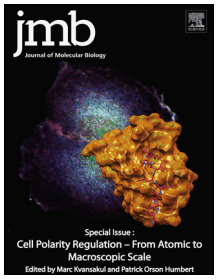
Review



Mapping the Polarity Interactome

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Abstract

Interactions between proteins are an essential part of biology, and the desire to identify these interactions has led to the development of numerous technologies to systematically map protein–protein interactions at a large scale. As in most cellular processes, protein interactions are central to the control of cell polarity, and a full understanding of polarity will require comprehensive knowledge of the protein interactions involved. At its core, cell polarity is established through carefully regulated mutually inhibitory interactions between several groups of cortical proteins. While several interactions have been identified, the dynamics and molecular mechanisms that control these interactions are not well understood. Cell polarity also needs to be integrated with cellular processes including junction formation, cytoskeletal organization, organelle positioning, protein trafficking, and functional specialization of membrane domains. Moreover, polarized cells need to respond to external cues that coordinate polarity at the tissue level. Identifying the protein–protein interactions responsible for integrating polarity with all of these processes remains a major challenge, in part because the mechanisms of polarity control vary in different contexts and with developmental times. Because of their unbiased nature, systematic large-scale protein–protein interaction mapping approaches can be particularly helpful to identify such mechanisms. Here, we discuss methods commonly used to generate proteome-wide interactome maps, with an emphasis on advances in our understanding of cell polarity that have been achieved through application of such methods.

Introduction

Cell polarity—the asymmetric distribution of components and functions in a cell—is a fundamental property of animal cells. In development, cell polarity plays a vital role in generating different cell types through asymmetric cell divisions, and many cell types need to establish functionally distinct domains along an axis of polarity to perform their functions. The archetypal polarized animal cell is the epithelial cell. Epithelial cells polarize to form apical and basolateral domains containing distinct assemblies of proteins and lipids that together enable them to act as selectively permeable barriers.

The establishment of polarity relies on an intricate network of molecular interactions between cortically localized proteins [1–3]. The best understood players in this network are the Partitioning defective (PAR) proteins, which are highly conserved and control polarity in many different cell types, and the Crumbs (CRB) and Scribble (SCRIB) protein modules, which have more restricted functions. Through mutual inhibitory interactions, these cortical polarity regulators become segregated into distinct domains. The PAR (PAR3/PAR6/aPCK) complex and the Crumbs (CRB/PALS1/PATJ) complex together promote apical domain identity, while the Scribble proteins (SCRIB, LGL, DLG), which were identified as tumor suppressors in *Drosophila*, define the basolateral domain [1,3–5]. The establishment of polarity, however, involves many other proteins, including the kinase PAR1, the 14–3–3 protein PAR5, and the Coracle group proteins (Yurt, Coracle, Neurexin IV, NaK-ATPase), as well as at least two types of

membrane lipids: phosphoinositides and glycosphyngolipids [3,6–8]. Moreover, cell polarity must be tightly coordinated and integrated with cellular events such as junction formation, cytoskeletal rearrangements, organelle positioning, organization of a polarized trafficking machinery, and functional specialization of membrane domains [1,9,10]. Finally, cells in polarized tissues often need to coordinate their polarity with cell divisions, with morphogenetic changes, or to align with the higher order structure of the tissue [11–14].

How cells achieve this complex integration is still relatively poorly understood, but a clue comes from the structure of the core cortical polarity regulators. Many of these are made up of multiple protein interaction domains, which enables them to act as protein scaffolds that can assemble different protein complexes (Fig. 1). Indeed, over the years, many links have already been uncovered between cell polarity and cellular signaling pathways that control processes such as tissue organization, growth, and cell division [16–21]. Identifying the full complement of polarity protein interactions is crucial to our understanding of how cell polarity integrates the diverse signaling pathways that control tissue homeostasis, and to understand how deregulation of cortical polarity contributes to disease.

Large-scale protein interaction mapping techniques excel at identifying potential functional interactions between proteins. Importantly, because of their unbiased nature, such approaches can yield unexpected novel links between cell polarity and other pathways. Most interaction networks, however, are static in nature, while both cortical polarity establishment and the cross-talk with other cellular pathways are context dependent and dynamic. Elucidating the dynamics of interactome networks in a systematic fashion is a major current challenge. In this review, we will provide an overview of large-scale protein interaction mapping approaches, highlighting several instances where the application of such techniques has led to new insights into cell polarity. We will also summarize strategies to elucidate the spatiotemporal dynamics of protein interactions, again with a focus on cell polarity.

Protein–protein interaction mapping

The identification of all protein interactions that take place in a cell or organism, referred to as the "interactome," is one of the major goals in modern biology. This has led to the development of large-scale protein–protein interaction (PPI) mapping, and the specialization of several groups in this field. Mapping protein interactions in a systematic fashion has several advantages. First, there is an economy of scale that allows for high efficiency and overall lower cost. Second, the quality of current interactome mapping data is very high, due to the development of rigorous experimental procedures, and the application of internal benchmarking tools that make use of defined sets of positive and negative control interactions [22]. Finally, systematic approaches can avoid the bias that is inherent when interactions are identified as part of studies that focus on a particular question [22].

Approaches to identifying physical relationships between proteins fall into two broad categories: binary interaction mapping and protein complex identification. These approaches yield orthogonal information: The first directly queries whether two proteins physically interact—without providing information on higher-order assembly. The second identifies proteins that act together in a macromolecular assembly—without discerning direct physical interactions or providing information on complex topology. Proteome-wide binary interaction mapping is currently dominated by the yeast two-hybrid (Y2H) system [23], although other methods such as split-ubiquitin Y2H [24–26], the Mammalian Protein–Protein Interaction Trap (MAPPIT) [27], the co-immunoprecipitation-based LUminescence-based Mammalian IntERactome (LUMIER) system [28,29], and bimolecular fluorescence complementation [30] are increasingly being used in high-throughput settings. Protein complex mapping is done using a variety of procedures that rely on mass spectrometry (MS) to determine protein identities, including affinity purification–mass spectrometry (AP-MS), co-fractionation, proximity labeling [biotin identification (BioID) and engineered ascorbate peroxidase (APEX)], and cross-linking mass spectrometry (XL-MS) [31–34]. Figure 2 and Tables 1 and 2 summarize the major techniques currently in use to map PPIs.

Each PPI mapping method has inherent benefits and limitations. For example, AP-MS excels at identifying stable protein complexes, while the Y2H system is better able to detect weak or transient protein interactions. Split-ubiquitin Y2H was developed to identify interactions at the cell membrane [36], while MAPPIT and LUMIER use mammalian cells as the host system to better mimic the natural environment of mammalian proteins [37]. XL-MS aims to provide information on the topology of protein complexes [33], while co-fractionation MS avoids the need to generate individually tagged proteins and can greatly speed up detection of protein complexes [40–43]. Finally, proximity labeling approaches were developed to provide spatial information [32]. Ultimately, each of these approaches identifies a different subset of the interactome, and the combination of interaction maps generated using many different approaches will be needed to generate a complete picture of the interactome.

The abundance of systematic high-throughput protein interaction data, not to mention the publication of thousands of interactions in small scale studies, led to the generation of dozens of public

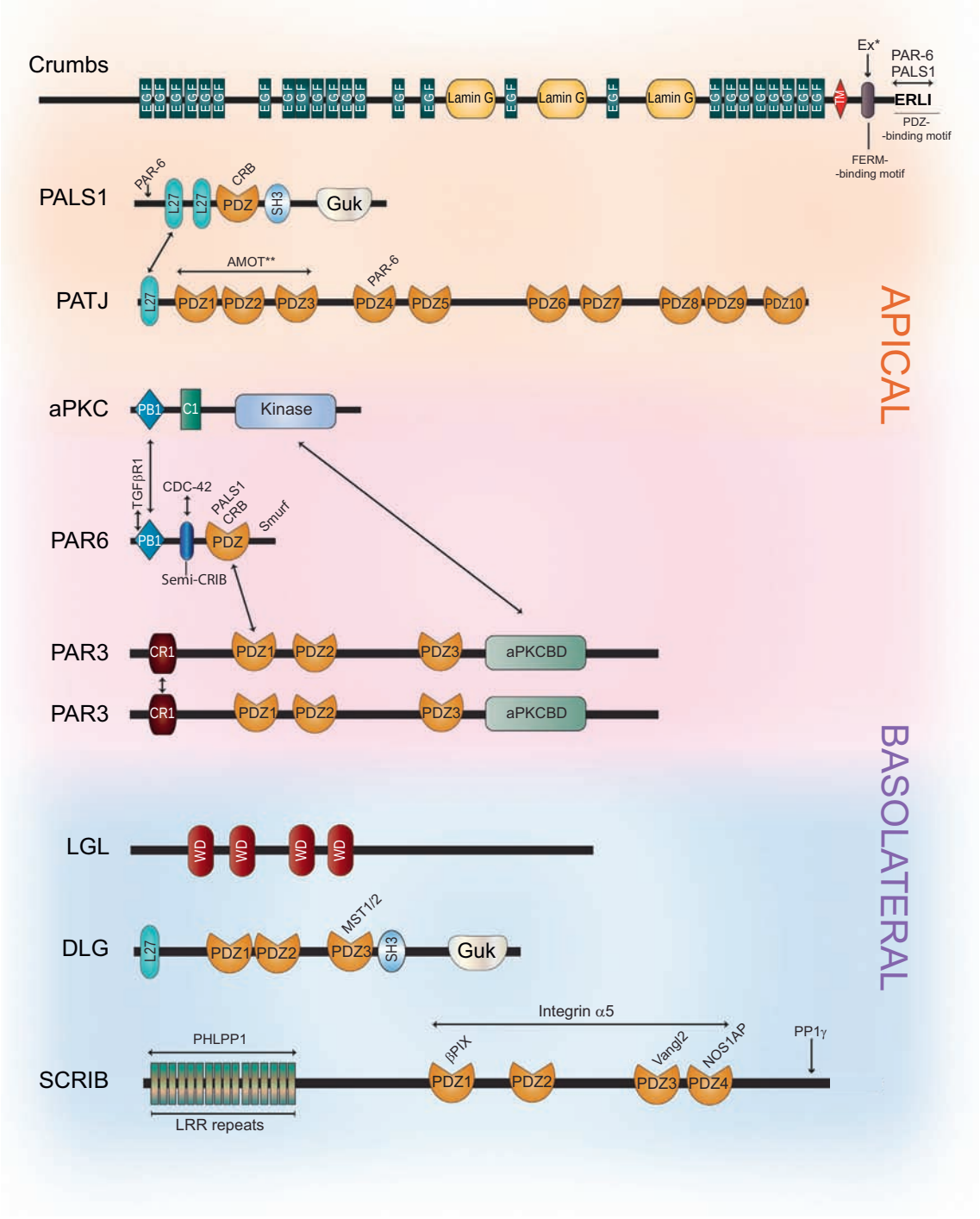

Fig. 1. Schematic showing domain structures of the PAR, Crumbs, and Scribble group proteins, and PPIs described in this review. Apical proteins Crumbs, PATJ, and PALS1 are represented in the top (apical) part of the scheme (orange background). Apical PAR group proteins PAR3, PAR6, and aPKC are represented below (pink background). Basolateral proteins LGL, DLG, and SCRIBBLE are represented at the bottom part of the scheme (blue background). Interactions between the polarity proteins themselves are also represented. *Interaction between Expanded and Crumbs is specific to *Drosophila* [15]. **Interaction between AMOT and PATJ was found specifically with mammalian proteins [15].

databases that catalog and archive this information, and make it readily accessible [45]. These databases currently list 200,000 to 400,000 human PPIs. Major archival databases include IntAct [46], MINT [47], biological general repository for interaction data sets (BioGRID) [48], and the STRING database [49].

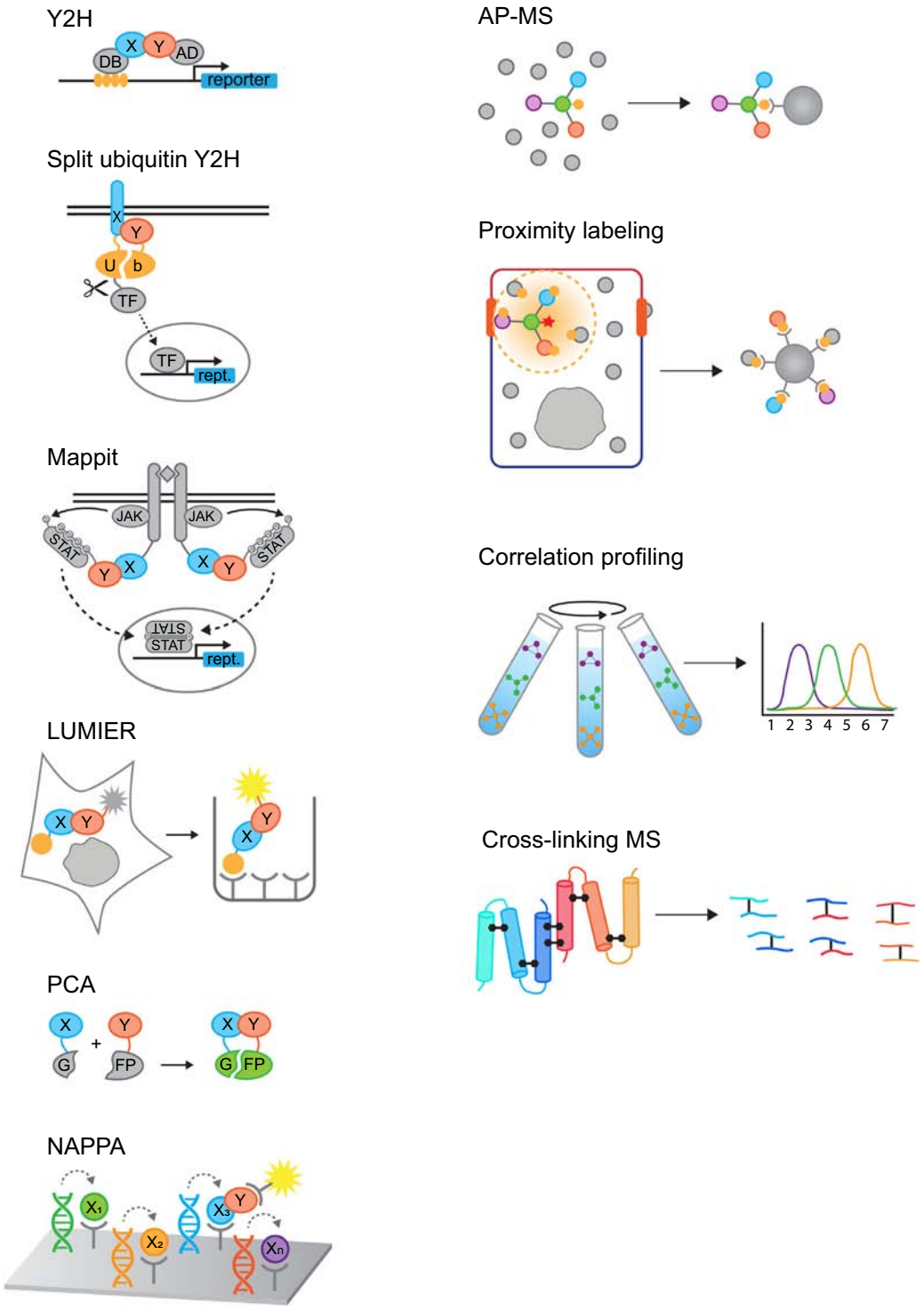

Fig. 2. Schematic representation of some of the most commonly used binary PPI approaches (left column) and protein complex mapping approaches (right column). For details, see Tables 1 and 2.

IntAct and MINT focus on direct physical interactions obtained through literature curation and direct submission, and aim to provide comprehensive coverage across all organisms. BioGRID contains genetic interactions, chemical associations, and post-translational modifications, in addition to physical interactions, but focuses on human and major model organisms. STRING emphasizes comprehensive

Table 1. Binary PPI mapping approaches

Technique	Approach	Readout	Host organism	Subcellular location	Reference
Y2H	Reconstitution of a transcription factor	Reporter gene activity	Yeast	Nucleus	[35]
Split-ubiquitin Y2H	Reconstitution of ubiquitin, resulting in cleavage and release of a transcription factor.	Reporter gene activity	Yeast or mammalian cell culture	Cytosol/ Cortex	[25,36]
MAPPIT	Reconstitution of a cytokine receptor signaling pathway	Reporter gene activity	Mammalian cell culture	Cytosol/ Cortex	[37]
LUMIER	Immunoprecipitation in multi-well plate	Detection of luciferase fused to prey protein in luminescence assay	Mammalian cell culture	Any	[38]
PCA	Reconstitution of two fragments into an active reporter	Most common: reconstitution of a fluorescent protein.	Multiple cell types and organisms	Any	[30]
NAPPA (nucleic acid programmable protein array)	Local binding of *in vitro* produced protein to array of proteins on a solid support surface.	Detection of bound protein with a specific antibody or anti-tag antibody	Proteins produced by reticulocyte lysate	N/A	[39]

coverage of physical interactions as well as functional associations, and includes interactions derived from literature curation, knowledge transfer between organisms based on protein orthology, and computational predictions. Several resources also focus on specific subsets of interactions, such as extracellular matrix protein interactions (MatrixDB) [50], innate immunity interactions (InnateDB) [51], host–pathogen interactions (HPIDB) [52], or interactions for which a high-resolution 3D structure is known (3DID) [53]. Several of the data provides listed here also share curation efforts in the IMEx consortium [54]. Finally, many published data sets are accompanied by a dedicated website tailored to the experimental methods and offering additional detail (see Table 3 for examples of major data set portals).

Challenges in interactome mapping

Two major challenges faced by interactome mapping approaches are quality and completeness. A third major challenge, elaborated on below, is how to move from static interaction networks to large-scale understanding of the dynamics of PPIs: when and where do interactions take place? How do interactions vary dynamically, and how are these dynamics regulated?

Large-scale interactome mapping approaches have steadily evolved to produce networks of high quality, largely by identifying and minimizing sources of technical artifacts. For example, Y2H screens now routinely eliminate false positives that are due to spontaneous auto-activation of reporter genes, and recent large-scale screens use a rigorous pipeline that utilizes sequence verified ORF clones and includes a re-testing phase to further eliminate false positives [23,58,59]. Similarly, the quality of MS-based interaction maps has improved tremendously both through experimental improvements, such as the development of quantitative MS technologies, and the development of sophisticated algorithms that distinguish true interactors from background proteins [60–62]. Comparisons of experimental results with gold standard sets of positive and negative binary protein interactions [63] and complexes [64] show that current implementations of the Y2H system and MS approaches generate highly

Table 2. Protein complex mapping approaches

Approach	Summary	References
AP-MS	Purification of a protein complex from a lysate through affinity purification, followed by the establishment of the identities of the purified proteins by MS	[31]
Proximity-based labeling	Covalent labeling of proteins in close proximity to a bait protein tagged with a reactive enzyme. Labeled proteins are purified and identified by MS. Current methods: proximity-dependent BioID and APEX	[32]
Protein correlation profiling	Identification of protein complex members based on the similarity of their behavior in extensive biochemical fractionation of cell lysates. Avoids need for protein tagging and rapidly identifies protein complexes with few MS runs	[40–43]
XL-MS	Amino acids in close proximity are covalently linked, yielding a pair of cross-linked peptides after proteolytic digestion. Cross-links within a protein inform on protein conformation, and cross-links between proteins on protein binding sites and complex topology. Covalent links enable stringent purification procedures.	[33,44]

Table 3. Examples of project websites and PPI dynamics websites

Description	URL	Reference
Major project portals		
Human Reference Protein Interactome Project (HuRI). Matrix-based Y2H screens of full-length sequence validated human ORFeome clones	http://interactome.baderlab.org/	[23]
BioPlex (biophysical interactions of ORFeome-based complexes). AP-MS of HA-FLAG tagged proteins expressed in human cells	http://bioplex.hms.harvard.edu/bioplexDisplay/	[55]
Metazoan complexes. Protein correlation profiling-MS of a panel of 8 model species, including 5 human cell lines	http://metazoa.med.utoronto.ca/	[43]
A census of human soluble protein complexes. Protein correlation profiling-MS of human HeLa S3 and HEK293 cells	http://human.med.utoronto.ca/	[40]
Human protein complex map (hu.MAP). Integration of two large-scale AP-MS data sets and a large-scale protein correlation profiling-MS data set	http://proteincomplexes.org/	[56]
The human proteome atlas. Antibody-based imaging, MS-based proteomics, transcriptomics and other 'omic' studies of human proteins	https://www.proteinatlas.org/	[57]
Websites of protein dynamics		
Encyclopedia of protein dynamics. "Open data" platform for display and sharing of proteomics data, with a focus on information on protein complex dynamics	https://www.peptracker.com/epd/analytics/	[196]
Integrated Interactions Database (IID). Database of known and predicted eukaryotic PPIs, in 30 tissues of model organisms and humans	http://iid.ophid.utoronto.ca/iid/	[197]
Human Integrated Protein–Protein Interaction rEference (HIPPIE). Context-specific PPI networks derived from integrated omics data sets	http://cbdm-01.zdv.uni-mainz.de/~mschaefer/hippie/	[198]
Compartmentalized protein–protein interaction database (ComPPI). Provides qualitative information on interactions, proteins, and their localizations integrated from multiple databases	http://comppi.linkgroup.hu/	[199]
GeneMANIA. Tool to find functionally related genes based on large set of functional association data	https://genemania.org/	[200]

reliable interactome data sets, with qualities equal to or higher than those of published interactions detected in small-scale assays [22].

The second major challenge is completeness. All experimental methods to map PPIs have inherent technical limitations that cause them to identify only a portion of the interactome. For example, the Y2H system is not well suited to identify interactions with transmembrane proteins, and in AP-MS, protein complexes must be soluble under the lysis conditions used. However, perhaps the main challenge is posed by the complexity of biological systems itself. Most genes produce more than one protein isoform through alternative splicing, and isoforms can have different interaction profiles. For example, splice isoforms of mammalian PAR3 and the related PAR3L differ in their ability to interact with aPKC and PAR6, respectively [65]. Interaction profiles can also be modified through posttranslational modifications, further increasing the potential complexity of the interactome. These levels of complexity are only just beginning to be taken into account in large-scale protein interactome maps.

Mapping the "polarity Interactome"

Binary interaction mapping of cell polarity

Most proteome-wide binary interactome mapping studies make use of the Y2H system, which is based on reconstitution of a transcription factor in yeast cells. A physical interaction between two proteins, one fused to a DNA binding (DB) domain and one fused to a transcription activation domain (AD), is detected through activation of reporter genes [35,66–68]. The most common DB and AD pairs used are derived from the yeast Gal4p transcription factor, or a combination of the bacterial *LexA* DB with the herpes simplex virus VP16 AD.

Y2H screens can be performed in a library format, where a single bait protein is used to query a library of prey proteins, or in a matrix format, where a defined set of proteins is systematically tested for interaction. The main advantage of a matrix screen is that the exact search space tested is known, and this approach is currently being used to generate a binary human interaction map [23]. Limitations of the Y2H system include a failure to detect interactions due to lack of an essential co-factor or a posttranslational modification, due to poor expression or folding of fusion proteins, or due to the need for interactions to take place in the nucleus. Because of the ease of handling yeast and the plasmids encoding the AD- and DB-fusion proteins, the Y2H system is highly scalable and remains a favorite for the detection of binary PPIs.

Recently, we employed the Y2H system to probe the polarity interactome of *Caenorhabditis elegans* [69]. We used a fragment-based Y2H strategy developed to increase the detectability of interactions by the Y2H system and to identify the minimal protein regions required for an interaction [70]. Our screens uncovered 439 interactions between 296

proteins, as well as the protein regions that mediate these interactions. To begin extracting functional relationships, we performed phenotypic profiling by RNA-mediated interference (RNAi) and identified 100 physically interacting protein pairs for which RNAi-mediated depletion caused a defect in a polarity-related process. We investigated a novel interaction between PAR-6 and the RhoGAP PAC-1 (ARHGAP21 in mammals), demonstrating a requirement for this interaction in radial polarization of the *C. elegans* embryo (Fig. 3a). Most of the interactions we identified were novel, and several have already been demonstrated to be biologically relevant. These include an interaction between PAC-1 and PICC-1 (mammalian CCDC85A-C), which has been shown to localize PAC-1 to adherens junctions by acting as a linker between PAC-1 and the p120 catenin JAC-1 [71] (Fig. 3a), and an interaction between the Polo kinase PLK-1 and PAR-3, which plays an important role in controlling PAR-3 oligomerization [72].

A link between cortical polarity and TGFβ-induced epithelial-to-mesenchymal transition identified by LUMIER screens

The LUMIER system provides an example of a high-throughput binary-interaction detection system that has been used to elucidate a link between cell polarity and extracellular signaling [28,73]. LUMIER was designed to map mammalian PPIs in the context of a mammalian cell environment and to map signaling-dependent PPIs. It is based on the co-expression in mammalian cells of a Luciferase-tagged protein and a protein fused with an affinity tag (e.g., HA, Flag, or the IgG-binding domain of *Staphylococcus aureus* protein A) [28,38]. The presence of an interaction between the two proteins is determined by immunoprecipitation of the affinity-tagged protein, followed by measuring Luciferase activity to determine the extent of co-immunoprecipitation. The LUMIER assay can be automated to perform high-throughput screens [28,63,74,75].

The first LUMIER screens uncovered a novel link between cell polarity and TGFβ-induced epithelial-to-mesenchymal transition (EMT) (Fig. 3b) [28,73]. During EMT, epithelial cells loose apical–basal polarity, cell–cell junctions are disassembled, migratory capacity is increased, and cells become resistant to apoptosis [76]. EMT likely plays an important role in tumorigenesis [77,78], and TGFβ is one of the major inducers of EMT [79,80].

A series of 10 Luciferase-tagged core TGFβ pathway members were screened against 518 Flag-tagged cDNAs in the presence or absence of TGFβ signaling [28]. The screens identified two novel interactors of the type I receptor TβRI: the tight junction (TJ) protein Occludin (OCLN) and PAR6. In mammary epithelial cells, the interaction with OCLN regulates the localization of TβRI to TJs (Fig. 3b), and

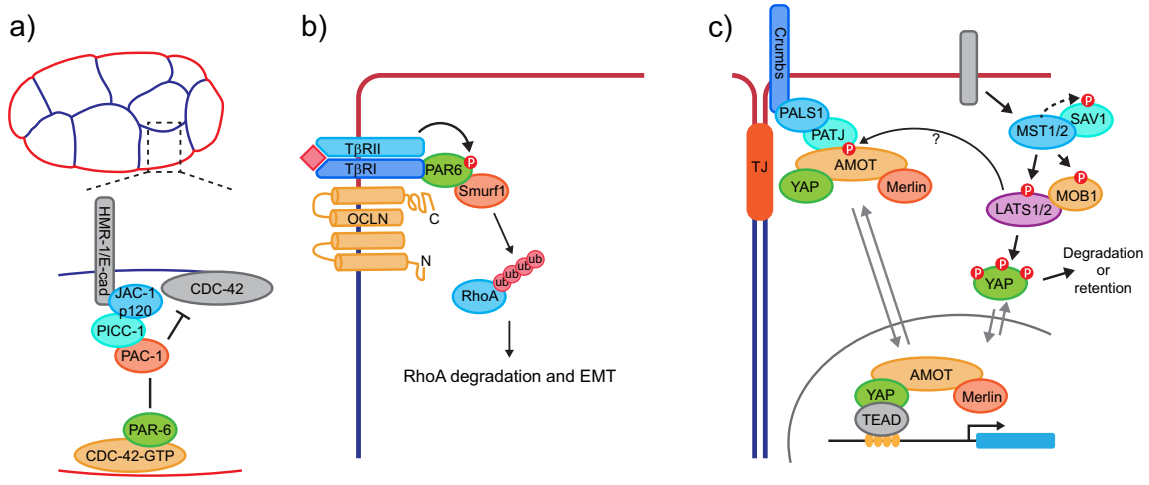

Fig. 3. Graphical representation of interactions described. (a) Interactions with the RhoGAP protein PAC-1, which mediates radial polarization of the *C. elegans* embryo by restricting the localization of PAR-6 to contact-free cell surfaces (red). The mechanistic details of how the PAC-1–PAR-6 interaction contributes to this process remain to be elucidated. (b) TGFβ/PAR6 interactions involved in EMT, discovered in high-throughput LUMIER screens. The activity of this TGFβ-PAR6 pathway results in degradation of RhoA through recruitment of the E3 ubiquitin ligase Smurf1 and is restricted to TJs through binding of TβRI to OCLN. (c) Control of the mammalian Hippo pathway transcription factor YAP through sequestering at cell junctions by an interaction with AMOT, which directly binds to the Crumbs complex component PATJ. The canonical Hippo pathway, which, when active, excludes YAP from the nucleus through phosphorylation by the LATS1/2 kinases, is also indicated. Note that control of the Hippo pathway is highly complex, with regulators interacting with the pathway at multiple levels, and only the main interactions described in this review are indicated.

is required for junction dissolution in TGFβ-induced EMT [28]. The TGFβ transcriptional response, however, was unaffected by loss of this interaction. The interaction with PAR6 similarly affects junction disassembly without interfering with transcriptional output. PAR6 is constitutively bound to TβRI at TJs and is a substrate for TβRII. Stimulation with TGFβ causes phosphorylation of PAR6 on Ser345, a mammalian-specific residue absent in, for example, *Drosophila* and *C. elegans* PAR-6. The phosphorylated form of PAR6 recruits the E3 ubiquitin ligase Smurf1, which degrades the small GTPase RhoA, causing dissolution of TJs. Further studies demonstrated that the PAR6/Smurf1/RhoA pathway is also involved in EMT in epicardial cells [81] and endocardial cells [82,83], and implicated TGFβ-induced phosphorylation of PAR6 in the progression of breast and prostate cancer [84,85]. In parallel to PAR6 binding and phosphorylation, TGFβ also downregulates PAR3 expression and induces translocation of PAR6 from TJs to the cytoplasm [86], suggesting that TGFβ signaling results in extensive remodeling of the PAR complex to promote EMT.

Using AP-MS to probe cell polarity

The predominant MS-based approach to map the composition of protein complexes is AP-MS. In this approach, a protein complex is purified from a lysate through affinity purification (AP), followed by the establishment of the identities of the purified proteins by MS [31]. Although antibodies against native proteins can be used, generally "bait" proteins are modified with an affinity tag so that a common purification reagent can be used. Initial high-throughput AP-MS approaches used tandem affinity purification (TAP) tags that facilitate a two-step sequential purification [87]. However, while greatly reducing background, the high stringency of the procedure makes it difficult to identify transient or weak interactions [31]. With the development of quantitative MS techniques, it became possible to use low-stringency single-step purifications, using increasingly sophisticated algorithms to distinguish true interactors from background proteins [60,62].

One of the earliest large-scale AP-MS networks generated for mammalian cells was focused on the human PAR proteins [88]. Using TAP, nine human homologs of the *C. elegans* proteins PAR-1, PAR-4, PAR-5, PAR-6, and PKC-3 were purified from HEK923 human embryonic kidney cells. The resulting network of about 60 proteins confirmed most interactions known at the time, including interactions between PAR3, PAR6, aPKC and LGL1, and the LKB1(PAR-4)/MO25/STRAD complex that had recently been identified and shown to be able to polarize single intestinal epithelial cells in culture [89–91]. The network also demonstrated extensive connections between the PAR-5 homologs 14–3–3ζ and 14–3–3η and several polarity proteins including PAR3 and the PAR-1 homologs MARK2 and MARK4. This proteomic investigation of the PAR network also stands out as one of the first studies to utilize prior AP-MS data to filter out "sticky" proteins that are found to co-purify with many different baits. This approach is currently a central part of most algorithms developed to discriminate true interactors from non-specific background [62].

The PAR interactome generated by Brajenovic et al. [88] is the most comprehensive example of an MS proteomics approach to elucidate the polarity interactome. Nevertheless, AP-MS approaches have been extensively used on smaller scales, often identifying unexpected connections between core cell polarity regulators and other cellular pathways. Below, we highlight a few of the more prominent of these.

Links between apical–basal polarity and Hippo growth control

Epithelial tissues rely on a highly evolutionary conserved signaling pathway that regulates cell proliferation, organ size, and tissue regeneration: the Hippo pathway. The core of the Hippo pathway is a kinase cascade in which the Hippo kinase (MST1/2 in mammals), in a complex with Salvador (SAV1), activates the kinase Warts (LATS1/2 in mammals). Warts in turn restrict the activity of the transcription factor Yorki (Yki, mammalian YAP/TAZ) (Fig. 3c) [92,93].

Several connections exist between the mechanisms that control polarity and the Hippo pathway [17,20]. One of the best known examples is the binding of the apical determinant Crumbs to the band 4.1 protein/ezrin/radixin/moesin (FERM) domain protein Expanded (Ex) in *Drosophila*, which acts as an upstream regulator of Hippo signaling by sequestering Yki from the nucleus [20]. In mammals, however, Crumbs appears to regulate Hippo signaling not through homologs of Expanded, but through Angiomotins (AMOT), a family of adapter proteins with broad roles in cellular functions [94]. TAP of YAP identified a strong interaction of YAP with all three AMOT family members (AMOT, AMOTL1, and AMOTL2), mediated by the YAP WW domain and PPxY motifs present in the N-terminal region of the AMOT proteins [95]. AMOT was shown to act as an inhibitor of YAP, possibly through sequestering of YAP from the nucleus to TJs [95]. A mechanism through which AMOT can be recruited to TJs was elucidated in an earlier proteomics study, which identified a direct association between the PSD-95/Dlg/ZO-1 (PDZ domain) of the Crumbs complex component PATJ and the C-terminus of AMOT (Fig. 1) [96]. A subsequent Y2H study confirmed the interaction between AMOT and PATJ, and also found PATJ to interact with AMOTL1 and AMOTL2 [97]. Thus, similar to Expanded, AMOT might be targeted to TJs, sequestering YAP from the nucleus.

One complication with this model is that both positive and negative effects of AMOT on Hippo signaling had been reported [94]. However, a recent study appears to reconcile these observations by finding that AMOT mediates formation of a YAP/Merlin/AMOT complex, which can exist both in the nucleus, or, upon phosphorylation of AMOT, be relocated from the nucleus and sequestered at the plasma membrane (Fig. 3c) [98].

The above interactions are only part of the complex relationship between AMOT and Hippo signaling, as AMOT also binds to MST2, and Kibra, another key regulator of Hippo signaling [94]. Moreover, AMOT plays multiple roles in cellular functions, including localizing RhoA activity to the leading edge of migratory cells [99], regulating TJ stability by controlling Rac1 and Cdc42 activity through the RhoGAP RICH1 [96,100], and modulating collective cell migration through a Merlin–Amot–Rich1–Rac1 pathway at TJs [101]. Thus, AMOT engages in numerous interactions in a context-dependent manner, highlighting the value of using unbiased proteomic approaches.

Another link between cortical polarity regulators and Hippo signaling was found in an AP-MS study centered on DLG5 [102]. AP-MS of DLG5 from HEK293T cells identified several binding partners, including MST1/2 and SAV1. Analysis of Hippo signaling in DLG5 knockout mice showed constitutive activation of the pathway in several tissues, and conditional knockout of MST1/2 in brain could rescue phenotypic defects caused by DLG5 knockout [102]. A role for DLG5 as a negative regulator of Hippo signaling appeared to be conserved in flies and human cell lines. Mechanistically, DLG5 appears to mediate the formation of a complex containing MST1/2 and mammalian PAR1 homologs, leading to hyperphosphorylation and inactivation of MST1/2 [102].

Scribble as a scaffolding protein

The members of the Scribble polarity module, Scrib, Lgl, and Dlg, were identified in *Drosophila* as regulators of basolateral domain identity and suppressors of neoplastic tumorous growth [103,104]. The Scribble module has been extensively studied in different model systems and found to play important roles in controlling apical–basal polarity, the polarity of migrating cells, actin cytoskeleton remodeling, vesicle trafficking, and the activity of several intracellular signaling pathways including the MAPK and PI3K–Akt pathways [105]. In mammals, both downregulation of SCRIB and mislocalization to the cytoplasm are associated with increased tumorigenic potential, but the exact mechanisms through which SCRIB acts as a tumor suppressor remain unclear [4,18,105,106].

Scribble is a member of the LAP family of proteins, which are characterized by the presence of either 1 or 4 PDZ domains in the C-terminal region (4 in case of Scribble), and 16 leucine-rich repeats at their N-terminus (Fig. 1) [107]. The leucine-rich-repeat domain is a 20- to 30-aa recognition motif [108] that is essential for Scribble basolateral membrane targeting [109], while the 4 PDZ domains enable Scribble to interact with various target proteins. Because of the broad and often context-dependent roles that Scribble plays, unbiased approaches to identify Scribble interacting proteins have been particularly valuable in elucidating its physiological functions. In fact, of all the cortical polarity regulators, the Scribble interaction landscape is the most extensively investigated by proteomics approaches.

Scribble in cell migration. Proteomics approaches were instrumental in identifying a link between mammalian SCRIB and the regulation of cell migration through the formation of a Scribble/β-Pix/PAK/GIT complex. No less than four AP-MS screens uncovered an association of SCRIB with p21-activated kinase 1 and 2 (PAK1/2), β-PAK interacting exchange factor (β-Pix), and G-protein coupled receptor kinase-interacting proteins 1 and 2 (GIT1/2) [110–113]. PAK1 and PAK2 are members of the PAK family of serine/threonine kinases that act as effector proteins of Cdc42 and Rac. PAK kinases function in a range of cellular processes, including migration, through remodeling of the actin cytoskeleton, and PAK overactivity is a common occurrence in cancer [114,115]. GIT proteins are GTPase activating proteins for ADP-ribosylation factor small GTPases, which control membrane traffic and remodeling of the actin cytoskeleton [116]. Finally, the mammalian Pix proteins α-Pix and β-Pix (also known as ARHGEF6 and ARHGEF7) act as exchange factors for the Rho-family GTPases Rac and Cdc42 [117].

GIT and Pix proteins form oligomeric scaffolds that can be recruited to different locations in the cell in response to specific signals, where they downregulate ADP-ribosylation factor signaling and increase Rac1 and/or Cdc42 activity [117]. One of the major roles of GIT/Pix complexes is to stimulate membrane dynamics and cytoskeletal reorganization associated with cell motility [118]. The PDZ domains of SCRIB bind a PDZ domain binding motif at the C-terminus of β-Pix (Fig. 1) [111], which in turn directly associates with GIT1 and PAK1 [112]. By targeting GIT/Pix/PAK to the leading edge of motile cells, SCRIB coordinates the activities of several signaling pathways to promote cell migration [112,119,120].

Two AP-MS screens identified a second SCRIB interaction partner that plays a role in cell motility, as well as in neuronal synapse formation: the nitric oxide synthase adaptor protein NOS1AP [110,113]. The phosphotyrosine binding domain of NOS1AP binds to the fourth PDZ domain of SCRIB (Fig. 1) [113]. However, the two studies disagree on the

nature of the complexes formed, possibly because different cell types and experimental conditions were used. One finds that SCRIB acts as a bridge between NOS1AP and the β-Pix/GIT/PAK complex [113], while the second finds that SCRIB is part of two distinct complexes: SCRIB/β-Pix/GIT/PAK and a complex containing SCRIB, NOS1AP, and the planar cell polarity (PCP) protein Van Gogh-like 1 (VANGL1) (Fig. 4a) [110]. In wound healing assays, the formation of this SCRIB/NOS1AP/VANGL1 complex played an important role in the establishment of front-rear polarity and migration of invasive breast cancer cells [110].

In the same AP-MS experiments, the PCP proteins Flamingo and VANGL2 were detected as SCRIB-associated proteins [110]. This is consistent with earlier studies demonstrating a role for Scribble in PCP and a direct association with *Drosophila* Van Gogh (Vang) identified by Y2H [121]. A Y2H screen of a library of 246 human PDZ domains also confirmed the interaction of VANGL2 with SCRIB and provides further evidence for a functional role of the SCRIB/VANGL2 interaction in promoting the pro-migratory activities of VANGL2 [122].

However, another mechanism through which Scribble can regulate cell migration was uncovered in a screen for SCRIB interactors in human umbilical vein endothelial cells (HUVEC), where SCRIB was found to interact with the integrin α5 (as well as β-Pix and GIT1 again) [123]. In migrating HUVEC cells,

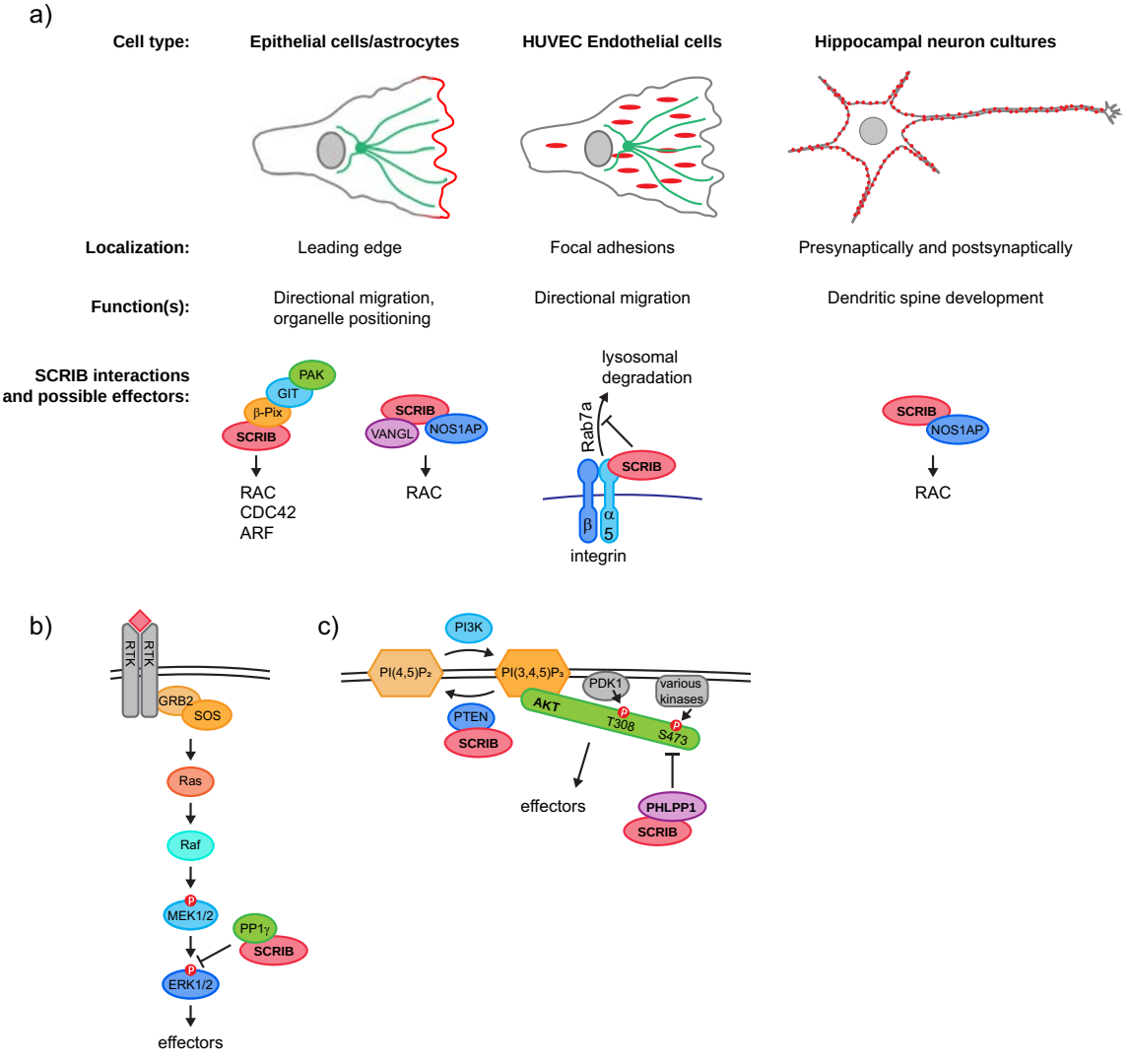

Fig. 4. Graphical representation of SCRIB interactions described. (a) Subcellular localization and protein interactions of SCRIB with functions in cell migration and neuronal functioning. Observed localization of SCRIB is indicated in red. (b and c) Control of the MAPK/ERK (b) and AKT (c) pathways through interactions between SCRIB and protein phosphatases.

SCRIB did not localize to the leading edge, but instead showed co-localization with α5 integrin at focal adhesions [123]. SCRIB was found to control surface levels of integrin α5, likely by protecting it from Rab7a-dependent sorting into the lysosomal degradation pathway (Fig. 4a [123]. The specificity of the interaction of SCRIB with integrin α5 was demonstrated by the observation that loss of SCRIB affected the directional migration of HUVEC cells on fibronectin, which is recognized by integrin α5, but not on collagen, whose binding is mediated by other integrins [123].

Regulation of MAPK and AKT signaling pathways by phosphatase recruitment. As mentioned above, SCRIB has been shown to regulate the activity of several intracellular signaling pathways, including the MAPK and PI3K–AKT pathways [105]. A direct physical interaction between SCRIB and ERK results in lowered levels of ERK phosphorylation, thus reducing MAPK signaling activity, and this activity of SCRIB likely contributes to its role as a tumor suppressor [119,124,125]. The reduction in ERK phosphorylation suggested involvement of a phosphatase, and indeed AP-MS of SCRIB from HEK293 cells identified protein phosphatase 1γ (PP1γ) as a major interacting partner of SCRIB (Fig. 4b) [126]. A conserved PP1-binding motif in the C-terminus of SCRIB directly interacts with PP1γ (Fig. 1), and the ability of SCRIB to downregulate phospho-ERK levels appears to depend on PP1γ. A similar phosphatase-dependent regulation was found to play a role in the regulation of AKT signaling by SCRIB, where SCRIB has been reported to recruit two phosphatases through direct binding: PTEN, which dephosphorylates phosphatidylinositol (3,4,5)-trisphosphate (PIP3), an upstream regulator of AKT activity, and PHLPP1, which dephosphorylates AKT itself [106,127] (Fig. 4c).

These studies highlight the important role proteomics approaches have played in uncovering the molecular mechanisms through which SCRIB exerts its diverse physiological roles and acts as a scaffolding protein controlling spatial–temporal aspects of cellular signaling. It also illustrates the broad and context-dependent roles that polarity proteins can play besides the establishment of cortical domains. Unbiased PPI mapping approaches will continue to be an important tool to uncover these functions.

Protein–lipid interactions

Interactions between proteins and lipids have been much less extensively mapped than protein–protein, protein–DNA, or protein–RNA interactions. Nevertheless, this important class of biomolecular interactions plays important roles in cell polarity [6,128,129]. For example, membrane targeting of mammalian and *Drosophila* PAR3 involves direct binding to phosphoinositides [128], and PAR3 recruitment by $PI(4,5)P_2$ was shown to be crucial for apical–basal polarity of the *Drosophila* follicular epithelium [130]. For mammalian PAR3, this interaction is mediated by the second PDZ domain, while the interaction of *Drosophila* Baz/PAR3 involves a C-terminal region that does not contain any known lipid binding domain. Recent studies indicate that multiple polarity proteins, including LGL and PAR1, associate with the lipid membrane through electrostatic interactions involving positively charged patches, which may be counteracted through phosphorylation events [131]. Hence, the identification of protein–lipid interactions in unbiased and large-scale efforts is likely to advance our understanding of cell polarity.

Several high-throughput methods have been developed for the identification of protein–lipid interactions. Lipid-overlay and lipid pull-down assays, in which binding of proteins to lipids immobilized on a solid support medium is tested, have been used in systematic screens in *Saccharomyces cerevisiae* and *Dictyostelium discoideum* [132,133]. In these approaches, the lipids tested are taken out of the context of a lipid bilayer. Two potentially more physiologic approaches utilize liposomes. In one, hundreds to thousands of proteins are arrayed on a solid support surface, such as a glass slide, and probed with fluorescently labeled liposomes containing the lipid of interest. This approach was used in one of the first systematic protein–lipid binding screens, which identified phospholipid binding activity for 150 out of 5800 *S. cerevisiae* proteins tested [134]. The second approach, termed liposome microarray-based assay, uses a microfluidics device in which hundreds of liposomes are arrayed on a thin agarose layer [135]. The liposome array is then probed for binding with a fluorescently labeled protein. Liposome microarray-based assay was recently used in a large-scale systematic study of the role of lipid cooperativity in the recruitment of Pleckstrin Homology domains [136]. Finally, several *in vivo* approaches have been developed for the identification of protein–lipid interactions. These include the use of AP followed by MS or thin-layer chromatography to identify soluble protein–lipid complexes [137,138], and a yeast-based Ras rescue assay, in which recruitment to the membrane of constitutively active Ha-Ras fused to a protein of interest rescues growth of a Ras-signaling deficient yeast strain [139]. This last assay contributed to the elucidation of the mechanism of PAR1 membrane association [140]. A C-terminal PAR1 domain, termed the kinase-associated domain 1 (KA1), had previously been identified as essential for membrane localization, but it was not clear how the KA1 domain mediated lipid binding [141]. A later study used the Ras rescue assay to identify a novel phospholipid binding-domain in yeast Septin-associated kinases. Crystallographic

studies of this domain revealed similarity to KA1 domains, and revealed that basic regions in the KA1 domain drive association with several classes of negatively charged phospholipids [141].

The studies listed here provide only a short overview of the field of protein–lipid interaction mapping. For a comprehensive historical overview of the development of such methods and their application, see the recent review by Saliba *et al*. [142].

From static to dynamic PPI networks

PPI networks are not static entities but vary between cell and tissue types, and undergo dynamic changes over time and in response to cues from the environment. Cell polarity itself is an excellent example for the need to determine protein interaction dynamics, as the spatial separation of components relies on tightly regulated protein interactions. To understand the establishment and maintenance of cell polarity and how it is coordinated with intracellular pathways and cues, it is important to know where and when interactions take place, and how they are regulated. Most interaction mapping approaches were not designed to provide information on the dynamics of interaction networks, and current interactome data are to a large extent static in nature. However, in recent years, efforts to resolve the spatiotemporal dynamics of protein interactions at large scale have increased in number. Below, we highlight several approaches to elucidate the temporal and spatial dynamics of protein interactions.

Integrative approaches to inferring PPI localization

Protein interactions can be regulated by controlling the expression pattern of proteins, the subcellular localization of proteins, and through posttranslational modifications that enable or block interactions from taking place. While systematic experimental approaches to directly probe the spatiotemporal dynamics of protein interactions are still limited, large-scale efforts to determine gene expression patterns and protein subcellular localization are more prevalent. Integrating such data with PPI network is an attractive way to provide important spatiotemporal information and to help determine when or where protein interactions are likely to take place. Table 3 lists several databases that integrate PPI networks with localization or expression data to provide contextual information.

Microscopy is the traditional method of choice to determine the localization of a protein. Although it is time consuming and requires the generation of reagents such as antibodies or transgenic organisms, microscopy has been used in large scale protein localization studies. For example, each of the 6234 predicted budding yeast ORFs was tagged with green fluorescent protein (GFP) in its chromosomal location, 4156 of which resulted in detectable GFP expression [143]. This ORF-GFP collection has been used extensively to examine the subcellular location and expression levels of proteins under normal conditions as well as in stress conditions or in specific mutant backgrounds [144,145]. For more complex model organisms such as *C. elegans* or *Drosophila*, such genome-wide strain collections do not yet exist, although genome-wide bacterial artificial chromosome or Fosmid resources for the generation of transgenic lines are available, and numbers of reporter lines continually increase [146]. In mammals, the largest effort to date uses immunofluorescence staining and confocal imaging. As part of the human protein atlas program[†], a panel of 13,993 antibodies and 22 human cell lines were used to map the localization of 12,003 proteins to 30 cellular structures and substructures [57].

Transcriptomic approaches have been widely used to determine cell-type or tissue-specific patterns of gene expression, as well as changes in gene expression under specific conditions. Integration of PPI networks with transcriptomics has long been used to extract more detailed models of protein functioning [147,148]. For example, expression data have been used to characterize the dynamics of protein complex formation during the yeast cell cycle [149] and to investigate changes in PPI networks during aging [150]. Correlation of PPI and expression data has also been used to extract more global properties of interaction networks. The yeast interactome was found to contain two types of "hub" proteins: those that interact with most of their partners simultaneously and those that bind their partners at different times or locations [151]. A similar analysis extended this observation to human protein interaction networks and showed that alterations in the interaction profile of the hub protein BRCA1 in breast cancer patients can predict disease outcome [152]. Finally, expression data are an excellent source of information to determine cell-type, tissue, or organism-specific differences in protein interaction networks.

A final localization method we would like to highlight uses separation of organelles by density gradient centrifugation coupled with quantitative MS to obtain proteome scale information on the subcellular localization of proteins [153–159]. In this approach, organelles are separated by density gradient centrifugation, and the distribution of proteins across fractions is compared with the distribution of known organelle markers to assign proteins to one or more cellular compartments. Different implementations of this approach have been used to map the localization of thousands of proteins to tens of subcellular localizations for *Arabidopsis*, HeLa cells, rat and mouse liver, mouse pluripotent stem cells, and retinal

pigment epithelial cells [153–159]. Moreover, similarity in subcellular localization patterns can be used to predict physical association, with results comparable to correlation profiling approaches based on separation of protein complexes (see below) [159].

In most instances, PPI networks and expression or localization data are generated separately. However, several groups have directly combined microscopy observations with PPI mapping. For example, by using purification tags incorporating a fluorescent protein, AP-MS and microscopy have been used to study protein complexes at the kinetochore in *C. elegans* and HeLa cells [160,161] and to identify and characterize a large series of mitotic protein complexes [162]. Similarly, in *Drosophila,* a random insertion-based protein trap method was used to tag 346 genes with yellow fluorescent protein and an affinity tag [163]. The strain collection was used to determine the expression pattern and subcellular location of the proteins and to identify associated proteins by AP-MS [163].

Experimental approaches to investigate protein interaction dynamics

Subcellular location mapping by proximity labeling

Proximity-dependent labeling is an MS-based approach to identify interaction partners based on their proximity to a protein of interest. Proximity labeling thereby intrinsically provides information of the subcellular location of interactions: if the subcellular localization of the "bait" protein is known, any labeled proteins can be postulated to be localized nearby.

Proximity-dependent labeling techniques are based on the covalent labeling with biotin of proteins under physiological conditions. The labeled proteins are identified by streptavidin purification and MS analysis. Since there is no need to keep complexes intact, stringent lysis and purification conditions can be used. Hence, proximity labeling facilitates the detection of transient interactions, interactions with poorly solubilized proteins, and detection of underrepresented proteins. A drawback can be that any proteins in close enough proximity to the bait protein are labeled, not just physically interacting proteins.

The two proximity labeling techniques that are currently in common use are proximity-dependent BioID and APEX labeling. In BioID, the bait protein is fused to a promiscuous variant of the *Escherichia coli* biotin ligase BirA (termed BirA*), which will biotinylate any proteins within a radius of ~20 nm [164,165]. APEX is a plant-derived ascorbate peroxidase that, in the presence of H_2O_2, catalyzes the oxidation of a biotin–phenol substrate to biotin–phenoxyl, a highly reactive radical that will covalently attach itself to the electron-rich sidechains of amino acids, primarily tyrosines [166–169]. Biotin–phenoxyl is short-lived (~5 ms), leading to a small labeling radius of ~20 nm. APEX was originally developed as a genetically encoded reporter for electron microscopy (EM) imaging [167]. APEX catalyzes the H_2O_2-dependent polymerization of 3,3′-diaminobenzidine into a local precipitate that can recruit electron-dense osmium to give EM contrast [167]. Hence, an APEX-tagged bait protein can be used both to identify protein interactors and for high-resolution subcellular localization studies.

The main difference between BioID and APEX is the timescale. While optimal labeling with BirA* takes hours, labeling with APEX occurs within tens of seconds. Hence, BioID provides a historic overview of all proteins having come into contact with the bait protein over a longer period of time, while APEX provides a snapshot of all nearby proteins at a particular moment in time. While both approaches label non-interacting proteins that are in close proximity, this effect is more pronounced with APEX due to the production of a diffusible radical.

Ludwig and colleagues have recently applied APEX tagging to study the spatial and molecular organization of apical polarity proteins in fully polarized MDCK-II cells (unpublished data; A. Ludwig, personal communication). Stable cell lines expressing APEX2 fusions of PAR3 and PALS1 and a combination of pair-wise quantitative proximity proteomics and EM imaging were used to address how these proteins are organized at the apical–lateral membrane border. In a second step, the PAR3 and PALS1 lines were compared to a line expressing APEX2 in the cytoplasm. This greatly reduced the number of cytoplasmic "contaminants" from the PAR3/PALS1 proximity map and permitted the identification of potentially novel PAR3/PALS1 interactors. Since APEX-mediated proximity biotinylation is rapid, this new technology may prove useful in dissecting the dynamic organization of the polarity network by time-resolved proximity proteomics.

Use of BioID in cell polarity studies. BioID has been used relatively frequently in studies of cell polarity, in particular to probe the composition of cellular junctions. Because of the connections to the cytoskeleton, many components of cellular junctions are insoluble, making BioID an excellent tool to probe these interactions. A total of four junctional components have been used as bait proteins in BioID experiments from tissue culture cells: the TJ scaffolding MAGUK protein ZO-1 [170], the TJ barrier proteins OCLN and Claudin-4 [171], and the adherens junction component E-cadherin [172,173]. Each of these studies identified hundreds of candidate TJ- or adherens junction-associated proteins, and particularly the most abundantly identified proteins contained many that were previously known to be localized to cell junctions or involved in cadherin functioning. Together, these four studies

provide a catalog of candidate junctional and junction-associated proteins.

A study of the mechanisms through which intestinal epithelial cells gain the ability to endocytose materials on their apical side highlights the ability of BioID to identify proteins that act together in a defined subcellular localization [174]. The MARVEL domain protein plasmolipin (PLLP) was found to be required for the differentiation of gut enterocytes in zebrafish, and the maturation of the apical recycling endosome (ARE) compartment. Using BioID in MDCK cells, the clathrin adaptor EpsinR and the SNARE protein Syntaxin-7 were identified as PLLP-associated proteins. PLLP was hypothesized to recruit EpsinR, which in turn promotes the polarized recycling of Syntaxin-7 to AREs, thus promoting endocytic uptake of apical cargo, including Crumbs and Notch.

Protein correlation profiling

Although AP-MS is a powerful approach to identify protein interactions, the necessity to tag, purify, and analyze each protein to be investigated complicates large-scale analysis of interactions across different conditions. A more recent development is the identification of protein complexes on a global scale based on biochemical fractionation coupled with quantitative MS [40–43]. In such protein correlation profiling approaches, cell lysates are subjected to extensive fractionation of protein complexes using techniques such as ion exchange chromatography, sucrose gradient centrifugation, isoelectric focusing, or size-exclusion chromatography. Proteins with similar elution profiles are considered likely to be associated in a complex. The major advantage of this approach is that large numbers of protein interactions are simultaneously assessed in a fixed number of MS runs. Of course, there are downsides, for example, in profiling insoluble complexes or complexes that do not survive the fractionation procedure. Nevertheless, the increase in scale makes it possible to address aspects of protein interaction dynamics that cannot easily be addressed using conventional AP-MS approaches. For example, changes were detected in the composition of the interactome upon epidermal growth factor stimulation [42], and Wan et al. [43] investigated the evolutionary conservation of protein complexes by profiling protein complexes from eight different species. This approach could readily be used to study dynamic aspects of cell polarity, for example, investigating complex formation during the development of a polarized tissue or cell type.

Differential interaction mapping

An intuitive way to identify dynamic changes in a protein interaction network is to experimentally determine protein interactions in different conditions, or over time as a cell or tissue develops or responds to a stimulus. In principle, any PPI mapping technology is suitable for such an approach, provided it identifies interactions under native conditions. The LUMIER system described above, for example, was developed to identify interactions in mammalian cells, specifically with the goal of determining protein interaction dynamics in mind. It was used to probe changes in the TGFβ PPI network in the presence or absence of signaling, and identified a dynamic interaction network that is remodeled upon TGFβ signaling [28].

Another binary PPI mapping approach used to probe interaction dynamics is the protein fragment complementation assay (PCA). In PCAs, a reporter protein is split into two fragments, each fused to a protein of interest. If the two proteins interact, the fragments of the reporter assemble into an active reporter protein. Several split reporter proteins are in use, including ubiquitin (see split-ubiquitin discussed above), luciferase, adenylate cyclase, and various fluorescent proteins such as GFP [30,175,176]. A PCA assay using split murine dihydrofolate reductase was recently used to investigate interactions between ~100 protein pairs in *S. cerevisiae* under different growth conditions [177]. The presence of intact murine dihydrofolate reductase can be readily detected as it allows growth of yeast in the presence of the yeast DHFR inhibitor methotrexate, making the system amenable for high-throughput applications [178]. A pooled competitive growth assay was used in combination with a genomic barcoding strategy to follow multiple interacting protein pairs. While this approach is specific for use in *S. cerevisiae*, other PCA assays can be adapted to investigate protein dynamics in other systems.

AP-MS strategies are in principle also highly suited to identify dynamics in interaction networks, as they isolate protein complexes from their native environment. However, MS is intrinsically not a quantitative technology, a prerequisite to accurately follow changes in protein interactions. Several approaches have been developed to enable quantitative MS studies. A common method is based on the differential labeling of proteins or peptides from different samples, enabling quantitative determination of their relative levels. Examples include metabolic labeling of proteins with stable isotopes (^{13}C and ^{15}N) (e.g., stable isotope labeling with amino acids in cell culture), and the chemical labeling of peptides or proteins after cell lysis with reagents such as iTRAQ, ICAT, or TMTs [31,179]. Label-free techniques that quantify proteins and peptides based on information contained in the MS data have also gained in popularity [180]. If the set of protein interactions whose dynamics is to be determined is known, a targeted MS approach (selected reaction monitoring, or multiple reaction monitoring) can be used to monitor a defined set of peptides with very high sensitivity and large dynamic range of quantification [179]. A more recent variant,

SWATH-MS, aims to combine the advantages of shotgun proteomics (the large-scale identification of proteins from complex mixtures) and targeted proteomics, by fragmenting all ions within a certain mass/charge range [181]. In combination with affinity purification, SWATH-MS in theory enables the reliable and consistent quantification of PPIs under different conditions or in a time-course experiment. This system was used, for example, to probe changes in the 14–3–3β interactome upon stimulation of HEK293 cells with IGF1 [182], and the GRB2 interactome in primary peripheral CD4+ T cells before or after activation with anti-CD3 and anti-CD4 antibodies [183].

Using MS to probe phosphorylation dynamics

Phosphorylation events mediated by serine/threonine protein kinases play an essential role in regulating the activities and dynamics of the protein networks that control cell polarity. A classic example is the exclusion of the basolateral polarity regulators PAR1 and LGL1 from the apical membrane domain through aPKC-mediated phosphorylation [184–189]. Although the accurate detection of phosphorylated peptides remains challenging, MS approaches provide an attractive way to probe the dynamics of phosphorylation. This approach was used, for example, to probe the mechanisms by which cell polarity is coupled to spindle positioning [190]. Spindle positioning in polarized cells relies on a conserved protein complex (LIN-5/GPR-1,2/Gα in *C. elegans*, Mud/Pins/Gα in *Drosophila*, and NuMA/LGN/Gα in humans), which anchors the dynein microtubule motor at the cell cortex [191]. MS analysis of LIN-5 purified from *C. elegans* embryos revealed extensive phosphorylation. To identify the responsible kinases, a quantitative AP-MS approach was used based on growth of whole animal populations on media with either ^{14}N or ^{15}N as the nitrogen source. Mixing control and kinase RNAi cultures allowed for determining the *in vivo* effect of candidate kinases. This resulted in the identification of PKC-3^{aPKC} as a kinase phosphorylating LIN-5^{NuMA}, as a mechanism contributing to the control of spindle positioning during asymmetric cell division [190].

Low-throughput methods to probe the spatiotemporal dynamics of PPIs

The focus of this review has been on high-throughput approaches, but there are times when the detailed observations afforded by low throughput methods to follow protein interactions are required. An example of a highly informative, though experimentally challenging, approach to follow the spatiotemporal dynamics of a protein interaction *in vivo* is Förster (or fluorescence) resonance energy transfer (FRET) [192]. In FRET, the energy emitted by a donor fluorophore excited by fluorescent light is transferred to an acceptor fluorophore. This results in the emission of light at the characteristic wavelength of the acceptor and reduced emission of light at the emission wavelength of the donor. The efficiency of FRET is highly dependent on distance and typically efficient when donor and acceptor fluorophores are 3–6 nm apart [193]. Hence, FRET can be used to monitor when and where two proteins come in very close proximity (i.e., interact).

One recent development we would like to highlight is that of single-molecule pull-downs, an adaptation of affinity purification that can be used to obtain time-resolved information on the dynamics of protein interactions in single cells [194,195]. In this approach, a cell lysate is introduced onto a surface to which an affinity capture reagent has been attached. The affinity reagent captures a protein of interest (bait), as well as any associated proteins (prey). The bait and prey proteins are labeled with a fluorescent tag, enabling detection of an interaction as co-localization in TIRF microscopy. By determining the fraction of spots in which two proteins co-localize, the presence of two proteins in a complex is quantitatively resolved. Moreover, the stoichiometry of protein complexes can be determined by performing photobleaching step counting, where the number of steps in which a fluorescent spot bleaches corresponds to the number of fluorescently labeled proteins present. Only a limited amount of sample is needed (down to a single cell), and measurements can start within a minute of cell lysis. As with any method, there are drawbacks of course, such as difficulty detecting weak interactions, incomplete labeling of bait or prey proteins, and an inability to identify novel interactions.

A single-molecule pull-down approach was recently used to investigate the dynamics of PAR-3 oligomerization and association with PAR-6/aPKC during polarization of the *C. elegans* embryo. This revealed that PAR-3 oligomerization is temporally regulated and essential for membrane localization of PAR-3, complex formation with PAR-6/aPKC, and efficient transport of the PAR-3/PAR-6/aPCK complex to the anterior cortex [72]. Moreover, the polo-like kinase PLK-1 was identified as a regulator of PAR-3 oligomerization. PLK-1 physically interacts with PAR-3 and phosphorylation by PLK-1 of PAR-3 on residues in the conserved region 1 oligomerization domain inhibits oligomerization [72]. Thus, PLK-1 may couple PAR protein segregation with cell cycle progression.

Summary and concluding remarks

The identification of all PPIs that occur in an organism, termed “interactome,” has been a long-standing goal and interactome mapping approaches have matured to generate networks of high confidence. PPI mapping approaches have been instrumental in

unraveling many biological processes, and the studies we included in this review highlight important contributions made to our understanding of cell polarity. Nevertheless, few high-throughput approaches have focused specifically on the interactions that underlie cell polarity. Moreover, most approaches that identify protein interactions from their native environment use non-polarized cells grown in tissue culture, when polarity is a process of which many aspects will only come to light when investigated in the right cell type, or in a proper 3D context. There are therefore ample opportunities yet to use interactome mapping approaches in a more directed fashion to uncover novel mechanistic details of how cells control polarity.

The next major challenges in interactome mapping are to address the complexities introduced by the presence of different protein isoforms and posttranslational modifications, and to develop high-throughput methods to investigate how PPIs are regulated, to determine at what developmental times or under what signaling conditions interactions take place, and to determine in what cells or tissues and at which subcellular location interactions take place. We have highlighted several efforts that are being undertaken in this direction, and undoubtedly, the next decade will see new and improved technologies to investigate PPI dynamics systematically. As a process that depends on protein interactions controlled in time and space, cell polarity studies stand to benefit greatly from such developments.

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†http://www.proteinatlas.org

Abbreviations used:
AP-MS, affinity purification–mass spectrometry; ARE, apical recycling endosome; BioGRID, biological general repository for interaction data sets; BioID, biotin identification; DB, DNA binding; EMT, epithelial-to-mesenchymal transition; FRET, Förster (or fluorescence) resonance energy transfer; GFP, green fluorescent protein; HUVEC, human umbilical vein endothelial cells; LUMIER, luminescence-based mammalian interactome; MAPPIT, mammalian protein–protein interaction trap; MS, mass spectrometry; PCA, protein fragment complementation assay; PDZ, PSD-95/Dlg/ZO-1; PPI, protein–protein interaction; TJ, tight junction; XL-MS, cross-linking mass spectrometry; Y2H, yeast two-hybrid.

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