PNAS Plus Significance Statements

Reducing posttreatment relapse in cleft lip palatal expansion using an injectable estrogen–nanodiamond hydrogel

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Patients with cleft lip and/or palate require palatal expansion to develop normal speech and fully functional occlusion. However, the expanded palate has a strong tendency to rebound to its original shape due to the patient's congenital lack of bone and diminished capability of bone regeneration in the cleft site. We propose an innovative method to combat this clinical challenge by utilizing estrogen, 17β-estradiol (E2), which has proven bone-building properties, within a nanodiamond–hydrogel (ND/G) complex vehicle. Our study shows that this targeted administration of E2 is able to markedly reduce postexpansion relapse. The demonstrated biocompatibility and efficacy of the E2/ND/G platform makes this a clinically promising solution in craniofacial care of patients. (See pp. E7218–E7225.)

Structural and functional studies of pyruvate carboxylase regulation by cyclic di-AMP in lactic acid bacteria

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Cyclic di-3′,5′-adenosine monophosphate (c-di-AMP) is a broadly conserved bacterial second messenger that has been implicated in a wide range of cellular processes. We report here structural, biochemical, and functional studies on the inhibition of *Lactococcus lactis* pyruvate carboxylase (LlPC) by c-di-AMP. The compound has a distinct binding mode in LlPC compared with that in *Listeria monocytogenes* PC. Mutations of residues in the binding site can abolish c-di-AMP inhibition. LlPC is required for efficient milk acidification through its essential role in aspartate biosynthesis. The aspartate pool in *L. lactis* is negatively regulated by c-di-AMP, and high aspartate levels can be restored by a c-di-AMP–insensitive LlPC. LlPC has high intrinsic catalytic activity and is insensitive to acetyl-CoA activation, in contrast to other PCs. (See pp. E7226–E7235.)

Fission yeast myosin Myo2 is down-regulated in actin affinity by light chain phosphorylation

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The separation of daughter cells during cell division, or cytokinesis, is a process that requires contractile rings which develop tension using actin and myosin. Current models of contractile ring dynamics are based on quantitative data from two decades of research using the tractable fission yeast system. However, it is unknown how fission yeast's essential myosin, Myo2, is regulated in the contractile ring. Here, we find that Myo2 does not assemble into minifilaments, consistent with its role in ring precursor nodes. Unphosphorylated Myo2 exhibits robust enzymatic and motor activity whereas phosphorylation of Myo2's regulatory light chain reduces its affinity for actin. This reduction likely weakens the tension in the contractile ring, potentially to delay cytokinesis until segregation of chromosomes is complete. (See pp. E7236–E7244.)

Kinetic and high-throughput profiling of epigenetic interactions by 3D-carbene chip-based surface plasmon resonance imaging technology

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In the era of functional proteomics, a myriad of new interactions, notably those modification-dependent ones, are widely suggested by advanced proteomic approaches and bioinformatic analysis. Therefore, there exists an urgent need to develop a technology for high-throughput mapping and quantitative characterization of biomolecular binding events. This study achieved the immobilization and kinetic detection of various biomacromolecules (including modified peptides and modified nucleic acids) in high throughput through the 3D-carbene chip-based surface plasmon resonance imaging (SPRi) technology. Modified histone peptides and nucleic acids, which are key epigenetic marks, could be efficiently probed by this platform. We envision that the 3D-carbene SPRi technology described here will have wide appeal in profiling and discovering biological recognitions in and beyond epigenetics. (See pp. E7245–E7254.)

Elucidating crosstalk mechanisms between phosphorylation and O-GlcNAcylation

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Nearly all proteins are posttranslationally modified, a phenomenon known to alter protein function. Recently, multiple posttranslational modifications (PTMs) have been documented to exist on the same proteins, revealing an additional level of complexity (named "PTM crosstalk") that, due to its dynamic nature, is challenging

to predict. Here, we propose a motif for PTM crosstalk between two of the most common PTMs: phosphorylation and O-GlcNAcylation. Through the use of a kinetic-based high-resolution mass spectrometry assay, we highlight specific residues that, when phosphorylated, hamper O-GlcNAcylation at nearby sites. In addition, we show that the Ser/Thr residues in one of the most common kinase motifs, PX(S/T)P, cannot be O-GlcNAcylated, demonstrating that reciprocal PTM crosstalk does not occur with Pro-directed kinases. (See pp. E7255–E7261.)

Two transmembrane dimers of the bovine papillomavirus E5 oncoprotein clamp the PDGF β receptor in an active dimeric conformation

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Highly specific protein–protein interactions between transmembrane domains play crucial roles in many biological processes, but are difficult to study because they occur within membranes. The E5 protein of bovine papillomavirus is a 44-residue transmembrane protein that transforms cells by binding the transmembrane domain of the PDGF receptor, resulting in receptor activation. By combining computational modeling, genetic analysis, and biochemical studies, we propose a quaternary structure of the complex between the E5 protein and the PDGF receptor, in which two dimers of the E5 protein clamp two molecules of the receptor transmembrane domain into an active dimeric conformation. These studies reveal the molecular mechanism of action of an unusual oncogene and provide a pathway to study biologically interesting transmembrane complexes. (See pp. E7262–E7271.)

Mathematical model reveals role of nucleotide signaling in airway surface liquid homeostasis and its dysregulation in cystic fibrosis

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The intrapulmonary airways conduct air to the alveoli and are defended from inhaled pathogens by a highly regulated protective system of mucus, cilia, and liquid. In healthy lungs, a well-hydrated mucus layer is cleared by cilia from airway surfaces. In cystic fibrosis (CF), airway surfaces are dehydrated, leading to a failure of cilia-mediated mucus clearance and accumulation of pathogen-infected mucus. In this study, we created a mathematical model of airway surface liquid regulation in normal and CF cells and used the model to investigate a potential therapy to rehydrate CF airways and restore proper mucus clearance. (See pp. E7272–E7281.)

Genomic evidence reveals a radiation of placental mammals uninterrupted by the KPg boundary

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We produced a genome-scale dataset from representatives of all placental mammal orders to infer diversification timing relative to the Cretaceous–Paleogene (KPg) boundary. Our sensitivity analyses show that divergence time estimates within placentals are considerably biased by the specific way in which a given dataset is processed. We examined the performance of various dating approaches using a comprehensive scheme of likelihood analyses and computational simulations, allowing us to identify the optimal molecular clock parameters, gene sets, and gene partitioning schemes for reliable dating. Based on the optimal methodology, we present a hypothesis of mammalian divergence timing that is more consistent with the fossil record than previous molecular clock reconstructions, suggesting that placental mammals underwent a continuous radiation across the KPg boundary. (See pp. E7282–E7290.)

Feedback amplification loop drives malignant growth in epithelial tissues

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Progression of epithelial tumors and successful colonization of target tissues rely in many cases on the presence of the tumor microenvironment (TME), which acts as a niche to provide secreted signaling molecules and growth factors to tumor cells. Here we used *Drosophila* to show that the TME is not an absolute requirement for the growth of epithelial tumors caused by chromosomal instability (CIN) or compromised cell polarity. Instead, tumor growth is driven by a feedback amplification loop between two well-defined—but not necessarily genetically different—tumor cell populations. As CIN or impaired cell polarity are frequently observed in human tumors of epithelial origin, our results will provide insight to the mechanistic understanding of their unlimited growth potential. (See pp. E7291–E7300.)

In vivo loss-of-function screens identify KPNB1 as a new druggable oncogene in epithelial ovarian cancer

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The poor prognosis of epithelial ovarian cancer (EOC) has not improved for several decades because of drug resistance to current anticancer drugs. Furthermore, few molecularly targeted agents are effective for EOC, likely because EOC has high tumor heterogeneity. Discovering new drug targets and mechanisms involved in the progression of EOC is therefore sorely needed. Our multiple CRISPR and RNAi-based in vivo loss-of-function screens have identified multiple new EOC candidate drug targets, including the druggable oncogene KPNB1, whose inhibition caused multiphased cell cycle arrest and induced apoptosis. Ivermectin, a Food and Drug Administration-approved antiparasitic drug, exerts KPNB1-dependent antitumor effects and synergistically inhibits tumor growth in combination with paclitaxel, and therefore represents a new potential combinatorial therapy for EOC through drug repositioning. (See pp. E7301–E7310.)

Phosphoantigen-induced conformational change of butyrophilin 3A1 (BTN3A1) and its implication on Vγ9Vδ2 T cell activation

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Gamma delta T cells, a group of immune cells that exhibit features from both innate and adaptive immunity, possess significant potential in clinical applications such as treatment of microbial infections and cancer immunotherapy. To fully understand their biology and harness them in the clinic it is imperative to dissect the molecular mechanisms involved in their recognition of infected and tumor cells. In this paper we focus on Vγ9Vδ2 T cells, a major subset of human gamma delta T cells in blood and investigate the phosphoantigen-induced,

MHC-independent molecular mechanisms governing their activation. (See pp. E7311–E7320.)

Multiplex, quantitative cellular analysis in large tissue volumes with clearing-enhanced 3D microscopy (C_e3D)

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Major biological processes rely on the precise positioning of diverse cell types in specific anatomical locations. Existing techniques for studying cellular spatial positioning in tissues, especially with robust identification of densely packed cells, have substantial time, cost, resolution, and multiplexing limitations. Here, we describe an easy-to-use and inexpensive tissue clearing technique for attaining high-quality images of cells and diverse molecules of interest in substantial tissue volumes, enabling simultaneous quantitative analysis of 3D organ structure and fine-grained cellular composition. This technology will enhance our capacity for acquiring a quantitative understanding of the relationships between cells and their microenvironments in the context of broader tissue organization and is directly applicable to diverse biological disciplines as well as diagnostic medicine. (See pp. E7321–E7330.)

Antitumor effect of *Batf2* through IL-12 p40 up-regulation in tumor-associated macrophages

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The therapeutic activity of checkpoint blockers and toll-like receptor (TLR) agonists, which show some efficacy against malignancies, appears to at least partially result from the secretion of type-I IFNs. Thus, we hypothesized that type-I IFN-inducible transcription factors, such as *basic leucine zipper transcription factor ATF-like 2* (*Batf2*), might play a role in tumor immunity. Here, we investigated the role of *Batf2*, especially its positive transcriptional activities, and evaluated its antitumor effect. This study shows that *Batf2* has an antitumor effect through the up-regulation of IL-12 p40 in tumor-associated macrophages, which eventually induces the activation of $CD8^+$ T cells and their accumulation within the tumor. *Batf2* may be an important target in anticancer treatment with immune checkpoint blockers and TLR agonists. (See pp. E7331–E7340.)

De novo mutations in inhibitors of Wnt, BMP, and Ras/ERK signaling pathways in non-syndromic midline craniosynostosis

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Craniosynostosis is a common congenital malformation resulting from premature fusion of the bones that comprise the cranial vault, requiring surgery in infancy to prevent adverse neurologic outcomes. Eighty-five percent of cases are non-syndromic and of unknown cause. By exome sequencing of families with non-syndromic midline craniosynostosis, we show that 5% of cases have de novo damaging mutations in negative regulators of the Wnt, bone morphogenetic protein (BMP), and Ras/ERK signaling pathways, developmental cascades that converge on common nuclear targets to promote bone formation. Another 5% have transmitted mutations in these pathways. Common variants near *BMP2* show genetic interaction with these rare mutations. The results provide insight into pathophysiology and have immediate implications for the diagnosis and genetic counseling of families with craniosynostosis. (See pp. E7341–E7347.)

Immunogenicity and structures of a rationally designed prefusion MERS-CoV spike antigen

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Coronaviruses such as Middle East respiratory syndrome coronavirus (MERS-CoV) cause severe respiratory distress with high fatality rates. The spike (S) glycoprotein is a determinant of host range and is the target of neutralizing antibodies and subunit vaccine development. We describe an engineering strategy for stabilization of soluble S proteins in the prefusion conformation, which results in greatly increased expression, conformational homogeneity, and elicitation of potent antibody responses. Cryo-EM structures of the stabilized MERS-CoV S protein in complex with a stem-directed neutralizing antibody provide a molecular basis for host-cell protease requirements and identify a site of immune pressure. We also defined four conformational states of the trimer wherein each receptor-binding domain is either packed together at the membrane-distal apex or rotated into a receptor-accessible conformation. (See pp. E7348–E7357.)

Recruitment of CRISPR-Cas systems by Tn7-like transposons

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CRISPR-Cas is an adaptive immunity system that protects bacteria and archaea from mobile genetic elements. We present comparative genomic and phylogenetic analysis of minimal CRISPR-Cas variants associated with distinct families of transposable elements and develop the hypothesis that such repurposed defense systems contribute to the transposable element propagation by facilitating transposition into specific sites. Thus, these transposable elements are predicted to propagate via RNA-guided transposition, a mechanism that has not been previously described for DNA transposons. (See pp. E7358–E7366.)

KCNE1 and KCNE3 modulate KCNQ1 channels by affecting different gating transitions

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Regulatory β-subunits associate with voltage-gated K^+ channels to modulate their biophysical properties and physiological roles. KCNE1 and KCNE3 β-subunits turn voltage-dependent KCNQ1 channels into delayed activating KCNQ1/KCNE1 channels and apparent voltage-independent KCNQ1/KCNE3 channels, respectively, which are important for cardiac action potentials and transport of water and salts across epithelial cells. Mutations in KCNQ1/KCNE1 and KCNQ1/KCNE3 channels are associated with diseases, such as cardiac arrhythmias, congenital deafness, secretory diarrhea, and tinnitus. Therefore, KCNQ1, KCNE1, and KCNE3 are potential drug targets. We here propose a model for how KCNE1 and KCNE3 differentially modulate KCNQ1 that will allow for a better understanding of how mutations in KCNQ1, KCNE1, and KCNE3 cause diseases and how to design drugs to treat these diseases. (See pp. E7367–E7376.)

A protein complex regulates RNA processing of intronic heterochromatin-containing genes in *Arabidopsis*

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How heterochromatin affects RNA processing is unclear. The chromatin regulators ASI1 and EDM2 function in regulating alternative polyadenylation at genes with intronic heterochromatin. We found that ASI1 and EDM2 are associated in planta through interactions with a putative RNA-binding protein, AIPP1. Protein interaction assays suggest that the RNA Pol II C-terminal domain phosphatase CPL2 and two other proteins (AIPP2 and AIPP3) are associated with the ASI1-AIPP1-EDM2 complex. Like ASI1 and EDM2, AIPP1 also functions in promoting the expression of heterochromatin-containing genes. However, the function of CPL2, AIPP2, and AIPP3 is antagonistic to that of ASI1, EDM2, and AIPP1. Our discovery of the ASI1-AIPP1-EDM2 complex and associated proteins is important for understanding how heterochromatin regulates RNA processing. (See pp. E7377–E7384.)

Signaling from the plasma-membrane localized plant immune receptor RPM1 requires self-association of the full-length protein

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Pathogen recognition first occurs at the plasma membrane, where receptor-like kinases perceive pathogen-derived molecules and initiate immune responses. To abrogate this immune response, pathogens evolved effector proteins that act as virulence factors, often following delivery to the host cell. Plants evolved intracellular receptors, known as NOD-like receptors (NLRs), to detect effectors, thereby ensuring activation of effector-triggered immunity. However, despite their importance in immunity, the molecular mechanisms underlying effector recognition and subsequent immune activation by membrane-localized NLRs remain to be fully elucidated. Our analyses reveal the importance of and need for self-association and the coordinated interplay of specific domains and conserved residues for NLR activity. This could provide strategies for crop improvement, contributing to effective, environmentally friendly, and sustainable solutions for future agriculture. (See pp. E7385–E7394.)

Separate mesocortical and mesolimbic pathways encode effort and reward learning signals

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Learning about multiple features of a choice option is crucial for optimal decision making. How such multiattribute learning is realized remains unclear. Using functional MRI, we show that the brain exploits separate mesolimbic and mesocortical networks to simultaneously learn about reward and effort attributes. We show a double dissociation, evident in the expression of effort learning signals in dorsomedial prefrontal and reward learning in ventral striatal areas, with this dissociation being spatially mirrored in dopaminergic midbrain. At the time of choice, these segregated signals are integrated in ventral striatum. These findings highlight how the brain parses parallel learning demands. (See pp. E7395–E7404.)