Chapter 5

Analyzing the Structure, Function and Information Flow in Signaling Networks using Quantitative Cellular Signatures

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THE CONCEPT OF LINEAR CASSETTES AND MODULARITY IN SIGNAL TRANSDUCTION

Cells communicate and respond to conditions in their local environment through signaling, a process consisting of a series of regulated steps that help propagate information across the external plasma membrane to the cell interior, and often to the nucleus, to regulate diverse cellular processes such as growth, proliferation, differentiation and apoptosis. The set of molecules recruited by a specific signal defines what is commonly referred to as a signal transduction pathway. Dissection of biological responses to similar families of ligands in various cell types and organisms revealed that these ligands regulate the activity of similar sets of downstream genes, a finding that led to the concept of 'evolutionarily conserved signal transduction cassettes' or modules [1]. A characteristic feature of these modules is the occurrence of a tight internal link between their individual components, and few, but well-defined connections to the rest of the system in which they operate. The concept of modules gained further acceptance when it became clear that these characteristic chains of events were iterated in the same pattern in different cellular and developmental situations. The extent of signaling modularity was underscored by studies of signal transduction in simple genetic model organisms, where signaling tends to be simpler than in more complex mammals (see below) [2]. The methodologies used to recognize and characterize these pathways relied mostly on the similarity in visible mutant phenotypes or screens in sensitized genetic backgrounds (see Box 5.1 for definition). The resounding conclusion from these studies was that of elegant simplicity: it is common for loss-of-function mutations (see Box 5.1 for definition) in different genes that constitute a specific pathway to result in identical phenotypes. For example, in the context of JAK/STAT signaling, mutations in the activating extracellular ligand (Unpaired/Upd), in the effector tyrosine kinase (Hopscotch/JAK), or in the effector transcription factor (STAT92E/Marelle) caused identical loss-of-function phenotypes, a feature consistent with linearity of the pathway [3] (Figure 5.1**A**). Furthermore, gain-of-function phenotypes (see Box 5.1 for definition) had strikingly opposite developmental phenotypes and could usually be reverted completely by removing the activity of a more downstream component of the pathway.

Handbook of Systems Biology Concepts and Insights. http://dx.doi.org/10.1016/B978-0-12-385944-0.00005-8
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Box 5.1 Key Terms and Concepts

- **Sensitized genetic background**: A sensitized genetic background is a mutant state in which an allele of a gene leads to a weak phenotype in the biological process under study (for example eye development in *Drosophila*). Thus, a weak allele for gene X may lead to fruit flies with abnormal/small eyes, whereas a strong/null allele would produce flies with no eyes. In the sensitized background carrying the weak allele for gene X it is possible to screen for new mutants that either enhance the phenotype (small eyes to no eyes) or suppress the phenotype (small eyes to normal eyes). Such sensitized genetic screens can lead to the identification of genes that function in the same pathway or genes that act redundantly in parallel pathways.
- **Saturation screen**: A saturation screen is a genetic screen that is performed to discover all genes that are involved in a particular biological process. One of the first saturation screens was performed in *Drosophila* by Christiane Nüsslein-Volhard and Eric Wieschaus (1980) to uncover genes that were associated with embryonic lethality and changes in cuticle morphology. In such genetic screens, a mutagen such as a chemical or radiation is used to generate mutations in the organism's chromosomes. Individuals that exhibit the phenotype of interest are selected and the mutant alleles are mapped and cloned to identify every gene involved.
- **Loss-of-function mutation**: Changes in the DNA sequence of a gene that leads to reduced or abolished function of the gene product.
- **Gain-of-function mutation**: Changes in the DNA sequence of a gene that confers a new and/or abnormal function to the gene product.
- **Epistasis analysis**: Epistasis is the interaction between two or more genes where the effect of one gene on a particular phenotype is modified by other gene(s). The gene whose phenotype is manifested is called epistatic, while the gene whose phenotype is modified as the result of the epistatic gene is called hypostatic.
- **Synthetic lethality**: Mutations in two genes are said to be synthetically lethal when cells with either of the single mutations are viable but cells with both mutations are lethal. A synthetic lethal genetic screen, starts with a mutation in gene X that does not kill the cell, but may confer a weak phenotype (such as, slow growth). This genetic background is then used to systematically test mutations in other genes that may lead to lethality.
- **SDS-PAGE (sodium dodecyl-polyacrylamide gel electrophoresis)**: SDS-PAGE is a technique that is widely used in molecular biology and biochemistry to separate proteins as a function of their length and charge by application of an electric field.
- **iTRAQ (isobaric tags for relative and absolute quantification) and TMT (tandem mass tags):** iTRAQ and TMT are used to identify differentially phosphorylated proteins between different samples. These amine-reactive molecules enable multiplexing of up to 4–8 samples: the small molecules, identical in structure and mass, differ in the isotopic substitution of atoms comprising their backbone. This altered distribution permits the unambiguous spectral identification of unique reporter ions generated from fragmentation of each tag during MS. The fragmentation of each tag during MS results in the release of a signature reporter ion that differs in mass from the other tags; the signature ions released from the six tag set differ successively by 1 Dalton and their relative levels can be considered to reflect differences in peptide levels between samples. iTRAQ and TMT enable the concurrent analysis of multiple samples, and the assessment of the relative levels of phosphopeptides.

Such properties have been instrumental in working out the epistatic (see Box 5.1 for definition) relationships between genes and in ordering components of a given pathway into linear, minimally branched cascades [4]. These pioneering studies have helped to reduce complex biological and developmental processes to a finite number of paradigms and clarified the identity and relationship of key components in evolutionary conserved pathways. Reflecting the implied linearity and independence of these pathways from each other, most are named after the activating ligand or a central effector protein, for example the Wnt/Wingless, Hedgehog (Hh), TGF-β, JAK/STAT, Toll, NFκB, Notch, receptor tyrosine kinase/extracellular regulated kinase (RTK/ERK), Akt/TOR, Jun Kinase (JNK), G protein-coupled receptor (GPCR) and steroid hormone pathways [5]. The analysis of signal transduction pathways in mammalian cells has presented a more complicated view, hinting at significant bridges or 'cross-talks' between various signal transduction modules/pathways. Cross-talk or interaction between pathways allows cells to respond in a coordinate manner to the combined extracellular and intracellular cues that they are exposed to in order to produce the appropriate response. Cross-talk between pathways accounts for many complex signaling behaviors, including signal integration, the ability to generate a variety of different responses to a signal, and/or to reuse proteins between pathways. For example, the SMAD proteins, which have been assigned to the TGF-β signaling cassette, can be phosphorylated by MAPK, which functions in a separate pathway in lower eukaryotes. Thus, rather than following a simple path, a signal received by a mammalian cell may be relayed through multiple channels. Similarly, other proteins such as Ras, protein kinase C (PKC), and protein kinase B (Akt) are also activated by multiple extracellular ligands. However, much of this knowledge has originated from studies in cell lines and in vitro

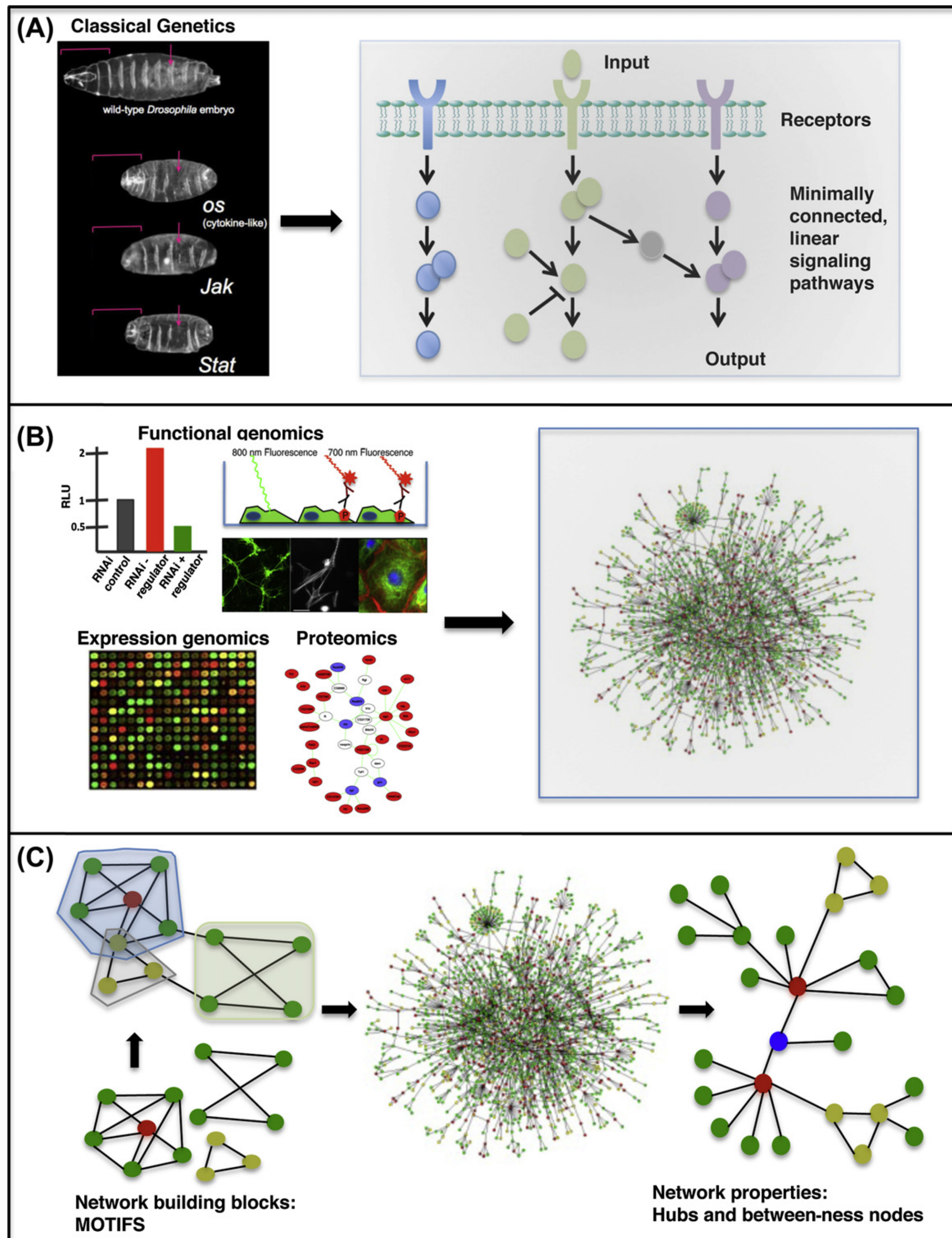

FIGURE 5.1 **Evolution of linear signal transduction pathways to highly organized signaling networks. A.** Mutant developmental phenotypes from classic genetics screens showed that mutations in the activating extracellular ligand (Unpaired/Upd), in the effector tyrosine kinase (Hopscotch/JAK), or in the effector transcription factor (STAT92E/Marelle) caused identical loss-of-function phenotypes, a feature consistent with linearity of signal transduction pathways. **B.** Systems-level, functional genomic, proteomic, and expression studies in the past few years have revealed that signaling is propagated within large networks consisting of hundreds or thousands of proteins. **C.** Structure—function analysis of signaling networks has led to the identification of network motifs, recurrent patterns of interconnections, that form the building blocks of networks as well as universal features such as Network Hubs (red circles) and between-ness nodes (blue circles) that are likely to be encoded by essential genes.

biochemical assays, and there is some question as to whether the same rules apply in vivo, or whether organisms have evolved tight controls to maintain modularity and prevent promiscuous cross talk.

More recently, newer findings from in vivo genetic studies have put the linear and simplistic model of signaling pathways increasingly at odds even in lower or simple metazoan model organisms. For example, during dorsal closure of the *Drosophila* embryo, the JNK, small GTPase and TGF-β pathways may act together or sequentially [6]. In addition, components that initially were thought to be unique to one pathway have now become implicated in others. For example, GSK3β and CK1α act as important regulators of both the Wingless and Hedgehog pathways in *Drosophila* [7]. Further, the Hippo pathway has recently been found to restrict Wingless/β-catenin signaling by promoting interaction between a canonical Hippo pathway target, the transcription factor TAZ/Yorkie, and Dishevelled, a canonical cytoplasmic component of the Wingless pathway [8]. In addition, the serine/threonine kinase Fused (Fu), a component of the canonical Hedgehog pathway, functions together with the E3 ligase Smurf to regulate the ubiquitylation and subsequent degradation of Thickveins (Tkv), a BMP receptor, during *Drosophila* oogenesis [9]. Altogether, an increasing number of examples escape the canonical view of linear signaling cassettes but rather argue in favor of more elaborate signaling mechanisms in which variations in both content and molecular interactions are a general feature of and between signaling pathways. In summary, our knowledge of the organization of signaling pathways is still rudimentary despite our extensive understanding of some of the players involved in signal transduction. Recognizing the flexible and interconnected nature of signaling cascades will promote a more systematic study of complex cellular signaling, which in turn may greatly improve our understanding of the origin of signaling versatility in development and pathology.

GENETIC DISSECTION OF SIGNAL TRANSDUCTION PATHWAYS

In 1958, G. Beadle and E. Tatum received the Nobel Prize in Physiology and Medicine for demonstrating that ‘body substances are synthesized in the individual cell step-by-step in long chains of chemical reactions, and that genes control these processes by regulating definite steps in the synthesis chain (http: //www.nobel.se). Since the realization half a century ago that genes encode the building blocks that make up cells, identifying their functions has become a priority in the life sciences.

Historically, identifying gene function has relied on genetic approaches whereby the function(s) of a given gene is inferred from the phenotype(s) associated with a mutation in that gene. The systematic application of genetics has led to a wealth of knowledge in processes such as pattern formation during development, and signal transduction. For example, saturation screens (see Box 5.1 for definition) have led to a global understanding of pattern formation in the early *Drosophila* embryo, and to the identification of the key genes involved in the process [10]. A major result of these seminal studies was that genes exhibiting the same or similar morphological mutant phenotypes were often found to be part of the same signaling pathway.

An important consideration to keep in mind when taking a genetic approach to deduce gene function is that one studies the global response of the organism to a genetic perturbation. Thus, the endpoint phenotype may be telling us more about the way an organism responds to a genetic perturbation rather than about the wild-type function of the gene itself. A telling example is found in the context of Wingless (Wg/Wnt) signal transduction in *Drosophila*. There, the seven transmembrane protein DFz2 (*Drosophila* frizzled 2), which encodes the Wg receptor, regulates the activities of the Dishevelled (Dsh), Glycogen Synthase Kinase 3 (GSK3) and β-catenin proteins [11]. In the absence of DFz2, a related receptor encoded by Frizzled (Fz) can substitute for DFz2, suggesting that these two related receptors can act redundantly. However, in the presence of DFz2, Fz does not appear to regulate the activity of the Dsh/GSK3/β-catenin pathway, but instead is involved in the regulation of the Planar Cell Polarity (PCP) pathway. Although Fz does not transmit the Wg signal in the wild-type context, the structure of the signaling network allows the activity of Fz to be hijacked to compensate for the absence of DFz2. In this case, the analysis of mutations in *DFz2* failed to reveal the bona fide physiological function of DFz2 in Wingless signaling.

This simple example illustrates a critical but often overlooked concept: when interpreting the results of a genetic approach, there is the danger that our conclusions about the purported wild-type function of a single gene product might be obscured by our existing (but incomplete) knowledge of the signaling network of which it is a component. This is analogous to Plato’s powerful Allegory of the Cave, which argues that our interpretation of the world around us is limited by observations made from our vantage point and current knowledge. Thus, in theory, the best way to fully evaluate the function of a single gene product would be to first have a global understanding of the cellular network in which they operate, then remove that component from the network and conclude about the function of that gene based on ‘network knowledge’.

Access to the full repertoire of genes encoded by different genomes has made it possible to design new systems-level approaches based on the principles of ‘reverse genetics’ to construct such global cellular networks. Reverse genetics is an approach to discover the function of

a gene by analyzing the effect of specific gene sequences on a phenotype, in contrast to forward genetics, which seeks to find the genetic basis of a particular phenotype. The availability of full genome sequences for all of the well-studied model organisms has given rise to the field of functional genomics, which attempts to describe gene sequences in terms of function (by perturbing the gene using, for example, RNA interference (RNAi) and examining the array of phenotypes associated with the perturbation), expression (by expression profiling using genome-wide microarrays) and protein–protein interactions (by proteomics studies, for example mass spectrometry (MS)). Functional genomic studies are generally carried out on a genome-wide scale using high-throughput methods rather than the more traditional 'gene-by-gene' approach, and can be implemented in both whole organisms and cell lines.

Importantly, functional genomic studies to analyze genotype–phenotype relationships using emerging technologies such as RNAi are based on quantitative (for example relative luminescence units from a luciferase reporter) rather than qualitative readouts (cuticle defects) to reflect effect on phenotype. The use of quantitative pathway reporters has led to the identification of hundreds of pathway components, each of which contributes to the measured phenotype, albeit in varying amounts. This can be best understood by ranking each gene by its effect (when knocked down by RNAi) on the quantitative phenotype being measured. In most cases this generates a continuous distribution from the strongest positive regulators to the strongest negative regulators [12]. In addition, large-scale interaction studies have shown that many proteins are involved in many interactions, both physical associations with other proteins, metabolites and nucleic acids as well as post-translational modifications, to regulate cellular and organismal functions. Thus, over the past decade or so, systems-level, functional, expression and proteomic studies have revealed that signaling is propagated within large networks consisting of hundreds or thousands of proteins (Figure 5.1B). This view is in direct contrast to the traditional reductive approaches discussed above, which focus on individual proteins and which had led to the consensus that signaling takes place largely within simple linear cascades. Furthermore, such analyses have led to the generation of network maps to represent cells as complex interconnected 'systems' rather than mere collections of individual molecules. In network representations of cells, 'nodes' represent proteins/metabolites/nucleic acids as 'parts' of the system [13]. The 'edges' represent relationships between the nodes. The generation of such network maps in the last decade has facilitated the identification of characteristic structural features or topologies inherent in most complex networks and has led to the realization that the structural organization of networks is key to their function. Topological analyses (Figure 5.1C) of different large-scale biological networks (metabolic networks [14], protein–protein interaction networks [15], and transcriptional regulatory networks [16]) have found that complex networks have a small-world property (that is, most nodes in the network can be reached from any other node by a small number of steps) [17] and are scale-free (that is, most nodes in the network have very few connections, whereas a few nodes have many connections) [18]. In at least three different eukaryotes – yeast, worm and fly [15, 19–21] – network hubs (nodes with a high degree of connectivity) are often encoded by essential genes. These shared network properties have been proposed to confer functional advantages such as robustness to fluctuations in the environment, tolerance to random mutations [22], and efficient information processing and flow [23]. Thus, the small-world and scale-free nature of biological networks is under positive evolutionary selection [24]. Another characteristic feature of biological networks are small, recurring patterns of interconnections called network motifs that are significantly more enriched in biological networks than in random networks [25,26]. These motifs form the basic building blocks of networks and are directly responsible for the dynamic information processing functions of biological systems (Figure 5.1C). For example, negative feedback loops increase the speed of response to incoming signals and help to reduce variations in protein levels across cells. On the other hand, positive feedback loops slow down the response time and can lead to a bi-stable (ON or OFF) switch-like behavior [27–30]. Another topological feature that has recently come into the limelight is the 'between-ness centrality' of nodes in a network [31,32]. Between-ness centrality measures the number of non-redundant shortest paths going through a given node [33,34]. Nodes with high between-ness are considered to represent bottlenecks within networks and are analogous to major intersections in a transportation network. Blockage of such intersections would cause a major traffic jam, leading to failure of the transportation system (Figure 5.1C), and so bottlenecks in signaling networks represent attractive targets for therapeutic intervention in the case of deregulated signaling. It has been shown that network bottlenecks are not necessarily hubs, but, like hubs, are more likely to be encoded by essential genes [19,35].

It is clear that to fully understand cellular responses to signaling will require approaches that go beyond the more classic genetic and biochemical studies. Indeed 'systems biology' approaches based on high-throughput, large-scale methods are needed to understand cellular responses to signals in toto. At the most basic level, a complete picture of signal transduction first requires a comprehensive 'parts list' of the components that participate in the cellular signaling network. The second step is to comprehensively identify the physical and functional relationships between the nodes and infer how information flows through the

network. Importantly, since the same network is deployed to achieve distinct cellular functions in a context-dependent manner, it is essential to extend the systems biology approaches to construct network models that incorporate information regarding the dynamics of molecular interactions within cells. Inferring the flow of information through a network provides directionality to the edges between the nodes of the network. This can be achieved by systematically perturbing nodes, either by gene knockouts or by RNAi knockdown, followed by measurements of a wide variety of quantitative phenotypes. The phenotypes measured can include changes in gene expression, post-translational modifications such as phosphorylation and acetylation of key proteins, and/or in cellular morphology or behavior. Quantitative phenotypic signatures provide insight into the information processing function of signaling networks, which is key to achieving a mechanistic understanding of how various cellular processes are regulated in time and space.

SYSTEMS APPROACHES TO IDENTIFY THE 'PARTS' OF CELLULAR SIGNALING NETWORKS

RNA Interference (RNAi)

In recent years, as full genome sequences have become available for *Drosophila* [36], human [37,38] and other organisms, large-scale analyses of gene functions have given rise to the field of 'functional genomics'. RNAi has emerged as a unique and powerful functional genomics tool to effectively suppress gene expression in many animal systems [39]. In contrast to other genomic-based approaches, RNAi provides a direct link from gene to function. The development of genome-scale RNAi libraries that contain clones for most genes in a genome in multiple organisms from *Caenorhabditis elegans*, *Drosophila*, mouse and human cells, to the flatworm *Planaria* [40,41] and *Arabidopsis* [42], permits the rapid identification of all genes involved in a particular process [43−49]. Because RNAi is applicable to high-throughput genome-wide analyses it provides a tool to extract functional information globally and comprehensively.

The phenomenon of RNAi was first identified in plants and worms [50]. In *C. elegans*, the process of target gene suppression by RNAi can be triggered by injecting long dsRNAs (~500 nucleotides) into worms, by feeding them bacteria that express the dsRNA or by simply soaking them in solution containing the dsRNAs [51−53]. In *Drosophila*, dsRNAs can be delivered into embryos via injection or by generating transgenic animals that carry RNAi hairpin constructs for in vivo screens [54]. Importantly, the addition of long dsRNAs to *Drosophila* tissue culture cells (dsRNA bathing) can efficiently reduce the expression of target genes [55,56]. Using RNAi in cell lines has led to an explosion of genome-wide cell-based RNAi data for diverse biological processes, including signal transduction, host−pathogen interactions and oncogenesis [47].

When dsRNAs are introduced into cells, they are recognized and degraded by the conserved RNAse III family of nucleases known as Dicer [52,56−60]. Dicer enzymes process the dsRNA into 21−23 nucleotide (nt) short-interfering RNAs (siRNAs) that are incorporated into a multi-protein RNA-induced silencing complex (RISC). This complex directs the unwinding of the siRNAs contained within RISC, and guides RISC to the corresponding mRNA to eventually degrade the targeted transcript. Different types of RNAi reagent have been developed to knockdown target genes in different types of cells and organisms. The four most commonly used RNAi reagents include long dsRNAs (~500 nt), siRNAs (21−23 nt), short-hairpin RNAs (shRNAs; 70 nt) that can be produced exogenously or carried on an expression vector, and endoribonuclease-prepared siRNA (esiRNAs) [61−63]. Typically, RNAi reagents are delivered into cells by virus-mediated transduction for shRNAs, or by lipid-mediated transfection or electroporation for shRNAs, siRNAs, esiRNAs, and dsRNAs [44,46,64,65]. In the case of many *Drosophila* tissue culture cells, dsRNAs are directly taken up from the surrounding medium without the need for transfection [55,56].

The success of RNAi screening depends on the robustness of the cell-based assay, especially its suitability to high-throughput screening (HTS). Almost all HTS cell-based assays provide a quantitative readout for the biological process under study. Many assays use transcriptional reporters where a well-characterized transcriptional regulatory element that is known to respond to the signaling pathway under study is linked to a reporter such as luciferase, green fluorescence protein (GFP) or the *E. coli* β-galactosidase (*LacZ*). The overall output of the reporter can be rapidly measured using a standard plate reader [66−70]. Candidate RNAi hits are identified by their ability to affect the basal or induced expression of the reporter driven by the pathway responsive promoter. Transcriptional reporter-based assays have been used to identify regulators of individual transcription factors such as NFκB [71], E2F [72], and FoxO [73]. In addition, several transcriptional reporter-based RNAi screens have been conducted to identify the regulatory network surrounding cellular signaling pathways, including the Wnt pathway [69,74−76], the Hh pathway [7,70] and the JAK-STAT pathway [66,77]. Further screens based on Oct4 expression level or Oct4 driven GFP expression level have been used to identify regulators of stem cell identity [78,79]. Transcriptional reporter-based assays have several advantages, including easy adaptability to HTS, rapid and automated data collection, and the ability to identify both positive and negative regulators of the process under study. However, transcriptional reporter-based

assays also suffer from a number of limitations that must be taken into careful consideration when interpreting the results from a given screen. For instance, the use of a single pathway-responsive promoter–reporter construct assumes that all signaling through the pathway converges on the single readout being assayed, and as a result components that do not converge on this readout will be missed. Furthermore, synthetic promoter constructs that are composed of a string of binding sites for a single downstream transcription factor do not reflect the endogenous context, as they lack sites for co-regulators or sequences that may be important for epigenetic regulation. These limitations contribute to the false negative rate (the number of true regulators missed) associated with the screen. Although it is not possible to completely eliminate false negatives in a genome-wide screen, one can estimate the false negative rate from benchmarking the data obtained with known pathway regulators. Perhaps a more serious problem is experimental variations due to non-specific factors affecting assay readout, leading to the accumulation of false positive hits in the screen. These include factors that affect the level of the reporter indirectly by having an effect on cell viability/proliferation, global transcription, protein translation and stability. Experimental variations are also introduced owing to differences in transfection efficiency in the case of screens where the reporter and/or RNAi reagents are transiently transfected into cells. Thus, appropriate normalization methods are required to account for false positives due to such non-specific factors. A commonly used normalization procedure is co-transfection of a control reporter (for example Renilla Luciferase) along with the experimental reporter (for example Firefly Luciferase). A good control reporter for assay normalization should include a constitutively active promoter that is inert to the pathway under study and achieves reporter expression significantly higher than background [80].

Transcriptional reporter assays have also been employed to identify transcription factor/signaling pathway regulators in a number of in vivo RNAi screens (i.e., in whole organisms rather than tissue culture cells). For instance, large collections of transgenic RNAi lines have been generated to conduct spatially and temporally defined in vivo screens in *Drosophila* [81,82]. Such resources can be used to systematically screen gene functions in specific tissues for phenotypes or effects on gene expression. For example, a *LacZ* reporter for Suppressor of Hairless (Su(H)) expression in the wing imaginal disc has been used as a readout in a screen for Notch pathway regulators [83]. In another case, serine proteases that are involved in the activation of the Toll pathway upon infection have been identified using a *drosomycin*-*LacZ* reporter assay [80].

Another quantitative cell-based assay makes use of specific antibodies that recognize protein modifications such as phosphoserine/tyrosine or methyl-lysine residues on key components of the signaling pathway itself or on proteins that form a part of the cell's response to activity through the pathway. For instance, genome-wide screens using phospho-specific antibodies have identified regulators of dually phosphorylated MAP kinase/ERK and phosphorylated Akt downstream of receptor tyrosine kinase (RTK) signaling [84–86]. The success of antibody-based assays is critically dependent on the availability of specific antibodies. Such assays can be performed using a simple plate reader to measure fluorescence emitted by the fluorescently coupled secondary antibody. Antibody selection and validation are critical for the development of any high-quality assay. The specificity of the antibody should be precisely evaluated by both Western blotting and immunocytochemistry (cell staining). An antibody that generates strong non-specific bands in a Western blot is not suitable for plate-based assays. Importantly, it must be determined beforehand that the antibody truly detects pathway activity in response to known stimuli and perturbations. A major difference between Western blots and plate-based assays is the context in which proteins are analyzed. In plate-based assays cultured cells are fixed to the bottom of a microplate, and therefore the immobilized antigens present a slightly different conformation than those that have been processed by SDS-PAGE (see Box 5.1 for definition) prior to Western blotting. Thus, it should be noted that although some primary antibodies perform well for Western blotting, they might exhibit poor binding characteristics on fixed antigens, resulting in low fluorescence signal in the plate-based format. As in the case for transcriptional reporter-based assays, the signal from the phospho-specific antibody must be normalized to account for variations in cell number across different wells in the plate. A wide array of fluorescent molecules, including DNA-binding dyes (DAPI, TO-PRO 3), actin-binding dyes (Phalloidin), antibodies to total protein and non-specific cytoplasmic protein stains, can be used for normalization. However, it must be first determined that the stain of choice does indeed provide a linear measure of cell number. These plate-based approaches provide an attractive alternative to high-throughput microscopy (also known as high-content screening, HCS) for assay readouts that are based on immunofluorescence detection. If high-resolution information is not central to the results of the screen, then plate reader assays [84–86] provide a significant advantage in terms of ease and speed of detection, as well as simplifying the downstream analysis to a single intensity measurement per well to report effect on pathway activity.

An alternative to the plate-based assays described above is the transfected cell microarrays that allow the miniaturization and simplification of high-throughput assays [87]. RNAi reagents are spotted on the surface of a standard glass microarray slide and are used to transfect cells. This generates a living cell microarray comprising locally

transfected cells in a mixture of non-transfected cells [87,88]. Transfected cell microarrays facilitate large screens with many replicates, as they offer the advantage of using minimal amounts of antibody compared to traditional plate-screening formats. For example, Lindquist et al. performed a genome-scale RNAi screen on microarrays of *Drosophila* cells to identify novel regulators of mTOR (mammalian Target of Rapamycin) complex 1 (TORC1) signaling by immunofluorescence [89]. A total of 70 novel genes were identified as significant regulators of RPS6, a TORC1 effector. Cell microarrays facilitate large screens with many replicates, as they offer the advantage of using minimal amounts of antibody compared to the traditional plate-screening format. The amount of immunofluorescence from the phospho-specific antibody can be normalized to cytoplasmic area using standard image analysis software packages such as those developed for HCS image analysis [90].

Although plate reader assays have the advantage of speed and ease of performance, HCS is undeniably one of the most powerful HTS assays because it allows multiple cellular features/parameters, such as protein abundance as well as localization, to be measured simultaneously. Image-based screens typically use either fluorescently conjugated primary or secondary antibodies to visualize proteins or cellular structures of interest (for example anti-Fibrillarin antibody to visualize the nucleolus) or fluorescently labeled dyes and GFPs tagged with the appropriate localization signal (for example nuclei, mitochondria, Golgi, and actin filaments). HCS has been performed to identify targets of small molecules/drugs [91–95] and also in a number of RNAi screens to identify genes that affect diverse cellular functions, including cell morphology [96–98], cell cycle progression [99], mitosis [100,101], endocytosis [102, 103] and host–pathogen interactions [104–107]. Quantitative image analysis has also been used to identify genes required for growth and morphology of fluorescently labelled primary neurons/glia and muscle cells in response to RNAi-mediated gene knockdown [108,109]. Although multi-parametric, quantitative image analysis applied to large-scale functional genomic screens promises to generate systems-wide insights into many fundamental cellular processes, automated image acquisition and analysis, feature extraction, and data storage can be challenging and are still undergoing rapid development [110,111]. Other cell-based assays include the use of flow cytometry to measure response to RNAi treatments [112–114].

RNAi HTS in various cell lines using the different cell-based assays discussed above, have been conducted for a diverse array of biological processes, including cell viability [68], cell morphology [96,98], cell cycle [112], cytokinesis [91], susceptibility to DNA-damaging agents [115,116], RNA processing [117,118], general and specialized secretion [67], calcium stores [119–121], factors influencing polyQ aggregation and toxicity [122], mitochondrial dynamics [123], circadian clock [124], hypoxia [125], phagocytosis [113,126], innate immunity [127–129], cell susceptibility to infection by viruses or other intracellular pathogens [104–107] as well as most of the major signaling pathways [7,47,66,69,70,75,77,85,86, 130,131]. Results from these screens have not only identified new components of the process under consideration but have also provided insights into the complexity of signaling networks. RNAi screens in mammalian cells [47] have led to the identification of novel oncogenes and putative drug targets for the development of therapeutics [64,132–136].

Although HTS based on RNAi has transformed the field of systems biology in the identification of gene functions, it is important to keep in mind that inhibition of gene expression by RNAi is not the same as gene inactivation by mutation. RNAi acts at the level of the messenger RNA (mRNA), either by reducing mRNA levels or by blocking mRNA translation [137–139]. Thus, RNAi-based assays can suffer from high rates of false negatives due to incomplete knockdown of mRNA levels (or knockdown of only specific splice forms). Another significant issue associated with RNAi reagents is that they can lack specificity due to suppression of unintended genes, leading to false positives. False positives due to sequence-dependent off-target effects (OTEs) have been shown for RNAi reagents with $\geq$ 19 nt regions of homology with unintended targets [140]. It has also been demonstrated that sequence-dependent OTEs are particularly problematic when the RNAi reagents target gene regions containing CAN repeats (where N can be any nucleotide) [75] that are found in many fly genes. In addition, siRNAs can also interfere with mRNA stability and/or translation through the microRNA pathway [137–139]. MicroRNAs (miRNAs) are non-coding RNAs that are encoded by the organism's genome and help regulate gene expression. Mature miRNAs are 22 nt RNAs and are similar in structure to siRNAs that are produced from exogenously introduced long dsRNAs. miRNAs bind to complementary sites that are 7–8 nt long within 3′UTRs (untranslated region) of target genes, leading to cleavage or translational repression [141]. The siRNA and microRNA pathways converge downstream of initial processing steps and share some of the same silencing machinery [118]. The 5′ region of the siRNA can act like the seed region of a microRNA, which extends from position 2 to position 8 of the guide strand and is complementary to sequences in the 3′ untranslated region (3′UTR) of target genes. Since a perfect match of only 7–8 nt is required between the seed region and the target mRNA for repression, it is difficult to identify all of the many putative targets in a cell [142,143]. Thus, sequence-dependent OTEs of siRNAs seem to result at least in part via microRNA-like off-target activity, which may result from siRNAs entering the microRNA pathway and functioning as microRNAs on targets with matches to the seed region in their 3′UTRs.

Although the prevalence of OTEs was underestimated in early RNAi screens, a number of approaches have now been developed to minimize their effects [47,144]. These include the development of computational tools to design RNAi reagents with limited or no homology to genes other than the intended target; the use of multiple independent RNAi reagents targeting the same gene; and the rescue of the RNAi-induced phenotype by an RNAi-resistant version of the gene. Further, with the availability of the catalogue of expressed genes in a wide array of commonly used cell lines by RNA sequencing [145], false positives associated with a screen performed in *Drosophila* cells can be identified and filtered based on whether the targeted gene is expressed in the cell line being screened. One can also filter out potential false positives by removing genes that score in a large majority of RNAi screens.

Large-scale epistasis or synthetic lethality studies (see Box 5.1 for definition) using sensitized genetic backgrounds can also uncover new components of signaling pathways [146] because they tend to reveal genes that are involved in redundant or parallel pathways/complexes. Such screens are similar in concept to the synthetic genetic array (SGA) analysis in yeast, where the viability of a set of gene deletions has been tested in backgrounds where other genes have been similarly deleted (synthetic lethal) or overexpressed [147−149]. The results from these studies showed that RNAi of many individual genes does not affect growth, but that many genes do have a synthetic genetic growth phenotype in combination with other genes. These genetic interactions include both negative (aggravating) interactions as well as positive (alleviating) ones, where the phenotype of eliminating one gene is attenuated by the loss of a second one. Combinatorial RNAi experiments where dsRNAs are screened for their ability to suppress or enhance the effect caused by another dsRNA (or by small molecules) are also becoming increasingly common [150,151]. Examples of HTS for multiple genes by RNAi include 17 724 combinations that identified regulators of *Drosophila* JNK signaling [150], and combinatorial RNAi of disease relevant genes in *C. elegans*, which identified ~1750 novel functions for genes in signaling [152]. RNAi microarrays facilitate the miniaturization of combinatorial RNAi screens and provide an effective and economical way to conduct large-scale screens in tissue culture cells [89,153,154].

In addition to identifying new genes involved in a particular biological process, comprehensive and quantitative genetic interaction data can be used to shed light on the organizing principles of signaling networks and the ways in which distinct signaling modules are interconnected. Schuldiner and colleagues [155] developed a strategy for building large-scale genetic interaction maps called 'epistatic miniarray profiles' (E-MAPs) that allows one to group sets of genes based on their signature/patterns of genetic interactions. Using this strategy, an E-MAP of genes involved in the early secretory pathway (ESP) in the budding yeast was constructed which robustly identified known pathways and relationships, such as the effect of the unfolded protein response (UPR) pathway on secretory functions, and the hierarchical relationships of the different stages of vesicular trafficking. This study also identified a strong link between endoplasmic reticulum-associated degradation (ERAD) pathway and lipid biosynthesis, a connection that had been previously poorly characterized. The E-MAPs strategy has been successfully extended to study the networks of genes involved in creating, maintaining and remodeling the chromatin in response to various cues [156], and also to identify novel components of the RNAi machinery in the fission yeast *Schizosaccharomyces pombe* [157]. The success of these studies highlights the power of E-MAPs to provide a systems-level view of the functional topology of networks that cannot be obtained by other methods. Recently**,** the concept of E-MAPs has been successfully implemented in *Drosophila* cells using combinatorial RNAi screens [158]. In this study, pairwise interactions between 93 genes involved in signaling were evaluated using two independent RNAi reagents for each per target. This set of 93 genes included components of the three MAPK pathways (Ras-MAPK, JNK and p38 pathway) and all expressed protein and lipid phosphatases. The pairwise knockdowns were analyzed for their effects on cell number, mean nuclear area and nuclear fluorescence intensity and resulted in 73 728 measurements, from which interaction scores were estimated. The success of the strategy was reflected in the high frequency of interactions observed between known components of the Ras-MAPK signaling pathway and a clear separation from regulators of the JNK signaling pathway. In addition, the authors identified connector of kinase to AP-1 (*Cka*), a scaffold protein in the JNK signaling pathway [159], as a positive regulator of Ras-MAPK signaling, and thus a putative point of cross-talk between the two pathways was identified.

Functional genomic approaches at the level of whole systems are powerful because they can identify most genes that affect a given signaling network, and have revealed that, contrary to previous views, hundreds of genes may be a part of a signaling network. However, genetic studies do not distinguish between direct and indirect effects, and therefore it is not clear where in the network the different genes identified act. Understanding how they contribute to the overall structure of the cellular signaling network requires the integration of genetic data with other datasets such as protein−protein interaction networks.

Protein−Protein Interactions

Large-scale protein−protein interaction (PPI) mapping complements genetic studies by revealing physical

associations and helps to define the physical signaling network. In PPI networks, nodes represent proteins and the edges represent a physical association between them. The methods most widely used to map PPI networks include the yeast two-hybrid (Y2H) system and its derivatives [160, 161], and affinity- or immunoprecipitation followed by mass spectrometry (AP/MS) [162–165]. PPI networks derived from Y2H methods are composed of binary (direct) interactions, whereas those derived from AP/MS techniques can be both direct and indirect, as they identify protein complexes.

Large-scale Y2H studies have been conducted with proteins from *Helicobacter pylori* [166], yeast [167–169], *C. elegans* [21,170,171], *Drosophila* [172–174], and human [175–179]. Strikingly, the three large-scale *Drosophila* Y2H mapping studies failed to fully recapitulate known signaling pathways. For example, querying the combination of these studies for Raf reveals only interactions with CG15422, Ras, Rhomboid, and Rap2L (http: //itchy.med.wayne.edu/PIM2/PIMtool.html), neglecting to identify most known targets, scaffolds, and co-regulators of Raf activity. Thus, these 'proteome-scale' approaches, although they identified highly abundant or strongly interacting cellular components, failed to identify many interactors of signaling components – most likely because of the absence of endogenous signaling contexts [180]. For this reason, MS-based approaches have become more popular, especially as the difficulty of implementation and costs have dropped dramatically.

Comprehensive MS-based PPI mapping has been applied in yeast [181–185]. A global protein kinase and phosphatase interaction network identified 1844 interactions between 887 proteins [181]. The success of MS approaches has been aided by the increased sensitivity of MS technology and implementation of tandem affinity purification (TAP) of protein complexes [186]. Recently, tandem affinity purification followed by MS has been used to isolate protein complexes from *Drosophila* tissue culture cells and tissues (http: //flybase.org/). ~5000 *Drosophila* proteins were fused to a FLAG-HA tag so that the fusion proteins could be expressed and recovered with their interacting partners from cells, or from whole transgenic flies. In addition to proteome-scale AP/MS, a number of smaller studies have been conducted in human cells on signaling pathways such as TNF-α and Wnt [187,188], biological processes such as autophagy [189], protein families such as the de-ubiquitinating enzymes [190], and protein complexes such as the RNA–polymerase II and PP2A complexes [191,192].

Both Y2H and AP/MS PPI mapping methods have been applied to the characterization of cellular networks with disease relevance, such as virus–host interactions [193–197]. These proteomic studies have confirmed that cellular processes take place within large networks of interconnected proteins.

PPI approaches, as implemented thus far, have been incomplete for investigations of signal transduction because they (1) do not provide functional information, and (2) often take place outside the context of endogenous signaling. These issues can be addressed by combining proteomics with RNAi. For instance, Y2H was used to identify interactors of the DAF-7/TGF-β pathway in *C. elegans*, resulting in a network of 59 proteins, and RNAi was used to show that nine novel interactors functionally interact with the TGF-β pathway [198]. Another major study used a pathway-specific approach with liquid chromatography/tandem MS to characterize the interactors of 32 TNF-α/NFκB pathway components in mammalian cells under endogenous signaling conditions [187]. Interactors were identified at baseline and under TNF-α stimulus, revealing 221 interactions. RNAi was then used to determine their influence on signaling output. This study demonstrated the power of pathway-directed proteomics in endogenous signaling contexts. One limitation of this study, however, was the lack of rigorous quantitation of the assembly of signaling complexes. Most signaling complexes are highly dynamic, with components often held in inactive complexes that can change dramatically following stimulation. For example, Raf and KSR are held in separate inactive complexes bound to PP2A core components and 14-3-3 proteins; following stimulation, Ras induces the recruitment of PP2A regulatory subunits to Raf, dephosphorylation of 14-3-3 binding sites, release of 14-3-3 proteins, membrane recruitment, KSR and Raf co-localization, Raf phosphorylation of MEK, and MEK phosphorylation of MAPK [199,200].

RNAi and MS can also be combined by first starting with RNAi and then following up with MS, as has been demonstrated for RTK/ERK signaling at baseline and under insulin stimulation [84,85]. All of the major known components of the pathway were tagged. In addition, a control cell line was engineered to subtract common interactors/contaminants. Altogether, 54 339 peptides were identified representing 12 208 proteins, encompassing an unfiltered network of 5009 interactions among 1188 individual proteins. To provide a ranked list of novel pathway interactors, filtering out sticky proteins found in control preparations and providing a probability that the observed interactor is real, the significance analysis of interactome (SAINT) method was applied to the PPI dataset [181]. Using a SAINT cut-off of 0.83 and a false discovery rate (FDR) of 10%, a filtered PPI network of 386 interactions among 249 proteins surrounding the canonical components of the RTK/Ras/ERK signaling pathway was generated [84]. In this network canonical baits have multiple common interactors, as would be expected from a well-connected signaling pathway (as opposed to unbiased PPI mapping of random protein

baits), as well as many unique interactors. Because the baits were purified under two conditions, baseline and insulin stimulation, the dynamics of the mini-proteome during signaling events was uncovered. As a measure of the sensitivity of the TAP/MS approach to network characterization, interactions among the canonical components and their known interactors were extracted. This canonical network recapitulated most of the known RTK-ERK signaling pathway.

Comparing the RTK-ERK PPI network to six unbiased genome-wide RNAi screens revealed that nearly half (119) of the proteins identified by PPI mapping scored in the RNAi screens, which is a significant enrichment relative to the entire genome (19%, $p < 7 \times 10^{-25}$) [187].

A major bottleneck in large-scale proteomics studies is the experimental validation of specific interactors or components of complexes. The combination of AP-MS with RNAi-mediated knockdown provides a way to directly validate specific PPIs. With differential labeling of the two proteomes to be compared (wild-type vs. RNAi knockdown) such analyses have the potential for accurate, highly quantitative results [201,202]. Currently, two major types of labeling technique are used for MS-based proteomics studies: metabolic labeling and chemical labeling [203]. Stable isotope labeling by amino acids in cell culture (SILAC) is considered to be the gold standard in the case of metabolic labeling. Here, isotopically labeled amino acids (for example arginine and lysine labeled with the stable ^{13}C and/or ^{15}N isotope) are incorporated into cellular proteins during normal protein biosynthesis [204]. Thus, the cells to be compared (for example wild-type vs. RNAi) are grown in media containing 'light' (normal) and 'heavy' (labeled) amino acids, respectively. After labeling, the two cell populations are mixed, fractionated, and subject to MS/MS analysis to quantify the differences between their two proteomes in a highly accurate manner [205,206]. Because the labels are carried by arginine and lysine residues tryptic digestion produces peptides that contain a labeled amino acid at the carboxy terminus. The heavy and light tryptic peptides elute together as pairs separated by a defined mass difference that allows the two proteomes to be distinguished in the MS/MS analyses. SILAC can be multiplexed to allow comparisons between three different proteomes simultaneously. SILAC-based differential labeling combined with RNAi, co-immunoprecipitation and quantitative MS analysis was used to detect and validate the cellular interaction partners of endogenous β-catenin and Cbl proteins in mammalian cells [202]. Alternatively, chemical labeling involves the use of isobaric tagging reagents such as iTRAQ (isobaric tags for relative and absolute quantification) [207] or TMT (tandem mass tags) [208] (see Box 5.1 for definitions) to label peptides after lysis and trypsinization. The peptides in samples to be compared are modified by covalent attachment of a unique tag or label, which enables the quantification of the same peptide across multiple samples. The uniquely labeled samples are combined and run through an MS analysis. Despite bearing distinct tags, the same peptides from the different samples are indistinguishable from each other in the first MS run, because the molecular weight of each tag is the same. However, during MS/MS each tag undergoes fragmentation, releasing a signature reporter ion. The signature reporter ions differ in mass between the tags and their relative levels serve as a measure of differences in the levels of a given peptide between samples. Labeling methods such as these provide a rapid means by which to quantitatively examine global proteome-level changes, and compare, for example, wild-type cells with those subjected to mutations, RNAi knockdown or small molecule treatments.

Transcriptional Profiling

Gene expression profiling using DNA microarrays, and more recently RNA-seq, has emerged as a valuable tool for broad correlation of gene activity with alterations in physiological or developmental states [209–211]. Transcriptional profiling experiments can be used to generate compendia of gene expression data across different cell types [212], diverse species [213], development times [214], and in response to distinct stimuli [215]. Such gene expression datasets have been commonly used to identify genes that function in common pathways or which encode components of the same complex. Studies in yeast have demonstrated that proteins that interact with each other show similar expression profiles to non-interacting proteins [216–219]. Gene expression profiling has been used to study signaling by wild-type and mutant receptor tyrosine kinases (RTKs) and has provided evidence for substantially overlapping immediate early transcriptional responses upon activation of PLCγ, PI3K, SHP2, and RasGAP proteins and their respective signaling pathways [220]. However, expression profiling studies do not provide details of how and where in this network pathways engage in cross-talk to specify the appropriate biological response.

Although expression profiling studies have become the gold standard for global responses to signaling, several recent studies have shown that correlation between transcriptome and proteome is only ~50% [221,222]. Proteomics approaches that directly measure the targets of signaling pathways — that is, the proteins — are more useful. For example, Yates and colleagues [223] compared protein abundance between wild-type *C. elegans* and those lacking the worm insulin receptor (InR) ortholog *daf-2*. This study revealed 86 proteins whose abundance changed following loss of InR, an important finding for a signaling

pathway with known effects at the translational rather than the transcriptional level.

QUANTITATIVE RNAI SIGNATURES OR PHENOPRINTS TO INFER CONTEXT DEPENDENT INFORMATION FLOW THROUGH CELLULAR SIGNALING NETWORKS

The network maps discussed so far are primarily static and do not provide information regarding the direction of information flow through the nodes; instead, they provide the framework required to begin to dissect the functional, logical and dynamical nature of cellular signaling networks. The topological features of signaling networks reflect the need to process multiple input cues received by a cell, interpret them correctly and transmit the information to coordinate cellular activity and generate the proper phenotypic response [224−226]. Thus, signaling networks are highly dynamic, exist in distinct states, and are capable of deploying the same or overlapping set of signaling molecules in different ways, depending on the context and the input cues received by the cell. It has been demonstrated that, depending on the cumulative effects of the signals received by a cell, JNK activity, for instance, can be either pro- or anti-apoptotic [224, 226]. The challenge of future studies is to gain a mechanistic understanding of the direction of information flow, the dynamic nature of cross-talk between signaling pathways, and the hierarchical relationship between network components in response to a distinct set of stimuli. Such mechanistic insights will allow the generation of predictive (testable) models of how this information processing capacity of signaling networks is coopted in disease conditions to produce aberrant phenotypes, and will lead to the identification of novel drug targets and the development of more effective therapeutics.

In recent years it has been demonstrated, albeit on a small scale, that systematically perturbing the components of the network and simultaneously measuring multiple quantitative phenotypes in the presence or absence of specific input cues can be used to infer information flow through signaling networks. The phenotypes measured can include changes in gene expression, in phosphorylation of key signaling or target proteins and/or in cellular morphology. These quantitative phenotypes result from multiplexed assays and are therefore different from the cell-based assays used in RNAi HTS (described above), such that, instead of measuring a single transcriptional reporter or changes in the phosphorylation status of a single protein in response to the knockdown of a gene, RNAi signatures/phenoprints are composed of multiple measurements ranging anywhere from tens to hundreds of genes or proteins. A compilation of such quantitative phenotypes provides a unique, context/signal specific 'signature' or 'phenoprint' for each perturbed network component. Network components that are deployed in the same or similar manner in response to the incoming signal would tend to have similar phenoprints [227,228].

Direction of Information Flow from Gene Expression Signatures

Transcriptional signatures resulting from the loss/reduction of individual network components by RNAi can be used to infer the flow of information through proteins that are interconnected within a cellular network. This approach was used successfully in analyzing the response of *Drosophila* cells to microbial infection and lipopolysaccharides (LPS) [229]. In these studies, the topology of network connections was retrieved from experimentally measured global transcriptional responses to successive perturbations in pathway components. Genome-wide expression profiling and loss-of-function experiments using RNAi were used to determine the identity of the signaling pathways that control microbial challenge-induced cellular responses. Differential gene expression signatures appeared with discrete temporal patterns after LPS stimulation and septic injury, and could be assigned to the activation of distinct signaling pathways by impairing pathway-specific components using RNAi. Specifically, the results indicated that in addition to signaling through the Toll and Imd pathways, microbial agents induce signal transmission through the JNK and JAK/STAT pathways. Altogether, this demonstrated how data obtained from microarray expression profiling combined with the RNAi technology could be used to extract interconnections between different signaling pathways downstream of an extracellular stimulus.

Whole genome expression profiling [230] has identified gene expression signature-based analysis of signaling networks in a number of different model systems. One of the first successful applications of this approach was the generation of a compendium of gene expression profiles in yeast for 300 different mutations and small molecule treatments [231]. The 300 different mutations and chemical treatments specifically included 276 deletion mutants, 11 tetracycline-inducible essential genes and 13 small molecule inhibitors (data available from Rosetta Inpharmatics). The assumption was that the cellular state can be deduced from the global gene expression response, and the transcriptional profile of a gene in response to a change in cellular state (disease, cellular activity such as cell division, response to drugs or genetic perturbation) constitutes a unique quantitative molecular phenotype [210,232−239]. The study showed that genes known to be co-regulated could be easily detected, and mutations in genes or treatments with small molecules that regulate similar cellular processes displayed

strikingly similar expression profiles. Most importantly, the study was able to assign eight unannotated genes to the regulation of pathways such as sterol metabolism, protein synthesis and mitochondrial function. Furthermore, the observation that gene expression profile in response to drug treatment phenocopies the loss-of-function profile of its target facilitated the identification of Erg2p, a sterol isomerase, as a novel target of the drug dyclonine [231].

Another study in *Saccharomyces cerevisiae* [240] analyzed the functional relationship between kinases and phosphatases by generating genome-wide expression signatures for 150 deletion mutants. Gene expression signatures were also generated for pairs of genes (kinases and phosphatases) that exhibit synthetic genetic interactions with the aim of investigating the mechanisms underlying the redundant relationships. The results of this study concluded that there are three types of redundant connection: (1) complete redundancy, where the two genes in a synthetic genetic interaction regulate the same set(s) of genes to an equal extent, such that the single mutants show no significant changes, but an effect on expression of regulated gene set(s) is seen only in the double mutant; (2) quantitative redundancy, where the two genes in a synthetic genetic interaction regulate the same set(s) of genes but to a quantitatively different extent. Here one of the single mutants shows no significant effect but the other does, and the effect on the expression of the regulated gene set(s) is amplified in the double mutant; and (3) mixed epistasis, where the two genes in a synthetic genetic interaction regulate some of the same gene set(s) via either complete or quantitative redundancy, while other gene set(s) behave in a completely different way. Mixed epistasis reflects only a partial overlap in function of the two genes in the synthetic genetic interaction. The authors concluded that such gene pairs share additional regulatory associations, such as inhibition of one by the other, and that mixed epistatic relationships provide the mechanisms to achieve signaling specificity in a context-dependent manner. Importantly, mixed epistasis was found to be the most common redundant relationship in signaling networks.

The Connectivity Map (Cmap) [241,242], identified functional connections between drugs, genes and diseases from expression profiles in a compilation of genome-wide expression data from cultured human cells treated with either bioactive small molecules or genetic perturbations. Cmap incorporates pattern-matching algorithms that decode differential gene expression data into functional relationships between drugs, genes and diseases to generate testable hypotheses. Again, the underlying assumption of Cmap is that common gene expression changes reflect functional connectivity between the gene products targeted by either small molecule or various genetic perturbations. Functional connectivity is expected to reflect the role of gene products in a common biological process, in particular components of a specific signaling pathway. Cmap was used to identify the target of two previously uncharacterized natural products (celastrol and gedunin) that had inhibitory activity towards androgen receptor activity, with implications for the treatment of prostate cancer [243]. Gene expression signatures for each of the drugs were generated and used to search the Cmap database for similar gene expression patterns. The gene expression signatures of celastrol and gedunin were most similar to the signatures of inhibitors of the chaperone HSP90. This finding predicted that HSP90 was the most likely target of celastrol and gedunin activity, a hypothesis that was tested and validated experimentally. In another study, rapamycin was found to reverse the effects of resistance to the glucocorticoid dexamethasone in acute lymphoblastic leukemia (ALL) [244].

A number of publicly available compendia of gene expression profiles are available for data mining purposes, including the Global Cancer Map [245], Gene Expression Atlas [246,247], and Oncomine Cancer Profiling Database [248].

The overall logic of establishing connectivity based on gene expression signatures of RNAi treated cells is simple and schematically presented in Figure 5.2. The current challenge is to go beyond proof-of-principle studies and establish robust experimental protocols and computational tools that will allow large-scale implementation. This presents three main challenges: (1) the generation of gene expression signatures for every biological state of interest (disease, cellular activity such as cell division, response to drugs or genetic perturbation); (2) a cost-effective high-throughput platform for screening genetic perturbations or small molecule treatment using gene expression signatures; and (3) the development of computational tools for data analysis.

Gene expression signatures serve as molecular surrogates for biological states, are composed of tens to hundreds of genes, and are distinct for different biological states [235,249]. The ability to identify a gene expression signature that can serve as a quantitative molecular phenotype for a specific biological state holds great promise for the development of high-throughput small-molecule or RNAi screens using the signature of interest as the readout. The first gene expression signature-based screening (now called gene-expression-based high-throughput screening, GE-HTS) was conducted [249] to identify small molecules that induce the differentiation of acute myeloid leukemia cells. Using gene expression analysis of primary cells from patients and unaffected individuals identified a number of differentiation-correlated genes to generate a five-gene signature for leukemia cell differentiation. This signature was then used to screen a library of 1739 bioactive small molecules to identify those that induce the expression of the

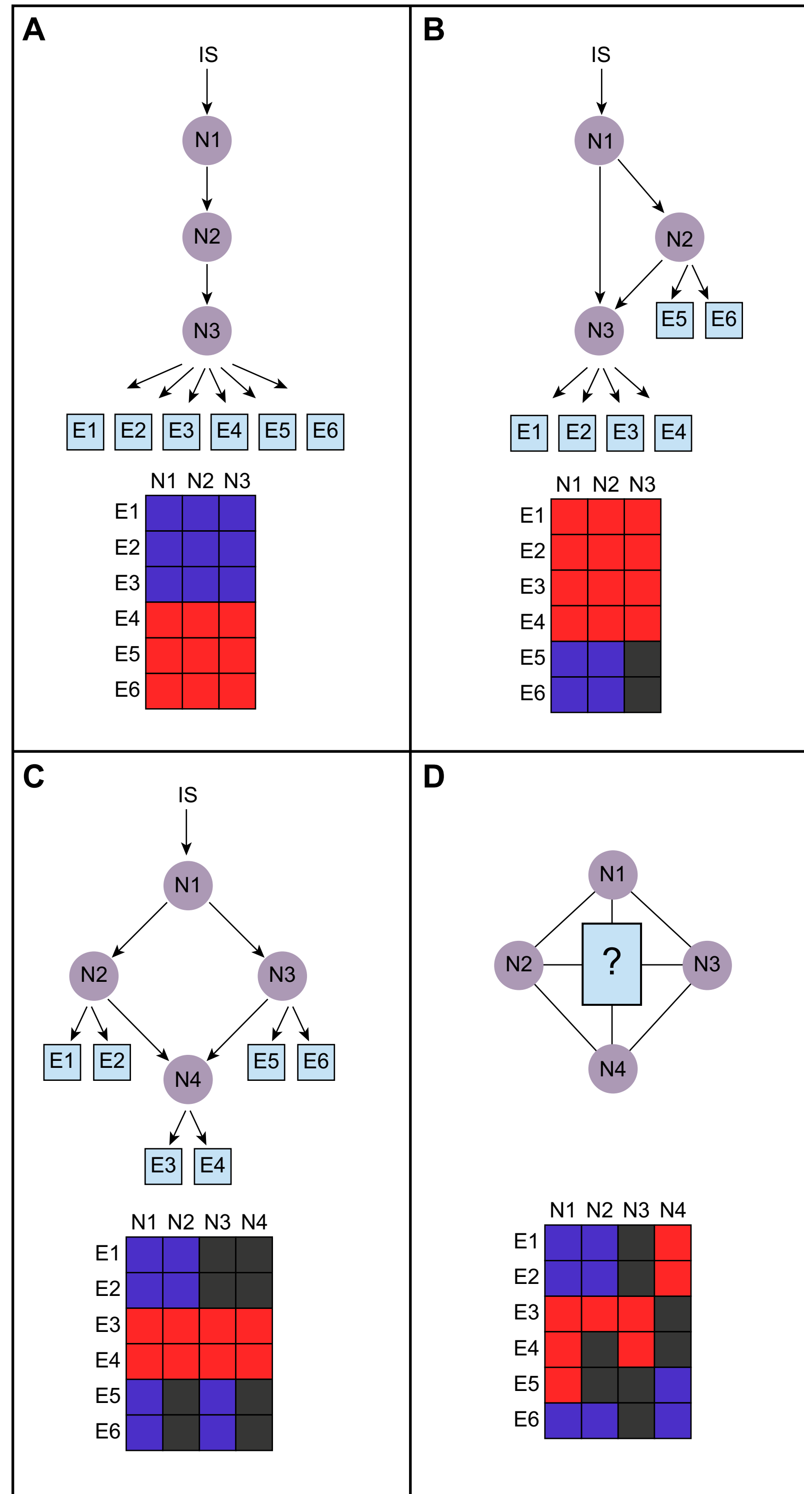

FIGURE 5.2 Inferring network structure from transcriptional signatures. A, B, C: Given the structure and knowledge regarding the flow of information through a network of n components that transduce the input signal (IS) received by a cell, one can predict the effects of perturbing individual network components on the expression of known target genes (E). For example, given the linear structure and flow of information of the network shown in **A**, perturbing any of the network components will affect the expression of target genes E1–E6 in a similar manner as depicted in the schematized heat map. **D:** Given a set of gene expression signatures generated by perturbing the components of a network, it should be possible to reconstruct the structure of the network and deduce the flow of information through its resident components.

differentiation-correlated signature genes in leukemia cells. This study identified eight bioactives that were further validated as bona fide differentiation inducers.

The success of the first GE-HTS [249] led to the development of the Luminex xMAP technology suitable for HTS (see Box 5.2 for details on the Luminex technology). The technology combines multiplex ligation-mediated amplification [250–252] with optically tagged and barcoded microsphere and flow cytometric detection. The technology is currently capable of measuring up to 500 transcripts

Box 5.2 Luminex xMAP technology for multiplex gene expression analysis

The Luminex xMAP Technology (http: //www.luminexcorp.com) can be used to perform a wide variety of multiplex assays on the surface of 5.6 µm polystyrene microspheres. Each microsphere or bead is uniquely color-coded internally using precise concentrations of red and infrared fluorescent dyes, resulting in 500 spectrally distinct beads. This feature allows multiplexing of 1−500 analytes in a single sample. The surface chemistry of the microspheres allows capture reagents to be efficiently coupled to the beads to facilitate the measurement of different kinds of analyte in the sample. For example, capture reagents may include oligonucleotides, antibodies, peptides, enzyme substrates, or receptors, thus offering a wide range of applications, including gene expression analysis, detection of single nucleotide polymorphisms, protein expression analysis, detection of protein−protein interactions, quantification of antibody affinity and epitope mapping, serum analyte profiling and detection of enzyme/substrate or receptor−ligand reactions. After the analyte of interest (transcript, antibody, antigen, ligand, or substrate) is captured from the sample on the surface of the beads, the reactions are quantified in the Luminex analyzer, an instrument that combines high-tech fluidics based on the principles of flow cytometry and laser optics for signal detection and processing. In the analyzer, the microspheres pass through the detection chamber in a single file such that the reaction between the surface coated capture reagent and the analyte of interest can be quantitatively measured for each bead. In the detection chamber, a red laser or light-emitting diode (LED) is first used to classify each microsphere to one out of the 500 spectrally different sets. A second laser or LED excites the fluorescent dye associated with the reporter molecule that is used to detect the analyte of interest.

Peck and colleagues [253] developed the Luminex xMAP technology for gene expression signature analysis. Messenger RNA (mRNA) transcripts from each sample are captured on immobilized poly-dT in 384-well plates and are reverse transcribed to complementary DNA (cDNA). For each gene/transcript of interest two oligonucleotide probes are designed. The 5′ probe contains a 20 nt sequence complementary to the T7 primer site, a unique 24 nt sequence that serves as a barcode, and a 20 nt sequence complementary to the transcript of interest. Each 3′ probe is phosphorylated at its 5′ end and contains a 20 nt sequence contiguous with the gene-specific fragment of the 5′ probe followed by a 20 nt T3 primer site. Probe pairs for the transcripts of interest are mixed with cDNA from each sample, unbound probes are removed, and probe pairs annealed to contiguous regions of target mRNAs are ligated together to yield synthetic 104 nt templates for amplification. Universal T3 and 5′-biotinylated T7 primers are used to amplify the templates by PCR. The resulting biotinylated and bar-coded amplicons are hybridized to a pool of spectrally distinct microspheres. Each microsphere presents on its surface a distinct capture probe complementary to one of the barcodes. The hybridization reactions are finally reacted with streptavidin-phycoerythrin to fluorescently label biotin labels. Captured labeled transcripts of interest are quantified and beads decoded in the Luminex analyzer as described above. Luminex xMAP assays are carried out in a 96-well plate format, with up to 500 genes being measured in each well/sample.

within a single reaction in thousands of samples in a cost-effective manner [253]. (http: //www.luminexcorp.com)

GE-HTS has also been recently adopted to dissect the regulatory network controlling the transcriptional response of mouse primary dendritic cells to pathogens [215]. A 118-gene signature that defines the response of mouse primary dendritic cells to infection by pathogens was established using expression profiling of dendritic cells exposed to different pathogen-derived components (virus, Gram-positive and Gram-negative bacteria). The gene expression signature was then used to screen 125 transcription factors, including proteins that modify chromatin and proteins that bind RNA, for their role(s) in coordinating cellular response to pathogen infection. The reconstructed network model composed of the transcription factors and their cognate upstream signaling pathways helps to explain how pathogen-sensing pathways achieve specificity in their response to different microbial populations. The study used a screening platform called NanoString nCounter Gene Expression Assay (see Box 5.3 for details on the Nanostring technology). The nCounter Gene Expression Assay is a robust and highly reproducible method for detecting the expression of up to 800 genes in a single reaction. The biggest advantages of this platform are its high sensitivity, requirement for very small amounts of total RNA as starting material, and the lack of any enzymatic reactions to convert total RNA to cDNA and amplification of resulting cDNA by polymerase chain reaction (PCR).

Direction of Information Flow from Phosphorylation Signatures

Similar to gene expression signatures, the biological state of a cell can also be inferred from the phosphorylation profile of proteins that are themselves components of the cellular signaling network, as well as of proteins that form a part of the cellular response to signals impinging on the cell. Protein phosphorylation is a widespread post-translational modification and plays important roles in most biological processes in eukaryotic cells. The addition of phosphate groups on substrate proteins by kinases modulates the overall function of the substrates by directing their activity, localization and stability. Extensive protein-phosphorylation-mediated signaling networks direct the flow of

Box 5.3 Nanostring nCounter System for Mulplex Gene Expression Analysis

The nCounter assay can be used to detect several types of nucleic acid molecule, including mRNA, DNA and microRNAs. The nCounter assay is based on direct imaging of mRNA molecules of interest that are detected using target-specific, color-coded probe pairs [273]. It does not require the conversion of mRNA to cDNA via reverse transcription or the amplification of the resulting cDNA via PCR. A pair of sequence-specific probes — the capture and reporter probes — detects each target gene of interest. The capture probe contains, from 5′ to 3′, a 35–50-base sequence complementary to the target mRNA, a short sequence common to all capture probes, and a biotin affinity tag that provides a molecular handle for the attachment of target genes to facilitate detection. The reporter probe contains, from 3′ to 5′, a second 35–50-base sequence (complementary to the same target mRNA, near or contiguous with the target-specific sequence in the capture probe partner), a short sequence common to all reporter probes, and a color-coded molecular barcode. The common sequences included in all capture and reporter probes facilitate the removal of unbound excess probes during post-hybridization steps. The barcode contained in each reporter probe is composed of a linearized single-stranded M13 DNA molecule annealed to a series of six complementary RNA segments, each labeled with one of four spectrally non-overlapping fluorescent dyes. The arrangement of the differently colored RNA segments creates a unique color code for each target gene of interest. The different combinations of the four distinct colors at six contiguous positions allows for a large diversity of color-based barcodes, each designating a different gene transcript, that can be mixed together in a single reaction for hybridization and still be individually resolved and identified. The methodology offers the flexibility of multiplexing up to 800 reporter–capture probe pairs within a single reaction.

The target mRNA is mixed in solution with a large excess of the reporter and capture probe pairs, so that each targeted transcript finds its corresponding probe pair. After hybridization, excess unbound probes are washed away and the complexes, comprising target mRNA bound to specific reporter–capture probe pairs, are isolated. The biotin label at the 3′ end of the capture probes is used to attach the complexes to streptavidin-coated slides. An electric field is applied to orient and extend the tripartite complexes on the surface of the slide to facilitate imaging and detection of the color-coded molecules. A microscope objective and a CCD camera are used to image the immobilized complexes. The number of molecules for a particular mRNA species is counted by decoding the unique pattern of the fluorescent colors encoded in each reporter probe. The protocol is performed from start to finish on the nCounter System, which is designed to provide hybridization, post-hybridization processing, and digital data acquisition capabilities in one simple workflow. The integrated system is composed of two instruments: the fully automated nCounter Prep Station for post-hybridization processing and the Digital Analyzer for imaging, data collection, and data processing.

information from cell surface receptors to effector molecules to regulate the response and functions of cells, tissues and organisms. Aberrant signaling due to misregulation of protein phosphorylation and dephosphorylation cascades is associated with many disease states, including most types of cancer.

A multiplex approach using phospho-specific antibodies and intracellular phospho-specific flow cytometry [254,255] to monitor changes in the level of phosphorylation of multiple key protein nodes in primary leukemic cells has been shown to have great potential in understanding how signaling through a network is co-opted in cancer cells to produce the aberrant phenotypes [256]. Phospho-specific antibodies to Stat1, Stat3, Stat5, Stat6, p38, and Erk1/2 were used to profile primary cells from patients with acute myeloid leukemia at basal state and following cytokine stimulation. Phosphorylation profiles of the six signaling proteins in acute myeloid leukemia cells were compared to those in normal blood cells to distinguish the leukemic signal transduction network from the healthy network. Using the same methodology, it is also possible to measure the effects of perturbations (genetic or small molecule) on these signaling events in cancer cells compared to normal cells with the aim of identifying potential drug targets. Furthermore, one could determine the effect of such perturbations in either attenuating or enhancing the response of the cancer cells to other environmental cues.

Multicolor flow cytometry [257] has been used to measure 11 phosphoproteins and phospholipids simultaneously in response to stimulatory or inhibitory perturbations (small molecule inhibitors of key signaling components) to determine the effects of each condition on the cellular signaling networks in naive $CD4^+$ T primary cells. Bayesian network analysis was applied in order to infer causal connections between components of the network. Key to the success of this application was the use of the phosphorylation signatures of the 11 phosphoproteins and phospholipids in response to stimuli and perturbations.

Both studies discussed above use phospho-specific antibodies to key signaling or response proteins [256,257]. Such studies are limited by the availability of specific antibodies that recognize phosphorylated residues on proteins of interest. Proteome-scale MS-based studies provide one of the most comprehensive analyses of phosphorylation and do not depend on antibodies [258–260]. MS-based technologies provide highly quantitative and direct measurements that can detect the activities of many phosphorylation pathways simultaneously. The KAYAK

(kinase activity assay for kinome profiling) method [261] can be used for the multiplexed measurement of phosphorylation events on 90 different peptides directly from cell lysates (see Box 5.4 for details on the KAYAK technology). Phosphorylated peptides are enriched using immobilized metal-ion affinity chromatography and analyzed by LC-MS techniques.

In addition to providing direct measurements on a proteome-wide scale, quantitative MS approaches facilitate the comparison of phosphoproteomes between wild-type cells and cells that have undergone manipulations of their signaling network components. In a recent study using mutant strains of *S. cerevisiae*, a label-free, quantitative phosphoproteomics approach was employed to determine the relationships between 97 kinases and 27 phosphatases and more than 1000 phosphoproteins [262]. Strikingly, inactivation of most (77%) kinases and phosphatases affects their immediate downstream targets as well as a large proportion of the overall signaling network. Owing to the inherent variation that exists between LC-MS experiments from run to run, label-free quantitation provides a relatively imprecise measurement of the differences in the phosphoproteome between wild-type and mutant yeast cells. Recent advances in techniques such as SILAC and chemical labeling, as well as improved sensitivity and dynamic range of peptide identification by current MS-based technologies, is enabling comprehensive and reproducible assessment of differences in phosphoproteomes [263]. Many groups have begun to apply this promising global approach to identify the effects of network perturbations on changes in the phosphoproteome [262,264−266]. iTRAQ labeling and phosphatase treatment was used to identify phosphorylation sites on the purified, auto-activated tyrosine kinase domain of fibroblast growth factor receptor 3 (FGFR3-KD) and to analyze complexes formed around the insulin receptor substrate homologue (chico) immunopurified from *Drosophila melanogaster* cells that were either stimulated with insulin or left untreated [267]. In two recent studies of the insulin signaling network in mammalian cells, Grb10 was identified as a mTORC1 substrate and was shown to be involved in feedback inhibition of the phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated, mitogen-activated protein kinase (ERK-MAPK) pathways [268,269]. iTRAQ has also been used to compare the phosphoproteomes of cells treated with insulin to activate the pathway and cells that were pretreated with Torin 1 before insulin activation [268]. Torin 1 is a novel adenosine 5′-triphosphate (ATP)-competitive mTOR kinase domain inhibitor that blocks all known activities of both mTORC1 and mTORC2 complexes [270]. Yu and colleagues [269] used SILAC to quantify differences in the phosphoproteome of $TSC2^{-/-}$ MEFs in the presence and absence of rapamycin, as well as in the absence or presence of a drug

Box 5.4 KAYAK (Kinase Activity Assay for Kinome Profiling) Method for Multiplex Analysis of Kinase Activities

KAYAK is a multiplexed, MS-based kinase assay developed to measure the activity of multiple kinases from the same sample lysate [261,274]. A single MS run directly measures the phosphorylation of 90 synthetic peptides, thus providing a multiplexed assay to simultaneously monitor kinase activities from multiple signaling pathways. Key to the success of the KAYAK method is the design and synthesis of substrate peptides that can represent activity through the different core cellular signaling pathways. This set of substrate peptides also includes synthetic peptides containing phosphorylation sites with no associated kinase. Such peptides are identified from large-scale phosphoproteomics studies of cellular signaling networks and can be used to identify the responsible kinase via perturbation assays. The peptides are composed of 10−15 amino acid residues, with five residues upstream and four residues downstream of the phospho-acceptor site, and a C-terminal tripeptide of proline−phenylalanine−arginine to facilitate the incorporation and quantification of the stable isotope. The set of peptide substrates whose phosphorylation reflects activity through multiple signaling pathways is incubated together with a cell lysate to allow for phosphorylation by active kinases in the lysate. The in vitro kinase reactions are quenched, followed by the addition of stable isotope-labeled phosphopeptides of identical sequence (as internal standards), at a known concentration. Immobilized metal-ion affinity chromatography is used to enrich the phosphorylated substrate peptides and internal standard phosphopeptides, which are then analyzed by LC-MS techniques. The light (product) and heavy (internal standard) peptide pairs differ in mass by 6 daltons (Da) and although they co-elute, they can be quantified by the ratio of light-to-heavy areas under the curve from the raw spectra. Since the amount of each heavy phosphopeptide added is known, the ratio of the light to the heavy phosphopeptide provides the absolute amount of each product formed during the kinase reaction. The in vitro kinase reactions are carried out in a reaction volume of 50 μL and require only nanogram to microgram amounts of cell lysate. 5 μM of each substrate peptide is used in the reaction to reduce cross-phosphorylation of peptides by different kinases.

The KAYAK method was first applied to profile the activity of kinases in different cellular contexts, including mitogen-induced cell proliferation, inhibition of signaling pathways by known small molecule inhibitors, and a number of breast cancer cell lines [261]. This study also identified that a peptide derived from a PI3K regulatory subunit was a novel Src family kinase site in vivo.

(Ku-0063794, an ATP-competitive mTor inhibitor) leading to the identification of rapamycin-insensitive substrates of mTORC1 and mTORC2.

CONCLUDING REMARKS

Building on several decades of targeted classic genetics approaches, unbiased high-throughput technologies are beginning to generate a systems-level view of cellular signaling networks. In this chapter we have reviewed a number of experimental methods available to generate a comprehensive 'parts list' of cellular signaling networks. Further, we have described various approaches that can be used to construct network models based on the phenotypic signatures of each component. These techniques give us the unprecedented opportunity to evaluate globally and systematically the contribution of all genes to a specific biological process. However, the implementation of these methods is technically challenging and in some cases they are best used in combination, as integration of data sets increases the quality of the networks. Although the false positive and false negative rates for networks generated from high-throughput methods are currently relatively high, new experimental techniques and new methods for integrating multiple interacting data types will allow these networks to become powerful predictive tools.

A global view of cellular networks holds great promise in advancing our mechanistic understanding of how individual genetic alterations, as well as combinations of gene mutations, lead to a disease phenotype. For example, sequencing of cancer genomes [271] and genome-wide association studies [272] have identified hundreds of genetic aberrations that are linked to different cancers and complex diseases such as diabetes, obesity, hypertension and Crohn's disease. Comprehensive structure/function analysis of networks should help to understand the biological functions of many of the affected genes. Importantly, network analyses will facilitate the selection of protein targets for therapeutic intervention based on the underlying mechanisms of action. Furthermore, network maps will shed light on how certain drug—target interactions may lead to toxic effects. Such a mechanistic understanding is critical to the development of effective and safe treatments. Eventually, generation of comprehensive dynamic models of protein networks in response to signals over time will allow scientists to quantitatively predict the outcome of various perturbations.

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