EXPERT REVIEWS

How do oncoprotein mutations rewire protein–protein interaction networks?

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The acquisition of mutations that activate oncogenes or inactivate tumor suppressors is a primary feature of most cancers. Mutations that directly alter protein sequence and structure drive the development of tumors through aberrant expression and modification of proteins, in many cases directly impacting components of signal transduction pathways and cellular architecture. Cancer-associated mutations may have direct or indirect effects on proteins and their interactions and while the effects of mutations on signaling pathways have been widely studied, how mutations alter underlying protein–protein interaction networks is much less well understood. Systematic mapping of oncoprotein protein interactions using proteomics techniques as well as computational network analyses is revealing how oncoprotein mutations perturb protein–protein interaction networks and drive the cancer phenotype.

KEYWORDS: cancer • mutation • oncoprotein • protein–protein interaction • signaling network

Mapping the molecular networks that govern biological systems is a key goal in systems approaches to biology and medicine [1]. Protein–protein interaction (PPI) networks are of particular interest because of their intrinsically functional nature; interactions between proteins assemble proteins into functional protein complexes or link them into pathways and larger networks, and these higher-order assemblages of proteins are at the heart of most cellular processes. Significant efforts have been made to map PPI networks, using both computational and experimental approaches. Experimental approaches such as yeast two-hybrid and mass-spectrometry proteomics have been applied on a large scale to map thousands of protein–protein interactions [2–4]. In most cases, however, these studies have been conducted under standard laboratory conditions, in which the dynamic nature of PPI networks is not considered [5]. Environmental signals, tissue type and genotype (e.g., mutation status) are some of the important features that determine cellular 'context', and the state and structure of PPI networks. In cancer, somatic mutations are important determinants of tumor phenotype, and although genome sequencing has created extensive catalogs of somatic mutations across diverse tumors, we know very little about the molecular effects of the vast majority of these mutations. Somatic mutations that affect protein function may result in stabilization or destabilization, altered interaction interfaces, and/or alter sub-cellular localization of the mutant protein. Gain or loss of interaction partners is an important consequence of protein mutations, and in turn may result in 'rewiring' of PPI networks, with associated alteration in cellular behavior. For example, although p53 is best known as a tumor suppressor, certain mutations promote p53 oncogenic properties [6]. These gain-of-function effects may be mediated via altered specificity or affinity for interaction partners, as illustrated by a recent study showing how an interaction between mutant p53 and Pontin (*RUVBL1*) promotes the invasion and migration of tumor cells [7]. Similarly, a proteomic comparison of the interaction partners of mutant and wild-type p53 identified several new mutant p53 partners, one of which, nardilysin, was shown to promote invasiveness [8]. Oncoprotein mutations frequently result in constitutive activation of signaling pathways and bypassing of normal regulatory controls. We recently showed how oncogenic variants of the p100α subunit of *PI3K* interact with insulin receptor substrate I (*IRS1*), and

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how mutant β-catenin interacts with, and stabilizes DNA methyltransferase I (Dnmt1); in both cases, the mutant interactions activate downstream signaling pathways [9,10]. In this review, we seek to bring together, and illustrate by examples, what we know about how mutations alter protein interactions and PPI networks with proteomic techniques that may be used to map these altered networks.

PPI network mapping: large-scale techniques

Binary interactions between pairs of proteins of interest have long been studied using the yeast two-hybrid (Y2H) technique [11], and large-scale applications of Y2H have enabled the construction of reference human interaction networks [4]. Applications of Y2H have contributed enormously to our understanding of oncoprotein PPI networks by identifying interaction partners for known oncoproteins [12] or by focusing on systematically mapping PPI networks that define specific cancer-relevant pathways, such as the MAPK pathway [13]. Although Y2H is a powerful tool for PPI analysis, interactions are by necessity identified in yeast cells rather than in a cancer-relevant context, and it cannot therefore provide a view of the complexity of the proteome in an endogenous cellular environment. This drawback has been somewhat mitigated by the development of similar methods in mammalian cells [14], and a recent application of a mammalian two-hybrid assay showed how changes in the interaction partners of wild type and oncogenic variants of epidermal growth factor receptor could be detected [15].

Mass-spectrometry proteomics methods are a highly complementary technique to Y2H for large-scale PPI analysis. Affinity-purification mass-spectrometry (AP-MS) is the method-of-choice for focused analysis of oncoproteins and their interaction partners. In AP-MS, 'bait' proteins of interest are isolated from biological samples using antibodies and then associated 'prey' proteins identified and quantified by mass spectrometry [16]. In principle, antibodies with high affinity and specificity for the bait proteins can be used to affinity purify associated protein complexes under physiological conditions. However, such antibodies are not available for most proteins and generation of high-quality antibodies is time consuming, expensive, and often not successful. More pragmatically, bait proteins are typically epitope-tagged, expressed in cultured human cells, and then associated protein complexes recovered using an antibody against the epitope tag [16]. This approach will be especially important for the analysis of oncoprotein PPIs since antibodies specific to different mutant or variant alleles of a target oncoprotein are typically unavailable. AP-MS has been widely used to analyze PPI networks associated with human disease. We previously used AP-MS to survey protein–protein interactions of 338 epitope-tagged bait proteins in human cells [17]. Most of the selected bait proteins have known disease associations, and 15% of them correspond to known tumor suppressors or oncoproteins in the Tumor Associated Gene database [18]. AP-MS has also been widely used to map PPI networks for specific diseases (for example, identification of the interaction partners for proteins linked to vascular conditions in the brain [19].

Proteomic and transcriptomic analyses of clinical samples (e.g., tumors) has contributed enormously to understanding the complexities of the cancer cell state [20]; however, pinpointing the specific effects of individual oncoprotein mutations in the resulting data is challenging, in part due to the genotypic complexity of the samples in which a multitude of mutations may contribute to the phenotype. To probe the effects of individual mutations on PPI networks, quantitative, phospho- and interaction proteomics techniques have been applied to cell lines expressing specific mutations. For example, proteomic analyses of the network-level effects of the adenomatous polyposis coli (*APC*) tumor suppressor have been performed using an isogenic pair of APC-null and -expressing cell lines [21]. Similarly, an integrated protein expression and phosphoproteomic approach was used to analyze isogenic cells edited with different *KRAS* mutations to reconstruct allele-specific PPI networks [22].

Oncoprotein mutations that constitutively activate signaling often cause modifications of protein stability or abundance in cancer cells. An important experimental consideration for AP-MS experiments therefore is to ensure that bait proteins are regulated and expressed at endogenous levels, and several methods that achieve this have been developed. For example, Bacterial Artificial Chromsome clone recombineering has been used to engineer epitope-tags into the target Open Reading Frame so that flanking regulatory regions are preserved. These clones are then transfected into the desired cells and AP-MS experiments performed [23]. Alternatively, the genome of target cells (e.g., cancer-cell lines or primary cells) may be directly engineered to knock-in epitope tags at loci of interest. To facilitate the identification of oncoprotein mutant-specific interaction partners, we developed a proteomic approach using cancer cell lines with endogenously epitope-tagged oncoproteins [24,25]. The singular advantage of 'knock-in AP-MS' is that specific alleles may be epitope-tagged thus providing a technique for analyzing allelic-specific (e.g., mutant) PPIs. This method has been applied to identify protein phosphatase (*PPP1CC2*) interaction partners in embryonic stem cells [26], and previously by us to identify mutation-specific partners for the p110α subunit of *PIK3CA*. The latter study showed that two mutations with different mechanisms of action and oncogenic properties also interacted differentially with their downstream partner, IRS1 [9]. These types of techniques, that allow endogenous PPI networks to be detected in relevant biological contexts (e.g., in the presence of oncogenic mutations), will become increasingly important as the field of proteomics moves toward understanding how biological context and cellular state impact PPI networks [5].

Re-constructing PPI networks

Assembly of protein–protein interactions into PPI networks is a pre-requisite for understanding the effects of mutations at the network-level, and significant effort has gone into developing tools and methodologies as the number of identified

protein–protein interactions has increased. PPI networks are typically constructed as weighted or unweighted graphs, with nodes representing individual proteins and edges representing the interactions between proteins [27]. Using this formulation, PPI networks can then be analyzed using graph-theoretic concepts such as node degree, which measures the number of edges connected to a node; clustering, which measures the interconnectivity of that node; and betweenness, which measures the centrality, or the number of shortest paths that transect a node [28]. An important question has been to understand how these network features correlate to biological function. For example, nodes with high degree represent hub proteins in the network, and sets of highly connected nodes may represent a functional module or protein complex [29]. Finally, the effects of mutations on PPI networks may be formalized as 'node removal' events, in which a mutation causes the loss of a protein and its associated edges in the network (e.g., a null mutation) or alternatively 'edgetic' mutations, whereby edges are selectively removed from the network, corresponding to mutations that impact specific interaction interfaces but not others [30].

A relatively new development in the field of PPI network analysis is to integrate the increasingly large volumes of protein structural information to create '3D' PPI networks [31,32]. This is particularly relevant for understanding how mutations impact PPI networks, since it places detailed structural descriptions of mutant proteins in their network context. By integrating gene–disease associations and known mutations with 3D PPI networks, it was found that certain classes of disease-associated mutations are enriched on interaction interfaces [33]. Furthermore, mutations on corresponding interaction interfaces of partner proteins were significantly more likely to be linked to the same disease, and mutations of different interfaces of the same protein were less likely to cause the same disease than mutations on the same interface, thus providing molecular explanations for the phenomena of gene pleiotropy and locus heterogeneity [33,34]. A long-standing challenge in constructing PPI networks has been to discriminate low confidence (or false positive) protein–protein interactions from bona fide ones. Homologous, co-crystal, and predicted protein structures, can all add to the confidence of mapped interactions by assessing their actual or predicted solvent accessible areas and therefore the likelihood of interactions between proteins [32,35]. Computational approaches can be used to combine functional and structural data that can be used to classify interactions according to their confidence, and these classifiers have prediction accuracy as high as experimental data in both yeast and human [36]. 3D PPI networks also enhance the understanding of a protein's position and interactions within its environment, facilitating identification of the central multi-domain proteins, commonly occurring interaction pairs and possible unspecific target effects [37,38].

Finally, given the large volumes of protein–protein interaction data, an important component of the protein interaction infrastructure are the databases that collate, curate and enable analysis of the data. These resources typically integrate primary data, literature and data analysis tools, allowing users to query both individual and lists of proteins, for physical, functional, and genetic interactions. Interaction databases such as BioGrid [39], STRING [40], and IntAct [41] all display graphical representations of interactions that can be modified by data source and interaction confidence scores.

How do mutations alter protein interactions & PPI networks?

Mutations that alter protein structure may have consequences at different levels, from the function and structure of the individual protein, knock-on effects on interacting partner proteins, broader global effects through the PPI network, and ultimately phenotypic effects at the cellular level and beyond. Here, we consider some of the important consequences of cancer-associated mutations on proteins and PPI networks. Primary protein sequence may be altered by mutations in diverse ways including substitution, insertion, deletion or truncation. In turn, this can have far-reaching effects on proteins, such as alterations of structural conformation, stability and interactions. Although there is a huge body of knowledge detailing how mutations and variants alter protein structure, much less is known about the PPI network-level consequences of mutations.

Changes in protein stability are an important consequence of mutations by altering the abundance and sub-cellular localization of proteins and their associated interaction partners. β-catenin, the primary effector of Wnt signaling, is a well-studied example of this phenomenon; mutations that alter or delete key serine residues in β-catenin allow the protein to escape phosphorylation and degradation by the destruction complex resulting in stabilization and aberrant accumulation [42]. This promotes β-catenin accumulation in the nucleus, with concomitant activation of Wnt gene-expression programs [12,43–45]. Stabilization also alters β-catenin interaction partners. For example, we found that stabilized mutant β-catenin interacts with DNA methyltransferase I (Dnmt1) in the nucleus of colorectal cancer cells. This interaction promotes the stability of both proteins, is associated with new β-catenin interaction partners including lysine-specific demethylase I, and impacts Wnt signaling activity and DNA methylation activity [10]. Importantly, mutations that constitutively activate or stabilize oncoproteins are likely to have more complex downstream activities that can be explained by simple activation of the associated signaling pathway [46]. For example, analyses of the network-level effects of oncogenic *RAS* mutations have revealed complex positive and negative feedback mechanisms [47].

Specific changes in physicochemical properties of substituted amino acids are an important determinant of the resulting effects. For example, large changes in amino acid charge typically result in protein destabilization, with the opposite being true for small changes [48]. Mutational phenotypes may also be dependent on their destabilizing energy changes, as is the case with RASopathies.

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RASopathies are a group of syndromes that display a wide and varied range of symptoms, occasionally including cancer as a minor component. They are caused by germline mutations in the components of the RAS/MAPK pathway, a pathway that is frequently involved in the development of cancer. There are 15 genes that when found mutated are involved in both RASopathies and cancer independently. Kiel and Serrano [49] found that mutations that were exclusively cancer-associated had a higher energy change and were randomly distributed within the protein sequence, whereas mutations causing RASopathies were clustered in specific structural regions responsible for protein signaling activation. There may not be a simple, linear relationship between the immediate molecular effects of a mutation and the phenotypic or network-level consequences. Through analysis of mutations in yeast, it has been found that mutations that subtly alter binding properties of interacting partners actually may have more serious phenotypic consequences than knock-out mutations [50].

Mutations that result in protein destabilization may not necessarily result in inactive or degraded protein, but rather in a dependence on other proteins for their stability. For example, wild-type B-Raf proteins do not require the stabilizing and folding activity of the chaperone Hsp90, whereas their cancer-associated mutant counterparts do [51]. Indeed, mutant but not wild-type B-Raf, can be completely silenced by targeted inhibition of Hsp90, leading to cell cycle arrest and apoptosis [51]. Protein dimers, such as IDH1, can be stabilized by point mutations resulting in a change of residue accessibility that impairs ligand interaction, locking the dimer in an inactive conformation lacking catalytic activity [52,53]. Mutations may occur in both functional and accessory regions of the protein, potentially directly affecting protein interactions through alteration of the favorability of electrostatic interactions [48]. Interaction site mutations can cause changes, such as hydrophobic destabilization, loss of electrostatic salt bridges, changes in the main-chain protein conformation, and formation of steric clashes [34].

An in-depth understanding of the effects of mutations on protein structure and function requires significant investment of time and effort, and is available for relatively few proteins. High-throughput techniques such as next-generation sequencing of whole genomes are able to identify large numbers of mutations very rapidly. This is nowhere more apparent than in the field of cancer genomics, where sequencing of whole tumor genomes is rapidly identifying thousands of somatic mutations across diverse cancers [54,55]. For the vast majority of these mutations, their effects on protein structure, function and interactions are unknown. Cancer mutations may be classified as 'drivers' or 'passengers' where drivers are those mutations that provide selective growth or other advantage to cancer cells and passengers are neutral mutations that accumulate in cancer cells [56], and several computational methods have been developed to classify mutations according to their functional effects. For example, the CHASM method uses a machine learning method trained on multiple predictive features to specifically identify deleterious missense cancer mutations [57]. CanPredict combines metrics that predict whether non-synonymous amino-acid substitutions are tolerant or intolerant, based on evolutionary conservation with domain-based conservation and gene annotations to create a classifier for identification of deleterious mutations [58]. While these methods aim to identify mutations that alter protein function, they do not explicitly consider the effects of mutations on PPI networks. A recent study focusing on cancer-associated SH2 domain–phosphotyrosine interactions sought to predict the effects of mutations on these interactions as well as their more global effects at the network level [59]. The authors used a statistical mechanics framework to predict the effects of mutations on SH2 domain–phosphotyrosine residues by integrating experimental peptide–domain interaction as well as known PPI networks. Intriguingly, the effect of mutations on SH2–phosphotyrosine interactions was not correlated to the mutation frequency, suggesting that many low-frequency somatic mutations in cancer may be functionally important.

Finally, how network topology relates to biological function is an important part of understanding the effects of mutations on PPI networks. For example, network hubs (highly connected proteins) have specific features in PPI networks, having been shown to be more essential, and with a higher likelihood of driver mutations in cancer [60,61]. Hub proteins may be further defined as 'party' hubs or as 'date' hubs. These categories were originally defined according to whether their interacting partners were co-expressed (party hubs) or incoherently expressed (date hubs) [62], although there has been considerable discussion about whether these categories represent real distinction in biological networks [63]. They are, at the very least, a useful framework for considering the function of proteins in PPI networks, since hubs may also be viewed as either single-interface nodes or multi-interface nodes. Single-interface nodes typically bind transiently to a wide range of partners, while multi-interface nodes are more likely to have high-affinity interaction partners and often are the central members of protein complexes [64].

Expert commentary & Five-year view

Understanding how cancer-associated mutations alter PPI networks requires integrated computational and experimental approaches. Applications of large-scale interaction proteomics have so far principally focused on coverage; identifying the interaction partners for as much of the proteome as possible. While these datasets provide a baseline interactome for thousands of different proteins, they are limited by the biological context in which they are performed. In the next phase of PPI network mapping, we can expect to see more insight into how PPI networks respond dynamically to the cellular state, or how PPI networks vary according to the tissue. This will be important in understanding cancer PPI networks, as the cancer cell state is determined by the cell's genotype (mutation landscape), its immediate environment and most importantly, the interaction between these features. Applications of interaction and quantitative proteomics in relevant cancer model systems will

provide detailed descriptions of cancer PPI networks. Continued developments in quantitative proteomics (accuracy, depth, and coverage) will enhance these networks, and, importantly, provide dynamic views of PPI networks.

Several different types of data will contribute to our growing understanding of mutations and their effects on PPI networks. First, we can anticipate growing repositories of (somatic) mutation information from large-scale analyses of tumors. Although we currently know relatively little about the effects (or not) of these mutations on proteins and protein interaction networks in cancer, we can anticipate that in the next few years, continued development of tools (both computational and experimental) that can better define or predict mutation function will occur. Second, 3D PPI networks, in which structural and PPI network data are integrated will continue to be developed through the acquisition of more high resolution protein structures, and high-quality protein interactions proteomics data. These efforts will bridge the current gap between genomics and functional proteomics and phenotype.

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Key issues

- Field must move toward mapping protein–protein interactions in specific contexts.
- More widespread adoption of techniques for analysis of protein–protein interactions in specific biological contexts such as cancer cells with specific mutations.
- Detailed biological context information should accompany protein–protein interactions in databases with appropriate query tools.
- Development of standardized integration methods for construction of 3D PPI networks.
- Improving the integration of interaction domain mutagenesis and binding coefficient data with 3D PPI networks.
- Better prediction, classification, and understanding of 'driver' and 'passenger' mutations in cancer.

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