

Simplistic pathways or complex networks?

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Signaling events are frequently described in textbooks as linear cascades. However, in reality, input cues are processed by dynamic and context-specific networks, which are assembled from numerous signaling molecules. Diseases, such as cancer, are typically associated with multiple genomic alterations that likely change the structure and dynamics of cellular signaling networks. To assess the impact of such genomic alterations on the structure of signaling networks and on the ability of cells to accurately translate environmental cues into phenotypic changes, we argue studies must be conducted on a network level. Advances in technologies and computational approaches for data integration have permitted network studies of signaling events in both cancer and normal cells. Here we will review recent advances and how they have impacted our view on signaling networks with a specific angle on signal processing in cancer.

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Introduction

Sequencing of cancer genomes has revealed a significant number of genetic alterations such as deletions, point mutations and amplifications [1–6,7^{**}]. While some of the genetic alterations reside in supposed well-characterized tumor-suppressors and oncogenes [4,7^{**}], a significant number of aberrations are located in genes with limited or no functional characterization. An emerging challenge is to identify causal relationships among these genome-wide changes, which simplistically can be described as identifying so-called driver and passenger mutations. Equally important, the combined effects of the genetic alterations on the signaling network and how the sum of these gives rise to the malignant phenotype

must be understood in order to target the diseased cells efficiently.

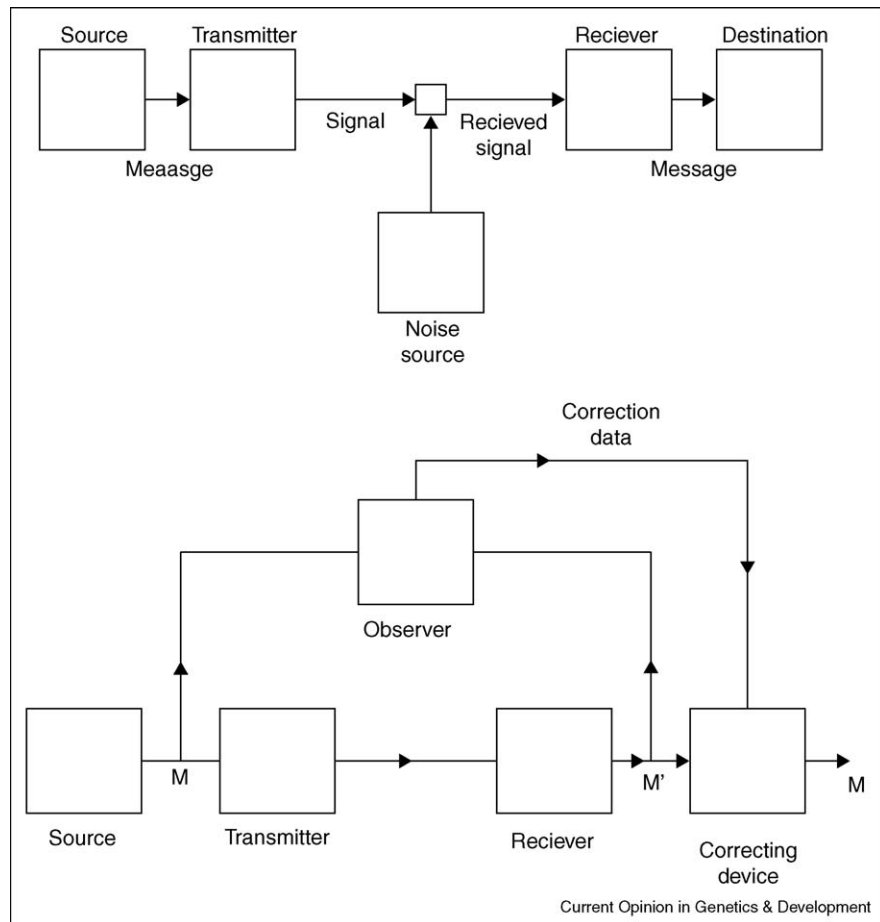
Correctly performed systematic studies of cellular behavior have provided significant insight into the structure and function of signaling networks, their capacity for signal processing and propagation as well as their impact on cellular decision processes (e.g. apoptosis versus proliferation). Quantitative data describing the state (e.g. the activity or level) of hundred to thousands of signaling nodes in combination with integrative analysis by novel computational methodologies have permitted analysis of cellular signal processes at the network level. Thus, these approaches hold great promise in advancing our understanding of cellular decision-making and how individual and collectively genetic perturbations affect cellular signaling networks. In particular, we argue these approaches will lead to the first global predictive models of cell behavior, something that will transform biology from descriptive and qualitative observations to a quantitative, causal and predictive science.

Rewiring of oncogenic signaling networks

Cellular signaling networks are information processing systems. They receive, interpret, correct and transmit or propagate input cues to other control layers in the cell, ultimately altering cell behavior or processes (Figure 1). Cellular signaling is in many aspects identical to a general communication system (Figure 1) where a message (signal) needs to be transferred to a recipient (e.g. a kinase) (upper panel). Since most real systems are exposed to noise, (biological) innovations are made to correct for scrambled messages (lower panel). There are two important observations that can be made from these two situations: First, as error correction becomes important we note that a nonlinear, branched and more complex network is required. Second, the information flow within the network becomes important as the ‘observer’ module needs to be able to process information fast enough in order for the correction device to ‘catch up’ with the message [8].

A hallmark of the ability of networks to process information, as compared to a linear pathway or cascade, is that the connectivity in networks makes them more robust toward inactivation or deleterious events of a few nodes [9,10,11^{*}]. Not surprisingly, the network connectivity (its ability to relay information between distant proteins) is more susceptible to the loss of highly connected proteins (hubs) [12]. These highly connected hubs can simplistically be divided into intramodular (or party hubs), which connect a larger number of local nodes (functional

Figure 1



Cellular information processing. The 1948 work from Shannon [8] laid the foundation of information theory. There are two important observations that can be made from his work: First, as error correction becomes important we note that a nonlinear, branched and more complex network is required. Second, the information flow within the network becomes important as the 'observer' module needs to be able to process information fast enough in order for the correction device to 'catch up' with the message [8]. We argue these fundamental principles are crucial for cellular signaling. Reprinted with permission of Alcatel-Lucent USA Inc.

modules), or intermodular (or data hubs), which connect the functional modules [10]. While intramodular hub proteins typically are coexpressed and reside in structural complexes, the function of intermodular hubs is context dependent and can for example be restricted by tissue-specific or time-dependent expression [10,11[•],13]. Using this knowledge of mammalian protein network structure, Taylor *et al.* were able to predict the outcome of breast cancer patients more accurately than from gene expression profiles alone [11[•]]. Although gene expression profiling has been widely used in attempts to categorize cancer patients, integration with orthogonal signal network information such as protein–protein interaction data increases the predictive power of the algorithm [11[•],14]. Interestingly, when expression profiles were analyzed in combination with network parameters, it became evident that the mRNA expression levels of proteins that interact with intermodular hubs were significantly different between

patients with good and poor outcome [11[•]]. This suggests that the network structure is extensively rewired in diseases such as cancer, and thus the altered signaling information processing has a significant impact on disease progression.

Genetic interaction studies from yeast and *Caenorhabditis elegans* [15,16[•],17,18^{••},19] have shown that the interaction between functional modules is highly conserved. Combining epitasis maps of genetic interactions from *S. pombe* or *S. cerevisiae* with protein–protein interaction networks further revealed that genetic interactions were highly conserved when the corresponding proteins also interact [16[•],17]. Importantly, although the functional modules were conserved, the interconnecting modules between them can vary significantly, which could be due to differences in the post-translational circuitry such as the phosphorylation networks. Phosphorylation sites are localized

in short linear motifs typically residing in disordered regions [20,21^{••},22[•]], which have been noted to change quickly during the course of evolution [21^{••},23^{••},24^{••},25,26[•]]. In fact, genetic interactions between kinases and substrates are less conserved on average, supporting the notion that evolutionary divergence of phosphorylations correlates with functional changes in protein kinases [25]. This is also supported by the notion that mammalian signaling networks have adapted to the relative low sequence specificity of tyrosine kinases by suppressing genomically encoded tyrosines [24^{••}]. Furthermore, we recently showed that tyrosine kinase domains that have less sequence specificity are more likely to become oncogenic [23^{••}]. Interestingly, phosphoproteins containing evolutionary conserved kinase–substrate interactions are more likely to be mutated in human diseases [21^{••}]. Thus, while network connections between kinases and substrates are evolutionary dynamic, they appear to be specifically adapted to the evolutionary constraint of a particular cellular context. As such, rewiring of these connections appears to be prone to diseases such as cancer. This was further supported by our recent observation that multiple diseases target a conserved core network of kinases and substrates, where more highly conserved networks correlated with an increased number of diseases affecting one or more of the nodes [21^{••}].

Global analysis of regulatory networks governing protein phosphorylation has undergone a massive expansion, primarily due to recent development in quantitative mass spectrometry [27,28]. Through its ability to specifically identify and quantify phosphorylation events in hundreds to thousands of proteins, a global signaling state can be identified [29,30,31[•],32–34]. Analyses of phosphorylation events in cancers have identified and quantified a significant number of dysregulated phosphorylation events [30,35,36[•],37]. These efforts have subsequently resulted in the identification of novel fusion proteins and deregulated kinases by mutational analysis [36[•]] and analysis of the network response following inhibitor treatment [35,37].

An unwanted side effect to inhibitor treatment is the development of resistance or tolerance to the drug treatment [7^{••},38]. While such effects frequently appear because of the accumulation of additional mutations in the target kinase (e.g. in BCR-Abl [39]), another underlying mechanism is rewiring and adaptation of the signaling network. For example, acquisition of MET amplification results in transactivation of EGFR and PI3K signaling, thereby causing resistance toward EGFR inhibitors [40]. In glioblastoma multiforme (GBM), so-called crosstalk between EGFRvIII and other receptor tyrosine kinases renders the cancer resistant to EGFR inhibitor treatment and thus requires simultaneous administration of several kinase inhibitors [37,41]. Thus, cancer-causing mutations frequently rewire signaling net-

works, which need to be taken into account during analysis and drug therapies. In particular it is essential to realize that loss of function in one part of signaling network can indeed lead to gain of function in another part of the network; thus the pathogenic effects of an oncogenic mutation might reside in a completely different node [42].

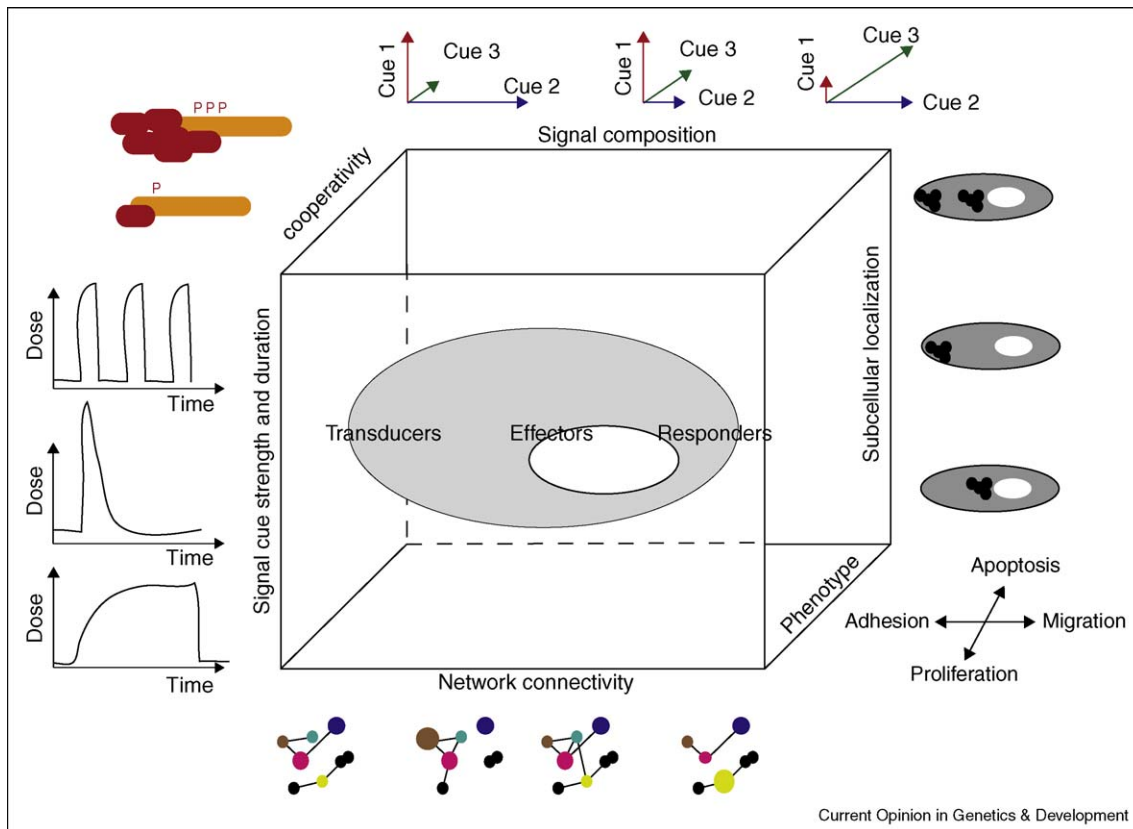
Further global assessments of signaling networks and their rewiring will have a significant impact on the development of effective and network-based drug therapies. Since kinase inhibitors in general bind and inhibit multiple kinases [43,44], assessment of their effects on normal and rewired signaling networks will aid in developing network medicine based strategies [45]. As an example, by employing a network model of Erb signaling a novel and effective antibody based treatment was recently developed [46^{••}]. By combining the effect of different EGFR ligands on the activation profiles of Erb receptors and Akt with mass action kinetic evaluation, ErbB3 was identified as the central targeting node. Subsequent targeting of this node by selective antibody therapy confirmed the model and showed efficient inhibition of tumor growth in a xenograft model [46^{••}].

Cell fate determination and the multivariate nature of networks

Integrative and data-driven modeling have now clearly shown that multivariate signaling networks govern cellular decision processes. Since cells are continuously exposed to multiple signals from growth factors, interacting cells and the extracellular matrix, a proper phenotypic response must take the cumulated effects of these signals into account [47^{••},48^{••},49[•]]. To accomplish such a challenge, signaling networks process this information in a multivariate manner [47^{••},48^{••}]. For example, JNK activity can be associated with both proapoptotic and anti-apoptotic behavior, depending on the combined effect of the molecular signals the cells is exposed to [47^{••},50–52]. Thus, the relative strength of the signal inputs was important to the predictive power of the models. This clearly shows that in order to generate relevant models the cellular phenotypic response should be determined using combinations of signal cues instead of one saturating signal [47^{••},53,54^{••}].

Using a similar approach, epithelial cell fate decisions were described according to a common effector model [48^{••}]. This model predicts that different cells, for example HeLa and HT29 cells use specific transducers, but similar effectors, to translate signaling cues into changes of the phenotypic output (apoptosis in this case). Importantly, since this model can accurately predict the apoptotic response of different epithelial cells to the same input cue, it is an essential tool in predicting common and specific network and phenotypic effects following drug treatment. Interestingly, as the model

Figure 2



Complexity in cellular signaling. Cellular response to cues and signal outcomes depends on several context-dependent internal and external factors that are integrated through a multivariate signaling network to alter the cellular phenotype.

failed to predict responses from different cellular lineages this would indicate that additional components such as expression patterns and levels of signal transducers and effectors impact the accuracy of the model.

Since the context of signaling cues has great impact on the cellular decision and phosphorylation events or enzymatic activities can be associated with several cellular phenotypic outcomes, this has profound impact on how we interpret markers (or biomarkers) to predict the cellular outcome. For example, one-sided utilization of the JNK phosphorylation site as a biomarker, without further analysis of the network state, could potentially be misleading [55]. Finally, as these data-derived models are capable of predicting cellular behavior, absolute quantitative (stoichiometric) measurements, information about subcellular localization and the presence of local scaffolding will likely improve the accuracy of such models [56,57**] (Figure 2).

Understanding the heterogeneous response of a cellular population is important to comprehend the underlying mechanisms whereby signaling networks develop resist-

ance to cancer therapeutics. Thus, the notion that individual cells from an apparent homogeneous cell population respond differently to molecular signals [58,59**] prompted the investigation of TRAIL-induced apoptosis under such conditions. Using a combination of single cell measurements and ODE modeling [58,59**], nongenetic components were identified as the primary reason for cell-to-cell variability. Intriguingly, variability in protein levels or signaling states between nonresponders and high responders appear to be the underlying cause of the heterogeneous effect [59**,60**], supporting the notion that the state of the network before treatment can influence the cellular outcome [47**]. These effects appear to be tightly linked with the cellular capability for signal transmission, which is further supported by the findings that signaling networks prefer dynamic range to signal strength [49*]. As such, in order to preserve the dynamic capability, network structures have evolved feedback loops as built-in reset mechanisms [61*,62,63*]. Whether oncogenic mutations are specifically selected to impact feedback loops to maintain a high basal signaling level or otherwise diminish the dynamic signaling capabilities of signaling networks is still not fully understood.

Signaling, genetic perturbation, and phenotype

Availability of small interfering RNA (siRNA) as a genetic tool to analyze signaling networks has provided valuable insight into the function of individual genes, genetic interactions, and has successfully been combined with small molecule inhibitors to identify gene drug target interactions [64–68]. One of the notions that has become increasingly evident from combinatorial siRNA screens and SGA studies in yeast is that signaling networks are extremely adaptive to genetic perturbation [15,16,17,69]. However, implementation of quantitative readouts has aided in our understanding of how genes contribute to a specific phenotypic outcome [57,69,70,71]. This was elegantly showcased when quantitative morphological signatures derived from classifier phenotypes were used to cluster siRNA phenotypes based on their individual morphological signatures [57]. As such, known as well as novel effectors of cellular morphology such as cellular protrusions, lamellipodia formation and adhesion assembly/disassembly were classified. By combining siRNA screening with network modeling, regulatory networks of JNK activity were recently identified [69]. Combining a dual RNAi screen (epistasis screen) for regulators of JNK activity with network modeling based on phospho-proteomics data, JNK was shown to be interconnected with many cellular functions, explaining why JNK activity is highly context dependent and is connected to different input cues [69]. The latter approach to modeling signaling networks is based on algorithms we have developed to re-assemble signaling networks from assigning kinases and phospho-binding domains to identified phosphorylation events [21,23,24,54,72]. The underlying principle of these algorithms connecting individual identified phosphorylation sites to kinases and phospho-binding domains is based on sequence consensus motifs [23] and probabilistic contextual network information [72] (NetworkKIN, <http://networkkin.info> and NetPhorest, <http://netphorest.info>). One of the advantages with such a molecular logic approach is that proteins are frequently modified on several sites, each of which can have independent molecular function, and thus the individual and combined effect of the modifications can be taken into account. Thus, this type of network analysis may provide insight to potential cooperative, or logic gate effects between multiple phosphorylation sites [56,73]. In addition, perturbation of signaling networks by siRNA or inhibitor treatment can be used to determine network connectivity when combined with computational approaches such as Bayesian modeling [74,75]. Moreover, by combining these approaches with network rewiring by synthetic biology there is great promise to obtain functional links between the structure and function of signaling networks and cellular decisions [76,77,78,79]. Finally, quantification of signaling networks is crucial for a deeper understanding of signal propagation [80,81].

Outlook

Although we here emphasize the power of integrative network biology approaches, it is clear that representing signaling information processing as networks currently has limitations. We argue more dynamic models are needed to reflect the network ensembles and states that can be occupied throughout a cellular decision process. This will require new mathematical formalisms and algorithms in addition to computational tools to fully visualize these. Although the studies discussed above are confronting these challenges, there is still a long way to complete models of cell behavior. This will require quantitative measurements of the metabolic, signaling, transcriptional and genomic state alongside multiple cellular phenotypes. Thus, multidisciplinary and highly collaborative research initiatives are needed. In addition to this, new scalable laboratory and computational infrastructures are needed. One might even argue network biology is becoming like a smaller version of subatomic particle physics in terms of technological and analytical requirements. Signaling network models are currently generated from homogeneous cellular populations or single cells, novel ways of identifying cell communication between heterogeneous cell types are needed in order to understand the multicellular environment [54]. Finally, models need to be tested in various disease and normal states to identify their limitations and predictive power. In many ways this is *similar* to the challenges structural biology faced decades ago, with the introduction of NMR spectrometry, new tools and analyses had to be developed to better describe and define the dynamics of protein structures [20,82]. Transforming the process of biological observation from a qualitative and hypothesis driven endeavor to systematic and quantitative measurements based on an integrated theory is necessary to enable predictive models of cell behavior and to define network drug targets.

We argue that the concept of linear cascades provide a limited and even misleading conceptual framework to determine how signal transduction is studied. This in turn underlines the importance of a shift in how we conceptualize information processes and a discontinuation in the use of simplistic pathway diagrams and instead move toward context-dependent and probabilistic concepts [56,83,84]. Cells utilize highly complex, dynamic networks which occupy ensembles of states during their lifetime, the challenge is to identify, quantify and link these network states to cell behavior with the aim of constructing predictive models of biological systems similar to those of other physical systems.

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