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We See the Light: Chemical-Genetic Protein Regulation

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The challenge of studying complex protein networks in whole animals has driven the development of new methods for manipulating protein function with spatial and temporal precision. A novel combination of chemical and genetic protein regulation (Rodriguez and Wolfgang, in this issue of *Chemistry & Biology*) achieves levels of control that will revolutionize the study of protein function.

Identifying the function of a protein has often relied on characterizing the impact of naturally occurring or directed mutations in the gene encoding that protein. Protein functions were first elucidated in microorganisms through the use of temperature-sensitive alleles, mutations, and deletions. In whole organisms, developmental biologists successfully described a variety of complex protein networks by designing ways to perturb specific proteins in model organisms and characterizing the resulting phenotypes. The past 20 years have seen the advent of homologous recombination techniques in mice that enable protein regulation through mutations, transcriptional control, and RNAi (Thomas and Capecchi, 1990). However, many of these technical advances have serious drawbacks. Attenuated gene expression, gene knockouts, and mutations in mammals frequently have lethal or detrimental outcomes during early development that prevent exploration of protein function on whole animal physiology and at later stages. Although RNAi has proven to be a powerful knockdown technique, designing functional RNAi probes can be problematic, because it is difficult to predict if a given sequence will attenuate mRNA levels. Further, changes in mRNA levels do not always produce a corresponding change in protein levels. Despite these challenges, it is critical to develop approaches that study the function of proteins in whole animals because complicated and overlapping factors such as secreted factors, cell-cell interactions, and metabolic state most certainly impact protein function but are impossible to replicate in less complex systems. Therefore, the future of functional protein studies in whole organisms lies in developing sophisticated tools that reversibly regulate proteins in a specific time and place.

Our understanding of complex biological networks would particularly benefit from improved capabilities in controlling the levels of any given protein with fine-tuned precision. For example, studies of lipid metabolism have been especially challenging because there are often many similar proteins (as defined by enzymatic properties) in the vertebrate genome that can act in concert with functionally-related lipid-modifying proteins. Elucidating the function of these proteins would be facilitated by designing experiments that discretely control expression of a protein. To this end, small molecule-mediated protein regulation has been the most successful approach to temporally regulate proteins due to its broad applicability to almost any protein of study, its specificity in targeting, and its reversibility. The basic principle of this approach is to fuse inherently unstable protein domains to a protein of interest, thereby conferring instability to the entire fusion construct. Introducing a biologically inert small molecule that binds specifically to the unstable protein domain in the fusion construct subsequently stabilizes the fusion protein in a dose-dependent manner, thereby effectively activating the protein of interest. Reversal of this process is achieved by removing the small molecule. Several groups have spent the better part of a decade modifying and perfecting a small protein domain of FK506 binding protein 12 (FKBP12) that can convey instability to any protein to which it is fused (Stankunas et al., 2003; Yang et al., 2000). Improvements in high throughput structure-based

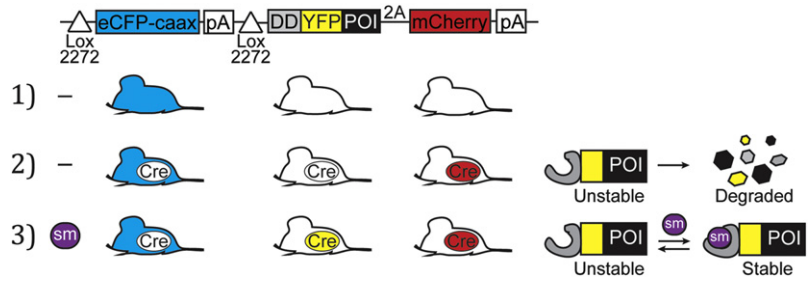

Figure 1. Spatial and Temporal Control of Proteins

CFP, mCherry, and YFP are used to trace incorporation, recombination, and activation of the construct, respectively. The use of fluorescent markers allows each of these steps to be monitored in real time. (1) CFP is used to indicate the incorporation of the construct into a mouse. YFP and mCherry are not translated. (2) Tissue-specific CRE induces homologous recombination that removes the CFP signal and activates the mCherry signal in the tissue of interest (represented by the oval). The unstable degradation domain (DD, in gray) causes the degradation of the DD-YFP-protein of interest (POI, in black) construct as indicated by the absence of YFP signal. (3) Similar to line 2, CRE removes the CFP signal and activates mCherry. The DD is stabilized by binding to a small, biologically-inert molecule (sm, in purple), penultimately resulting in a stabilized fusion construct indicated by a YFP signal.

design, protein mutagenesis, and small molecule screening enabled predictions of how modifications of FKBP12 convey significantly higher affinity to a variety of stabilizing ligands, including the highly specific and biologically inert small molecule Shield-1.

In this issue, Rodriguez and Wolfgang (2012) have taken advantage of the FKBP12/Shield-1 regulatory mechanism to design a tractable method to control malonyl-CoA decarboxylase (MLYCD) expression. The authors cleverly employ three fluorescent proteins to trace the incorporation and activation of a construct (Figure 1). First, MLYCD was fused to the previously published YFP-tagged version of the mutated degradation domain of FKBP12, called FKBP, so that the stable fusion protein can be observed via YFP fluorescence in real time (Banaszynski et al., 2006). They verified that the N-terminal fusion to MLYCD does not affect the enzymatic function of stabilized MLYCD. Second, because the YFP signal is only produced in the presence of the stabilizing small molecule Shield-1, the authors incorporated mCherry into the fusion construct through a 2A peptide linker, which allows the production of two proteins from a single mRNA at a 1:1 stoichiometric ratio (Szymczak et al., 2004). In doing so, the expression of this construct can be verified by analyzing mCherry fluorescence even in the absence of Shield-1. Finally, the authors incorporated CFP, flanked by loxP sites, 5′ of the YFP-FKBP-MLYCD-2A-mCherry so that integration of the construct into cells and tissues can be monitored. Incorporating the CRE/Lox approach creates a "dual inducible system" that allows both temporal (small molecule-mediated inhibition) and spatial (CRE-mediated tissue expression) control of the overexpression of a protein of interest. The authors' elegant use of fluorescent proteins to trace the various key steps in this process (e.g., integration, recombination, and protein stabilization) sets it apart from previous studies employing small molecule-mediated inhibition.

Rodriguez and Wolfgang (2012) validate a novel technique that has several advantages over the currently favored approach for temporal control over overexpressed proteins in mice, the rtTA tetracycline system (Gossen et al., 1995). These advantages include the following. (1) Typically only one transgenic mouse line needs to be created as opposed to multiple lines because of the large number of tissue-specific CRE lines as compared to rtTA lines. (2) Not all tissues are accessible to doxycycline (e.g., brain, testis), the antibiotic used to induce expression (Beard et al., 2006). (3) Using the FKBP12 fusion results in protein degradation when Shield-1 is withdrawn, whereas removal of doxycycline only shuts down transcription, leaving existing proteins intact. This is not a problem for proteins with a short half-life but can be an issue for more stable proteins. (4) The use of the viral 2A sequence allows for real-time visual identification of transgene-expressing cells irrespective of the presence of Shield-1. For the rtTA approach, there is no secondary marker to visually confirm recombination in the absence of doxycycline. (5) mCherry expression can serve as a lineage marker for studies in which Shield-1-regulated protein expression could alter cell fate.

Rodriguez and Wolfgang (2012) have pieced together cutting-edge aspects of small molecule-mediated chemical regulation and mouse recombinant genetics to generate a technique that is broadly applicable to any protein that is unchanged by a fusion to a small protein domain. Importantly, the authors carefully instruct how their approach is broadly applicable to many genes and proteins, not just to MLYCD. In their proof of principle article, the authors successfully demonstrate the most exciting possibility of their targeted genetic-chemical technique: rapidly and reversibly regulating the activity of a protein in a tissue-specific and temporal manner. Using their favorite protein as an example, they are able to control the stability of MLYCD in the skeletal muscle of mice. However, they have kept us in suspense as to the physiological effect of this inducible MLYCD tissue-specific activation. Rodriguez and Wolfgang (2012) have succeeded in whetting our appetites for future experiments that will surely capitalize on this technique. The ability to regulate proteins in such a finely-tuned manner will likely open up a world of new possibilities for future studies.

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