Research Digest

Synopses of Research Articles

Using Biology to Create Complex Patterns

In his seminal exploration of the properties of living organisms, *What Is Life?*, Erwin Schroedinger concluded that life depends in large part on storing and processing information. For genetic material to carry the diverse instructions required for living processes, he proposed, it must be stored in an aperiodic crystal. Just nine years later, it was clear that DNA is indeed an aperiodic crystal and that genetic information is conveyed through this irregular pattern. Much like computers, biological systems are programmed to follow a precise set of rules, or algorithms, to store information and solve problems. These biological algorithms direct all manner of biochemical processes to create complex patterns and structures by chemically modifying and assembling individual components.

Of course, cells use biochemical circuits not electronic circuits. Single tubulin proteins, for example, follow precise rules of chemistry and physics to spontaneously self-assemble, or polymerize, into the microtubules essential for cell transport and motility. The proteins' binding interactions effect rules that specify how the pieces fit together to form the resulting structure. They also specify when and how tubulins assemble from a nucleation complex—a molecular algorithm governing the logic of polymerization. These complex structures self-assemble with remarkably few mistakes. Though considered quite simple, little is understood about the principles that govern programmable structural order underlying this type of spontaneous self-assembly.

In crystals, the simplest example of spontaneous self-assembly, subunits of the whole are arranged in a repeating pattern that extends indefinitely in all directions. If you know the position of one unit in the pattern, you can tell the exact position of every other unit. In a new study, Rothemund and colleagues use DNA to show that crystal growth can be programmed to create specific aperiodic patterns. Inspired by a model of crystal growth as a computational process, they have programmed DNA molecules to act as molecular building blocks, arranging themselves according to local rules that in turn create a complex global pattern. The resulting two-dimensional structures, which self-assemble from knotted DNA complexes (called tiles), grow to create a fractal pattern known as a Sierpinski triangle.
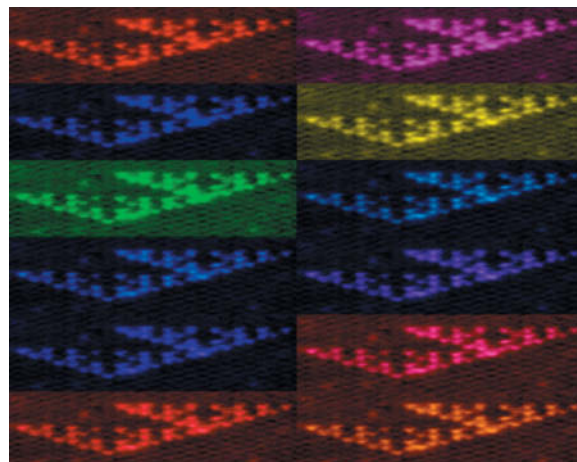
DOI: 10.731/journal/pbio.0020448.g001

Fractal patterns from DNA

These DNA structures—neither periodic (as in quartz), nor random (as in glass), nor pseudorandom (as in quasicrystals with "forbidden" five-fold symmetries)—demonstrate a form of self organization in crystalline materials determined by programmable growth rules, and are hence dubbed "algorithmic crystals."

How can such growth algorithms be encoded in biological molecules? The rules of chemical base-pairing follow regular, predictable patterns, allowing the authors to use DNA to determine the tiles' binding interactions.

Desired binding interactions between tiles were programmed by endowing each tile with single-stranded "sticky ends" whose sequence was complementary to the sticky ends of tiles it should stick to. Each tile was either white (0) or black (1): a black tile can fit at any site where the two neighboring tiles are opposite colors, while a white tile can fit at any site where the two neighboring tiles are the same color. Logically, the new tile's color is the exclusive-or (XOR) of the tiles in the previous layer.

That such logical layer-by-layer iteration of XOR computations will produce the Sierpinski triangle is well known. What's remarkable is that DNA molecules can be programmed to grow according to this logic. With this programmable algorithmic crystal, Rothemund and colleagues demonstrate a method for designing DNA molecules capable of implementing any pattern of abstract logical tiles. What's more, the authors argue, any algorithmic crystal growth process can, in principle, be experimentally investigated using DNA self-assembly.

So how is algorithmic self-assembly related to biology? Like the algorithmic crystals, many of the self-assembled structures in biology are ordered but aperiodic. The hope is that the theoretical insights of computer science—well-honed for describing, analyzing, and programming computational systems—can direct investigations of biochemical self-assembly and information processing. And with a method for demonstrating how simple chemical and physical elements can create complex organization, Rothemund and colleagues have added a concrete experimental framework to bolster that work. (For more on DNA and complexity, see "The Emergence of Complexity: Lessons from DNA" by Chengde Mao.)

Rothemund PWK, Papadakis N, Winfree E (2004) Algorithmic self-assembly of DNA Sierpinski triangles. DOI: 10.1371/journal. pbio.0020424

Computer Simulation of the Movement of *Listeria* Bacteria

Each of the cells in our body is a complex machine. Within each cell, thousands of proteins and other molecules interact to produce the highly organized cellular events that are needed for life. However, there is no cellular line manager telling the individual proteins where to go and what to do. It's rather like an ant colony. No one tells the individual ants how to build a nest. They just do it. Similarly, no one tells the individual actin molecules, for example, to get together and polymerize to form the cytoskeleton that is essential for cell movement and other cellular processes. It just happens.

Ant colonies and actin polymerization are both examples of emergent behavior. Broadly defined, emergent behavior is when a collection of individuals interact without central control to produce results that are not explicitly programmed. Scientists are only just beginning to understand how complex behaviors of this type can emerge from a myriad of individual interactions, many of which are well-defined experimentally.

Jonathan Alberts and Garrett Odell have turned to an unlikely ally—the bacterium *Listeria monocytogenes*—in their study of the complexities of cellular actin polymerization. This rod-shaped microbe, which lurks in well-ripened brie and other unpasteurized cheeses, can cause the sometimes fatal disease listeriosis, particularly in young babies or people with weakened immune systems.

When *Listeria* microbes in food reach a person's gut, they penetrate the cells lining the gut, reproduce, and then spread from cell to cell without ever exposing themselves to the extracellular environment. In this way, they avoid the host's immune system. One particular protein produced by *Listeria* is central to this sneaky intracellular lifestyle: ActA. Expression of ActA allows the microbes to hijack the machinery in the host cell that controls the growth of actin networks. Some of the cellular actin, instead of forming the cytoskeleton of the cell, polymerizes around the bacterium, forming a dense "comet tail" of actin that generates a force to move the bacterium around the cell and push it into neighboring cells. This clandestine use of our cellular machinery for actin polymerization is far simpler to understand than the elaborate use our cells normally make of it, so the study of *Listeria* propulsion provides a scientific stepping-stone to understanding the involvement of the actin cytoskeleton in normal cell movements.

Alberts and Odell have used established data on the biochemical and mechanical details of actin polymerization and the physiological concentrations of the proteins involved in the process to build a computer simulation of how an actin network assembles around a moving, rod-shaped bacterium. Their "in silico" reconstitution, which considers the behavior of individual actin filaments and requires a cluster of 80 computer processors to be run for several days at a time, produces realistic bacterial motion in terms of speed and persistence, and models the actin tail shape. The model also reproduces smaller scale emergent behavior. Real *Listeria* cells do not move smoothly. Instead, the microbes move jerkily, with runs interspersed with pauses. The simulation faithfully reproduces this type of movement and offers a mechanistic explanation for it.

The approach described by Alberts and Odell can now be used to investigate many other, more complex cell mechanics problems, such as how the cellular movements involved in cell division are achieved. With the availability of detailed biological information and powerful computers, we may at last start to solve the mystery of how interactions among maybe 100,000 gene products can produce the organized cellular processes that cell biologists have been watching under the microscope for years.

Alberts JB, Odell GM (2004) In silico reconstitution of *Listeria* propulsion exhibits nano-saltation. DOI: 10.1371/journal.pbio.0020412

When Immune Defenses Turn Traitor

When pathogens enter your body, most wind up engulfed (by phagocytes), poisoned (by stomach acids), or flushed out of your system. These defenses kick in when a bacterium's toxic secretions bind to cellular receptors that trigger a chain of events ending in an innate response. A wide variety of cells and chemicals, including phagocytes and cytokines, initiate the inflammatory reaction that redirects innate defenses in the bloodstream to the infected site.

If all goes well, the innate system successfully contains and eliminates a pathogen at the site of infection. But if infection persists, so will the inflammatory response, and these first responders turn traitor. It's thought that the immune response rather than the bacteria, for example, causes diarrhea in food poisoning. And when infection leads to death—as happens in septic shock—the immune response may be just as culpable as the infectious microbe.

To better understand the interplay between pathogen and host in the onset of infection and disease, David Schneider and colleagues turned to the genetically compliant fruitfly *Drosophila* and the food-borne pathogen *Salmonella*. Working with mutant strains of both fly and bacteria, the authors identified genes important to the development of infection and disease and showed that the host's reaction can indeed be lethal.

Pathogens possess various means to infect their host, unleashing toxins and secreting molecules that enhance virulence by breaching cell membranes and altering

Adult fruit fly infected with flourescent *Salmonella*

the intracellular environment. Fruitflies combat these intrusions with various innate responses, including phagocytosis. In this study, the authors investigated how *Drosophila* phagocytes find, engulf, and kill invading microbes and then alert the rest of the immune system—and how *Salmonella* circumvents these defenses to initiate disease.

Schneider and colleagues used a *Salmonella* strain (*S. tyhphimurium*) that produces two pathogenicity complexes, called type III secretion apparatuses, which shuttle virulence molecules through the host's cell membrane and into its cytoplasm. One complex, SPI1, facilitates cell entry while the other, SPI2, retools the intracellular environment to suit bacterial growth. The authors created a series of less virulent *Salmonella* strains and examined their effects on wild-type (nonmutant) flies. In addition, they looked at infections of wild-type *Salmonella* in flies carrying mutations in two critical immune response pathways (called Toll and imd, for immune deficiency).

Imd mutants infected with nonmutant *Salmonella* died much faster than the Toll mutants and had far more bacteria in their blood. When Schneider and colleagues correlated *Salmonella* population numbers with fly fate, they discovered something surprising. In other bacteria models, flies die when bacterial pathogen numbers reach a critical mass. Here, *Salmonella* populations hit a ceiling and the flies died with comparatively few bacteria. In flies infected with *Salmonella* strains lacking either one or the other virulence complex, the flies survived despite increased bacterial growth.

The authors explain this surprising finding with a model in which the fly's immune response produces substances that ultimately engineer its own destruction. Their model is supported by experiments showing that flies carrying a mutation in the *eiger* gene—a homolog of the human TNF gene—live longer with *Salmonella* infections. This is the same phenomenon seen in TNF-induced septic shock, when patients die as much from their immune system's response to infection as from the bacteria itself. Since many of the proteins involved in the *Drosophila* imd pathway have counterparts in the mammalian antibacterial immune response, the model described here can help identify the genetic agents of metabolic collapse associated with bacterial infections in humans.

Brandt SM, Dionne MS, Khush RS, Pham LN, Vigdal TJ, et al. (2004) Secreted bacterial effectors and host-produced Eiger/ TNF drive death in a *Salmonella* infected fruitfly. DOI: 10.1371/journal.pbio.0020418

Norovirus Cultured for the First Time

Norwalk virus and related noroviruses cause a short but unpleasant illness known variously as stomach flu, viral gastroenteritis, and winter-vomiting disease. They are the major causative agent of epidemic gastroenteritis worldwide. Norwalk virus was first described in 1968 when teachers and pupils at a school in Norwalk, Ohio, succumbed to acute gastroenteritis. The causative agent was identified in 1972, and since then, many other Norwalk-like viruses have been recognized. Noroviruses are usually picked up from contaminated food or water but can also be spread by person-to-person contact. There are no cures for norovirus infections and no vaccines. Worse still, noroviruses survive freezing, heating to 60 °C, and the amounts of chlorine added to public water supplies. Little surprise, then, that around 23 million Americans get a norovirus infection annually.

To reduce this disease burden, better prevention and control strategies for noroviruses are urgently needed. However, the development of such strategies has been hampered by the inability of scientists to find a way to grow noroviruses in cultured cells. Like all viruses, noroviruses replicate inside host cells, and they are choosy about which cells they will grow in. The discovery by Herbert Virgin and colleagues that a
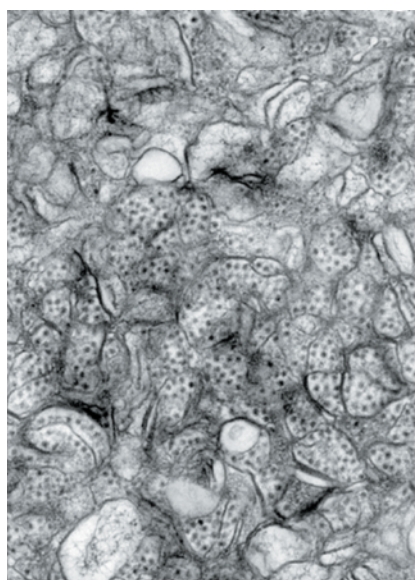
The first norovirus cell culture system

mouse norovirus can be grown inside dendritic cells and macrophages, two types of immune system cells, opens the door to a better understanding of norovirus biology and better disease control strategies.

In 2003, Virgin's team discovered MNV-1, a mouse norovirus, and reported that the innate arm of the immune system (as opposed to the adaptive arm) was important in combating MNV-1 infection. The adaptive immune system involves cells that respond to a disease-causing bacterium or virus by making "adaptations" to their genomes that result in specific anti-pathogen responses. The innate immune system, on the other hand, contains cells that recognize general features of pathogens and rapidly attack them when they enter our bodies. It is our first line of defense against bacteria and viruses, and Virgin and coworkers found that mice with defective innate immune systems were particularly sensitive to MNV-1.

Now, by examining tissues taken from mice infected with MNV-1 infection, the researchers show that in live animals the virus infects macrophages (cells that engulf and kill pathogens) and dendritic cells (cells that display pathogen proteins to the adaptive immune system). This observation provided the clue needed to grow a norovirus in cultured cells for the first time: when the researchers took uninfected dendritic cells or macrophages out of animals with defective innate immune systems and grew them in the laboratory, they found that MNV-1 could replicate within these cells. The researchers then used physical and biochemical techniques to verify that what they were growing in culture was indeed MNV-1 and also determined the cellular factors that control MNV-1

growth in culture, thereby confirming that the innate immune system is important for combating norovirus infection. Analysis of the sequence of a virus attenuated by growth in vitro identified an important part of the viral capsid that plays a role in MNV-1 virulence, potentially opening up an avenue to vaccination with attenuated viruses.

The researchers speculate that dendritic cells in the gut may provide the route by which noroviruses gain access to cells deep within the lining of our guts and thus cause disease. The next step will be to see whether human noroviruses can also grow in macrophages and dendritic cells. If they do, researchers should at last be able to elucidate the lifecycle of human noroviruses and identify the cellular factors needed for their replication. Hopefully, this new knowledge will swiftly suggest ways to prevent and control the human diseases caused by noroviruses.

Wobus CE, Karst SM, Thackray LB, Chang KO, Sosnovtsev SV, et al. (2004) Replication of a *Norovirus* in cell culture reveals a tropism for dendritic cells and macrophages. DOI: 10.1371/journal.pbio.0020432

A Structural Analysis of Eukaryotic Membrane Evolution

It took nearly 200 years for biologists to redefine the plant/animal dichotomy set up by Linnaeus in 1758. Among the defining traits used in the new five kingdom model of the 20th century was the presence of a nucleus. Possession of a nucleus is one of the chief characteristics that earns an organism, even a single-celled organism, the name of eukaryote. Those not similarly blessed are prokaryotes. Biologists today classify life into three domains (of which microbes lay claim to two), yet the evolution of many fundamental features of eukaryotic biology remains a mystery.

A pivotal moment in the evolution of early eukaryotes was the emergence of elaborate, interconnected membrane-bound compartments that make up the Golgi apparatus, endoplasmic reticulum, and nuclear envelope. The nuclear envelope, with its inner and outer membrane, forms a barrier between the cytoplasm and nucleus. Embedded in this envelope are nuclear pore complexes (NPCs), massive (over 400 subunits),

Early eukaryote?

cylindrically shaped protein assemblies that connect the outer and inner nuclear membranes via sharply curved sections of pore membranes. The NPC's central ring-like structure is sandwiched between a cytoplasmic ring, with fibrils extending into the cytoplasm, and a nuclear ring, with a "basket" extending into the nucleoplasm. NPCs police traffic flow between the nucleus and cytoplasm, routinely allowing entry to small molecules while providing only selective passage to macromolecules.

How eukaryotes evolved complex membrane-mediated trafficking systems from their stripped down prokaryotic contemporaries is a fundamental question in biology. Michael Rout's team investigates one aspect of eukaryotic evolution—the origin and evolution of NPC proteins (nups)—by examining the structure of nups. In a new study, Rout and colleagues report the structure of a core building block of the NPC in yeast, and propose how the complex could have evolved from organisms with no such system.

The researchers first tackled the structures of the seven protein components of a core NPC subcomplex, called the yNup84 subcomplex in yeast (and the vNup107-160 subcomplex in vertebrates). Rout and colleagues used algorithms that predict secondary structures to generate three-dimensional models of the component nups. Each nup, they found, consists mostly of either repeating alpha helixes (in an alpha-solenoid fold), zigzagging beta sheets (in a beta-propeller fold), or a distinctive arrangement of an amino-terminal beta-propeller followed by a long stretch of alpha-solenoid. Next, the authors compared the structural conformations of the homologous nups found in humans and plants, and showed that the overall architecture of the subcomplex has been conserved throughout eukaryotic evolution.

A search for evidence of the distinctive propeller/solenoid arrangement in other organisms shed light on the function and origin of the yNup87/vNup107-160 subcomplex. Neither bacterial nor archaebacterial proteins contain such an arrangement; it appears to exist only in eukaryotes. Moreover, proteins containing this arrangement function only as components of the coated vesicle complexes that operate in intracellular vesicular transport systems or as part of the NPC. That these complexes are linked by common architecture, the authors argue, suggests an "intimate connection between vesicle coating complexes and the yNup87/vNup107-160 subcomplex." It's likely that both complexes function in curving membranes: when components of this subcomplex are disrupted in yeast, NPCs form abnormal clusters that impair nuclear membrane interactions.

How did this shared molecular architecture evolve? Rout and colleagues propose that both nups and vesicle coating complexes developed from a common early eukaryotic ancestor—a primitive coating component with a simplified version of the repetitive folds described here. This molecular carpenter specialized in carving and remodeling membranes, and was repurposed to support the many specialized functions that facilitate molecular transport through the elaborately connected, highly specialized internal membrane systems of the modern eukaryote.

Devos D, Dokudovskaya S, Alber F, Williams R, Chait BT, et al. (2004) Components of coated vesicles and nuclear pore complexes share a common molecular architecture. DOI: 10.1371/journal.pbio.0020380
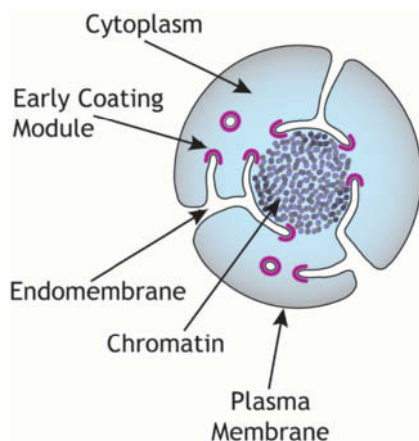
A Fly Enzyme for Motor Control

Long-range communication requires special technology. Although we usually think of nerve cells communicating over distances measured in millimeters, they must also stretch over centimeters and even meters to enable movement and sensation throughout our bodies. In a disorder known as spastic paraplegia, people experience stiffness and loss of function in their legs because the far ends of the longest nerves in the spinal cord degenerate. One form of this disease, autosomal dominant hereditary spastic paraplegia (AD-HSP), is most frequently caused by mutations in a gene that codes for the enzyme Spastin.

Until recently, the function of Spastin has only been inferred by its similarity to another protein, Katanin, which chops up microtubules. The microtubules in neurons, as in all types of cells, provide a network to transport materials from one place in the cell to another. For neurons to communicate with each other and with muscles, neurotransmitters packaged at one end of the cell make the journey through the cell's long, narrow axon on the backs of microtubules. At the other end of the cell, the neurotransmitters reach small cellular projections (boutons) where they are released as a signal to the receiving cell. Since long spinal cord axons are most often affected by AD-HSP, researchers have suggested that transport through axons might be culpable.

Is there a link between Spastin, microtubule severing, and AD-HSP? To address this question, Nina Tang Sherwood and colleagues studied the function of Spastin in the fruitfly *Drosophila* by identifying the *spastin* gene in this species and manipulating its expression.

They found that indeed *Drosophila* Spastin in neurons regulates microtubule networks. Overexpressing *spastin* caused collapse of the embryonic central nervous system and also eliminated the microtubule network, as expected based on the related Katanin protein's microtubule-severing activity. But—surprisingly—knocking out *spastin* did not yield the opposite result. *spastin*-null flies had fewer microtubule bundles, particularly at the far ends of the neurons. They also exhibited smaller and more numerous boutons that were unusually clustered together.

On the basis of their results, the authors speculate that Spastin cuts microtubules to a manageable size. Too much Spastin chops the microtubules into useless fragments, but too little Spastin may leave microtubule polymers too large to be efficiently moved into newly forming boutons. With an intermediate amount, microtubule pieces are the right size for transport throughout the neuron.

Without Spastin, normal motor function ceases. In *spastin*-null flies, neurotransmitter release is impaired and flying is impossible. Flies even tend to drag their hind legs. This weakness in the legs is just one compelling parallel to human AD-HSP. The severity of symptoms in people is highly variable, similar to the variability in phenotypes exhibited by *Drosophila* with intermediate *spastin* gene mutations. The authors caution, however, that they do not prove that the fly phenotypes observed arise through the same mechanisms that cause human AD-HSP. The utility of *Drosophila* as a model for human AD-HSP has yet to be demonstrated, but the importance of Spastin in regulating neuronal microtubule networks in vivo is no longer in doubt.

A Neuron Survival Protein May Give Directions, Too

Anyone who's ever had a physical exam knows the monosynaptic stretch reflex arc. When the doctor taps your patellar tendon with a hammer, your quadriceps muscle briefly stretches and your knee responds with a quick kick. This involuntarily reflex is mediated by muscle spindles, specialized muscle structures containing both proprioceptive (sensory) and motor neurons. Proprioceptive neurons send information about how much the muscle is stretched to the spinal cord, and motor neurons emanating from the spinal cord tell the muscle to contract, which corrects the stretch.

This reflex circuit is established in the developing embryo, when neurons migrate through and around developing tissues and send their axons to their signaling targets. Many neurons will successfully extend their axons into a target—which might be a muscle, another neuron, or some other tissue—but not all survive the process. Whether a neuron lives or dies depends on a family of growth factor proteins called neurotrophins. If a sensory neuron doesn't get enough neurotrophin-3 (NT-3), it will die.

Proper development of the sensory/ motor circuit also depends on NT-3, which is expressed in limb buds, muscle spindles, and the ventral spinal cord: muscle spindle development depends on sensory axons, and motor neuron connections depend on developing limb buds. It has not been clear, however, whether NT-3 simply ensures the survival of proprioceptive neurons or whether it also helps establish the proprioceptive reflex arc. In a new study, Reha Erzurumlu and colleagues demonstrate a clear role for NT-3 in axon guidance.

Developing nerves in the mouse spinal cord

Attempts to investigate the axon guidance theory have been difficult since sensory neurons die without NT-3. To circumvent this problem, the authors developed a "double knockout" mouse

model that deletes both the NT-3 gene and the apoptosis-promoting gene *Bax*, which activates the cell death pathway for sensory neurons. This system removes NT-3 signaling without killing the sensory neurons, so the researchers can investigate any effects NT-3 may have on axon behavior.

Erzurumlu and colleagues showed that sensory neurons, in the absence of NT-3 signaling, project to the right places but never reach their final destination. In normal development, sensory neuron axons travel into the ventral spinal cord and form synapses with motor neuron dendrites in the ventral horn to establish the reflex arc circuit. In the double knockout mice, sensory neurons manage to extend into the spinal cord, but then get lost; they can't find the ventral horn, so they never form a synapse with the motor neuron dendrites. The failure to establish connections between the sensory axons and motor neurons in mice lacking NT-3, the authors argue, indicates that NT-3 is required for proper axon targeting.

Similarly, deprived of NT-3 signaling, sensory neuron axons fail to reach their ultimate targets in peripheral muscle. They project down toward the muscle but don't recognize the muscle and thus cannot enter or innervate it. Consequently, the muscles' sense organs, the muscle spindles, cannot differentiate. The authors argue that these findings, along with the results of tissue culture experiments, show that NT-3 acts as a short-distance cue for proprioceptive axons, which travel in the right direction but ultimately lose their way without NT-3.

Altogether these results show that proprioceptive axons require NT-3 not just for survival, but to reach their proper endpoints in the peripheral and central nervous system. NT-3 also helps proprioceptive axons initiate muscle innervation and spindle differentiation. Researchers developing therapies to treat neurodegenerative injuries have increasingly focused their attentions on growth factors like NT-3. By identifying the molecules and mechanisms that establish connections between sensory and motor neurons during development, it may be possible to engage similar processes to attenuate neurodegeneration and even repair damaged nerves.

Genç B, Özdinler PH, Mendoza AE, Erzurumlu RS (2004) A chemoattractant role for NT-3 in proprioceptive axon guidance. DOI: 10.1371/journal.pbio.0020403

This Is Your Fly's Brain on Drugs

Cocaine addiction wreaks profound changes on the brain, hijacking reward circuits and depressing inhibitory loops to the point that drug seeking and taking become central drivers of behavior. Lying at the core of these behavioral changes are molecular ones; at its most basic level, addiction alters the sensitivity of neurons. While primates and rats are useful for mapping out the neural complexity of these behavioral manifestations, insights into the molecular basis of drug abuse can be garnered more easily from simpler models, such as the fruitfly, *Drosophila*.

The reigning model of cocaine's effects on the brain has highlighted its ability to block reuptake of dopamine by cells of a brain region called the nucleus accumbens. But numerous experiments show this is not the whole story. Ulrike Heberlein and colleagues describe their discovery of a new gene that modulates sensitivity to cocaine within the cells of the fruitfly's internal clock. They further show that the cells' role in regulating cocaine sensitivity is distinct from its function as a timekeeper.

One known effect of cocaine on *Drosophila* is loss of "negative geotaxis," or wall climbing, in response to startle. Using this behavior to screen 400 different mutants, the researchers identified seven with an increased response to cocaine, and for two of these, the disrupted gene was the same, *Lmo*. The *Lmo* protein, whose levels were reduced by the mutations, is known to regulate certain transcription factors during development. Despite this, no developmental defects were detected in the loss-of-function mutants that might explain the cocaine effect. The researchers also found that a third mutation in the same gene, previously associated with disruption in wing formation, *increased* levels of the *Lmo* protein, and *decreased* response to cocaine. Thus, *Lmo* appears to play a central role in regulating cocaine sensitivity.

While *Lmo* is found throughout the body, it is enriched in the brain, and by expressing normal *Lmo* in oversensitive mutants, Heberlein and colleagues discovered that its cocaine-related effects were localized to the ventral lateral neurons (LN_vs). Comprising about ten cells per hemisphere, these neurons provide the fly with an internal clock, driving circadian activities even in the absence of light. Not surprisingly, *Lmo* mutants had weaker circadian rhythms than normal flies.

But is increased cocaine sensitivity a simple consequence of a broken clock? Apparently not. To date, the only known output of the LN_v is a small peptide called PDF, and PDF mutation causes circadian disruptions. It does not, however, alter cocaine sensitivity. Furthermore, completely obliterating the LN_v or blocking its ability to fire, disrupted circadian rhythmicity but *reduced* cocaine sensitivity, rather than increasing it. These results indicate than the LN_v normally enhances sensitivity to cocaine, a function enhanced further by *Lmo* mutants, and does so independently of circadian regulation.
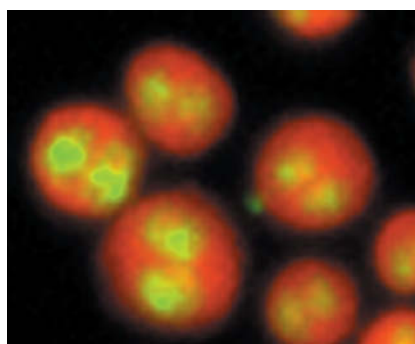
Based on their results, Heberlein and colleagues propose a possible model for *Lmo*'s role in modulating cocaine sensitivity. Drawing on recent evidence that a subset of LN_v cells possess dopamine receptors, they suggest that *Lmo* expression normally regulates the density of these receptors on LN_v cells. Loss of *Lmo* would raise the number of receptors, thereby increasing the sensitivity to cocaine. A key prediction from their findings is that the LN_v has another output, as yet undetected, in parallel with PDF that mediates responsiveness to cocaine.

Because *Lmo*-related proteins are found in key areas of mammalian brains, these results may have important implications for understanding innate differences in sensitivity to cocaine in humans, and potentially provide targets for development of drugs to treat or prevent addiction.

Tsai LTY, Bainton RJ, Blau J, Heberlein U (2004) *Lmo* mutants reveal a novel role for circadian pacemaker neurons in cocaine-induced behaviors. DOI: 10.1371/journal.pbio.0020408

Chemical and Genetic Screens Hit the Target in Cytokinesis

Cytokinesis, in which newly formed daughter cells separate, is the culmination of the cell cycle. It is necessary for normal growth and development, and it is also a sine qua non in the pathogenesis of cancers—cells that can't divide can't form tumors, can't metastasize, and can't kill. Therefore, understanding the full range of proteins involved in cytokinesis has both deep theoretical and immediate practical applications. In this issue, Ulrike Eggert and colleagues report results from two complementary screening approaches to identify those proteins and to discover molecules that inhibit them.

A cell cannot divide if it lacks a protein vital for cytokinesis or if that protein is inhibited. When that occurs, the cell retains both nuclei, and can be quickly identified by an automated process. Working with *Drosophila* cells, the first screen used almost 20,000 double-stranded RNAs, representing virtually the entire *Drosophila* genome. A double-stranded RNA pairs with, and causes the destruction of, a matching messenger RNA, thus preventing the encoded protein from being formed, a process called RNA interference (RNAi). The authors identified 214 proteins whose absence prevented cytokinesis. While some of these, including actin and Myosin, were already known to be essential for the process, others were not. The latter included a new discovery,

Drosophila cells that have failed to divide

CG4454 (named Borealin-related or Borr), which was found to be one of the handful of proteins deemed most critical to cytokinesis.

The second screen also treated *Drosophila* cells, but this time used over 50,000 "small molecules," a catchall term for molecules small enough to pass easily into cells. The vast majority of drugs currently in clinical use are small molecules. This screen revealed 50 cytokinesis inhibitors, of which 25, dubbed binucleines, were selected for further characterization. Not surprisingly, several inhibited actin, whose role in cytokinesis is key in contraction of the cytokinesis furrow.

For the purposes of this study, however, binucleines affecting other proteins were even more interesting. By comparing the appearances of binucleate cells from the

small-molecule screen with those from the RNAi screen, Eggert et al. identified one molecule and three proteins that caused a similar phenotype, suggesting that the three proteins acted within a single pathway, which the molecule could disrupt. One of the three proteins was CG4454/Borr, and the researchers' results indicated it interacts with Aurora B, an essential but still poorly understood protein that is needed for proper division of the chromosomes. The identified binucleine will be a valuable reagent for exploring the details of the Aurora B pathway.

While these results are from *Drosophila*, the insights they provide into the cell cycle are likely to be applicable to humans as well. Equally importantly, they provide the proof of principle for a new drug discovery method. A major bottleneck in drug development is target identification—determining which of the cell's thousands of proteins is the right one to inhibit with a drug. The unique aspect of this study is the parallel use of the two approaches—small molecules and RNAi—to provide a "stereoscopic" view of cytokinesis and its inhibition. Working together, they provide a set of proteins and a matched set of inhibitors, the target and the bullet at the same time.

Eggert US, Kiger AA, Richter C, Perlman ZE, Perrimon N, et al. (2004) Parallel chemical and genome-wide RNAi screens identify cytokinesis inhibitors and targets. DOI: 10.1371/journal.pbio.0020379

Repetitive Editing and RNA Splicing

The so-called "central dogma" of biology—DNA makes RNA makes protein—is a simple statement that subsumes a wealth of complexity. In particular, the past decade has shown that after RNA is made, it is run through a gauntlet of processes that strip it of introns and splice its exons, add a cap and tail, and even chemically modify one or more bases along the way. This last possibility includes deamination—removal of an NH_2 group—from adenosine, converting it to inosine. During translation, the ribosome reads an inosine as a guanosine; thus, an A-to-I edit in RNA can even cause an amino acid change in the resulting protein. A new study in this issue by Stefan Maas and colleagues shows that A-to-I editing is remarkably widespread among human genes, and commonly targets a ubiquitous repetitive sequence, the Alu repeat.

A-to-I editing has been recognized for several years, but the known targets have been few, far fewer than the number predicted by measuring the inosine content in messenger RNA. To identify more targets, Alekos Athanasiadis, Alexander Rich, and Maas compared genomic sequences to cDNA sequences. Adenosines are unchanged in genomic DNA, while in complementary DNA (cDNA), which is derived
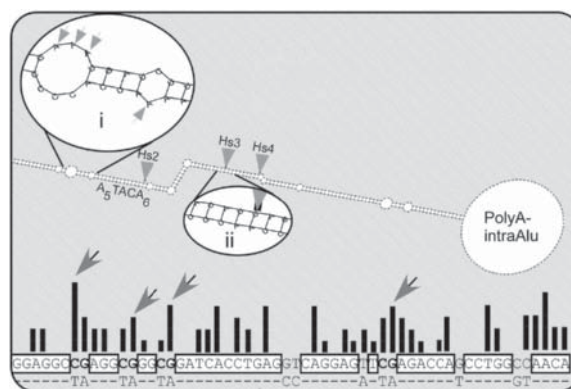
Sequence and structure preferences of editing in Alu repeats

from reverse transcribing mRNA, any adenosines that were converted to inosines during RNA editing show up as guanosines. Thus, A-to-G discrepancies revealed candidate editing sites. To reduce the number of false positives, the researchers confined their search to regions with multiple A-to-G discrepancies. In an initial screen of 3,000 cDNAs, they found 26 A-to-I edited genes. In all but one case, the editing occurred in an Alu sequence.

There are approximately 1.4 million Alu sequences in the human genome, each about 300 base-pairs in length, which together comprise about 10% of the entire genome. Not all of them occur in genes, but those that do are typically found in transcribed but untranslated regions (introns), either upstream (3′) or downstream (5′) of the translated region. In many genes, they are found in pairs, ordered head to head or tail to tail, separated by a short intervening sequence. Once transcribed, the Alu sequences can pair up, forming a stretch of double-stranded RNA that makes an ideal target for the A-to-I RNA editing machinery, called ADAR (adenosine deaminase acting on RNA).

A typical gene contains between one and two dozen Alu sequences. Based on this and the frequency of editing found when analyzing more than 100,000 mRNAs in the human transcriptome, Athanasiadis, Rich, and Maas estimate that the probability that any particular mRNA undergoes A-to-I editing is between 85% and 95%.

While the bulk of edited Alu sites are in introns, a small fraction of them are in exons. Here they can lead to alternative forms of the same protein, expressed in different cell types or at different times; this appears to be especially common in the nervous system. Alu editing can also convert introns to exons, and vice versa, through creation or destruction of splice sites. It is possible A-to-I editing may be used to reduce the creation of deleterious new exons, although more work will be needed to explore this possibility, as well as what role, if any, A-to-I editing plays in promoting new exon creation.

Athanasiadis A, Rich A, Maas S (2004) Widespread A-to-I RNA editing of Alu-containing mRNAs in the human transcriptome. DOI: 10.1371/journal. pbio.0020391

Computational Mapping of Complex Traits in the Mouse

The past few years have seen an explosion of data on the mouse genome, widely hailed as a guidebook to the genetic origins of human disease. Scientists are particularly interested in charting the location of SNPs—single nucleotide polymorphisms—throughout the genome. SNPs are DNA sequence variations, or mutations, that change a single nucleotide in the genome, replacing a cytosine (C) base, say, with thymine (T). Many SNPs, even those found within genes, have no functional effect. Others, however, can increase risk of specific diseases or alter a person's response to pathogens and drugs. Whether or not they are involved directly in a disease, SNPs are attractive markers for population studies aimed at identifying the multiple genes underlying complex diseases like diabetes and cancer.

Extensive data exist on multiple inbred strains of mice linking their genetic makeup (genotype) to physical traits (phenotype), and scientists have used these data to guide investigations of gene function and disease. Many data have been gathered by crossing mouse strains and painstakingly analyzing their progeny, to tease out the relative contributions different genes make to pathogenesis. But these efforts take time. Investigators would greatly benefit from high-throughput methods to scan the mouse genome and flag markers for candidate disease genes. In 2001, Andrew Grupe et al. introduced an "in silico" (computational) approach to do that very thing. The method scanned mouse SNP data to home in on chromosomal areas regulating complex traits and reduce the time needed to analyze mouse disease models from "many months" to "milliseconds."

For a number of reasons, however, it wasn't clear whether in silico mapping could deliver on its promise. For one thing, the density of SNP maps was sufficient to provide meaningful markers for only a few mouse strains, and phenotype information was lacking for many strains. In a new study, Tim Wiltshire and colleagues have addressed these limitations by mapping nearly 11,000 SNP probes to 48 mouse strains. They have also been able to use this dataset for in silico mapping to predict genomic regions with functionally important phenotypes.

Wiltshire and colleagues first show that their method can predict the genomic location of a Mendelian trait

In silico mapping for mouse genetics

(controlled by a single gene), in this case coat color, which the authors acknowledge is a "minimum requirement for a viable in silico mapping method." They go on to map complex "quantitative" traits (controlled by differential contributions from multiple genes at what are called quantitative trait loci, or QTLs)—gallstone development and plasma levels of high-density lipoprotein cholesterol—and find that their predictions fall in line with loci identified by traditional mouse disease studies. Noting a high correlation between QTLs predicted in silico and those identified experimentally, the authors argue that loci predicted using this method are "very likely to be biologically relevant."

Wiltshire and colleagues are careful to point out that in silico mapping is meant to complement, not replace, traditional gene mapping models. After all, computers are no match for living organisms in modeling the subtleties inherent in biological reactions. But this approach is a good starting point for identifying significant genomic areas in a new strain. And as new strains are genotyped and phenotyped, and refinements are made to the SNP database, the robustness of this method should only get better.

Pletcher MT, McClurg P, Batalov S, Su AI, Barnes W, et al. (2004) Use of a dense single nucleotide polymorphism map for in silico mapping in the mouse. DOI: 10.1371/journal.pbio.0020393

All HEPped Up about Methylation

DOI: 10.1371/journal.pbio.0020433

For a recipe to become a meal, it's often necessary to embellish or modify the basic instructions—and to keep a note of the changes that work, so that it can be just as delicious next time around. The same is true for a gene, whose basic recipe—its nucleotide sequence—can be heritably annotated to "epigenetically" influence its level of expression without altering its sequence. Among the many epigenetic influences at work in the genome, methylation of cytosine is one of the most versatile and powerful. Addition of a methyl (-CH$_3$) group to cytosines within a gene's regulatory regions can reduce its transcription. In its extreme form, methylation is involved in silencing one of the two X chromosomes in female mammals. Aberrant methylation underlies susceptibilities to several forms of cancer, and is likely to be involved in numerous other human diseases.

The goal of the Human Epigenome Project (HEP) is to map the methylation patterns of human genes, and to determine how they vary: among individuals, among tissues within an individual, and even over time within a single tissue. In this issue, Stephan Beck and colleagues describe the execution and results of a HEP pilot project, in which they analyzed methylation within the major histocompatibility complex (MHC), the set of genes that establish an individual's self-identity within the context of immune surveillance.

The key to any such large-scale project is high throughput—a rapid, efficient set of technologies that produce the needed data with minimal human intervention. The strategy used by Rakyan et al. included bisulfite sequencing of DNA, in which unmethylated cytosines are chemically converted to uracils, while methylated cytosines are not. Software they developed detects the methylated sites and provides an overall measure of the methylation level within any given sequence. They confirmed the accuracy of their method with mass spectrometry, an alternative method also suitable for high-throughput screening.

They initially analyzed 253 sequences within 90 genes in the MHC, about two-thirds of the total, from multiple tissues in multiple individuals. They found that most genes were either completely methylated or completely unmethylated, while relatively few had an intermediate value. The significance of this distribution pattern is not yet clear, although it does confirm similar results in smaller samples from other research groups. The
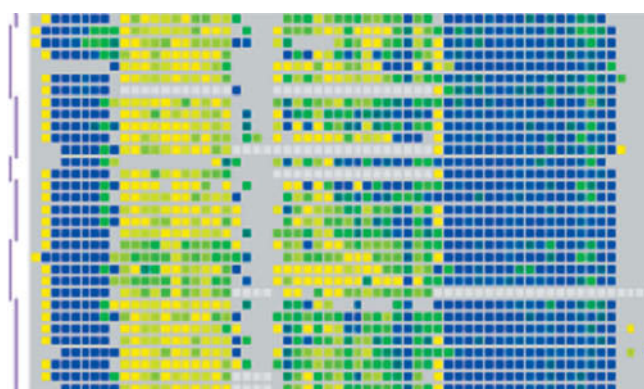
DOI: 10.1371/journal.pbio.0020433.g001

Methylation levels in a region of the human genome

researchers also confirmed that so-called CpG islands, regions rich in CG dinucleotides, are relatively hypomethylated, especially when they occur at the upstream end of a gene.

Rakyan et al. also found differences in methylation levels among tissues, with some suggestion that the variations influence tissue-specific alternative splicing, at least in some genes. Intriguing inter-individual differences were also found, with median methylation levels differing significantly between individuals for at least one tissue at almost half the sites analyzed. For instance, such differences were found in liver for the regulatory region for the tumor necrosis factor gene.

A major goal of the HEP is to identify methylation variable positions, sites whose methylation state is linked with some important biological state, be it tissue type, developmental stage, or disease state. The pilot project described here begins this undertaking, which will be greatly expanded as the HEP progresses. The first phase of the full-scale HEP, an analysis of 5,000 DNA sequences, is currently underway.

Rakyan VK, Hildmann T, Novik KL, Lewin J, Tost J, et al. (2004) DNA methylation profiling of the human major histocompatibility complex: A pilot study for the human epigenome project. DOI: 10.1371/journal.pbio.0020405

Retroviral Gene Vectors Show Clear Target Preferences

DOI: 10.1371/journal.pbio.0020443

Despite some high-profile setbacks in gene therapy over the past few years, scientists have not lost hope that targeted gene transfer will one day treat a wide range of acquired and congenital diseases. After two young gene therapy patients developed a leukemia-like disorder last year— apparently because the viral vector used to carry the corrective gene activated a cancer-causing gene—researchers redoubled their efforts to develop safer, more effective retroviral gene delivery methods. Such efforts depend on

understanding how and where retroviral vectors integrate into the genome.

In a new study, Cynthia Dunbar and colleagues describe a nonhuman primate model that mimics gene therapy protocols in humans, and report the integration biases of two classes of retroviral vectors being developed for clinical trials. The retroviruses, the authors show, display clear preferences that not only suggest different genomic integration mechanisms but also have different implications for safety.

Retroviral gene therapy exploits

a retrovirus's skill at entering a cell, infiltrating its genome, and hijacking its molecular machinery for its own reproductive advantage. In gene therapy, therapeutic genes largely take the place of viral genes, and so the "infected" cell churns out beneficial gene products, not viruses. Before a retrovirus can integrate into a host cell, however, it must copy its genome—which is encoded in RNA— into DNA, so the cell's copying machinery will recognize it. After generating this "pre-integration complex," the virus must access the cell's chromosomal

DNA, which lies behind a nuclear barrier. Different retroviruses accomplish this task in different ways.

Lentiviruses—which include AIDS and SIV (simian immunodeficiency virus)—can infect nondividing cells simply by slipping through nuclear pores. Oncoretroviruses—such as murine leukemia virus, or MLV, the vector type used for the vast majority of previous clinical trials, including the trial complicated by leukemia in two patients—must wait until the nuclear membrane dissolves during cell division. Once integrated into the host genome, the provirus—and its therapeutic gene—will persist through each new cell division—a trait that underlies its usefulness as a vector as well as its risk. Retroviruses that insert near proto-oncogenes can activate these genes and set the cell on the path to tumorigenesis. Until recently, researchers assumed this risk was extremely low because retroviral integration was thought to be random—an assumption recently undercut by a number of studies that mapped retroviral integration in different cell lines.

Dunbar and colleagues take these studies a step further by mapping the integration patterns of MLV and SIV vectors in hematopoietic stem cells (HSCs) of rhesus monkeys. HSCs are the cells typically used to carry these vectors for therapeutic applications involving any of the cell types, such as red blood cells, produced by the bone marrow. The monkeys had received infusions of HSCs carrying either the SIV or MLV vectors between six months and six years earlier. Two types of white blood cells (granulocytes and mononuclear cells) were harvested from the monkeys and evaluated for proviral insertion sites.

Of nearly 1,000 integration sites identified, 760 could be mapped to unique corresponding sites in the human genome (432 MLV and 328 SIV). While both MLV and SIV vectors tended to integrate within genes, MLV showed a strong preference for the starting end of genes, most likely to result in gene activation. In contrast, SIV showed a preference for genomic regions of high gene density, but not for specific sites within a gene. Surprisingly, MLV targeted one gene—known previously to be involved in spontaneous leukemias and in murine retroviral oncogenesis—seven times, a "highly nonrandom" result suggesting that such insertions may occur far more often than previously thought. About 40 genes, including seven known oncogenes, were targeted more than once by one or both vectors. Such differences, Dunbar and colleagues note, "likely reflect the vectors' distinct mechanisms for accessing DNA and integrating," which could in turn affect their risk of causing insertional mutagenesis. Even though the vectors tend to integrate nonrandomly and can target oncogenes, however, none of the monkeys showed signs of ill effects such as leukemia.

But before any widespread applications of retroviral gene therapy can proceed, Dunbar and colleagues argue, potential risks of proviral insertion must be assessed in the specific cell types associated with different gene therapies. And with a model for long-term, genome-wide retroviral integration analysis that mimics human gene therapy protocols, the authors have made an important contribution toward that end.

## A Global View of Gene Expression in the Aging Kidney

Four years ago in *Science*, Stuart Kim, a Stanford developmental biologist, made the case for laying down the broad strokes of a complex physiological process before defining its mechanisms. "A powerful, top-down, holistic approach," he wrote, "is to identify all of the components of a particular cellular process, so that one can define the global picture first and then use it as a framework to understand how the individual components of the process fit together." To get a broad view of gene expression in the aging nematode, Kim's lab turned to DNA microarrays and functional genomics. In a new study, Kim and colleagues apply this same approach to the decidedly more complex problem of human aging and "present a molecular portrait" of the aging kidney.

Scientists have identified a wide range of molecular pathways and mechanisms associated with aging. Many have been found in evolutionarily distant organisms, suggesting they have been conserved and could shed light on human aging. Yet other studies suggest that since few animals reach old age in the wild, any aging-related physiological changes aren't likely to impact the fitness of a population and so aren't likely to be conserved. Consequently, aging pathways in worms, for example, would have little bearing on humans. To investigate the molecular pathways associated with human aging, the authors focused on human tissue—in this case, the kidney.

Kidneys came from 74 patients, ranging in age from 27 to 92. Samples were extracted from donated kidneys or "meticulously harvested" from kidneys with localized disease to ensure only normal tissue was taken. Two structures that are critical to kidney function (removing toxins from
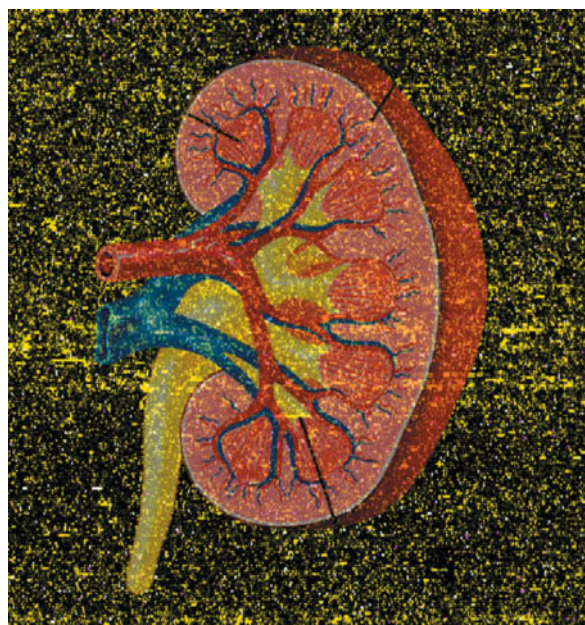
DOI: 10.1371/journal.pbio.0020451.g001

Transcriptional profiling to study aging in the kidney

blood) were removed from each sample: the renal cortex, which filters plasma, and the medulla, which alters urine composition to maintain fluid balance. Both deteriorate with age. An extensive clinical history was noted for each sample to account for any potentially confounding medical factors.

Kim and colleagues then isolated RNA transcripts from the samples to determine the activity of every gene, broken down by age and kidney section, through microarray analysis. Looking for differences in gene expression across the genome, they identified genes that showed a statistically significant change in expression as a function of age. Of 33,000 known human genes on the microarray, 985 showed age-related changes, most showing increased activity. These changes are truly age-regulated, the authors conclude, since none of the medical factors impacted the observed changes in gene expression.

Although cortex and medulla have different cell types and perform different functions, their genetic aging profile was very similar, suggesting a common aging mechanism operates in both structures. In fact, these mechanisms may function broadly, as most of the age-regulated kidney genes were also active in a wide range of human tissues. Other organisms appear to lack these changes, however, prompting the authors to argue that understanding aging in humans will require human subjects.

Most importantly, the genetic profile of the tissue samples correlated with the physiological and morphological decline of an aging kidney. An 81-year-old patient with an unusually healthy kidney had a molecular profile typical of someone much younger, while a 78-year-old with a damaged kidney had the profile of a much older person. Using the power of functional genomics, this study has identified a set of genes that can serve as molecular markers for various stages of a deteriorating kidney and predict the relative health of a patient compared to their age group. These gene sets can also serve as probes to shed light on the molecular pathways at work in the aging kidney, and possibly on the process of aging itself.

Rodwell GEJ, Sonu R, Zahn JM, Lund J, Wilhelmy J, et al. (2004) A transcriptional profile of aging in the human kidney. DOI: 10.1371/journal.pbio.0020427

Genes, Genomes, and the Road to Diversity: How Regulatory Networks Evolve

Evolutionary biologists have long been interested in understanding the molecular basis for the great diversity in size, shape, and behavior seen in life on earth. Recent attention has focused on the role that gene expression changes play in organismal evolution. Tracing the evolution of gene regulation, however, has proved difficult. This is in large part due to the difficulty in identifying and comparing the regulatory elements that control gene expression in different species.

Gene expression depends on *cis*-regulatory elements, short sequence motifs embedded in the DNA that flank a gene's coding region. Regulatory proteins bind to specific *cis*-regulatory sequences, and command the activation or repression of the corresponding gene. The challenge in studying the evolution of *cis*-regulatory elements lies in identifying those elements in multiple species. Unlike protein sequences, which are typically a few hundred amino acids long and relatively straightforward to identify in related organisms, *cis*-regulatory elements are often short and can have variations in sequence. This makes it very difficult to distinguish the regulatory elements from the nonfunctional DNA that surrounds them. It is even harder to identify corresponding regulatory elements across species. As the evolutionary distance between species increases, so, too, does the difficulty in identifying corresponding cis-elements in those species.

In this issue of *PLoS Biology*, Audrey Gasch and her colleagues describe a comparative genomics approach that allows them to identify potential *cis*-regulatory elements in thousands of genes across 14 ascomycete fungi whose diversity represents the effects of several hundred million years of evolution. Ascomycetes are a large class of fungi with extremely diverse morphologies, reproductive strategies, and habitats. A divergence dating back 500 million to 1 billion
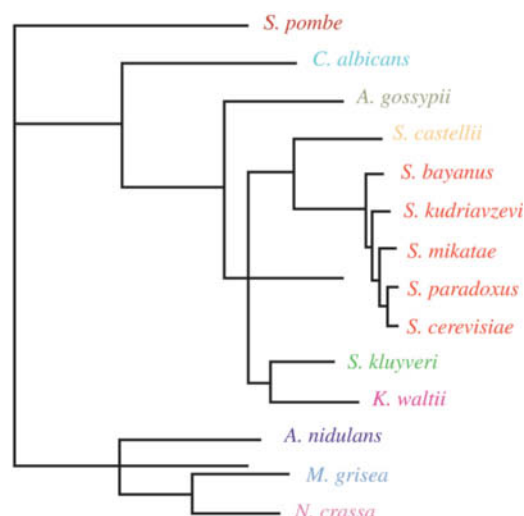
Phylogeny of fungi used to study evolution of gene regulation

years ago gave rise to three groups: Archaeascomycetes, Euascomycetes, and Hemiascomycetes. The genome of the brewer's yeast, *Saccharomyces cerevisiae*, a hemiascomycete, was completely sequenced in 1995, and that of fission yeast, *Schizosaccharomyces pombe*, an archaeascomycete, in 2002. Since that time, complete genome sequences have been released for more than nine additional hemiascomycetes and three euascomycetes. This gives the authors an opportunity to compare regulatory systems among progressively more distantly related species, on a genomic scale.

Genome-wide expression studies in the yeast *S. cerevisiae* have revealed groups of genes whose expression levels vary simultaneously under varying experimental conditions. Such co-regulated genes, the authors reasoned, must harbor common regulatory elements that coordinate their response to experimental triggers. Gasch and colleagues looked for such *cis*-elements and found 35 groups of co-regulated *S. cerevisiae* genes with at least one shared *cis*-element. The authors then argued that co-regulation may reflect selection pressures that also apply to other ascomycetes, and so they identified the equivalent of the 35 co-regulated gene groups in each of the 13 other species.

They then looked for shared *cis*-elements within each group and in each species independently, and compared the regulatory systems across the species.

The results of this study show that the majority of *cis*-elements first identified in yeast are retained in the equivalent gene groups in other species, in a manner that reflects the species' evolutionary distance from yeast. One *cis*-element, in a group of co-regulated genes that control the cell cycle, is found all the way from budding yeast to fission yeast, suggesting a selection pressure on the co-regulation of these genes that has withstood greater than 500 million to 1 billion years of evolution.

In contrast, there were other examples in which the same gene groups contained different putative *cis*-elements in each species, suggesting that the regulation of those genes has evolved. In the case of *cis*-elements found in genes controlling protein degradation, a related element was identified in all of the hemiascomycetes, whereas the euascomycetes appear to have adopted a novel *cis*-element for this gene group. Interestingly, the hemiascomycete element displays a sequence variation in *Candida albicans* that is not found in *S. cerevisiae*. The two species diverged 200 million years ago. Gasch and colleagues showed that the protein that binds to the hemiascomycete element has evolved to have slightly different DNA interactions in the two species, allowing the *C. albicans* protein to bind the novel sequence found only in the *C. albicans* genes. This provides evidence for co-evolution between a transcription factor and its target *cis*-element.

Overall, this analysis has uncovered striking cases of conservation and innovation of gene regulatory systems, and therefore provides important insight into the evolutionary forces that have shaped the evolution of gene regulation.

Gasch AP, Moses AM, Chiang DY, Fraser HB, Berardini M, et al. (2004) Conservation and evolution of *cis*-regulatory systems in Ascomycete fungi. DOI: 10.1371/journal. pbio.0020398

In Times of Stress, Mutate Early and Often

For a human, the normal response to stress is to reduce it through some purposeful action, be it indulging in chocolate or calling in sick, at a rate which we can vary to fit the circumstances. For a strain of bacteria faced with stress, the choice is often more stark: it must mutate or die. Among evolutionary theorists, an important question has been whether the rate of mutation is fixed, or instead can adaptively increase in response to stress, thereby increasing the likelihood of a favorable mutation. Something like this latter possibility was envisioned by Darwin, but fell out of favor among some neo-Darwinists, for whom a steady rate of mutation was more in keeping with their overall model of evolutionary gradualism. This debate is taken up in a new study in this issue by P. J. Hastings and colleagues, who examined the mechanism by which *Escherichia coli* lacking the ability to digest lactose, called *lac−* mutants, regain that ability when presented with lactose as their only food source.

It has been known for some time that the reversion of *lac−* mutants to a *lac+* state can be achieved by either of two genetic events: amplification, which creates numerous copies of the nonfunctional *lac* gene, and point mutations, which give rise to functional versions of the gene (many non-useful mutations also occur; thus, there is no directed mutation, in keeping with standard Darwinian evolution). According to the gradualist view, amplification precedes mutation, and the rapid appearance of *lac+* cells is explained by a normal mutation rate acting on multiple copies of the gene. In contrast, according to the hypermutation view, amplification and mutation are independent events, and *lac+* cells arise quickly because the mutation rate has increased.

While some results from previous studies have supported the gradualist interpretation, the experiments of Hastings et al. show that hypermutation is the most plausible explanation. A variety of procedural improvements allowed them to analyze more individual cells at an earlier stage of colony development. For instance, they analyzed colonies composed of as few as one hundred cells, rather than the ten thousand cells in prior
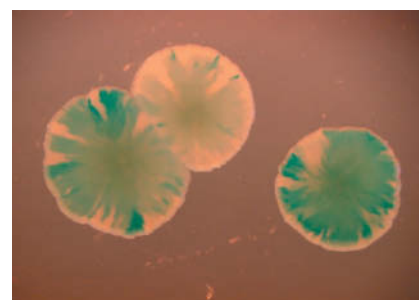
Sectored colonies of *lac+* and *lac− E. coli*

experiments, and even nascent colonies at the two-cell stage.

The study produced clear evidence that point mutations arise very early in the development of *lac+* colonies, before amplification can account for the number of *lac+* revertants observed. Amplification is not only independent from mutation, but occurs relatively late under starvation. The researchers found that amplification, but not point mutation, requires the presence of a particular DNA polymerase, further strengthening the case that amplification need not precede mutation. They also showed that amplification by itself does not induce a so-called SOS response. The SOS system includes a group of genes that cause an increase in mutation in response to stress, and one hypothesis arising from the gradualist model was that amplification turned on the SOS response.

Based on their data, the authors reject the strict gradualist model for the adaptive mutation mechanism in the Lac system. They propose that amplification and hypermutation are independent responses to stress, each of which increases the likelihood of adaptive change. They also suggest that a stress-induced increase in the rate of point mutations may have implications for a variety of mutation-related phenomena, from tumor formation to development of resistance to antibiotics and chemotherapeutic drugs.

Hastings PJ, Slack A, Petrosino JF, Rosenberg SM (2004) Adaptive amplification and point mutation are independent mechanisms: Evidence for various stress-inducible mutation mechanisms. DOI: 10.1371/journal. pbio.0020399

The History of the Intron—Balancing Gains and Losses

In the 25 years since they were first discovered, introns have puzzled molecular biologists because of their uncertain function and mysterious origin. Introns are non-coding DNA sequences that reside inside a gene, splitting it into discrete units called exons. The resulting disruption of coding sequence continuity would wreak havoc in protein assembly if eukaryotic cells did not dispose of introns in messenger RNAs—the intermediates in the decoding of gene sequences to produce protein chains—in a now well-described process known as splicing.

At first glance, introns may seem like pesky parasites for which eukaryotes have cleverly evolved bypass mechanisms. But introns may also benefit their hosts. Evolutionary advantages of introns include the possibility to create new genes by cutting and pasting exons from existing genes or to diversify the protein output of a single gene by splicing the exons together in different ways. Thus, balancing intron gains and losses clearly has important evolutionary implications for a host.

Yet different organisms strike that balance differently. The budding yeast *Saccharomyces cerevisiae* averages less than one intron per gene, whereas mammalian genes routinely have 10 or more. Whether these differences reflect different propensities for gaining or losing introns is the subject of ongoing debates.

Organisms with low intron density display a bias for insertions at the beginning (5' end) rather than the end (3' end) of genes. A popular hypothesis is that in these organisms, genes lose their introns through a process that rewrites genomic DNA using as template the messenger RNAs purged of intron sequences. This process might preferentially remove 3' introns because it relies on an enzyme called reverse transcriptase that can be primed to read RNAs starting at their 3' end. The hypothesis has gained experimental support in yeast. It also presents the advantage of potentially explaining intron paucity and 5' position bias in one stroke. In a new study, Cydney Nielsen and her colleagues present evidence that challenges this model.

They address intron dynamics with a genome-wide survey of intron distribution among four Ascomycete fungi with recently completed genome sequences. The four fungi (*Neurospora crassa, Magnoporthe grisea, Fusarium gramineum,* and *Aspergillus nidulans*) form an evolutionary tree with branching points estimated at 200, 230, and 330 million years ago. While they diverged from yeast some 500 million years ago, they share with yeast a low intron density (one to two introns per gene) and a 5' position bias. The authors' approach is to tally intron gains and losses during the evolution of these four species and then plot their positions along the genes' length.

They identify 3,450 gene regions that are clearly conserved in all four species and harbor an intron in at least one of them. To distinguish intron gains from losses, they rely on a simple parsimony principle, which they refine with additional probability analyses. In brief, an intron present in only one species counts as a gain; an intron absent from one species but present in its closest relative and in a cousin counts as a loss.

Nielsen and colleagues record between 150 and 350 intron losses in each lineage. Surprisingly, losses do not occur preferentially at the genes' 3' end. The authors conclude that while a 3' reverse transcriptase-based mechanism might be a factor, it cannot be the sole reason for the introns' 5' bias. The other surprising result is that intron gains occur at almost the same rate as losses in all lineages. Intron gains therefore play an important role in the evolution of even intron-poor genomes. Clearly, intron distribution in fungi owes to forces more complex than simple 3' intron elimination, forces that the authors propose may also shape evolution of other eukaryotic genomes.

Nielsen CB, Friedman B, Birren B, Burge CB, Galagan JE (2004) Patterns of intron gain and loss in fungi. DOI: 10.1371/journal. pbio.0020422

# Fungus Holds Clues to the Evolution of Sex Chromosomes

It's a basic biological principle that living things share certain fundamental traits. That's why understanding the mechanisms of cell division in single-celled yeast, say, can offer insight into cell division in humans. Now Joseph Heitman and colleagues report that the evolutionary events that spawned sex chromosomes in yeast resemble those that shaped sex chromosomes in animals.

Strictly speaking, yeast—the common name for single-celled fungi—don't have sex chromosomes; they have sex-determining regions within chromosomes, called mating type, or MAT, loci. In a comparative genomic analysis of the MAT locus in three species of the human pathogenic fungus *Cryptococcus*, Heitman and colleagues found that this fungal sex-determining region arose via a series of discrete events that echo those that shaped mammalian sex chromosomes.

A primary benefit of sexual reproduction is the genetic diversity gained from reshuffling genetic material during meiosis, which creates gametes. Yeast sex, such as it is, accomplishes the same thing. Of course, sexual identity for a fungus does not take the form of sperm or egg but of mating type **a**, for example, and mating type alpha. Still, yeast manage a measure of complexity and considerable elegance in the systems they deploy to sexually reproduce.

In ascomycetes, like baker's yeast, the MAT locus is small and includes just a few genes. The genes that determine a cell's **a**
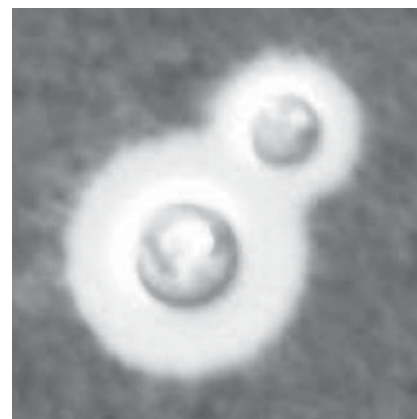
Human pathogenic fungus *Cryptococcus*

or alpha mating status are alleles (variants) of a single MAT locus. Cells with the MAT**a** allele are mating type **a**, while cells with the MATalpha allele exhibit mating type alpha. A cell can switch its mating type when genetic exchange, or recombination, between two mating loci occurs.

In basidiomycetes, like the corn smut *Ustilago maydis*—a maize pathogen that some consider a culinary delicacy—mating is more complex, and sexual identity is determined by two unlinked genomic regions with distinct classes of genes. Cells must be of different mating types at both loci to allow sexual reproduction. To their surprise, Heitman and colleagues discovered that the mating locus of *Cryptococcus neoformans*—a basidiomycete fungus that infects humans and is associated with transplant recipients, patients with AIDS, and other immune-compromised patients—exhibits several unique features, common to neither ascomycetes or their basidiomycete relatives.

Unlike most basidiomycetes, the *C. neoformans* locus occupies a single region and is unusually large, spanning more than 100 kilobases and containing over 20 genes, including those typically segregated in separate locations in other basidiomycetes. Like on the human Y chromosome, the sex-determining genes of *C. neoformans* are interspersed with non-sex-related genes. And unlike ascomycetes, which also have a single active MAT locus and two mating types, no mating type switching occurs as there are no silent mating type cassettes in the genome.

Heitman and colleagues sequenced the **a** and alpha alleles of *C. neoformans'* closest relative, *C. gattii*, and compared these variants to four already characterized variants derived from two *C. neoformans* subspecies. All six MAT alleles share characteristic features, including a fairly large size, a common gene set, and dramatic genomic migration during evolution (which is unusual compared to other genomic regions in the three strains). Each MAT allele has genes with different evolutionary histories, ranging from ancient to recent, that fall into distinct patterns based on shared nucleotide composition and mating type. The patterns correlate with how long the genes have occupied the MAT locus, suggesting how it evolved.

The authors hypothesize that this novel structure was formed by chromosomal rearrangements that linked two unrelated genomic regions into a single region. Recombination between these sex-determining regions was suppressed after other events blurred their boundaries. Specific genes in the once separated loci then attracted mobile elements in the genome to their sites, thus precipitating expansion of the locus. Because the *Cryptococcus* MAT locus resembles the evolution and structure proposed for the ancient Y chromosome, the authors argue that *Cryptococcus* can serve as a valuable model to study the molecular dynamics of sex chromosomes.

Fraser JA, Diezmann S, Subaran RL, Allen A, Lengeler KB, et al. (2004) Convergent evolution of chromosomal sex-determining regions in the animal and fungal kingdoms. DOI: 10.1371/journal.pbio.0020384

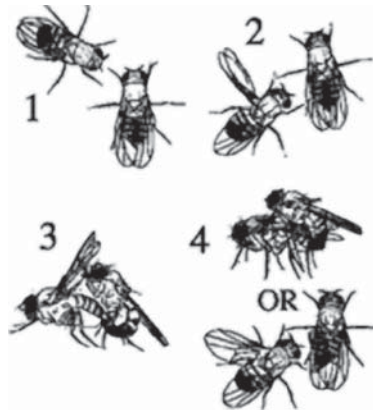# Getting a Whiff of Speciation by Reinforcement

Creating a new species is a bit like climbing a greased flagpole—it's hard to get started and even harder to keep going. Random genetic variations may introduce slight differences between two groups but, without some means to keep them apart, sexual interbreeding will quickly remix the genes and obliterate the differences. Accidents of geography—the rising of mountains or a course-changing river, for instance—can provide physical isolation, which then enables genetic divergence through the accumulation of mutations, either through natural selection or genetic drift.

In contrast, speciation without geographic separation relies on the direct action of natural selection to complete the speciation process by strengthening behavioral differences, a process called reinforcement. One of the most powerful means of completing speciation is through the evolution of mate discrimination.

A study by Daniel Ortiz-Barrientos and colleagues focuses on the genetic underpinnings of mate discrimination in *Drosophila*. They identify two loci that influence the likelihood that a female will choose to mate with a conspecific male, rather than one of a closely related species.

Drosophila pseudoobscura and *D. persimilis* exist together along the west coast of the United States (sympatry), but separately elsewhere (allopatry). When together, they hybridize and produce sterile males. While *D. pseudoobscura* males will court females of both species, females prefer conspecific males. This female preference is stronger in sympatric females, an enhancement that presumably evolved by the direct action of natural selection to prevent females from wasting their reproductive efforts producing sterile sons. This variation allowed the authors to conduct a series of genetic crosses among flies of the same species but from different locations. Because the daughters of discriminating *D. pseudoobscura* females were just as discriminating as their mothers, Ortiz-Barrientos and colleagues concluded that female mating discrimination was inherited as a dominant trait. Further crosses showed that genes responsible for female preference were on the X and fourth chromosomes, and high-resolution mapping refined their locations sufficiently to allow the identification of likely candidate genes. While more work remains to be done, the most promising genes in both regions appear to be involved with olfaction. The fact that one of them, *CG13982*, is known to up-regulate the other, *bru-3*, strengthens the case that these are indeed promising candidates.

***Drosophila* choosing their mates**

According to their findings, the authors propose a novel model of mating discrimination in *D. pseudoobscura* based on the combined response to auditory and olfactory cues. The first of these two layers, weak, or "basal" mating discrimination, has previously been associated with a set of traits for acoustic recognition and mapped to chromosomal regions that are inverted between the two species. Such inversions prevent recombination from purging alleles, thereby contributing to hybrid male sterility. As a consequence, when these species interbreed, they inexorably produce sterile males. The second layer, elucidated in the current study, is "reinforced" mating discrimination, which appears to be related to olfactory cues. This additional system of discrimination helps the first layer to fully eliminate the inevitable cost of producing sterile males. Once the nascent species have started up that slippery pole, reinforced discrimination could provide the traction needed to reach its top.

Ortiz-Barrientos D, Counterman BA, Noor MAF (2004) The genetics of speciation by reinforcement. DOI: 10.1371/journal.pbio.0020416

Cro-Magnons Conquered Europe, but Left Neanderthals Alone

After miners unearthed a skull and bones in a Neander Valley cave in Germany in 1856—three years before the publication of *On the Origin of Species*—the remains were initially described as either those of a "brutish" race or of someone disfigured by disease. As Darwinian evolution caught on, so did the realization that these fossils were evidence of an earlier human species. Scientists have been debating Neanderthal's place in human evolution ever since.

An ongoing question concerns the possibility that Neanderthals and early humans mated, since they likely crossed paths during thousands of years of European cohabitation. In a new study, Mathias Currat and Laurent Excoffier present a simulation model based on what we know about the population density and distribution of Neanderthals and Cro-Magnons. Their results complement recent genetic and morphological evidence indicating that early human and Neanderthal interbreeding was unlikely.

The notion that modern Europeans directly descended from Neanderthals has mostly yielded to two competing models: One postulates that modern humans arose in Africa about 130,000 years ago and completely replaced coexisting archaic forms with no interbreeding, while the other proposes a gradual transition with interbreeding.

Though mounting genetic evidence (based on mitochondrial DNA extracted from fossils) suggests Neanderthals and early humans did not breed, the evidence has been inconclusive. It's possible, for example, that any Neanderthal gene "leakage" could have been lost through genetic drift if the mating populations were small. And because so few fossils are available to analyze, previous studies could rule out only Neanderthal contributions over 25%.

Currat and Excoffier's model refines various parameters—such as geographic boundaries, local population variations, range expansion, and competition for resources—based on archeological and demographic data for both populations. Evidence suggests modern humans replaced Neanderthals over 12,500 years, for example, which constrains the speed at which modern humans could expand.

The authors started with a scenario based on a set of "plausible" parameter values—their basic scenario—and then

Reconstruction of Neanderthal woman

varied the local interbreeding rate and, for example, the population size and location of Cro-Magnons, to produce eight alternate scenarios describing how Cro-Magnon colonization of Europe might have proceeded. They estimated the likely proportion of Neanderthal gene contributions to the modern gene pool using "coalescent simulations," which generate the genealogies and diversity of genes in local populations based on simulations of their population densities and migration histories. The simulations

show that if Neanderthals bred with Cro-Magnons without constraints over thousands of years, Neanderthal contributions to the modern gene pool "would be immense." Surprisingly, the simulations also show that even a very small mixing should lead to high levels of Neanderthal DNA in modern humans.

What could account for this counterintuitive result? Given a low population density with small local breeding populations, any introduction of Neanderthal genes would decrease the frequency of Cro-Magnon genes of that population; if these Neanderthal integrations take place as the Cro-Magnon population is expanding, newly acquired Neanderthal genes would also be amplified.

Since no Neanderthal mitochondrial DNA has been found in modern-day Europeans, the authors modeled the maximum number of interbreeding events that would support this observation. The estimated maximum number of events, it turns out, falls between 34 and 120—extremely low values, Currat and Excoffier conclude, "given the fact that the two populations must have coexisted for more than 12,000 years."

While the authors acknowledge their simulations suggest rather than reflect reality, their model does incorporate real historical data such as Cro-Magnon expansion over time and local population growth. At a value of only 0.1%, their new estimate of the rate of interbreeding is about 400 times lower than previous estimates and provides strong support that Neanderthals and Cro-Magnon didn't interbreed and may even have been different species.

Currat M, Excoffier L (2004) Modern humans did not admix with Neanderthals during their range expansion into Europe. DOI: 10.1371/journal.pbio.0020421

An Evolutionary View of Tiger Conservation

> Tyger! Tyger! burning bright
> In the forests of the night
> What immortal hand or eye
> Could frame thy fearful symmetry?

When William Blake wrote these words in the late 1700s, the deforestation and habitat destruction that would decimate wild tiger populations had already begun. In 1900, an estimated 100,000 wild tigers lived throughout much of Asia, from India in the west to Sumatra and Indonesia in the south to Siberia in the east. Today, the ongoing stresses of habitat loss, hunting, and an illegal trade in tiger parts have spared fewer than 7,000 tigers. Of eight traditionally classified subspecies of *Panthera tigris*, three have gone extinct since the 1940s.

Conservation strategies to combat this grinding attrition are tailored to each subspecies. But several lines of evidence suggest that subspecies designations—based on geographic range and morphological traits such as body size, skull traits, coat color, and striping patterns—may be flawed. An earlier molecular study of 28 tigers found little evidence of genetically distinct subspecies, while surveys of tiger habitat found few physical barriers sufficient for subspecies isolation.

To get a clearer picture of the genetic structure of existing tiger populations, Shu-Jin Luo, Jae-Heup Kim, Stephen O'Brien, and nineteen colleagues performed a comprehensive genetic analysis of mitochondrial and nuclear genes from over 130 tigers. By identifying distinct patterns of variation within these gene families, the authors reconstructed the evolutionary distribution and ancestry of the tiger. Their results support many of the traditional subspecies designations and identify further subdivisions in others.

Luo et al. collected "voucher specimens" (taken from animals of verified wild ancestry and geographic origin) of blood, skin, and hair from 134 tigers representing the entire tiger range, and examined them, along with samples of preserved pelts and hair, for three molecular markers. The markers—a stretch of mitochondrial DNA (mtDNA) sequence, a gene with highly variable DNA sequence called *DRB* that's involved in pathogen recognition, and short repeating genetic elements called microsatellites—act as unique signposts that flag significant demographic and evolutionary events in the tiger populations.

mtDNA sequences were extracted from tigers originating in the Russian far east (Siberian, or Amur, tigers), south China, northern Indochina, the Malaya Peninsula, Sumatra, and the Indian subcontinent. The mtDNA analysis identified 30 haplotypes—characteristic regions on a chromosome—that could be clustered. Some of the clusters supported traditional classifications—e.g., for the Sumatran (*P. t. sumatrae*) and (*P. t. tigris*) Bengal tigers—but others suggested that the Indochinese subspecies (*P. t. corbetti*) should be divided into two groups, representing a northern Indochinese and a peninsular Malaya population (which the authors designated respectively as *P. t. corbetti* and *P. t. jacksoni*, after the tiger conservationist Peter Jackson). Interestingly, clusters for the captive South China tigers also fell into two distinct lineages—*P. t. amoyensis*, the traditional grouping, and *P. t. corbetti*, though the designation is still tentative. These subdivisions were largely supported by the other genetic analyses.

The distinct genetic patterns found in the tiger populations suggest six rather than five living subspecies. Reduced gene flow and genetic drift in isolated populations, as well as human activity, likely caused these partitions. The low genetic variability seen in the Siberian tigers, for example, might be explained by severe population declines: the animals were nearly exterminated in the early 1900s, and today only 500 remain. Sumatran tigers, on the other hand, show relatively high genetic variability and uniqueness, possibly reflecting a historically large breeding population that was later isolated.

Whether recent population and habitat declines, as opposed to earlier events, can fully explain these patterns is not clear. But these results offer valuable data for conservation strategies and captive breeding programs that rely on distinctions in subspecies taxonomy and geographic provenance. Evoking both the darker side of creation and humanity, Blake could not have imagined the modern fate of his "Tyger." Scholars have long debated the multilayered meaning of his poem, including the second stanza, which starts,

> In what distant deeps or skies
> Burnt the fire of thine eyes?

Will we reduce future generations to a literal reading?

Luo SJ, Kim JH, Johnson WE, van der Walt J, Martenson J, et al. (2004) Phylogeography and genetic ancestry of tigers (*Panthera tigris*). DOI: 10.1371/journal.pbio.0020442
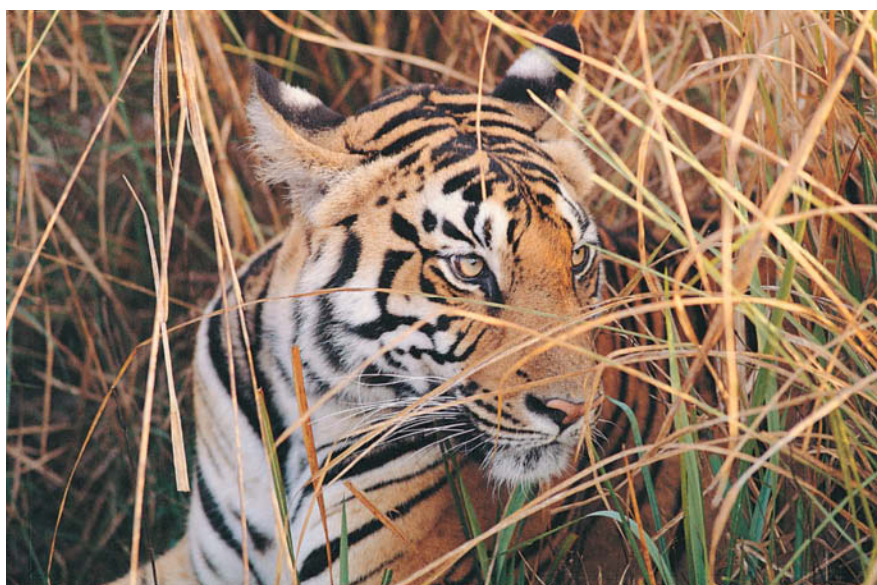
A Bengal tiger in the tall grassland (Photo: Ullas Karanth, WCS-India)

Taking Stock of Biodiversity to Stem Its Rapid Decline

Far more species exist in the fossil record than inhabit Earth today. An estimated 94% of all bird species that ever lived, for example, are now extinct. So why is species extinction of urgent concern today? Though species come and go over evolutionary time, mass extinctions are relatively rare. Biologists believe they have occurred only five times, arising from relatively short-lived cataclysmic natural forces like astronomical or volcanic events. We are now on the brink of the Sixth Great Extinction, and we humans are largely to blame. For thousands of years, humans have retooled the landscape, an endeavor that has rarely coincided with the life history needs of local flora and fauna: over 150 bird species alone have vanished since 1500.

As our capacity to alter the landscape has mushroomed, species have started disappearing faster than biologists can identify and document them. Mindful of this crisis, nearly 200 countries (under the Convention on Biological Diversity, or CBD) agreed to staunch the loss of biodiversity by 2010, with the European Union raising the bar to *halt* biodiversity loss by that time. To meet this goal, biologists need reliable metrics to monitor global trends in biodiversity. Stuart Butchart et al. describe a new model for generating such indices to measure trends in extinction risk for complete classes of organisms, starting with the world's 10,000 bird species. Their "Red List Index" measures changes in overall extinction risk over time for all bird species worldwide. Similar indices are already being developed to track other groups, including mammals and amphibians, and in the future will hopefully be developed for some plant and invertebrate groups.

In 2002, the CBD proposed that efforts to monitor global trends in biodiversity start by developing indicators to evaluate trends in biodiversity components, such as ecosystems and habitats, abundance and distribution of selected species, and change in threat status of species. Butchart et al. focus on evaluating trends in changes in threat status (extinction risk), relying on categories developed by the World Conservation Union (IUCN) Red List. Species are placed in categories on the Red List ranging from extinct to

Rufous-collared Kingfisher: Deforestation threatens its survival (Photo: Jakob Wijkema)

"least concern," according to criteria that take into account their population size, population trends, and range size. Thousands of scientists from around the world feed these assessments, which have been widely used to measure the degree of degradation of biodiversity.

To use the Red List to track biodiversity trends over time, Butchart et al. collected data from four complete assessments of the world's birds over sixteen years, supplemented by other sources. The number of threatened and near threatened species increased from 1,664 species in 1988 to 1,990 species in 2004, but many species moved between categories. To calculate the real net increase in extinction risk for the world's birds over this time, the authors first identified reasons for these category switches to remove biases introduced by factors irrelevant to genuine changes in species status; category changes owing to better knowledge, for example, do not reflect real changes in conservation status. They also accounted for time lags between status changes, and category changes owing to delays in knowledge becoming available to Red List assessors.

The authors argue that the Red List Index provides a simple measure of trends in the status of avian species worldwide, in terms of their overall extinction risk. Overall, the index shows "a steady and continuing deterioration in the threat status of the world's birds between 1988

and 2004." Though the extinction risk has improved for some species, it has deteriorated for others, with "particularly steep declines" in recent years for Asian birds—resulting from massive deforestation in Indonesia—and for seabirds such as albatrosses and petrels, which drown on the hooks of commercial long-line fisheries.

Butchart et al. argue that Red List Indices complement indicators based on population trends, because although the indices show coarser temporal resolution, they have much better geographic representation; they're based on nearly all species in a group worldwide rather than on a potentially biased subset. Both types of species-based indicators show finer ecological resolution in tracking biodiversity loss than indicators like habitat or biome trends. Thus, the Red List Index provides a baseline for tracking progress toward the 2010 target. But having a reliable indicator is only the first step. Without an international commitment to halt the advancing extinction crisis, biodiversity will continue to decrease. The United States is the only industrialized nation that has not signed on to this effort.

Butchart SHM, Stattersfield AJ, Bennun LA, Shutes SM, Akçakaya HR, et al. (2004) Measuring global trends in the status of biodiversity: Red List Indices for birds. DOI: 10.1371/journal.pbio.0020383

Are Animals As Irrational As Humans?

Animals in the wild are constantly confronted with decisions: Where to nest? Who to mate? Where's the best forage? To explore the mechanisms underlying such decisions, animal behavior studies often incorporate concepts from economic theory. Mainstream models of choice in both economics and biology predict that preferences will be rational, or consistent across contexts, as a result of being motivated by self interest or, in the case of animals, reproductive success. Yet many studies report that when making decisions people often take shortcuts, using cognitive heuristics that may lead to economically irrational decisions, with similar claims now showing up in animal behavior studies.

In a new study, Cynthia Schuck-Paim, Lorena Pompilio, and Alex Kacelnik ask whether studies applying economic rationality to animal behavior are controlling for potentially confounding effects inherent in such approaches. The authors suggest that observed "breaches of rationality" may stem from differences in the physiological state of animals "unwittingly imposed" by experimental design rather than from real irrational decisions.

Choice studies typically offer subjects a range of choices that include clearly superior and inferior alternatives. While humans can simply hear about the various alternatives and their respective properties, animals must be trained to learn about the different choices. This difference is far from trivial, Schuck-Paim et al. argue, and could well require different interpretations of results in animal and human studies. In fact, economic theory states that optimal choices depend on both the properties of the option and the chooser's state. Training animals to learn of different choices typically involves giving them food rewards, which means that an animal's energetic state—that is,

hungry versus sated—will change over a day of training. A bird that's eaten an ounce of birdseed is more likely to opt for an "irrational" option—say, a choice that dispenses little food—than one that's hungry.

To examine this theoretical constraint under experimental conditions, Schuck-Paim et al. trained European starlings to choose between two rich food sources (called focal options) and one of two poorer "decoys" in different contexts. One of the focal options offered more food while the other offered a shorter delay between pecking a key and receiving the
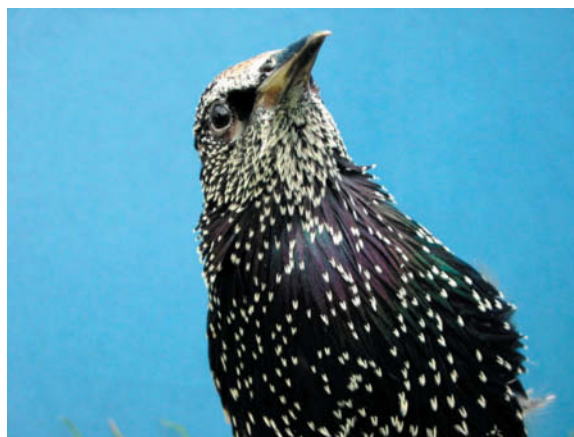
European starlings make rational decisions

food, but their amount/delay ratios were equal. The decoys were considered less preferable because their ratio of amount to delay was lower than that of the focal options. But the decoys could potentially confound the results because repeated training to each decoy could sate a bird's appetite to different degrees: although amount/delay was equal among the decoys, their long term energetic consequences differed.

The authors tested for preference between the focal options under three experimental conditions: altering the birds' food intake/energetic state with no decoys; changing the decoy and

not controlling for its corresponding energetic contributions; and changing the decoy but controlling for its energetic consequence (by supplemental feeding).

Schuck-Paim and collaborators show that the birds' preferences between the focal options differed significantly between treatments, in apparent breach of economic rationality; the preference for the larger reward option over the shorter delay option was much stronger when the trial involved lower accumulated intake than when the accumulated intake was high. Introducing the decoys resulted in an "irrational" preference only when the decoys were allowed to have an effect on food intake, suggesting that the choice resulted from the birds' energetic state rather than from cognitive mechanisms of choice similar to those used to explain irrationality in human subjects.

The authors offer an evolutionary and mechanistic explanation for why animal preference might be governed by energetic state, including the possibility that animals are less motivated to focus exclusively on the richest option when they are well fed. But they are careful to disabuse the notion that "state-dependence accounts for all reported inconsistencies in animal choice" or that animals do not employ cognitive mechanisms of choice similar to those of humans. Altogether, Schuck-Paim and co-authors argue, these results warn that studies appropriating ideas from other disciplines can introduce confounding effects. And that researchers would do well to carefully examine the underlying causes of observed animal behaviors when testing ideas formulated in a nonbiological framework.

Schuck-Paim C, Pompilio L, Kacelnik A (2004) State-dependent decisions cause apparent violations of rationality in animal choice. DOI: 10.1371/journal.pbio.0020402