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` **2013: Signaling Breakthroughs of the Year**
Jason D. Berndt and Nancy R. Gough (7 January 2014)
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CELL BIOLOGY

# 2013: Signaling Breakthroughs of the Year

Jason D. Berndt[1*] and Nancy R. Gough[2*]

**The editorial staff and distinguished scientists in the field of cell signaling nominated diverse research as advances for 2013. Breakthroughs in understanding how spatial and temporal signals control cellular behavior ranged from filtering high-frequency stimuli to interpreting circadian inputs. This year's nominations also highlight the importance of understanding cell signaling in the context of physiology and disease, such as links between the nervous system and cancer. Furthermore, the application of new techniques to study cell signaling—such as optogenetics, DNA editing with CRISPR-Cas9, and sequencing untranslated regions of transcripts—continues to expand the realm and impact of signaling research.**

After polling our Board of Reviewing Editors and other prominent researchers to bring you this 12th edition of "Signaling Breakthroughs of the Year," we received several nominations with important implications for human disease and focus on four main areas: (i) the increasing recognition of the importance of temporal dynamics in signaling pathways; (ii) new understanding of the mTOR pathway; (iii) previously unknown roles for lipids, metabolites, and posttranslational modifications; and (iv) several techniques that provide unprecedented insights into signaling biology.

We had an outstanding list of contributors, including George P. Chrousos (University of Athens Medical School, Greece; Georgetown University and National Institute of Child Health and Development, USA); Henrik Dohlman (University of North Carolina Chapel Hill, USA); James Ferrell Jr. (Stanford University, USA); Anne-Claude Gavin (European Molecular Biology Laboratory Heidelberg, Germany); Toby Gibson (European Molecular Biology Laboratory Heidelberg, Germany); Tony Hunter (Salk Institute, USA); Stephen P. Jackson (Gurdon Institute University of Cambridge, UK); Norbert Perrimon (Harvard Medical School, USA); Steve J. Smerdon (Medical Research Council National Institute for Medical Research, UK); Solomon Snyder (Johns Hopkins University, USA); Arthur Weiss (University of California San Francisco, USA); and Michael B. Yaffe (David H. Koch Institute for Integrative Cancer Research, The Broad Institute, and Massachusetts Institute of Technology, USA).

Timing is everything in cells and in signaling networks. Complex molecular cascades, feedback mechanisms, and crosstalk are coordinated to enable cells to translate signals into physiological responses. We begin our list of breakthroughs at the cellular level, focusing on the nervous system in cancer and mood disorders. Magnon *et al.* noted that neurons are commonly present in tumors, leading the authors to ask whether nervous system activation could play a role in tumor progression (*1*). Using orthotopic xenografts of human prostate cancer cells in mice, they found that tumors were infiltrated with both sympathetic and parasympathetic nerve fibers. Severing sympathetic nerves that normally innervate the prostate inhibited the growth of established tumors, as did inhibition of adrenergic or muscarinic acetylcholine receptors. Analysis of prostate cancer samples from patients revealed that the density of sympathetic and parasympathetic nerve innervation correlated with poor prognosis.

The nervous system also contributes to circadian rhythm, a biological process in which cells respond to daily fluctuations in light. Disruptions in circadian rhythm have profound effects on animal behavior and mood, as exemplified by seasonal affective disorder, which is related to the decrease in light during winter. Dulcis *et al.* found that rodents exposed to periods of light mimicking long summer days and then switched to short periods of light mimicking winter days exhibited a reversible switch in neurotransmitters in the hypothalamic neurons that control the release of corticotropin-releasing hormone (CRH) (*2*). This study not only provides a molecular mechanism by which stress hormone signaling, and thus mood and disease resistance, changes with the circadian cycle, it also is one of only a few examples of neurotransmitter switching in adult neurons.

It is well known that cells in the nervous system respond differently depending on the precise spatial and temporal combinations of inputs. Temporal information can be encoded by biochemical events that take place across multiple systems or pathways. Chrousos recommended a study in which a cell receiving an initial signal responds by altering its ability to respond to another signal through regulation of alternative splicing. This work by Lal *et al.* (*3*) illustrates "cross-talk between … two receptor systems that allows seamless integration of extracellular and intracellular signaling pathways and genomic effects to fine-tune biological responses." The authors found that estrogen-mediated transcription regulates the splicing machinery, which ultimately affects the function of G protein–coupled receptors (GPCRs) that respond to other hormonal signals. Although chronic stress increases the risk of disease, and stress hormones can contribute to disease pathology, stress hormones are also essential in the response to injury and inflammation and can inhibit disease progression. CRH is a stress hormone that can either increase the proliferation and invasion of breast cancer cells or inhibit estrogen-mediated cell proliferation in breast cancer. Estrogen inhibited the expression of the mRNA encoding serine- and arginine-rich splicing factor 55, ultimately stimulating the expression of the gene encoding the CRH type-2 receptor and altering the splicing of another gene encoding the CRH type-1 receptor. These changes were associated with decreased invasion of cultured breast cancer cells and with estrogen receptor status in patients with breast cancer, suggesting that this switch may contribute to tumor progression. These results exemplify a potentially new paradigm in the regulation of GPCRs through alternative splicing and show how the cell's "history" and "previous experiences" set the responsiveness to subsequent signals. Similar to CRH, transforming growth factor–β (TGF-β) is both a tumor suppressor and tumor promoter. The study by Vizán *et al.* (*4*) revealed how differences in the dynamics of the trafficking of subunits of the TGF-β receptor altered the cellular response to subsequent exposure to TGF-β. As with CRH, the effect of TGF-β on tumor cells may depend on the cell's history.

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In addition to crosstalk between pathways, temporal information encoded in the signal itself can influence cellular responses (Fig. 1). Toettcher *et al*. showed that this principle exists in signaling networks at the molecular level by analyzing phosphorylation cascades downstream of receptor tyrosine kinases through the use of optogenetic techniques to directly stimulate Ras in cells with light (*5*). High-frequency, short-duration bursts of Ras activation failed to stimulate extracellular signal–regulated protein kinase (ERK), a downstream target of Ras, indicating that the pathway from Ras to ERK suppresses noise in favor of more robust signals, thus effectively acting as a low-pass filter. Both transient and sustained pulses of Ras activity stimulated ERK, but promoted phosphorylation of different targets, suggesting that ERK can decode not only amplitude but also temporal components of input signals and then convey that information to downstream targets.

How might the temporal features of inputs be decoded by signaling proteins like ERK? A paper nominated by Weiss suggests that scaffolding proteins may integrate information over time. In the work of Zheng *et al*. (*6*), the differential, temporally regulated phosphorylation of the scaffolding protein Shc1 (Src homology 2 domain containing 1) coordinated the biochemical events after activation of the EGF (epidermal growth factor) receptor tyrosine kinase. Using quantitative mass spectrometry, the authors found that different sites in Shc1 were phosphorylated or dephosphorylated at different times after stimulation with EGF, and this effect correlated with interactions between Shc1 and groups of proteins with distinct cellular functions. Early waves of Shc1 phosphorylation promoted its association with promitogenic and survival pathway proteins, which was followed by feedback phosphorylation events and the subsequent recruitment of a phosphatase. Changes in the residues that were targeted for phosphorylation switched Shc1 interactions to those with proteins involved in terminating the mitogenic signal and promoting cytoskeletal rearrangement, cell migration, and invasion. Thus, a single scaffolding protein can serve as a both a network hub protein and a central processing node to manage the timing of diverse functional outputs. Weiss noted that this study, "opened our eyes to more early temporal regulation and feedback than we previously appreciated took place."

Another mechanism by which hubs decode different inputs is exemplified in a study by Kiel *et al*. (*7*). The authors showed that competition among proteins binding to a hub can govern how cells produce different responses to the activation of the same receptor. For proteins with a similar affinity for a common hub that bind in a mutually exclusive fashion, the relative abundance of these proteins determines which protein complex forms. Therefore, changes in the abundance of competing binding partners could provide a cellular memory of a previous stimulus and a mechanism to change the flow of information downstream of signaling hubs.

Understanding the temporal aspects of signaling could have clinical ramifications, as noted by Gavin: "Some signaling hubs rely on stimulus-specific signal dynamics to selectively activate downstream cellular processes. Behar *et al*. (*8*) used a theoretical approach to demonstrate that the dynamics features of signaling can be targeted pharmacologically to achieve therapeutic specificity." Therapies that target individual proteins can have limited clinical use due to the convergence of multiple signals on hubs, and thus the repression or activation of a hub protein's function can result in undesirable side effects. A virtual screen of perturbations to parameters in an idealized signaling module surrounding a theoretical hub indicated that targeting nodes responsible for the "dynamic signaling code" could represent a novel strategy of developing therapeutics for various human diseases.

Temporal encoding in molecular signaling is not limited to receptor tyrosine kinases and ERK. In fact, the sequential biochemical events that occur during the cell cycle have long been known to require exquisite timing. Gibson and Ferrell recommended two studies that refine our understanding of how multisite phosphorylation can direct this process, and Gibson suggested that "signaling researchers might want to take a fresh look at the distributions of serines and threonines in their favourite proteins." Cyclin-dependent kinases (CDKs) phosphorylate various substrates, and changes in the activity of CDKs toward different substrate proteins control the cell cycle "clock." Yeast Cdk1 is composed of (i) cyclins, (ii) catalytic subunits, and (iii) the regulatory subunit Cks1p. The catalytic subunit recognizes two consensus motifs, one with high affinity and the other with low affinity. In addition, the cyclin subunits dock onto motifs of certain substrates to increase CDK specificity and activity. In a pair of companion papers, Kõivomägi *et al*. (*9*) and McGrath *et al*. (*10*) showed that yeast Cks1p recognized a consensus sequence containing residues that had been "primed" by CDK-mediated phosphorylation and thus promoted processive multisite phosphorylation by Cdk1. Through this molecular recognition mechanism, Cks1p lowered the threshold for complete phosphorylation and contributed to the timing by which specific cell cycle regulatory substrates are activated or deactivated by CDK-mediated phosphorylation. Gibson also nominated a related paper by Lyons *et al*. (*11*), who found that the degradation of the acetylase Eco1, which is required for cohesion between sister chromatids during S phase, required sequential phosphorylation by three kinases. Similar to recognition of phosphorylation-primed motifs by Cks1p, recognition of Eco1 by the ubiquitin E3 ligase Cdc4 required precise spacing between phosphorylated residues. Gibson concludes, "Since clustered phosphorylation sites are very common in natively disordered polypeptide[s], the take-home message is that sequential priming is likely to be an abundant cell regulatory mechanism and there is no reason for it to be limited to protein destruction phosphodegron signals...." Thus, understanding how the specific spacing and sequential phosphorylation of substrates functions as an intrinsic molecular clock is a breakthrough in our knowledge of the molecular mechanisms that control the timing and flow of biochemical information through a signal transduction pathway.

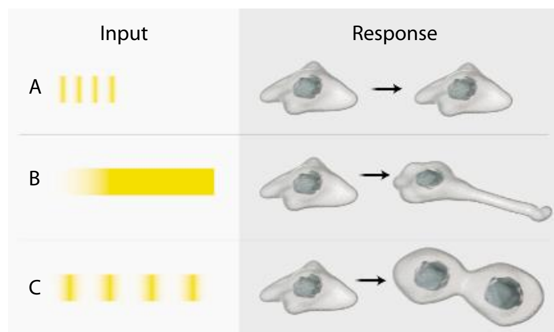


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**Fig. 1. Temporal coding regulates cell signaling and cellular behavior.** (**A** to **C**) The schematic represents a theoretical outcome of cells exposed to different forms of input. (A) Short-duration, high-amplitude spikes are filtered by signaling molecules, resulting in no change in the cell's behavior, whereas (B) sustained activation results in cell migration and (C) repeated transient activation results in cell division.

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In other developments related to cell cycle, a study by Chang and Ferrell suggested how biochemical events that were initiated on one side of a large frog egg cell undergoing mitosis could be coordinated with those on the opposite side to enable rapid spatial and temporal coordination of processes over "large" distances relative to the size of the molecules involved (*12*). Using a combination of mathematical modeling and imaging of mitotic events in a mitotic egg extract in vitro, the authors identified a mechanism that relied on a network of positive and negative feedback loops to produce a bistable system that propagated through "trigger waves." The concept of trigger waves is the basis for the propagation of action potentials along an axon, and this study provides another molecular system in which trigger waves control biochemical processes that need to occur faster than the rate of molecular diffusion.

Target of rapamycin (TOR in yeast or mTOR in mammals) is a phosphoinositide 3-kinase (PI3K)–related kinase that once again was featured in several nominations this year. This kinase is part of two protein complexes, mTORC1 and mTORC2. mTORC1 integrates information regarding the presence of growth factors with energy and nutrient status to inhibit autophagy and promote ribosome formation and protein translation. mTORC2 responds to growth factors to enhance cellular metabolism, survival, proliferation, and cytoskeletal rearrangements. mTOR is implicated in human diseases and genetic disorders, such as cancer, diabetes, schizophrenia, and tuberous sclerosis. Rapamycin is an mTOR inhibitor with specificity for mTORC1 that slows aging in yeast, flies, and mice, and rapamycin is used clinically as an immunosuppressant during kidney transplantation and a chemotherapeutic for Kaposi's sarcoma.

Hunter nominated a paper that examined the substrate specificity of mTORC1 (*13*). Rapamycin binds to the 12-kD FK506-binding protein FKBP12 and acts as an allosteric inhibitor of mTORC1, but it only inhibits the phosphorylation of some substrates. In contrast, inhibitors that bind directly to the active site of mTOR affect the phosphorylation of all substrates equally. Kang *et al*. found that mTORC1 has a preferred consensus recognition motif and that substrate affinity was inversely related to the inhibition of phosphorylation by rapamycin. As Hunter describes, "mTORC1 has a hierarchy of substrate affinities, with only 'efficient' substrates being phosphorylated when mTORC1 activity is low either through starvation or rapamycin inhibition, [and] with serine being preferred over threonine at efficient target sites. A single protein can have both 'good' and 'poor' mTORC1 sites illustrating that this is a property of the sequence.…A similar hierarchy may well exist for other protein kinases dictating which substrates are phosphorylated as kinase activity increases over different thresholds in response to a stimulus."

Yang *et al*. published the structure of mTOR (*14*), which according to Smerdon is a "structural tour-de-force and something of a holy grail in kinase signaling … [and] shed[s] new light on signaling by the whole family of 'giant' PI3K-like kinases, many of which are well-validated drug targets." mTOR was cocrystalized with mLST8 (mammalian lethal with SEC13 protein 8), a subunit of both mTORC1 and mTORC2. The structure revealed an intrinsically active kinase domain formed by N- and C-terminal lobes separated by the catalytic cleft. The positioning of the ATP- and substrate-binding residues was remarkably similar to that of other protein kinases, such as CDK2. The active site was situated in a deep groove created by mLST8 and the FRB and LBE domains of mTOR, and portions of the kα9b helix hinder substrate access (Fig. 2). Many residues that when mutated result in increased activation of mTOR clustered near the distal end of the catalytic cleft, which is blocked by the kα9b helix in the crystal. Superimposition of the mTOR-mLST8 structure with that of rapamycin bound to FKBP12 suggested that rapamycin inhibits mTOR by creating a cap across the catalytic cleft and that conserved residues in the FRB domain formed a secondary substrate recruitment motif, which may explain the differences in substrate affinity seen by Kang *et al*. (*13*).

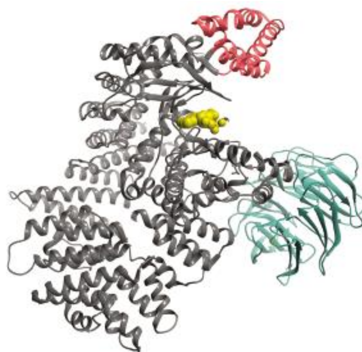

**Fig. 2. The structure of mTOR bound to mLST8 reveals a deep catalytic cleft.** Ribbon structure from PDB 4JSV shows the N- and C-terminal lobes of mTOR flanking the active site containing an analog of ATP (yellow). The FRB domain (red) of mTOR and the mTORC1 subunit mLST8 (green) extend from the sides of the catalytic cleft to restrict substrate access.

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Plants also utilize TOR signaling to regulate growth. Xiong *et al*. (*15*) found that light-activated glucose production in the root meristem was required for root development at the point when plants transition from heterotrophic (seed nutrient–dependent) to photoautotrophic (photosynthetic) growth. The authors found that glucose promoted phosphorylation of the TOR substrate S6-kinase and induced root growth in a TOR-dependent manner. Glucose-induced transcriptional responses were mediated in part by phosphorylation of the S phase–associated transcription factor E2Fa by TOR. Thus, this study expands the functions of TOR signaling to include transcriptional regulation and shows that E2Fa is phosphorylated by TOR, perhaps bypassing the need for CDK-retinoblastoma–mediated phosphorylation.

Protein kinases were not the only signaling components making the breakthrough list. New signaling roles for lipid kinases and their association with disease were also revealed. Yaffe nominated research that identified a link between cancer and the enzyme that phosphorylates a specific position of the inositol ring of phosphoinositide (PI)–type lipids. Although most therapeutic studies have focused on PI3K, which generates the lipid to which proteins such as Ras and Akt bind and are thus recruited to the plasma membrane and activated, Yaffe selected work by Emerling *et al*. (*16*) as a breakthrough because this study suggests that enzymes in the initial steps of the phosphoinositide polyphosphate pathway may also be important in cancer. Emerling *et al*. found amplification of *PIP4K2B* and an increased abundance of PI5P4Ks in breast cancer cell lines and human epidermal growth factor receptor 2 (HER2)–positive human breast tumors. Knockdown of PI5P4Kα and PI5P4Kβ in a p53-deficient breast cancer cell line (genetically lacking *TP53*) enhanced the production of reactive oxygen species and cell senescence, inhibited glucose metabolism, and slowed the growth of cells in culture or xenografts. *TP53*$^{-/-}$ *PIP4K2A*$^{-/-}$ *PIP4K2B*$^{+/-}$ mice had fewer tumors than did *TP53*$^{-/-}$ mice, sug-

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gesting that PI5P4Ks may be a good drug target in *TP53*-deficient breast cancer.

The roles of lipids in cellular regulation can be technically difficult to assess because of the complexity of the lipid species present in the cell and the lack of tools to selectively manipulate or visualize specific lipids. Montefusco *et al*. (*17*) devised an approach to analyze the role of specific groups of ceramide species, which differ in N-acyl chains and hydroxylations, to identify specific functions of these bioactive lipids in yeast. Their work demonstrated that lipid diversity has functional consequences in the cellular response to stress and in transcriptional regulation.

In addition to proteins and lipids, many metabolites can serve regulatory functions. Sutter *et al*. (*18*) found that the amino acid methionine was sufficient to inhibit non-nitrogen starvation (NNS)–induced autophagy in yeast. Methionine increased the S-adenosyl methionine (SAM)–dependent methylation of the protein phosphatase PP2A. Methylated PP2A dephosphorylated Npr2p (Nitrogen permease regulator 2p), a protein that is required for NNS-induced autophagy and is a negative regulator of TORC1. Laxman *et al*. (*19*) found that the abundance of methionine and SAM also controls tRNA thiolation, which enables tRNAs to read more than one codon and facilitates translation of genes associated with rRNA processing, ribosome biogenesis, and translation. Thus, like glucose and arginine, methionine plays a key regulatory role in cellular metabolism.

Cyclic nucleotides, such as cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP), were the first identified second messenger signaling metabolites. Snyder and colleagues recommended a pair of papers that identified cyclic guanosine monophosphate–adenosine monophosphate (cGAMP) as a new second messenger that functions in the mammalian immune response to microbial pathogens, which includes interferon (IFN) production triggered by the presence of foreign DNA in the cytosol. Sun *et al*. (*20*), Wu *et al*. (*21*), and Gao *et al*. (*22*) found that cGAMP and the enzyme that produces it, cGAMP synthase, were critical mediators of the innate immune response to infection. Indeed, cGAMP produced in response to the presence of DNA in the cytosol bound to the adaptor protein STING (stimulator of interferon genes), leading to the activation of the transcription factor IRF3 and IFN production.

Complexity in signaling can arise through multiple types of posttranslational modifications of proteins. In addition to investigations of phosphorylation, studies looking at other posttranslational modifications, including ubiquitylation and methylation, made this year's list of signaling breakthroughs. Protein ubiquitylation is a pervasive posttranslational modification that regulates virtually all aspects of cellular function. Ubiquitin ligase complexes conjugate ubiquitin moieties to lysines in target proteins and to one or more lysines in ubiquitin itself to formed branched chains with diverse linkage patterns. $Lys^{48}$-linked polyubiquitylation targets proteins for proteasomal degradation, whereas monoubiquitylation or other patterns of polyubiquitylation, such as $Lys^{63}$-linkage, are generally associated with regulation of protein trafficking and protein-protein interactions. Deubiquitylating enzymes (DUBs) fall into two categories: (i) the ovarian tumor (OTU) class and (ii) ubiquitin-specific proteases (USPs). Jackson recommended the study by Mevissen *et al*. (*23*), which characterized 16 catalytically active OTUs and showed that, unlike the nonspecific USPs, OTUs recognize only one or a few of the eight possible types of ubiquitin linkages. Structural analysis of related OTUs with different linkage preferences revealed four distinct mechanisms used to achieve linkage specificity. Understanding the specificity of OTUs can be used to determine unknown linkage patterns on proteins of interest by performing in vitro reactions with different OTUs.

Jackson and Yaffe both selected studies that identified interplay between ubiquitylation and methylation in the DNA damage response. Fradet-Turcotte *et al*. (*24*), recommended by Jackson, found that the DNA repair protein 53BP1 recognized a bivalent epigenetic mark consisting of dimethylation of histone-H4 on $Lys^{20}$ and ubiquitylation of histone-2A on $Lys^{15}$ through a previously uncharacterized methyl-lysine binding Tudor domain and an extension on its C terminus. Yaffe nominated work by Watanabe *et al*. (*25*) showing that the demethylase JMJD1C associated with the E3 ubiquitin ligase RNF8 at DNA double-strand breaks. JMJD1C demethylated MDC1 (mediator of DNA-damage checkpoint 1) and promoted its ubiquitylation by RNF8, leading to the recruitment of the RAP80-BRCA1 E3 ligase complex, which further ubiquitylated MDC1. Thus, recognition and repair of DNA damage requires orchestration of multiple types of posttranslational modifications.

The interplay between protein methylation and phosphorylation was selected as a breakthrough in the understanding of signaling downstream of the bone morphogenetic protein (BMP) receptors. BMP receptor activation promotes phosphorylation of Smads 1 and 5 (Smad1/5), which partner with the cofactor Smad4 and translocate to the nucleus to drive gene expression. Smad6 inhibits Smad1/5 by blocking their interaction with the BMP receptor. Xu *et al*. (*26*) found that treating cultured keratinocytes with BMP4 promoted an interaction between the arginine methyltransferase PRMT1 and Smad6. PRMT1 methylated Smad6, was required for BMP-mediated phosphorylation of Smad1/5, and increased the expression of target genes. Overexpression of the homolog of PRMT1 in flies prevented phenotypes resulting from overexpression of Smad6. Thus, methylation emerges as a key posttranslational modification that is involved in the response to metabolic signals, DNA damage, and morphogenetic stimuli.

This year, we received several nominations representing technical innovations that have yielded or have the potential to yield important discoveries in cell signaling biology. Optogenetics, the ability to genetically encode proteins that can be activated or inhibited by light, was applied to understand temporal encoding of the Ras-ERK pathway by Toettcher *et al*. (*5*). Gibson nominated a study by Baarlink *et al*. (*27*) showing that optogenetic activation of the formin mDIA induced nuclear actin filament formation. The results of this optogenetic study continue a thread from our 2011 Breakthroughs, highlighting Mouilleron *et al*. (in the Archives), which showed that monomeric nuclear actin binds to the cofactor myocardin-related transcription factor A (MRTF-A, also known as MAL) to inhibit activation of gene transcription in response to serum response factor.

Dohlman and Hunter both noted papers that advanced antibody technologies. Dohlman nominated a study that demonstrated the use of genetically encoded antibodies, "nanobodies," to visualize the subcellular location of active GPCRs in living cells. Endocytosis can be associated with receptor down-regulation or can contribute to signaling from endosomal membranes. Irannejad *et al*. (*28*) expressed green fluorescent protein (GFP) fused to a conformation-specific, single-domain camelid antibody (Nb80-GFP) that selectively binds to the agonist-bound $\beta_2$-adrenergic receptor ($\beta_2$AR) and then tracked the location of the agonist-

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bound receptors. In cells stimulated with the adrenergic agonist isoprenaline, Nb80-GFP localized to both the plasma membrane and early endosomes. Likewise, a similarly constructed antibody that recognized the guanine nucleotide–free form of G$\alpha_s$, representing an intermediate state of G protein activation, localized to $\beta_2$AR-containing endosomes in stimulated cells. As Dohlman states, "the use of nanobodies represents a novel approach for genetically-encoded biosensor design. In the future, one can easily imagine that nanobodies could be directed against other cellular targets, such as metabolic control points, and thus it opens up a new avenue to elucidate signaling mechanisms and the identification of new drug targets."

In his nomination of the study by Kee *et al*. (*29*), Hunter notes, "Histidine kinases and a pHis [phosphorylated histidine-specific] phosphatase have been reported, but a challenge in detecting phosphohistidine is the chemical instability of its phosphoramidate linkage at low pH…. The availability of antibodies that could detect pHis would clearly facilitate the detection of histidine phosphorylation, just as pTyr antibodies accelerated study of tyrosine phosphorylation." Kee *et al*. used a stable phosphohistidine mimetic to develop a pan-specific phosphohistidine antibody and used it to explore changes in the phosphorylation of metabolic enzymes in bacteria deprived of nitrogen. Hunter cautions, "These antibodies will undoubtedly be valuable, although their cross-reactivity with pTyr may limit their usefulness."

Two nominations focused on advances in the application of deep sequencing. Perrimon nominated the study by Shalek *et al*. (*30*), which used RNA sequencing to analyze gene expression in murine bone marrow–derived dendritic cells stimulated with lipopolysaccharide. By sequencing mRNA from single cells, they found bimodal variation in mRNA abundance and splicing in hundreds of immune-responsive genes, indicating extensive cellular transcriptional heterogeneity. Variation in gene expression correlated with distinct cellular maturation states or with the expression of master regulatory genes including *Irf7* and *Stat2*. Perrimon notes, "This approach is 'game changing' for investigating signaling heterogeneity and temporal responses."

Yaffe nominated a study that investigated the mechanism by which ubiquitously transcribed genes show tissue-specific expression. Lianoglou *et al*. (*31*) created a method to make libraries of the 3′-untranslated regions (3′-UTRs) of mRNAs from different tissues and isogenic cell lines and used deep sequencing to quantify alternative cleavage and polyadenylation. Tissue-specific transcribed genes tended to produce transcripts with a single 3′-UTR, whereas ubiquitously transcribed genes produced transcripts with multiple 3′-UTRs (Fig. 3). The ratio of 3′-UTR isoforms of ubiquitously transcribed genes varied among tissues and between different states of isogenic cell lines undergoing transformation or differentiation. Both mechanisms of restricted gene expression contributed to genes involved in the same molecular pathways or cellular program. Alteration in the ratio of the 3′-UTRs contributed to differential gene expression by changing the number of recognition sites for microRNAs. Thus, differential 3′-UTR production represents another layer of regulation to enable tissue-specific protein production.

Transitioning from RNA to genome editing, CRISPR (clustered regularly interspaced short palindromic repeats)–based techniques were used extensively in various cellular and organismal model systems this year (*32–35*), as noted by Snyder and colleagues. CRISPR sequences are fragments of viral or plasmid DNA incorporated into the bacterial genome by the RNA-guided nuclease Cas9 as part of the bacterial immune response. Researchers have exploited the ability of Cas9 to create site-specific DNA double-strand breaks by creating synthetic guide RNAs that correspond to the target sequence. Because of the imprecise nature of nonhomologous end joining, DNA repair often results in insertions or deletions that ultimately produce truncated or nonfunctional proteins. In addition, providing a template with homologous sequences to the targeted area can result in recombination and enable site-specific modification or insertion of novel sequences. Unlike similar technologies, such as zinc finger nucleases (ZFNs) or transcription activator–like effector nucleases (TALENs), the CRISPR-Cas9 system does not rely on nucleotide recognition by amino acids. Therefore, construction of targeting vectors is both easy and inexpensive.

Unlike RNAi or pharmacological perturbation, CRISPR-Cas9 enables complete genetic ablation in most cultured cells without the need to create a knockout animal. In addition, CRISPR-Cas9 can be used to create specific amino acid substitutions in endogenous proteins. Schwank *et al*. (*36*) used CRISPR-Cas9 and homologous recombination in intestinal stem cells isolated from patients with cystic fibrosis to correct the disease-causing mutation in the gene encoding the cystic fibrosis transmembrane conductor receptor (CFTR). Unlike the parental cells, CFTR-corrected intestinal stem cells grown as organoids in three-dimensional culture swelled in response to cAMP, indicating that the corrected allele was functional and opening the possibility for further studies in this system.

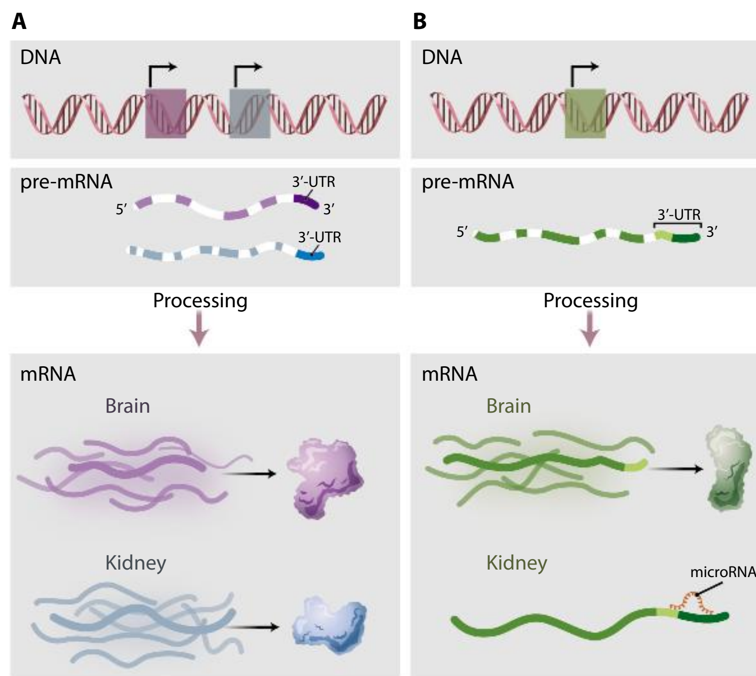


**Fig. 3. Alternative 3′-UTRs confer tissue specificity to ubiquitously transcribed genes.** (**A**) Tissue-specific transcription results in the tissue-specific presence of proteins. (**B**) Ubiquitously transcribed genes undergo alternative cleavage and polyadenylation of the 3′-UTR that enables differential targeting by microRNAs to prevent protein translation in some tissues.

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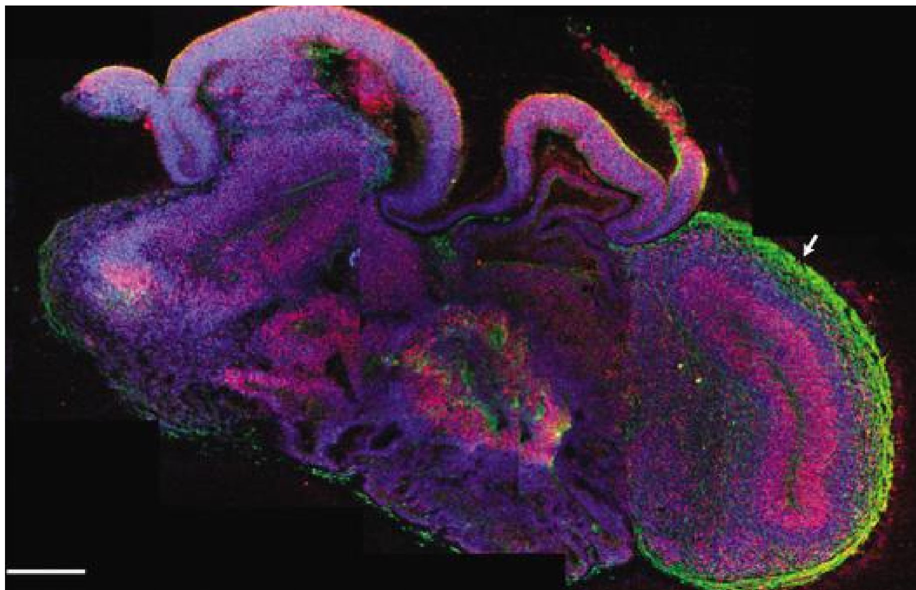

**Fig. 4. Human PSCs grown as organoids recapitulate disease and development.** Image of a cross section of a cerebral organoid stained for nuclei with Hoechst (blue), neural progenitor cells (red), or mature neurons (green). Organoids display regional specification and laminar organization similar to the brain (*37*)

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One particularly intriguing application for CRISPR-Cas9 is in the study of development. CRISPR-Cas9 has been used to create single-gene and even multigenic mutations in cultured pluripotent stem cells (PSCs) and to generate mutant mice and fish. In another recommendation by Snyder and colleagues, Lancaster *et al*. (*37*) demonstrated the ability to grow three-dimensional organoids from PSCs that faithfully recapitulated many aspects of the development and architecture of the human brain (Fig. 4). Cerebral organoids from PSCs reprogrammed from the fibroblasts of a patient with severe microcephaly or from human embryonic PSCs expressing short-hairpin RNAs targeting *CDK5RAP2*, the gene that was mutated in this patient, had epithelial hyperplasia and reduced numbers of neural progenitor cells. Overexpressing CDK5RAP2 in patient-derived cells rescued these defects. Thus, the studies by Schwank *et al*. and Lancaster *et al*. suggest that combining advanced genome-editing with sophisticated cell culture techniques could yield important information about normal development and disease.

The nominations for breakthroughs in signaling this year uncover new regulatory molecules and mechanisms and new interactions among physiological systems and highlight the development of a growing repertoire of tools to dissect signaling pathways. The science of signal transduction continues to expand into vast areas of biology embracing the molecules, cells, and systems that control cellular and organismal behavior—and whose disruption forms the etiology of human disease.

## Related Resources

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*Research Articles*

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