Nov 1, 2012 (Vol. 32, No. 19)

Visualizing Cell Signaling Pathways

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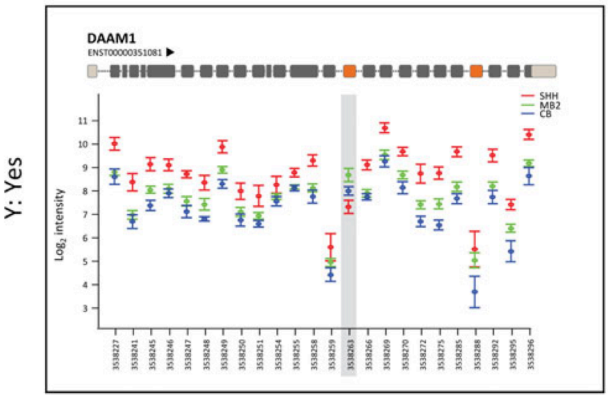
As multiple lines of evidence reveal, signaling pathways originally thought to be primarily involved in activating transcriptional programs and specifying cell fates during development may assume central roles in disease pathogenesis later in life.

The Sonic hedgehog, a critical embryonic signaling pathway conserved from fruit flies to humans, which is involved in cell differentiation, proliferation, and anterior-posterior patterning during development, provides an illustrative example. Signaling in this pathway decreases, but remains active in the adult, where it is involved in stem cell maintenance, homeostasis, and tissue repair.

But its aberrant or uncontrolled activation was reported in a growing number of human malignant tumors. Small molecules that target this, and other signaling pathways involved in tumorigenesis, emerge as attractive therapeutic options.

"We developed an approach to look at Hedgehog signaling in pediatric neuroblastoma," says Mike Hubank, Ph.D., senior lecturer in molecular hematology and cancer biology at University College London.



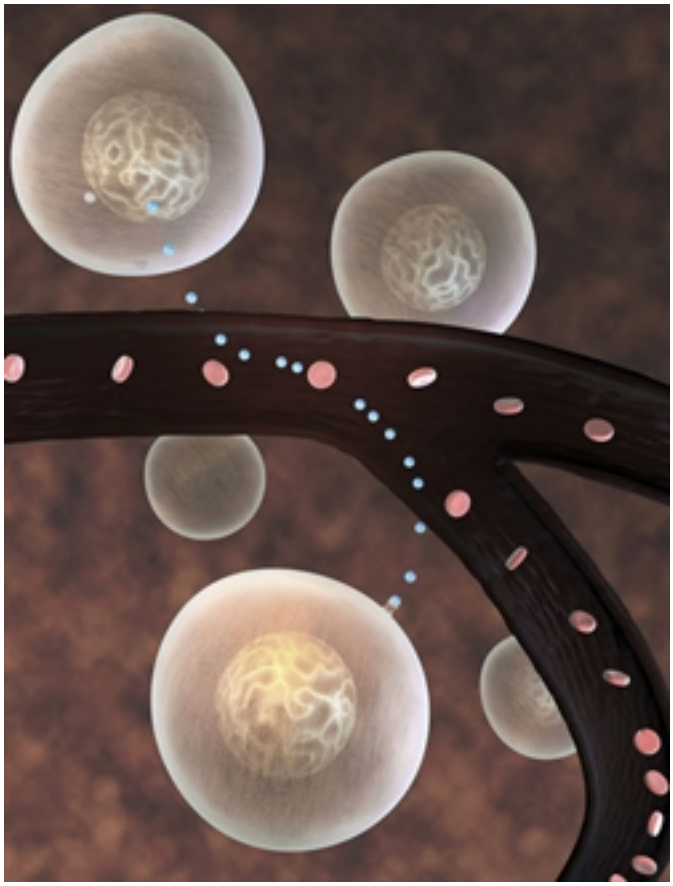
Each point represents an expression intensity value for each exon in an example gene. Previously reported variably spliced axons are highlighted in red. It shows that DAAM1 is more highly expressed overall in SHH tumors than other medulloblastomas or controls, and that exon measured by code set 3538263 is spliced out in SHH but not the others. [University College London: Adapted from Menghi et al. Cancer Res, 2011. 71(6): p. 2045-55.] Combined experimental and theoretical tools that include biochemical and molecular biology approaches, genome, transcriptome, and proteome analyses, and comparative methods, have advanced our understanding of signaling pathways that shape development, homeostasis, and disease.

In a collaborative research initiative, investigators from Dr. Hubank's laboratory and those from the lab of his colleague Jonathan Ham, M.D., compared the genomic profiles of samples from pediatric medulloblastoma patients with those from normal cerebellar tissues by using Affymetrix Exon arrays. They reported that different splicing patterns exist between the two groups and between different molecular subgroups among the medulloblastoma patients.

"By examining exon variants with sequencing-based approaches and microarrays, we can analyze splicing and design new experiments to better understand heterogeneities in cell populations," says Dr. Hubank.

The analysis of 1,262 genes that were found to have at least one exon differentially included between samples from the two groups revealed 174 alternative splicing events, and 11 exons were confirmed by RT-PCR to be differentially spliced.

Similar Splicing Patterns



Computer artwork of cell signaling molecules (blue) traveling via the bloodstream between cells. Signaling molecules allow cells to respond to changes in their environment and to coordinate this response. They can trigger a number of reactions, including changes in metabolism and gene expression. [Gunilla Elam / Science Source]

In addition, the investigators found, in a subgroup of medulloblastoma patients, splicing patterns that were similar to the ones encountered in undifferentiated cerebellar cells, indicating that a disruption in alternative splicing, and the lack of differentiation of these cells, could provide the link between defective or aberrant signaling and the activation of oncogenic pathways.

"It would have been impossible to perform this type of analysis five or six years ago, and the technologies have really helped pull apart the system in such intricate detail," reports Dr. Hubank.

An important cell-cell communication system is established when ephrin (Eph) receptors, the largest subfamily of receptor tyrosine kinases, interact with their ligands, known as ephrins (Eph receptor interacting proteins). Six GPI membrane-anchored ephrin-A ligands (A1-A6) that preferentially bind ephrin-A receptors (A1-A10), and three transmembrane ephrin-B ligands (B1-B3) that preferentially bind ephrin-B receptors (B1-B6) were described, although some crosstalk is possible.

Ephrin receptors and ligands are expressed on almost all embryonic cell types, and distinct expression patterns are established in adult tissues. The bidirectional signal transduction events that they mediate shape intercellular communication during key events from development, tissue homeostasis, and disease. Ephrin ligand-receptor engagement initiates signal transduction, which is referred to as "forward signaling" when it occurs in the cell bearing the receptor, or "reverse signaling" when it occurs in the cell harboring the ligand.

"We are interested in understanding how ephrin B-type ligands send cellular signals," says Ira O. Daar, Ph.D. senior investigator and head of the developmental signal transduction section of the laboratory of cell and developmental signaling at the National Cancer Institute. Investigators in Dr. Daar's lab use *Xenopus laevis*, a manipulable organism for which a detailed fate map exists for the 32-cell stage, providing an ideal system to dissect cellular and molecular events during development.

Dr. Daar and colleagues revealed that ephrin B1 interacts with a protein called Dishevelled. "We found that, as a result of this interaction, ephrin B1 activates a non-canonical Wnt signaling pathway that is also known as the planar cell polarity pathway and allows retinal progenitor cells to move into the developing eye field," he explains.

In addition to shedding light on the involvement of ephrin B1 in cell migration, investigators in Dr. Daar's lab recently unveiled another role of this protein, related to the establishment of cell and tissue boundaries.

"We have shown that ephrin B1 regulates tight junctions by its ability to bind Par6 and prevent Cdc42 from binding," says Dr. Daar.

Par6, a scaffolding protein of the Par polarity complex that is involved in organizing tight junctions at the apical-lateral border of the polarized epithelial cells, is constitutively bound to the atypical protein kinase C (aPKC). Upon binding the GTP-bound form of Cdc42, it undergoes a conformational change that results in aPKC activation.

Ephrin B1 competes with the active, GTP-bound form of Cdc42 for association with Par6. As a consequence of this competition, aPKC is no longer activated, leading to the disintegration of the tight junctions.

An additional layer of complexity is provided by the presence, in the intracellular domain of ephrin B1, of six conserved tyrosine residues that can be phosphorylated. Phosphorylated ephrin recruits Grb4, a small adaptor protein that binds other cytoskeletal signaling molecules.

Ephrin B1 phosphorylation may occur by multiple mechanisms, and phosphorylated ephrin B1 is impaired in its ability to interact with Par6. As a result, Par6 becomes more available for Cdc42 binding, the power polarity complex can reform, and the aPKC pathway is activated, allowing tight junctions to reform.

Clinical Implications

One of the clinical implications of these findings is related to the involvement of ephrins in the intestinal epithelium homeostasis and in colorectal cancer progression. Ephrin B signaling blocks the acquisition of malignancy characteristics in colorectal cancer by tumor cell compartmentalization, through an E-cadherin-dependent mechanism, and loss of ephrin B receptor expression is a key step in the transition from adenoma to adenocarcinoma.

Downregulation of the ephrin B1 receptor, which results in lower ephrin B1 phosphorylation levels, could increase its ability to interact with Par6, and result in reduced aPKC signaling and the disruption of tight junctions.

“This is one potential implication that forward and reverse signaling through the ephrin receptor has for understanding tumor metastasis,” explains Dr. Daar.

“One of the nice things about the current technology, especially single molecule in situ fluorescence hybridization, is that one can look at individual mRNA molecules, and this is another example of how techniques are allowing a very refined glimpse of what is going on in single cells and at the single-molecule level,” says Martin Chalfie, Ph.D., professor of biological sciences at Columbia University and co-recipient of the 2008 Nobel Prize in Physiology or Medicine.

In a recent study that examined genes that are overexpressed in *C. elegans* mechanosensory neurons (FLP) and touch receptor neurons (TRNs), two sets of cells that have different features but use the same selector genes, mec-3 and unc-86, Dr. Chalfie and colleagues found that FLP neurons express approximately 23% of the TRN neuron-specific mRNAs at levels that are low, do not undergo translation, appear to be well tolerated, and do not change the differentiation of FLP cells.

There are two possibilities to explain this low level of mRNA expression. “It may be that this is the only background that the cell tolerates, and it does not cause any real effects, which is what we think is going on,” says Dr. Chalfie. “But, on the other hand, it may represent the consequence of an active process that has to be controlled and could create potential problems for the cell, and at this time we do not really know.”

This low level of the TRN cell-specific mRNA expression in FLP neurons reveals, nevertheless, that transcriptional control is somewhat inexact, and indicates that interpretations based solely on cellular mRNA levels may be misleading.

By ectopically expressing the ALR-1 transcription factor, which ensures TRN cell differentiation, Dr. Chalfie and colleagues revealed that FLP neurons may acquire a TRN-like fate, a change that is similar to the one that was previously shown to occur, by an independent mechanism, when the EGL-44 and EGL-46 transcription factors, known to inhibit touch cell fate, are mutated. This pointed toward the existence of multiple independent regulatory pathways that operate in FLP neurons to induce FLP cell characteristics and suppress TRN cell characteristics.

Challenges Remain

Despite recent methodological advances, the study of protein-protein interactions still presents multiple types of experimental challenges, particularly when membrane proteins are involved, due to their biochemical intractability and the highly transient nature that typifies many of their interactions.

"We developed a new approach to address the paucity of existing methods that existed for studying this class of protein-protein interactions," says Gavin J. Wright, Ph.D., team leader at the cell surface signalling laboratory, the Wellcome Trust Sanger Institute.

To identify and study low-affinity interactions between membrane-embedded receptor proteins and their ligands, Dr. Wright and colleagues designed a method called AVEXIS (AVidity-based EXtracellular Interaction Screen). "We hope that by applying this technology, we will facilitate the identification of interactions involved in cellular recognition processes that are difficult to detect using other approaches," explains Dr. Wright.

AVEXIS was created by generating libraries of proteins that consist of only the extracellular regions of the receptors, which are expressed as soluble recombinant proteins in mammalian cells.

"The idea was to take two cell types that are known to interact, make a protein library that represents the cell surface receptor repertoire of each cell type, and then screen within the two libraries to identify new interactions in a very systematic and unbiased way," notes Dr. Wright.

To circumvent the challenges raised by the low affinity of the interactions, Dr. Wright and colleagues multimerized the protein by using a peptide from the cartilage oligomeric matrix protein, which induces formation of pentamers, increasing the local concentration of the protein of interest. This helps increase the half-life of the interaction, which is often less than a second, to tens of minutes or several hours, enabling the detection of interactions that would otherwise be challenging to capture.

"The appreciation that the interaction affinities can be weak is important, because regular biochemical purification methods aren't likely to work for these very transient interactions," explains Dr. Wright.

By using AVEXIS, researchers in Dr. Wright's lab recently found that basigin, an erythrocyte surface receptor, binds RH5, a *Plasmodium falciparum* ligand, and that the basigin-RH5 interaction was essential for erythrocyte invasion by the parasite. This finding now holds promise for the development of an antimalarial blood-stage vaccine.

Dr. Wright and colleagues are also focusing on dissecting receptor-ligand interactions involved in platelet aggregation, an area that promises to unveil key molecular events in cardiovascular diseases and stroke, and on understanding molecular events involved in sperm-egg recognition.

"Understanding the molecular basis of how sperm and egg recognize each other and interact is a remarkably understudied area with important clinical applications, and represents a fundamental biological problem that has gone largely unaddressed due to the lack of technology," explains Dr. Wright.

Enzyme Positioning

The idea that enzymes are positioned in precise locations in the cell is "something that has become more and more appreciated over the past 20 years, and now we are able to recognize the importance of this concept in understanding pathophysiological effects," says John D. Scott, Ph.D., professor of pharmacology at the University of Washington.

Dr. Scott and colleagues revealed that when protein kinase C is anchored to the membrane by the human A-kinase anchoring protein AKAP79 (corresponding to murine AKAP150), it becomes resistant to certain standard ATP-competitive inhibitors. This indicates that in vitro enzymatic assays might not reliably inform about the intracellular pharmacological profile of an enzyme.

One possible explanation is that the active site of the enzyme is modulated when it is bound to other proteins, so that small molecules may not be able to access the active site in the same way.

“This finding is tremendously important for drug design and drug targeting, because some of the drugs that work on a soluble enzyme do not necessarily work in the same way on an anchored enzyme,” explains Dr. Scott.

Most recently, he and his colleagues showed that AKAP150 is involved in organizing insulin secretion by pancreatic b-cells, and the loss of AKAP150 reduces calcium influx and impairs the oscillatory cAMP production, affecting insulin secretion. AKAP150 knockout mice also showed reduced circulating insulin levels and increased insulin sensitivity.

Furthermore, the metabolic profiling of mice harboring a seven amino-acid deletion in AKAP150, which disrupts the in vivo anchoring of protein phosphatase 2B (PP2B), revealed glucose homeostasis changes similar to the ones seen in AKAP150 knockout mice, indicating that PP2B targeting to the plasma membrane by AKAP150 is an important aspect of its function in insulin-responsive tissues.

If these findings can be translated to the human condition, it would suggest that therapeutic targeting of this AKAP-PP2B interaction could boost insulin action in a manner that could be advantageous for the treatment of diabetes and metabolic disorders.

“This is a very important concept for drug design as one considers the importance of knowing not only the protein structure, but the cellular context as well,” emphasized Dr. Scott.

Increasingly, technological advances are providing opportunities to visualize, at a higher resolution than ever before, key components of intra- and inter-cellular signaling pathways and networks. As more information is becoming available, additional layers of complexity are unveiled, fueling new areas of research and new clinical applications.

These strides would not have been possible without the interdisciplinarity that has become the defining feature of every biomedical area, and it is this approach that is the one that promises to capture the dynamic complexity of the cellular and molecular universe.