PNAS Plus Significance Statements

Solution structure of the TLR adaptor MAL/TIRAP reveals an intact BB loop and supports MAL Cys91 glutathionylation for signaling

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Toll-like receptor (TLR) signaling pathways are targeted to limit inflammation in immune cells. TLRs use adaptor proteins to drive inflammatory signaling platforms for effective microbial clearance. Here we show that MyD88 adaptor-like (MAL), an adaptor protein in TLR signaling, undergoes glutathionylation in response to LPS, driving macrophage responses to proinflammatory stimuli. We also determined the solution structure of MAL in the reduced form without disulfides, revealing a typical BB loop observed in adaptor proteins, in contrast to previously reported crystal structures. This alternate solution structure reveals the inherent flexibility of MAL, supporting the hypothesis that glutathionylation may reposition the MAL BB loop for MyD88 interaction to drive inflammation. This discovery could lead to novel approaches to target MAL glutathionylation in dysregulated TLR signaling, limiting inflammation. (See pp. E6480–E6489.)

Lipophilic siRNA targets albumin in situ and promotes bioavailability, tumor penetration, and carrier-free gene silencing

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Small interfering RNA (siRNA) has the capacity to silence traditionally undruggable targets, but in vivo delivery barriers limit clinical translation of siRNA, especially for nonhepatic targets such as solid tumors. Most delivery strategies for RNAi cancer therapies focus on synthetic nanocarriers, but their shortcomings include limited delivery to and variable distribution throughout the target site and low therapeutic indices due to nonspecific, carrier-associated toxicities. A diacyl lipid-modified siRNA can leverage albumin as an endogenous carrier, resulting in comprehensively enhanced pharmacokinetic properties that translate to greater quantity and homogeneity of tumor accumulation relative to nanocarriers. The albumin-binding siRNA conjugate strategy is synthetically simple and safe at high doses, and thus is a translatable and potentially transformative option for RNAi oncology therapies. (See pp. E6490–E6497.)

Genomic landscape of human diversity across Madagascar

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The origins of the Malagasy raise questions about ancient connections between continents; moreover, because ancestors are fundamental to Malagasy society, Malagasy origins is also a heated topic around the country, with numerous proposed hypotheses. This study provides a comprehensive view of genomic diversity (including maternal lineages, paternal lineages, and genome-wide data) based on a sampling of 257 villages across Madagascar. The observed spatial patterns lead to a scenario of a recent and sex-biased admixture between Bantu and Austronesian ancestors across the island. Moreover, we find geographical influences creating subtle signals of genetic structure that are independent of the Bantu/Austronesian admixture, suggesting that recent history has a role in the genomic diversity of the Malagasy. (See pp. E6498–E6506.)

Serum amyloid A forms stable oligomers that disrupt vesicles at lysosomal pH and contribute to the pathogenesis of reactive amyloidosis

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Although acidic conditions favor the misfolding of various proteins in amyloid diseases, the molecular underpinnings of this process are unclear. We used an array of spectroscopic, biochemical, and electron microscopic methods to unravel the effects of pH on serum amyloid A, a protein precursor of reactive amyloidosis, the major complication of chronic inflammation and one of the major human systemic amyloidoses worldwide. We found that at lysosomal pH this protein forms unusually stable proteolysis-resistant soluble oligomers that have solvent-accessible apolar surfaces, disrupt lipid vesicles, and undergo an α-helix to β-sheet transition in the presence of lipids. Such

oligomers are likely to escape lysosomal degradation, accumulate in the lysosomes, and disrupt cellular membranes, thereby contributing to the development of amyloid A amyloidosis. (See pp. E6507–E6515.)

Effect of ATP and regulatory light-chain phosphorylation on the polymerization of mammalian nonmuscle myosin II

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Nonmuscle myosin II (NM2) filaments have essential roles in many cellular processes that require the dynamic relocation of filamentous myosin. We find that addition of ATP to polymerized regulatory light chain (RLC)-unphosphorylated NM2s in vitro results in the formation of dimers, tetramers, hexamers, and monomers, which is reversed by phosphorylation of the RLC. Our data suggest that assembly of NM2 filaments proceeds from folded monomers to folded antiparallel dimers, tetramers, and hexamers that unfold and polymerize into antiparallel filaments. This could explain the dynamic relocalization of NM2 filaments in vivo by dephosphorylation of RLC-phosphorylated filaments, disassembly of the dephosphorylated filaments to monomers, dimers, and small oligomers, diffusion of these species, and their reassembly into filaments at the new location following rephosphorylation of the RLC. (See pp. E6516–E6525.)

Structural basis of a histidine-DNA nicking/joining mechanism for gene transfer and promiscuous spread of antibiotic resistance

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Nearly 90% of lethal antibiotic-resistant infections in the United States are caused by Gram-positive pathogens, with *Staphylococcus aureus* accounting for more than one-half of these. Antibiotic resistance is often encoded by plasmids and integrative elements that are exchanged between bacteria through conjugative DNA transfer. During conjugation, a relaxase protein binds, nicks, and covalently attaches to the 5′-end of the DNA, guiding it to the recipient cell, where it restores its circular closed form. We show that relaxase MobM from the promiscuous plasmid pMV158 uses a hitherto unseen mechanism for DNA nicking/closing that is based on the formation of a protein-DNA phosphoramidate adduct. Moreover, our analysis reveals that MobM-like histidine relaxases account for 85% of all relaxases in *S. aureus* isolates. (See pp. E6526–E6535.)

Unique structural features of the AIPL1–FKBP domain that support prenyl lipid binding and underlie protein malfunction in blindness

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Mutations in the gene encoding aryl hydrocarbon receptor-interacting protein-like 1 (AIPL1) disrupt the ability of this protein to function as a chaperone of prenylated photoreceptor phosphodiesterase 6, and cause a severe form of childhood blindness. Our discovery of two features—the unique structure of the AIPL1–FKBP domain essential for its binding of prenyl lipids, and the unusual conformational dynamics altered by pathogenic mutations—advances our understanding of both the protein structure and dynamics required for prenyl binding. Moreover, our studies provide a molecular mechanism underlying the blindness disease. (See pp. E6536–E6545.)

Protein diversity in discrete structures at the distal tip of the trypanosome flagellum

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The distal end of the eukaryotic flagellum/cilium has critical functions, yet due to its small dimensions and association of tip structures with the axoneme is rather intractable to studying. We have developed biochemical approaches to identify a cohort of proteins specific for the flagellum tip structures. We sublocalized these proteins into individual structures. Using functional studies, we elucidated how the identified proteins contribute to the function of the flagella connector, the mobile membrane junction at the tip of the trypanosome flagellum. (See pp. E6546–E6555.)

CDCP1 drives triple-negative breast cancer metastasis through reduction of lipid-droplet abundance and stimulation of fatty acid oxidation

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Approximately 34% of triple-negative breast cancer (TNBC) patients relapse with local recurrence or metastasis within 5 y of radiation and chemotherapy treatment. There are currently no targeted therapies to treat TNBC. This study offers therapeutic targets for blocking TNBC metastasis: cell-surface antigen CUB-domain containing protein 1 (CDCP1) and proteins in the lipid metabolism pathway. CDCP1 regulates lipid metabolism by reducing cytoplasmic lipid droplet abundance, stimulating fatty acid oxidation and oxidative phosphorylation. This metabolic pathway likely contributes to the energy production required for cell migration and metastasis of TNBC, and represents a potential therapeutic target. (See pp. E6556–E6565.)

Embryo implantation evolved from an ancestral inflammatory attachment reaction

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Our data suggest that implantation in eutherians is derived from an ancestral inflammatory reaction to embryo attachment in the therian ancestor. These results explain the paradoxical role of inflammation at the beginning and the end of pregnancy in humans: Inflammation is necessary for implantation and parturition, but for most of pregnancy, inflammation threatens the continuation of pregnancy. We argue that the role of inflammation during implantation is an ancestral response to the embryo as a foreign body. By changing the way investigators think about implantation, we expect this research to contribute to new ways to study and treat implantation disorders, the most vulnerable step of assisted reproductive technology, in women. (See pp. E6566–E6575.)

Two functionally distinct E2/E3 pairs coordinate sequential ubiquitination of a common substrate in *Caenorhabditis elegans* development

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Ubiquitination—the covalent attachment of ubiquitin to substrates—is a posttranslational modification that regulates virtually every aspect of cellular function in eukaryotes. The final step of substrate ubiquitination requires the coordination of two types of enzymes: ubiquitin-conjugating enzymes (E2s) and ubiquitin ligases (E3s). Whereas E3s can function with different E2s, coordination between E3s has been reported only for E3s of the same class. Here we

show that two distinct E2/E3 pairs (UBC-18/ARI-1 and UBC-3/CUL-1) coordinate to ubiquitinate a common substrate and regulate its steady-state levels in *C. elegans*. Failure to regulate the substrate's levels leads to a serious developmental defect and lethality in worms. Our work provides evidence that cross-talk between two classes of E3s and their respective dedicated E2s occurs in an organism. (See pp. E6576–E6584.)

Cortical actin recovery at the immunological synapse leads to termination of lytic granule secretion in cytotoxic T lymphocytes

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Cytotoxic T lymphocytes (CTLs) destroy virally infected and tumor cells through the directed secretion of specialized lysosomes called "lytic granules." Because a single CTL must direct its cytotoxic activities only against specific targets but can sequentially kill multiple cells, granule secretion must be tightly controlled. We demonstrate here that dynamic regulation of the cortical actin cytoskeleton is critical for both the initiation and termination of secretion. We further link actin dynamics with phosphatidylinositol 4,5-bisphosphate (PIP_2) levels and provide evidence that lytic granule delivery initiates cortical actin recovery and the cessation of secretion. Our results suggest that actin both regulates and is regulated by secretion, providing a mechanism by which CTLs control their ability to kill targets serially during immune responses. (See pp. E6585–E6594.)

Protein nanocages that penetrate airway mucus and tumor tissue

Xinglu Huang, Jane Chisholm, Jie Zhuang, Yanyu Xiao, Gregg Duncan, Xiaoyuan Chen, Jung Soo Suk, and Justin Hanes

In designing new nanoparticle drug delivery systems, it is critical to identify simple formulations that overcome multiple biological barriers while being safe, reproducible, and scalable. We modified human ferritin nanocages using a unique PEGylation strategy, which provides a highly uniform, stable, and compact nanocarrier platform capable of overcoming multiple biological barriers, specifically penetration of airway mucus and tumor tissue, selective uptake by cancer cells, and drug release triggered only upon cell uptake. Surprisingly, PEGylation of ferritin to overcome the mucus barrier did not interfere with the ability of the nanocages to form particles, penetrate tumor tissues, and enter cells. Proof-of-concept of the system is provided in the treatment of an aggressive orthotopic model of lung cancer. (See pp. E6595–E6602.)

Rho-associated kinase is a therapeutic target in neuroblastoma

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Despite intensive therapy, the cure rate for children diagnosed with high-risk neuroblastoma is still below 50%, accentuating the need for more effective therapies. Recurrent somatic mutations are relatively infrequent in neuroblastoma. We show that approximately 30% of neuroblastoma contains mutations in genes regulating Rho/Rac signaling. The mutations may be associated with activation of downstream Rho-associated kinases (ROCKs). High ROCK2 expression is associated with poor patient survival. Inhibition of ROCK activity suppresses the growth of neuroblastoma in preclinical in vivo models. ROCK inhibitors induce differentiation of neuroblastoma cells partly by glycogen synthase kinase 3β-mediated phosphorylation and degradation of MYCN proteins. These findings suggest that inhibitors of ROCK may represent a therapeutic opportunity for children with high-risk neuroblastoma. (See pp. E6603–E6612.)

Mitogenic stimulation accelerates influenza-induced mortality by increasing susceptibility of alveolar type II cells to infection

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Influenza is a recurring global health threat that preferentially targets vulnerable groups such as the very young, the pregnant, the elderly, and the infirm. The spread of influenza A virus (IAV) from the epithelium of the conducting airway to the alveolar epithelium is a pivotal event in the pathogenesis of primary viral pneumonia. Host susceptibility to IAV pneumonia is often attributed to altered immunity, and cell autonomous vulnerability states of the alveolar epithelium, such as proliferative tone, are rarely considered. Here we demonstrate that mitogenic stimulation of alveolar epithelial type II cells renders them susceptible to IAV infection in an mTOR-dependent manner. (See pp. E6613–E6622.)

Therapeutically targeting glypican-2 via single-domain antibody-based chimeric antigen receptors and immunotoxins in neuroblastoma

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Neuroblastoma is a childhood cancer that remains an important clinical challenge. It is fatal in almost half of the patients despite advances in multimodal treatments. In this report, we show that the cell surface glycoprotein glypican-2 (GPC2) is overexpressed in neuroblastomas when compared with normal tissues and that a high expression level is correlated with poor survival of neuroblastoma. We also describe that GPC2 has proliferative effects in neuroblastoma via activating Wnt signaling and its downstream target genes including N-Myc, a major driver for neuroblastoma tumorigenesis. We have produced the immunotoxins and chimeric antigen receptor T cells that target GPC2 and exhibit promising antitumor activities in cell and mouse models. This study suggests GPC2 as a promising target in neuroblastoma. (See pp. E6623–E6631.)

Nontypeable *Haemophilus influenzae* releases DNA and DNABII proteins via a T4SS-like complex and ComE of the type IV pilus machinery

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Extracellular DNA and DNABII proteins are essential structural components of the extracellular polymeric substance, or matrix, of the nontypeable *Haemophilus influenzae* biofilm; however, the mechanisms by which these elements are released from the bacterial cell for incorporation into the biofilm matrix are not yet characterized. Here, we propose a mechanism for active DNA release during biofilm formation that involves an inner-membrane complex (TraCG) and the ComE pore through which the type IV pilus is typically expressed. Knowledge of how and when DNA and DNABII proteins are released into the extracellular

milieu for integration into the biofilm matrix will further our understanding of biofilm formation and maturation and, in turn, guide development of directed therapies for diseases with a biofilm etiology. (See pp. E6632–E6641.)

HEMO, an ancestral endogenous retroviral envelope protein shed in the blood of pregnant women and expressed in pluripotent stem cells and tumors

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Endogenization of retroviruses has occurred multiple times in the course of vertebrate evolution, with the captured retroviral envelope syncytins playing a role in placentation in mammals, including marsupials. Here, we identify an endogenous retroviral envelope protein with unprecedented properties, including a specific cleavage process resulting in the shedding of its extracellular moiety in the human blood circulation. This protein is conserved in all simians—with a homologous protein found in marsupials—with a "stemness" expression in embryonic and reprogrammed stem cells, as well as in the placenta and some human tumors, especially ovarian tumors. This protein could constitute a versatile marker—and possibly an effector—of specific cellular states and being shed, be immunodetected in the blood. (See pp. E6642–E6651.)

Antibacterial photosensitization through activation of coproporphyrinogen oxidase

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Skin and soft tissue infections (SSTIs) account for a majority of visits to hospitals and clinics in the United States and are typically caused by Gram-positive pathogens. Recently, it was discovered that Gram-positive bacteria use a unique pathway to synthesize the critical cellular cofactor heme. The divergence of the heme biosynthesis pathways between humans and Gram-positive bacteria provides a unique opportunity for the development of new antibiotics targeting this pathway. We report here the identification of a small-molecule activator of coproporphyrinogen oxidase (CgoX) from Gram-positive bacteria that induces accumulation of coproporphyrin III and leads to photosensitization of Gram-positive pathogens. In combination with light, CgoX activation reduces bacterial burden in murine models of SSTI. (See pp. E6652–E6659.)

Thalamocortical synchronization during induction and emergence from propofol-induced unconsciousness

Francisco J. Flores, Katharine E. Hartnack, Amanda B. Fath, Seong-Eun Kim, Matthew A. Wilson, Emery N. Brown, and Patrick L. Purdon

General anesthesia is a drug-induced state of altered arousal associated with profound, stereotyped electrophysiological oscillations. Here we report evidence in rats that propofol, an anesthetic drug frequently used in clinical practice, disrupts activity in medial prefrontal cortex and thalamus by inducing highly synchronized oscillations between these structures. These oscillations closely parallel human electroencephalogram oscillations under propofol. Disruption of activity in medial prefrontal cortex by these oscillations implies an impairment of self-awareness and internal consciousness. During recovery of consciousness, these synchronized oscillations dissipate in a "boot-up" sequence most likely driven by ascending arousal centers. These studies advance our understanding of what it means to be unconscious under anesthesia and establish principled neurophysiological markers to monitor and manage this state. (See pp. E6660–E6668.)

SIK3–HDAC4 signaling regulates *Drosophila* circadian male sex drive rhythm via modulating the DN1 clock neurons

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Physiology and behavior are subject to daily cycles. In *Drosophila melanogaster*, two clusters of clock neurons—morning (M cells) and evening (E cells) oscillators—are largely responsible for activity bursts at dawn and dusk. In contrast, male–female pairs of flies follow a distinct pattern: low activity at dusk, followed by male courtship activity during the night, referred to as "male sex drive rhythm" (MSDR). Here we report that males lacking Salt-inducible kinase 3 (SIK3) expression in M cells exhibit a short period of MSDR but a long period of single-fly locomotor rhythm (SLR) because circadian nucleocytoplasmic shuttling of Histone deacetylase 4 (HDAC4) is disrupted. We conclude that SIK3–HDAC4 signaling in M cells regulates MSDR by regulating the molecular oscillation in DN1 neurons. (See pp. E6669–E6677.)

Structural organization of the actin-spectrin–based membrane skeleton in dendrites and soma of neurons

Boran Han, Ruobo Zhou, Chenglong Xia, and Xiaowei Zhuang

Actin, spectrin, and associated molecules form a quasi-1D periodic membrane skeleton in neurons, which organizes membrane proteins in periodic distributions and provides mechanical stability for axons. Here, we provide detailed quantifications of this periodic structure in neurons and show that it develops substantially more slowly in dendrites than in axons. Moreover, we observed a 2D, polygonal lattice structure of these molecules in the somatodendritic compartment. The diverse structural organizations and different developmental courses of the membrane skeleton in different neuronal compartments suggest the membrane skeleton is differentially regulated across these neuronal compartments. The observation of the polygonal lattice structure in cells in addition to erythrocytes suggests a potentially general presence of this structure across diverse cell types. (See pp. E6678–E6685.)

SUMOylation determines the voltage required to activate cardiac I_{Ks} channels

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The slow delayed rectifier K^+ current (I_{Ks}) determines the length of each human heartbeat because it activates after myocytes are excited and depolarize. This sensitivity to voltage, as well as dynamic regulation by hormones and second messengers, underlies the essential role of I_{Ks} in determining the duration and rhythm of cardiac action potentials. Here, we demonstrate the unexpected mechanism that establishes the voltage-dependent operation of I_{Ks} channels: SUMOylation. When native I_{Ks} channels are resident in the plasma membranes of neonatal mouse ventricular myocytes, or human channels are reconstituted in CHO cells, each of the four KCNQ1 pore-forming subunits is subject to monoSUMOylation in a manner that depends on KCNE1 accessory subunits, leading to stepwise depolarizing shifts in the activation voltage. (See pp. E6686–E6694.)

PIF3 is a negative regulator of the *CBF* pathway and freezing tolerance in *Arabidopsis*

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PHYTOCHROME-INTERACTING FACTORS (PIFs) are central integrators of plants' responses to various environmental signals. In this study, we show that PIF3 acts as a negative regulator of plant cold acclimation by directly repressing the expression of *CBF* genes, whereas its protein stability is negatively regulated by two F-box proteins, EBF1 and EBF2, via the 26S proteasome pathway. Moreover, EBF1 and EBF2 are degraded under cold stress, which enhances the stability of PIF3 protein. Collectively, our study establishes an important regulatory paradigm for PIF3 in preventing runaway expression of the *CBF* genes at low temperature, which allows plants to adapt to and withstand harsh environments. (See pp. E6695–E6702.)

Stem parasitic plant *Cuscuta australis* (dodder) transfers herbivory-induced signals among plants

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Cuscuta spp. (i.e., dodders) are plant parasites that connect to the vasculature of their host plants to extract water, nutrients, and even macromolecules. Knowledge of ecologically meaningful communications between host plants and *Cuscuta*, or between *Cuscuta* bridge-connected hosts, has remained obscure until now. Here we show that herbivore attack on one of the *Cuscuta* bridge-connected plants induces gene expression and increases the activity of trypsin proteinase inhibitors, and thus elevates the resistance to insects in other undamaged but *Cuscuta*-connected plants. This *Cuscuta*-mediated interplant signaling is rapid, conserved, far-reaching, and partly requires the plant hormone jasmonic acid. Although *Cuscuta* parasites can negatively influence their host plants, under certain circumstances, they may also provide ecologically relevant information-based benefits. (See pp. E6703–E6709.)

LEC1 sequentially regulates the transcription of genes involved in diverse developmental processes during seed development

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Seed development is biphasic, consisting of the morphogenesis phase when the basic plant body plan is established and the maturation phase when the embryo accumulates storage reserves and becomes desiccation tolerant. Despite the importance of seeds as human food and animal feed, little is known about the gene-regulatory networks that operate during these phases. We identified genes that are regulated genetically and transcriptionally by a master regulator of seed development, LEAFY COTYLEDON1 (LEC1). We show that LEC1 transcriptionally regulates genes involved in photosynthesis and other developmental processes in early and maturation genes in late seed development. Our results suggest that LEC1 partners with different transcription factors to regulate distinct gene sets and that LEC1 function is conserved in *Arabidopsis* and soybean seed development. (See pp. E6710–E6719.)

The critical phase for visual control of human walking over complex terrain

Jonathan Samir Matthis, Sean L. Barton, and Brett R. Fajen

The physical dynamics of the body are central to the generation and maintenance of the human gait cycle. The ability to exploit the force of gravity and bodily inertia increases the energetic efficiency of locomotion by minimizing the need for internally generated muscular forces and simplifies control by obviating the need to actively guide each body segment. Here we explore how these principles generalize to situations in which foot placement is constrained, as when walking over a rocky trail. Walkers can exploit external forces to efficiently traverse extended stretches of complex terrain provided that visual information about the upcoming ground surface is available during a particular (critical) phase of the gait cycle between midstance of the preceding step and toe-off. (See pp. E6720–E6729.)