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Navigating the network: signaling cross-talk in hematopoietic cells

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Abstract

Recent studies in hematopoietic cells have led to a growing appreciation of the diverse modes of molecular and functional cross-talk between canonical signaling pathways. However, these intersections represent only the tip of the iceberg. Emerging global analytical methods are providing an even richer and more complete picture of the many components that measurably interact in a network manner to produce cellular responses. Here we highlight the pieces in this Focus, emphasize the limitations of the present canonical pathway paradigm, and discuss the value of a systems biology approach using more global, quantitative experimental design and data analysis strategies. Lastly, we urge caution about overly facile interpretation of genome- and proteome-level studies.

Past is prologue

The ability of cells to respond dynamically to their environment through engagement of cell surface receptors is a fundamental principle of biology, and a breakdown in cellular information processing involving these receptors and their linked intracellular signal transduction events underlies many diseases. This combination of basic and clinical importance has led to a substantial effort over the past 50 years to elucidate the mechanistic principles of cellular signaling.

The concept of a ‘receptor’ dates back to the first decade of the twentieth century and studies of the action of adrenergic agonists derived from “supra-renal” extract[1]. Classical pharmacological analyses of dose–response relationships dominated cellular research for the next 50 years (ref. [2]). Biochemical methods emerging in the 1960s and 1970s led to seminal breakthroughs in the field, with the identification of cyclic AMP as the first ‘second messenger’[3] and the purification and functional characterization of the components of the first G protein–coupled receptor pathways[4–6]. These studies identified reversible modification of proteins, through serine/threonine phosphorylation in this case, as a primary mechanism of signal relay and amplification, giving birth to the concept of the linear signal transduction pathway (Fig. 1a).

The emergence of molecular cloning in the 1980s accelerated the identification of signaling pathways, and the discovery of tyrosine phosphorylation[7] and the consequent identification of adaptor proteins with modular phosphotyrosine binding domains introduced the concept of signal propagation through modification-induced protein-protein interaction[8]. Receptors or noncovalent receptor complexes with many sites for stimulus-induced interaction[9], severalof which were identified in hematopoietic cells[10,11], were shown to initiate the formation of multicomponent complexes. Such assemblies could activate many signaling events, and their characterization revealed the prevalence of branched signaling pathways (Fig. 1a). Molecular methods to screen for protein-protein interactions[12], in conjunction with fluorescent protein–based technology to observe the subcellular localization and dynamics of signaling proteins in live cells[13], provided further insight into the topology of these branched pathways.

Advances in mass spectrometry and proteomics permitted a more global assessment of the protein[14] and lipid modifications[15,16] that result from cell stimulation. This led to the realization that single stimuli often provoke complex responses not predictable from known signaling schema, heralding the beginnings of network biology (Fig. 1a). Initial networks emerged from the addition of discrete points of cross-talk between canonical signaling pathways involving shared components[17–19], but the growing scale of the data sets generated by high-throughput methods and the density of interconnections[20,21] revealed by these new ‘omic’ approaches have necessitated more sophisticated methods for data analysis and display[22–25]. This has taken the field into the realm of computational systems biology, network analysis and display, and statistical inference of signal propagation routes[26–29].

This brings us back to the subject of the articles in this issue of *Nature Immunology*. They cover in substantial detail a variety of subfields in the area of immune signal transduction, with an emphasis on the frequent and diverse nature of cross-talk and component sharing among signaling pathways in hematopoietic cells (Ivashkiv[30]; Wilson, Dixit and Ashkenazi[31]; Bezbradica and Medzhitov[32]) and on current approaches for genetic (Saveliev and Tybulewicz[33]) or therapeutic (Ghoreschi, Laurence and O'Shea[34]) manipulation of signaling components. Here we try to place the main themes of these articles in the context of the emerging network-based view of cellular signaling, and to emphasize the growing need for careful experimental design to maximize the physiological relevance and proper interpretation of data acquired using modern data-rich technologies.

From pathways to networks

Canonical signaling pathways provide a fundamental organizational and intellectual framework for understanding the nature of biological responses at the cellular level. However, there are also substantial drawbacks to the dominance of the canonical pathway paradigm. The principal problem is that the experimental design of most studies is highly biased by what we presume to already ‘know’; as a result, the perceived importance of canonical pathway components is reinforced by their repeated use as the primary or only readout in many experiments. Thus, just as each of the six blind men can interpret only the part of the elephant he touches, reductionist biological approaches based narrowly on existing pathway dogma fail to provide a comprehensive account of complex cellular responses because these approaches are inherently self-reflexive.

Modern technology now allows investigators to probe the activities of a cell in a much broader, less dogmatic manner. Multiplex fluorescent technologies permit the assessment of multiple readouts from a stimulated cell[35], RNA interference can perturb gene expression on a genome-wide scale[36,37], new-generation sequencing technologies promise to substantially improve the accuracy and data output from transcript profiling[38,39], and advances in mass spectrometry are refining our knowledge of biochemical events in stimulated cells by assessing in a highly

refined manner a very large fraction of a cell's constituents and their modifications during signal transduction[40].

A recurrent theme emerging from recent studies is the marked increase in the number of components that influence a phenotypic outcome that was previously linked to operation of a 'canonical pathway'[41,42]. Rather than the tens of components previously thought to participate in signaling through a typical pathway, recent screens often suggest the involvement of hundreds. Moreover, these components are often identified based on arbitrary thresholds set for practical purposes, suggesting that the actual number of potential regulators could be even higher. In a prescient essay on the implications of these data[41], Friedman and Perrimon recently highlighted reports showing that screens with quantitative phenotypic readouts often produce a 'continuous' gene–versus-phenotype relationship, one in which every tested gene has some degree of influence on the measured outcome (Fig. 1b). One interpretation of these findings is that the cell operates as a single mega-network with limited isolation between the hubs of what would traditionally be considered 'pathways'. However, these data alone do not rule out a core signaling architecture based on canonical, relatively linear, isolated pathways.

The application of such global screens to immune cells could provide a new picture of how single and conjoint stimuli are processed within the cell to result in the measurable changes we associate with such perturbations. As just one example, the present analysis of cytokine signaling in instances that involve Jak–STAT pathways may be a classic example of 'canonical bias,' wherein the role of new components in cytokine responses remains unappreciated because a small, standard set of Jak–STAT components is repeatedly used as an assay readout. Such 'hidden' components may help to explain how particular cytokine receptors have the capacity to drive cell type–specific responses, despite their shared usage of a small set of Jak and STAT proteins. This issue is discussed in some detail in the review by Bezbradica and Medzhitov[32]. The argument in favor of a less 'pathway-centric' view of signaling is also supported by the identification of components in large-scale screens that are well established participants in 'other' canonical signaling pathways[41]. This principle is especially relevant to the review by Wilson, Dixit and Ashkenazi[31], which discusses the involvement of members of the tumor necrosis factor receptor family and death domain proteins in both apoptotic and non-apoptotic pathways.

A noteworthy feature of many immune cell networks is their convergence on common downstream transcriptional modules (Fig. 1a). How do different upstream signaling modules share the same downstream components when both inputs are activated simultaneously? Do the signaling modules draw from a common pool, or are preformed complexes dedicated to a given pathway? The latter case would actually argue against the use of common components as cross-talk nodes, and certain examples of immunoreceptor tyrosine-based activation motif (ITAM)-based pathway interactions support this notion. The NF-κB and MAPK signaling complexes are activated by ITAM-based signaling, but also by tumor necrosis factor receptor family signaling and many innate immune signaling pathways. Nonetheless, as illustrated in the review by Ivashkiv[30], ITAM pathway cross-talk more commonly involves components of the Ca^{2+} pathway activated by ITAM signaling. This situation may be somewhat specific to ITAM-based signaling, as these pathways depend heavily on co-stimulation through heterologous pathways to achieve signal flow. Further studies on pathway cross-talk will be required to determine whether shared components are more likely or less likely to represent network nodes or hubs.

A word of caution

Having emphasized the potential value of a 'connected network' view of cellular signaling, some caution about overinterpretation of global studies is warranted. Large-scale connectivity

among signaling components may not always be the case. For example, recent studies have identified roles for death receptor (DR) signal transducers in non-DR innate signaling pathways. This may simply represent reuse of a modular signaling adaptor molecule in an alternative pathway, rather than a point of cross-talk between DR and non-DR signaling, as there is no evidence yet to demonstrate that the use of DR transducers in the non-DR pathway directly influences DR signaling. It is possible that the lack of experimental evidence of cross-talk between DR signaling and other immune cell pathways indicates a requirement for hematopoietic cells to limit influence on the apoptotic cascade because of this pathway's fundamental importance in removal of autoreactive lymphocytes.

Such findings raise questions about the notion of a fully connected network of the type pictured by Friedman and Perrimon[41], suggesting instead that there remains some value in the more conventional ‘pathway’ picture of signal transduction and hinting that effective inter-pathway insulation, possibly facilitated by scaffold proteins (Fig. 1a), is needed to avoid spurious responses to particular stimuli. Continuous function graphs assign a degree of influence to every gene in a given response if the measured effect is above some statistical threshold. However, such analyses do not directly address the question of whether the effect measured is physiological, in the sense that it has evolved for a specific, necessary role in cellular behavior. It is quite possible that the effect represents only some inescapable degree of ‘spillover’ or ‘noise’ between evolved pathways, in which the energetic and other costs of establishing greater interpathway isolation are higher than the selection benefit. As a simple example, consider a kinase whose catalytic activity on two substrates differs by more than tenfold, with the more sensitive target being within a canonical signaling pathway and the less sensitive one in a distinct pathway. At modest levels of input that activate only a limited amount of the kinase, only the more sensitive target will be modified to an extent that changes cell behavior. Under most physiological circumstances, this means that only the canonical pathway will be engaged in a biologically meaningful way. However, saturating supraphysiologic inputs may induce modification of the second substrate sufficient to result in a measurable phenotypic effect (Fig. 1c). Thus, assays using supraphysiologic inputs may not necessarily reflect how the cell behaves in its normal state. Put another way, it is important to distinguish between that which can happen and that which evolution has specifically selected in ensuring proper organismal functioning under nonpathologic circumstances. In many cases, there may be no selective advantage to the evolution of strict pathway isolation of the type highlighted in the DR and death-domain pathways; indeed, the development of protein structures that enforce such isolation might actually be deleterious to the operation of the ‘canonical’ pathway to which these elements belong. The upshot is that we may be able to measure weak signal spreading within the ‘network’ in very precise experiments without such ‘signaling ripples’ being fundamental to the cell's proper interpretation of incoming stimuli.

The importance of context and state

These considerations point to a key challenge in improving our understanding of signal integration in mammalian cells—the development of enhanced experimental design practices that address the importance of cellular context, physiological conditions of stimulation, and minimal perturbation of cellular content and molecular structure. Although it is clear that immune cell signaling pathways are connected by physical and functional interactions, how do we more accurately assess the physiological relevance of these connections? Most published studies are characterized by one or all of the following experimental strategy elements: acute stimulation, (near to) saturating ligand concentration, overexpressed components, and/or use of a heterologous cell system. Because physiological stimuli are typically subsaturating with respect to available receptor pools, often transient or pulsatile in nature, and have consequences that are highly susceptible to the effects of cellular composition[43], the experimental methods

in common use raise the obvious question of the extent to which the results reflect the behavior of the cell under more physiologic conditions versus the outcome of extreme perturbations.

To a large extent, this experimental design paradigm has been driven by practical necessity. Such unphysiological conditions are often required to elicit a consistently measurable response from cells that invest heavily in dampening and negative regulation, a characteristic that may be an even greater factor in immortalized cell lines. Although this is helpful in generating robust data that are convincing in publications, there is great value to developing and using more sensitive multiplex methodologies with a broad range of readouts that permit experimentation under more realistic conditions of cell stimulation. Emerging sensor-based assays using 384- and 1,536-well microplates to facilitate genome-wide perturbation screens[44] and the use of microfabricated devices to permit single cell measurements with high resolution[45] will also allow an increase in the breadth and dynamic range of measurements involving non-transformed cells available in limited number. Application of such methods to network analysis will permit an increase in sensitivity, analysis at the single-cell level with time resolution, and an increased number of tested ligand concentrations and ligand combinations, which together should markedly enhance the likelihood that we will observe events relevant to physiology. The next step in the evolution of such analyses will be making the same sets of observations *in vivo*, using imaging methods that permit observation of cells interacting within the tissue environment and involving physiologic amounts of mediators in that natural setting[46,47].

Assessment of cellular responses to combined rather than single stimuli is also a generally neglected but vitally important experimental design parameter in efforts to unravel complex signaling events[48]. For example, there is growing evidence that cytokine receptors activate non–Jak-STAT signaling effectors (reviewed by Bezbradica and Medzhitov[32]), but little is known about how this influences activation of these effectors through their canonical pathways when they become activated by non-cytokine stimuli also present in the cell's environment. Also, pathogens represent a complex stimulus, invariably activating several pathways. These stimuli often combine to produce a synergistic response that is not predictable from the output evoked by an individual canonical pathway downstream of a single receptor.

Careful modeling of quantitative data would also be valuable for explaining observed phenomena. For example, Ghoreschi, Laurence and O'Shea[34] discuss the unexpected success of broad-spectrum kinase inhibitors such as staurosporine for treatment of various malignancies, achieved even though these drugs target a substantial proportion of the entire kinome. This success may relate to differences in network flux between normal and transformed cells. Normal cells may be capable of tolerating, or recovering from, inhibition of large portion of their cellular kinases. Tumor cells, by contrast, may require more kinase-driven pathways operating close to their maximum capacity to maintain the transformed state, and they may thus be more susceptible to a partial reduction in signaling capacity through multiple routes. A comprehensive recent assessment of the kinase dependencies of many cell types shows notable heterogeneity in kinase requirements[49], pointing to a need for caution in predicting the physiological effects of kinase inhibitors. This also highlights the possibility, discussed in the review[34], that combination therapies targeting multiple kinases are likely to be more fruitful, considering the interconnected nature of at least portions of the signaling network. Dual perturbation increases the likelihood that the drug treatment will inhibit signal flow (because of the branched nature of the signaling network) and lead to a significant effect, and it also may reduce the incidence of drug resistance as the network is perturbed at multiple points. The potential of this strategy has led to the recent development of screens specifically designed to identify dual-target inhibitors[50].

On a related note, Saveliev and Tybulewicz[33] describe the value of partial loss-of-function mutants in assessing the various functions of the ZAP70 tyrosine kinase in T cell antigen

receptor (TCR) signaling, demonstrating that different amounts of kinase activity are required for different aspects of T cell development. These latter studies emphasize two key points. First is the importance of quantitative parameter recording. The contribution of ZAP70 is not a digital phenomenon, but instead resembles a rheostat in its control of signaling output from the TCR complex. Second, partial loss-of-function can provide important quantitative insights into signal flow, emphasizing the value of RNA-mediated interference (RNAi) technology—especially viral short hairpin RNA (shRNA) expression systems that permit the conditional knockdown of targets and reexpression of mutant proteins even in moderately intractable hematopoietic cells[51–53]—as a complement to knockout and knock-in strategies.

Finally, on the qualitative side of the equation, the more sophisticated gene perturbation technologies discussed by Saveliev and Tybulewicz[33] hold particular promise for improving our ability to identify the normal physiologic function of a protein and its domains: the selective ablation of a single property in a multifunctional signaling protein will more accurately describe its function within a network than the complete removal of this protein from the cell by classical knockout methods. The findings emerging from such research highlight the value of therapies that combine inhibitors of catalytic function with inhibitors of protein-protein interactions.

Concluding remarks

There is now a debate about whether we should abandon the canonical pathway concept for a network picture of cellular function in which nearly all molecular elements influence one another to some measurable degree. However, caution is needed to avoid confusing what our techniques are now capable of measuring and what actually guides cell behavior under physiologically relevant conditions.

Refined experimental approaches need to be combined with recent advances in mathematical tools and computer software that permit the generation of quantitative computer models of complex biological systems across scales of organization and with a high degree of correspondence to biological reality—from molecular interactions underlying intracellular signaling pathways, to gene regulatory networks interfacing with these signaling events and also regulated by epigenetic modifications, to the cross-talk between cells as is critical in the immune system in particular, to organ behavior, and finally, to the overall response of an organism[54–57]. The resulting simulations will allow us to make predictions about the anticipated results of experiment and to refine our understanding in an iterative manner. An exciting path lies ahead for ‘systems immunology’, but we must tread carefully.

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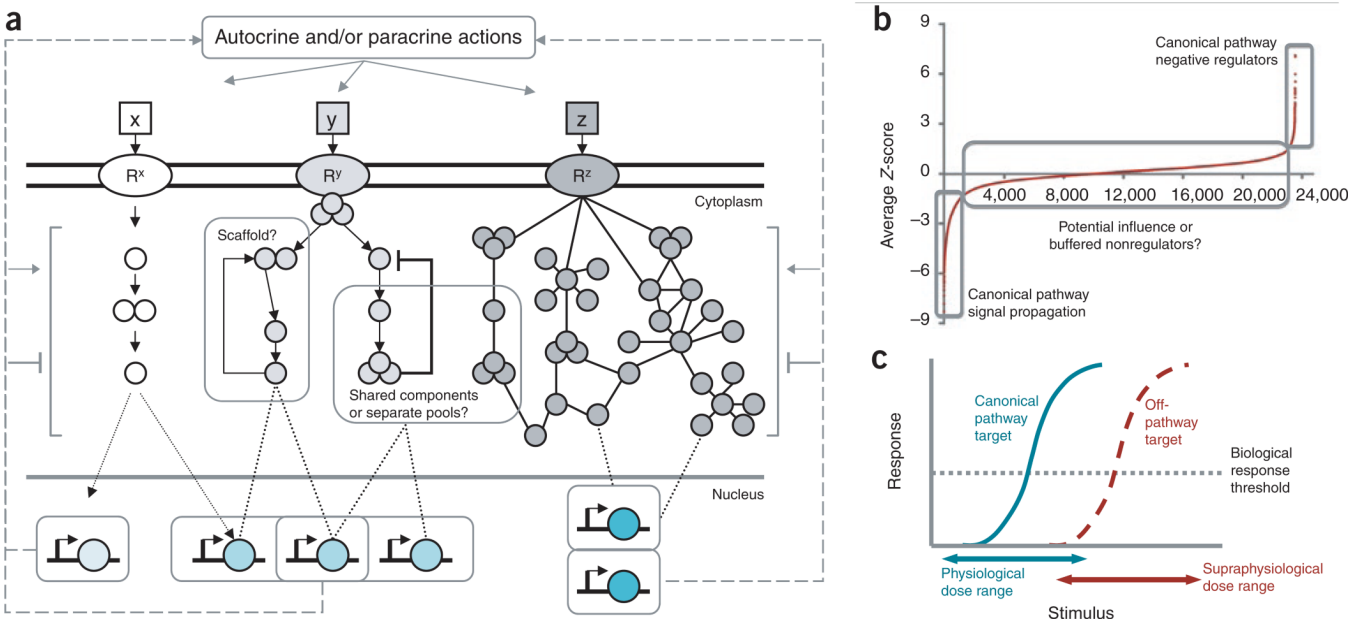

Figure 1.

Topology of signaling networks and interpretation of experimental readouts. (**a**) Representation of signaling pathway schema from simple linear pathways (R^x), to branched pathways downstream of multicomponent receptor complexes (R^y), to signaling networks (R^z). Multiple pathways can either induce distinct transcriptional signatures or converge on common genes. Feedback from induced transcripts can occur at the level of autocrine and/or paracrine actions of secreted proteins or at the level of direct effects on cytoplasmic signaling components. Several aspects of network architecture include the importance of scaffold proteins for interpathway insulation and how multiple pathways may ‘share’ common signaling components. (**b**) Continuous gene-versus-phenotype relationship from genome-wide RNAi screen for signaling regulators in *Drosophila melanogaster* cells (reproduced from ref. [41]). The effect of dsRNA targeting 24,000 genes is represented as a *Z*-score relative to control Erk activation. Canonical pathway signaling components and regulators are identified at the extremes of the distribution. The physiological influence of genes in the central section is discussed in the text. (**c**) Effects of various degrees of stimulus on a hypothetical signaling pathway ‘canonical’ target and an ‘off-pathway’ target. Whereas the off-pathway target can induce a measurable response under high-dose conditions, only the canonical target induces a response in the physiological dose range.