Biochemical and Biophysical Research Communications 445 (2014) 739–745

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Review

Analyzing protein–protein interactions in the post-interactomic era. Are we ready for the endgame?

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ARTICLE INFO

Article history:
Available online 15 February 2014

Keywords:
Protein interaction
Constraint interaction networks
SPLIFF
Split-Ubiquitin

ABSTRACT

Mapping protein–protein interactions in genome-wide scales revealed thousands of novel binding partners in each of the explored model organisms. Organizing these hits in comprehensive ways is becoming increasingly important for systems biology approaches to understand complex cellular processes and diseases. However, proteome wide interaction techniques and their resulting global networks are not revealing the topologies of networks that are truly operating in the cell. In this short review I will discuss which prerequisites have to be fulfilled and which experimental methods might be practicable to translate primary protein interaction data into network presentations that help in understanding cellular processes.

Contents

1. Introduction

Protein interactomics started with the publication by Uetz et al. reporting on a massive identification of novel protein–protein interactions by a systematic application of the two-hybrid system in the yeast *Saccharomyces cerevisiae* [1]. The publication was accompanied by a graphic visualization of these interactions where proteins were shown as dots and the interaction between them as edges [2]. As many dots were targeted by more than one edge the display of all interactions resulted in a very complex network. Besides listing all binary interactions this and the following protein interaction networks of the yeast proteome allowed some global statements about the general organization of proteins and their interactions but often eluded any further biologically meaningful interpretations [3–7]. Already the central element of these networks, the edge, left much room for interpretation. Due to the technical limitations of genome-wide applicable interaction techniques, it was impossible to distinguish whether edges represented direct or indirect interactions. An indirect interaction implies the existence of a third, unknown protein that divides the original edge into two. Furthermore, depending on the thermodynamic and kinetic properties of the interaction, an edge can represent a stable

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http://dx.doi.org/10.1016/j.bbrc.2014.02.023
0006-291X/

or a fleeting association, an association that is constitutive or regulated. Presentations that ignore these aspects of protein–protein interactions fall short to reveal the biologically relevant and meaningful topologies of these networks. Unraveling these topologies will however be an essential requirement to recognize recurring motifs within these networks, to associate interaction with function and to draw the flow of information through these networks.

Many recent approaches are based on computational tools and modeling to structure large protein interaction networks [8–11]. The complexity of the task seems to exclude any significant contribution of experimental work to reveal the relevant structural features of these networks. This short review introduces several experimental approaches to analyze the changing topologies of small-scale protein interaction networks and emphasizes their significance for understanding these and also large-scale networks. I will confine the discussion on recent examples from our own work. The discussed interactions were all discovered by the Split-Ubiquitin assay in the yeast *S. cerevisiae* (from here on yeast). As the meaning of "interaction" critically depends on the technique used for its identification, I will first give a short introduction into this method.

2. The Split-Ubiquitin technique

The Split-Ubiquitin (Split-Ub) system is based on the reassociation of an artificially fragmented Ubiquitin. When coupled to proteins that interact their binding to each other will enhance the reassociation between the N-terminal (N_{ub}) and the C-terminal (C_{ub}) fragment of Ub to its native-like structure and consequently will stimulate the release of a reporter protein from the C-terminus of the C_{ub} [12,13]. The altered activity of the cleaved reporter signals the interaction between the N_{ub}- and C_{ub}-coupled proteins [6,14]. The Split-Ub system like all other subsequently developed split-protein sensors is able to detect direct as well as indirect interactions of proteins. Due to the rapid refolding of the fragmented Ub, the system is also capable of detecting transient and weak interactions [15]. Very similar to the genome-wide yeast two hybrid screens mating based Split-Ubiquitin and other split-protein sensor systems allow for large scale and genome-wide investigations of protein–protein interactions [6,14,16]. The traditional output for the interaction of this and most other split-protein sensors is the ability of the cells expressing a pair of interacting proteins to grow on a specifically designed medium [17,18]. If the proteins are screened in their original host for example yeast proteins in yeast, the found hits do not necessarily implicate a direct interaction. This feature makes the obtained interaction network ambiguous but information rich. As with most other high through put interaction technologies the yes/no output ignores spatial and temporal aspects of the measured interactions.

3. Two simple but effective strategies to deconvolute interaction networks

Fig. 1 highlights the shortcomings of the traditional presentation of a small interaction network that was recently established through a systematic Split-Ub interaction screen [14]. Here the analysis linked the phosphatase Ptc1p with the SH3 domain-containing protein Nbp2p, and both proteins with the histone chaperone Nap1p, and the protein kinases Bck1p and Cla4p (Fig. 1A). For clarity only a subset of all discovered interaction partners are shown [14]. It is important to realize that this presentation is a superposition of many possible configurations that could explain the measured interactions. The true topology of the organization of this ensemble of proteins is still hidden among these alternative network configurations (Fig. 1B and C).

Two simple experimental approaches can be applied to exclude already many of these potential interaction states. An interaction state is defined as a collection of proteins that, depending on the state of knowledge, are able to associate with each other at the same time. Measuring the interactions between two proteins (A and B) in the absence of a third protein (C) of this ensemble will allow to distinguish between direct and most probably indirect interactions. If the interaction between A and B is lost upon deletion of C one can in a first approximation conclude that the interaction between A and B is mediated by C. Applying this reasoning to the Ptc1p/Nbp2p-ensemble revealed that the interaction between Ptc1p and Cla4p, Nap1p as well as Bck1p are mediated by Nbp2p (Fig. 1D) [14]. The resulting motif in Fig. 1D now reflects this knowledge. The motif does not distinguish whether certain of the edges might be able to coexist in the same interaction state

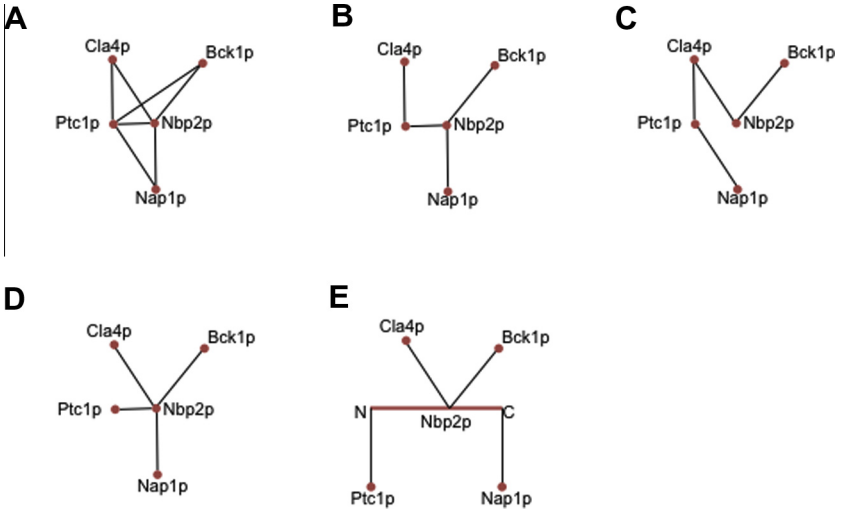

Fig. 1. (A) A graphical presentation of a subset of interaction hits discovered by a Split-Ub based screen for binding partners of Ptc1p and Nbp2p. (B and C) Possible manifestations of the found interactions. (D) Measuring protein interactions in the absence of a certain member of the ensemble leads to a first constraint for the possible interaction states. (E) Fragmentation of Nbp2p and measuring the interaction of its fragments leads to further constraints that are better reflected by presenting Nbp2p as a horizontal line. The line is drawn in red to better distinguish it from the edges. Edges that converge on one node or on one site of a horizontal line might indicate competition between the respective binding partners. Edges contacting horizontal lines at different points can coexist. When known the order of the edges reflects the order of the binding sites within this protein.

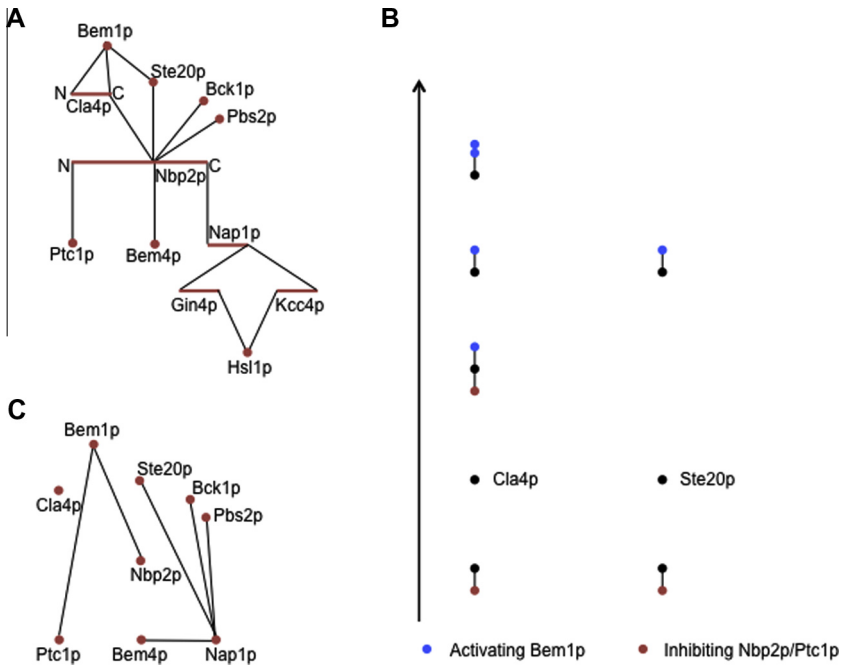

Fig. 2. (A) Constraint interaction network of the Nbp2p/Ptc1p ensemble of proteins. (B) The constraints in (A) result in five different interaction states for the kinase Cla4p. Each state is associated with a different number of activators or inhibitor that influence the central kinase activity. Different constraints lead to only three interaction states with different activating potential for the kinase Ste20p. (C) A "negative" image of the interaction network shown in (A). Edges indicate indirect interactions that were not experimentally verified. Only the indirect interaction to the spatially closest member of a connected group of proteins is indicated by an edge.

or exclude each other to form individual interaction states. The observation that no interactions were detectable among the proteins Cla4p and Bck1p, or Nap1p seem to imply that their binding to Nbp2p cannot occur at the same time. Alternatively, the interactions might exist but are missed due to technical limitations.

The question is more directly addressed by the second experimental approach. Here the protein that is known to mediate the interaction between the different binding partners is fragmented and the different pieces of the protein are tested for interactions with the original binding partners of the full-length protein. Cutting Nbp2p into three pieces and measuring their interactions revealed three different binding sites on Nbp2p (Fig. 1E). Ptc1p binds to the N-terminal fragment, Bck1p and Cla4p to the central SH3 domain and Nap1p to the C-terminal fragment of Nbp2p [14]. A mutation in the SH3 domain of Nbp2p that disrupts the interaction with its classical PXXP ligands also interfered with the binding to Cla4p and Bck1p. It can be concluded that Bck1p and Cla4p compete for the same binding site and are therefore exclusive ligands of Nbp2p.

The information obtained from both approaches can no longer be accommodated by the traditional dot/edge presentation. Proteins that are shown to have more than one binding sites are better represented by horizontal lines. Interactions from its different binding partners are placed on either different positions on this line or, when known to bind to the same site, on a common position (Fig. 1E). By adding new proteins, Fig. 2A shows a more expanded version of the Nbp2p/Ptc1p interaction state [14].

4. Insights from constraint interaction networks

The additional insights about the workings of the network should justify the extra work of both experimental approaches. I have listed four immediate advantages of the constraint interaction network:

(1) The new presentation reveals the basic function of Nbp2p in connecting the phosphatase Ptc1p with different kinases to down regulate their activities (Fig. 2A).
(2) The kinases Bck1p, Ste20p, Pbs2p, Cla4p and Bem4p, a protein of unknown function, might compete for binding to Nbp2p/Ptc1p (Fig. 2A).
(3) The kinases Kcc4p, Gin4p and Hsl1p are attached through Nap1p on a separate site of Nbp2p. Nbp2p might act as scaffold to foster a crosstalk between the kinases binding to the SH3 domain and the kinases attached to the C-terminus of Nbp2p (Fig. 2A).
(4) The homologous kinases Cla4p and Ste20p are linked to the SH3 domains of Bem1p and Nbp2p in slightly different ways. An additional, Bem1p-specific PXXP-motif allows Cla4p to simultaneously bind Bem1p and Nbp2p, whereas Ste20p can only bind either of them at the same time (Fig. 2A) [14,19]. Cla4p and Ste20p are activated by the GTP-bound form of Cdc42p. Bem1p is a scaffold protein involved in the production of $Cdc42_{GTP}$ [20]. The direct binding to Bem1p is thought to be necessary to fully activate both kinases [20]. The binding motif identified for Ste20p might thus function as a switch between two interaction states that either fully activate (bound to Bem1p) or inhibit (bound to Nbp2p/Ptc1p) the Ste20p-controlled signaling cascade (Fig. 2B). The motif found for Cla4p can realize at least five interaction states that could allow for a more graded transition between full activation and complete repression: Cla4p is either fully activated (only bound to Bem1p), simultaneously linked to Nbp2/Ptc1p and Bem1p or fully repressed (only bound to Nbp2p/Ptc1p) (Fig. 2B).

The presentation in Fig. 2A is still problematic and has a severe shortcoming in not clearly distinguishing between proven indirect interactions and possible but not experimentally verified interactions. For example, the Split-Ub assay monitored a link between Ptc1p and Gin4p but not between Gin4p and Bck1p, or Gin4p

and Cla4p. The presentation makes no difference between these edges. At the same time, ruling out that a certain interactions might occur is very difficult. A slightly cumbersome solution for this dilemma is to present an additional negative image of the interaction network where only indirect interactions that were not experimentally proven are shown as edges (Fig. 2C).

5. Competition between edges

The presentation of the network in Figs. 1E and 2A prompts the question whether competition among the different SH3 ligands occurs in the cell. A logical prerequisite for competition is that the number of binding sites provided by the central SH3-domain of Nbp2p is limiting [21]. This condition seems to apply to the Nbp2p/Ptc1p interaction network as the sum of the ligands for the SH3 domain outnumbers the Nbp2p molecules in the cell by a factor of 10 [22]. Likewise, the sum of Bem1p and Nbp2p molecules per cell also exceeds the cellular concentration of Cla4p [22]. Whether and how competition occurs in the cell and which kinases are mostly affected by it depends on the affinities of their PXXP motifs to the SH3-domain of Nbp2p as well as the individual local concentrations of Ptc1p and Nbp2p towards each of their binding partners. Furthermore equating the measured concentration of a protein with the concentrations of its relevant binding sites might be misleading as these binding sites could be occupied by known or unknown ligands that very much reduce their free concentrations. All these parameters are technically very difficult to measure or to calculate. It might be thus more straightforward and certainly desirable to directly study the interaction between two proteins in the living cell to find out whether their interaction might be influenced by the presence or absence of any other third party [15,23].

6. What drives the transition between interaction states?

The Nbp2p/Ptc1p ensemble of proteins will probably never reach a stable equilibrium that applies for the complete cytosol. It is more likely that the distribution between its different interaction states is very dynamic and changes over time and cytosolic space. One obvious force is the unequal synthesis of components of the network that will displace certain competitive members from the network hub and thus disturb the equilibrium between the different interaction states. Secondly, posttranslational modifications are well known to enforce or disrupt protein complexes and thus are able to favor the prevalence of a certain interaction state. Furthermore, a sudden increase in the local concentrations of at least two members of the ensemble might change the composition of the dominant interaction state at this position. During the establishment of cell polarity both Bem1p and Cla4p are independently recruited to a membrane patch below the site of new cell growth [20]. This co-localization will increase the local concentrations of both proteins and thus will drive the ensemble to the interaction state where Cla4p is exclusively linked to Bem1p and thus fully activated (Fig. 2B). The energy to drive this transformation would be derived from the GDP/GTP exchange and GTP-hydrolysis of $Cdc42_{GTP}$. $Cdc42_{GTP}$ is the major determinant for localizing Bem1p and Cla4p to sites of polar growth [20]. It follows that the increased production of $Cdc42_{GTP}$ could indirectly influence the topology of the Nbp2p/Ptc1p protein ensemble through affecting the cellular distribution and increasing the local concentration of two members of this ensemble.

This example emphasizes the need to measure the interactions between its critical members in living cells as only this might ensure that all important influences on this network will be considered including those that are too difficult to foresee let alone to calculate.

7. How to characterize and display the dynamics of interaction networks?

It is often stressed that protein interactions are dynamic. It is thus considered a major shortcoming that the fast majority of known protein interactions were sampled by techniques that do not reflect their dynamics.

A superficial glance at the Nbp2p/Ptc1p constraint interaction network already hints at its dynamic features. Certain interaction can only be realized by replacing alternative interaction states (Fig. 3A). The shown constraint interaction network is thus a projection of different interaction states that might come into existence at different places during different phases of the cell cycle (Fig. 3A). The question whether different interaction states are realized at the same time in perhaps different locations or are successive transformations that follow each other during time can only be experimentally answered. Measuring protein interactions in time and space is not yet possible in large-scale formats. Instead robust methods are becoming available to transfer the hits obtained from large-scale screens into time and space dependent interaction profiles of proteins in single cells. Fusing the protein pair of interest to the complementary fragments of autofluorescent proteins and watching the appearance of fluorescence during time allows to restrict the window where and when a certain interaction might occur [24,25]. The method was readily implemented in a wide variety of different cell lines. However, the considerable time lag between the interaction and its visualization limits the resolution of this method. Alternatively, a novel fluorescent reporter configuration (SPLIFF) that is based on the Split-Ub technique enables to follow the formation of a protein complex during the cell cycle of single yeast cells (Fig. 3B and C) [23]. Here a GFP fused to the C-terminal half of Ub is cleaved after the reconstitution of the native like Ub. A second fluorescent protein with different spectral properties (for example m-Cherry) stays attached to the C_{ub} fusion protein. As a consequence the interaction between N_{ub}-X and Y-Cherry-C_{ub}-GFP is reported by the decrease in the ratio of green to red fluorescence (Fig. 3B). By expressing the N_{ub}- and the C_{ub}-fusion in haploid yeast cells of different mating types the interaction between the N_{ub}- and C_{ub}-fusion can be initiated by mixing both cell types and observing the fate of the red and green fluorescence by time-lapse microscopy of the obtained diploids (Fig. 3C). The transfer from a high-throughput-compatible format of the Split-Ub technique to the SPLIFF analysis was successfully demonstrated [23]. However, the necessity to perform time-lapse analysis and its careful quantitative analysis will set limits to the amount of interactions that can be measured by a single lab.

8. Limits in determining the exact composition and structure of interaction networks

The dissection of a further SH3-domain based protein interaction network posed an interesting dilemma. The characterized network consisted of a core of four proteins involved in the cytokinesis of yeast: Three SH3-containing proteins, Hof1p, Cyk3p, and Sho1p and Inn1p, a protein displaying three PXXP acceptor sites being specific for Cyk3p, Hof1p, and Hof1p/Sho1p respectively. Beside its SH3-domain Hof1p displays an additional PXXP acceptor site for the SH3-domain of Sho1p, and Cyk3p possesses an additional PXXP acceptor sites for the SH3-domains of Sho1p and Hof1p. All four proteins interact with each other exclusively through their SH3-domains and their corresponding PXXP acceptor sites [26]. By assuming that the complex consists of one copy of each protein this small constraint interaction network can be realized by seven different configurations of edges connecting the same number of

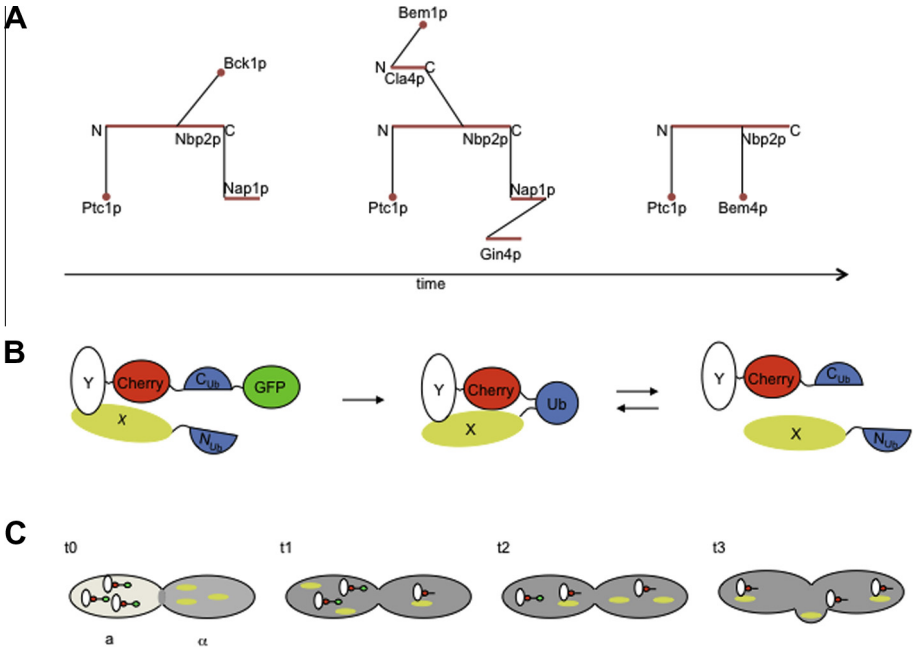

Fig. 3. (A) Hypothetical time line of transformations between different interaction states. (B) The SPLIFF method to measure protein–protein interactions in a time and spatially resolved manner. Protein Y coupled to the Cherry-C_{ub}-GFP (Y-CCG) interacts with the N_{ub}-fusion of protein X (N_{ub}-X). Upon reassociation of N_{ub} and C_{ub}, the GFP is cleaved off and Y-CCG is converted to Y-CC. The N-terminally exposed arginine leads to rapid degradation of GFP. (C) Two yeast cells of the a- and α-mating type expressing Y-CCG (connected red and green circles) and N_{ub}-X (yellow ellipsoid) mate at t0. The cytosols mix, Y-CCG and N_{ub}-X interact, leading to the progressive conversion of Y-CCG to Y-CC at t1, t2 and t3.

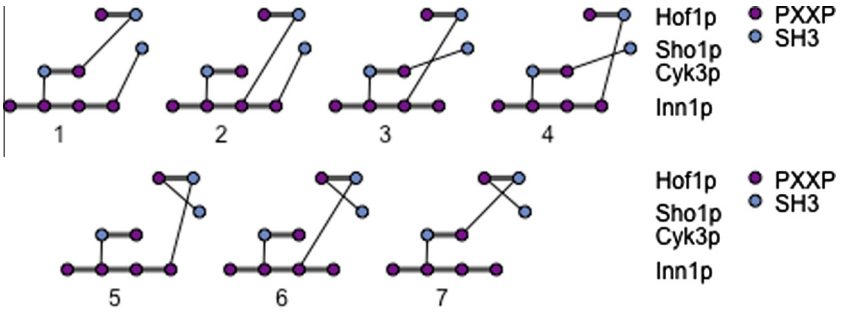

Fig. 4. The seven isomeric interaction states of a protein ensemble where each member is connected through the interaction between at least one SH3-domain and one PXXP-motif. A 1:1:1:1 stoichiometry is assumed. Hof1p contains one SH3 and one PXXP motif. Cyk3p contains one SH3 and ones PXXP motif. Sho1p harbors one SH3 domain, and Inn1p contains 3 relevant PXXP motifs.

PXXP sites with the same number of SH3-domains (Fig. 4). Whether any of these isomeric interaction states is preferred or whether a constant rotation between the isomeric interaction states is an inherent and important property of the complex is neither known nor easy to experimentally address. However this example makes it already very likely that more of these inherent uncertainties of network configurations will be encountered, as more different topologies will be discovered.

9. From structure to function

The yeast type II protein phosphatase Ptc1p and its binding partner Nbp2p both belong to the class of pleiotropic proteins. Loss of either gene leads to a variety of phenotypes including osmo-sensitivity, decreased cell wall integrity, temperature sensitivity, and the delay of organelle inheritance [27–32]. The majority of these phenotypes can now be understood by the links between Ptc1p/Nbp2p and those kinases that are known to be involved in at least one of these processes [14]. Other edges of this interaction network still wait to be functionally annotated. The observed multi-functionality of Nbp2p and Ptc1p correlates with multiple genetic interactions that place both genes as hubs into similar but non-identical genetic interaction networks. [28,33–37]. Using the knowledge of the constraint interaction network of Nbp2p/Ptc1p, alleles of *NBP2* can now be constructed that affect only a subset of its known protein interactions. Probing these alleles for epistasis with the deletions of already known genetic interaction partners of *NBP2* might then point to the specific functions of the disrupted edges.

10. A Lego-approach to build larger networks from bottom-up

It is unlikely that global approaches alone will suffice to structure networks in enough detail that are interesting and informative for cell biologist. Only a combination of computational and experimental approaches will unravel the topologies and the dynamics of protein–protein interaction networks. If we are convinced that this knowledge is important for understanding cellular processes

we face not only the challenge of performing the experiments but also to collect and organize their outcomes as well as the results of those experiments that were already done and documented in the past. A molecular description that strives for completeness should be able to associate different steps in a cellular process with the underlying protein interaction states and their transformations. This will only happen when tools to address these questions become practicable and affordable. The realization that the herein described experimental approaches also work in other split-protein-sensor settings is an important step [38,39]. Once a small network is described in sufficient detail it can be treated as an autonomous module. Identifying common proteins in different modules and analyzing where the individually constructed modules overlap, exclude or complement each other will then lead to the assembly of larger networks from these smaller units. The decision whether to combine these units into larger structures or to keep them apart should follow pragmatic reasons: Does the topology of the larger network helps to better explain the associated processes and does it allow to formulate novel hypotheses about the interplay between them?

Deciphering the code of protein interactions was once optimistically called the endgame of biochemistry [40]. As there a by far too many figures on the board, only a concerted effort might prevent that we lose it.

Acknowledgments

I like to thank present and past members of the lab for their contributions to the development and application of the Split-Ubiquitin technique. The author was supported by a DFG Research Grant (JO 187/5-1), and the BMBF Initiatives SysTec (0315690B), and GerontoSys2 (SyStaR, 03158 94A).

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