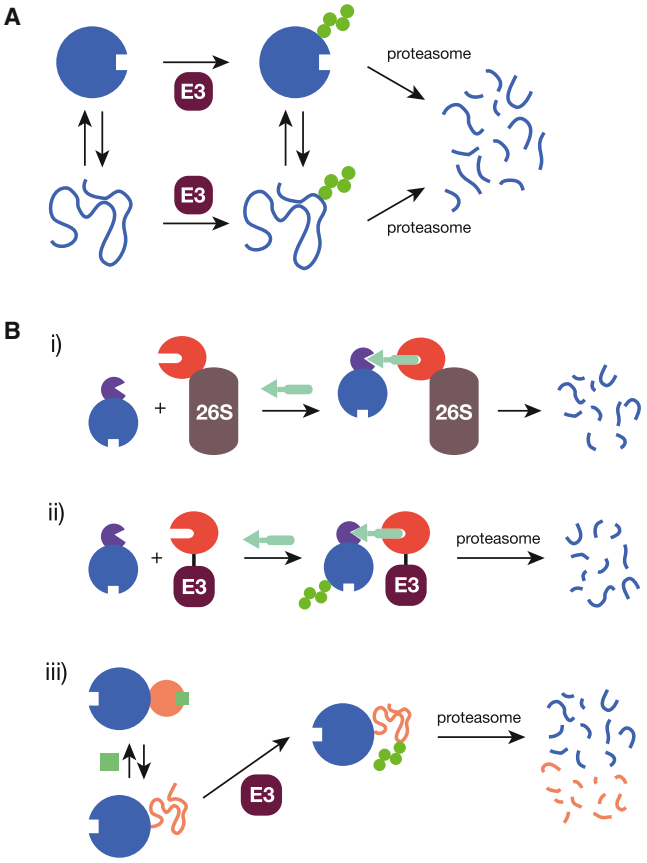

Figure 6. Degradation Mediated Control of Protein Abundance
(A) Schematic of the UPS. Normal proteins can be targeted for degradation by their regulated ubiquitylation or if they are damaged or misfolded. Addition of a minimum of four ubiquitin moieties targets the protein to the proteasome for degradation.
(B) Each step in the UPS can be manipulated for regulated degradation of a protein-of-interest. (i) Directly targeting a protein to the proteasome obviates the need for ubiquitylation for degradation. (ii) Recruitment of an ubiquitin E3 ligase by the addition of a dimerizer causes ubiquitylation of the protein-of-interest substrate, followed by its subsequent targeting to the proteasome and degradation. (iii) Relying on natural ubiquitylation processes. Conditional regulation of natural ubiquitylation processes by triggering the N-end rule or recognition of destabilizing domains by protein quality control ubiquitin E3 ligases.

protein-of-interest (Dohmen et al., 1994). The N-end rule is an evolutionarily conserved pathway that targets proteins with certain N-terminal amino acids (Arg, Lys, etc.) for ubiquitylation and degradation; to alter the N-terminal amino acid, they rely on the rapid cleavage of linear ubiquitin fusion proteins by intracellular deubiquitinating enzymes. Their temperature-sensitive degron (td) technique used the conditional temperature accessibility of the N-terminal arginine residue to control the intracellular stability of Cdc28 and Ura3.

A significant advance to the robustness of this technique was made by Labib and Diffley (Labib et al., 2000), who showed that overexpression of the ubiquitin E3 ligase that facilitates the N-end rule in yeast, Ubr1, can greatly increase the efficiency of clearance of td-tagged substrates; inducible expression systems are used when possible to minimize potentially pleiotropic effects of Ubr1. More than 100 genes have been tagged in this manner. Because large changes in temperature can have pleiotropic effects, an isothermal method might be desirable. In order to achieve this, Taxis and coworkers instead expressed a tripartate fusion of a reporter-TEV target cut site-protein-of-interest (Taxis et al., 2009). Coexpression of the TEV protease in these cells causes cleavage and de novo exposure of the N-end rule substrate and subsequent degradation of the fusion protein. It should be noted that both these techniques also lowered the expression of their target proteins prior to degradation by altering the promoter used.

Because polyubiquitin chains target a protein to the proteasome for degradation, directly controlling the recruitment of a protein to the proteasome could also mediate its degradation. Church and coworkers used a conditional dimerization system to localize a Tor-His3 fusion to the proteasome by the addition of rapamycin to cells expressing a Fpr1-Rpn10 fusion (Janse et al., 2004). Localization of the proteasome caused fast degradation of the His3 fusion protein. Degradation of His3 produced a loss-of-function auxotrophic phenotype, though it was somewhat leaky. Another method to directly tether a protein-of-interest to the proteasome is by fusing it to a fragment of ornithine decarboxylase (ODC); GFP-ODC is constitutively degraded in the absence of proteasome inhibition. Taxis and coworkers fused ODC to the C-terminal J helix of Lov2 to produce a light-dependent conditional degradation signal (Renicke et al., 2013). This modular degradation signal was able to confer loss-of-function phenotypes to a variety of target proteins in yeast but produced leaky phenotypes in some cases. In a similar approach, our group concurrently showed that fusing a degron to the C-terminal J helix of Lov2 could produce blue-light-dependent conditional protein regulation in mammalian cells and in zebrafish embryos (Bonger et al., 2014).

Instead of directly recruiting a protein-of-interest to the proteasome, researchers have demonstrated that recruitment a protein-of-interest to a ubiquitin E3 ligase is often sufficient to drive its ubiquitylation and degradation. Crews and colleagues first demonstrated in cell-free extracts that synthetic molecules, called bifunctional proteolysis targeting chimeras (protacs), can bind to both a target protein-of-interest, and a known E3 ligase is sufficient to drive its degradation (Sakamoto et al., 2001). Several other protac molecules have since been developed that allow conditional degradation of particular target proteins in cells by the addition of cell-penetrating peptides (Schneekloth et al., 2004). Importantly, protacs allows the degradation of endogenous proteins without the need for genetic manipulation, but its use is limited to proteins with known small-molecule ligands that can also be functionalized while retaining reasonable solubility and cell permeability. In another protac-like approach, Wang and colleagues recently demonstrated that a bifunctional molecule that binds to a protein-of-interest and acts as a signal for chaperone mediated autophagy can also degrade proteins in a proteasome independent manner (Fan et al., 2014). Although there was still significant undegraded protein, this approach could be very important in directing the degradation of proteins under conditions of proteotoxic stress and insufficient proteasome function.

If using a chemical genetic strategy, it should be possible to simply fuse a known ligand-binding domain with a protein-of-interest and use an established protac molecule. Analogously,

Kanemaki and coworkers used small-molecule plant hormones called auxins to bridge the protein-of-interest with its target E3 ligase (Nishimura et al., 2009). A fusion of a protein-of-interest with an auxin inducible degron could be ubiquitylated by coexpressing an adaptor F-box protein that also binds to SCF family ubiquiting ligases in the presence of either of two synthetic auxins, IAA or NAA. This yielded fast, tunable control in a variety of experimental model systems.

Two recent reports also demonstrated degradation of a target endogenous protein. In each case, single-chain Fv domains that specifically bind to GFP are fused to either an F-box E3 ligase adaptor protein (Caussinus et al., 2011) or directly to the promiscuous E3 ligase CHIP (Portnoff et al., 2014). When these molecules are coexpressed with GFP fusion proteins, the single-chain Fv binds to the GFP, which is subsequently ubiquitylated by the E3 ligase and degraded by the proteasome. Although these have been demonstrated only as genetically encoded engineered E3 ligases, it should be possible to combine these with other conditional techniques highlighted here in order to produce reversible control of the endogenous protein-of-interest.

Intracellular protein folding is subject to strict quality control, where misfolded or damaged proteins are targeted for ubiquitylation and degradation. When attempting to control the activity of GSK3β in mice using a conditional dimerization system, Crabtree and colleagues noticed that the GSK3β-FRBmutant protein they were using was being degraded, but that could be rescued by the addition of the small-molecule dimerizer MaRap, with the additional recruitment of FKBP (Stankunas et al., 2003). Indeed, Varshavsky and colleagues had previously shown that methotrexate binds to and suppresses the degradation of td-degron-tagged proteins (Johnston et al., 1995). Our group then demonstrated that a genetically compact system requiring only the expression of a protein-of-interest fused to an unstable mutant of FKBP could be rescued by the addition of the specific ligand Shield-1(Banaszynski et al., 2006). These "destabilizing domains" conferred instability to a variety of proteins through the processive degradation at the proteasome. Destabilizing domains also conferred tunable protein regulation in a several organisms, including in living mice (Banaszynski et al., 2008; Iwamoto et al., 2010). Using different protein-ligand pairs now allows the orthogonal control of several proteins simultaneously in the same cell (Iwamoto et al., 2010). Perhaps because of its modular and generalizable uses, destabilizing domains have been widely adopted (Armstrong and Goldberg, 2007; Brooks et al., 2010; Campeau et al., 2009; Dolan et al., 2012; Dvorin et al., 2010; Gong and de Lange, 2010; Kwan et al., 2011; Madeira da Silva et al., 2009; Muralidharan et al., 2012; Pruett-Miller et al., 2009; Raj et al., 2014). Some limitations to the use of destabilizing domains remain, including the necessity for genetic manipulation and mixed utility in the yeast *S. cerevisiae* (Rakhit et al., 2011).

Where fusion of the destabilizing domain to the protein-of-interest may interfere with its function, the protein-of-interest may be combined with an excisable degron to release a free complemented or unmodified protein. Although an intein could be used for this purpose, Pratt and Muir and colleagues exploited the propensity of linear ubiquitin fusions to be rapidly hydrolyzed by intracellular deubiquitinating enzymes (Pratt et al., 2007). The protein-of-interest if fused to a degron, a dimerizer, and a ubiquitin fragment and expressed concurrently with a second protein fused to the dimerization domain and the other ubiquitin fragment. Upon dimerization and ubiquitin fragment complementation, deubiquitinating enzymes cleave the dimerization domains and ubiquitin to release the free, unmodified protein-of-interest. Although it is not reversible and requires dimerization, this system allows tunable control over the abundance of a resultant unmodified (other than its N-terminal amino acids) protein-of-interest (Lin and Pratt, 2014).

In theory, the application of genome-editing technology allows site-specific addition of protein tags that enable many of the posttranslational strategies outlined here. For example, it has now been demonstrated that destabilizing domains can be inserted upstream of exon 1 of Treacher Collins-Franceschetti syndrome-1 (TCOF1) to conditionally regulate it by the addition of Shield-1 (Park et al., 2014). Because of the high efficiency of gene targeting, both alleles can be modified in a single cell leading to conditional regulation without interference from endogenous sources. As the efficacy of gene editing techniques improve, the conditional systems described above will become more valuable to investigators who design control over biological perturbations.

Future Directions and Outlook

The direct control of protein activity through chemical genetics encapsulates many of the desirable properties of an ideal perturbation strategy, including specificity, speed, reversibility, and tunability; however, they also present unique limitations. For example, the direct regulation of an engineered protein has been demonstrated in several cases but can require substantial investment in optimization for the particular protein being studied. Conditional dimerization techniques offer significant advantages in speed compared with degradation tools but are limited to those proteins with subcellular compartment-specific activity. Degradation-based systems are more generally applicable but are somewhat slower and limited to proteins with access to the proteasome.

When choosing an experimental perturbation strategy, a researcher must weigh the precision that an experiment requires against the available tools. Chemical genetic approaches to posttranslationally control protein activity combine the strengths of biologically active small molecules and the specificity of genetic manipulation without having to screen for a particular small molecule. As alluded to earlier, a primary concern with any manipulation, especially in the context of protein-level control, is the robustness of the technology. Although genetic deletion is slow, cumbersome, and spatiotemporally imprecise, it remains the gold standard for many biologists in understanding a gene's function, because it yields unambiguous results. The burden of proof falls on the chemical biology community to improve the robustness of posttranslational, protein-level perturbation technologies to move them from niche to more mainstream biological applications, as seen with ligand-inducible transcriptional systems.

Although the past few years have witnessed tremendous advances in the breadth of protein control technologies, more specific manipulations are desirable. An area that has seen

limited development is the conditional regulation of posttranslational modifications. Because kinases, phosphatases, and so on, may have many downstream targets, regulating their activity does not allow careful dissection of these networks. When posttranslational modifications are present, it is not clear which modification, and in which contexts, are biologically relevant to the control of that protein's activity. In order to study this, biologists currently make phosphoanalog mutants by replacing a serine, threonine, or tyrosine residue with one of the negatively charged amino acids, such as aspartic or glutamic acid. Although combining a phospho mutant with an established conditional protein regulation tool may provide more precise control of this target, the perturbation is likely to be slow relative to the timescale of the biological process under study, such as its downstream signaling or its role in chromatin remodeling. A more elegant solution would enable the study of these biologically important processes.

Another key area of potential improvement to these chemical genetic tools is in experimental systems in which fewer tools are available. First, conditional tools to study mitochondrial or extracellular proteins remain relatively underexplored (Sellmyer et al., 2012). Strategies that target these proteins for degradation are also lacking, largely because they are compartmentally sequestered from the proteasome. The study of clinically relevant apicomplexan parasites, such as *Toxoplasma gondii* and the *Plasmodium* species, is limited by the availability of conditional techniques to control essential protein function, because they are haploid. Destabilizing domains have been used to conditionally degrade proteins in these organisms, but have not been as effective as they are in mammalian cells (Jimanez-Ruiz et al., 2014). The development of improved conditional protein regulation systems would allow more efficient characterization of protein signaling in these organisms.

Moving beyond these specific applications, one aspect of these conditional control systems remains to be exploited, which is their ability to deepen our mechanistic understanding of biological systems. In theory, the speed and specificity these technologies offer should help control for any cellular compensatory mechanisms leading to an observable phenotype; however, the timescale at which most of these tools operate lags behind the speed of signaling processes in vivo, which can still complicate quantitative assessments. More generally, mechanistic studies are further complicated by the fact that the most well-characterized and commonly used tools are far from ideal. A case in point here is the use of rapamycin-based approaches, which continue to be implemented today. This ultimately highlights the need for significantly improved, general small-molecule-based tools. Another factor that complicates in-depth biological studies is that a given protein may have more than one function or binding partner, which would be difficult to identify using current technologies. Although the parallel application of these tools—to study multiple proteins in a single system—can help elucidate such confounding factors, such studies may be difficult to execute from a practical standpoint. The development of efficient and practical ways to simultaneously study multiple proteins will ultimately give us a more quantitative look at the interactions that orchestrate complex biological behavior.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two tables and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2014.08.011.

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