Structure
Perspective

Structural Data in Synthetic Biology Approaches for Studying General Design Principles of Cellular Signaling Networks

Christina Kiel[1,2,*] and Luis Serrano[1,2,3]
[1]EMBL/CRG Systems Biology Research Unit, Centre for Genomic Regulation (CRG), Dr. Aiguader 88, 08003 Barcelona, Spain
[2]Universitat Pompeu Fabra (UPF), 08003 Barcelona, Spain
[3]ICREA, Pg. Lluís Companys 23, 08010 Barcelona, Spain
*Correspondence: christina.kiel@crg.eu
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In recent years, high-throughput discovery of macromolecular protein structures and complexes has played a major role in advancing a more systems-oriented view of protein interaction and signaling networks. The design of biological systems often employs structural information or structure-based protein design to successfully implement synthetic signaling circuits or for rewiring signaling flows. Here, we summarize the latest advances in using structural information for studying protein interaction and signaling networks, and in synthetic biology approaches. We then provide a perspective of how combining structural biology with engineered cell signaling modules—using additional information from quantitative biochemistry and proteomics, gene evolution, and mathematical modeling—can provide insight into signaling modules and the general design principles of cell signaling. Ultimately, this will improve our understanding of cell- and tissue-type-specific signal transduction. Integrating the quantitative effects of disease mutations into these systems may provide a basis for elucidating the molecular mechanisms of diseases.

Introduction

There is growing three-dimensional (3D) structural information about proteins, protein complexes, and homology models available (Stein et al., 2011). Structural information has contributed to the prediction and analysis of protein interaction networks (Kiel et al., 2008; Bhardwaj et al., 2011; Clarke et al., 2012; Tuncbag et al., 2009). In addition, structural information has been used on a large scale to predict the effects of disease mutations on the interactome properties, especially of those mutations located in the physical interface between proteins (Zhong et al., 2009; Wang et al., 2012). This has provided insight into human genetic disease at a global level. However, some challenging questions still remain, such as why a mutation often functionally affects only a few tissues, or why the same mutation causes disease in one person, but not in another. For example, mutations that affect the affinity of the Ras-Raf complex were shown to affect downstream signaling only weakly in a cell line with strong negative feedback from ERK to Sos1 but strongly in a cell line with weak negative feedback (Kiel and Serrano, 2009). To tackle these questions, recent work has focused on describing the proteome in different cell types and tissues (Pontén et al., 2009; Geiger et al., 2012; Lamond et al., 2012), with the aim of defining common and variable elements in different cell types (Burkard et al., 2011; Geiger et al., 2012). In parallel, approaches in the field of signal transduction, often combined with mathematical modeling, aim to unravel mechanisms of achieving cell-type-specific signaling responses (Kholodenko et al., 2010; Kiel and Serrano, 2012). One of the main problems in elucidating cellular signal transduction is the high level of crosstalk and existence of multiple feedback loops within and between signaling branches. Mechanistically, this is even further complicated by the fact that often proteins recruit binding partners using several domain or linear motifs. However, it is unlikely that all interaction possibilities are explored in one cell, and at the same time. Thus, organizational patterns (i.e., protein complexes, localization, expression levels, splice variants) need to be identified in order to reduce the complexity, and for this, the main challenge lies in finding the right balance between reductionism and necessary details (Rollié et al., 2012).

This Perspective article aims to give a structural view to signaling networks and synthetic biology (Figure 1). Structural information can help to reduce complexity in cellular networks, by allowing functional units (modules) and larger signaling complexes to be analyzed. Furthermore, 3D structures mapped on signaling networks, together with absolute protein abundance and binding affinities, can be highly informative about competing protein interactions (Kim et al., 2006). This, together with quantitative mathematical modeling (i.e., using ordinary differential equations), can predict signaling flows through different downstream signaling branches. We propose that identifying and analyzing modules to discriminate between general and cell-type-specific modules, followed by analysis in orthogonal systems using synthetic biology approaches (Di Ventura et al., 2006; Collins, 2012), will allow network behavior to be tested after protein levels are altered or after disease mutations in proteins have been introduced. Thus, we can expect that structure-based synthetic biology approaches will ultimately help to unravel general design principles of signaling. This could provide a basis for understanding the molecular mechanisms underlying different diseases, for example, by analyzing alternations in cell-type- and tissue-specific protein levels into predictive models for pathways that are frequently altered in diseases.

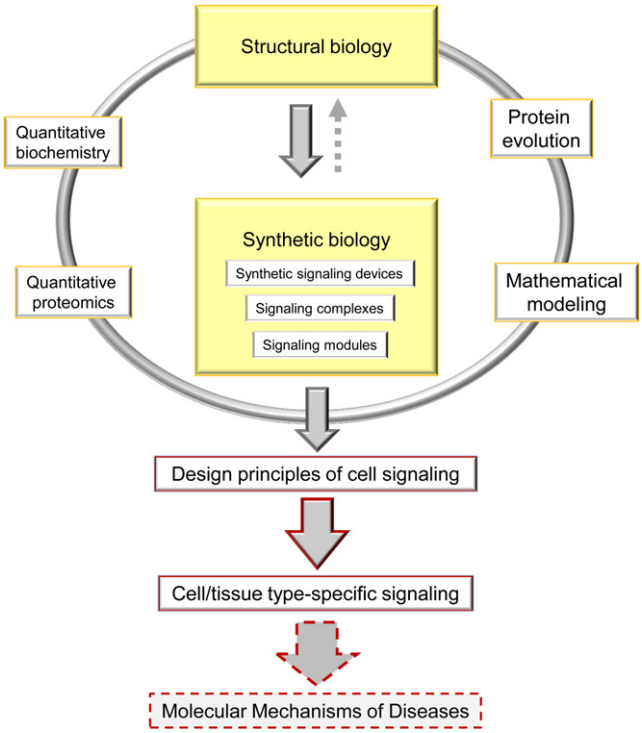

Figure 1. Overview of Combining Structural Biology with Synthetic Biology to Provide Insights into Cell Signaling

Structural information provides a valuable tool in engineering synthetic signaling devices. Combined with additional information from quantitative biochemistry and proteomics, gene evolution, and mathematical modeling, this can provide insights into signaling modules and the general design principles of cell signaling. Altogether, this will improve our understanding of cell- and tissue-type-specific signal transduction. In the future, this knowledge can be the basis for understanding the molecular mechanisms of disease by predicting the effect of disease mutation using mathematical modeling.

Structural Information Combined with Protein Design in Signaling and Synthetic Biology Approaches

Structure-based protein design is an important approach for understanding and modifying biological systems (Van der Sloot et al., 2009). There are two ways of using structural information and protein design to analyze signaling networks. One is to modify existing proteins to either eliminate interactions or to change kinetic or binding constants, with the aim of probing the network behavior. The other way is to design and engineer new parts that can be directed and therefore perturb a signal transduction pathway in a controlled way. An example of the first application is the structure-based design of mutations with altered binding or kinetic constants (Selzer et al., 2000; Kortemme et al., 2004; Kiel et al., 2004). Using the concepts of electrostatic steering, "mild/subtle" mutations were designed for the Ras-Raf1 complex (Kiel and Serrano, 2009). By introducing these mutations into different cell types and monitoring the downstream effects of them on signal transduction, important underlying network topologies were unraveled using a combination of both experiments and deterministic mathematical modeling based on differential equations (Kiel and Serrano, 2009). In another approach using mathematical modeling predicted that increasing the receptor specificity of the cytokine tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), without changing its binding parameters, would lead to faster receptor activation and enhanced signaling. This hypothesis was experimentally validated using a structure-based design approach and was subsequently tested in cell lines (Szegezdi et al., 2012). The important biological conclusion from this combined experimental and computational approach was that by modulating the relative amount of the different receptors for the ligand, signaling processes like apoptosis can be accelerated, decelerated, or even inhibited. In another set of experiments, the GTP-binding protein Ras, a central protein in the regulation of various cellular processes, was targeted by structure-based design (Kiel et al., 2009). Ras is a molecular switch cycling between GTP- and GDP-bound forms, and only Ras-GTP is able to interact with effector proteins (such as CRaf) with high affinity; the lower affinity of Ras-GDP for effector proteins decreases the stability of complexes and interrupts signaling. To analyze whether the ability of effectors to bind to Ras only in the GTP-bound state is controlled by the affinity of the interaction, mutations were designed that have high affinity in complexes with Ras-GDP. Implementing these mutations in cell lines and measuring downstream signal processing showed that higher-affinity Raf mutants can signal in the context of Ras bound to GDP; thus, signaling appears to be only controlled by the stability of the complex, and not by whether Ras is bound to GTP per se. Recently, it was demonstrated that some natural disease mutations located on the surface of proteins and domains, and in the interface of protein complexes, affect only some of the binding partners ("edgetic mutations" versus node removal) (Zhong et al., 2009; Wang et al., 2012). In analogy to these natural mutations, structure-based protein design could also be used to redesign mutations of an upstream key hub protein, which specifically binds to only a subset of downstream effector molecules. This would allow signaling that flows through only one or a few branches downstream of a key hub protein ("branch pruning") to be analyzed. In this case, no orthogonal systems would be necessary to study signaling flows through different branches.

The second application of structure-based design is to engineer and design new parts. Synthetic biology depends on the concepts of interchangeable parts and modularity (Smolke and Silver, 2011; O'Shaughnessy and Sarkar, 2012). Until now, structural biology has been mainly used in synthetic biology approaches to design new parts/components and tools (Channon et al., 2008; Gurevich and Gurevich, 2012). Many of the characterized components can be found in the Registry of Standard Biological Parts (http://partsregistry.org/). Of special importance is the recently developed "SYNZIP protein toolbox" because it contains a complete biophysical quantitative description (i.e., affinities) of 22 heterospecific synthetic coiled-coil domains (Thompson et al., 2012). Coiled-coil peptides were also designed to be used for controlled protein assembly, and range from dimer to higher oligomer states (Fletcher et al., 2012). Other structure-based design approaches have combined synthetic peptides with PDZ domain design to generate light-controlled interacting protein tags (TULIPs) (Strickland et al., 2012). The remarkable novelty of this approach is that equilibrium and kinetic binding constants can be fine-tuned by mutagenesis and thus are adaptable to studying signaling pathways with different response

times. In another elegant approach that uses structure-based design, a synthetic GTPase/GEF pair was designed to have a completely orthogonal interface (Kapp et al., 2012). This is especially important because it allows individual pathways to be studied without influence from the extensive crosstalk within a cell. Finally, using a 3D structure-based design, short hairpin RNA was constructed that could respond to specific proteins and subsequently induce the desired gene expression (Kashida et al., 2012). This approach could be applied to developing new synthetic circuits.

A Perspective for Understanding Cellular Design Principles and Cell-Type-Specific Signaling Using Protein Design and Synthetic Biology

It is evident that the number of signaling proteins and genes is smaller than the multitude of biological processes. Thus, the challenge is to identify general design principles of how precise cellular regulation through signaling networks is achieved. In recent years, important general design principles have been worked out, such as the use of scaffold interactions to provide specificity, receptor clustering into microdomains, and localized signaling of proteins in different cellular compartments (Kholodenko et al., 2010). One important design principle is that, despite apparent high complexity and interconnectivity (''everything does everything to everything,'' Dumont et al., 2001), cellular signaling networks can be decomposed into functional modules, which perform certain functions and are often highly connected (Hartwell et al., 1999; Lauffenburger, 2000). Challenges in identifying signaling modules arise due to the high level of crosstalk and feedback between different modules, and because a signaling protein can often participate in more than one module. Thus, modules defined from connectivity analysis of PPI data from high-throughput data (Sharan et al., 2005; e.g., the MINE [module identification in networks] web server; Rhrissorrakrai and Gunsalus, 2011) do not necessarily reflect functional modules (Szallasi et al., 2006). Another problem is that pathways without extensive interconnectivity can be missed in such approaches. Different approaches are used to identify modules prior to these being tested to ensure that they are indeed ''functional modules.'' One of them is based on evolutionary conservation. Recent mass spectrometry-based studies have aimed at defining a so-called ''central proteome'' (Schirle et al., 2003; Burkard et al., 2011), including those proteins expressed in most/all cell types. The central proteome was found to be enriched in evolutionary-conserved proteins, including signaling-related proteins (Burkard et al., 2011). Together with the finding that tissue- or cell-type-specific proteins often interact with core cellular components and modules (Ryan et al., 2012; Bossi and Lehner, 2009), this suggests that signaling pathway modules exist that contain conserved and highly expressed proteins in all cell types. Thus, comparative analyses that search for conserved proteins in a pathway could help to identify functional modules. Recently, a web server that uses evolutionary conservation (BioXGEM.MoNetFamily, http://monetfamily.life.nctu.edu.tw) was developed based on using BLASTP and 3D structural information, which infers homologous modules in vertebrates (*Homo sapiens*, *Mus musculus*, and *Danio rerio*) (Lin et al., 2012). Furthermore, advances in mathematical modeling tools, such as elementary flux modes, can help in defining and analyzing modules (Conradi et al., 2007; Kaltenbach and Stelling, 2012).

Prior approaches to characterize module constituents use structural biology in combination with high-throughput protein-interaction network analyses, like TAP-tag (Aloy et al., 2004), to determine protein complexes that could constitute functional modules. However, transient and weak complexes, which often occur in signaling processes involving interactions (for example, with phospho-peptide/SH2 domains), are often lost during the purification process. Lately, great advances in combining crosslinking with TAP purification and mass spectrometry open the possibility now to specifically target transient complexes (Leitner et al., 2012). 3D structures of protein/domain complexes or of homologs can inform which proteins/domains interact in a compatible (''AND''), and which ones in a mutually exclusive (''XOR''), manner (Kim et al., 2006). Adding ''XOR''/''AND'' structural information into signaling networks is important because it helps to identify competing interactions; for example, we found that competitors may dynamically connect to different modules in rhodopsin signaling networks (Kiel et al., 2011). Another important aspect of signaling networks and modularity is that complexes and modules can change under different conditions (Ideker and Krogan, 2012). Thus, complexes should be ideally measured under different conditions, as was done in a recent quantitative approach of the Grb2 complexes by Bisson et al. (2011). Interestingly, whereas some of the partner proteins associated with Grb2—as expected—during growth factor stimulation, others, such as dynamin family members, were permanently associated with Grb2. In addition, subsequent changes in the concentration of one component involved in a competing upstream protein interaction could result in activation of different pathways (Kiel et al., 2011). This, together with cell-type-specific protein abundance information, could help to discriminate between modules that occur in all cell types and those that are cell and tissue specific (Sharan et al., 2005).

Posttranslational modifications (PTMs) provide another layer of complexity in signaling networks (Nussinov et al., 2012). Keeping in mind that more than five PTMs are found on average per protein (as experimentally identified; UniProt Consortium, 2009) and that PTMs are recognized by specific domains, it is clear that different combinations of PTMs could lead to assemblies of different complexes. One of the key challenges here is understanding the geometrical fitting of several partner proteins binding to the same central node through several PTMs, i.e., to identify how different shapes fit together (Nussinov et al., 2012). In a pioneering study, geometrical fittings at PTMs were exemplified at the EGFR, for which a 3D structural model was generated for binding of four SH2/PID-containing partner proteins, namely Grb2, PLCg1, Stat5, and Shc (Hsieh et al., 2010). Subsequently, agent-based modeling was used to evaluate the effects of reaction kinetics, steric constraints, and receptor clustering. An interesting conclusion from this theoretical study was that simultaneous docking of multiple proteins is highly dependent on the stability between receptor and partner proteins. Although this might initially seem to add an additional layer of complexity, it could help to define the (possible) functional assemblies and modules in the long term. Thus, 3D modeling and docking combined with cell-type-specific

identification of PTMs could provide new clues in functional assemblies of proteins containing multiple PTMs.

The best proof of a proposed module is if function is preserved when analyzed in isolation, such as in synthetic biology design approaches in orthogonal systems (Collins, 2012). Analyzing signaling modules and reengineered modules in artificial environments also has great value because often mechanistic aspects, such as the role of protein level perturbation on signal propagation, can be studied in isolation (O'Shaughnessy and Sarkar, 2012). For example, synthetic biology approaches, led by pioneering work from the W. Lim laboratory, had an important contribution to unraveling the importance of scaffold interactions for achieving signaling specificity (Good et al., 2011). In recent work, W. Lim and colleagues systematically analyzed to which extent kinase components can be rewired to functionally signal to distinct combinations of its natural interaction partners (Won et al., 2011). Interestingly, their work on four MAPK pathways in the yeast *Saccharomyces cerevisiae* suggested that protein recruitment interactions have a larger impact for achieving specificity than the catalytic specificity. In another fundamental study, it was shown that the Ste5 scaffold proteins (also in *S. cerevisiae*) not only serve as an assembly platform to guide information flow but also derive input signals and act as conformational switches to passage information flow between two distinct output responses (Zalatan et al., 2012).

Based on defined modules, advances in mathematical tools can contribute to the automatic design for experimentally implementing synthetic (signaling) circuits (Marchisio and Stelling, 2011). Once synthetic circuits are implemented into orthogonal systems, as outlined above, several system properties can be analyzed. Robustness (i.e., stability/persistence), despite perturbations, such as environmental changes or intracellular noise, is a key property of cellular systems (Stelling et al., 2004). Robust behavior is achieved through backup systems, feedback control, hierarchies, and modularity (Kitano, 2002; Stelling et al., 2004). To analyze robust points in a signaling module, protein level perturbations can be performed. Furthermore, based on structural information, subtle mutations can be designed to gain insight into network and feedback properties of the module. Subtle mutations in protein complexes could be those, for example, that retain a similar affinity (K_D) but have compensating changes in association (k_{on}) and dissociation (k_{off}) rate constants ($K_D = k_{off}/k_{on}$). Association rate constants can be increased by increasing the electrostatic charge complementarity at the edge of the interface (long-range interactions, "electrostatic steering") (Selzer et al., 2000). Dissociation rate constants can be increased by mutating amino acid residues in the interface (short-range interactions). These mutants could be used to test if a network is under kinetic control (Kiel and Serrano, 2009). However, this type of experimental parameter sensitivity analysis needs some prior knowledge of the network because, for example, strong negative feedback effects will be dampened if the modified interaction is located inside (Kiel and Serrano, 2009). Recently, an automatic web tool based on a program for inducing disturbances into protein interaction networks was developed (NEXCADE), which calculates the changes in global network topology and connectivity as a function of the perturbation (Yadav and Babu, 2012).

In addition to module identification, another key issue is to define the robust and sensitive parts in a module (Beisser et al., 2012). Synthetic signaling devices, ideally implemented in orthogonal systems, are ideal for this kind of analysis (O'Shaughnessy and Sarkar, 2012). The interesting question is whether the robust and sensitive parts relate to natural protein level variation. If there is a relation (e.g., conserved modules show low-protein level variation between tissues and cell lines), one would not need to test all signaling modules experimentally. Rather, one could focus on analyzing the effect of the varying parts on network behavior and on crosstalk between modules. For example, in a recent elegant approach, an experimental network-perturbation approach was used to investigate crosstalk between signaling modules during the neutrophil polarization process (Ku et al., 2012). This work was interesting with respect to a surprisingly simple circuit that influenced and affected all crosstalk and signaling module interactions during the polarization response. This suggests the possibility that a few key (perhaps cell-type-specific) proteins could control several modules and their crosstalk. Thus, with a common basic conserved module in all cell types, the present cell-type-specific proteins can rewire and influence different modules, which could explain how a large repertoire of different signaling responses can be achieved using the same core MAPK module.

As outlined above, defining modules is a first step for designing synthetic signaling systems to be analyzed in orthogonal systems (Figure 2). To understand cell-type-specific signaling, one would ideally analyze the ~200 human cell types using quantitative proteomics to define all common modules and variable elements connecting or affecting them in each cell. Mass spectrometry is now ready to achieve this, and the latest work described the identification of about 10,000 proteins in 11 different cell lines (Geiger et al., 2012). Thus, this approach is technically feasible. The main problem is to avoid using transformed cell lines, which often have their peculiarities and do not always resemble cells in their natural environment in the organism; thus, and instead, use material from primary cells and tissues (e.g., mice). Altogether, to define and investigate modules, a multitude of information about structure, protein abundance, analysis of competition, protein evolution, and mathematical analyses should be considered.

Is There Potential for Synergy between Structural and Synthetic Biology?

As outlined above, 3D structural information can help in designing and analyzing biological systems. At first glance, it is less obvious how synthetic biology efforts can be used to tackle challenging structures. Nonetheless, this has been achieved recently, using a system in which a eukaryotic signaling pathway was reconstituted in bacteria that specifically generated ubiquitylated eukaryotic proteins (Keren-Kaplan et al., 2012). Ubiquitylated complexes were subsequently purified in quantities suitable for crystallographic analysis and biophysical characterization. In this case, reconstitution in a bacterial system was crucial to tackle a specific conformation, which otherwise would have been impossible to capture due to the rapid dynamics of ubiquitylation/deubiquitylation in eukaryotic cells. However, application of this system on a systemic level for other

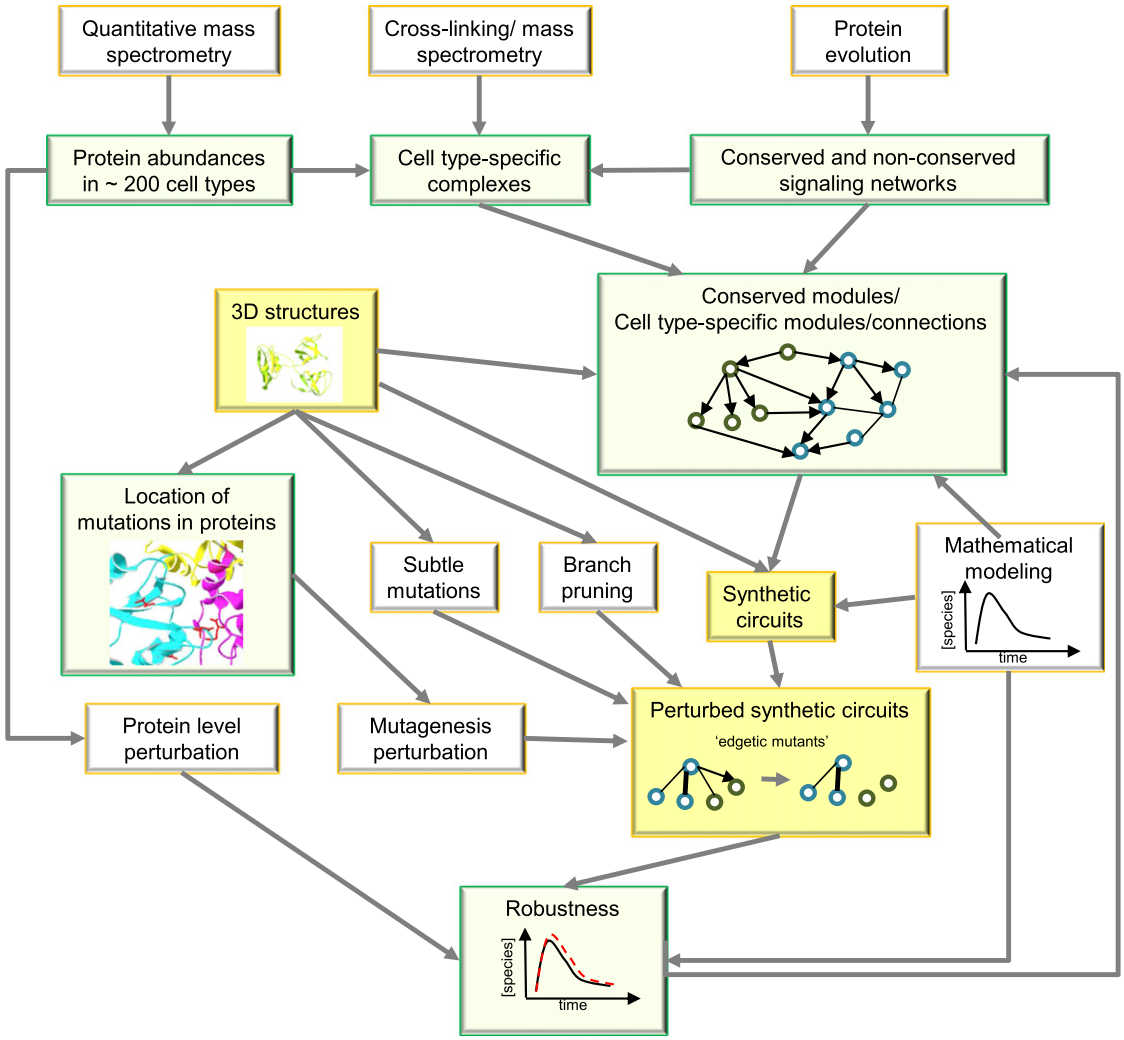

Figure 2. Proposed Flow Chart of Combining Different Scientific Disciplines to Study Cellular Design Principles and Cell-Type-Specific Signaling

Structural information is a key component in proposing network modules, predicting the localization and effect of disease mutations, and designing perturbed protein complexes to be used in synthetic biology approaches.

pathways will face several challenges. For example, one of the reasons that this design was successful is that the ubiquitylation cascade works in a modular fashion. However, other signaling pathways, such as MAPK signaling, usually operate with a high level of crosstalk as well as feedback regulation. Furthermore, the expression of larger proteins, such as kinases and membrane proteins, will be difficult to achieve in sufficient amounts suitable for crystallization. In these cases, alternative host platforms need to be considered. For instance, in recent approaches, the mammalian MAPK pathway was reconstituted into yeast cells to study signal processing (O'Shaughnessy et al., 2011), and human p53-Mdm2 interactions were studied in yeast (Di Ventura et al., 2008).

A requirement for crystallization is the availability of a large amount of purified protein or protein complexes. In a recent synthetic biology approach, the fraction of the soluble/insoluble protein was increased using *E. coli* as the host organism for recombinant protein production (Dragosits et al., 2012). This was achieved by a self-regulatory mechanism, which coupled a stress-induced promoter with a negative feedback to down-regulate protein expression. Thus, this represents another example of how synthetic biology approaches can help in protein expression efficiency and thereby tackling challenging structures.

Setting the Basis for Understanding the Molecular Mechanisms of Diseases?

Experimentally analyzing and predicting the effect of disease mutations are key challenges for the future. It is well known that mutations in one protein that is expressed in many tissues cause a disease only in some of them (i.e., dystrophin in brain and muscle) (Lage et al., 2008). The reasons behind this are related to the environment of the cell, the network of interactions within it, and splice variants and relative protein concentrations

that are specific to every cell type. Having detailed structural reconstruction of interacting networks allows the mutant to be localized in the 3D structure of the protein. This in turn makes it possible to distinguish between folding mutants that will kill all interactions or mutants that will affect enzyme activity or perturb one or more interactions ("edgetic mutation") (Zhong et al., 2009; Wang et al., 2012). Here, structural information available for proteins in the Protein Data Bank and of complexes (3DID database) and using larger-scale docking and proteome scale structural modeling approaches (Kuzu et al., 2012) are of great importance. Once the structural effect is analyzed, information about the concentration of the partners and spliced variants could be integrated into signaling modules and in different cell types, which could provide some ideas about the functional cell specificity of the mutation. In parallel, the (predicted) effect of disease mutations could be experimentally analyzed either in orthogonal synthetic biology devices (modules), or in different cell types, by stably expressing the mutant protein at endogenous levels and performing pull-down experiments. The exciting challenge will be to capture transient interactors, possibly through the use of crosslinking approaches during the purification process (Leitner et al., 2012).

Conclusions

The last 20 years have seen great achievements in unraveling diverse general design principles of biological systems using different systems biology, synthetic biology, and genome-wide approaches. However, it has lately become more and more obvious that cell-type-specific properties and variation between individuals need to be taken into account to fully understand how living systems operate. Now, improvements in mass spectrometry- and antibody-based proteomics have reached a technical level necessary to measure protein abundance in different cell types and tissues. Nonetheless, for predictive models, we need a reduction of complexity, with the challenge of finding the right balance between reductionism and essential details. The growing amount of information from structures can help in defining modules. There is also increasing evidence that modules contain conserved proteins and that these are connected through less-conserved proteins; thus, we also need to integrate evolutionary aspects into the picture.

The time is now right for the different disciplines—structural biology, quantitative biochemistry and proteomics, evolution, synthetic biology, mathematical modeling, and the network and signaling fields—to join forces to tackle ambitious projects. For instance, similar to mapping protein interactions on a large scale in different organisms, one could move to quantitatively analyzing the ~200 different cell types in the human body. Cutting-edge approaches, such as combining pull-down experiments with crosslinking (to capture transient interactions) and mass spectrometry, will be of great value (Herzog et al., 2012). To understand how different cell types operate is of fundamental interest for basic science and has great potential for unraveling molecular disease mechanisms.

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