PNAS Plus Significance Statements

Elucidating the mechanism of fluorinated extender unit loading for improved production of fluorine-containing polyketides

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Polyketide natural products represent a rich source for discovery of new bioactive compounds. However, the optimization of polyketide structure for medicinal purposes can be difficult using chemical methods given their complexity. Site-selective introduction of fluorine into polyketides and other natural products is a particularly interesting area of exploration given the demonstrated effectiveness of fluorine in modulating the behavior of small-molecule therapeutics. Here, we show that fluorine can be inserted site-selectively by an engineered polyketide synthase system via a fluorinated monomer that becomes covalently tethered to the enzyme to complete a canonical reaction cycle. By increasing the throughput of fluorinated extender units, we can produce multiply fluorinated polyketide products by chemoenzymatic synthesis and target the production of complex structures. (See pp. E660–E668.)

Unlocking Tn3-family transposase activity in vitro unveils an asymetric pathway for transposome assembly

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The Tn3 family of transposons, discovered in the early 1970s, represents a serious threat to human health because of its prevalence in the dissemination of antibiotic resistance and, indirectly, because of its involvement in xenobiotic metabolism and in the transmission of plant pathogenicity determinants. Astonishingly, their transposition mechanism has yet to be elucidated. We have started to unravel this mechanism by reconstituting the transposition reaction of the Tn3-family transposon Tn4430 in a cell-free in vitro system. The assays also were used to characterize transposase mutants affected in target immunity, a phenomenon whereby a given transposon avoids inserting more than once into the same DNA target. The data support a tentative model linking target immunity with transposition complex assembly and activation. (See pp. E669–E678.)

Recognition of protein-linked glycans as a determinant of peptidase activity

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Protein glycosylation is one of the most abundant and important posttranslational modifications where the protein-linked glycans can impart specific physiochemical properties to the glycoprotein and/or the glycans themselves can mediate particular biological functions. The degradation of glycosylated proteins in normal or pathogenic processes, therefore, is an important biological process. This study reveals the molecular basis of how peptidases can use the O-glycans present on glycoproteins as a critical determinant of peptidase activity and, in doing so, provides unique insight into how peptidases may directly use post-translational modifications present on their substrates to influence recognition and peptide bond cleavage. (See pp. E679–E688.)

Kinetic assay shows that increasing red cell volume could be a treatment for sickle cell disease

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Sickle cell disease can be treated by preventing polymerization of the mutant hemoglobin to form fibers during the time that red cells are transiting the smallest vessels of the tissues. However, most drugs in clinical trials are aimed at reducing the sequelae of fiber formation, such as inflammation and adhesion to the vascular endothelium. Searching for drugs that increase the delay before polymerization, which allows more cells to escape the small vessels before fibers form, has been hampered by the lack of a quantitative and sensitive assay, which we describe in this work. With this assay, we show that increasing the delay time by increasing red cell volume to reduce the intracellular hemoglobin concentration is a viable approach to therapy. (See pp. E689–E696.)

Structure of eukaryotic CMG helicase at a replication fork and implications to replisome architecture and origin initiation

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All cellular life forms use a ring-shaped hexameric helicase during DNA replication. CMG (Cdc45, Mcm2–7, GINS) is the eukaryotic replicative helicase. CMG contains the ring-shaped hexameric Mcm2–7 that harbors the helicase motors. CMG is known to bind many other proteins, including a leading and lagging polymerase and primase. Thus, the threading of DNA through the CMG helicase at a replication fork determines the orientation of the associated polymerases at the replication fork, an important structural feature with many consequences that may direct future experimentation. This report uses cryo-EM single-particle reconstruction to image CMG that motored to a block site at a forked junction, enabling direct visualization of DNA threading through CMG. (See pp. E697–E706.)

β-Catenin haploinsufficiency promotes mammary tumorigenesis in an ErbB2-positive basal breast cancer model

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Although the oncogenic potential of β-catenin as a transcriptional factor is well-established, its role as a critical component of adherens junctions during tumorigenesis remains elusive. Using two transgenic mouse models of ErbB2-induced mammary tumorigenesis that recapitulate either luminal or basal human breast cancer, we show that β-catenin is required for proper adherens junction formation and that, consequently, β-catenin haploinsufficiency promotes aggressive mammary tumorigenesis. This haploinsufficient phenotype is unique to a basal ErbB2-driven model with a preexisting aberrant activation of β-catenin signaling, highlighting a tumor suppressor role of β-catenin, similar to other adherens junction proteins, in maintaining junctional integrity and a complex interplay between its junctional and transcriptional roles in facilitating tumor progression. (See pp. E707–E716.)

Transcriptomic, proteomic, and metabolomic landscape of positional memory in the caudal fin of zebrafish

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In vertebrates, proper patterning during appendage regeneration is regulated by positional memory—a cellular property hypothesized to rely on gradients of molecules present in uninjured limbs. Only one gene, exclusive to salamanders, has been shown to regulate positional memory and be expressed in a gradient in the uninjured limb. To identify new candidate effectors of positional memory, we mapped the abundance of RNAs, proteins, and metabolites along the uninjured zebrafish tail fin. We identified hundreds of molecular gradients and generated a high-confidence list of 32 genes and 42 metabolites that are candidate effectors of positional memory in zebrafish. Furthermore, expression patterns discovered here may help to explain how size-homeostasis and patterning are maintained in a complex adult tissue. (See pp. E717–E726.)

Selection against variants in the genome associated with educational attainment

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Epidemiological studies suggest that educational attainment is affected by genetic variants. Results from recent genetic studies allow us to construct a score from a person's genotypes that captures a portion of this genetic component. Using data from Iceland that include a substantial fraction of the population we show that individuals with high scores tend to have fewer children, mainly because they have children later in life. Consequently, the average score has been decreasing over time in the population. The rate of decrease is small per generation but marked on an evolutionary timescale. Another important observation is that the association between the score and fertility remains highly significant after adjusting for the educational attainment of the individuals. (See pp. E727–E732.)

Human transposon insertion profiling: Analysis, visualization and identification of somatic LINE-1 insertions in ovarian cancer

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Much of our genome is repetitive sequence. This property poses challenges for investigators because differences in repetitive sequences are difficult to detect. With hundreds of thousands of similar repeats, it has been difficult to discern how one person's genome differs from another person's genome or how tumor DNA differs from normal DNA. To solve this issue, we developed methods to target next-generation sequencing to the insertion sites of the most variable repeats. Computational pipelines to make these studies scalable and more widely accessible were needed, however. Here, we report a pipeline that accomplishes this goal. We use it to demonstrate insertions of the long interspersed element-1 (LINE-1) acquired in ovarian cancer that may contribute to the development of these tumors. (See pp. E733–E740.)

Development of chronic allergic responses by dampening Bcl6-mediated suppressor activity in memory T helper 2 cells

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It has been suggested that the transcriptional repressor Bcl6 suppresses T helper 2 (T_H2) immune responses underlying allergic diseases. However, the molecular role of B-cell CLL/lymphoma 6 (Bcl6) in T_H2 cells is incompletely understood in pathophysiological settings. We found that Bcl6 suppressed cytokine production in memory T_H2 cells through binding to intron 2 of the *Interkeukin 4* (*Il4*) locus using murine models. Furthermore, IL-33 controlled Bcl6 function at the chromatin level and consequently, augmented cytokine production in memory T_H2 cells. Therefore, pro-T_H2 cytokines, such as IL-33, play a role in chronic allergic diseases via the functional breakdown of Bcl6. This study identifies a relationship between T_H2-promoting factors and Bcl6 in T_H2

cells, which may lead to therapeutic strategies against chronic allergic diseases. (See pp. E741–E750.)

Ornithine decarboxylase regulates M1 macrophage activation and mucosal inflammation via histone modifications

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The pathogenesis of many bacteria is enhanced by the ability to establish persistent infection. Macrophages, particularly classically activated M1 macrophages, provide essential functions in the initiation of antibacterial immune responses. The regulation of macrophage activation is still poorly understood. Here, we demonstrate that ornithine decarboxylase (ODC), the rate-limiting enzyme in polyamine synthesis, regulates M1 activation during *Helicobacter pylori* and *Citrobacter rodentium* infection. Deletion of *Odc* in macrophages resulted in increased inflammation and decreased bacterial persistence in mouse models. The enhanced M1 response was due to alterations in histone modifications, resulting in changes in chromatin structure and upregulated transcription. These findings represent a novel mechanism by which ODC directly regulates macrophage activation and provides new insights into understanding bacterial persistence. (See pp. E751–E760.)

Mitochondrial activation chemicals synergize with surface receptor PD-1 blockade for T cell-dependent antitumor activity

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Although PD-1 blockade has innovated cancer therapy, a novel combinatorial strategy is required to save less sensitive cancer patients. Mitochondria are key cytoplasmic organelles that efficiently supply the ATP necessary for the rapid proliferation and differentiation of T cells. We found that reactive oxygen species (ROS) strongly activate mitochondrial function of tumor-reactive T cells and synergize tumor regression by PD-1 blockade. ROS appear to activate both AMP-activated protein kinase (AMPK) and mechanistic target of rapamycin (mTOR), which subsequently induce the PPAR-gamma coactivator 1α (PGC-1α) transcription factor. Small-molecule activators of AMPK and mTOR, or PGC-1α, also synergistically enhance tumor-growth suppression by PD-1 blockade therapy. These findings not only open a new aspect of immune metabolism but also pave a way to developing a combinational strategy of PD-1 cancer immunotherapy. (See pp. E761–E770.)

Atrial natriuretic peptide regulates adipose tissue accumulation in adult atria

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Atrial fibrillation is the most frequent cardiac arrhythmia and is a major cause of stroke. Recently, it has been shown that the adipose tissue that accumulates at the surface of the heart contributes to the pathogenesis of atrial fibrillation by favoring fibrosis of the neighboring myocardium. However, the cellular origin of adult cardiac fat tissue is unknown. Here, we show that resident progenitor cells of the external layer of the heart, referred to as the "epicardium," are a source of adipocytes through an epithelial-to-mesenchymal transition process. The atrial natriuretic peptide, which is secreted by atrial myocytes, is a potent factor in the differentiation of epicardial progenitors in adipocytes. Our data uncover cross-talk between myocardial mechanical properties and adipose tissue expansion. (See pp. E771–E780.)

Pathogen-mediated manipulation of arthropod microbiota to promote infection

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The importance of arthropod microbiota in the capacity of pathogens (including malaria and flaviviruses, among others) to persist in vectors and cause infection is just beginning to be appreciated. The influence of pathogens, either directly or indirectly, to manipulate vector microbiota for their own benefit, has not been described. In this study, we demonstrate that a pathogen can use an arthropod molecule to alter vector microbiota and enhance infection. We believe that this work will help others consider that pathogens are not passive microbes when they enter the arthropod vector but actively influence vector gene expression that can manipulate the local environment (in this case the microbiota) and facilitate pathogen infection of the vector. (See pp. E781–E790.)

Tissue dual RNA-seq allows fast discovery of infection-specific functions and riboregulators shaping host–pathogen transcriptomes

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Our knowledge of the functions required by extracellular bacterial pathogens to grow in host tissues is still limited. Most available information refers to studies conducted under laboratory growth conditions that mimic host environments but exclude the influence of the host immune system. Tissue dual RNA sequencing allows simultaneous transcript profiling of a pathogen and its infected host. This sensitive approach led to the identification of host immune responses and virulence-relevant bacterial functions that were not previously reported in the context of a *Yersinia* infection. Application of this tool will allow transcript profiling of other pathogens to unravel concealed gene functions that are crucial for survival in different host niches and will improve identification of potential drug targets. (See pp. E791–E800.)

Continual renewal and replication of persistent *Leishmania major* parasites in concomitantly immune hosts

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Persistent parasites contribute to the maintenance of protective immune responses through concomitant immunity, but our understanding of how they do so has been limited by the difficulties associated with their low numbers. Our studies indicate that for *Leishmania* substantial parasite replication occurs in persistent infections, with most parasites found within activated antigen-presenting cells. Parasite replication serves to maintain the infection and likely also provides a constant source of parasite antigens for immune stimulation and the maintenance of protective immunity. Collectively, these studies suggest a framework to understand concomitant immunity that may be applicable to other persistent pathogens. (See pp. E801–E810.)

Antiviral screening identifies adenosine analogs targeting the endogenous dsRNA *Leishmania* RNA virus 1 (LRV1) pathogenicity factor

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The endogenous double-stranded RNA virus *Leishmaniavirus* (LRV1) has been implicated as a pathogenicity factor for leishmaniasis in rodent models and human disease, and associated with drug-treatment failures. As a first step toward the identification of therapeutic LRV1 inhibitors, we identified two adenosine analogs that selectively inhibited LRV1 replication. These analogs were used as tools to confirm that viral inheritance is by random segregation, as well as to generate LRV1-cured lines of *Leishmania guyanensis*, which correspondingly lost the increased pathogenicity conferred by LRV1. These compounds hold promise as leads to ameliorate the severity of LRV1-bearing *Leishmania* infections, and raise the possibility of targeting other protozoal infections whose pathogenicity may be exacerbated by similar endogenous viruses. (See pp. E811–E819.)

Defining recovery neurobiology of injured spinal cord by synthetic matrix-assisted hMSC implantation

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We developed a platform technology to determine therapeutic mechanisms of human mesenchymal stromal stem cells (hMSCs) in a dorsal root ganglion coculture system and an intraspinal cord implantation model. The unique poly(lactic-*co*-glycolic) acid scaffolding augments hMSC stemness, engraftment, and function without neural transdifferentiation or mesenchymal lineage development, resulting in robust motosensory improvement, pain and tissue damage mitigation, and myelin preservation in adult rat spinal cord after injury. The scaffolded hMSC-derived neurotrophism, neurogenesis, angiogenesis, antiautoimmunity, and antiinflammation support the propriospinal network, neuromuscular junctions, and serotonergic reticulospinal reinnervation to activate the central pattern generator for restoring hindlimb locomotion. Our findings illuminate "recovery neurobiology"—i.e., the injured spinal cord may deploy polysynaptic neural circuits different from normal adulthood pathways for postinjury improvement. (See pp. E820–E829.)

Bilateral recruitment of prefrontal cortex in working memory is associated with task demand but not with age

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One principle of human cerebral cortex is its lateralized functional architecture supporting processes such as language, precise motor control of the hands, and working memory. It has been shown that in elderly subjects such lateralized activations of dorsolateral and anterior prefrontal cortex vanish in working memory tasks, which is due to the corecruitment of corresponding regions in the other cerebral hemisphere. We show that such corecruitment of cross-hemispheric counterparts in prefrontal cortex is associated with subjectively demanding working memory tasks but not with age. This result suggests that prefrontal areas support us to maintain performance in challenging circumstances by additionally recruiting their non-specialized counterpart in the contralateral hemisphere. (See pp. E830–E839.)

Harmonic template neurons in primate auditory cortex underlying complex sound processing

Lei Feng and Xiaoqin Wang

Harmonicity is a fundamental element of music, speech, and animal vocalizations. How the brain extracts harmonic structures embedded in complex sounds remains largely unknown. We have discovered a unique population of harmonic template neurons in the core region of auditory cortex of marmosets, a highly vocal primate species. Responses of these neurons show nonlinear facilitation to harmonic complex sounds over inharmonic sounds and selectivity for particular harmonic structures. Such neuronal selectivity may form the basis of harmonic processing by the brain and has important implications for music and speech processing. (See pp. E840–E848.)

Increased mitochondrial nanotunneling activity, induced by calcium imbalance, affects intermitochondrial matrix exchanges

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Nanotunnels are long, thin mitochondrial extensions that have been implied in direct long-distance (1 to >5 μm) communication between mitochondria of cardiac myocytes. The engineered RyR2A4860G$^{+/-}$ mutation, resulting in loss of function of the sarcoplasmic reticulum calcium release channel and arrhythmia, induces a striking increase in the frequency of long-distance intermitochondrial communication via nanotunnels without involvement of obvious mitochondrial migration. We use this model for exploring the significance of mitochondrial nanotunneling in myocardium and the contribution of microtubules to the formation of these unusual organelle extensions using EM tomography and live confocal imaging. This study constitutes an approach to arrhythmia investigations that focuses on a new target: the mitochondria. (See pp. E849–E858.)

Mitochondrial fusion dynamics is robust in the heart and depends on calcium oscillations and contractile activity

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Mitochondrial function is supported by dynamic quality control processes, such as mitochondrial fusion. Cardiac contractility depends on mitochondrial metabolism, yet in cardiomyocytes, mitochondria are confined among myofibrils, raising questions about the possibility of mitochondrial physical communication. Here we demonstrate that mitochondrial continuity is robust and fusion is frequent in freshly isolated rat ventricular myocytes, manifesting both as rapid content mixing events between adjacent organelles and slower, often long-distance events. We show that mitochondrial fusion decreases dramatically in culture because of the decay in contractile activity and, more specifically, the underlying calcium oscillations, which involve mitofusin 1 (Mfn1) abundance. In addition, we show that attenuation of cardiac contractility in vivo in alcoholic animals is also associated with depressed mitochondrial fusion. (See pp. E859–E868.)

Competition of calcified calmodulin N lobe and PIP_2 to an LQT mutation site in Kv7.1 channel

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Voltage-gated potassium 7.1 (Kv7.1) channel and KCNE1 protein coassembly forms the I_{KS} K^+ current that repolarizes the cardiac action potential, and mutations in *Kv7.1* and *KCNE1* genes cause cardiac arrhythmias. The proximal Kv7.1 C terminus binds calmodulin and the phospholipid phosphatidylinositol-4,5-bisphosphate (PIP_2); however, it is unknown whether their binding sites overlap physically and functionally. Here, we reveal the competition of PIP_2 and the calcified form of the calmodulin N lobe to a previously unidentified site in helix B of the proximal Kv7.1 C terminus. Notably, this site bears a mutation causing a cardiac arrhythmia called the long-QT syndrome. Our results suggest that, after receptor-mediated PIP_2 depletion and increased cytosolic Ca^{2+}, calcified calmodulin N lobe interacts with helix B in place of PIP_2 to limit excessive I_{KS} current depression. (See pp. E869–E878.)

Phosphorylation by PKC and PKA regulate the kinase activity and downstream signaling of WNK4

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The kinase WNK4 (with-no-lysine kinase 4) is an important regulator of the Na-Cl cotransporter (NCC) in the renal distal convoluted tubule (DCT). Volume depletion induces angiotensin II, activating PKC, which prevents WNK4 degradation by phosphorylating the KLHL3/CUL3 ubiquitin ligase. We now show that PKC also directly phosphorylates WNK4 at multiple sites in cell culture. Phosphorylation of two of these sites, S64 and S1196, promotes increased WNK4 kinase activity by increasing autophosphorylation of the WNK4 T-loop at S332. Volume depletion also induces phosphorylation of WNK4-S64 in the DCT in vivo, promoting NCC activity. These findings provide insights into the mechanisms regulating activity of NCC and the promotion of renal Na-Cl reabsorption without concomitant K^+ secretion in volume depletion. (See pp. E879–E886.)

Dynamic PIN-FORMED auxin efflux carrier phosphorylation at the plasma membrane controls auxin efflux-dependent growth

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The distribution of the hormone auxin controls most processes in plant development. Auxin distribution within the plant requires PIN transporters and efficient PIN-mediated transport requires PIN phosphorylation. Phosphorylation seemingly also controls auxin transport by targeting PINs to specific sides of the cell. Understanding how auxin is directed and activated through phosphorylation is essential to understand plant growth. Two different protein kinases targeting the same phosphosites in PIN1 can activate auxin efflux. Surprisingly, however, only one affects PIN1 polar distribution. Here, we show that the differential effects of the two kinases on PIN1 cannot be explained by phosphorylation at the established phosphosites, and suggest that a more complex model is needed to explain the effects of the kinase on PIN1 polarity. (See pp. E887–E896.)

TALE-induced bHLH transcription factors that activate a pectate lyase contribute to water soaking in bacterial spot of tomato

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AvrHah1 [avirulence (avr) gene homologous to avrBs3 and hax2, no. 1] is a transcription activator-like (TAL) effector (TALE) in *Xanthomonas gardneri* that enhances water soaking in its known hosts tomato, pepper, and *Nicotiana benthamiana*. We observe that the water soaking conferred by AvrHah1 is due to the movement of water into the infected apoplast from a wet environment. RNA sequencing identified two basic helix–loop–helix (bHLH) transcription factors that we confirmed as targets of AvrHah1. We discovered that a pectate lyase was upregulated by both of the bHLH transcription factors. Designer TALEs (dTALEs) for both bHLH transcription factors and the pectate lyase complemented the water-soaking phenotype of *X. gardneriΔavrHah1*. This report demonstrates virulence activity from an indirect TALE target. (See pp. E897–E903.)