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Human protein–protein interaction networks and the value for drug discovery

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Systematic genome-wide and pathway-specific protein–protein interaction screens have generated a putative, organizing framework of the spatial interconnectivity of a large number of human proteins, including numerous therapeutically relevant disease-associated proteins. The intrinsic value for drug discovery is that these physical protein–protein interaction networks may contribute to a mechanistic understanding of the pathophysiology of disease and can aid in the identification and prioritization of tractable targets and generate hypotheses on how to best drug non-tractable, disease-associated targets. Here, we review the 'therapeutic potential' of the 1st generation sub-genome-scale human interaction networks and disease-associated protein networks generated by yeast two-hybrid screens and affinity purification-mass spectrometry approaches.

Introduction

Traditionally, the spotlight in drug discovery has been on a relatively small number of validated therapeutic target classes, such as G-protein coupled receptors and protein kinases, with well characterized enzymatic and cellular activities that are chemically tractable. The identification and validation of novel therapeutic targets for any disease indication is an inherently difficult, time consuming and expensive process. The biopharmaceutical industry has to date not yet exploited and capitalized on the proteome information encoded by the human genome sequence for novel, innovative therapeutic strategies, which has become available in 2001 by the seminal effort of the human genome project. In the post-genomic era the classic paradigm of tractability and, inherent to that, the scope of drug discovery projects is slowly shifting from a protein-centric view towards a more holistic, pathway-centric view [1]. Ensembles of up to 100 distinct proteins that are physically interconnected and functionally act in concert to transduce extrinsic and intrinsic information are viewed as the target modules for therapeutic intervention. The conceptual advantage of this paradigm shift is that it may not only provide for a molecular taxonomy of disease but may also enable rational, mono- or multi-target therapeutics based on biologicals for extracellular targets and small molecules and RNA interference (RNAi) strategies for intracellular targets. Large-scale, genome-wide protein–protein interaction screens have great potential to expand pathway sub-networks, identify novel therapeutic targets and provide the basis for a molecular understanding of the etiology and progression of disease. In this review, we highlight recent advances in methods analyzing protein interactions, with an emphasis on mass spectrometry-based methods, point out some salient features of the 1st generation human protein interaction networks and use some examples of disease protein networks to illustrate the potential value for drug discovery.

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Methods for the identification of protein–protein interactions

A number of experimental methods, based on distinct, physical principles have been developed to identify protein–protein interactions such as the yeast two-hybrid method (Y2H), affinity purification-mass spectrometry (AP-MS) approaches and protein microarrays [2–5]. The primary modus operandi to identify and map binary, physical protein–protein interactions is the Y2H assay. This method is easy to implement, amenable to automation and relatively cheap. Nearly any protein, including most therapeutically relevant target proteins, expressed as *Bait* protein fused to a DNA-binding domain can be screened against the full complement of a proteome, expressed as individual *Prey* proteins fused to a transcriptional activator domain. The Y2H method has been

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GLOSSARY

Interactome a protein–protein interaction network that describes the complement of physical interactions detected by a given method.
Genome-scale all proteins encoded by the genome.
Sub-genome-scale a fraction of proteins encoded by the genome.
Hard-wiring constitutive physical interactions that are easy to detect with methods described.
Soft-wiring transient, induced physical interactions, resulting in protein modification that are typically difficult to detect with methods described.
Orthogonal data integration comparison with different high-throughput datasets.
Node protein connected in a network.
Hub protein with many physical interactions (highly connected).
Centrality-lethality rule describes the notion that deletion of a hub protein is more likely to be lethal than deletion of a non-hub protein.

used to generate global protein–protein interaction networks for the bacterial strain *Helicobacter pylori*, the yeast *Saccharomyces cerevisiae*, the fruit fly *Drosophila melanogaster*, the worm *Caenorhabditis elegans* and recently a significant fraction of the human proteome [6–12].

The second major modus operandi, AP-MS is particularly suited to identify and map multi-protein complexes under near to physiological conditions and is therefore complementary to the Y2H method. AP-MS is based on immuno-affinity purification methods, such as the tandem affinity purification (TAP) or other single affinity tags, in conjunction with mass spectrometric protein identification strategies. Epitope-tagged proteins are transduced in immortalized cells and immuno-purified with reagents specific for the tag. All co-purifying, specific ('interactors') and non-specific ('false positives') proteins are identified by mass spectrometric analysis. Alternatively, specific antibodies can be employed to purify endogenous protein complexes from physiological and pathophysiological conditions. The advantage of AP-MS-based approaches is that they provide real-time *in vivo* snapshots of protein assemblies. AP-MS strategies have been applied to generate protein–protein interaction maps for the bacterial strain *Escherichia coli*, the yeast *S. cerevisiae* and recently a small fraction of the human proteome [13–18].

In addition, protein microarrays are being developed to identify binary protein–protein interactions. The concept is based on high-density immobilization of purified, recombinant proteins onto a surface-coated glass slide, which is then probed with fluorescence-labeled target proteins to detect physical interactions. Although of great future potential, a comprehensive proteome array has thus far only been described for *S. cerevisiae* to detect calmodulin interactors [19]. A comparison of the different methods is provided in Table 1.

In general, large-scale datasets suffer from an intrinsically high rate of false positive identifications, necessitating *a posterior* curation by statistical and bioinformatic methods. Various methods, including iterative clustering algorithms have been used to rigorously assess the validity of pair-wise physical interactions [15]. In addition, integration of orthogonal molecular datasets, including data derived from single deficiency and synthetic lethality screens, co-localization and co-expression analyses and co-occurrence of gene ontology terms have been used to further substantiate the validity of individual physical interactions and general network properties (Figure 1).

One major challenge constitutes the experimental validation of specific interactors or complex components as this is mostly a difficult and time-consuming task. The combination of AP-MS with RNAi-mediated knockdown, albeit a low throughput procedure for now, has the potential to validate directly target protein-specific interactions (Figure 2). This method, which has been dubbed quantitative immunoprecipitation combined with knockdown (QUICK) has been successfully used to identify and validate interaction partners of CBL and β-catenin, two signal transduction

TABLE 1

Comparison of different methods used to identify protein–protein interactions

	Yeast two-hybrid method	AP-MS method	Protein microarray method
Key features			
Scaleabilty/throughput	High throughput	Low/medium throughput	Medium/high throughput[a]
Assay set-up	*In vivo* (yeast)	*In vivo* (e.g. human cells)	*In vitro*
Interactions			
Physical interactions	Binary	Binary, protein complexes	Binary
Identification of PTMs	No	Yes	Indirect
Quantitation	No	Yes (e.g. SILAC, iTRAQ)	Yes
Available interactomes (scale)			
Model organisms	Genome-scale	Genome-scale	Genome-scale
	Yeast, *Drosophila*, *C. elegans*	Yeast	Not published yet
Human	Sub-genome-scale	Sub-genome/pathway-scale	Protein-scale
Drug discovery utility			
Applications	Target identification	Target identification	Target identification
	Mechanism of disease	Mechanism of disease	Mechanism of disease
	Protein-compound interaction[b]	Biomarker discovery (e.g. phospho signatures)	Diagnostics[b]
		Protein-compound interaction[b]	Protein-compound interaction[b]

[a] Dependent on the availability of proteome complement of purified proteins (currently only published for yeast) [19].
[b] Applications not covered by this review.

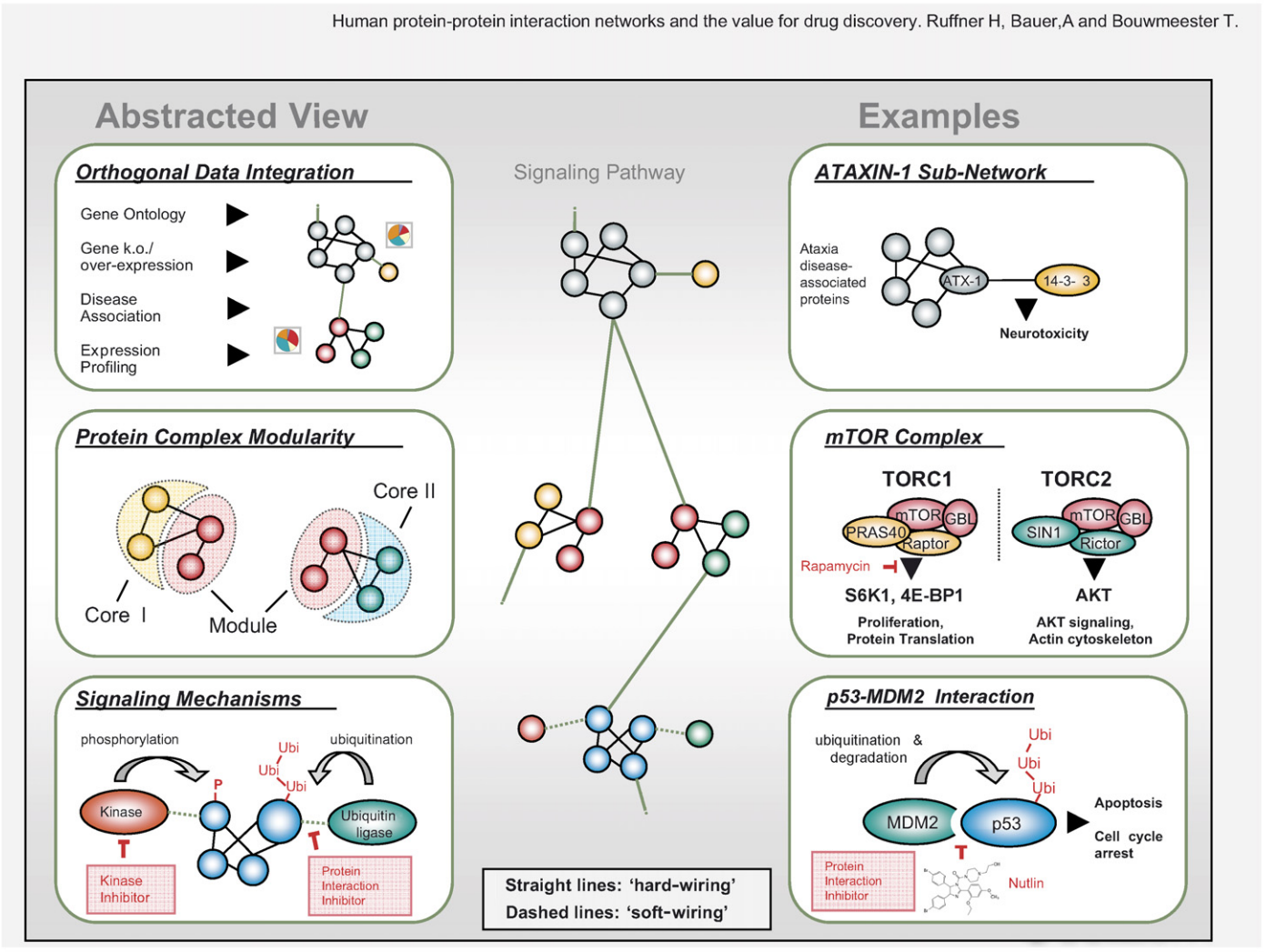

FIGURE 1

Abstracted and exemplary principles of protein–protein interaction networks. A hypothetical canonical signal transduction pathway (therapeutic target module) is shown in the center, with 'hard-wiring' interactions represented by solid lines and 'soft-wiring' interactions indicated by dashed lines. To the left three features of network topology are indicated, (1) orthogonal data integration (see Glossary) for network interpretation, (2) protein complex modularity, whereby modules are shared by distinct cores to multiply their functionality and (3) signaling mechanisms and to the right three case studies, (1) the ATAXIN-1 sub-network, derived from ataxia-associated proteins (2) the mTor complexes for complex modularity and (3) the p53-MDM2 interaction for small molecule inhibition that underscore these distinct network properties. See text for more detail.

nodes (see Glossary) in tyrosine kinase receptor and canonical Wnt pathways, respectively [20]. This method is a powerful asset as more and more validated genome-scale (see Glossary) RNAi reagents have become available [21,22].

Many interactions in cellular signaling networks, however, are subject to dynamic temporal and spatial regulation, which are dependent on signal-induced or context-dependent post-translational modifications (PTM) such as phosphorylation or ubiquitination. The enzymes involved in these processes, protein kinases and E3 ubiquitin ligases constitute major therapeutic target classes. These enzymes typically only interact transiently with substrates and the modifications are often only substoichiometric. PTM-dependent interactions and sites of modification are not covered by the Y2H method and under-sampled by qualitative AP-MS approaches. Conceptually, these functional, transient physical interrelations can be viewed as 'soft-wiring' (see Glossary) interactions, which need to be integrated with the constitutive 'hard-wiring' (see Glossary) physical interaction framework in order to understand the mechanisms of signal transduction akin to an electrical circuit. The identification and quantitation of dynamic, signal-dependent interactions is being facilitated by novel highly sensitive, quantitative AP-MS-based approaches. Stable isotope labeling with amino acids in cell culture (SILAC) or chemical peptide labeling with isobaric tags for relative and absolute quantitation (iTRAQ) can be applied for peptide and protein quantitation from up to four distinct physiological or patho-physiological samples, based on the comparison of differential ion intensities [23]. Phospho-tyrosine antibodies have been used to immuno-purify and quantitate signal-dependent tyrosine phosphorylated protein networks by these methods [24–26].

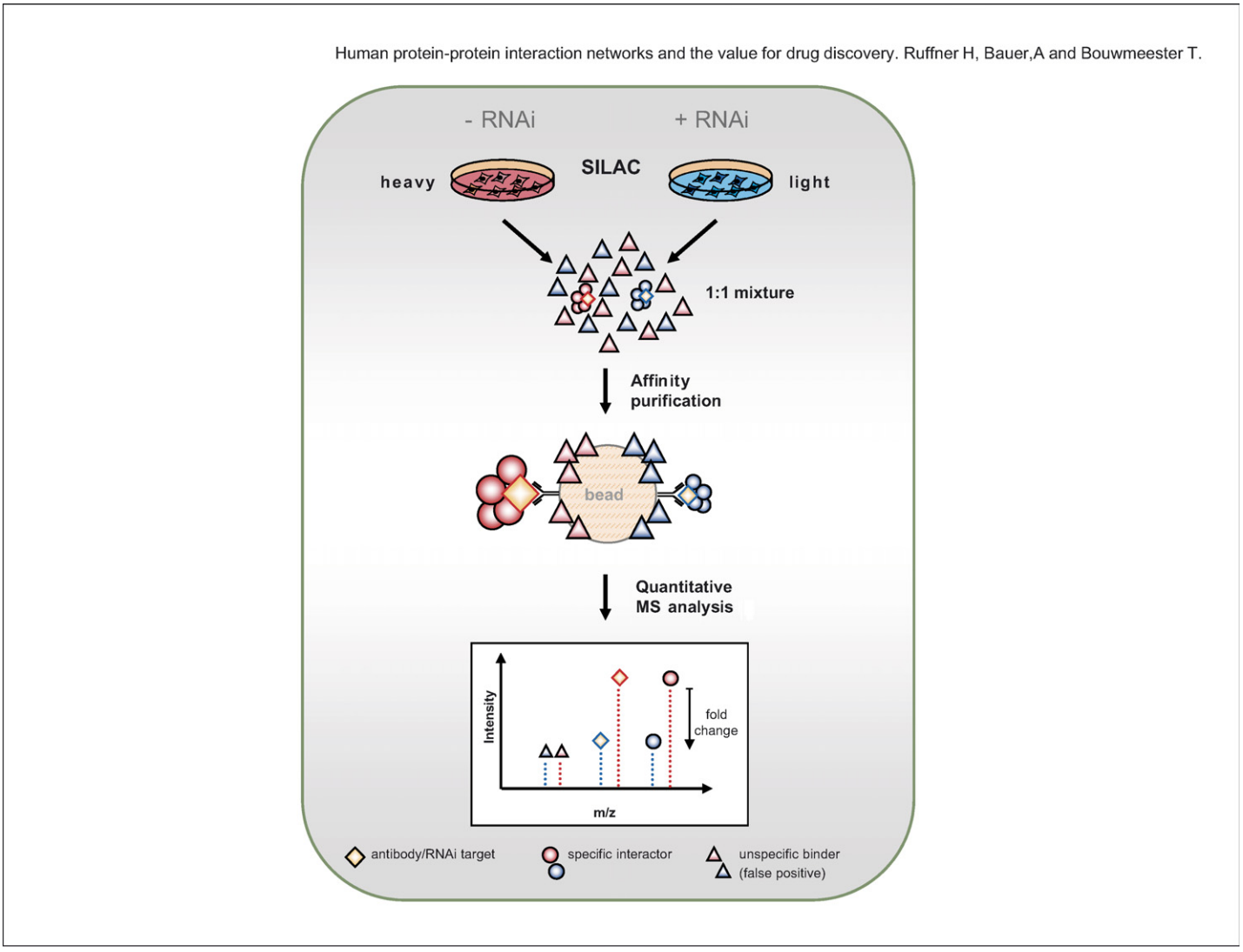

FIGURE 2

Schematic overview of the QUICK AP-MS method. Non-treated (indicated in red) and RNAi-treated (indicated in blue) cells are metabolically labeled with heavy and light isotopes, respectively. Lysates are pooled 1:1, the target protein is immuno-purified and eluates are subjected to liquid chromatography–tandem mass spectrometry (LC–MS/MS). Fold changes can be inferred by comparing ion peak intensities. Specific interactors have the same fold change as the target protein whereas unspecific interactors (false positives) do not change.

General properties of protein–protein interaction networks

The Y2H and AP-MS-based *S. cerevisiae* global protein interaction networks have provided pivotal insights into the macromolecular and spatial arrangement of the expressed cellular proteome and the topology and modularity of multiprotein complexes. These interaction networks are often depicted as one large connectivity network with embedded functional sub-networks. Conceptually these sub-networks can be viewed as the physical organizing framework of signaling pathways. Proteins are depicted as nodes and interactions as edges. The interconnectivity of individual proteins can be inferred from the number of edges. It has to be mentioned though that pair-wise interactions cannot always be inferred from AP-MS-based networks as supposed interactor proteins may have been indirectly purified via accessory proteins.

A key feature of all interaction networks is that they display scale-free properties with hierarchical modularity [27]. Network 'nodes' or 'hubs' (see Glossary) are highly conserved, essential proteins, which tend to be more interconnected than non-essential proteins and are therefore central and of structural importance to the network [28]. This phenomenon is referred to as the centrality-lethality rule (see Glossary). First-pass bioinformatic analysis of the human interaction networks supports the notion that human disease-associated proteins, too, are more interconnected than non-disease proteins [29]. Therefore, human protein interaction networks can be used to drive hypotheses on how to 'drug' non-tractable disease-associated proteins (see p53 example). This potential is likely to incite new initiatives to drug protein–protein interactions in case both disease-relevant proteins are non-tractable by classical chemistry approaches.

The AP-MS approaches have provided fundamental insights in the topology and modularity of multi-protein complexes [15,17]. Complexes are composed of core elements, which are unique protein assemblies composed of up to 23 components, and mod-

TABLE 2

Protein–protein interaction studies and their relevance to drug discovery

Studies	Human protein interaction studies	Relevance to drug discovery	Refs
Proteome-wide interaction screens	Sub-proteome: Y2H AP-MS in human cells	Contextual information, relevance to human disease Hypotheses for disease association *RNT4/SPG21 (Mast syndrome)* *ANP32A, CRMP1 (Wnt pathway)*	[18] [12] [11]
Therapeutically relevant pathways	TNFα pathway: TAP-MS in human cells TGFβ pathway: Y2H; LUMIER in human cells	Pathway connectivity Candidate targets of disease pathway *TRAF7 (TNFα pathway)* *OCLN (TGFβ RI localization)*	 [34] [33]
Disease-based protein networks	'Ataxia-causing' proteins (inherited ataxias): Y2H	Contextual information for disease proteins, phenotype-pathway correlation Key mediators of pathogenesis *Candidate disease genes, modifiers*	[35]
Disease-associated, tractable and non-tractable proteins	*Tractable proteins, example:* mTOR: AP-MS in human cells *Non-tractable proteins, examples:* p53: AP-MS in human cells ATAXIN-1: AP-MS in human cells	Inhibitors of protein–protein interaction *p53: MDM2 – Nutlins* Hypotheses to generate directed intervention approaches *mTOR: SIN1 (MAPKAP1)* *PRAS40 (AKT1S1)* Identification of target candidates *p53: HAUSP* *ATAXIN-1: 14-3-3/AKT*	[40,41] [44,45] [46] [42] [36]

ules, which are protein entities of up to nine polypeptides that can interconnect with distinct cores. The differential interconnectivity of modules with distinct cores may provide for efficient and dynamic means to multiply functionality of the 'core' assemblies and may enable rapid adaptation to different physiological states and possibly contribute to pathophysiological conditions in disease (Figure 1).

In the next section we will illustrate how protein–protein interactions have provided new molecular insight in therapeutically relevant human pathways, disease-associated proteins and non-tractable and tractable targets (Table 2).

Human large-scale protein–protein interaction networks

Two independent sub-genome-scale (see Glossary) Y2H screens have been performed to systematically explore the interconnectivity of human proteins [11,12]. In one study, ~7000 human proteins were screened resulting in an interaction network encompassing ~2800 high confidence interactions including ~300 novel connections for over 100 disease-associated proteins [12]. In the second study, 5500 human protein were screened resulting in an connectivity graph depicting more than 3200 protein interactions connecting ~1700 human proteins including 45 disease nodes that engaged in ~160 interactions [11]. Furthermore, an AP-MS-based approach has recently been performed in human cells to identify interactions for 338 medically relevant target proteins resulting in a network depicting ~6500 interactions between 2235 distinct proteins [18]. These 1st generation human interactomes (see Glossary) have topological similarities to other protein networks with respect to scale-free properties, hierarchical organization and hyper-interconnected hubs, as exemplified by Ewing sarcoma related protein EWSR1, which is connected to 94 proteins [12].

Pathway-based protein interaction networks

Pathways can be viewed as ensembles of functionally distinct proteins that act in a concerted manner to transduce extrinsic and intrinsic information. An outline of the interaction skeleton of most cellular signal transduction pathways has been elucidated by forward genetic screens in model organisms [30]. The TGFβ pathway is such an evolutionarily conserved signal transduction cascade, initiated by ligand–receptor ligation which triggers subsequent intracellular propagation by tiers of physical protein–protein interactions and post-translational modifications among the SMAD family of signal transducers, ultimately resulting in differential transcriptional responses. From a therapeutic perspective, this pathway has been implicated in various disease indications such as fibrosis, osteoporosis and cancer [31].

Both the Y2H approach and luminescence-based mammalian interactome mapping (LUMIER), a novel affinity purification method based on luminescence detection of physical interactions have been used in focused applications to expand the TGFβ interaction network [32,33]. In the Y2H set-up, 11 proteins were screened in a genome-wide manner resulting in an interaction network of 755 protein associations connecting 591 proteins. Functional validation of selected candidate interactors, including siRNA knockdown experiments, demonstrated that eight of the identified novel proteins are implicated in Smad signaling [32]. Independently, the LUMIER method was applied to map signal-dependent interactions following pathway activation by the TGFβ cytokine. Core members of the TGFβ pathway were tagged with the Renilla luciferase enzyme and screened against 518 Flag-tagged cDNA-encoded proteins by transient co-transfection into mammalian cells under different signaling conditions. Cluster analysis and validation of candidate interactors revealed connections to other signaling networks, including those involving the protein OCLN (Occludin), which is associated with the regulation of cell polarity [33].

The classic TAP-MS approach has been used to map interactions in the pro-inflammatory TNFα-induced NF-κB signaling pathway, which has been implicated in chronic diseases, such as rheumatoid arthritis and Crohn's disease. TAP-tagging of 32 known and candidate TNFα/NF-κB transduction components revealed a highly

interconnected sub-network with more than 220 molecular associations including 80 previously unknown interactions [34]. Functional validation using RNAi demonstrated a modulatory role on TNFα signaling for 10 of the newly identified candidates, including a putative, RING-domain containing E3 ubiquitin ligase, TRAF7.

As is the case for most other signal transduction pathways, relay within both the TGFβ and the TNFα/NF-κB signaling cascades is driven by tiers of phosphorylation and ubiquitination. The congenial enzyme pairs that control these reversible modifications in a 'yin-yang type mechanism', kinases and phosphatases for dynamic phosphorylation and E3 ubiquitin ligases and deubiquitinating proteases for dynamic ubiquitination constitute important therapeutic target classes.

Disease-based protein interaction networks

Genome-wide linkage and association studies aim at systematically identifying loci that correlate with human inherited disorders. Inherited cerebellar ataxias are a cluster of diseases with common clinical and pathological features. Many dominant ataxias are caused by intragenic expansion of CAG triplets, resulting in stretches of polyglutamine residues in translated proteins. Despite a large body of evidence suggesting a strong correlation between polyglutamine expansion and neurotoxicity, the molecular mechanism of pathogenesis is still ill-defined. Several unrelated genes have been associated with the etiology of inherited cerebellar ataxias. The Y2H method has been used to identify proteome-wide, physical interactors of ~20 distinct ataxia-associated proteins [35]. Strikingly, most of the ataxia-associated proteins were shown to form an interconnected sub-network, suggesting that these proteins may operate functionally in a concerted manner (Figure 1). Furthermore, human orthologs of genetic modifiers identified in model organisms such as *Drosophila melanogaster* also interact with the principal ataxia-associated proteins. To unravel the molecular mechanism of ataxia pathogenesis, poly-glutamine-expanded, epitope-tagged ATAXIN-1 has been subjected to AP-MS analysis and was shown to interact with the multifunctional signaling adaptor protein 14-3-3 [36]. This interaction is dependent on phosphorylation of serine residue 776 by the protein kinase AKT. Binding of 14-3-3 leads to stabilization of the polyglutamine expanded ATAXIN-1, resulting in neurotoxicity. This observation suggests that AKT conceptually could be considered a therapeutic target for ATAXIN-1-dependent cerebellar ataxia.

The strength of phenotype- or disease-protein networks lies in the propensity to identify key mediators of pathogenesis that could be targeted therapeutically. In-depth molecular analysis of novel disease proteins will result in an understanding of disease-causing mechanisms and create drug discovery opportunities. Such disease-based protein interaction approaches are applicable to many human disorders with common pathological and clinical manifestation. Computational methods have provided support for the notion that disease-causing proteins with similar clinical and pathological hallmarks are more likely to physically interact and form interconnected sub-networks [29,37]. This again illustrates the importance of physically defined signal transduction pathways as the fundamental organizing principles of physiological and pathophysiological processes, which may enable a targeted therapeutic exploitation in the near future.

Interaction networks of non-tractable targets

Many disease-associated proteins are non-tractable to small molecule therapeutics and have therefore posed a conundrum for the drug discovery process. An example is the transcriptional regulator p53, a critical tumor suppressor that lacks enzymatic activity [38]. p53 is mutated in about 50% of human cancers and p53 protein levels are frequently downregulated in the remainder. Both Y2H and AP-MS approaches have been used to identify interaction partners and have shown that p53 is highly interconnected, reflecting its functional centrality. The E3 ubiquitin ligases MDM2 and the paralog MDM4/MDMX have been shown to physically and functionally interact with p53. *In vivo* data revealed that they are negative regulators of p53, MDM2 mainly regulating p53 stability and MDM4 controlling p53 activity [39]. These observations have sparked campaigns to identify small molecule inhibitors of the MDM2-p53 protein interaction surface. One class of small molecules, the Nutlins, specifically inhibit the interaction between MDM2 and p53, leading to stabilization and activation of p53 and apoptosis of cancer cells *in vitro* and *in vivo* [40,41] (Figure 1, Table 2). Various other proteins interact with p53, MDM2 and MDM4 and are likely regulating the stability and activity of these proteins. One important protein is the ubiquitin-specific protease 7 (USP7), which interacts with and deubiquitinates all three proteins, counteracting MDM2-mediated ubiquitination and therefore increasing their stability [42]. Ultimately, the balance between ubiquitination by MDM2 and deubiquitination by USP7 controls protein homeostasis of p53 and therefore MDM2 and USP7 constitute emerging therapeutic targets.

Interaction networks of tractable targets

Drug targets can be party to distinct multiprotein complexes with different physiological functions, as is exemplified by the mammalian target of rapamycin (mTOR). This conserved serine/threonine kinase is part of at least two distinct multiprotein complexes, TORC1 and TORC2. The immunosuppressive activity of rapamycin is believed to be mediated via the inhibition of TORC1 only. Aberrant mTOR signaling has been implicated in a variety of human cancers. Blocking mTOR function by rapamycin or its derivative RAD001 in these tumors ablates cell growth [43]. From a therapeutic perspective, it is conceivable that one may want to target TORC1 independent of TORC2, to increase the therapeutic window for certain indications. A prerequisite for such a targeted approach is to analyze the composition and function of these distinct complexes and the pathways in which they operate. AP-MS approaches have been very informative in dissecting TORC complex composition. For instance, SIN1 (MAPKAP1) has recently been identified as an essential, specific TORC2 component [44,45]. Multiple SIN1 isoforms can be generated by alternative splicing that assemble into at least three distinct TORC2 subcomplexes, adding another layer of modularity and functionality. In a similar manner, PRAS40 (AKT1S1) has been identified as a TORC1-specific complex component [46–48]. Inhibition of TORC1 kinase activity by PRAS40 is relieved upon insulin stimulation, leading to phosphorylation of TORC1 substrates 4E-BP1 and S6K1 (Figure 1). The precise mechanism how TORC1 becomes activated by insulin is unclear, but it may involve AKT-mediated phosphorylation of PRAS40. Together, these observations provide insight into the modularity of mTOR complexes. Common between TORC1 and

TORC2 are mTOR and GβL, suggesting that they have evolved as a module that can combine with distinct accessory proteins such as Raptor and PRAS40 on one hand and Rictor and SIN1 on the other hand to reconstitute TORC1 and TORC2, respectively (Figure 1).

Conclusion and outlook

The Y2H method and AP-MS approaches have matured sufficiently to systematically interrogate the interconnectivity of disease-relevant signal transduction pathways and disease proteins as exemplified by the ataxia sub-network, TGFβ and TNFα/NF-κB pathways and p53. Future incarnations of the AP-MS methods may allow for determining protein interaction dynamics and integrate the identification of post-translational modifications. The latter will not only provide evidence for signal relay but also serve as a basis to functionally assess the extent of pharmacological intervention on pathway nodes/targets, for example substrate phosphorylation mediated by upstream kinases, and to assess global pathway activity under disease conditions. These 'phospho-markers' may allow for patient stratification with regard to appropriate treatment regimen as well as for *in vivo* assessment of the treatment efficacy, for example using phospho-specific antibodies to probe patient tissue sections before and after treatment.

In abstracted terms, protein–protein interaction networks embody a systems-level description of the proteome and will be invaluable for systems biology approaches to model complex biological systems such as pathway modules. Knowledge about protein–protein interactions will enable a better understanding of the physiology of signal transduction at the molecular level and the manifestation of multi-facetted diseases that originate due to disturbances of overlapping pathways. Intervention strategies that specifically target sub-complexes of therapeutic nodes may allow for more effective treatment.

How many pair-wise interactions can we expect in the human interactome? Statistical evaluation of the intersection between the available large-scale Y2H and AP-MS datasets, respectively has been used to predict the dimension of the human interactome [49]. Although preliminary, the analysis predicts in excess of 250 000 interactions in the human interactome indicating that so far only 5–10% of pair-wise interactions have been charted. It has been proposed that multiple coverage or over-sampling is required to complete the human interactome. How many of these pair-wise interactions would have direct therapeutic potential? Although the chemical space is believed to be infinite, the identification of drug-like small molecule modulators of protein–protein interactions has so far occupied only niche status in pharmaceutical companies. With the emergence of some tangible success stories such as the p53-MDM2 interaction modulators, it is tempting to speculate that they could be the 'tipping point' for more targeted approaches to modulate protein–protein interactions in the near future.

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