Synopses of Research Articles

Automated Imaging Screen Reveals Promising Drug Candidates

DOI: 10.1371/journal.pbio.0030165

The birth of combinatorial chemistry in the early 1990s held out the promise that scientists would soon synthesize trillions of compounds at a time and screen up to a million a day, revolutionizing the process of drug discovery. But synthesizing a vast library of compounds is just the first step in the historically painstaking process of determining whether a compound has the desired effect on a target. In addition to an ever-growing library of candidate therapeutic compounds, advances in genome analysis have produced a growing list of potential drug targets—drowning drug researchers in an excess of riches.

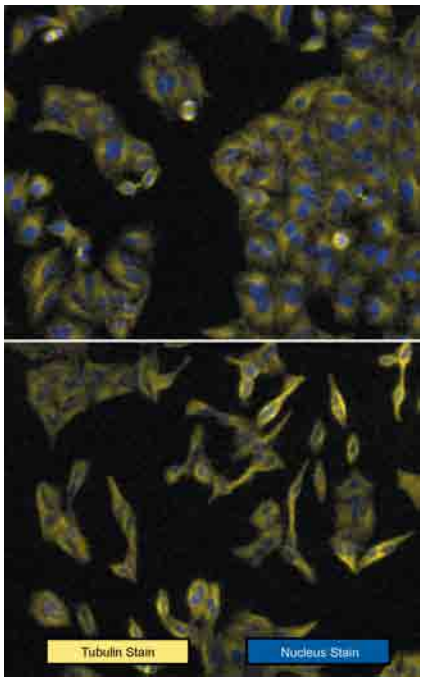
In a new study, Kevan Shokat and colleagues report a high-throughput screening method that substantially narrows the field of candidate therapeutic agents. Their approach takes advantage of a recently developed automated system (called Cytometrix) that combines advanced imaging and bioinformatics approaches to classify cells according to small-molecule-induced changes in cell size, shape, and structure (morphology). Their analysis identified a novel compound with promising potential as an anticancer agent.

The Cytometrix system offers a high-throughput, unbiased (that is, machine-rendered) approach to identifying molecules that induce changes in cell processes, molecules that could be used to probe cells or to test for therapeutic effect. High-tech imaging equipment, combined with statistical analysis, extracts the biological effects of small molecules as "phenotypic readouts" based on the physical and structural characteristics of the cells. Using this system, the authors tested 107 small-molecule compounds with structural similarities to four types of protein kinase inhibitors—used in anticancer therapies—by injecting them into human cancer cell lines (and one noncancerous cell line). The phenotypic readouts produced by each compound were classified based on a statistical analysis of cell morphology, staining intensity (staining aids visualization), and the spatial distribution of subcellular structures like nuclei, microtubules, and the Golgi compartments. This analysis could also identify inhibitors of cell components not targeted by known kinase inhibitors.

From the library of screened compounds, Shokat and colleagues identified a molecule (hydroxy-PP) that, though structurally related to a known kinase inhibitor, induced morphological changes distinct from any known kinase inhibitor. What does hydroxy-PP target? An enzyme, called carbonyl reductase 1 (CBR1), that acts on xenobiotics like anticancer drugs and is thought to cause the heart damage associated with daunorubicin chemotherapy.

To better understand how compound and enzyme interact, the authors solved the structure of hydroxy-PP and CBR1 bound together. Knowing their respective structures also suggests ways of enhancing a molecule's effect on a target. In this case, Shokat and colleagues used their structural analysis to increase hydroxy-PP's inhibition of CBR1 in cell culture so they could further explore the enzyme's biological function. These experiments revealed a previously uncharacterized role for CBR1 in programmed cell death.

Given the enzyme's suspected role in chemotherapy-related cardiotoxicity, inhibiting CBR1 activity might enhance the efficacy of chemotherapy treatments by reducing their debilitating side effects—a possibility that future studies can explore. But for now, Shokat and colleagues have demonstrated the power of using high-throughput image-based screening to identify small molecules both for probing cell biology and for identifying promising drug candidates.



DOI: 10.1371/journal.pbio.0030165.g001

Two closely related compounds produced the morphological differences evident in these lung cancer cells and reveal biological activity that could be important for drug discovery (Photo: Cytokinetics, Inc.)

Tanaka M, Bateman R, Rauh D, Vaisberg E, Ramachandani S, et al. (2005) An unbiased cell morphology–based screen for new, biologically active small molecules. DOI: 10.1371/journal.pbio.0030128

A Novel Data-Mining Approach Systematically Links Genes to Traits

DOI: 10.1371/journal.pbio.0030166

With exponential advances in computing power over the past ten years, data-generating capacity has far outpaced anyone's ability to mine the rich seams of information. This is especially true in the field of genomics. So far, over 222 prokaryote (bacteria) genomes have been sequenced, 21 archaea (primitive bacteria-like extremophiles), and 17 eukaryotes (from yeast to fly and rat to human), according to the Center for Biological Sequence Analysis in Denmark (http://www.cbs.dtu.dk/services/GenomeAtlas/). All these genomes promise to provide powerful insights into the biological processes of life, but such insights come with painstaking analysis by trained experts. Matching genotype to phenotype—the visible or measurable characteristics of species—is a major challenge in what Francis Collins, Director of the United States National Human Genome Research Institute, has called the "post-genomic era."

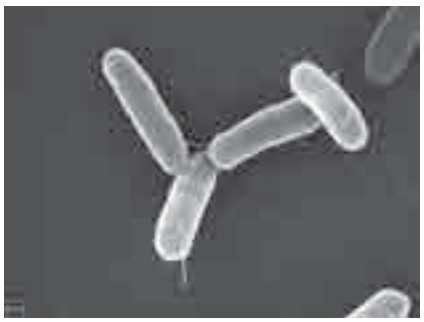
In a new study, Peer Bork and a team of bioinformatics-savvy molecular biologists tested a new approach to extracting biologically meaningful information from the massive MEDLINE database. The US National Library of

Medicine's MEDLINE contains over 12 million abstracts from thousands of publications dating back to 1965. Combining automated literature mining with comparative genomics—which compares genome sequences of different organisms to discern differences and similarities in gene content—the authors conducted a systematic search for associations between genes and phenotypic traits. Their approach automates tasks that typically require human curation.

Recognizing that the best source of information on species phenotypic traits is the scientific literature where biologists describe them, the authors first ran a search to identify associations between species and traits in MEDLINE abstracts. Words that tended to occur with subsets of species, the authors reasoned, were more likely to reflect particular traits. From a total of 255,249 MEDLINE abstracts showing any connection to 92 prokaryotic species with sequenced genomes, 172,967 nouns showed meaningful associations related to the species' traits. "Flagellum" and "motility" showed up more often in self-propelling species, for example, and "endosymbiont" aptly appeared with the intracellular bacteria (*Buchnera aphidicola*) that inhabits aphids.

Next, Bork and colleagues detected the presence or absence of over 200,000 evolutionarily conserved genes across the 92 species and sorted the results into species–word and species–gene groups. The analysis revealed a number of words and genes with similar distribution in related species, leading to over 2,700 significant associations between trait-descriptive words and orthologous (evolved from a common ancestor) groups of genes. These genes encode over 28,000 proteins. Many were already known—including genes involved in pathogenicity, biodegradation and biosynthesis, and photosynthesis—but many, the authors note, are "novel" or of "unexpected character and complexity."

And it is the ability to uncover unexpected relationships across numerous genes and genomes—patterns likely to escape human analysis—that makes this approach so powerful. Among these unexpected match-ups, Bork and colleagues linked a number of food and food-poisoning-related terms with metabolic-enzyme-coding genes. All 37 genes predicted to play a role in food spoilage and toxicity are present in food-borne pathogens but not in most other prokaryotes. By assigning functions to these previously uncharacterized genes, the authors could also assign new roles for pathways that use the genes. For example, by linking two genes with pathways that metabolize propanediol and ethanolamine—compounds found almost exclusively in highly hazardous food-borne pathogens—the authors predict that propanediol and ethanolamine pathways are "crucial genomic determinants of pathogenicity associated with food poisoning."



DOI: 10.1371/journal.pbio.0030166.g001

Many predicted genes were tied to food poisoning and bacterial pathogens, such as *Salmonella typhimurium* (Photo: Volker Brinkmann, Max Planck Institute for Infection Biology, Berlin, Germany)

That their analysis linked so many predicted genes with bacterial pathogenicity might be expected, the authors note, since both genome sequencing and biological research are heavily focused on human health. Given the weekly increase in the number of genomes sequenced and in MEDLINE entries, the method outlined here should provide a valuable tool to help researchers narrow the gap between the promise and payoff of the genomic revolution.

Korbel JO, Doerks T, Jensen LJ, Perez-Iratxeta C, Kaczanowski S, et al. (2005) Systematic association of genes to phenotypes by genome and literature lining. DOI: 10.1371/journal.pbio.0030134

The Hand That Protects: Structural Insights into a Porphyrin-Binding Protein

DOI: 10.1371/journal.pbio.0030167

Eukaryotic cells have an organizational problem. The specialized proteins found in cellular organelles (structures with specific functions such as energy production) are mostly encoded within the nucleus. To build and maintain a cell that works efficiently under all conditions, each type of organelle needs to be able to send signals to the nucleus to say "Send more protein X" or "hold back on enzyme Y." Think of it as the cellular version of grocery store clerks' restocking orders to the warehouse.

In plant cells, the chloroplasts (the photosynthetic organelles that convert light excitation energy into chemical energy) send signals to the nucleus to control the expression of the genes that encode chloroplast-localized proteins such as the enzymes that fix carbon dioxide, make chlorophyll, or perform photosynthesis. The accumulation of the chlorophyll precursor Mg-protoporphyrin IX provides one of these signals. A protein called GUN4 both enhances the activity of Mg-chelatase, the enzyme that makes Mg-protoporphyrin IX, and plays a role in the chloroplast-to-nucleus signaling activity of Mg-protoporphyrin IX in *Arabidopsis*, a well-studied plant.

To discover how GUN4 has these effects, Mark Verdecia in Joseph Noel's laboratory and Rob Larkin, formally in Joanne Chory's laboratory, determined the crystal structure of the GUN4 equivalent in the cyanobacteria *Synechocystis*. Cyanobacteria are the evolutionary ancestors of chloroplasts, so whatever GUN4 does in these cells is likely to be important in plant cells. The researchers' crystallographic studies, together with nuclear magnetic resonance and other studies, indicate that the porphyrin-binding region of *Synechocystis* GUN4 has a unique three-dimensional shape that resembles a cupped hand, the inner concave surface of which is highly hydrophobic. Because of this tendency to repel water, the researchers call this region the "greasy palm" of the cupped hand.

This structure suggests how GUN4 is involved in the chloroplast-to-nucleus signaling activity of Mg-protoporphyrin. By wrapping Mg-protoporphyrin IX in its cupped, greasy palm, the GUN4 structure provides a novel vehicle for binding Mg-protoporphyrin IX and may be involved in transporting signals from the chloroplast to the nucleus. In addition, the structure

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A "greasy palm" in the cupped hand of a porphyrin-binding protein reveals how this hydrophobic protein repels water. (Illustration: Mark Verdecia)

also suggests that GUN4 may be involved in photoprotection. Although light drives photosynthesis, which is essential to green plants, light has a downside—porphyrins combine with the oxygen released during photosynthesis to generate reactive oxygen species, which are generally damaging to the cell. GUN4, by cocooning Mg-protoporphyrin IX in its protective hand, may provide a way to safely move porphyrin around the chloroplast without exposing it to oxygen. Finally, the detailed structural and functional studies described by Noel and colleagues explain how GUN4 enhances the activity of Mg-chelatase. GUN4 binds Mg-protoporphyrin IX, the product of the chelatase, much better than protoporphyrin IX and so will tend to enhance the enzymatic reaction by removing its product.

Verdecia MA, Larkin R, Ferrer JL, Riek R, Chory J, et al. (2005) Structure of the Mg-chelatase cofactor GUN4 reveals a novel hand-shaped fold for porphyrin binding. DOI: 10.1371/journal.pbio.0030151

Where Do All Those Genes Come From?

DOI: 10.1371/journal.pbio.0030169

An important source of genetic novelty is the introduction of new genes. Since most genes in an organism's genome are under selective constraint, opportunities for the evolution of new gene functions—which in turn might confer selective advantage—most often arise when new genes enter the genome. In eukaryotes—a category that includes humans and rice—novel genes typically arise when existing genes undergo duplication. Extra copies of genes can be created when normal DNA replication hiccups and erroneously duplicates entire regions of DNA. These extra gene copies reside in species' genomes for generations and might eventually mutate to code for novel proteins, adding new genes to the species' repertoire. The new genes, along with the rest of the genome, are passed down from one generation to the next in a process known as vertical transmission.

In prokaryotes—which include unicellular organisms in the bacteria and archaea domains—novel genes can appear through multiple routes. In addition to gene duplication, prokaryote genomes can change when DNA fragments are taken up directly by cells, passed from cell to cell, or transferred to new cells with the help of viruses. All three scenarios provide a means for whole genes to move directly from one bacterial genome to another, a process called lateral gene transfer (LGT) or horizontal transmission.

Until now, the importance of vertical versus horizontal transmission in the evolution of any large prokaryote group was unknown. In a new study, Emmanuelle Lerat et al. capitalized on the availability of complete genome sequences within the diverse γ-Proteobacteria, a group of prokaryotes that includes *Escherichia coli*, *Salmonella* spp., and some nitrogen-fixing bacteria, to pursue that question.

Sorting out the issue is no simple task. If the same gene is present in more than one species, it could have been inherited from a common ancestor or it could have jumped from one lineage to another by LGT. Even if the same gene appears twice in one species' genome, the copies could have different histories—one copy could have been acquired vertically from its ancestors, while the other could have come from a different species.

Though previous studies have looked at the distributions of genes across species phylogenies, information about gene origin appeared contradictory. To create a clearer picture, Lerat et al. accounted for the possibility of widespread LGT by statistically comparing the phylogenies of many different gene families with a benchmark phylogenetic tree that reflected the accepted evolutionary history of γ-Proteobacteria.

The authors found that LGT plays a substantial role in generating the diversity of genes found in γ-Proteobacteria genomes. Members of the group are constantly acquiring and losing genes, although the extent of LGT can vary greatly among species. In contrast, gene duplications play a much smaller role in explaining γ-Proteobacteria genome diversity, although duplications have been shown to be important for short-term adaptation.

Genes that have arrived by LGT within a single genome do not necessarily share a common history with each other. Many of the genes that are found only in a single genome and are not widely distributed across the γ-Proteobacteria were recently acquired from distant sources. Most of these acquired genes will likely be lost soon after joining a genome; those that persist are then inherited vertically. This helps to reconcile why gene trees tend to provide valid phylogenetic inferences about the relationships among different bacterial lineages, despite the potential mixing that could result from LGT. Phylogeneticists aiming to reconstruct a phylogeny for a group look at variations in genes distributed in the species, and these are largely vertically transmitted.

Lerat et al. propose that LGT is a common source of genes in γ-Proteobacteria because it has the potential to introduce functionally different genes into the genome with immediate contributions to fitness. Gradual evolution of gene duplicates doesn't provide the same type of immediate reward.

Lerat E, Daubin V, Ochman H, Moran NA (2005) Evolutionary origins of genomic repertoires in bacteria. DOI: 10.1371/journal.pbio.0030130

Genomes Offer Ecological Clues to Viruses That Target Ubiquitous Ocean Bacteria

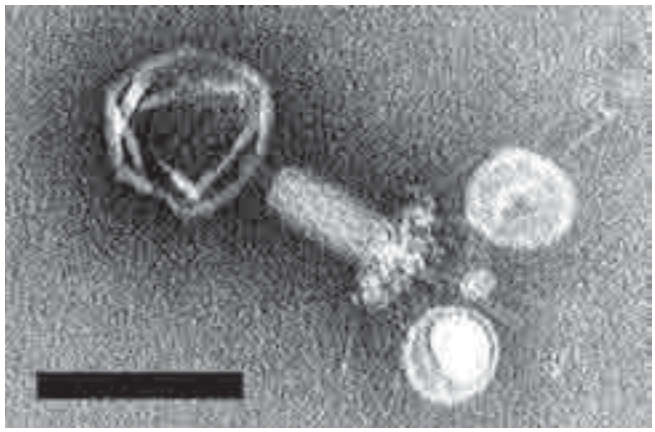
DOI: 10.1371/journal.pbio.0030184

Cyanobacteria have a long and checkered past. When their ancestors first appeared some 3 billion years ago, earth's atmosphere likely contained mostly carbon dioxide, along with hydrogen sulfide, ammonia, nitrogen, and water vapor. Thought to be the first photosynthesizers, cyanobacteria forebears used water from their ocean habitat, carbon dioxide, and sunlight to make sugar, and produced oxygen as waste—the kiss of death for most ancient microorganisms, which eventually died from oxygen poisoning.

Modern cyanobacteria continue to exert disproportionate influence for their size. The *Prochlorococcus* group of cyanobacteria—which measure in at less than a micron in diameter, allowing 500-plus individuals to fit comfortably on the head of a pin—account for a significant fraction of global photosynthesis by virtue of their ubiquitous presence in nutrient-depleted ocean waters. Even tinier agents—the viruses that infect these bacteria, called cyanophages—appear capable of wielding equally surprising influence on global cycles by affecting the population dynamics and evolutionary path of *Prochlorococcus*.

To better understand the nature of virus–host interactions at sea, Sallie Chisholm and colleagues investigated the genetic makeup of three cyanophages. The marine phages resemble two terrestrial phages—called T4 and T7—that infect *Escherichia coli* but carry genes that appear specially adapted to infecting photosynthetic bacteria in nutrient-poor oceans.

Of over 430 completed phage genomes, only one (P60) infects cyanobacteria. Since marine phages likely face different selection pressures than their terrestrial equivalents, the authors explain, genome analysis can shed light on the agents of selection, besides providing a survey of marine phage types. Chisholm and colleagues chose to sequence three marine phages—one podovirus (P-SSP7) and two myoviruses (P-SSM2 and P-SSM4)—based on their morphology and host range, and characterized their genomes. The P-SSP7 virus has genes that closely match many of T7's so-called core genes—signature genes required for that virus's mode of infection, which involves killing its host. P-SSP7 also has the same genome structure as other T7-like phages, though it appears capable of coexisting with its host (based on the presence of an integrating enzyme) while T7 kills as it infects. Chisholm and colleagues go on to characterize the two myoviruses and find that both viruses share most of the core genes found in T4-like phages. And like T4 phages, both myoviruses lack the integrating enzymes, suggesting they share T4 phages' homicidal approach to infection.



DOI: 10.1371/journal.pbio.0030184.g001

Despite a striking resemblance to an *E. coli* virus, this marine virus appears to have evolved genes adapted to infecting photosynthetic bacteria inhabiting low-nutrient oceans (Scale bar indicates 100 nm) (Photo: Peter Weigele)

Beyond the core phage genes, Chisholm and colleagues also present a survey of genes likely derived from cyanobacteria that "could play defining functional roles" in marine phage–host interactions. All three cyanophages contain photosynthesis-related genes, some of which, the authors propose, may mean the virus helps the host maintain photosynthesis during infection. The podovirus also has a candidate gene involved in DNA synthesis, which the authors speculate might help the virus reproduce in nutrient-poor environments, and all three cyanophages carry genes involved in metabolizing carbon. The absence of such genes in terrestrial phages, the authors argue, lends support to the notion that marine phages have evolved different adaptive mechanisms in response to the ocean environment.

Given the intimate relation between virus and host, the effects of gene swapping between virus and host is likely to be a two-way street. Just as cyanophages may help shape the fate of their hosts, it's likely that cyanobacterial genes influence phage ecology and perhaps even its range. The cyanophages characterized here take after two phages that were central to many fundamental breakthroughs in molecular biology, including the discovery that genes are made of DNA. It remains to be seen how the marine versions of these legendary laboratory viruses contribute to our understanding of phage infections in one of the most abundant, ecologically diverse primary producers in the open seas. See also the related Primer "The Third Age of Phage" (DOI: 10.1371/journal.pbio.0030182).

Sullivan MB, Coleman ML, Weigele P, Rohwer F, Chisholm SW (2005) Three *Prochlorococcus* cyanophage genomes: Signature features and ecological interpretations. DOI: 10.1371/journal.pbio.0030144

Exon Silencing Regulated by a Trio of Short RNA Motifs

DOI: 10.1371/journal.pbio.0030173

Our cells make many more kinds of proteins than can be accounted for by the relatively modest number of genes in our genome. The key to this protein-coding bounty is alternative splicing, in which one or more transcribed exons—nucleotide sequences that code for a specific segment of the protein—are excluded from the final messenger RNA before it is translated into protein. While the majority of human genes are alternatively spliced, little is known about specific RNA sequences that dictate exclusion of these exons. In a new study, Paula Grabowski and colleagues show that three short sequences, two within the excluded exon and one in an adjacent intron, or non-coding nucleotide sequence, trigger exclusion in at least one gene, and probably a large handful of others as well.

Grabowski and colleagues studied this process in a class of proteins essential for brain function called glutamate receptors. As the name implies the glutamate receptors bind to glutamate, the principal excitatory neurotransmitter in the brain. NMDA glutamate receptors, which play a role in memory formation and neuronal development, are composed of multiple subunits. Within the NR1 subunit, exclusion or inclusion of the C1 cassette exon has dramatic functional consequences. The C1 exon appears in the forebrain but is virtually absent in the hindbrain. How this differential splicing is regulated is poorly understood.

The authors noted an atypical but highly conserved GGGG motif in the intron just downstream from the splice site that ends the C1 exon. When they introduced point mutations in this motif, the exon was included up to four times as often. The rate of exclusion, or silencing, could be dramatically increased by the addition of another GGGG tetrad farther inside the intron.

Systematic mutation within the exon identified a pair of UAGG motifs that also promoted exon silencing, an effect that could be enhanced even further by introducing a third, artificial, UAGG. The pair of UAGG tetrads appears to work in combination with the GGGG tetrad, since without the former sequences, the latter had little power to silence C1 expression. Silencing is mediated by binding of UAGG to the ribonucleoprotein hnRNP A1, which also apparently interacts with the GGGG within the intron.

The authors next did a series of genomic database searches, to identify these motifs in other genes. They reasoned that if the triad was a common means of exon silencing, it should be overrepresented among genes known to undergo alternative splicing. In more than 90,000 exons in human and mouse genomes, they found 16 with the motif pattern, of which three (19%) were known skipped exons. In contrast, among those without the pattern, the proportion of skipped exons was only 5%. They also found that the GGGG motif by itself was overrepresented among skipped exons, indicating it probably plays a significant role in exon exclusion even without its UAGG partners.

These results alone cannot explain why one cell type includes an exon while another excludes it, since the primary transcript in different cell types is the same. Instead, these differences are likely explained by tissue-specific differences in levels of splicing factors or binding proteins. With such small absolute gene numbers, it is clear that the specific trio identified by Grabowski and colleagues is only one of many likely to regulate exon inclusion. In the search for others, this study indicates the value of bioinformatics strategies that employ not only specific sequences, but also spatial configurations.

Han K, Yeo G, An P, Burge CB, Grabowski PJ (2005) A combinatorial code for splicing silencing: UAGG and GGGG motifs. DOI: 10.1371/journal.pbio.0030158

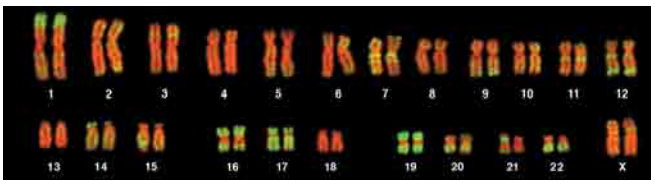
The Nuclear Landscape: A 3D Map of Human Chromosomes

DOI: 10.1371/journal.pbio.0030188

On this, theologians, philosophers, and biologists can agree: we are more than the sum of our genes. Biological complexity arises not from gene number but from patterns of gene expression, which change under the direction of both genetic and so-called epigenetic mechanisms. Epigenetics, broadly defined, concerns heritable changes in gene function that don't involve changes in DNA sequence. Until recently, studies of heritable traits have focused largely on mutations in DNA. But it's become increasingly clear that how DNA is packaged in the nucleus also impacts heritability.

Epigenetic changes are mediated largely by proteins that shape and remodel chromatin—the association of DNA and histone proteins that condenses the genome into compact bundles inside the nucleus. Different cell types have different chromatin arrangements during development and cell differentiation that appear to regulate gene expression, which possibly accounts for the unique gene expression patterns associated with specific cell types. Such phenomena have been well-studied for specific genes or chromosomal regions, but to understand the full impact of epigenetic mechanisms on gene regulation, we need a more panoramic view of gene organization within the nucleus.

In a new study, Thomas Cremer together with Andreas Bolzer and an interdisciplinary team of German physicists, bioinformaticians, and geneticists created 3D positional maps of each human chromosome simultaneously in a single nucleus to investigate the link between chromatin structure and cell-specific gene expression. Working with human fibroblasts, cultured from a skin biopsy from a two-year-old boy, the authors were able to visualize and study the order of the full genetic complement within a human nucleus.



DOI: 10.1371/journal.pbio.0030188.g001

In this karotype from a female human lymphocyte, the gene-rich areas are stained green and the gene-poor areas are red (Photo: Irina Solovei)

Cremer and colleagues first produced a 3D topological map of all 46 chromosomes in different cell types at key points in the cell cycle—a landmark achievement—using a fluorescent staining technique that preserves chromosome shape during visual inspection under the microscope. Next, they established that small chromosomes in quiescent (nondividing) fibroblasts hewed close to the center of the nucleus while the large

chromosomes were preferentially found at the nuclear rim, regardless of their gene density. Nuclei from cells entering the prometaphase stage of the cell cycle—just before chromosomes are aligned along the center of the nucleus prior to segregation—revealed a size-correlated chromosomal distribution akin to that seen in the quiescent nuclei. Statistical modeling analyses indicated that these size correlations do not simply reflect the geometric constraints of fitting into the nucleus, but likely hint at some degree of functional order within the nucleus.

Because previous studies of cells with sphere-like nuclei correlated chromosomal arrangements with gene density, the authors investigated how shape affects chromosome position along the nuclear radius. Fibroblast nuclei are somewhat flat and ellipsoidal. Chromosomes in similarly shaped amniotic fluid cells assumed the same size-related positions taken by chromosomes in fibroblast nuclei. But when the authors examined the higher-order chromatin arrangements in fibroblasts and lymphocytes, they found that, even though the cell types differ in nuclear shape and radial chromosomal arrangements, they both show a nonrandom higher-order chromatin architecture correlated with gene density. Many questions remain concerning the functional and physiological significance of these observations: Do shape changes produce changes in chromosomal arrangements and vice versa? Do shape changes produce changes in gene expression patterns?

Cremer and colleagues conclude that, although nonrandom chromosome positions occur, these appear to be governed by a degree of uncertainty and more likely reflect probabilistic preferences inside the nucleus. Still, deterministic mechanisms in higher-order chromatin structure may exist—sequestering gene-rich chromatin areas in the nuclear interior, for example, protected from malevolent agents entering the nucleus. And given the coexistence of size-correlated features with gene-density-correlated features seen in this study, it may well be that both random and deterministic factors combine to create the nuclear landscape.

Bolzer A, Kreth G, Solovei I, Koehler D, Saracoglu K, et al. (2005) Three-dimensional maps of all chromosomes in human male fibroblast nuclei and prometaphase rosettes. DOI: 10.1371/journal.pbio.0030157

A Developmental Switch in Neuronal Differentiation

DOI: 10.1371/journal.pbio.0030180

Building an embryo is like building a house: everything has to be done at the right time and the right place if the plans are to be translated faithfully. On the building site, if the roofer comes along before the bricklayer has finished, the result may be a bungalow instead of a two-story residence. In the embryo, if the neurons, for example, start to make connections prematurely, the resultant animal may lack feeling in its skin.

On the building site, the project manager passes messages to the subcontractors, and they tell the laborers what to do and where. In the embryo, the expression of specific transcription factors (molecules that tell the cell which DNA sequences to convert into proteins) at different stages of development and in different places controls the orderly construction of the body.

Silvia Arber and her colleagues are studying the protracted process of neuronal differentiation in mice. Early in development, neurons are generated from dividing progenitor cells. Cell division stops soon after, and long extensions called axons grow out of the neurons in specific directions. When these axons reach their targets—peripheral tissues like the skin at one end, in the case of sensory neurons, and the spinal cord at the other—they form characteristic terminal branches. Finally, the nerves form contacts with other neurons so that they can pass messages on to the brain.

Many aspects of neuronal character are acquired through the expression of transcription factors in the progenitor cells or immediately after cell division stops. But Arber and her colleagues have been investigating whether an even later wave of transcription programs is needed for neuronal differentiation and circuit assembly in the sensory neurons of the dorsal root ganglia (DRG), structures containing the cell bodies of the sensory neurons. Previous work indicates that the release of molecules called neurotrophic factors by the neuron's target tissues directs the late expression of Er81 and Pea3. These ETS transcription factors (so called because they contain a region known as the erythroblast-transformation-specific domain) control late aspects of the differentiation of DRG neurons. What would happen, the researchers asked, if ETS proteins were expressed earlier? Would precocious ETS expression in DRG neurons also direct the appropriate neuronal developmental programs?

Arber's team made genetically engineered mice in which ETS signaling occurred either at the correct time or earlier, and examined the development of the proprioceptive sensory neurons, which are involved in the coordination of body balance. In vivo, they found that early initiation of ETS signaling disrupts the axonal growth of the DRG neurons, both to their peripheral targets and into the spinal cord, and perturbs the acquisition of terminal differentiation markers. In vitro, premature ETS signaling allows the DRG neurons to survive and grow in the absence of the neurotrophins normally required for these processes.

Arber and her coworkers conclude that the late onset of expression of ETS transcription factors induced by target-derived signals is essential for many of the later aspects of neuronal differentiation and circuit formation. During their differentiation, the researchers suggest, DRG neurons undergo a temporal switch in their ability to respond to ETS signaling. Further analysis of the mechanisms by which responses to transcription factor programs are altered over time during development will advance our understanding not only of neuronal differentiation but of other aspects of embryogenesis.

Hippenmeyer S, Vrieseling E, Sigrist M, Portmann T, Laengle C, et al. (2005) A developmental switch in the response of DRG neurons to ETS transcription factor signaling. DOI: 10.1371/journal.pbio.0030159

Growth Disorder Gene Plays a Big Role in Normal Size Variation

DOI: 10.1371/journal.pbio.0030163

The presence of a small number of discrete forms—as you find in a classic Mendelian trait like eye color—suggests that the phenotype is controlled by a very small number of genes. In contrast, a complex trait such as body size is influenced by multiple genes as well as environmental factors, giving rise to a continuous spectrum of phenotypes. This causal complexity makes discovery of the genetic determinants of the trait—so-called quantitative trait loci (QTLs)—very difficult. In this issue, Fiona Oliver, Julian Christians, and colleagues extend their work on a single QTL with a large effect on body size variation in mice, and show that the responsible gene is one already linked to a Mendelian growth disorder in humans.

In previous work, mice were divergently selected for large or small body size, revealing a QTL on the X chromosome that causes a 20% difference in growth rate. In this study, the authors further refined the map of the area to 660 kilobases, and found it contains only four genes: glypican-3 and glypican-4 (Gpc3 and Gpc4), and two other genes of unknown function. The glypicans are membrane-bound growth regulators; loss of function of Gpc3 in humans causes a rare syndrome of overgrowth, skeletal and other abnormalities, and neonatal death. Since

DOI: 10.1371/journal.pbio.0030163.g001

Variation in the expression of a protein involved in a rare human overgrowth syndrome contributes to size variation in mice (Photo: Lutz Bünger, Scottish Agricultural College)

none of the four genes showed coding-region variations that might explain the differences between the large and small mice, the authors examined expression levels. In Gpc3, but not the others, size correlated with a significant difference in gene expression: larger mice had low levels of the messenger RNA, and smaller mice had high levels—the same pattern seen in the human loss-of-function disorder. The authors identified several non-coding polymorphisms in Gpc3 that differed between the two forms, although it remains to be seen whether the differential effect on growth is due to these or other DNA differences nearby.

The results from this study point out an important feature of inheritance, namely, that a gene implicated in a Mendelian trait can also contribute to quantitative variation. While catastrophic expression failure, such as a loss of function, can cause disease, smaller changes in expression of the same gene may simply help fill out the bell curve of normal variation.

Oliver F, Christians JK, Liu X, Rhind S, Verma V, et al. (2005) Regulatory variation at glypican-3 underlies a major growth QTL in mice. DOI: 10.1371/journal.pbio.0030135

Virus Subverts Cellular Defense for Reproduction and Escape

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Against the constant threat of infection by bacteria or viruses, one line of defense for the eukaryotic cell is the autophagosome. This double-membrane structure, which buds off from the endoplasmic reticulum, traps cytoplasmic intruders and, upon maturation, merges with a lysosome to destroy them. In this issue, however, Karla Kirkegaard and colleagues show that for one class of viruses, the autophagosome is not a holding cell but a breeding ground, and may even provide a novel escape route.

The viruses in question are picornaviruses, which include polioviruses and rhinoviruses. Infection of human cells with poliovirus is known to induce proliferation of double-membrane cytoplasmic vesicles that are morphologically similar to autophagosomes, but the origin and ultimate identity of these vesicles has not been resolved. To test whether these viral-laden vesicles are truly autophagosomes, the authors visualized two proteins: LC3, a specific marker for autophagosomes, and 3A, a part of the poliovirus RNA replication complex. After infection, these proteins colocalized, indicating the poliovirus was indeed within the autophagosome-like vesicles. LC3 also colocalized with LAMP1, a marker for lysosomes, indicating these vesicles mature in a manner similar to that of autophagosomes. This same effect could be induced simply by expressing two viral proteins.

All these results indicate that the virus stimulates production of vesicles that bear the traits of autophagosomes and contain the virus, but they don't indicate what the consequence is for viral replication. To determine that, the authors increased autophagosome production with two known stimulators of autophagy, tamoxifen and rapamycin. But rather than protecting the cell, this stimulation increased viral yield either 4-fold, in the case of tamoxifen, or 3-fold, for rapamycin. Conversely, inhibiting autophagosome production reduced viral yield. From these results, it seems the virus has subverted the components of the autophagy pathway for its own uses.

Inhibiting autophagosome production reduced viral yield inside the cell, but even more so outside. While they were not able to exclude other mechanisms, the authors argue that one possible explanation is that these vesicles are used by the virus to exit from the cell. Supporting this view, they produced electron micrographic images consistent with the fusion of the autophagosome with the plasma membrane and the extracellular release of its contents. This suggests that the virus, which is known to lyse cells to release new viral particles, has another, less lethal means of escape. This may increase the virus's chance of avoiding immune system detection as it infects new cells.

Jackson WT, Giddings TH Jr, Taylor MP, Mulinyawe S, Rabinovitch M, et al. (2005) Subversion of cellular autophagosomal machinery by RNA viruses. DOI: 10.1371/journal.pbio.0030156

Assessing Consciousness: Of Vigilance and Distractedness

DOI: 10.1371/journal.pbio.0030179

Have you ever walked smack into a parking meter or tripped over something on the sidewalk? Embarrassing as such incidents may be, they're the product of normal brain function. The brain is continuously bombarded with sensory information about the environment but perceives just a fraction of these inputs. The rest—pertinent details or not—is filtered out. It's thought that consciousness emerges from the activity of multiple spontaneous neural processors that run in parallel and connect to a higher order cognitive network that mediates the conscious perception. But this higher order network has limited processing capacity. That means if you're distracted, your brain can't accommodate additional sensory information, like "there's a parking meter in front of you, look out!"

To understand how spontaneous brain processing interacts with higher order cognition, Stanislas Dehaene and Jean-Pierre Changeux modeled the dynamic properties of brain activity with computer simulations. Their simulations show that while spontaneous brain activity sometimes facilitates processing, more often it competes with external stimuli for access to consciousness. Intriguingly, the results of the computer simulations very closely match physiological and psychophysical experimental data and thus shed new light on how intrinsic brain activity modulates conscious perception.

Neurons are simulated in their model as single "integrate and fire" units, integrating the signals received from all connected cells and firing an action potential as soon as their threshold is exceeded. These units are nested in columns, which are multiply linked among themselves and thereby form hierarchical assemblies. Lower columns increase their firing activities upon perception of external stimuli. This excitation propagates upwards to higher processing areas in a bottom-up activation process, but the model also includes, critically, top-down connections that can amplify incoming inputs. Eventually, if the input is strong enough, the reverberating excitation results in "ignition" of the global workspace with all areas simultaneously displaying sustained high firing activities. In the ignited network, the information of the stimulus is globally available; in this simplified model ignition corresponds to the access to consciousness. Most interestingly, the ignition threshold is variable and depends on the intensity of spontaneous activity in the network prior to perception of the stimulus.

In their study, Dehaene and Changeux changed the values of only one input parameter: the ascending neuromodulatory current. This model parameter simulates the effect of various diffuse neuromodulatory systems located in lower regions of the brain, which regulate the transition between awake and asleep states by liberating a diversity of neurotransmitters in the cortex and thalamus, the upper brain regions. The dynamics of the neuronal network could clearly be separated into two broad states of activity. Below a certain threshold for the neuromodulatory current, ignition of the global workspace is not possible, no matter how strong the stimulus—this can be compared to the brain states of comatose patients lacking any sign of consciousness. Above the threshold, however, spontaneous neural activity emerges: firing signals are amplified in feedback loops in single columns of the neuronal network.

With higher vigilance states, weaker external stimuli are able to ignite the global workspace. But paying attention to one thing narrows your perceptive capacity. Once ignited by one stimulus, the network cannot consciously process any others. Dehaene and Changeux propose that spontaneous activity—which operates within an "anatomically distinct set of workplace neurons"—offers an organism a measure of autonomy relative to the external world. While this decoupling of internal thought and external stimuli does have its disadvantages—like that pesky parking meter—it also provides the opportunity for introspection and creativity, which the authors argue is likely to "play a crucial role in the spontaneous generation of novel, flexible behavior."

Dehaene S, Changeux JP (2005) Ongoing spontaneous activity controls access to consciousness: A neuronal model for inattentional blindness. DOI: 10.1371/journal.pbio.0030141

To a Zebra Finch: How the Brain Cultivates Birdsong

DOI: 10.1371/journal.pbio.0030162

From the "ecstatic sound" of Thomas Hardy's thrush to the "full-throated ease" of Keats's nightingale, the dulcet tones of songbirds have long inspired poetic explorations of the human spirit. Scientists have more recently found inspiration in songbirds, but it is their behavior and not their song that tickles the scientific imagination. Just as the vocal explorations of toddlers reflect the (no doubt) consequential conversations of their elders, the highly variable chirps and warbles of juvenile songbirds echo the precise melodies of the adult songbird. Through trial and error and random forays into harmolodic dissonance, the young bird patterns his performance after a tutor song (usually performed by dad) until he produces a workable facsimile. It is this behavior— known as reinforcement learning—that makes songbirds an ideal model for studying the interplay between experience, brain activity, and learning.

Michale Fee's lab studies the neural basis of song learning in the zebra finch, the organism of choice for birdsong researchers. In a new study, Bence Ölveczky, Aaron Andalman, and Fee study just how young songbirds generate the vocal explorations that help the apprentice master its song.

Two major neural pathways control zebra finch song. The motor pathway

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The vocal explorations of young zebra finches shed light on the neural basis of learning motor tasks (Photo: Daniel D. Baleckaitis)

controls vocal outputs through the RA (for robust nucleus of the arcopallium) neuron cluster, which indirectly stimulates vocal and respiratory muscles. When adult birds sing, RA neurons show a signature sequence of bursts during each syllable. Another pathway, called the anterior forebrain pathway (AFP), appears to be critical for song learning. AFP shares characteristics with the mammalian basal ganglia, which regulates movement and motor learning in mammals.

To explore the nature of the AFP's contributions to song learning, Fee and colleagues recorded brain activity from young zebra finches (54–79 days old) learning to sing. Then they injected young birds with drugs that temporarily blocked activity in a brain region that is part of the AFP called LMAN (lateral magnocellular nucleus of the nidopallium). Zebra finch songs typically contain three to seven syllables—the basic acoustic units of zebra finch songs—that follow a specific sequence. Thirty to 90 minutes after LMAN inactivation, the birds sang with less syllabic variation. This effect was especially dramatic in the youngest birds, which normally exhibit the greatest acoustic variation. LMAN inactivation, the authors note, "eliminated 75% of the difference in mean variability between juvenile song and adult directed song [wooing a mate, for example]—the most stereotyped form of song." LMAN inactivation also reduced the birds' variation in syllable sequence, which again hewed closer to the orthodoxy of adult song than to the exuberance of youthful experimentation.

The authors go on to show that changes in the firing patterns of LMAN neurons projecting into the motor pathway accompany changes in song. That LMAN inactivation reduces song variability quickly and reversibly, the authors argue, indicates that LMAN supports experimental behavior and controls song variability by providing rapid inputs to the motor pathway. This model requires that LMAN neurons show high variability across different song motifs—which is what Fee and colleagues found. As the bird sings, some as yet unknown brain areas must also evaluate the song against a template, modulating the actions of the motor pathway as a conductor might correct a performer's mistakes in note and pitch until she masters the tune.

It's thought that birdsong serves multiple purposes—staking a territorial claim, for example, and attracting a mate—though precisely how the song relates to fitness is still an open question. Whether inducing the type of exploratory motor behavior that's so critical to motor learning is a fundamental feature of basal ganglia circuits also remains to be determined. But it does seem clear that these circuits play a significant role in generating the variability that songbirds need in order to acquire the communication skills of their parents—a finding that may shed light on how the brain produces the fluctuations required for learning other tasks. For more on song learning, see the primer by Fernando Nottebohm (DOI: 10.1371/journal.pbio.0030164, available online May 2005).

Ölveczky BP, Andalman AS, Fee MS (2005) Vocal experimentation in the juvenile songbird requires a basal ganglia circuit. DOI: 10.1371/journal.pbio.0030153

Infant Sleep: A Precursor to Adult Sleep?

DOI: 10.1371/journal.pbio.0030168

Sleep is absolutely essential for well-being. Just ask one of the 40 million Americans with sleep disorders who suffer crippling fatigue, impaired judgment, irritability, moodiness, and myriad health problems. Still, its precise function remains unclear. An intriguing role for REM sleep—the stage most closely associated with dreaming—was suggested almost 40 years ago when sleep researchers Howard Roffwarg and William Dement discovered that babies spend far more time in REM sleep than adults—prompting their hypothesis that infant REM sleep plays a role in central nervous system development.

A central element of their hypothesis revolves around the nature of infant sleep and whether the neural mechanisms of infant sleep differ significantly from those of adult sleep. Infant rats, like the offspring of other "altricial" species (born naked, helpless, and blind), spend most of their time in what's now called active sleep, indicated by intermittent muscle twitching and low muscle tone (atonia)—behaviors characteristic of adult REM sleep. At issue is whether infant mechanisms are primitive, undifferentiated, and distinct from adult mechanisms or whether they contain elementary components that are integrated into the developing sleep system.

In a new study, Karl Karlsson, Mark Blumberg, and their colleagues tackle the technical difficulties involved in studying the tiny neonatal brain to investigate the neural activity associated with infant sleep states. The active sleep of week-old rats, they show, bears a striking resemblance to the conventional definitions of adult sleep. What's more, the neural mechanisms underlying the infant sleep state contain the primary components of adult sleep.

In previous studies, Karlsson and Blumberg discovered a brainstem region in the ventromedial medulla, which they called the medullary inhibitory area (MIA), that appears functionally equivalent to the region that generates REM atonia in adults. They also found that the MIA doesn't generate infant sleep on its own but depends on a network that spans both lower brainstem and midbrain regions. In this study, the authors set out to identify the neural structures that project to the MIA and better characterize the network.

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Though naked, helpless, and blind, this week-old rat (pictured with a quarter) already has the fundamental neural components of adult sleep (Photo: Mark Blumberg)

Karlsson et al. first established that there are neurons that connect to the

MIA from areas in the medulla and pons. Then, by recording from neurons in these areas, they found neurons that are active only during sleep or wakefulness and that appear to control muscle tone and twitching. Neurons active mostly during atonia—indicating sleep—concentrated in the subcoeruleus (SubLC) region of the pons; those active mostly during wakefulness clustered in an area within the dorsolateral pontine tegmentum (DLPT) in the midbrain. The authors went on to link different sets of neurons with specific behaviors and brain regions. A group of neurons within the DLPT, for example, showed distinct bursts of activity just before muscle twitching. And a subset of SubLC neurons fired at much higher rates when atonia was accompanied by bouts of tail and neck muscle twitching.

Introducing lesions in the SubLC and another pontine nucleus, called the pontis oralis, caused significant changes in muscle tone and twitching. Lesions in the two pontine nuclei reduced periods of atonia but not the number of muscle twitches—in effect decoupling the key components of REM sleep, twitching and atonia. Lesions in the DLPT had the opposite effect: increased atonia and significantly less muscle twitching.

Altogether, the authors argue, these results show that sleep development elaborates on elementary components already in place soon after birth. If the neural mechanisms of infant and adult sleep were entirely different, then sleep might serve different purposes in infancy and adulthood. But the striking parallels outlined in this study suggest a developmental continuity between the two states. They also chart a course for future study that might even test Roffwarg's view that the neonatal brainstem primes the central nervous system for the sensory challenges that lie ahead—and could even be the stuff that dreams are made of.

Karlsson KÆ, Gall AJ, Mohns EJ, Seelke AMH, Blumberg MS (2005) The neural substrates of infant sleep in rats. DOI: 10.1371/journal.pbio.0030143