Direct protein control

Monya Baker

Light and chemicals offer precise ways to manipulate proteins.

Knocking out a gene may be the most straightforward way to probe a protein's function, but such experiments are blunt instruments. Removing a protein from a cell altogether can be both too much and too little, explains Kevan Shokat, a chemical biologist at the University of California, San Francisco. "You can either get no phenotype because another protein will replace it, or you'll destroy a whole complex because it's so central."

In response to this and other challenges, researchers have developed multiple systems in which proteins are engineered to respond to small molecules or light. Depending on the technology, scientists can inhibit proteins, activate them, bring them together or send them to specific cellular locations. Though less familiar than adding or removing genes, these techniques offer faster and more subtle ways to perturb protein function.

Adapting enzymes to their inhibitors

Shokat has developed a way to specifically disable proteins' catalytic activity while leaving the protein itself in place. He has focused on the cell's chief workhorses for signaling, the superfamily of hundreds of proteins called kinases.

Though each kinase has its own functions and interaction partners, all carry out similar chemical reactions. A small molecule that inhibits one kinase is likely to inhibit several if not dozens of others, and so their roles can be hard to distinguish experimentally. To circumvent this problem, Shokat took a small molecule that reliably inhibited kinases and enlarged it so that it would no longer fit the kinase active site. Then he and his colleagues engineered the active site of the kinase he wanted to study to fit the inhibitor by replacing a key amino acid with a smaller one[1].

Kevan Shokat engineers kinases to fit a specific inhibitor.

Shrinking the 'gatekeeper' amino acid worked well for most but not all kinases. In about 3 of 10 cases, the mutation destabilized the enzyme and significantly reduced its activity. Eventually, Shokat and colleagues encountered enough kinases that couldn't be probed by the bulky inhibitor that they decided to search for other options. This time, instead of shrinking the gatekeeper residue, Shokat's lab replaced it with a gatekeeper that would trap an inhibitor in place.

The amino acid cysteine was a good candidate because it occurs very rarely as the gatekeeper residue in natural kinases and because it can form covalent bonds with electrophilic small molecules. In work published last September, Shokat showed that the kinase Src, which becomes unstable with a smaller gatekeeper, maintains normal activity with a cysteine gatekeeper and is potently inhibited by an electrophilic inhibitor. Screens of electrophilic inhibitors against a panel of 307 other kinases show very little off-target inhibition[2].

There are still some inconveniences, says Shokat. The main one is that electrophilic inhibitors can be difficult to synthesize and are unlikely to be available commercially. However, Shokat's lab has recently improved and scaled up the synthesis of the best inhibitor, and he plans to supply researchers on request.

One appealing aspect of these chemical genetics systems is that they simultaneously elucidate biological processes and show that they can be modulated with small molecules, says Shokat. "I'd like to keep using tools that are potentially convertible to a therapeutic."

Proteins that need small molecules

Thomas Wandless at Stanford University was part of one of the earliest demonstrations that small molecules could be used to bring proteins together and control signal transduction[3]. Chemically induced dimerization is now a common technique, with the most popular components being a protein called FKBP and derivatives of the small molecule rapamycin.

Tom Wandless designs proteins that are destroyed in the absence of certain small molecules.

Several years ago, a puzzling observation in a collaborator's lab sent Wandless in a new direction. Gerald Crabtree, also at Stanford, wanted to use a version of the FKBP system to bring proteins together on cue, but realized that one of the proteins was being quickly degraded. In certain situations, the protein tags that trigger dimerization act as 'destabilizing domains' that trigger cellular machinery designed to eliminate misfolded proteins by the proteasome—an effect that is quickly reversed by the addition of small molecules that can stabilize the inserted domain. Wandless realized that this property could be fashioned into a new tool: a way to control protein activity outside of gene expression.

Since then, he has crafted three separate destabilizing domains, each paired with

its own small molecules. The most recent one, published earlier this year, uses a portion of the estrogen receptor and can be regulated by several estrogen-receptor antagonists[4]. A fourth system uses a domain that is destabilized by a small molecule[5]. Because each domain relies on distinct ligands, multiple systems can be combined to probe activity of proteins in the same cell, says Wandless.

Klaus Hahn uses both light and small molecules to activate proteins.

Conceptually, the systems work much like conditional alleles that can be turned on by adding tetracycline or doxycycline, says Wandless. Destabilizing domains can be used on a wide range of proteins, including membrane proteins, he says, and the reagents are either commercially available or easy for Wandless's lab to make in large enough quantities to share. Though scientists must first make sure the modified protein still functions, these destabilizing domains can be particularly useful when conditional alleles are not an option, such as in organisms like the malaria parasite. In any case, manipulating the protein itself, rather than a transcription factor, has advantages, says Wandless. "Because the drug is controlling the protein directly, we have more tunable control." It is speedy, too. The protein starts to accumulate or decay within five minutes after the ligand is added or washed out, says Wandless.

Small molecules can also be used to specifically activate enzymes, as shown by Klaus Hahn and colleagues at the University of North Carolina. Hahn inserted domains of FKBP12 into the kinase he wanted to study. In contrast to Wandless's approach, Hahn's goal was to use FKBP not to trigger the protein's destruction but to selectively inactivate it. Rapamycin, a small molecule that binds the FKBP12 domain and attracts another, larger protein, was supposed to block the protein's activity. "We thought we'd be putting a big blob over the active site," says Hahn. Unexpectedly, the FKBP insertion on its own blocked the enzyme's catalytic activity, and rapamycin restored it.

Intrigued, Hahn and colleagues investigated further. It turned out that the FKBP12 domain destabilized the kinase, and that the interaction with rapamycin allowed it to work again. They went on to minimize and optimize the inserted domains, creating rapamycin-activated versions of focal adhesion kinase (more commonly known as FAK) as well as of Src, a widely studied target[6]. What's more, the inserted domain does not involve altering the active site itself. "It works through the backbone of the protein," explains Hahn.

Hahn believes the approach is generalizable: models suggest that the same strategy can also be applied to other kinases to produce stable, inactive enzymes that can be restored to activity using rapamycin or similar molecules.

Light-activated proteins

But small molecules are not the only way to control a protein's function. Light travels faster than small molecules can diffuse and can also be directed at specific parts of an animal or cell. Plant and microbe researchers have discovered a wide range of proteins that respond to light directly. Perhaps most famously, light-sensing proteins that act as ion channels or pumps have been introduced into neurons and muscle cells so that their excitation can be both prompted and repressed by light.

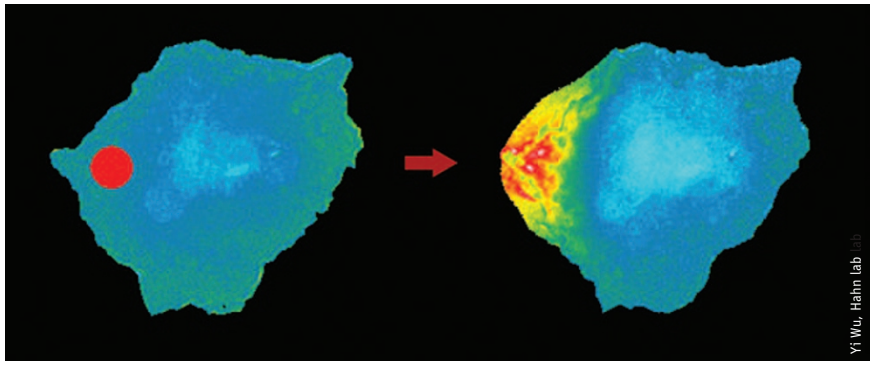
But light-sensitive proteins can be used more generally than that. The ability to fuse a light-sensitive domain onto other functional domains can be used for the control of many cellular functions even in cells that don't contract or have action potentials, says Hahn, one of the few researchers to have worked on controlling proteins with both small molecules and light. "It opens the door to all the other protein families that are important in cells."

Hahn's work on light-based system uses the LOV (light, oxygen, voltage) domain of the oat photoreceptor phototropin, which can serve as a photactivatable protein switch. When the protein is exposed to blue light, a helix in this domain unwinds. Hahn thought this movement could be used to block key sites in another protein. He attached the LOV domain to a protein called Rac1, a GTPase that controls components of the cytoskeleton. In the dark, the helix from the LOV domain was held tight against Rac1, blocking the protein from interacting with its downstream targets. In the light, the helix relaxed, allowing Rac1's partner to bind and activate the enzyme. In the dark, the LOV domain closes again over about 20 seconds, says Hahn.

In cells containing photoactivatable Rac1, ruffles and protrusions appear in the spots illuminated by blue light, causing cells to crawl in that direction[7]. Other scientists have used photoactivatable Rac1 in a variety of organisms, including zebrafish, flies and mice. "You can lead cells around like a cat following a laser beam," says Hahn, who expects photoactivatable versions of related proteins to be reported in the near future.

Light-induced interactions

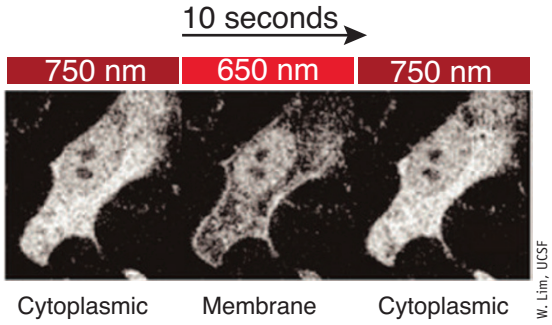
Light can also trigger protein-protein interactions directly. In 2009, a team of researchers at the University of California, San Francisco was able to drive proteins to the cell membrane by exploiting a pair of mustard plant proteins that come together under red light. They could attach a membrane-localization domain to one of the light-sensitive proteins and a protein of interest to the other, and then drive the protein of interest to the cell membrane using red light. Infrared light causes the



Mammalian cells expressing photoactivated proteins will crawl toward light.

proteins, called phytochrome B and PIF, to dissociate[8].

The phytochrome B system requires a cofactor that is easy to deliver to cell lines but is hard to synthesize and must be extracted from cell cultures of cyanobacteria. This can provide an extra layer of control by preventing interactions that might occur even in the dark, but it can also be an inconvenience. Soon after that publication, researchers led by Ricardo Dolmetsch at Stanford University demonstrated a similar system that relies on a cofactor that occurs naturally even in non-photosynthetic cells. They showed that the mustard plant proteins FKF1 and GIGANTEA can be brought together in mammalian cells under blue light. They used this property to bring Rac1 to the cell membrane on demand: Rac1 was fused to FKF1, and GIGANTEA was fused to a peptide sequence that moved it to the cell membrane. Even a quick flash of light can cause an interaction that lasts for around 90 minutes. This minimizes the amount of light that must be used, but it also means researchers cannot stop or redirect the interaction on cue[9].



Different colors of light can drive engineered proteins together and apart.

In 2010, Chandra Tucker, now at the University of Colorado, Denver, developed a system that uses a third pair of proteins, cryptochrome 2 and CIB1. Proteins of interest can be fused to the light-sensitive proteins so that their interaction is stimulated by blue light[10]. Tucker has used the system to reconstitute the activity of enzymes. Her strategy is to split the gene encoding a protein, with one portion fused to the gene encoding each light-sensitive protein. When the resulting fusion proteins are exposed to light, the light-sensitive proteins bring their attached portions together and the original protein's function is restored. It ceases after a few minutes in the dark as the light-sensitive proteins dissociate.

Though the light-driven aspect is new, technology to split and reconstitute proteins is well established, says Tucker. "All sorts of protein activities can be regulated this way," she says. "I don't think every protein is going to be able to be split, but in theory, the sky's the limit."

In a demonstration of light-induced dimerization this year, Michael Glotzer and colleagues at the University of Chicago used a clamshell-like protein called ePDZ that captures a peptide ligand between its two halves. The team appended this ligand to a helix within the LOV domain. In the dark state, the ligand is packed against the protein's surface. Light releases the helix and lets the ligand dangle out, where it can come into contact with ePDZ. When that happens, the clamshell latches on, bringing the proteins of interest together[11].

The project got its start at a departmental retreat a few years ago, recalls Glotzer. Fellow faculty member Tobin Sosnick and his graduate student Devin Strickland were making an assay that used the LOV domain to control a bacterial transcription factor, and they asked Glotzer rhetorically what protein he would most want to control. Glotzer had an immediate, non-hypothetical answer: he wanted to manipulate a small cell-signaling protein called Rho. Strickland began working on the idea and later joined Glotzer's lab as a postdoc to see the project through. This resulted in a general way to use light for controlling protein-protein interactions, dubbed TULIPs (tunable, light-controlled interacting protein tags).

Glotzer and colleagues found that they could fuse the modified LOV domain to peptides that anchor it in specific parts of the cell, such as the plasma membrane. When exposed to light, ePDZ grabs on to LOV wherever it is anchored. In fact, by shining light on only certain parts of the cell membrane, it was possible to bring ePDZ specifically to the illuminated regions.

Researchers can tune the system by picking differently engineered proteins, says Glotzer. He and his colleagues have created five distinct modified LOV domains that have different affinities for ePDZ in the light and dark, so that researchers can choose how big of an activation jump occurs when the light is turned on, as well as how little activity there is in the dark.

Using this system, Glotzer and colleagues were able to manipulate several signaling pathways that require proteins to be recruited to the cell membrane. This kind of control will allow researchers to map out the order of distinct signaling events within cells, says Glotzer. "With light, since you know where you activated the system and you would know exactly when, you could order the activation events. You would know who got there first."

Sorting through a signaling cascade

Orion Weiner at the University of California, San Francisco believes researchers have not yet come close to realizing the potential of light to puzzle out complex mechanisms behind cell signaling. The people who have developed the light-gated systems have used them in a largely binary fashion, he says. "They turn it on full strength and just ask what happens."

But subtler manipulations could be valuable for interrogating both signal amplification and negative feedback loops, Weiner says. In mammalian chemotaxis, for instance, it is not known where in the signaling cascade cells convert small differences in ligand binding into a large downstream response, and the question is hard to address experimentally. "People haven't had a way of fine-tuning inputs," Weiner explains.

Orion Weiner believes that light offers new ways to study both positive and negative feedback cascades.

In 2011, Weiner and colleagues described a system to do just that. They used a light-driven interaction using the phytochrome B system to recruit the enzyme PI3K to the cell membrane and monitored its activity by fluorescently labeling its lipid byproduct. The amount of this product could be kept constant by continuously adjusting enzyme recruitment to the membrane using different wavelengths of light[12].

Weiner thinks such systems could let researchers "walk down the signaling cascade" and interrogate its components. A light-gated system would be used to keep some output of a signaling cascade at a constant level, much as a thermostat will turn on the heater or air conditioner to maintain a constant temperature. "It's analogous to a voltage clamp," explains Wendell Lim at the University of California, San Francisco, who co-led development of the phytochrome B system. "Seeing how the system responds to stimuli and how much correction you need to hold it at that constant state can give you information."

Wendell Lim believes light can be used to study how the frequency of a signal affects cell response.

Weiner believes that much of the necessary technology is already in place for both monitoring specific components of signaling pathways and controlling them with light. For monitoring, a variety of biosensors and fluorescent labels are already well established. To control the components, Weiner hopes to adapt chemically driven interaction systems in which proteins of interest are fused to protein domains that come together in the presence of small molecules such as rapamycin. "People have put dozens if not hundreds of systems onto rapamycin," he says. "We should be able to port most of them to light control."

These systems have already been subjected to considerable engineering, but researchers trying to bring them into their own labs should expect to do considerable tinkering. New protein fusions may affect protein function, and some systems are more tractable than others. Both light-based and chemical systems have their own sets of artifacts and inconveniences. For example, proteins that interact in light often have some affinity in the dark, and small molecules can have off-target effects.

Researchers like Weiner and others believe that more researchers will start to use light to manipulate cell biology, and that paired systems will develop such that the same proteins or pathways can be controlled both by light and by small molecules, depending on what an experiment requires. Assuming that they can be synthesized in large enough quantities and penetrate tissues, small molecules can be used to study effects in the whole body, and they are more accessible to researchers without microscopes. However, light can allow exquisite control over processes within isolated single cells, and systems for shining light into desired organs within living animals are becoming more sophisticated.

But although no one system is perfect, together they are expanding the kinds of questions cell biologists can ask, says Lim. "We can control things over time. We can change the amplitude. We can ramp it up and ramp it down, we can do zigzags," he says. "We're still trying to figure out how to use all this power."

1. Bishop, A.C. *et al. Nature* **407**, 395–401 (2000).
2. Garske, A.L. *et al. Proc. Natl. Acad. Sci. USA* **108**, 15046–15052 (2011).
3. Spencer, D.M., Wandless, T.J., Schreiber, S.L. & Crabtree, G.R. *Science* **262**, 1019–1024 (1993).
4. Miyazaki, Y. & Imoto, H., Chen, L.C. & Wandless, T.J. *J. Am. Chem. Soc.* **134**, 3942–3945 (2012).
5. Bonger, K.M., Chen, L.C., Liu, C.W. & Wandless, T.J. *Nat. Chem. Biol.* **7**, 531–537 (2011).
6. Karginov, A.V., Ding, F., Kota, P., Dokholyan, N.V. & Hahn, K.M. *Nat. Biotechnol.* **28**, 743–747 (2010).
7. Wu, Y.I. *et al. Nature* **461**, 104–108 (2009).
8. Levskaya, A., Weiner, O.D., Lim, W.A. & Voigt, C.A. *Nature* **461**, 997–1001 (2009).
9. Yazawa, M., Sadaghiani, A.M., Hsueh, B. & Dolmetsch, R.E. *Nat. Biotechnol.* **27**, 941–945 (2009).
10. Kennedy, M.J. *et al. Nat. Methods* **7**, 973–975 (2010).
11. Strickland, D.S. *et al. Nat. Methods* **9**, 379–384 (2012).
12. Toettcher, J.E., Gong, D., Lim, W.A. & Weiner, O.D. *Nat. Methods* **8**, 837–839 (2011).

Monya Baker is technology editor for *Nature* and *Nature Methods* (m.baker@us.nature.com).