2014: Signaling Breakthroughs of the Year

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The 2014 breakthroughs fell into four main areas: innate immunity, host-microbe interactions, cell death signaling, and methodological advances in the study of cell signaling. Nominations included new discoveries about signaling in innate immune cells, innate immune functions for lymphoid and nonhematological cells, and the importance of host-microbe interactions for the regulation of host physiology. Also this year, we received nominations highlighting molecular mechanisms by which p53 contributes to the pathology of chronic inflammation and how signaling pathways mediate programmed necrotic cell death. Finally, 2014 saw the use of new techniques to study cell signaling and identify drug targets, such as the in vivo use of RNA interference to study signaling in T cells and new computational methods to study large datasets of different data types.

We invited nominations from our Board of Reviewing Editors for this 13th edition of “Signaling Breakthroughs of the Year” and received recommendations largely focusing on four main areas: innate immunity, host-microbe interactions, cell death, and methodological advances in the study of cell signaling.

This year’s contributors included Mark Ansel (University of California San Francisco, USA), Henrik Dohlman (University of North Carolina Chapel Hill, USA), George Dubyak (Case Western Reserve University, USA), Kevin Janes (University of Virginia Charlottesville, USA), Rune Linding (University of Copenhagen, Denmark), Samuel Miller (University of Washington Seattle, USA), Gabriel Núñez (University of Michigan Health System, USA), Ute Römling (Karolinska Institute, Sweden), John Silke (Walter and Eliza Hall Institute of Medical Research, Australia), and Eric Vivier (INSERM-CNRS-Université Méditerranée, France).

The immune system was a hot topic for 2014. Recognition of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) can occur through cell surface and endosomal receptors, such as TLRs (Toll-like receptors), or through cytosolic protein complexes known as inflammasomes. Inflammasomes are typically found in myeloid lineage cells, including monocytes, macrophages, and dendritic cells. Most characterized inflammasomes comprise the protease caspase 1, the adaptor protein ASC, and one of several molecular pattern recognition sensors, such as members of the nucleotide-binding oligomerization domain (NOD)–like family of receptors (NLRs), including NLRP3. Stimulation of inflammasome sensors by PAMPs or DAMPs promotes caspase activation, leading to the production of inflammatory cytokines, such as interleukin (IL)–1β and IL-18, and programmed cell death (pyroptosis). Excess activation of this process can lead to septic shock and death.

In addition, there are “noncanonical” inflammasomes, which contain other caspases and can be experimentally activated independently from canonical inflammasomes using distinct stimuli. Noncanonical inflammasomes in mice contain caspase 11, homologous to human caspase 4 and caspase 5, or caspase 8. In primary mouse macrophages, deficiency for caspase 11 inhibits activation of caspase 1 and IL-1β production in response to infection by several species of Gram-negative bacteria. Unlike caspase 1, activation of caspase 11 does not require NLRs or ASC. Moreover, caspase 11 can elicit macrophage pyroptosis independently of caspase 1, suggesting that there are multiple pathways downstream of noncanonical inflammasome activation.

Lipopolysaccharide (LPS) is an abundant structural molecule in the outer membrane of Gram-negative bacteria that stimulates TLR4 on the surface of host immune cells. However, mice deficient for caspase 11 are resistant to a lethal dose of LPS, suggesting that noncanonical inflammasome activation is required to mount a full immune response to LPS. Miller and Núñez both nominated a study by Shi et al. (1) showing that LPS directly binds to mouse caspase 11, or human caspase 4 or caspase 5, leading to caspase oligomerization and activation. Thus, this study provides a mechanism showing how caspases can function as pattern recognition receptors and be activated independently of NLRs.

A major function of macrophages and dendritic cells is the phagocytosis of pathogens before they can infect cells. Few of the internalized pathogens actually reach the cytosol; instead, the pathogens are sequestered in endosomes. So, an open question has been how these sequestered pathogens activate NLRs in the cytosol. Nakamura et al. (2) show that two peptide transporters, SLC15A3 and SLC15A4, present in the endosomes of dendritic cells and macrophages, enable MDP to escape endosomes and recruit NOD2-containing signaling complexes to these endosomes (Fig. 1), thereby facilitating inflammasome activation.

Small proteins in the blood form the complement component of the innate immune system. During pathogenic stimulation, these proteins, such as complement C3, are cleaved to produce cytokines that amplify the inflammatory response. Tam et al. (3) show that during infection with virus or bacteria, complement C3 enters cultured nonimmune cells together with the pathogen. Intracellular C3 triggers both immune signaling and degradation of the internalized pathogen, suggesting that immune cells can use the presence of C3 to indicate infection and thereby induce a response without requiring recognition of specific ligands from pathogens.

Innate lymphoid cells (ILCs) constitute an important defense mechanism against infection, particularly in mucosal tissues. ILCs do not produce cytokines in response to activation of conventional antigen-specific receptors. In nominating a group of papers published in 2014 on ILCs, Vivier noted that “the field is literally exploding…. Harnessing ILC activity may lead to improved immunotherapy and vaccines.” Papers by Klose et al. (4), Constantinides et al. (5), Geiger et al. (6), and Serafini et al. (7) identify the precursor populations for ILCs and characterize the transcription factors required for the differ...
ferentiation of distinct subsets of these cells. The transcription factor RORγt (retinoid-related orphan receptor γt) is required for the development of the ILC3 subset, which produces the cytokines IL-22 and IL-17 in response to pathogenic bacterial infection in the intestine. Guo et al. (8) found that signaling through the transcription factor STAT3 (signal transducer and activator of transcription 3) in RORγt+ ILCs is required for these cells to produce IL-22 in mice infected with *Citrobacter rodentium*. Infection of mice with *C. rodentium* is a model for understanding the pathogenesis of *Escherichia coli* strains that cause diarrhea in humans. Drugs that suppress STAT3 activity are used in chemotherapy, and the findings of Guo et al. (8) suggest that these drugs could render patients susceptible to pathogen-induced diarrhea and may provide a possible explanation for one of the common side effects of the tyrosine kinase inhibitor sunitinib, which blocks the activity of STAT3.

Inflammasome-like protein complexes can function in cells outside the hematopoietic system to deter infection. Mucus produced by intestinal goblet cells serves as a physical barrier to separate potentially infectious organisms from the host intestinal epithelium. The outer layers of mucus are replete with both pathogenic and commensal bacteria, and much attention has focused on understanding how these organisms can influence host physiology. Mice deficient for NLRP6 have alterations in the intestinal microbiota. Wlodarska et al. (9) [see the Perspective by Chen and Stappenbeck (10)] showed that mice lacking NLRP6, caspase 1 and 11, or ASC had reduced ability to clear intestinal infection by *C. rodentium*. The amount of IL-1β and IL-18 and the number of neutrophils and T cells in the colon were normal in infected NLRP6-deficient mice. Nlrc4 and Asc were expressed in intestinal epithelial cells near the mucosal layer, and mice lacking these inflammasome components had reduced mucus production. In intestinal goblet cells in NLRP6-deficient mice, mucin granules failed to fuse with the membrane and undergo exocytosis and instead were present intact in the extracellular space (Fig. 2). Autophagy is important for secretion, and autophagy-associated proteins are found on mucin granules. Intestinal goblet cells from mice deficient in NLRP6, caspase 1 and 11, or ASC showed reduced autophagic flux. Thus, rather than playing a role in immune cells, NLRP6 appears to be required in intestinal goblet cells for autophagy that promotes mucus secretion.

Römling nominated a paper showing another example of how pathogen-associated stimuli can affect host physiology by acting outside the immune system. Bacteria use cyclic di-guanosine monophosphate (c-di-GMP) as a second messenger to regulate diverse processes, and the mammalian immune system has evolved mechanisms to detect c-di-GMP and mount an antibacterial response. Intriguingly, Lolicato et al. (11) found that c-di-GMP may affect cardiac function. The authors identified a binding site for c-di-GMP in hyperpolarization-activated cyclic nucleotide (HCN)-gated channels. The activity of these channels, which underlie the pacemaker current in the heart, is enhanced by cyclic adenosine monophosphate (cAMP), a nucleotide that is generated in response to various stimuli that increase heart rate (such as β-adrenergic stimulation). Application of c-di-GMP suppressed the ability of cAMP to increase HCN activity, leading the authors to characterize c-di-GMP as an means of counteracting β-adrenergic stimulation that could potentially be used therapeutically.

In addition to influences on basal physiology, changes in host-microbe interactions can promote disease. Inflammatory bowel disease is an autoimmune disorder that reduces the diversity of enteric commensal bacteria, leading to chronic inflammation and increased risk of colorectal cancer. Diets rich in carbohydrates also increase the risk of colon cancer. Belcheva et al. (12) report that changing the composition of intestinal microbiota with antibiotics or reducing dietary carbohydrates reduces poly-p formation in colons of mice genetically predisposed to colon cancer. Gut microbes convert dietary carbohydrates into metabolites, such as butyrate, that act on intestinal cells to enhance oncogenesis in these mice.

Two other papers [Fenalti et al. (13) and Pila et al. (14)] that made the Breakthroughs list this year exemplify how constituents of our diet affect our physiology. Dohlman noted that the crystal structures in these papers “provide a detailed picture of protein ‘sensors’ for physiological sodium and chloride” that may illustrate the mechanisms underlying the “connection between the consum-
tion of table salt (sodium chloride) and hypertension.” Fenalti et al. (13) discovered a partially hydrated sodium ion in the middle of the seven-transmembrane domains of the δ-opioid receptor (Fig. 3), reminiscent of several other class A G protein–coupled receptors. The sodium ion allosterically modifies the binding of a peptide agonist to the δ-opioid receptor, producing the “sodium effect” that has been used to differentiate opioid agonists from antagonists. The bound sodium ion also determines whether the receptor signals through Gs or β-arrestin. The second paper, by Piala et al. (14), provided a crystal structure of WNK1 [with no lysine (K)], a protein kinase that is involved in the regulation of chloride cotransporters and ion channels that help to determine blood pressure. Piala et al. found that chloride stabilized the inactive conformation of WNK1, preventing the kinase from phosphorylating itself and becoming activated. As Dohman explained, “These data provide a molecular explanation for how cells detect and respond to changes in intracellular chloride, and why mutations in WNK1 can lead to hypertension as occurs in pseudohypoaldosteronism II.”

Nitric oxide (NO) and the targeted modification of proteins through the reaction of NO with cysteines through a process called S-nitrosylation are important regulators of blood pressure and mediators of inflammatory responses. Inflammation leads to the production of NO, which is a free radical that can damage infectious microbes. In contrast, the targeted and specific modification of proteins by S-nitrosylation is a well-controlled regulatory event that affects the localization and function of modified proteins. Because NO is a highly reactive free radical, it has been unclear how site-specific S-nitrosylation occurs. Jia et al. (15) found that inflammatory agents increase the cytosolic concentration of calcium, which promotes the association of the inflammatory mediators S100A8 and S100A9, inducible nitric oxide synthase (iNOS), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in peripheral blood monocytes. The interaction with S100A8 is required for the site-specific transfer of NO from S100A9 to Cys247 of GAPDH, suggesting at least one mechanism by which site-specific S-nitrosylation may occur.

The targets of S-nitrosylation are diverse and may be modified by multiple other types of posttranslational modifications or may be involved in the posttranslational modification of other targets. Thus, S-nitrosylation, along with phosphorylation, ubiquitylation, acetylation, and methylation, participates in a highly complex set of regulatory events. The complexity of these events is exemplified by Shinozaki et al. (16), who showed that S-nitrosylation of the CXXC motif of the protein deacetylase SIRT1 inhibits its ability to deacetylate and inactivate two transcription factors involved in stress responses: p53, a transcription factor that can induce apoptosis, and p65 (also known as RelA), a subunit of the proinflammatory mediator NF-κB. In mouse models of septic shock, Parkinson’s disease, and age-related muscle wasting, all of which involve chronic inflammation, S-nitrosylation of SIRT1 correlated with acetylation and activation of p53 and p65, suggesting that tipping the balance among posttranslational modifications may mediate the pathological effects of chronic inflammation.

Although p53 is best characterized as a transcription factor that promotes cell cycle arrest or apoptosis, two studies show additional roles for p53. Exposure to ultraviolet (UV) light induces DNA damage in skin cells, and this DNA damage activates p53-dependent transcription of the gene encoding proopiomelanocortin (POMC), which is translated and cleaved to produce various bioactive peptides, including the pigment-inducing hormone α-MSH and the endogenous opioid β-endorphin. Fell et al. (17) found that moderate UV exposure increased systemic concentrations of β-endorphin and caused addiction-like behaviors in mice that could be reversed with opioid antagonists or deletion of the gene encoding p53 (TP53). Thus, the human predilection for sun and tanning, despite known health risks, may be explained, at least in part, by the ability of UV to stimulate production of endogenous opioids.

TP53 produces several isoforms. Senturk et al. (18) identified a p53 variant called p53β that is produced by alternative splicing. This variant lacks the nuclear translocation and DNA binding domains and is transcriptionally inactive. Instead, p53β localizes to mitochondria and promotes opening of the mitochondrial permeability transition pore, thereby increasing the production of reactive oxygen species (ROS). Increased ROS is associated with epithelial-to-mesenchymal transitions (EMTs) in several tissues, including the EMT-like transition that occurs during cancer metastasis. p53β promotes mesenchymal phenotypes in lung cancer cells, and increased expression of the mRNA encoding p53β is associated with reduced survival in human lung cancer patients.

Cells die by different mechanisms depending on timing and context, and the manner by which cells die results in different outcomes. Whereas apoptosis is a self-contained form of cell death that results in little inflammation, necrotic cells undergo membrane breakdown, releasing their cytosolic contents, which can trigger inflammation. Initially, the process of necrosis was thought to be passive and unregulated. But evidence obtained with both genetic and pharmacological approaches led to the realization that programmed necrosis, also called necroptosis, occurs in response to specific stimuli. As Dubyk noted, “Necroptosis is emerging as a regulated cell death signaling cascade which is as important as apoptosis.” Necroptosis triggered by tumor necrosis factor (TNF) requires that receptor-interacting kinase 1 (RIPK1) activates a related kinase, RIPK3. Besides stimulating necroptosis, RIPK1 can also stimulate apoptosis by interacting with the proteins FADD (Fas-associated protein with death domain) and so on.

**Fig. 3. The seven-transmembrane domains of the δ-opioid receptor contain a sodium ion.** The crystal structure of a fusion protein containing residues 36-338 of the human δ-opioid receptor in complex with the ligand naltrexone was obtained at 1.8 Å resolution. The sodium ion is shown as a blue sphere; water molecules are shown as red and pink spheres; hydrophobic residues are shown as the orange surface; naltrexone is shown as orange sticks; and hydrogen bonds are shown as dashed lines.
and caspase-8. RIPK1 triggers necroptosis if caspase-8–mediated apoptosis is blocked. However, several studies nominated by Silke, including one from his own group, emphasize that RIPK1 can also inhibit rather than promote cell death by apoptosis or necroptosis in certain contexts. Genetic analysis performed by Rickard et al. (19) and Dannappel et al. (20) using mice with a global or tissue-specific deficiency of Ripk1 indicates that RIPK1 suppresses necroptosis (Fig. 4), a finding supported by Kearney et al. (21). On the other hand, the results of Takahashi et al. (22) and Dillon et al. (23), along with those of Rickard et al. and Dannappel et al., show that RIPK1 prevents apoptosis in the intestine. These studies, together with Kaiser et al. (24), collectively reveal the importance of RIPK1-mediated regulation of both necrotic and apoptotic cell death pathways in different tissues in vivo. Berger et al. (25) and Newton et al. (26) found that mice expressing a catalytically inactive form of RIPK1 develop normally but are resistant to TNF-induced necroptosis, and Berger et al. showed that this mutation alleviates the chronic proliferative dermatitis in a mouse model with features reminiscent of autoimmune skin disorders. These findings emphasize the complex roles that RIPK1 plays in regulating cell death pathway, and, as Silke points out, open “the possibility that necroptosis can be targeted in human auto-inflammatory diseases, such as psoriasis and IBD.” The final papers nominated by Silke focus on RIPK3. Unlike RIPK1, the function of RIPK3 was considered to be limited to the promotion of necroptosis. However, Newton et al. (26) and Mandal et al. (27) suggest that the kinase activity of RIPK3 could be necessary to prevent cell death through apoptosis.

The effector of necroptosis is mixed lineage kinase domain-like (MLKL), which, when phosphorylated by RIPK3, induces the membrane permeabilization characteristic of necrotic cells. The papers nominated by Dubyak highlight the mechanism by which MLKL causes membrane breakdown. Wang et al. (28) showed that RIPK3-mediated phosphorylation causes MLKL to oligomerize. Both Wang et al. and Dondelinger et al. (29) showed that oligomerization of MLKL is required for necroptosis and that the four-helical bundle domain in the N terminus binds to negatively charged lipids, such as phosphatidylinositol phosphates (PIPs), enabling recruitment of MLKL to membranes. Both groups demonstrated that MLKL can permeabilize liposomes containing PIPs. Su et al. (30) [as well as work by Hildebrand et al. (31) and Cai et al. (32), as noted by Silke] provided further evidence for the ability of the four-helical bundle domain to mediate membrane breakdown during necroptosis. Su et al. (30) also showed by nuclear magnetic resonance spectroscopy that the C-terminal helix in MLKL can act as a “plug” for the pore formed by the four-helical bundle domain.

Every year, we receive nominations for breakthrough methods that address difficult biological questions. New technologies not only facilitate research but also provide data that lead scientists to pose questions that were not previously evident. Cancer drug discovery often begins by screening large libraries of small molecules for the ability to kill cultured cancer cells. One bottleneck in the development pipeline is the identification of the molecular target or targets of these molecules. Janes suggested a study by Savitski et al. (33) that combines quantitative mass spectrometry analysis of cell lysates with ligand-induced changes in protein thermal stability to identify protein–drug interactions (Fig. 5). Janes wrote, “This paper introduces a remarkably simple method for monitoring protein conformational changes triggered by small molecules, such as kinase inhibitors and second messengers. The technique provides informa-

![Fig. 4. RIPK1 deficiency results in excessive necroptosis.](http://stke.sciencemag.org/) Fluorescence microscopy of skin sections from mice of the indicated genotypes was immunolabeled for keratin-6 or keratin-14 (red). Nuclei were counterstained with Hoechst (blue). The aberrant distribution of keratin-6 and keratin-14 in the hyperplastic keratinocytes in the epidermis of Ripk1−/− mice, which is an indicator of excessive cell death in the skin, is partially rescued by deficiency in Ripk3 or Mlkl.

![Fig. 5. Thermal proteome profiling is a method for detecting protein targets of drugs.](http://stke.sciencemag.org/) Schematic representation of an experiment used to identify unknown targets of a drug of interest (39). Cells are exposed to the drug (red) or vehicle control, and then cells or cell lysates are heated. Drug binding to target proteins (orange) increases protein thermal stability. Heat-denatured proteins aggregate and precipitate from solution. Quantitative mass spectrometry analysis identifies the relative abundance of target proteins in drug-treated and untreated cells across a range of temperatures, enabling determination of target proteins.
tion about signaling networks that is complementary to that obtained by other proteomic methods. One of its appeals is that thermal stability profiling can be implemented in an unbiased way by using isobaric tags and mass spectrometry, or it can be done in a focused way by immunoblotting for specific target proteins. I found it especially striking that the technique identified a heme biosynthesis enzyme [FECH] as an off-target of protein kinase inhibitors with recognized phototoxicity side effects in the clinic. I would be eager to see thermal proteome profiling applied systematically across all FDA-approved kinase inhibitors.” Moreover, genetic defects in FECH are associated with photosensitivity in humans, suggesting that drug-mediated inhibition of FECH may be a mechanism for this common side effect.

New computational and genomic screening methods may help to identify proteins and networks for the development of targeted therapies. For example, Ansel nominated Zhou et al. (34) and Chen et al. (35), which “tackled the difficult technical hurdles to performing RNAi screens for genes involved in immune responses in vivo.” Both studies used a pooled approach to screen short hairpin RNAs (shRNAs) that affected T cell biology in mice. Zhou et al. transplanted mice with T cells expressing a pool of shRNAs and determined which genes were required for T cell proliferation, survival, and function in the microenvironment of mouse melanoma. In contrast, Chen et al. transplanted mice with pools of T cells, each with a different individual shRNA, to identify factors involved in the differentiation of cytotoxic and helper T cells in response to viral infection. Both approaches relied on RNA deep sequencing to identify enriched shRNAs in the isolated cell populations.

Linding recommended two studies that deal with the integration of large datasets of diverse types. Vinayagam et al. (36) addressed the problem of how to know whether a given protein-protein interaction in a network positively or negatively affects the function of the proteins. The authors integrated protein-protein interaction networks from various sources and phenotypic data from RNA interference screens for ~50 different phenotypes in Drosophila or cultured Drosophila cells. This approach enabled annotation of “sign” to greater than 6000 protein-protein interactions across a wide range of biological functions. A related study by AIQuRaishi et al. (37) used a computational method to integrate knowledge of cancer-associated mutations with a protein-protein interaction network focused on the interaction between SH2 (Src homology 2) domain-containing proteins and proteins with phosphorylated tyrosines, which bind to SH2 domains. This analysis revealed that mutations in tyrosine-phosphorylated proteins frequently enable new protein interactions, whereas mutations in SH2 domains selectively inhibit protein interactions. Because SH2 domain-containing proteins are often network hubs for multiple signaling pathways, this observation suggests a specific mechanism for the pathway remodeling and acquired drug resistance that occur in cancer.

As with every year, we appreciate the input from the distinguished scientists who made nominations, and we anticipate that the highlighted studies will advance signal research and lead the study of cellular and organismal regulation in new directions to advance both basic science and human health.

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