Cell

Genetic Screening for Signal Transduction in the Era of Network Biology

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DOI 10.1016/j.cell.2007.01.007

In contrast to animal-based mutant phenotype assays, recent biochemical and quantitative genetic studies have identified hundreds of potential regulators of known signaling pathways. We discuss the discrepancy between previous models and new data, put forward a different signaling conceptual framework incorporating time-dependent quantitative contributions, and suggest how this new framework can impact our study of human disease.

Cell biology and biochemistry have provided insights into the mechanistic understanding of the nature, location, and kinetics of the protein interactions underlying signal transduction. The identity of proteins that transduce signals within cells originates, in part, from forward genetic screens in model organisms in addition to biochemical techniques. For example, in the fruit fly *Drosophila*, arguably the most successful genetic screens were the early large screens isolating genes that, when mutated, were associated with embryonic lethality and striking changes in cuticle morphology (Nusslein-Volhard and Wieschaus, 1980). The outcome of these screens was that genes with similar visible morphological mutant phenotypes were subsequently found to be involved in the same signaling pathway. Later screens using mosaic techniques, overexpression, and sensitized genetic backgrounds and concurrent biochemical approaches completed the current list of signaling components.

Extensive signaling studies have yielded a limited repertoire of canonical signaling pathways, including the Wingless/Wnt, Hedgehog, receptor tyrosine kinase/extracellular regulated kinase (RTK/ERK), Akt/Tor, Jak-STAT, Notch, TGFβ, G protein-coupled receptor (GPCR), NF-κB, Toll, and steroid hormone pathways. Since the discovery of founding members of these pathways, new components continue to be identified steadily either through biochemistry or genetic screening.

The current dominant model for signal transduction is that of a signaling pathway, a cassette consisting of tens of proteins, compartmentalized, hierarchical, and independent from the rest of the proteome. These pathways are frequently depicted in the literature as wiring diagrams with linear flows from the input signal, through the cascade of reactions, to the output. The analogy, either implicitly or explicitly, is that of an electrical circuit. Although cell biologists have long recognized that some connections between pathway components and other pathways appear in particular cell types, this crosstalk is relatively limited and dependent upon the circuit models that rarely consider the dynamic and quantitative contributions of individual components. This canonical pathway model has been extremely useful as an organizational and didactic tool to explain the properties of some biological systems. However, the limited linear conceptual framework determines how these pathways are studied and implicated in human disease states. Therefore, new models may be necessary to respond to emerging technologies that suggest a very different view of signal transduction and thus the pathophysiology of disease.

A New Look at Signaling

The wealth of genetic and biochemical data suggests two major revisions to the traditional, canonical view of signaling pathways: (1) a massive increase in the number of components linked to a particular pathway and (2) an appreciation for the variable quantitative contribution of each of these new components to dynamic signal propagation.

Systematic yeast two-hybrid and coimmunoprecipitation assays to explore several eukaryotic proteomes—including those of the budding yeast *Saccharomyces cerevisiae*, fruit fly *D. melanogaster*, worm *Caenorhabditis elegans*, and human—have revealed thousands of new protein complexes and connections among many functionally unannotated proteins (for example, see Giot et al., 2003; Krogan et al., 2006). These data collectively suggest that cellular processes are orchestrated by a much larger protein network than previously thought. As most of these screens are neither saturating nor within endogenous signaling contexts, this conclusion is likely to be revised and expanded in the near future.

Unbiased genetic studies have provided validation that these new interactions are often functionally relevant and frequently demonstrate an even wider collection of genes that affect previously well-defined simple processes. Synthetic genetic array (SGA) analysis of yeast single-gene

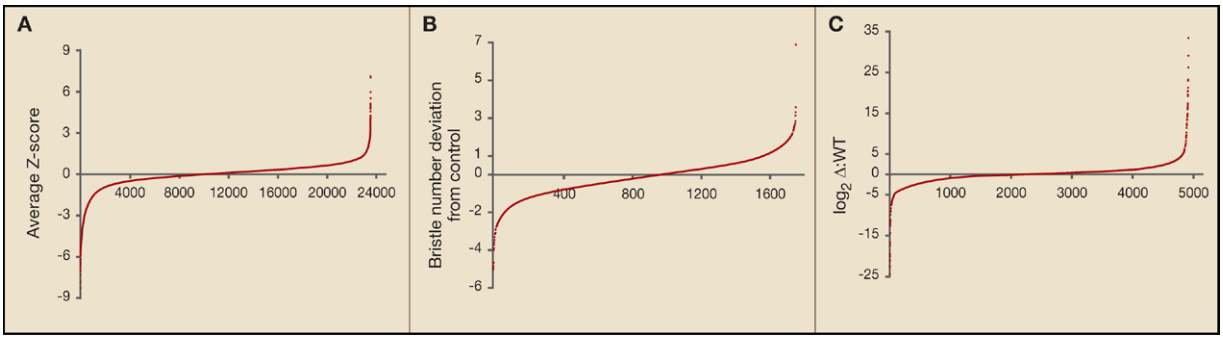

Figure 1. Continuous Distributions of Quantitative Signaling Readouts

(A) Quantitative assay for ERK activation in a cell-based *Drosophila* RNAi screen (Friedman and Perrimon, 2006). Effect of each double-stranded RNA is represented as a *Z* score relative to control ERK activation.

(B) In vivo quantitative P element collection screen in *Drosophila* assaying deviation of bristle number (Norga et al., 2003).

(C) Genome-wide quantitative deletion screen for G protein/MAPK signaling in *S. cerevisiae* (Chasse et al., 2006). Effect of each deletion on mating factor stimulation relative to an internal wild-type control. Unpublished dataset is courtesy of H. Dohlman.

deletions suggests that 10%–50% of genes without a significant effect on growth alone may have a synthetic genetic interaction growth phenotype (Tong et al., 2004). In yeast metabolic systems, although only 13% of metabolic genes are essential by single-gene knockout studies, computational methods demonstrate that 74% of the genes contribute to metabolic function (Deutscher et al., 2006). Advances in RNA interference (RNAi) technology, notably the recent creation of genome-wide RNAi screening libraries, have helped to speed up the rate of gene discovery. Similar to the SGA studies in yeast, synthetic genetic RNAi interactions between disease-relevant mutations in *C. elegans* and ~1750 genes similarly uncovered new roles for genes in signaling (Lehner et al., 2006). Although *C. elegans* screens often assay developmental phenotypes, *Drosophila* and mammalian RNAi screens are performed in cell culture, with many of these focused on dissecting the major signaling pathways. A hallmark of these RNAi screens is that the number of new hits is in the hundreds. As an example of the application of genome-wide RNAi screening technology to signaling, we recently completed a screen for regulators of ERK/MAPK activity following RTK stimulation and identified 331 potential regulators of RTK/ERK (Friedman and Perrimon, 2006). This rapid expansion of functional annotation provides a starting point for detailed mechanistic analyses of the new regulators in the setting of animal development and disease.

As cell-based RNAi screens for signaling pathways use quantitative pathway reporters, genome-wide screens can also reveal the individual contribution of each gene to the signaling output, as measured at the given point in the pathway. This can be best understood by observing total assay distribution plots that rank the score of each double-stranded RNA tested (and thus the contribution of each gene when knocked down) from the strongest positive to the strongest negative regulators (Figure 1A). An under-appreciated feature of these distributions is that, strikingly, they are continuous functions. Frequently the known canonical components are the strongest regulators, but, unexpectedly, they are not a discrete cluster. Sequence-specific off-target effects associated with long double-stranded RNAs used in these RNAi screens (Kulkarni et al., 2006; Ma et al., 2006) may contribute to this distribution. However, in our view, curation of false positives from the assay distributions may not change the overall picture of a continuous function for proteome regulation of particular pathways.

Given concern over RNAi off-target effects, it is important to note that continuous distributions of genetic contributions to signaling output have also been observed in systems other than *Drosophila* cells and RNAi, as long as a quantitative trait is being measured. Examples include several mammalian overexpression and RNAi screens, a quantitative screen for defects in bristle number in *Drosophila* (Norga et al., 2003), a process linked to Notch and EGFR signaling (Figure 1B), and a genome-wide gene deletion screen in yeast for modifiers of the mating-type response signaled by G protein/MAPK pathways (Chasse et al., 2006) (Figure 1C). The emerging view is that there is not a specific number of regulators of a pathway. In fact, the definitions of a finite number of potential hits in such screens (e.g., 331) are based on arbitrary thresholds frequently used for practical reasons.

Recent data from systematic unbiased biochemical and genetic experiments and cell-based, pathway-specific quantitative functional genomic screens argue (1) against signaling pathways as being limited to a handful of canonical components and (2) that the large regulatory network has a graded effect on signaling output.

Genetic Screens and Robustness

If recent technological advances suggest a newer model of signal transduction cascades, perhaps our current view is an artificial product of previous methodologies used to identify and organize them—namely, traditional mutagenesis screens for

developmental defects. How have developmental screens in model organisms yielded a different view of signaling than that arising from unbiased quantitative screens?

The difference between animal and cellular activity-based screens may reflect the robustness of biological networks. Signaling networks underlying animal development are well-known examples of robustness in biology (Stelling et al., 2004; Wagner, 2005)—that is, the developmental vector toward the wild-type is highly resistant to both extrinsic (environmental influences such as temperature) and intrinsic (mutations) perturbations. Examples of robustness (alternatively called buffering or decoupling) can be dramatic: *Drosophila* embryos from females with five additional copies of the *bicoid* gene have an enlarged head and compressed parasegments but, amazingly, develop into "fairly normal" adults (Busturia and Lawrence, 1994). Robustness is a long-recognized phenomenon, a generalization of Waddington's "canalization" of animal development: "the genotype can, as it were, absorb a certain amount of its own variation without exhibiting any alteration in development" (Waddington, 1942). An alternative view of robustness is that developmental signaling networks inhabit a large neutral space of qualitative output, tolerating quantitatively measurable differences without affecting the end-point phenotype (Wagner, 2005). Technological advances have enabled explicit experimental and computational investigation of Waddington's strikingly prescient insights revealing extensive robustness in cell growth and metabolism.

Both theoretical and experimental studies have identified multiple potential sources of robustness, including modularity and redundancy, network architecture, and feedback control (Stelling et al., 2004). Redundancy of individual genes or modules of groups of proteins is a relatively straightforward mechanism to buffer genetic change. Theoretically, the structure of biological networks, such as protein-protein interaction networks, provides some resistance to perturbation. The probability that a given node in nearly all of these biological networks has a particular number of links to others follows a power law distribution, referred to as a scale-free network (Barabasi and Oltvai, 2004). A consequence of this structure is that random attacks (mutations) have little effect as most genes affect few others, but an attack on a hub can cause a dramatic breakdown, that is, a vast increase in network path length. It is likely, however, that the critical component of robustness in signal transduction is feedback control, as the information in signaling networks is propagated over time. For example, positive feedback ensures that weak input stimuli in the network evolve into discrete outputs (switch-like behavior), whereas negative feedback control suppresses undesired stochastic effects on the final output.

Robustness toward one outcome—the wild-type—is balanced by fragility to certain kinds of attacks (on feedback controls, for example). Other robust characteristics of the network (such as other positive feedback loops) may remain after an attack and therefore can be hijacked in a cascading failure (Stelling et al., 2004). The behavior of the attacked network may itself be robust, with an entirely abnormal outcome, and analysis or perturbation of this network may show little resemblance to the original network.

Given the robustness of signaling networks, the success of genetic screens in elucidating a number of signaling pathways is surprising. Indeed, for a mutant to alter morphology sufficiently to be observed, the developmental network has to be perturbed to cause complete breakdown. A decade ago, the special characteristics of zygotically lethal genes were appreciated in terms of developmental switches or controlling elements that logically had spatially restricted expression patterns (Wieschaus, 1996). Within the language of networks, genes that, when mutated, result in lethality must disrupt the robustness of developmental signaling pathways. Thus, a special characteristic of these genes may be that they are hubs—a fragility in scale-free networks. In support of this model, in yeast, essential genes are among the most highly connected in the global protein interaction network (Barabasi and Oltvai, 2004; Krogan et al., 2006). However, recent studies question this "centrality-lethality" concept. Hubs can be separated into those that tend to be coexpressed with their interaction partners ("party" or intramodule hubs) or not coexpressed ("date" or intermodule hubs). Only attacks on date hubs have dramatic effects on network path length despite both types being equally essential, and so network connectivity can be uncoupled from the essentialness of proteins (Han et al., 2004). Reinterpreting network architecture within a dynamic context (correlated gene expression) provides a more complicated view of centrality-lethality. Further inclusion of dynamics within the timescale of signaling—for example, seconds and minutes—may refine the contribution of network architecture to signaling robustness. Within this context, robustness imparted by feedback control may be more compelling. Mutations in genes outside of major canalizing feedback loops may have little effect because these loops maintain the same quantitative output of the overall intercellular network, even if mutation of the gene does cause quantitative effects when measured elsewhere in the intracellular network. On the other hand, mutation of genes that break major feedback loops destroy the canalizing forces. Thus, genes with similar lethal, morphologically mutant endpoints have been closely associated within networks as pathways. Other, similarly "close" regulators of that network with slightly different connections within feedback loops may have different, but not recognizable, outcomes.

Even if the genetic buffering of particular mutations is not complete, another major cause of the failure to identify other potential pathway regulators is pleiotropy. Pleiotropic genes either function in multiple pathways (e.g., casein kinase and protein

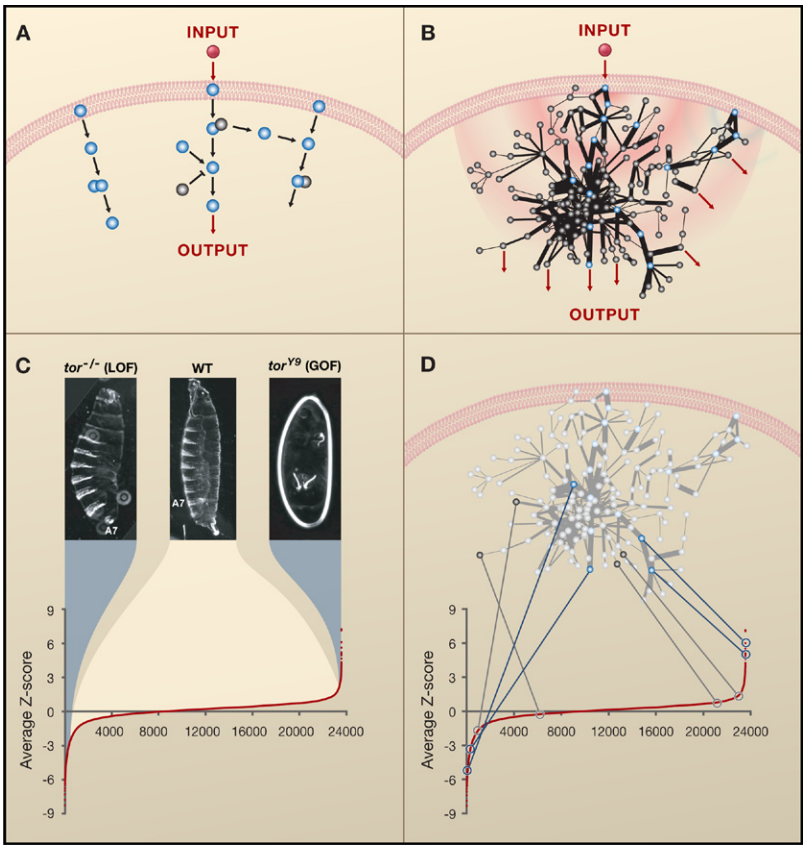

Figure 2. Traditional Developmental and Recent Cell-Based Views of Signaling

(A) Simplified cell circuit diagram for a signaling pathway depicting a few, compartmentalized components without quantitative information.

(B) Network model for signaling. Following a stimulus, information is propagated throughout the network, leading to multiple outputs. Relative contribution, or flow rate, of each protein to the maximal output is represented by line weights (thicker implies larger contribution of that protein or interaction to the output). Canonical components (blue) from genetic screens may make many of the quantitatively significant connections, as measured at a particular protein within the network. Model complexity is magnified when quantitative contributions are considered in the context of multiple signals.

(C) Mutant developmental phenotypes may appear only at the extremes of genetic contribution to the signaling pathway due to robustness. Shown is the correlation between extreme positive *Z* scores (negative regulators) in an RTK/ERK screen (Friedman and Perrimon, 2006) and a gain-of-function (GOF) Torso RTK phenotype (tor^{Y9}) and extreme negative *Z* scores (positive regulators) and a loss-of-function (LOF) Torso phenotype ($tor^{-/-}$). All other gene contributions are buffered by the developmental context and appear as wild-type (WT).

(D) Relationship between cell-based quantitative screen output and a network signaling model. In this screen, canonical components have the greatest impact on signaling output and thus are represented at the extreme ends of the distribution. More distant and quantitatively less important proteins appear internally along the distribution. Measuring signaling output at one of these proteins may result in canonical components having weak effects in the assay.

(Images in panel C courtesy of Willis Li.)

phosphatase 2A family members) or are used in modules that are redeployed multiple times (e.g., MEK acting downstream of RTKs). Because developmental outcomes were used in traditional genetic screens, the role of a pleiotropic gene may be obscured by an earlier, perhaps unrecognizable phenotype. Lastly, proteins that are party hubs or components of feedback circuits may genuinely interact with and control many other genes or proteins in a single pathway and therefore result in phenotypes when attacked. On the other hand, genes such as heat-shock proteins or general transcription factors, while connected to many other proteins and modifiers of multiple networks, may not transmit information among them (they have unidirectional information flow). These latter genes are expected to be pleiotropic and may correspond in part to intermodule date hubs; indeed, genetic interactions among date hubs are twice as likely as party hubs (Han et al., 2004). Given that hubs are more likely than non-hubs to result in phenotypes when attacked, some proportion of these may be pleiotropic and thus missed during genetic screening.

Experimental evidence supports widespread pleiotropy. Heat-shock protein Hsp90 was one of the first experimentally defined genetic buffers for morphological variation (Rutherford and Lindquist, 1998). More recently, the *C. elegans* RNAi screen for modifiers of disease-linked pathway genes reached the same conclusion: genetic buffers including general transcriptional machinery can modify multiple disease-linked pathway mutants in vivo (Lehner et al., 2006). A study of cell growth in yeast revealed that pleiotropy may be much more common than is appreciated: 216 out of 767 deletion strains affected growth under more than two conditions (Dudley et al., 2005). An assay for wing shape in *Drosophila* found that mutations in genes nominally in the same pathway had distinct effects on morphology, implying that these genes do not operate in the same pathway to control wing shape, and hence "quantitative cross-talk between pathways may be complex" (Dworkin and Gibson, 2006).

More broadly, it is obvious that a screen for developmental morphological alteration must rely on phenotypes that are visible. Indeed, only 580 out of 4500 mutations analyzed in the *Drosophila* embryonic lethal screens had a morphology that was sufficiently altered to be classified (Wieschaus, 1996). In addition, theoretically, mutant phenotypes may be more variable than the "constancy of the wild-type" (Waddington, 1942). Although not universal (Dworkin and Gibson, 2006), it may be true in some cases that mutant variability reduced the likelihood of further functional characterization by the investigator. More importantly, if regulators have a

weaker quantitative effect on signaling, they may not have been observed. Hence, assay sensitivity itself is a barrier to isolation of genes.

Thus, the practical constraints of traditional screening and pleiotropy may have led to the isolation of a few canonical pathway components. The robustness of biological networks transforms the continuous distribution of gene effects on signaling networks into discrete developmental outcomes in mutagenesis screens (Figure 2C).

New Methods to Study Signaling

If traditional genetic screening is unlikely to comprehensively identify new components of signaling networks, how can we uncover these links? Two major problems hinder this discovery: sensitivity and scale. The robust developmental network includes both intracellular signaling networks and intercellular communication and coordination. Developmental robustness, for which the final outcome is organismal organization, is on a different scale than specific intracellular signaling networks, which may be able to buffer biochemical noise but not abrogation of feedback control (Stelling et al., 2004). Such perturbations may or may not be buffered on the scale of multicellular developmental morphogenesis. Thus, to identify new pathway regulators, we must focus genetic screening below the layer of the canalizing elements, at the scale of the cellular network itself. In addition, screening should be sensitive and quantitative rather than relying on discrete or qualitative outputs so that the relative contribution to the network can be appreciated.

Functional RNAi screening of signaling networks using quantitative cell-based assays approaches this goal. Such assays using reporters measure output of specific signaling networks removed from the buffering developmental context. Phospho-specific antibody assays, in particular, offer insight into the network signal level at specific points within the network, an advantage over transcriptional reporters at the bottom of networks (their output). RNAi screening still suffers from time lags that may amplify network failure. However, the delay is necessary for depletion of the gene rather than the appearance of a phenotype.

Although the isolation of specific networks in cells may be an advantage for identifying new pathway components, some may consider this system to be less relevant to signaling in vivo. Perhaps another approach would be to perform quantitative in vivo genetic screening, such as done recently in *Drosophila* for bristle number (Norga et al., 2003) and wing shape (Dworkin and Gibson, 2006). Notably, when a sensitive quantitative trait is being measured, the resulting similarity of the assay score distribution plots for these in vivo screens and RNAi screens is striking (Figures 1A and 1B), suggesting that noncanonical proteins are also isolated in vivo as well as in cell culture. These studies using a collection of P-element insertion lines could be performed in a more systematic manner using a genome-wide collection of transgenic *Drosophila* RNAi lines. In this way, large-scale synthetic phenotypic screens could be conducted in *Drosophila* similar to RNAi screens that have proven successful in *C. elegans* (Lehner et al., 2006).

New Models for Signal Transduction

Recent data demonstrate that extracellular cues are transmitted through a dense network of proteins with variable contributions to the network output. The circuit-like signal transduction models of compartmentalized linear pathways (Figure 2A) consisting of only a few components have been defined as such precisely because of the robustness of the developmental context in which their organization was described. The importance of these components as central players becomes reinforced when they are repeatedly used as reagents (in epistasis experiments, for example) for testing new regulators. Their centrality may be further examined by proposing that, following their disruption, the remaining signaling network and its robust character is hijacked, producing a developmental vector toward a completely abnormal morphological endpoint. As we suggested above, analysis or perturbation of this (new) network may reveal little about the properties of the original wild-type network. The time lags (days or weeks) incorporated into developmental screens compound cascading failure on the timescale of intracellular signaling networks as the delays allow dramatic network failure to be amplified. Therefore, the simple hierarchical or network structural relationships established in a particular developmental mutant (for example, by epistasis) for distantly connected pathway components may be questionable.

As opposed to electric circuits, a model we favor for signal transduction (Figure 2B) is that of a gradient of quantitative information propagated outward throughout the dense protein network following an input through an individual receptor. A central maximum of information transfer can include the old canonical pathways (Figure 2C), but the propagation throughout the signaling network may be in part stochastic and influenced by the local network composition. Information, usually in the form of phosphorylation events, is propagated through each protein at a particular rate, quantitatively influencing network output, thus requiring a dynamic signal transduction model. The rates are likely to be highly variable depending on cell type, context, or disease state. Subcellular compartmentalization of segments of the signaling network can restrict information flow to particular subnetworks. Network architecture and feedback control determine how the network responds to perturbation: removal of a protein through which 20% of the input signal propagates when measured locally may lead to compensation through other proteins resulting in little change when measured distantly. The continuous distribution observed when a network measurement is made in cell-based screens may be due to a combination of the individual protein's biochemical activity within the network and the

distance in the interactome between the knocked-down protein and the protein used for the assay. Although physical protein-protein interactions are the simplest to visualize within this model, other interactions (protein-DNA and protein-RNA) must be incorporated to explain isolation of particular functional classes (e.g., RNA splicing factors) from screens. That similar distributions are observed in yeast, where gene deletions have been used, and in vivo argues against degree of knockdown (hypomorphic states) as an explanation for the continuous distributions. However, there may be other models that emphasize other features to explain current data. Although network models of signal transduction, in the form of crosstalk, are not new, our model incorporates a much larger scale and considers quantitative contributions of individual components and network dynamics. We note that this framework contrasts drastically with previous views recently expressed by some of us (Noselli and Perrimon, 2000), reflecting the impact that new methodologies can have on our thinking.

Although current cell-based pathway-specific screens do isolate the canonical components as the strongest regulators, it is important to remember that these screens use the canonical components as reagents (e.g., well-characterized enhancer elements for transcriptional reporters or phospho-specific antibodies directed toward canonical component isoforms). Performing unbiased screens using noncanonical reagents would measure signaling output at different points within the network and may not necessarily still show that the canonical components are the strongest regulators following the same stimulus.

Given that quantitative unbiased screens measuring points within the signaling networks have uncovered many new regulators, a major challenge going forward will be to reconstruct the dynamic structure of the networks using computational and experimental methodologies. Although large-scale, global static interaction maps are useful initially (for example, Giot et al., 2003; Krogan et al., 2006), these rarely recapitulate the often transient connections of known signaling modules. More helpful for understanding signal transduction networks would be time-dependent experimental and computational reconstructions of local signaling networks.

Finally, while we argue for recontextualizing signal transduction within network biology rather than canonical pathways, the comparison between the findings of signaling RNAi screens and traditional developmental screens itself provides crucial information about the origin of developmental robustness. If the former provides a much more comprehensive picture of local signaling networks, then the failure to isolate particular genes from the latter may suggest the general mechanisms that organisms use to canalize development and explore neutral signaling space. Both computational (Deutscher et al., 2006) and experimental (Lehner et al., 2006; Rutherford and Lindquist, 1998) approaches have already begun such work.

Signaling Networks and Human Disease

Changing the language—and experimental approaches—of signal transduction research from cell-circuit pathways to network propagation impacts how we approach human disease and its treatment. Genome-wide association studies isolating common genetic variants that influence disease susceptibility—quantitative trait loci (QTL)—are essentially equivalent to unbiased in vivo genetic screens with quantitative readouts, such as those in *Drosophila* observing effects on bristle number (Norga et al., 2003) or wing shape (Dworkin and Gibson, 2006). Understanding signal transduction from a network perspective allows an appreciation of how variants of proteins (by mutation or concentration) within the network can lead to quantitatively different outputs. Thus, for diseases known to be linked to disruption of particular signaling networks, comparison of QTLs with functional genomic RNAi screens of those networks synergistically provides a mechanistic insight into disease etiology. More powerfully, given that for most diseases the underlying cellular mechanisms are incompletely known, comprehensive searches for enrichment among QTLs for genes that regulate particular networks (discovered by RNAi screens) may help to uncover the underlying cellular signaling pathology. As the number of quantitative traits from these studies starts to soar, ranking traits for future analysis and understanding their mechanism of action will become a necessity.

Cancer research is likely to benefit most from a network appreciation of signal transduction. Cancer is indicative of both the fragility of ordinarily robust signaling networks and is itself a robust phenomenon, reflected by the paucity of effective treatments (Stelling et al., 2004). Efforts to sequence cancer genomes, exemplified by the recent proof-of-principle sequencing of a large number of genes from breast and colorectal cancers (Sjoblom et al., 2006), projected an average of 93 mutated genes per tumor, 17 of which are causative, suggesting that many mutations are needed to attack the robust cell signaling network. It is logical that signal transduction and transcriptional machinery components are extremely common functional categories mutated in these cancers (Sjoblom et al., 2006). Indeed, targeting of these hubs through mutations is likely to result in dramatic changes in network structure. Thus, identification of networks for the most common cell signals (for example, by RNAi screening) may help to elucidate the underlying reasons these mutations occur and how they disrupt robustness. Monitoring the signaling networks at multiple points and under multiple stimuli simulating endogenous signaling can help to predict outcomes of mutations and, more importantly, responses to treatment by individual tumors (Janes et al., 2005). An alternative model for cancer, "oncogene addiction," speculates that mutation of single genes rather than networks of genes may underlie cancer progression (Weinstein, 2002). Even if

this model applies in some circumstances, network modeling could predict "Achilles' heel" genes in addition to a few well-characterized oncogenes and predict if targeting of these oncogenes could lead to cancer regression. Thus, identification of the components, contribution, and structure of the signaling networks through experimental approaches will help us to understand how hijacking of network robustness by oncogenic stimuli buried within the network generates distinct mutant (carcinogenic) outputs and how we can target the robustness of tumors to design better therapeutics.

ACKNOWLEDGMENTS

We thank R. Dasgupta, B. Mathey-Prevot, M. Kulkarni, and C. Bakal for helpful comments and H. Dohlman for sharing unpublished data. A.F. is supported by the Medical Scientist Training Program (MSTP). N.P. is an investigator of the Howard Hughes Medical Institute.

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