# Chapter 16
# Reconstruction and Comparison of Cellular Signaling Pathway Resources for the Systems-Level Analysis of Cross-Talks

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**Abstract** Signaling pathways control a large variety of cellular processes and their defects are often linked with diseases. Reliable analyses of these pathways need uniform pathway definitions and curation rules applied to all pathways. Here, we compare KEGG, Reactome, Netpath and SignaLink pathway databases and examine their usefulness in systems-level analysis. Further on, we show that the integration of various bioinformatics databases allows a comprehensive understanding of the regulatory processes that control signaling pathways. We also discuss the drug target relevance of cross-talking (i.e., multi-pathway) proteins and signal transduction regulators (e.g., phophatases and miRNAs). Accordingly, modern integrated databases are not only essential for studying signaling processes at the systems level, but will also serve as invaluable tools for pharmacology and network-based medicine.

**Keywords** Signaling · Cross-talk · Regulation · Drug discovery · Network · Pathway · miRNA · Drug targeting · Pathway database

**Acronyms**

| | |
|---|---|
| HTP | High-throughput |
| PPI | Protein-protein interaction |
| TF | Transcription factor |
| TFBS | Transcription factor binding site |
| miRNA | microRNA |

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## 16.1 Signaling Pathways and Cross-Talks

Intracellular signaling, from the simplest cascades to the highly intertwined networks of kinases, contributes to the diversity of developmental programs and adaptation responses in metazoans [1]. In humans, defects in intracellular signaling can cause various diseases, e.g., cancer, neurodegeneration, or diabetes. Thus, understanding the structure, function, and evolution of signal transduction is an important task for both basic research and medicine.

Signaling pathways, the functional building blocks of intracellular signaling, transmit extracellular information from ligands through receptors and mediators to transcription factors, which induce specific gene expression changes. In contrast to the wide variety of signaling functions and the macroscopic and microscopic diversity of living forms, the number of signaling pathway types are relatively low (a few dozen) [2]. The basic mechanisms of each pathway are conservative, characteristic to large taxon groups, and present ubiquitously in different tissues [1, 2]. Interestingly, most of the pathways have a maximum of 10–20 protein components [1]. These numbers apparently contradict to the number of cell types that signaling pathways can create and maintain. The major sources to generate diverse and complex signaling flow with such few pathways are specific co-factors and positive/negative feedback loops [3, 4]. Over the past decade, it has been realized that signaling pathways are highly structured and rich in cross-talks (where cross-talk is defined here as a directed physical interaction between pathways) ([84], [5]). Cross-talks can form and change more frequently than the interactions within pathways [6, 7]. As the number and combination of transducable signals are limited, new cross-talks between pathways can create novel input/output combinations, which increase the possible ways of signaling flow and thus contribute to diverse phenotypes.

However, to ensure that an appropriate response is elicited, the signaling system has to maintain the pathways' output specificity (inputs preferentially activate their own output) and input fidelity (outputs preferentially respond to their own input) [8]. Thus, new interactions between pathways need to be precisely regulated. Regulation of cross-talks to prevent 'leaking' or 'spillover' can be achieved with different insulating mechanisms [8]. Signaling cross-talks are controlled mainly by scaffold proteins, cross-pathway inhibitions, kinetic insulation, and the spatial and temporal expression patterns of proteins [4, 9–11]. One can find all these mechanisms in the concept of critical nodes, defined by Kahn and co-workers, and demonstrated for the insulin pathway [12]. Critical nodes are defined as protein groups, where the members are (1) essential in the signal transduction of a given pathway, (2) related to each other (isoforms), (3) regulated and function in a partially different way, and where (4) at least one of the members participates in a cross-talk with another pathway [12]. The relative concentration of the critical node members and their differential regulation determine the way of the signaling flow [12]. The default way of signaling flow is from a pathway-specific ligand via a critical node to a pathway-specific transcription factor. But when a critical node contains multiple protein

isoforms, which include a member that can cross-talk, the signal can be switched to another pathway, i.e., generating another output. Consequently, two pathways can specifically cross-talk with a shared protein group, where the partially regulated protein isoforms serve as a source of divergence [12].

## 16.2 Challenges to Study Cross-Talk at the Systems-Level

Despite the general prevalence of network approaches, the definition of pathways has seen little change. As structural, functional, tissue- and disease-specific aspects come into consideration while identifying individual pathways, these differing aspects also have to be taken into account when studying cross-talks. Different studies address the role of cross-talks in the context of distinct cell fates, cell types, single or multiple pathways. In Table 16.1 we list some examples for these different approaches.

Nowadays, systems-level and network-based methods have started to dominate the study of signaling pathways, accordingly the systems level analyses of cross-talks has become a major task. First of all, this requires a precise definition of pathways and pathway borders. By reviewing the major issues of studying cross-talks at the systems level, Gerstein and colleges point out that pathways compiled from different systems and constructed for distinct purposes are not suitable for examining cross-talks [13]. Bauer-Mehren et al. came to the same conclusion while testing the cPath integrated database [14] and argue the need of new databases that make the study of cross-talks possible at the systems level [15]. These require a compilation based on general principles and importantly, the use of standardized methods. Among these, high-throughput (HTP) methods provide the greatest number of protein–protein interactions (PPIs) and are therefore commonly used in network biology research. However, for methodological reasons, these HTP screens are unable to reveal interactions of extracellular, membrane-bound and nuclear proteins—all of them important players in signal transduction. A further problem of PPIs from HTP data is that they are mostly undirected, while most of the reactions in the signaling network are directed.

Due to these limitations, manually curated databases have emerged as indispensable tools for systems-level research of signaling pathways. Although usually containing less information, they are more detailed and reliable. However, most of these curated signaling databases both lack a precise definition of the pathways and a standardized curation protocol. Consequently, it is difficult to compare the distinct pathways even within the same database, or to analyze interactions between pathways. For extensive cross-talk analysis, a signaling database is required, that: (1) has a structure fulfilling the modern requirements of systems biology; (2) is objective and contains uniformly defined pathways; (3) contains sufficient and reliable network information. Additionally, if the above criteria apply to multiple species, this further allows prediction of new proteins, protein functions, and PPIs based on orthology.

**Table 16.1** Examples of different approaches for the analysis of cross-talks ordered in growing complexity

| Cross-talk studies | Cross-talking signaling pathways | References | Type and details of the reference(s) |
|---|---|---|---|
| Modeling cross-talks in a single pathway | Hyperosmolar and pheromone MAPK pathway | [70] | Research article on mathematical modeling and experimental validation |
| Cross-talks of a single pathway in healthy cell types | Cross-talks of TGF-$\beta$/BMP with MAPK, PI3K/Akt, WNT, Hh, notch, IL/TNF-$\beta$/IFN-$\gamma$ pathways; Cross-talks of notch with Hh, JAK-STAT,TGF-$\beta$, RTK, WNT pathways | [71, 72] | Review articles |
| Cross-talks of a single pathway in stem-cells | Cross-talk of WNT pathway with FGF, notch pathways | [73] | Review article |
| Cross-talks of two pathways | EGF and Insulin pathways; PI3K and ERK(MAPK) pathways | [74, 75] | Research articles on computational modeling and experimental validation |
| Cross-talks of specific pathways in a specific tissue | (many) | [76] | Research article |
| Interaction of multiple pathways in stem cells | Notch, WNT, TGF-$\beta$, BMP pathways | [77] | Review article |
| Coordination of multiple pathways during organ development | Hh, WNT, FGF, WNT, IGF; EGF, notch, WNT | [86], [78, 79] | Review article |
| Cross-talk of multiple pathways in an organ | Notch and WNT pathways | [80] | Research article on experimental data |
| Cross-talk of multiple pathways in the development of tumors | WNT, BMP, FGF, notch and Hh pathways | [81] | Review article |
| Interaction of multiple pathways in normal and stem cell differentiation | JAK-STAT, notch, MAPK, PI3 K/AKT, NF-$\kappa$B, WNT, TGF-$\beta$ pathways | [85] | Review article |
| Cross-talk of multiple (9) pathways in a general protein network | MAPK, TGF-$\beta$, notch, WNT, Hh, mTOR, TLR, JAK-STAT, VEGF pathways | [13] | Review article |
| Extensive cross-talk (580) of multiple pathways in a general protein network | (many) | [82] | Research article on bioinformatic data |
| Cross-talks in intercellular communication of two pathways in an organ | FGF and BMP pathways | [83] | Research article on experimental data |

## 16.3 Benchmarking Signaling Resources to Study Cross-Talks

We examined 3 widely used, freely available general signaling pathway databases, KEGG, Reactome and Netpath [16–19], and compared it with SignaLink, a recently developed signaling pathway database intended for the analysis of signaling cross-talks [6]. All four databases were constructed by utilizing different sources and applying distinct methods, hence they greatly vary in a number of aspects. KEGG contains pathway information from a large number of species, whereas SignaLink deals only with data from the model organisms *Caenorhabditis elegans, Drosophila melanogaster* and from human. In contrast, the data collected in the Reactome and Netpath databases are restricted to human signaling pathways. In case of KEGG there is no clear pathway definition, thus, what is considered as an individual pathway is decided by the curator. In Netpath 10 immune and 10 cancer signaling pathways were curated based on PPI data from the HPRD resource [18, 19]. In contrast, the Reactome and SignaLink databases feature a unified and available protocol for data collection. The pathways in SignaLink are biochemically and evolutionarily defined and are identical with the pathway grouping of [1]. It is important to note, that solely in virtue of the number of pathways, these databases are not comparable. For example, in the SignaLink database, the EGF/MAPK pathway contains the proteins and interactions between the EGF ligand and the terminal MAPK proteins. While the grouping of these interactions and proteins into a single pathway is biochemically and evolutionarily reasonable, many databases scatter this pathway across many (sub)pathways (e.g., EGFR, RAS, p38, JNK, ERK, ASK). Although a relevant and objective pathway definition decreases the overall number of pathways in the database, it avoids artificial and biased pathway grouping.

An important aspect in manually curated databases is the assignment of proteins to signaling pathways. In the Reactome and Netpath databases, this is entirely dependent on individual experts who construct the pathways, but no references are provided for the users. Similarly, in the KEGG and SignaLink pathways, to which pathway a protein is annotated is decided by curators, but importantly, their decision is based on published review papers from experts of the given pathway. While KEGG collects the information from only a few (usually 5–10) reviews, SignaLink uses 20–25 reviews per pathway and also adds additional PPI information based on orthology. The reliability and utility of databases greatly depends on the availability of published references, which underlie every single protein–protein interaction. This is accessible for every interaction in the Reactome, NetPath and SignaLink databases, however, KEGG only refers to review papers.

By comparing all 4 databases, SignaLink showed the largest overlap with the other databases and contained the most references from the literature. Therefore, we set SignaLink against the other databases comparing 7 human signal transduction pathways in SignaLink (EGF/MAPK, IGF, Hedgehog, JAK/STAT, Notch, TGF-$\beta$, WNT) with 7 human signaling pathways from KEGG (MAPK, Insulin,

Hedgehog, JAK/STAT, Notch, TGF-$\beta$, WNT), 5 pathways from Reactome (EGFR, Insulin receptor, Notch, TGF, WNT) and 5 pathways from NetPath (EGFR1, Hedgehog, Notch, TGF, WNT). Regarding the number of proteins found in the pathways, the 7 pathways in KEGG have 17 % less, the 5 pathways in Reactome have 84 % less, while the 5 pathways in Netpath contain approximately the same number of proteins as in the corresponding pathways in SignaLink. In the case of so-called multi-pathway proteins [20], which participate in multiple pathways and function in cross-talks, KEGG contains about the same number as SignaLink, whereas only half of these types of proteins can be found in Reactome and Netpath.

In comparing the number of PPIs, KEGG contains 52 % more interactions than SignaLink, but notably, most of these interactions are artificial, as they were obtained indirectly using a matrix method [21]. Interestingly, the number of cross-talks linking distinct signaling pathways is about the same in SignaLink and KEGG, therefore, the relative amount of cross-talks in SignaLink is probably higher. In Reactome, when including all interactions within protein complexes, this database has up to two times as many PPIsas SignaLink in overall, however, without the protein complexes, the number of interactions is roughly equal. Regarding the number of cross-talks, SignaLink contains almost three times as many as Reactome, and this is not influenced by the presence of interactions within protein complexes. In comparison to Netpath, SignaLink contains about three times as many cross-talks, 1.5 times as many PPIs, while the number of proteins is approximately the same, albeit with only about 30 % overlap between the two databases [6].

Based on this comparison we can conclude, that the major advantage of SignaLink over the other three databases is that it features precisely defined signaling pathways, has detailed criteria for assigning proteins to pathways and uses a unified curation method which makes a systems-wide analysis of signaling pathways possible. Furthermore, within the signaling pathways shared by all four databases, SignaLink contains the most proteins, interactions and references. This makes SignaLink an excellent resource for taking on the new challenges of signal transduction research and for the efficient study of cross-talks.

## 16.4 Extending Signaling Pathways with Regulatory Processes

Signaling networks can be divided into upstream and downstream subnetworks. The upstream subnetwork contains the intertwined network of signaling pathways, presented earlier, while the downstream, gene regulatory subnetwork (GRN) contains transcription factor binding sites, transcription factors and microRNAs, ultimately controlling global gene expression and the dynamics of protein output in a living cell [22] (Fig. 16.1). The GRN can further be divided into

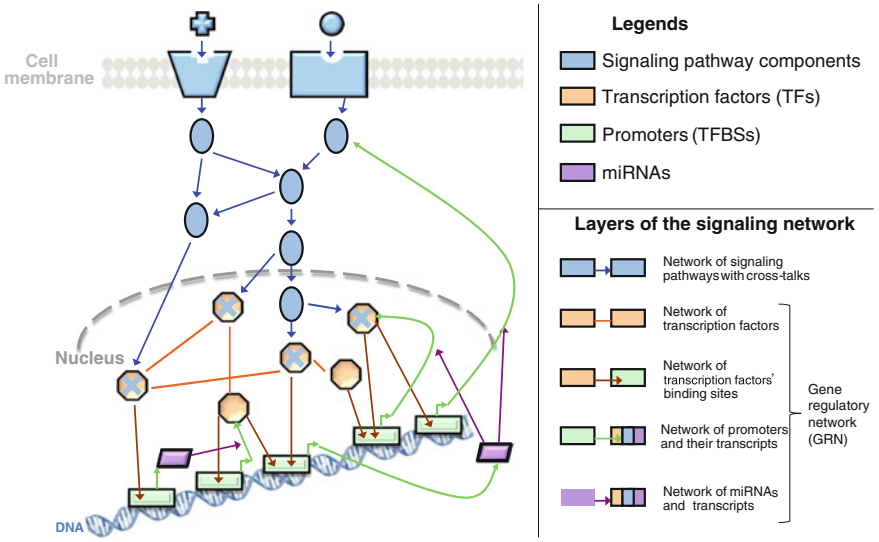


**Fig. 16.1** Layers of the signaling network. In the upstream part the network of signaling pathways contains the incoming signals (ligands) that activate receptors and mediator proteins to reach the transcription factors in the nucleus. In the nucleus, the downstream, gene regulatory network (*GRN*) contains four layers (networks): the network of transcription factors (*TFs*), the network of TFs and their binding site in the promoter region of certain genes, the network between these regions and their transcripts, and the network of microRNAs (*miRNAs*) and their target mRNAs

transcriptional and post-transcriptional subnetworks. At the transcriptional level, transcription factors (TFs) bind specific regions of DNA sequences (called transcription factor binding sites (TFBS) or response elements) and regulate the mRNA expression of transcription factor target genes. Post-transcriptionally, microRNAs (miRNAs) regulate gene expression by binding to complementary sequences (i.e., miRNA binding-sites) on target mRNAs. The specific binding of a miRNA to its target mRNA can suspend or permanently repress the translation of a given transcript, thereby specifically inhibiting protein production [23, 24]. Despite the difficulties of identifying miRNA targets, it is predicted that nearly all human genes can be controlled by at least one miRNA [25] and mutations in many miRNA coding genes have pathological consequences [26]. The importance of miRNAs in the regulation of protein–protein networks was highlighted by a positive correlation between the number of repressing miRNAs and the protein partners (i.e., degree) of a given protein [27]. Thus, proteins having many interactors (i.e., protein hubs) are more tightly regulated than proteins with less interactors [27]. In addition, a comprehensive analysis suggested that specific biological processes are regulated by miRNAs through targeting the hub and bottleneck proteins of the protein interaction network [28].

Recently, many databases comprising the downstream regulatory subnetwork components of signaling pathways have been created. A compendium of human

TFs have been collected and analyzed in [29], while their regulatory interactions can be acquired from the resources JASPAR, MPromDB, PAZAR and OregAnno [30–33]. Experimentally validated miRNA-mRNA interactions are available from TarBase [34], while predicted interactions can be accessed at TargetScan and PicTar [35, 36]. TransMir and PutMir contain TF-miRNA regulatory information to examine how miRNAs are regulated [37, 38]. In addition, miRecords and miRGen provide an integrated resource from where different miRNA-related resources can be accessed [39, 40]. To examine the signaling network in a unified fashion, integrated resources including IntegromeDB and TranscriptomeBrowser 3.0 have been developed, which allow the examination of all layers from signaling pathways to miRNAs through TFs [41, 42].

As an update for SignaLink, we have recently developed an integrated database on the regulation of signaling, containing information from *C. elegans*, *D. melanogaster*, and humans (BMC Syst Biol. 7:1752-0509-7-7). Signaling pathway information from SignaLink was integrated with major processes that regulate signaling. First, on the bases of manual curation of primary literature and reviews, we linked scaffold proteins, specific ubiquitin-ligases, and proteins involved in endocytosis to pathway proteins. Next, we extended the network with the first neighbors of the proteins based on directed protein–protein interactions (PPI). The PPI data was retrieved from BioGRID, DroID, and WI8. The direction and the confidence for each interaction was calculated based on domain–domain and domain-motif interactions. In the next step, we included the underlying regulatory network: (1) downstream transcription factors and their subnetworks, based on manual curation of primary literature; (2) interactions between transcription factors and transcription factor binding sites of genes, using OregAnno, JASPAR, and MPromDB; (3) mRNA transcripts (from ENSEMBL), miRNA transcripts (from miRBase, miRGen and PutmiR), and their interactions (from miRecords and Tarbase). The database can be freely downloaded for academic purposes in various network file formats (BioPAX, SBML, CSV, etc.) via a BioMART-like download page, where users can filter the datasets.

## 16.5 Pharmacological Relevance of Signaling Networks

Understanding the structure and mechanism of normal signaling networks can reveal important targets for drug discovery. In many cases, these targets have no direct relation to a particular disease but their stimulation or inhibition can have beneficial systems-level effects on the cellular network, and lead to the survival of the organism. Pharmacological modulation of key proteins of the signaling network can influence the robustness of the cells for therapeutic purposes, e.g., increasing robustness in healthy cells and decreasing robustness in cancerous cells during chemotherapy [43, 44]. Three members of the insulin signaling pathway (PI3 kinase, AKT and IRS families) have already been identified as ‘critical nodes’ having distinctive roles in the junctions of signaling pathways and effecting the

behavior of the cell during diabetes [12]. Hwang et al. developed the network parameter bridging centrality to identify key proteins in signal flow-modulation as promising drug targets [45]. The major strength of bridging centrality is that it effectively combines local and global network properties. Proteins with high bridging centrality (i.e., bridging nodes) are located in the critical sites of the signaling network and connect different parts (regions or modules) to one another [45]. They also found that many bridging nodes (e.g., SHC, JAK2, cortisol) had a track record as effective drug targets [45].

On the other hand, gene expression and sequencing studies on pathologically altered signaling networks can uncover possible drug targets whose malfunction directly cause disease. For example, during tumorigenesis when cells acquire continuous cell division and often increased mutation rate [46] most of the (driver) mutations affect a limited number of central pathways [47]. Drug targeting of these specific pathways could potentially prevent tumor growth. However, the development of aggressive and malignant tumor cells cause a systems-level change in the signaling network [48], thus their therapeutic treatment poses a major challenge. The pathological rewiring of the signaling network allows the appearance of cancer hallmarks, including sustained angiogenesis and metastatic tissue invasion capabilities [49], as well as the deregulation of cellular metabolism and avoidance from immune destructions [50]. The effect of signaling rewiring on cancer hallmarks was shown in prostate cancer [51]. Several works demonstrated that changes of cross-talk (i.e., multi-pathway) proteins are important for the rewiring of the signaling network [48, 52, 53]. Mutation even in one multi-pathway protein can have a systems-level effect as it can significantly alter the signaling flow, for example, transducing a ‘death’ signal to a ‘survival’ transcription factor [49, 54]. Similarly, we found a significant change in the expression level of multi-pathway proteins in hepatocellular carcinoma [6]. Accordingly, multi-pathway proteins are often altered in systems diseases such as cancer, thus, they are among the most promising drug targets [20]. Pharmacological modification of these proteins can re-transform the rewired cancerous signaling network.

Kinases are traditionally among the most targeted proteins of the cellular signaling network [55] although their selective targeting is a challenge for drug development. Kinase domains and their target motifs (i.e., specific amino acid sequences in the substrate proteins) are well-known and comprehensively compiled in resources such as Phosphosite [56], NetworKIN and NetPhorest [57, 58]. Regulatory domains of these kinases and scaffold proteins are also important to maintain kinase-substrate or scaffold-substrate specificity [59] but our systems-level knowledge on these (undirected) protein–protein interactions are less limited than the directed phosphorylation data.

It is important to highlight that less attention has been taken on the other players of the phosphorylation system: the protein phoshatases. As reviewed by Kholodenko and colleagues [60], protein phosphatases can play a dominant role in determining the spatio-temporal behavior of protein phosphorylation systems in the cell as both immediate and delayed negative regulators. Thus, pharmacological

targeting of phosphatases can modify the signaling network at the systems-level. Despite their promising effect, only a few protein tyrosine phosphatases are currently used as therapeutic targets [61]. The development of drugs specifically targeting phosphatases is much more complicated than the development of anti-kinase drugs for the following reasons: (1) high-level of homology between phosphatase domains limits the development of selective compounds; (2) contrary to kinases, phosphatase substrate specificity is achieved through docking of the phosphatase complex at a site distant from the dephosphorylated amino acid [62, 63]; (3) the targeted sequences are highly charged, and many of the developed drug compounds cannot cross the membrane [64]. Resolved phosphatase-complex structures and detailed knowledge of their enzymatic activity will allow effective drug development and their utilization as systems-level drug targets.

Recently, miRNAs have been recognized as highly promising, non-protein intervention points in the signaling network. Therapeutic targeting of regulatory components is a challenging task because of specificity and pharmacological availability (i.e., therapeutic agents often have off-target effects and hardly enter the nucleus). Pharmacological modulation of protein and miRNA expression with an antisense strategy appears to be more specific than targeting TFs, TFBSs and miRNA promoters [65]. Specificity comes from the fact that antisense strategy affects single miRNAs and miRNA families that are specific for a given mRNA (or mRNA cluster), while TFs and promoters have less specific effects on the whole transcriptome [65].

Besides specificity, miRNAs can be important therapeutic targets, as their down- or up-regulation is implicated in more than 270 diseases according to the the Human MicroRNA Disease Database [66]. The diseases where altered expression of miRNAs have been reported include cardiovascular, neurodegenerative diseases, viral infections like HIV and various types of cancer [67]. The development of therapeutic strategies involving miRNAs requires the exploration of the signaling network. Therapeutic miRNAs can only be selected if their mRNA-interactions have been confidently identified and experimentally validated. These interactions can be accessed in specific and integrated resources listed in the previous section. In addition, evaluation of the cellular processes that are affected by the given miRNA is also necessary to avoid side-effects and unwanted drug effects. Web-services, such as Pathway Linker (http://PathwayLinker.org; [68]) have been developed for this purpose. As miRNAs often have multiple targets analysis of the network of the affected proteins (encoded by target mRNAs) can facilitate pharmacological development: identification of proteins whose knock-down has limited side-effects and toxicity profiles can be promising agents for miRNA-based therapeutics [65]. Such side-effects can be analyzed by databases such as SIDER [69].

## 16.6 Conclusion

The study of cross-talks has emerged as an important field in signal transduction research. To identify cross-talks and understand their roles in development and disease, one needs to analyze signaling networks at the systems level. Decades of research on signaling pathways and modern high-throughput methods have provided large data sets on the signaling components. Still, only a small number of databases fulfill the requirements of analyzing cross-talks at the systems level. By comparing 4 signaling databases (KEGG, Reactome, Netpath and SignaLink) in terms of pathway definition, curation methods, protein number, PPI number and the number of cross-talks, we point out that the SignaLink database is a valuable resource for cross-talk research. Signaling pathways are strictly regulated by downstream components, including transcription factors and miRNAs. Information on this gene regulatory subnetwork has been compiled into various databases which serve specific needs. For a comprehensive analysis of signaling from ligand binding to alterations in gene expression, integrated databases containing a great number of regulatory components (including both posttranscriptional and post-translational modifications) of signaling proteins are needed. These will contribute to the understanding of systems biology diseases such as cancer, and help predict more efficient drug targets for fighting against these diseases.

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