Spotlight

Red-Green Color Spliceness

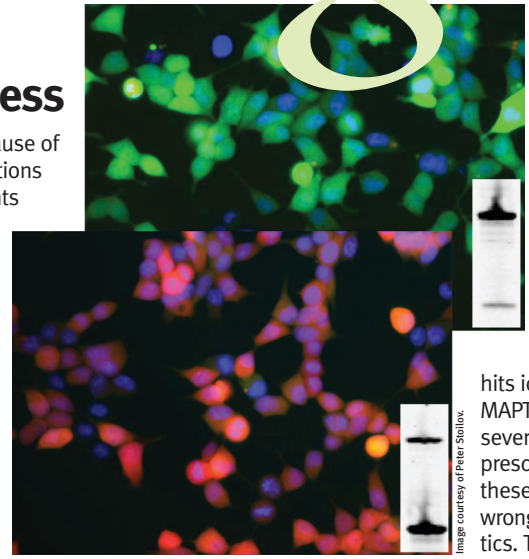


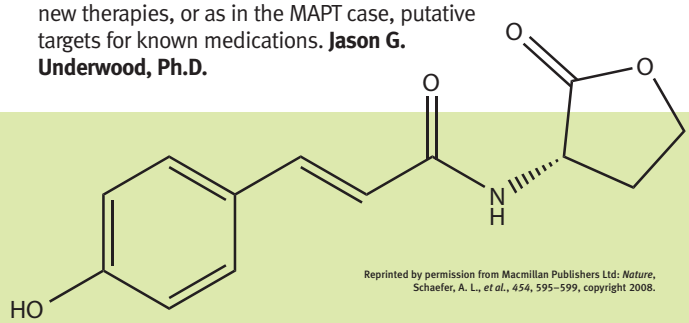
Image courtesy of Peter Stoilov.

A stumble in proper RNA metabolism is the root cause of many human genetic diseases. In particular, mutations underlying numerous neuronal or muscular ailments are linked to RNA splicing defects. Among these defects, the mechanisms that trigger improper splicing are as numerous as the afflictions. Some mutations cause loss of the pre-messenger RNA (pre-mRNA) splicing signals, whereas others change the regulatory sequences of the RNA, mucking with splice site choice or resulting in intron retention. Since nearly all protein-coding RNAs require accurate splicing, a magic pill to correct splicing defects has proven an impossible task. A study by Stoilov *et al.* (*Proc. Natl. Acad. Sci. U.S.A.* 2008, *105*, 11218–11223) uses a small-molecule screening method to look for compounds that shift the splicing pattern of one particularly problematic exon. In the mRNA encoding microtubule-associated protein tau (MAPT), misregulation of exon 10 splicing is associated with one type of frontotemporal dementia. After thousands of compounds were screened, a number of candidates emerged and several of these actually grouped within a class of drugs already prescribed by physicians.

To search for compounds that altered alternative splicing, the authors developed a series of sophisticated reporter constructs. Exon 10 of MAPT was the first test case, but the constructs incorporated features that are adaptable to other cases of mis-splicing. The reporters encoded the open reading frames for both the green and red fluorescent proteins (GFP and RFP), with splice site choice determining which protein is translated. Skipping of the test exon caused GFP to be translated, whereas inclusion of the exon resulted in RFP expression. With this reporter stably integrated in human tissue culture cells, small-molecule libraries were screened in multiwell plate format in a hunt for compounds that could shift the color from green to red. Multiple hits identified from the screen altered MAPT exon 10 splicing. These included several cardiotonic steroids, drugs long prescribed for heart failure. However, these shifted splicing of exon 10 in the wrong direction to be useful therapeutics. The study went on to show that digoxin and several other candidates can modulate splicing not just in the artificial reporter context, but also in the endogenous MAPT mRNA. Further, a microarray gauging hundreds of splicing events in human cells showed that these drugs affect alternative splicing of numerous mRNAs. The next challenge will be uncovering the direct and secondary mechanisms that lie between these drugs and the spliceosome. Are signaling pathways being stimulated and altering splicing regulatory networks, or could these drugs directly influence the splicing machinery, like an antibiotic working on the ribosome? Finally, this methodology paves the way for screening more clinically relevant splicing events for new therapies, or as in the MAPT case, putative targets for known medications. **Jason G. Underwood, Ph.D.**

Sensing Something Different

Quorum sensing is a process used by bacteria to regulate gene expression based on the local density of their population. Fatty acyl-homoserine lactone (HSL) is a common quorum-sensing signal that is generated by acyl-HSL synthases from fatty acids within bacterial metabolic pools. In the soil bacterium *Rhodopseudomonas palustris*, a protein homologous to fatty acyl-HSL synthase, RpaI, was surprisingly activated in response to growth on *p*-coumarate, an aromatic compound abundant in plants but not synthesized by bacteria. Investigating RpaI activity, Schaefer *et al.* (*Nature* 2008, *454*, 595–599) discover a new class of quorum sensing molecules.



To explore RpaI function, an assay was developed in which activation of the *rpaI* promoter could be detected. While synthetic fatty acyl-HSLs did not activate the *rpaI* promoter, extracts from cultures containing *p*-coumarate did. High-performance liquid chromatography and mass spectrometry indicated that the active molecule within the

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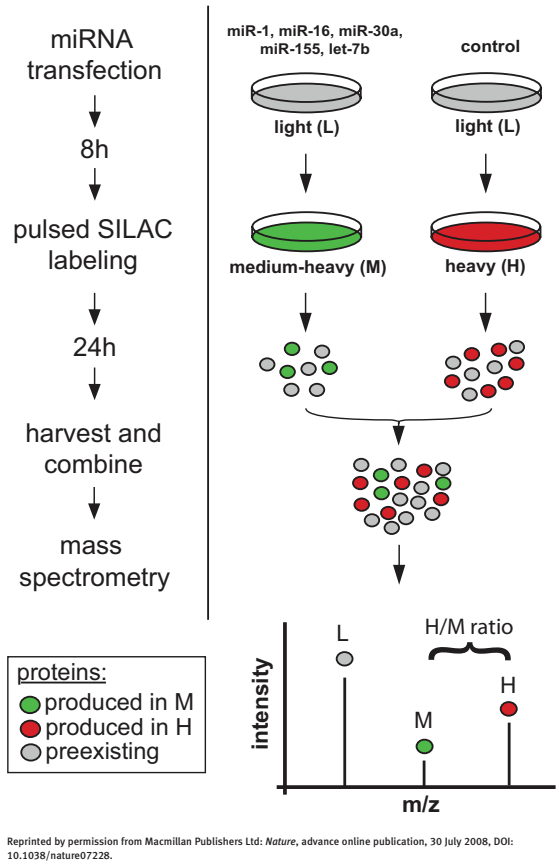
MicroRNAs Have Macro Effects

Gene expression is regulated in part by microRNAs (miRNAs), short strands of RNA that function by both promoting messenger RNA (mRNA) degradation and repressing protein production. Though studies have shown that miRNA overexpression results in down-regulation of many mRNAs, little is known about the control that miRNAs have over protein translation. Selbach *et al.* (*Nature*, published online July 30, 2008; DOI: 10.1038/nature07228) used a novel pulse-labeling strategy to measure changes in production of ~5000 proteins in response to miRNA expression.

In SILAC (stable isotope labeling with amino acids in cell culture), mass spectrometry is used to distinguish peptides derived from metabolically labeled proteins within a sample. The authors hypothesized that, by pulse-labeling with two different heavy stable isotopes in a process they deemed pulsed SILAC (pSILAC), changes in protein production between two samples could be measured at the level of the proteome. When this approach was used in cells overexpressing specific miRNAs, the authors found proteins with reduced synthesis to be enriched in direct miRNA targets. Moreover, comparison between changes in protein production and changes in mRNA levels, measured by pSILAC and microarray analysis, respectively, indicated that whereas the distribution of changes was similar, miRNAs can mediate direct repression of gene translation. Finally, modulation of the expression of a specific miRNA resulted in complementary effects on the proteome, indicating that miRNAs can affect protein production from thousands of genes. This study sheds light on an important aspect of miRNA function, contributing to our rapidly expanding knowledge of RNA interference. **Eva J. Gordon, Ph.D.**



extracts was *p*-coumaroyl-HSL, and NMR analysis and testing of synthetic *p*-coumaroyl-HSL confirmed this structure and activity. Transcriptome analysis in response to *p*-coumaroyl-HSL revealed that expression of 17 genes was altered, several of which are linked to chemotaxis. In addition, characteristic of most quorum-sensing systems, *p*-coumaroyl-HSL levels increased late in growth and correlated with *rpaI* expression. Moreover, the cognate signal-receptor transcription factor, *rpaR*, functioned as a responsive repressor of the *rpaI* promoter, analogous to the corresponding receptor in fatty acyl-HSL quorum sensing systems. When *rpaI* was expressed in *Escherichia coli* and *P. aeruginosa*, *p*-coumaroyl-HSL was produced but only in the presence of exogenous *p*-coumarate, offering additional evidence that RpaI is a *p*-coumaroyl-HSL synthase. Furthermore, two other bacterial species were found to be capable of producing *p*-coumaroyl-HSL when grown in the presence of *p*-coumarate. This finding redefines the types of molecules thought to be capable of acting as quorum-sensing signals, and intimates that bacteria and plants may use quorum sensing as a method for interkingdom communication. **Eva J. Gordon, Ph.D.**

RNAi Chooses the Nuclear Option

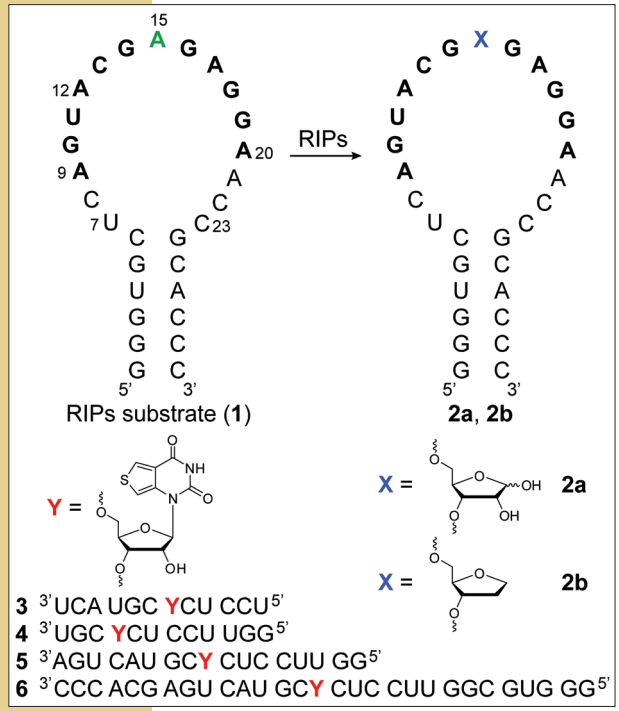
In the field of RNA interference (RNAi), the controversy often lies in what happens in the cytoplasm. It is well-established that double-stranded RNAs (dsRNAs) get processed in the nucleus and go to the cytoplasm to be loaded into another processing complex, but the end result is often a point of contention. Do RNAs get destabilized, directly degraded, or translationally halted in some manner? Now, a new study points a big bold arrow back into the nucleus for yet another piece to the RNAi puzzle. In *Caenorhabditis elegans*, there are clear cases where RNAi takes place in the nucleus, but how this regulation occurs has remained a mystery. Using a clever genetic screen that looked for mutant worms that could perform standard cytoplasmic RNAi, but not nuclear RNAi, the researchers uncovered the function of yet another Argonaute protein in the organism.

Guang *et al.* (*Science* 2008, *321*, 537–541) report the discovery of the gene NRDE-3 (nuclear RNAi defective-3), a specialized Argonaute family member that takes small RNAs from the cytoplasm and transports them to the nucleus to then regulate select nuclear events. A majority of the mutant worms displayed a mutation in this gene, and often these lesions disrupted key RNAi-related protein motifs, the PAZ or PIWI domains. Of key interest, the PAZ domain harbors the siRNA in Argonaute proteins, and NRDE-3 was no exception. However, a key difference with NRDE-3 was that the binding of RNA to the PAZ domain was coupled with transport to the nucleus. This was elegantly shown by using PAZ-mutant worms and also worm mutants that cannot produce their own endogenous small-interfering RNA (siRNA) molecules, and thus rely upon outside sources of RNA. Thus, the NRDE-3 protein gets programmed for nuclear RNAi in the cytoplasm and then brings its siRNA cargo back to the nucleus to carry out its gene regulatory business. In particular, some sleuthing work on the 5′ phosphate status of the NRDE-3-bound siRNAs indicated that the protein is probably programmed by small RNAs generated by a special cellular mechanism. Worms use RNA-dependent RNA polymerases to generate secondary effector siRNAs by copying portions of mRNA substrates in the cytoplasm, cutting up these dsRNAs, and putting the secondary siRNAs back into Argonaute proteins. This appears to be the case for NRDE-3, so an interesting communication loop between cytoplasm and nucleus seems to run in both directions. Though this discovery adds to the well-studied RNAi pathway in worms, expect more complexity ahead. The *C. elegans* genome encodes a whopping 27 Argonaute proteins. **Jason G. Underwood, Ph.D.**

A Mission of Emission

Ribosome-inactivating proteins (RIPs), such as ricin and saporin, are exceptionally poisonous toxins whose straightforward accessibility facilitates their potential use as bioterrorism weapons. Ricin and saporin function by removing the purine moiety from a specific nucleotide residue on a conserved ribosomal RNA sequence, which deleteriously affects protein synthesis and ultimately causes cell death. Alarmingly, methods to detect these toxins and treat those exposed to them are considerably lacking. Srivatsan *et al.* (*Angew. Chem., Int. Ed.* 2008, *47*, 6661–6665) now report the development of synthetically modified oligonucleotides that, through enhanced fluorescence emission, enable the detection of RIP activity.

Central to the strategy used for detecting RIP activity is a fluorescent ribonucleoside analog that exhibits enhanced emission upon interaction with abasic RNA sites, that is, those without nucleobases. Initial model studies were conducted with short oligonucleotides containing the fluorescent ribonucleotide strategically located opposite an abasic site in a complementary RNA strand. Relative to hybridization to the perfect complementary strand, hybridization of the fluorescent oligonucleotide to the strand containing the depurinated residue resulted in significant fluorescence enhancement. Armed with this fluorescent molecular detector, the authors created an oligonucleotide whose sequence was complementary to the saporin target sequence, with the emissive nucleotide located opposite the site of saporin activity. Reaction of saporin with the RNA substrate and subsequent quenching with the emissive oligonucleotide revealed a time-dependent increase in fluorescence, indicating that the emissive oligonucleotide can signal the depurination process of the RNA substrate. This simple, innovative hybridization assay is amenable to high-throughput screening methods and thus could facilitate the discovery of RIP inhibitors and antidotes. Importantly, it also points to a strategy for developing a biological warfare detection device for toxins of this type. **Eva J. Gordon, Ph.D.**



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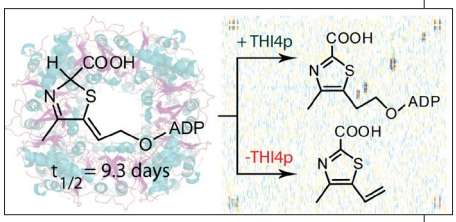
Better Late Than Never

Thiamin pyrophosphate is an essential cofactor involved in amino acid and carbohydrate metabolism. Though mammals lack the ability to biosynthesize this compound, prokaryotes and some eukaryotes can. Thiamin is composed of a thiazole linked to a pyrimidine, and these components are synthesized separately and then linked. Unlike the prokaryotic biosynthesis of the thiazole component, which is relatively well-understood, the eukaryotic pathway is just beginning to be elucidated. Chatterjee *et al.* (*J. Am. Chem. Soc.*, published online July 25, 2008; DOI: 10.1021/ja802140a) contribute to this effort by characterizing a late intermediate in thiamin thiazole biosynthesis in the yeast *Saccharomyces cerevisiae*.

The enzyme THI4p is required for generating thiamin thiazole in yeast. In previous studies, three adenylated metabolites copurified with THI4p. One of the metabolites was identified to be an adenylated thiazole, which suggested that thiamin thiazole is biosynthesized from NAD; determination of the structure of the second metabolite enabled the generation of a hypothetical mechanistic pathway to the adenylated thiazole. The third metabolite, however, remained a mystery. Altering experimental conditions to optimize the generation of this compound facilitated its identification and characterization. First, exposure of this metabolite to THI4p resulted in the generation of the adenylated thiazole, suggesting that it was an intermediate en route to that compound. Next, extensive spectroscopic analyses indicated that it was in fact a seemingly unstable isomer of the adenylated thiazole. In the absence of THI4p, the compound slowly decomposes to yield ADP and a carboxylated thiazole derivative, which further decomposes to the decarboxylated thiazole. Notably, nonenzymatic decomposition of the metabolite did not produce any adenylated thiazole, suggesting that THI4p is required for this isomerization. Identification of this metabolite provides an insightful view into the complex biosynthetic route to this important biomolecule. **Eva J. Gordon, Ph.D.**



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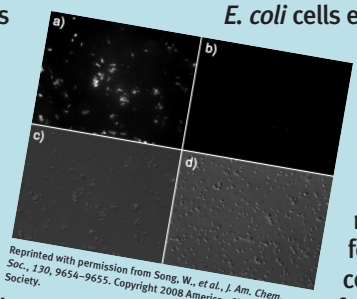
Chemical Reporting for Duty

Chemical reporters that can participate in bioorthogonal reactions are powerful tools for site-selective labeling of proteins, offering a unique approach for exploring protein function. The limited number of reactions that have been developed for this purpose so far have made clear the power of the technology, but more are needed to expand the structural diversity and increase the potential applications. Song *et al.* (*J. Am. Chem. Soc.* 2008, *130*, 9654–9655) report the use of "photoclick chemistry" to achieve a site-selective reaction between a protein-linked alkene and a diaryltetrazole in bacterial cells.

Thanks to the ability to site-specifically incorporate alkene-containing amino acids into proteins, a Z-domain protein containing an *O*-allyl tyrosine residue was generated in *Escherichia coli*. Exploiting the "click chemistry" philosophy in which only practical, reliable chemical reactions are used, a photoactivated nitrile imine was chosen to participate in a 1,3 dipolar cycloaddition with the protein-appended alkene unit. Twelve diaryltetrazoles, which are the nitrile imine precursors, were examined for their ability to react with purified alkene-containing Z-domain upon photoactivation, yielding a protein containing a fluorescent pyrazoline cycloadduct. Fluorescent gel electrophoresis imaging indicated that three of the tetrazoles selectively reacted with the protein, and cycloadduct formation was confirmed by mass spectrometry. Taking it a step further, the authors incubated *E. coli* cells expressing the alkene-containing Z-domain protein with a diaryltetrazole and exposed them for a short time to UV light. The reaction was monitored *in vivo* using fluorescence microscopy, and cell lysis followed by in-gel fluorescence analysis confirmed formation of the fluorescent pyrazoline-Z-domain product. This reaction will be a welcome addition to the toolkit of bioorthogonal chemistry used for selective functionalization of proteins. **Eva J. Gordon, Ph.D.**



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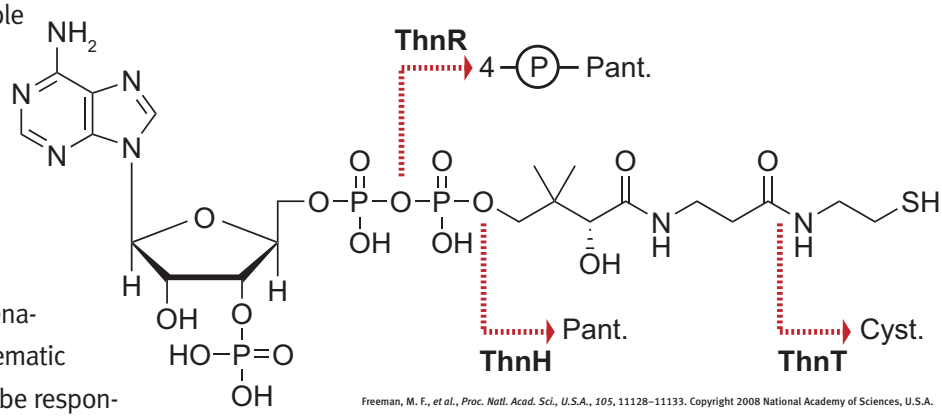
Stepwise Side Chain Synthesis

Thienamycin is a member of the carbapenem class of β-lactam antibiotics. Structurally distinct from other β-lactams such as penicillin and cephalosporin, carbapenems are resistant to most classes of β-lactamases and are thus of growing importance for treating bacterial infections. Although early studies established the metabolites involved in generation of the thienamycin core, little is known about how the various side chains in thienamycin and other family members are incorporated into the compounds. Recent sequencing of the thienamycin gene cluster has facilitated investigation into thienamycin biosynthesis, and now Freeman *et al.* (*Proc. Natl. Acad. Sci. U.S.A.* 2008, *105*, 11128–11133) report the identification of four enzymes responsible for the step-wise generation of the cysteamine and *N*-acetylcysteamine side chains of thienamycin and *N*-acetylthienamycin, respectively.

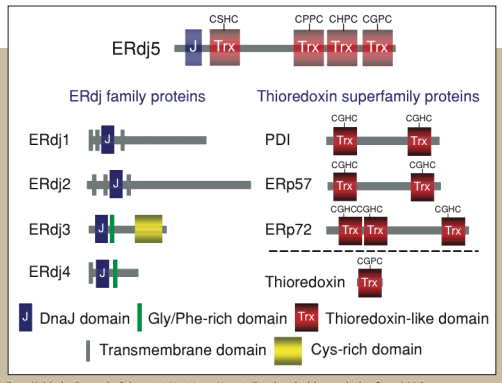


Freeman, M. F., *et al.*, *Proc. Natl. Acad. Sci., U.S.A.*, *105*, 11128–11133. Copyright 2008 National Academy of Sciences, U.S.A.

Careful examination of the thienamycin gene cluster enabled systematic deduction of which genes might be responsible for constructing the side chain. The process began with the search for a protein capable of hydrolyzing an amide bond in a potential biosynthetic precursor to cysteamine, pantetheine. With sequence similarity to other amidohydrolases, the protein ThnT was identified as a candidate enzyme. Overcoming several challenges during the cloning process, ThnT was expressed in *Escherichia coli* and indeed found to be capable of hydrolyzing pantetheine to generate cysteamine. Notably, 4-phosphopantetheine and coenzyme A (CoA) are present in considerably higher levels than pantetheine, and neither were substrates for ThnT. Thus, in a similar fashion, the researchers uncovered two additional enzymes, ThnR and ThnH, capable of metabolizing CoA to 4-phosphopantetheine and 4-phosphopanththeine to pantetheine, respectively. The final known modification to the thienamycin side chain is acetylation of the amine. With weak homology to an *N*-acetyltransferase superfamily, the protein ThnF was found to be capable of acetylating cysteamine derivatives to their corresponding acetylated products. In addition to putting forth a biogenetic pathway for the cysteaminyl side chain of thienamycin, this study opens the door to protein engineering methods for the synthesis of novel carbapenem analogs. **Eva J. Gordon, Ph.D.**

Reducing ER-Associated Degradation to a Science

When proteins cotranslationally enter the endoplasmic reticulum (ER), protein chaperones that reside in the ER ideally step in to help them fold properly. However, if for any reason a protein does not adopt the proper conformation, it is shuttled back into the cytosol and destroyed through a process known as ER-associated degradation (ERAD). ER degradation-enhancing α-mannosidase-like protein (EDEM) is an ER-resident protein that enhances misfolded protein degradation through recognition of N-linked glycans. To discover more about how EDEM participates in ERAD, Ushioda *et al.* (*Science* 2008, *321*, 569–572) use an ER-membrane yeast two-hybrid system to search for EDEM binding partners, and uncover the ER-resident protein ERdj5.



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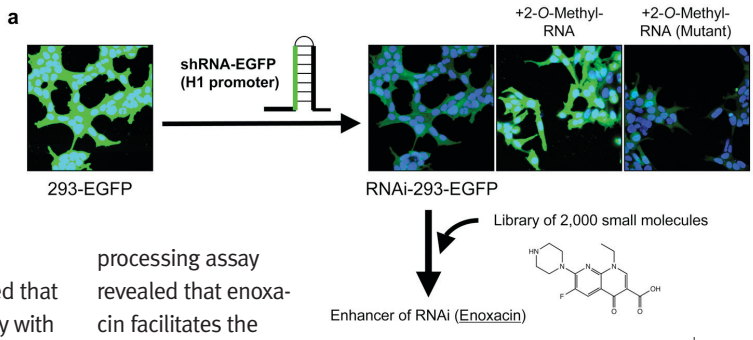
ERdj5 is unique among its five family members in that it contains thioredoxin-domains. Misfolded proteins can end up as large oligomers because of misguided disulfide bonding, which can hinder their retrotranslocation into the cytosol. The authors hypothesized that ERdj5 may function in ERAD to reduce such disulfide bonds, breaking up the oligomers and facilitating retrotransport of the protein. Indeed, purified recombinant ERdj5 acted as a potent disulfide reductase under the redox conditions of the ER. In addition, in cells, ERdj5 accelerated ERAD substrate degradation by catalyzing disulfide bond cleavage and preventing disulfide-induced oligomerization of misfolded proteins. Further investigation into ERdj5 structure revealed the presence of a DnaJ domain, and surface plasmon resonance confirmed that ERdj5 interacted with the resident ER, DnaJ-binding chaperone BiP. In fact, the reductase activity of ERdj5 and its interaction with BiP and EDEM were all shown to be required for efficient ERAD of misfolded proteins. The authors propose a model in which the presence of a supramolecular ERAD complex composed of EDEM, ERdj5, and BiP work in concert to facilitate the untangling and transport of misfolded proteins during the ERAD process. Eva J. Gordon, Ph.D.

Small-Molecule Interference in RNAi

RNA interference (RNAi) is a gene silencing mechanism pivotal in the regulation of gene expression in eukaryotic cells. RNAi employs two RNA-based agents, small-interfering RNAs (siRNAs) and microRNAs (miRNAs), to achieve its objectives. siRNAs target messenger RNA (mRNA) for degradation, and miRNAs suppress translation and can also trigger mRNA degradation. Though scientists have extensively hijacked this pathway for research purposes, our understanding of RNAi pathway regulation is murky at best. Taking a chemical-biological approach to probe RNAi regulation, Shan *et al.* (*Nat. Biotechnol.* 2008, *26*, 933–940; DOI: 10.1038/nbt.1481) discover a small molecule capable of modulating the RNAi pathway.

To find a small molecule that could perturb RNAi, 2000 approved drugs were screened using a reporter assay designed to enable identification of both RNAi inhibitors and enhancers. From this screen, the antibiotic enoxacin was found to enhance siRNA-mediated mRNA degradation. Structure–activity and gene expression analysis suggested that enoxacin interacts specifically with nucleic acids and proteins involved in the RNAi pathway. Investigation of enoxacin's mechanism of action indicated that the antibiotic promotes the processing of pre-miRNAs and enhances the loading of siRNA duplexes onto the RNA-induced silencing complex (RISC). The protein trans-activation-responsive region RNA-binding protein (TRBP) mediates miRNA and siRNA loading onto the RISC, and indeed, testing in an *in vitro* processing assay revealed that enoxacin facilitates the interaction between TRBP and RNAs. Notably, enoxacin also enhanced siRNA-mediated mRNA degradation in mice. Thus, the authors demonstrate that small molecules can modulate the RNAi pathway, and moreover, provide a path to find such compounds. Their results point to the potential of small molecules as tools for exploring RNAi pathway regulation and in development of RNAi-based therapeutics. **Eva J. Gordon, Ph.D.**

Figure 1- Shan et al.



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