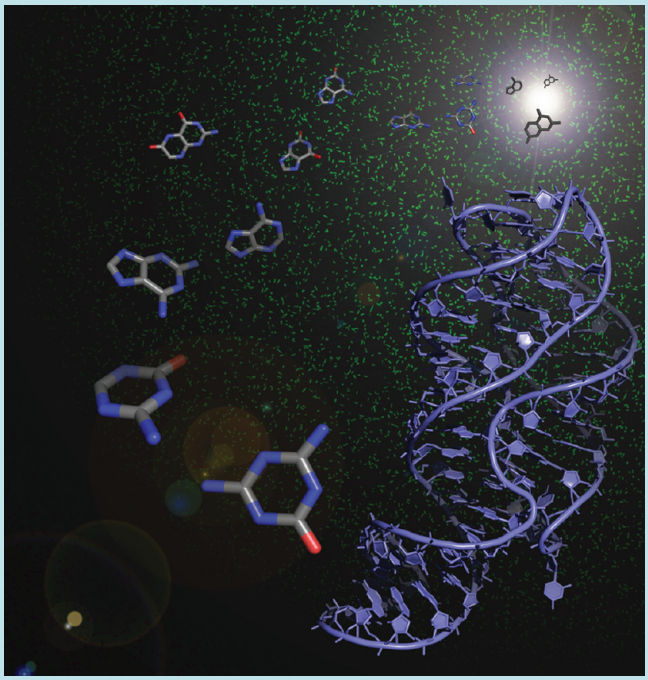
Spotlight

Riboswitch Switch Hitters



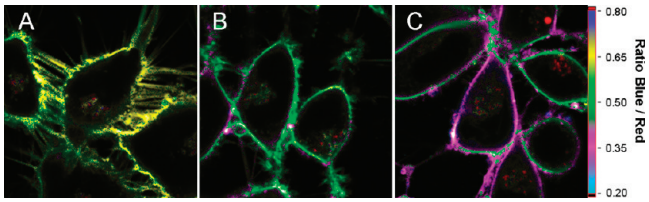
Riboswitches are structured RNA scaffolds that adopt different tertiary structures in the presence or absence of a small molecule effector. These RNA sensors, first discovered in bacteria, detect the levels of a metabolite and regulate genes involved in metabolite biosynthesis or catabolism. Riboswitches can alter the neighboring gene's expression via a variety of mechanisms, affecting either translation or transcription, and thus play critical roles in autoregulating numerous metabolic pathways. Using a riboswitch as an artificial sensor or structural switch would be an attractive tool for biologists, but unfortunately the cognate ligands are often common cellular currency like thiamine pyrophosphate, adenine, or amino acids and would therefore lead to leaky basal expression levels.

Now, Dixon *et al.* (*Proc. Natl. Acad. Sci. U.S.A.* 2010, *17*, 2830−2835) has used one well studied riboswitch to serve as a blueprint in the search for a new RNA switch that no longer responds to the parent ligand but does respond to new ligands not found naturally in cells. The *add* A switch usually senses adenine levels and upregulates expression of the neighboring adenine deaminase gene. The RNA structural arrangement upon binding to adenine was previously solved by X-ray crystallography, and the new study used this information to guide mutations that might alter that specificity. With panels of mutant riboswitches in hand and a battery of almost 80 small molecules, a clever screen in bacteria searched for the RNA/small molecule combinations capable of upregulating a neighboring reporter gene. Mutant *add* A riboswitches that could now respond to synthetic heterocyclic compounds ammeline and azacytosine were chosen for further characterization. Using these RNA switches upstream of GFP allowed for easy screening of additional mutants within bacterial cells. Further refinement of the new M6 riboswitches improved the maximum yield of GFP produced, allowing for a tunable system that gives low, medium, or high expression levels. Finally, to bring the study full circle, the authors solved the X-ray structure of the M6C′′ mutant aptamer domain complexed with azacytosine. They observed how the mutant *add* A-riboswitch has lost selectivity of binding for adenine but gained it for the new ligand. Since the identified ligands are not naturally found inside of cell, these new riboswitches and future ones selected via similar methods could become interesting candidates for engineered switches to control gene expression inside of living cells. **Jason G. Underwood, Ph.D.**

To the Nile for Membrane Characterization

The lipid components of cell membranes are key players in directing membrane structure and function. In particular, strong interactions between cholesterol and the phospholipid sphingomyelin result in a highly packed state in lipid bilayers, called liquid ordered (Lo) phase. Lo phase is distinct from the loosely packed phase formed by cholesterol and other lipids, referred to as liquid disordered (Ld) phase. However, few molecular tools exist that effectively distinguish between Lo and Ld phases *and* selectively target the outer leaflet of cell membranes, which contains the majority of sphingomyelin. Toward gaining a better understanding of the structural and functional relevance of Lo domains in cell membranes, Kucherak *et al.* (*J. Am. Chem. Soc.* 2010, *132*, 4907−4916) developed a fluorescent probe, referred to as NR12S, that is highly selective for the outer leaflet and effectively discriminates between Lo and Ld phases.

Published online April 16, 2010 • 10.1021/cb100086a

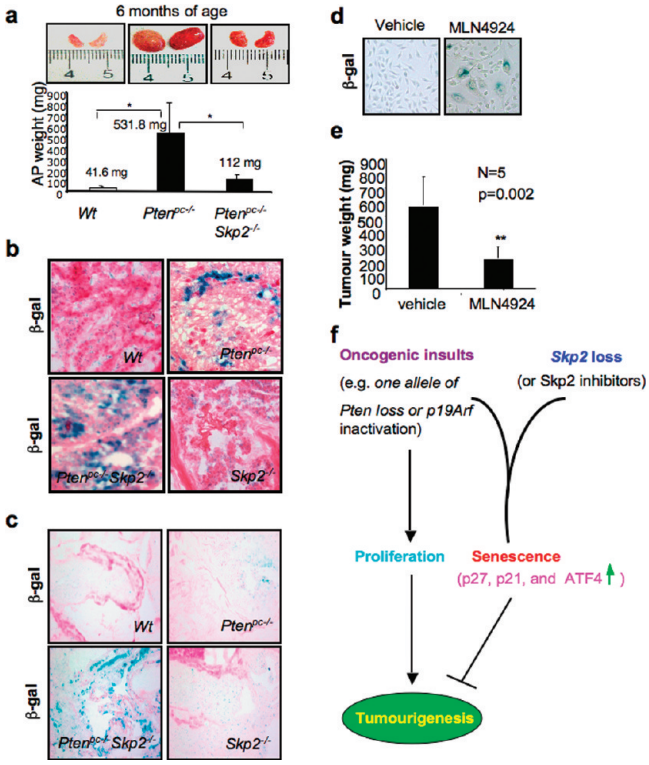


Based on the fluorophore Nile Red, NR12S also contains a long alkyl chain and a zwitterionic group, which presumably enhance its interactions with lipid membranes. Characterization of NR12S demonstrated its high selectivity for the outer leaflet of both model lipid vesicles and live cell membranes. In addition, in contrast to Nile Red itself, NR12S is not internalized, nor does it flip-flop between the inner and outer leaflet. Experiments in model vesicles revealed a significant blue shift in the emission maximum of NR12S in the presence of Lo phase versus Ld phase, illustrating its ability to distinguish the two phases. When examined in live cells, the emission maximum was in between those of Lo and Ld phases, consistent with what is known about composition of plasma membranes. Importantly, the emission color of NR12S was sensitive to increases or decreases in the cholesterol content of the membranes, demonstrating its utility for monitoring cholesterol content as well. These properties make NR12S a valuable tool for investigation of lipid order in model and cell membranes selectively at one leaflet. **Eva J. Gordon, Ph.D.**

Skp-ping to a Cancer Cure

Cellular senescence refers to a process in which cells become unable to divide. Recent evidence has linked cellular senescence to tumor suppression mechanisms, hinting that researchers might be able to hijack the process for the purpose of fighting cancer. Studies have suggested that senescence is dependent on activation of the p19Arf-p53 tumor suppressor pathway, but the frequent mutations in this pathway that are associated with human cancers have confounded the pursuit of senescence as an anticancer strategy. Now, Linet *et al.* (*Nature* 2010, *464*, 374−379) revive senescence in the fight against cancer by discovering the anticancer potential of inhibiting Skp2, a key component of the ubiquitin E3 ligase Skp2-SCF.

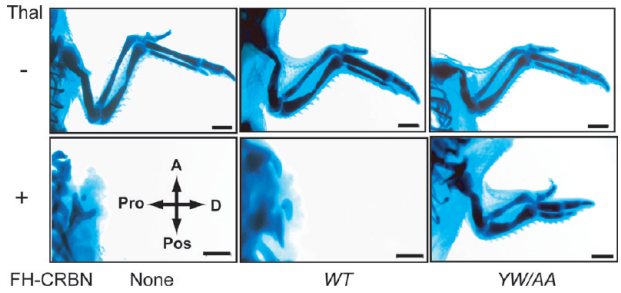
Skp2 is overexpressed in many human cancers but is not necessary for survival in mice, making it an intriguing cancer target. Interestingly, it was found that inactivation of the *Skp2* gene in mouse embryonic fibroblasts does not trigger senescence. However, when strong oncogenic signals are present, *Skp2* deficiency triggers a powerful, tumor-suppressive senescence response. Notably, unlike other senescence processes, this response was not associated with DNA damage, nor did it depend on the p19Arf-p53 pathway. Rather, Skp2-triggered senescence was dependent on upregulation of the endoplasmic reticulum stress protein Atf4 and the cyclin-dependent kinase inhibitor proteins p21 and p27. Importantly, inactivation of *Skp2* significantly reduced tumor progression in mouse models of prostate cancer, and investigation into the mechanism of action suggested that in the presence of an oncogenic stimulus inactivation of *Skp2* enhances and prolongs the senescence response. Finally, exposure of prostate cancer cells and mouse models of prostate cancer to a small molecule inhibitor of the Skp2-SCF complex resulted in induction of senescence and slowed tumor growth, respectively. These exciting findings implicate Skp2 and the senescence response as important cancer targets, and point to small molecule inhibitors of Skp2 function as potential cancer drugs. **Eva J. Gordon, Ph.D.**



Thalidomide Makes a Molecular Debut

In the 1950s and 60s, many pregnant women battled morning sickness with what they thought was a sedative drug, thalidomide. Sadly, the unseen effects of thalidomide on the fetus would not be fully realized until over 10,000 children were born with birth defects, mostly involving limb formation or size abnormalities. Decades later, the molecular mechanism of the drug's ill effects remained controversial and largely unsolved.

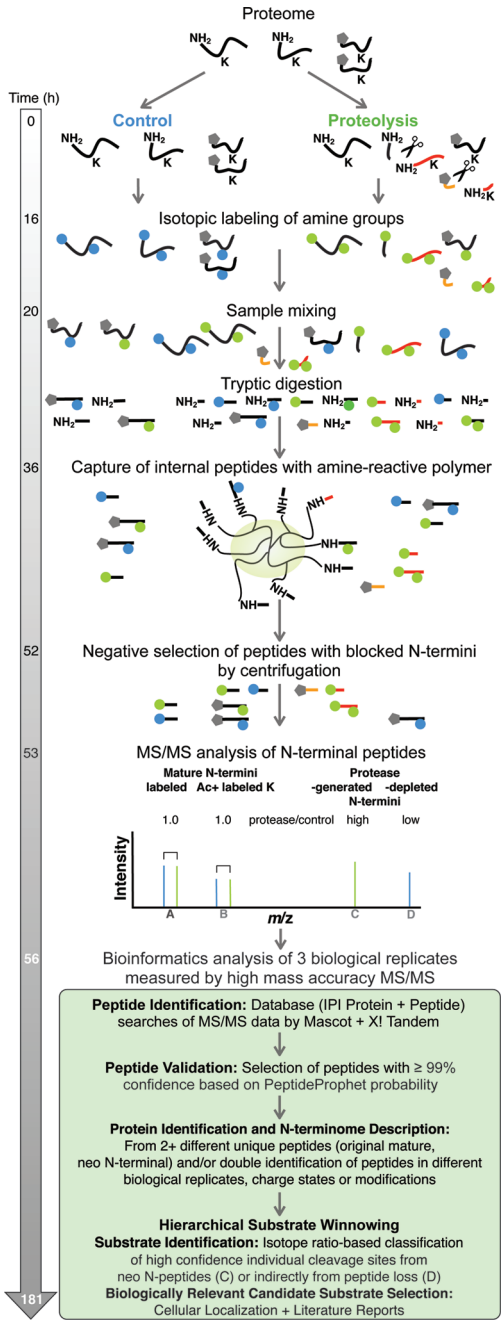
Now, a new study by Ito *et al.* (*Science* 2010 327, 1345−50) has used a powerful combination of biochemistry and developmen-



From Ito, T., *et al.*, *Science*, 2010, *327*, 1345–1350. Reprinted with permission from AAAS.

tal biology to show the first *bona fide* direct target of thalidomide. Using special beads coupled with a thalidomide derivative, the researchers fished out two proteins from mammalian cell extracts that specifically bound to the drug. The candidate proteins, CRBN and DDB1, both bound to the beads, but further tests proved that CRBN was the directly associated target. Luckily, the DDB1 not only came along for the ride by binding to CRBN but also gave a clue to one possible effect of thalidomide. DDB1 was previously found to be associated with a protein complex that plays a key role in protein turnover by ubiquitination, a mark that sends proteins to the proteasome for degradation. When the researchers tested the associated protein, CRBN, they found that it was a component of an E3 ubiquitin ligase complex and CRBN also displayed ubiquitination. Moreover, addition of thalidomide caused specific inhibition of ubiquitination.

After localizing the thalidomide-binding region of CRBN, a valuable mutant of the protein was constructed. The YW/AA mutant could still interact with DDB1 and function as an E3 ligase but could no longer bind to thalidomide. Armed with this mutant, two highly divergent models systems of refined embryology, the zebrafish and chicken, were both employed to show that CRBN is the target of thalidomide. Knockdown of zebrafish CRBN protein caused a similar phenotype to thalidomide treatment, a loss of proper fin formation. Protection from the drug was not observed by expression of the wild-type (*WT*) CRBN but was strikingly apparent with the thalidomide-immune YW/AA rescue in both fish fins and chick limbs. Further experiments demonstrated that FGF proteins could be the main downstream targets of the CRBN/DDB1 complex. These results bring a half-century old mystery into a new molecular limelight, but unlock a new mystery to tackle. Since CRBN is present in most known cell types, why are the limbs the main target of this devastating drug? **Jason Underwood, Ph.D.**



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Getting to the End of the N-Terminus

The N-terminus of a protein can be modified or altered in numerous ways, which can result in profound effects on protein activity, regulation, and location. Examples include acetylation, which changes the chemical reactivity of the terminal amino group and (as recently shown) determines protein half-life in yeast models, and N-terminal truncations, which produce different protein isoforms that can each have distinct functions. Degradomics methods to characterize protein N-termini at the proteomic level, or N-terminomics, offer a global view of this important component of protein structure and function. A significant challenge in N-terminomics is the discrimination between the N-termini of ma-

ture proteins and those generated from proteolytic cleavage. Taking on this challenge, Kleifeld *et al.* (*Nat. Biotechnol.* 2010, *28*, 281−288) report the development of terminal amine isotopic labeling of substrates (TAILS), in which N-terminal peptides are labeled and isolated before and after exposure to a protease of interest.

In TAILS, a proteome is subjected to proteolysis, along with a control sample that is not. The samples are differentially isotopically labeled on all primary amines, which includes all free N-terminal and lysine amino groups. This enables cleavage events specific to the protease of interest to be quantified and thereby distinguished from background proteolysis products. Next, the labeled proteomes are mixed and subjected to proteolysis by trypsin, which cleaves only after arginine, rather than after both arginine and lysine, in peptides in which the lysine residues have been labeled. In these cases, trypsin cleavage generates longer peptides, which aids in the identification of the shortened N-terminal peptides generated in the original cleavage reaction. Enrichment of the blocked N-terminal peptides by negative selection is accomplished using a novel class of hyperbranched polyglycerol-aldehyde polymers, which reacts with all free amino groups generated by the trypsin cleavage. This enables all the unreactive products, which include acetylated, cyclized, and isotopically labeled N-terminal peptides, to be isolated and identified by tandem mass spectrometry. This impressive strategy was used to characterize the N-termini of the secreted proteins from mouse fibroblasts, as well as in the identification of substrates for the poorly understood breast cancer protease MMP-11. The massive sample simplification achieved also enables identification of proteins covering 6 orders of magnitude in highly complex samples, such as BALF or serum, so representing a new approach to solve the dynamic range problem in the analysis of highly complex proteomes. **Eva J. Gordon, Ph.D.**

Making the E1 Mechanism Crystal Clear

Post-translational modification of proteins with ubiquitin (Ub) and ubiquitin-like (Ubl) proteins is a key regulator of diverse cellular processes such as cell differentiation and apoptosis. Most well-known for targeting proteins for proteosomal degradation, ubiquitination is a multistep process that begins by activation of Ub/Ubl by enzymes called E1s. This activation step can itself be broken into two steps: adenylation of the C-terminal Ub/Ubl carboxylate, followed by thioester bond formation between the C-terminus and the E1 active site cysteine. Though impressive progress has been made in the quest to unravel the entire process of ubiquitination, unresolved questions remain over the mechanism of action of E1 enzymes. Now, two studies (Lu *et al.* (*J. Am. Chem. Soc.* 2010, *132*, 1748−1749) and Olsen *et al.* (*Nature* 2010, *463*, 906−912)) report significant advances in our understanding of E1 structure and function with the design and synthesis of mechanism-based E1 inhibitors and the crystal structures of E1-Ub/Ubl-inhibitor complexes.

Toward creating new molecular tools with which to probe E1 mechanism of action, Lu *et al.* designed two classes of mechanism-based inhibitors inspired by 5′-sulfonyladenosine-derived inhibitors of other enzymes that catalyze adenylation reactions. Both classes of inhibitors are comprised of a Ub/Ubl having a C-terminal modification designed to bind the E1 and mimic a key reaction intermediate. The first set of inhibitors targets the first step of the reaction, in which adenylation of the Ub/Ubl C-terminus generates a Ub/Ubl-AMP intermediate. These compounds incorporate a sulfamide that sits between the Ub/Ubl and adenosine, which serves as a nonhydrolyzable analogue of the phosphate in Ub/Ubl-AMP. Indeed, these inhibitors prevent thioester bond formation, as well as conjugation of the Ub/Ubls to a substrate protein. The second set of inhibitors target the second step of the reaction, in which the E1 cysteine displaces the AMP group resulting in the formation of the thioester bond. These inhibitors contain a vinylsulfonamide that connects the Ub/Ubl to adenosine. This functional group can trap the E1 cysteine nucleophile to generate a covalent bond between the E1 and the Ub/Ubl-based inhibitor, resulting in a stable tetrahedral intermediate analogue. Indeed, incubation of the vinylsulfonamide inhibitors with E1 enzymes resulted in a stable thioether adduct and also prevented conjugation to a substrate protein.

In a related study, Olsen *et al.* use the Ub/Ubl-based sulfamide- and vinylsulfonamide inhibitors created by Lu *et al.* to probe the mechanism of action of the E1 for the Ubl SUMO. Crystal structures of SUMO E1 in complex with inhibitors were solved, offering unique insight into the adenylate and tetrahedral intermediates that are formed over the course of the reaction. It was found that profound conformational changes accompany the transition between the adenylate intermediate and the tetrahedral intermediate, which illuminated the remarkable process by which E1 functions. Specifically, an astounding 130° rotation of the domain harboring the active site cysteine (Cys domain) of E1 proceeds after the adenylation reaction, concomitant with the release of interactions between E1 side chains and PPi·Mg and significant remodeling of numerous structural elements. As a consequence of these conformational changes, close to half of the active site residues that participate in the adenylation reaction are replaced with residues from the Cys domain that are used in the thioester bond formation reaction. Notably, mutational and biochemical studies indicated that other E1 enzymes likely use a similar process for Ub/Ubl activation.

Together, these studies provide valuable new tools for studying the earliest steps in the ubiquitination process and put forth a striking revelation regarding the mechanism of E1 enzymes. The results also have numerous implications in the near ubiquitous processes touched by post-translational modification by ubiquitin and ubiquitin-like proteins. **Eva J. Gordon, Ph.D.**