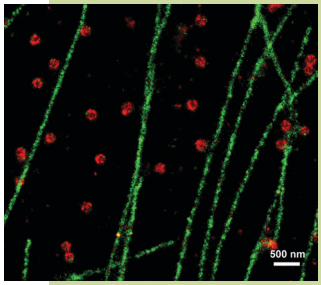
Spotlight

A Perfect, Multicolor STORM

Fluorescence microscopy is an extraordinarily powerful imaging method that enables single and multicolor visualization of biomolecules in cells and tissues. However, the spatial resolution of conventional optical microscopy, limited by the diffraction of light to ~300 nm, is 1–2 orders of magnitude above the typical molecular length scales in cells. This has prompted development of several "super-resolution" techniques, including stochastic optical reconstruction microscopy (STORM), which have 20–50 nm resolution capabilities. However, the development of multicolor super-resolution imaging has been challenging. Now, Bates *et al.* (*Science*, published online Aug 16, 2007; DOI: 10.1126/science.1146598) describe the development of a family of photoswitchable probes that use STORM to enable multicolor nanoscale imaging of biomolecules.



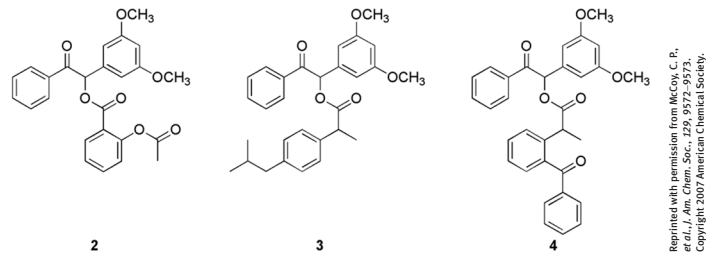
From Bates, M., *et al.*, *Science*, Aug 16, 2007, DOI: 10.1126/science.1146598. Reprinted with permission from AAAS.

STORM employs photoswitchable probes to detect single fluorescent molecules so that only an optically resolvable subset of fluorophores is activated at any given moment. High-resolution images are constructed from images collected over the course of multiple activation cycles, and the result is high-accuracy, nanoscale localization. Combining multicolor imaging concepts with STORM technology, the authors have developed a series of dye pairs that could be deactivated and reactivated upon exposure to appropriate illumination conditions. The pairs consisted of an activator dye and a reporter dye that can be independently selected and, therefore, allow the number of distinguishable fluorescent probes to increase in a combinatorial manner. The authors first use three dye pairs to demonstrate the much-improved resolution of individual DNA molecules. Next, taking the technology a step further, they imaged microtubules and clathrin-coated pits in cells. Remarkably clear spatial resolution of microtubule filaments and clathrin-coated pits not possible with conventional fluorescence microscopy imaging was achieved. Further development of this method will provide additional colors and even better resolutions and facilitate application to more complex biological systems, such as imaging in live cells. **Eva J. Gordon, Ph.D.**

Enlightened Drug Delivery

An ideal drug delivery system encompasses control over the dose, timing, and location of drug release to achieve an optimal therapeutic response while side effects are kept to a minimum. Though several methods, including irradiation with light, have been explored for controlling drug release, they all rely on a global change in the matrix into which the drug is incorporated, ultimately diminishing control over important aspects of the delivery process. McCoy *et al.* (*J. Am. Chem. Soc.* 2007, *129*, 9572–9573) now report a novel, light-triggered method for directly controlling drug release at the level of the drug, rather than the matrix.

3,5-Dimethoxybenzoin (3,5-DMB) derivatives are commonly used in organic synthesis as protecting groups for carboxylic acids and secondary amines, and their removal is achieved by irradiation with UV light. In an effort to extend the utility of this group to drug delivery, the authors synthesized 3,5-DMB conjugates of three common drugs, acetyl salicylic acid, ibuprofen, and ketoprofen. Upon UV exposure, the amount of drug liberated correlated with the duration of light exposure. In addition, no drug was formed in the absence of irradiation, and this provided a solid basis for translating the molecules into a drug delivery setting. The 3,5-DMB–drug conjugates were loaded into a specific polymer scaffold that retained the 3,5-DMB–drug conjugate and that was itself unaffected by UV exposure; the scaffold enabled the photolysis reaction to occur on the encapsulated molecule and allowed diffusion of the drug after removal of the 3,5-DMB group. Indeed, irradiation of the conjugate-loaded polymers resulted in the controlled, localized release of the drugs into aqueous solution, while the 3,5-DMB byproduct remained trapped in the scaffold. This general strategy can be expanded to other drugs, and varying the wavelength, duration, intensity, and location of light could enable exquisite control over drug release for a variety of challenging drug delivery applications. **Eva J. Gordon, Ph.D.**



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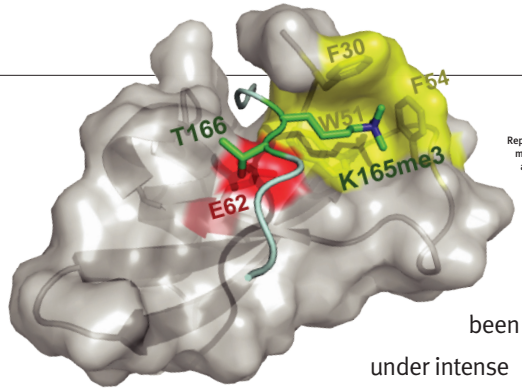
acs chemical biology

Cracking the Histone Code



Post-translational modification of histones, the major protein components of chromatin, is a key mechanism used by cells to control gene expression. For example, trimethylation of Lys9 on histone 3 (H3K9) signals the cell to repress expression of certain genes, and phosphorylation of a neighboring serine antagonizes this signal. The potential for a combinatorial assembly of post-translational modifications in this gene-regulation system, sometimes referred to as the "histone code", has been under intense investigation in recent years. However, the focus in these studies has largely been on modifications that occur on the histone proteins themselves. Now, Sampath *et al.* (*Mol. Cell* 2007, *27*, 596–608) demonstrate that another protein component of the histone modification-recognition system, the H3K9 methyltransferase G9a, is itself methylated and participates in interactions and regulatory processes similar to those in histone proteins.

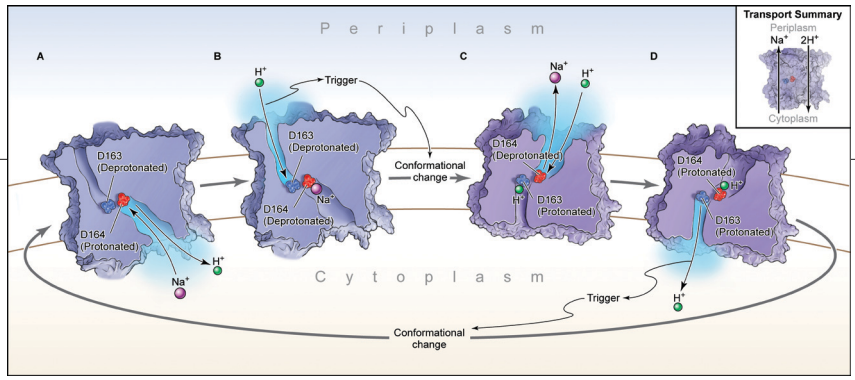
An eight-amino-acid stretch within the amino terminus of G9a that strongly resembled the H3K9 methylation site targeted by G9a led the researchers to speculate that Lys165 (K165) of G9a might also be methylated. Using immunoprecipitation, mass spectrometry, and G9a-deficient cells, the authors determined that indeed G9a methylates its own K165 residue. In addition, it was found that methylated G9a K165 interacts with members of the heterochromatin protein 1 (HP1) family, which also bind to methylated H3K9. Notably, methyl-G9a is the first nonhistone protein found to associate with HP1 proteins in a methylation-dependent manner. Structural and biochemical studies of the interaction revealed that a similar set of residues in HP1 is involved in binding both H3K9 and G9a K165, and that the interactions have comparable affinity. Similar to histone 3, it was also found that phosphorylation of an adjacent threonine in G9a significantly weakens its interaction with HP1. These results demonstrate that the systems that govern histone activity apply to other proteins as well, potentially extending the "histone code" model to what someday may more generally be referred to as the "protein code". **Eva J. Gordon, Ph.D.**

A Tunable Selection Strategy

In vivo genetic selection strategies that lead to the generation of new and possibly even improved proteins are powerful methods for investigating structural and mechanistic aspects of protein function. When applied to enzymes involved in the synthesis of small molecules, such strategies offer new insights into the biosynthetic pathways of various metabolites. In practice, however, the design of such selection systems can be tricky, because numerous possibilities exist in the metabolism of living organisms to compensate for defects engineered into a given pathway. Kleeb *et al.* (*Proc. Natl. Acad. Sci. U.S.A.* 2007, *104*, 13,907–13,912) now describe a selection system that enables the creation of new versions of the enzyme prephenate dehydratase (PDT) while gracefully steering clear of key stumbling blocks inherent in other selection strategies.

PDT catalyzes the conversion of prephenate to phenylpyruvate, which is the penultimate step in the biosynthesis of L-phenylalanine. Prephenate, however, can spontaneously convert to phenylpyruvate in the absence of PDT and is also an intermediate in the biosynthesis of L-tyrosine. These alternative transformations for prephenate affect its concentration in the cell and encumber the ability to control selection stringency. The authors cleverly managed to increase and even fine-tune the selective pressure on PDT by regulating the production of another enzyme, cyclohexadienyl dehydrogenase, which irreversibly converts prephenate to the L-tyrosine precursor 4-hydroxyphenylpyruvate. By controlling the intracellular substrate concentration, the authors could characterize PDT mutant enzymes spanning a 50,000-fold range in activity and sort them by genetic selection experiments. Combining this approach with combinatorial mutagenesis, they also explored the mechanism of PDT. Analysis of the mutants generated revealed insights into the potential contributions of several conserved residues to the mechanism of action and structural stability of PDT. The innovative selection system described here can be applied to other metabolic reactions, adding another resource to the arsenal of genetic engineering and selection strategies for probing metabolic reactions. **Eva J. Gordon, Ph.D.**

Simulated Antiporting



From Arkin, I. T., *et al.*, *Science*, Aug 10, 2007, DOI: 10.1126/science.1142824. Reprinted with permission from AAAS.

The Na^+/H^+ antiporter NhaA is a membrane-spanning protein that transports Na^+ and Li^+ from the cytoplasm and plays an essential role in maintaining cellular salt and pH homeostasis. Despite the recent determination of the crystal structure of *Escherichia coli* NhaA, little is known about the mechanisms by which the protein transports ions and regulates the pH and salinity of the cell. Using molecular dynamics (MD) simulations, Arkin *et al.* (*Science* 2007, *317*, 799–803) now propose a detailed molecular model of NhaA function and describe mutagenesis experiments whose results are consistent with the predictions of their model.

High-speed MD simulations of membrane-embedded NhaA were used to explore ion transport, pH regulation, and cation selectivity. On the basis of previous studies, two aspartic acid residues, D163 and D164, were pegged as likely candidates to drive Na^+ transport. MD simulations performed with these residues in different protonation states and with the Na^+ adjacent to either D163 or D164 suggested that D164 is the binding site for Na^+, while D163 is the accessibility-control site whose protonation state is the presiding factor over whether the Na^+-binding site is exposed to the periplasm or cytoplasm. pH sensitivity was found to be regulated by a third aspartic acid residue, D133. MD simulations suggested that the protonation of D133, but not other carboxylic acid containing residues, results in a conformational change that inactivates the protein, accounting for its pH sensitivity. Finally, the authors examined the protein's selectivity for Na^+ and Li^+ over K^+ by using free-energy perturbation calculations and found it to be explainable on the basis of thermodynamics. The approach used in this study enabled the development of a compelling model for NhaA function that was not possible to extract from existing structural data. **Eva J. Gordon, Ph.D.**

Micromanagers of Translation

Members of a large family of small RNAs, termed microRNAs (miRNAs), have emerged as central players in eukaryotic gene regulation. These 21–23 nucleotide RNAs are cleaved out of longer hairpin RNA structures and then join with the protein machinery in the cytoplasm to form an RNA-programmed particle. Ever since the earliest miRNA was identified in worms, it has been known that miRNAs can form imperfect base pairing with the 3′ untranslated (UTR) region of a messenger RNA (mRNA) and tone down that mRNA's protein output. But how could an RNA duplex formed at the 3′-end of an mRNA change how much protein is made? After all, the ribosome starts at the 5′-end of the RNA and is capable of opening RNA secondary structures as it marches along in polypeptide production.

Although this protein synthesis phenomenon has been seen with many miRNAs *in vivo*, the precise mechanism by which miRNAs inhibit protein production has remained controversial. Two camps have formed in the field. One believes that the miRNAs bind to the 3′ UTR region and directly inhibit translation through interactions of the miRNA-bound protein factors and the translational machinery. Numerous lines of evidence in the translation field have indicated crosstalk between the 5′- and 3′-ends of an mRNA, so this plays into that observation. The second camp posits that the miRNA-programmed particle binds and targets the mRNA for destabilization, and thus, less protein is produced. Consistent with this view are the observations that miRNA-bound factors can flag mRNAs to be shipped away to cytoplasmic RNA degradation bodies. Obviously, these two camps are not mutually exclusive, and in fact, recent studies by several groups indicate that a combined scheme may be at work. To get to the heart of this mechanistic issue, numerous labs have been working diligently to develop *in vitro* systems to study the relationship between miRNAs and translation.

In a recent paper, Wakiyama *et al.* (*Genes Dev.* 2007, *21*, 1857–1862) employ a translation-competent extract from the human embryonic kidney cell line HEK293, but with special modifications. This cell line has a low amount of the miRNA let-7, so this RNA could be added to make a "programmed extract". In addition, a number of known miRNA processing and binding proteins were overexpressed from exogenous plasmids to potentiate the more efficient maturation of the precursor miRNA. The HEK293 extracts were incubated with luciferase mRNAs that contained two flavors of synthetic 3′ UTR sequences. When wild-type binding sites for the

"Reducing" Stress on the Heart

In matters of the heart, "reducing" stress now appears to be more important than ever. It is known that a mutation in the heat shock protein (Hsp) αB-crystallin leads to cardiomyopathy, or a deterioration of the function of the heart muscle. Aberrant folding of this αB-crystallin mutant triggers conditions that can lead to heart failure, but the molecular mechanisms behind this process have not been defined. Rajasekaran *et al.* (*Cell* 2007, *130*, 427–439) now report the generation of a mouse model of human αB-crystallin cardiomyopathy that suggests that "reductive" stress contributes to the pathogenesis of this disease.

Transgenic mice expressing the human form of mutant αB-crystallin experienced cardiac hypertrophy (an increase in the size of the heart), progressive heart failure, and premature death; this signifies that this animal model represents an accurate recapitulation of cardiomyopathy in humans. Investigation into the effects of expression of mutant αB-crystallin in these mice revealed an increase in stress-inducible Hsp's, especially Hsp25, a chaperone implicated in modulating glutathione levels in the cell. Further probing indicated that levels of reduced glutathione (GSH) and the ratio of GSH to oxidized glutathione (GSSG) were significantly elevated, signs that the animals were under reductive stress. In addition, increased activity of the enzyme glucose-6-phosphate dehydrogenase (G6PD), which is a key component in the generation of GSH, was also observed. Crossing mutant αB-crystallin mice with mice deficient in G6PD activity produced animals with reduced protein aggregation and no evidence of cardiac hypertrophy. The finding that reductive stress contributes to the pathogenesis of cardiomyopathy indicates that methods to control the reductive state of the cell, such as targeting G6PD activity, could lead to new treatment strategies for cardiac and perhaps other degenerative diseases. **Eva J. Gordon, Ph.D.**

let-7 miRNA were present in the UTR, ~30% less protein was produced compared with a UTR with mutant sites. By dosing the mixture with extra GW182 and Argonaute 2, known miRNA-associated proteins, the authors enhanced the translation difference to an impressive 70%. They further dissected the effect by varying both the 5′ cap and the 3′ polyadenylation states. They found that both the cap and the poly(A) tail were important to see a translation effect from the miRNA. This implies that miRNAs interfere with the crosstalk between the 5′- and 3′-ends of the mRNA. In addition, this study found a destabilizing effect on the mRNA when the 3′ UTR contained the let-7 sites. Binding of let-7 to the UTR caused deadenylation of the mRNA, consistent with a role in destabilization as well.

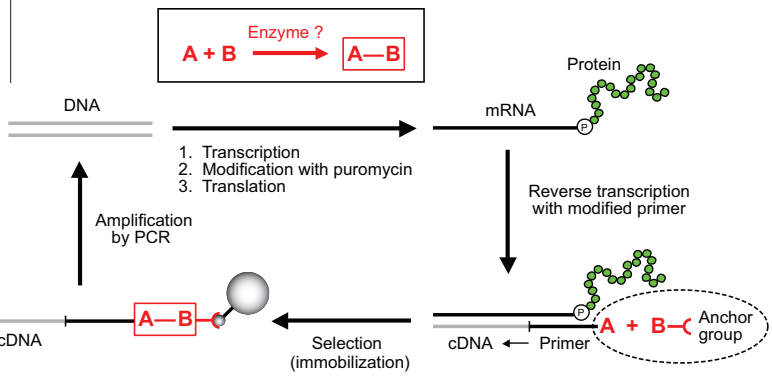
In another biochemical effort, Mathonnet *et al.* (*Science*, published online July 30, 2007; DOI: 10.1126/science.1146067) generated a translation extract from the Krebs-2 mouse ascites cells. Using a similar approach, they fused the luciferase mRNA to 3′ UTR sequences containing let-7 binding sites or mutant sites. In contrast to the HEK293 extract, the Krebs-2 extract contained a significant amount of let-7, and an impressive inhibition of luciferase translation was shown without any additional RNAs or protein components. Sequestering the let-7 miRNA with a complementary oligonucleotide increased the translation of the mRNA containing the let-7 site UTR. This study used rigorous kinetics to look at inhibition and RNA stability. They found that, at early time points, translation was inhibited, but RNA stability could not account for this difference. After a 1-h incubation, the mRNA containing let-7 sites did begin to display less stability than the same mRNA with mutant sites. This group also found that the cap was an important factor in the translation inhibition, and they went on to specifically test the cap-binding complex, eIF4F. The addition of this factor to the extract stimulated translation and seemed to titrate away the effect of the let-7 miRNA. This indicated that the miRNA probably disrupts an event in the cap recognition that is important for translation initiation.

These studies make big strides forward in understanding the mechanism that miRNAs employ in eukaryotic cells, and they make it clear that both sides in this controversy have valid points. The real news seems to be that both camps are correct. A more thorough biochemical characterization and a look at other miRNAs and natural UTRs will be the next direction for this increasingly interesting field. **Jason G. Underwood, Ph.D.**

A New Evolution

Enzymes exquisitely catalyze an impressive range of chemical reactions, many of which can be quite challenging to accomplish through chemical methods alone. The ability to create enzymes with novel activities would provide additional tools with valuable research and therapeutic applications, but thus far, this has only been achieved when extensive knowledge of the mechanism of the reaction is in hand. Using messenger RNA (mRNA) display, Seelig and Szostak (*Nature* 2007, *448*, 828–831) now report the creation of a new enzyme capable of performing a previously unknown RNA ligase activity.

mRNA display technology and *in vitro* directed evolution were employed to create enzymes that could ligate a 5′-triphosphorylated RNA oligonucleotide to the terminal 3′-hydroxyl group of a second RNA strand. In mRNA display, proteins are covalently linked to their encoding mRNA, so a convenient decoding mechanism based on reverse transcription of the mRNA template exists for the identification of proteins of interest. Adding a clever twist to standard mRNA display methodology, the authors carried out the reverse transcription step by using a primer modified with the 5′-triphosphorylated RNA oligonucleotide substrate, and the result was a substrate–cDNA conjugate hybridized to the mRNA displayed protein. The attachment of a biotin moiety to the 3′-hydroxyl group of the second RNA substrate provided a method for identifying mRNA-displayed proteins capable of ligating the two substrates. When product is formed, it becomes covalently attached to the cDNA encoding the protein, and the product–DNA conjugate can be captured by streptavidin affinity chromatography. Subsequently, the cDNA of interest is amplified by using the polymerase chain reaction, and the entire cycle is repeated for further optimization. Using this strategy, the authors isolated several new enzymes with the desired RNA ligase activity. Structural and biochemical characterization of the most active clones revealed a dependence on zinc and pH, and evidence of a folded structure. This innovative application of mRNA display can be translated to the creation of other enzymes with both bond-making and bond-breaking activities. **Eva J. Gordon, Ph.D.**



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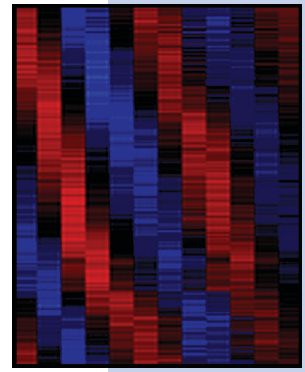
Hormones on the Clock



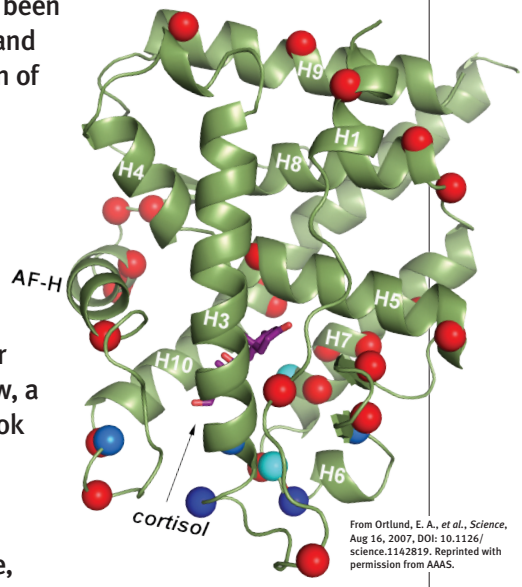
Reprinted from *PLoS Biol.*, *5*, Covington, M. F., and Harmer, S. L., The circadian clock regulates auxin signaling and responses in Arabidopsis, e222.

Ever since the first experiments investigating circadian rhythms in plants were documented in the early 1700s, scientists have studied numerous temporal and spatial regulatory mechanisms used by plants to control their growth, development, and response to their ever-changing environment. The circadian clock refers to the temporal regulation instilled by the ~24-h rhythm that organisms rely on to anticipate regular changes in their environment. In contrast, the auxin class of plant hormones, of which indole-3-acetic acid is the most prominent member, controls many spatial aspects of plant growth and development, including direction of growth and plant embryogenesis. Now, Covington and Harmer (*PLoS Biol.* 2007, *5*; e222) uncover an intriguing link between circadian rhythms and auxin signal transduction.

Genome-wide transcriptional profiling experiments provided initial evidence that several auxin-induced genes, but not genes induced by another plant hormone, brassinolide, are under circadian regulation, because a suspiciously high number of genes involved in auxin signaling exhibited circadian fluctuations in messenger RNA expression. The authors confirmed this observation by using a bioluminescence assay in which transgenic plants expressed the firefly luciferase gene under the control of an enhanced version of an auxin-responsive promoter. In this assay, plants were also found to have differential sensitivity to exogenous auxin at different times of day. Notably, both transcriptional and growth responses to exogenous auxin were found to be clock-regulated. This connection between circadian and auxin signaling pathways intimates the existence of an intricate web of temporal and spatial factors that influence plant growth, development, and environmental response. **Eva J. Gordon, Ph.D.**

Prehistoric Protein Resurrected

Viewing molecular evolution in action can be a difficult task, because many of the selection and optimization events that led to modern species are hidden in ancient history. To turn back the clock and look at these events, computational biologists align the genomic sequences of present-day organisms and then infer the sequences of the ancestors. Even the genes of an extinct organism can be reliably reconstructed if the sequence data from descendent living organisms are available. If a gene family is relatively conserved in a diverse set of living organisms across the tree of life, computer algorithms can trace the evolution of the genes backwards through evolutionary time to determine the sequence of a founding gene. Having more relatives leads to more confidence and a better chance at correctly determining the eldest ancestor shared by all. Once the ancestral gene sequence has been inferred, molecular techniques can be used to biochemically synthesize and experimentally study it, and hypotheses about the evolution and function of ancient genes can be tested in the laboratory.



From Ortlund, E. A., *et al.*, *Science*, Aug 16, 2007, DOI: 10.1126/science.1142819. Reprinted with permission from AAAS.

Recently, this technique was applied to the glucocorticoid and mineralocorticoid hormone receptor. These two genes arose from duplication of a single ancient receptor gene, referred to as the ancestral corticoid receptor (AncCR), ~450 million years ago. In modern organisms, the glucocorticoid receptor (GR) mediates the stress response and regulates blood glucose levels in response to the hormone cortisol, and the mineralocorticoid receptor (MR) controls electrolyte levels and proper kidney function in response to aldosterone and deoxycorticosterone. Now, a high-resolution look at the AncCR shows that the descendent families took an interesting evolutionary path. In a new paper, Ortlund *et al.* (*Science* 2007, published online Aug 16, 2007; DOI: 10.1126/science.1142819) express, crystallize, and determine the X-ray structures of the AncCR ligand-binding domain complexed with aldosterone, deoxycorticosterone, and cortisol. The hydrogen bonding pattern of AncCR with all of the ligands mimics the interactions seen in the modern human MR, and like the modern MR, it is more sensitive to aldosterone and deoxycorticosterone than to cortisol. The group then reintroduced mutations that occurred during the evolution of the GR from AncCR and tested the effect of each on the receptor's hormone sensitivity. Mutation of just two amino acids pushed the receptor toward cortisol recognition and away from the other hormone ligands. This study provides 3D support for the notion that a single mutation is often not just tolerated, but can actually potentiate the ability for a second mutation to change the function for a protein. In this case, the first mutation unwound a helix and moved the second site into a new position near a hydroxyl group that is present on cortisol but not the other hormones; the second substitution then formed a new hydrogen bond to that hormone, uniquely stabilizing the receptor complex with cortisol. This phenomenon, termed conformational epistasis, renders the protein functionally flexible should a new job arise. The first new amino acid cocks the hammer for a second mutation to change the function of the protein. This study also proves that structural information on ancestral proteins can be extremely helpful in understanding the diversifications displayed in modern proteins. **Jason G. Underwood, Ph.D.**