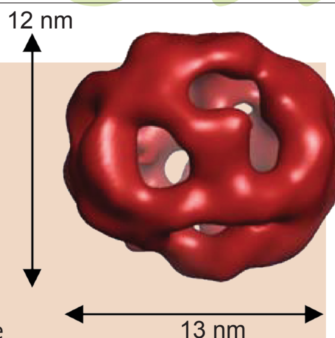
Codename Q: Enzymatic Double Agent



Reprinted from *PLoS Biol.*, *5*, Muzzolini, L., *et al.*, Different quaternary structures of human RECQ1 are associated with its dual enzymatic activity, DOI: 10.1371/journal.pbio.0050020.

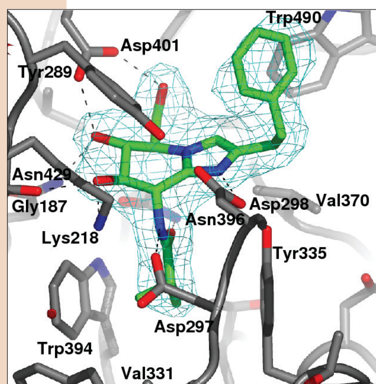
Pulling apart two complementary strands of RNA or DNA is critical in many cellular processes. On the classical path, beginning at the chromosome and ending at the ribosome, the gene expression machinery repeatedly separates base pairs with the help of specialized enzymes. These proteins, called helicases, couple ATP hydrolysis and strand dissociation. In all kingdoms of life, the RecQ helicases act as watchdogs to monitor genome stability. Human RecQ enzyme can dissociate DNA strands like a conventional helicase, but interestingly, it can also catalyze the annealing of complementary strands. Now, a new study by Muzzolini *et al.* (*PLoS Biol.* 2007, *5*, published online January 16, DOI: 10.1371/journal.pbio.0050020) zooms in on this double-agent enzyme and provides a surprising explanation.

By titrating ATP, DNA, and RecQ concentrations, the authors could effectively push the dual-activity enzyme to favor one activity over the other. Then, using size exclusion chromatography and electron microscopy, they found that two different sizes of RecQ multimers are responsible for the two different activities. With ATP present, the enzyme displays the hallmarks of monomer or homodimer, and it readily catalyzes strand dissociation. In contrast, addition of single-stranded DNA further stabilizes interactions between RecQ proteins, and larger complexes are seen by both chromatographic methods and electron microscopy. A 3D reconstruction from microscopy data reveals complexes of ~12 nm in diameter. This volume is consistent with five or six RecQ proteins bound together by homotypic interactions. These larger RecQ particles are competent for strand annealing. Upon addition of ATP, the RecQ multimers quickly dissociate into the smaller helicase form, and this reveals the dynamic, reversible nature of the RecQ quaternary structure. Although the helicase activity of RecQ has been investigated extensively, this study provides a fresh perspective on the elusive annealing activity of the RecQ family of helicases. Higher-resolution views of the annealing complex and a look at where double-stranded DNA might fit into the picture will be of primary interest. **Jason G. Underwood, Ph.D.**

"Sweeter" Proteins

Post-translational modification of serines and threonines by *N*-acetylglucosamine (*O*-GlcNAc) is an important regulator of various cellular events, and misregulation of this process has been linked to diseases such as diabetes and Alzheimer's disease. The enzyme *O*-GlcNAcase (OGA) is responsible for removing *O*-GlcNAc from proteins; thus, OGA inhibitors effectively "sweeten" proteins by preventing the removal of *O*-GlcNAc groups from their surface. These inhibitors are useful tools for deciphering the role of this modification, but development of potent and selective compounds has been challenging. Dorfmueller *et al.* (*J. Am. Chem. Soc.* 2006, *128*, 16,484–16,485) use the crystal structure of OGA in complex with the known inhibitor PUGNAc to guide the design of a novel OGA inhibitor termed GlcNAcstatin.

Scrutiny of the OGA–PUGNAc complex revealed the presence of a deep pocket not present in the related enzymes human lysosomal hexosaminidases HexA and HexB. In addition, the authors noted that glycoimidazoles were effective mimics of the transition state of the sugar ring in the natural substrate of OGA. Mingling these two notions led to the design and synthesis of GlcNAcstatin, a glucoimidazole with structural similarities to PUGNAc but incorporating a larger isobutanamido group intended to occupy the OGA pocket. GlcNAcstatin was found to be a picomolar inhibitor of OGA and 100,000× more selective for OGA than for HexA and HexB. Determination of the crystal structure of GlcNAcstatin in complex with OGA further characterized the molecular details of the interaction. Finally, the compound was demonstrated to be a more effective GlcNAcase inhibitor than PUGNAc, as assessed by its ability to prevent the removal of *O*-GlcNAc from proteins in cell lysates and to raise *O*-GlcNAc levels in a human cancer cell line. These studies will facilitate development of additional tools to study this important cellular process, indeed sweetening the prospects for future *O*-GlcNAcylation investigations. **Eva J. Gordon, Ph.D.**



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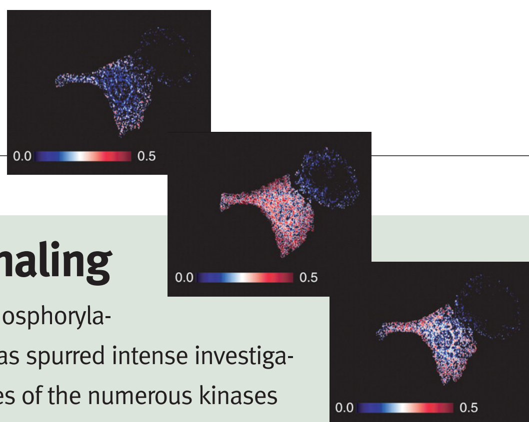
Spaced Out Signaling



From Yudushkin, I. A., *et al.*, *Science*, Jan 5, 2007, DOI: 10.1126/science.1134966. Reprinted with permission from AAAS.

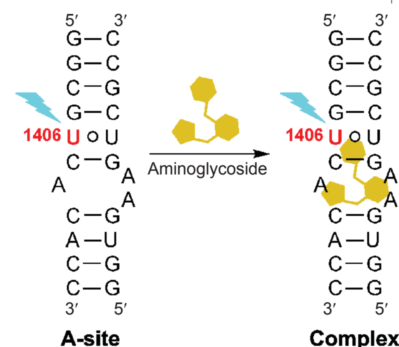
The significance of protein phosphorylation in cell signaling events has spurred intense investigations surrounding the activities of the numerous kinases and phosphatases that reside in the cell. The fact that phosphatase activity essentially reverses the action of kinases and thus results in signal termination has fueled interest in the dynamic interplay between these signaling molecules. Recent evidence suggests that receptor tyrosine kinase signaling terminates at the endoplasmic reticulum, which just happens to be the location of protein tyrosine phosphatase-1B (PTP1B). In search of the molecular basis for this spatial segregation of kinases and phosphatases, Yudushkin *et al.* (*Science* 2007, *315*, 115–118) developed a FRET-based imaging method to spatially resolve interactions between PTP1B and its substrate.

The imaging approach relies on the enzyme–substrate (ES) interaction between an enhanced GFP-tagged PTP1B and a lissamine rhodamine B-conjugated, phosphotyrosine-containing synthetic peptide. The authors first demonstrated that interaction between the phosphatase and its substrate could be detected by FRET *in vitro*. In addition, the clever use of a caged version of the substrate to manipulate its ability to bind the phosphatase revealed that a steady-state concentration of the phosphorylated substrate was maintained in mammalian cells. Subsequent analysis of the spatial distribution of the ES complex indicated that the steady-state concentration of the complex was higher at the cell periphery than in the perinuclear region. This concentration gradient was retained across varying cell shapes and during growth factor activation, an indication that PTP1B activity is indeed spatially regulated. This innovative approach for imaging the PTP1B-substrate enabled the authors to propose that spatial regulation of PTP1B may contribute to the establishment of appropriate cell signaling environments in the cell. Moreover, this method has potential applications for investigating other ES interactions *in vivo* as well. **Eva J. Gordon, Ph.D.**

All in the Nucleoside Family

Fluorescent nucleosides are valuable molecular tools for investigating RNA structure, dynamics, and recognition. For example, 2-aminopurine (2-AP) is a commonly used fluorescent purine analogue that has been used to explore various processes, including ribozyme folding and RNA interactions with proteins and small molecules. Unlike their purine-derived siblings, however, few fluorescent pyrimidines are available for studying RNA interactions; this has made cytidine and uridine derivatives the black sheep of the fluorescent nucleoside family, so to speak. After a recent hunt for fluorescent pyrimidines fit to marry into this exclusive clan, Srivatsan and Tor (*J. Am. Chem. Soc.* published online Jan 26, 2007; DOI: 10.1021/ja066455r) now report the synthesis and biophysical characterization of a furan-modified pyrimidine derivative, referred to as nucleoside **2**, and its triphosphate counterpart, ribonucleotide **2TP**.

Synthesis of **2** and **2TP** was straightforward, and characterization of their fluorescence properties revealed a strong emission at 440 nm, providing a signature for the use of these compounds in biophysical studies. **2TP** was efficiently incorporated into RNA oligonucleotides *via in vitro* transcription reactions, an indication that oligonucleotides containing **2** could be exploited for exploration of RNA interactions. The bacterial decoding A-site, which is part of the bacterial ribosome and is the target of aminoglycoside antibiotics such as paromomycin and neomycin, was chosen to test the utility of **2** for investigating RNA binding interactions. Binding of paromomycin or neomycin to A-site RNA containing **2** in place of a uridine resulted in a large increase in fluorescence intensity, an indication that the binding event results in a change in the environment around **2**. Intriguingly, only binding of paromomcyin, but not neomycin, could be monitored in the analogous experiments that used 2-AP, and this underscores the need for multiple fluorescent nucleosides for comprehensive RNA interaction studies. The fluorescent pyrimidine derivatives described here validate the use of fluorescent pyrimdines for RNA studies and are promising leads toward the generation of future members of the fluorescent pyrimidine side of the family. **Eva J. Gordon, Ph.D.**



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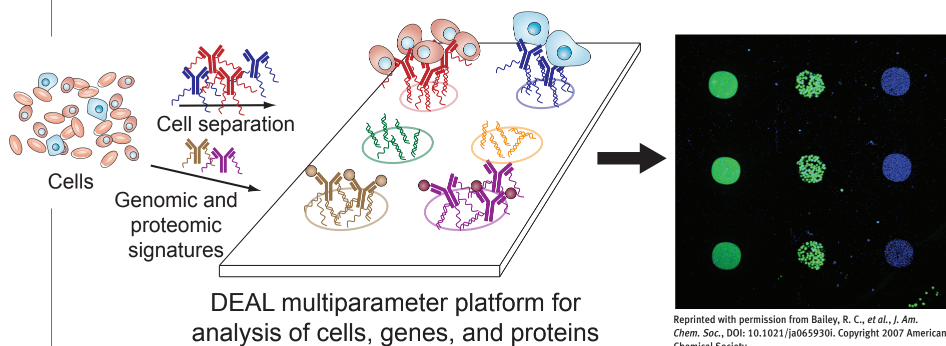
A Good DEAL

Dealing with extraordinarily complex biological samples, especially those affected by diseases such as cancer, is greatly facilitated by multiparameter analysis both for diagnostic and for therapeutic applications. Recent advances in genomics and proteomics have spurred various methods for multiplexed detection within a given class of biomolecules, but chemical incompatibilities between the platforms have previously made the simultaneous detection of genes, proteins, and cells impossible. Bailey *et al.* (*J. Am. Chem. Soc.*, published online Jan 30, 2007; DOI: 10.1021/ja065930i) now describe a DNA-encoded antibody library (DEAL) approach, which enables multiparameter analysis of cells, proteins, and genes on the same surface.

The DEAL strategy is based on the covalent attachment of single-stranded DNA sequences to antibodies, which can then be spatially segregated on DNA microarrays containing the complementary sequences. Conversion of lysine residues on the antibody to hydrazide groups, followed by coupling to DNA sequences modified at the 5′-end with an aldehyde *via* hydrazone linkages, yielded DNA-tagged antibodies. An initial demonstration of the DEAL concept showed that three identical goat anti-human antibodies, each containing a different fluorophore and a unique DNA strand, could be correctly assembled and detected on a DNA microarray. DEAL was also adapted to a sandwich immunoassay format, with or without microfluidics technology, where protein antigens of interest could be detected with increased sensitivity and speed over traditional formats. Furthermore, DNA labeling of antibodies that recognize cell surface markers enabled the use of DEAL to efficiently sort and spatially stratify components within both immortalized cultures and primary cell populations. Finally, simultaneous detection of a DNA sequence, a protein, and cells was accomplished on the same microarray slide, demonstrating the incredible versatility of this method. Expansion and refinement of this approach will indeed be a good deal for the multiparameter analysis of complex biological samples in the future. **Eva J. Gordon, Ph.D.**



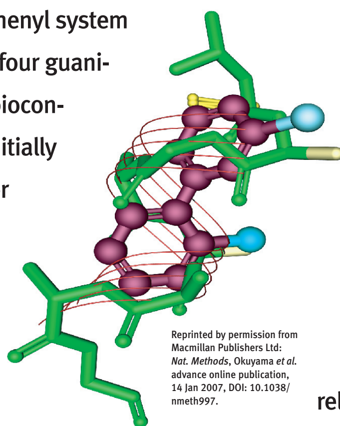
DEAL multiparameter platform for analysis of cells, genes, and proteins

Reprinted with permission from Bailey, R. C., *et al.*, *J. Am. Chem. Soc.*, DOI: 10.1021/ja065930i. Copyright 2007 American Chemical Society.

“SMoC”king Proteins into Cells

Although many methods exist to take proteins out of cells, getting them into cells is an entirely different ball game. Researchers have tackled this problem from many angles, including microinjection, lipid membrane-permeablizing agents, and protein transduction domains (PTDs), which are α-helical peptides that can be fused to proteins of interest. These methods, however, can be tedious, expensive, or toxic or require the use of reagents that are subject to degradation. Now, Okuyama *et al.* (*Nat. Methods* 2007, *4*, 153–159, DOI: 10.1038/nmeth997) present the design, synthesis, and biological evaluation of small-molecule PTD mimics, which cleverly maintain the ability to transport proteins into cells yet lack many of the troublesome properties inherent in the PTDs themselves.

Coached by molecular modeling studies, the researchers devised two small-molecule α-helix mimics termed 2G-SMoC and 4G-SMoC. The PTD mimics are composed of a biphenyl system strategically displaying two or four guanidine groups and a handle for bioconjugation. These SMoCs were initially demonstrated to rapidly deliver fluorescein into both the cytoplasm and the nucleus of cultured and primary cells with no evidence of toxicity. Next, the nuclear protein geminin, which regulates entry into the S phase of the cell cycle, was coupled to 4G-SMoC. The conjugate was incubated with various cell types, which were evaluated for the presence and function of the protein. The geminin conjugate was observed to preferentially localize to the perinuclear and nuclear areas of the cells. In addition, the cells were prevented from entering S phase, and this demonstrated biological activity of the exogenously added protein. Notably, significantly reduced 4G-SMoC–geminin–Alexa Fluor 488 uptake at 4 °C or in the presence of chlorpromazine indicated that the delivery process is energy-dependent and relies upon clathrin-mediated endocytosis. The SmoCs described here are a solid hit for efficient delivery of proteins into cells; expansion of their utility for transport of other biomolecules such as peptides and oligonucleotides will be a home run. Eva J. Gordon, Ph.D.



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Handling Phosphoproteins

The importance of protein phosphorylation in cell signaling mechanisms is irrefutable, but the multitude of phosphorylated proteins in the cell can make it difficult to get a handle on them, figuratively speaking. Now, Green and Pflum (*J. Am. Chem. Soc.* 2007, *129*, 10–11) present an enzymatic approach that literally appends a molecular handle onto phosphoproteins, enabling the inspection and detection of these important signaling molecules.

Perusal of ATP binding interactions in the structures of various kinases led the researchers to speculate that the γ-phosphate of ATP, the phosphate that is transferred to kinase substrates, could be chemically modified and still act as an efficient cosubstrate in kinase-catalyzed reactions. Thus, they exploited an ATP derivative containing a biotin group appended to the γ-phosphate such that a phosphobiotin moiety, rather than just a phosphate, would be transferred to the kinase substrate. The authors first demonstrated that capable kinases could use ATP–biotin to biotinylate serine-, threonine-, and tyrosine-containing peptides and that the biotinylation reactions proceeded with similar efficiency to the native phosphorylation reactions. Next, they showed that the purified, full-length protein β-casein was biotinylated by the kinase CK2. In addition, they used protein kinase A and ATP–biotin to biotinylate the cAMP response element-binding (CREB) protein in bacterial and mammalian cell lysates, demonstrating that the method tolerates the presence of endogenous ATP and other proteins. Finally, this approach was extended to proteomics applications where, in the presence of ATP–biotin, endogenous kinases were shown to biotinylate various proteins in mammalian cell lysates. The versatility of this method combined with the potential for development of analogous tools, such as fluorescent ATP derivatives, will enable many researchers in the phosphoproteomics world to get a better handle on their proteins as well. **Eva J. Gordon, Ph.D.**

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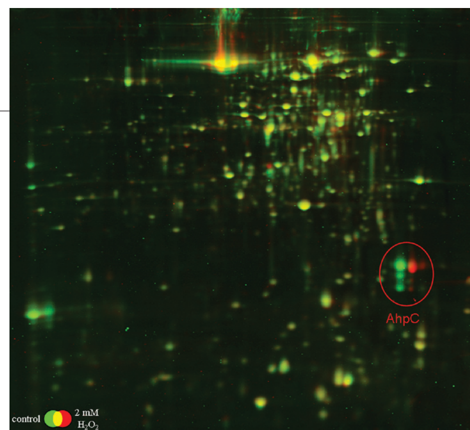
Spotlight

Cultivating the Unculturable



From Markert, S., *et al.*, *Science*, Jan 12, 2007, DOI: 10.1126/science.1132913. Reprinted with permission from AAAS.

Though it may seem a staggering statistic, <1% of known bacteria can actually be grown in the lab *via* traditional culture methods. Thus, exploration of the biology of these microbes has been considerably hindered. One of these bacteria, the endosymbiont of the deep-sea tube worm *Riftia pachyptila*, is the main source of carbon and energy for its host. Side-stepping traditional methods for investigating culturable bacteria, Markert *et al.* (*Science* 2007, *315*, 247–250) use a functional genomics approach to delve into the physiology of these unique *Riftia* symbionts.

In pursuit of the molecular basis for symbiont activity, the authors isolated the bacteria from the host tissue, and they used 1D and 2D gel electrophoresis to separate the bacteria's intracellular and membrane proteins. These gels were representations of the quantity and thus potential relevance of protein expression under natural growth conditions and enabled the identification of >220 of the proteins in the bacteria's proteome. The authors found that three major sulfide oxidation proteins make up >12% of the total cytosolic proteome; this finding underscores the importance of these enzymes for the bacteria's energy metabolism. In addition, examination of the symbiont's carbon metabolism indicated surprising but convincing evidence that it uses the reverse tricarboxylic acid cycle, which is an alternative to other cycles that require more energy. Finally, experiments using altered growth conditions suggested that the bacteria can adjust their protein levels in response to different environments, such as low-energy situations or oxidative stress. Unlike the unculturable bacteria it is directed toward, this approach for analyzing bacterial physiology has substantial growth potential. Eva J. Gordon, Ph.D.

MicroRNAs Flex Their Muscles

Gene expression in many eukaryotes is modulated by short RNAs called microRNAs (miRNAs). Large-scale cloning efforts and computational predictions have uncovered hundreds of new miRNAs, but finding the messenger RNA (mRNA) targets for these molecules has proven difficult. Unlike their small interfering RNA cousins, the miRNAs do not bind to the target with perfect base complementarity, and this makes predictions trickier. Computational screens have identified putative targets, but validation in actual cells has been on a case-by-case basis, so widespread conclusions are difficult to make. To add to the complexity, many miRNAs are expressed in a tissue-specific or developmentally timed manner. Now, a new study by Boutz *et al.* (*Genes Dev.* 2007, *21*, 71–84) finds that a tissue-restricted protein involved in mRNA splicing regulation is itself the target of a miRNA.

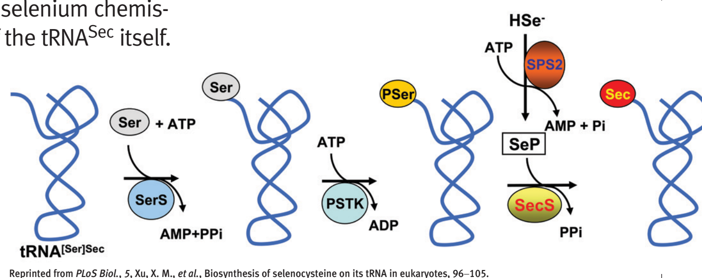
The authors noticed that mouse myoblast cells expressed detectable levels of the splicing regulator, neuronal polypyrimidine tract binding protein (nPTB), a factor that is normally expressed in the adult brain and testes. Upon differentiation of these precursor cells into the more muscle-like myotube form, nPTB protein disappeared, yet the mRNA levels remained unchanged. This observation implied that a post-transcriptional

(continued on page 87)

Searching for SecS

All living things build their proteins from a toolkit of 20 amino acids, but in a special subset of these organisms, including humans, a 21st amino acid makes a proteomic appearance. This special case is selenocysteine, an amino acid that resembles cysteine but with a selenium atom in place of the usual sulfur. An added peculiar property of selenocysteine is that it is synthesized on its transfer RNA (tRNA), rather than the standard pathway where amino acids are first synthesized and then charged onto the cognate tRNA in a ready-for-ribosome form. The enzyme that makes selenocysteine in *Escherichia coli*, SecA, uses $tRNA^{Sec}$ as starting material for the addition of an activated selenium. To date, functional SecA enzymes had not been found in archaea or eukaryotes, even though a wide range of creatures in these kingdoms do indeed make selenocysteine-containing proteins. Now, Xu *et al.* (*PLoS Biol.* 2006, *5*, 96–105) use sequenced genomes that span the far reaches of the tree of life to help solve the mystery of selenocysteine synthase (SecS).

The genomes of 24 eukaryotes and 3 archaea that encode the selenocysteine-containing machinery are now fully sequenced. Using bioinformatics, the authors compared these genomes with a set of 24 eukaryotes and 24 archaea that do not contain selenocysteine proteins. This generated a handful of proteins, including known Sec pathway components and an additional candidate protein. The mouse version of the additional protein was cloned and shown to be the elusive eukaryotic selenocysteine synthase. This enzyme, termed SecS, was further characterized and shown to bind to the *O*-phosphoserine form of the tRNA and dephosphorylate the amino acid to generate the modified serine intermediate necessary for selenium addition. This is in contrast to the bacterial SecA, which binds and performs the selenium chemistry on the serine form of the $tRNA^{Sec}$ itself. The primary sequences of the bacterial and mouse proteins are vastly different, but they share a pyridoxal phosphate cofactor. This finding also demonstrated the function of *O*-phosphoseryl-$tRNA^{Sec}$ kinase (PSTK) in selenocysteine biosynthesis. PSTK was previously identified by these investigators, and like SecS, it also occurs in organisms with an active selenocysteine pathway. The authors also identified the mouse enzyme SPS2, which is responsible for making the monoselenophosphate donor molecule. Interestingly, this enzyme is a selenoprotein itself. By combining the two key enzymes, the tRNA, and a selenium source, the authors readily synthesized the charged $tRNA^{Sec}$ *in vitro*. Overall, this study determined the selenocysteine biosynthetic pathway with a clever medley of new genomics techniques and classical biochemistry. **Jason G. Underwood, Ph.D.**



Reprinted from *PLoS Biol.*, *5*, Xu, X. M., *et al.*, Biosynthesis of selenocysteine on its tRNA in eukaryotes, 96–105.

MicroRNAs Flex Their Muscles, *continued from page 86*

mechanism must be controlling the nPTB protein levels. Candidate binding sites for several miRNAs were found in the 3′-untranslated region of the nPTB mRNA, and the putative regulator miRNAs were then tested. Interestingly, the expression of several miRNAs was switched on during the same differentiation that resulted in nPTB loss. Blocking one of these miRNAs with a complementary strand of nucleic acid resulted in the reappearance of the nPTB protein. Most striking, the study goes on to look at the downstream effects of blocking a miRNA and the consequence of nPTB protein in muscle cells. Alternative splicing events that were known targets for the PTB family of proteins were assayed for splice site choice upon differentiation treatment. In parallel, the same splicing events were assayed in differentiated cells where the muscle miRNA of interest was blocked. The result was a muscle cell that displayed alternative splicing patterns that were more similar to undifferentiated cells. Given that PTB genes as far reaching as *Drosophila* and human contain conserved miRNA binding sites, a miRNA effect on splicing is probably a general phenomenon. This study indicates the increasing complexity of gene regulation and the effect of a few meddling RNAs. **Jason G. Underwood, Ph.D.**