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How to cite

ROUAUD, Florian et al. Scaffolding proteins of vertebrate apical junctions: structure, functions and biophysics. In: Biochimica et biophysica acta. Biomembranes, 2020, vol. 1862, n° 10, p. 183399. doi: 10.1016/j.bbamem.2020.183399

This publication URL: https://archive-ouverte.unige.ch/unige:173706
Publication DOI: 10.1016/j.bbamem.2020.183399

BBA - Biomembranes 1862 (2020) 183399

Contents lists available at ScienceDirect

BBA - Biomembranes

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

Review

Scaffolding proteins of vertebrate apical junctions: structure, functions and biophysics

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ARTICLE INFO

Keywords:
Tight junction
Adherens junction
ZO-1
Cingulin
Paracingulin
PLEKHA7
Afadin
MAGI
Par3
Par6
PATJ
Pals1
Angiomotin

ABSTRACT

Tight and adherens junctions are specialized sites of cell-cell interaction in epithelia and endothelia, and are involved in barrier, adhesion, and signaling functions. These functions are orchestrated by a highly organized meshwork of macromolecules in the membrane and cytoplasmic compartments. In this review, we discuss the structural organization and functions of the major cytoplasmic scaffolding and adaptor proteins of vertebrate apical junctions (ZO proteins, afadin, PLEKHA7, cingulin, paracingulin, polarity complex proteins, and a few others), focusing on their interactions with cytoskeletal and signaling proteins. Furthermore, we discuss recent results highlighting how mechanical tension, protein-protein interactions and post-translational modifications regulate the conformation and function of scaffolding proteins, and how spontaneous phase separation into biomolecular condensates contributes to apical junction assembly. Using a sequence-based algorithm, a large fraction of cytoplasmic proteins of apical junctions are predicted to be phase separating proteins (PSPs), suggesting that formation of biomolecular condensates is a general mechanism to organize cell-cell contacts by clustering proteins.

1. Introduction

Multicellular organisms require specialized structures and mechanisms to assemble cells into tissues, to allow them to communicate and operate as a functional unit. Epithelial and endothelial cells interact together through cell-cell junctions, which comprise tight (TJ) and adherens junctions (AJ/ZA), desmosomes and gap junctions. Junctions are organized in a modular fashion: transmembrane proteins are anchored to the cytoskeleton through scaffolding and adaptor proteins. Research in the last four decades has led to the identification and functional characterization of a large number of these proteins and their interactors, often associated with signaling functions. Here we outline the main functions of apical junctions, and we summarize our current understanding of how specific scaffolding and adaptor proteins contribute to the biology of vertebrate cells, tissues, and organisms. We also discuss recent results on biophysical mechanisms of regulation of these proteins, which open new questions and perspectives for future studies. We focus on "zonular" vertebrate proteins, e.g. cytoplasmic scaffolding proteins which in polarized epithelia are localized exclusively either at TJ, or at the underlying circumferential cadherin-based junction (*zonula adhaerens*). We refer the reader to excellent reviews for further information about scaffolding proteins, such as the catenin family of proteins, which in epithelial cells are also abundantly distributed along lateral contacts [1–4], as well as information about cytoplasmic proteins of apical junctions in invertebrate model systems [5–9], and additional mechano-sensing proteins of non-zonular cell-cell and cell-substrate adhesions [10–12].

2. The functions of apical junctions

The apical junctional complex (AJC) of vertebrate epithelial cells comprises tight junctions (TJ), also known as *zonulae occludentes* (ZO), and *zonulae adhaerentes* (ZA). These two junctions form continuous circumferential belts ("*zonulae*") around the apico-lateral region of polarized epithelial cells and are associated with a highly organized network of cytoskeletal filaments. TJ and ZA were first identified by transmission electron microscopy, which revealed the morphology of the junctional membranes, and the presence of distinct electron-dense membrane and cytoplasmic domains in each type of junction [13].

The TJ is the most apical component of the epithelial AJC and is always located immediately above the ZA. The TJ and ZA are molecularly interconnected in their biogenesis: on the one hand, the formation of the AJ and then the ZA is a pre-requisite for TJ formation [14], and on the other hand ZO-1, the major scaffolding protein of TJ, is

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https://doi.org/10.1016/j.bbamem.2020.183399
Received 13 January 2020; Received in revised form 5 June 2020; Accepted 11 June 2020
Available online 15 June 2020

first assembled into cadherin-based AJ, and is required for correct integration of myosin in the ZA and the formation of a continuous ZA belt [15,16]. ZO-1 interacts at AJ/ZA with the actin-binding proteins α-catenin and afadin, and the formation of TJ involves the segregation of ZO-1 from AJ/ZA to TJ, concomitant with assembly of claudin-based TJ fibrils apically, and the establishment of a spatially distinct ZA immediately below [17–20]. Different lines of evidence indicate that conformational changes in ZO-1, resulting from actomyosin contractility that develops at the ZA and specific protein-protein interactions, promote TJ assembly [21,22]. Important factors in the formation and separation of AJ and TJ are also the level of expression of cell adhesion molecules, and their apical polarity protein-dependent repositioning [23,24]. Although the biogenesis of TJ and ZA are coordinated, TJ and ZA proteins are effectively segregated into distinct macromolecular complexes in most mature polarized epithelia, with some exceptions, one being for example the heterotypic junctions between sensory and non-sensory cells of the inner ear [25].

The main canonical function of TJ consists in establishing and maintaining a selective permeability barrier for the passage of water, ions and solutes through the paracellular pathway. This barrier is crucially important not only to protect the organisms from the entry of pathogens, antigens and molecules [26,27], but also to mediate the establishment and maintenance of physiological gradients between extracellular compartments [28]. Thus, the TJ barrier function is crucial for the physiology of major organs, including the kidney, the gastrointestinal tract, exocrine glands, the epidermis, and vascular endothelial cells involved in tissue barriers, such as the blood-brain, blood-testis, blood-retina, and blood-placental barriers [28,29]. The main molecular constituents of the TJ barrier are claudins, a family of > 27 isoforms, which are differentially expressed in cells and tissues, according to their specific physiological requirements [30,31]. Claudins assemble in cis into polymers scaffolded by ZO proteins [32], and claudin polymers associate in trans with polymers from adjacent cells, to form the TJ strands visible by freeze-fracture electron microscopy, which are the structural hallmark of the TJ barrier [33,34]. In addition to claudins, TJ membrane proteins include additional 4-pass membrane proteins, such as occludin and tricellulin, and Ig-like adhesion molecules, such as JAM-A (Junctional Adhesion Molecule), CAR (Cocksackie and Adenovirus Receptor) and ESAM (Endothelial cell Selective Adhesion Molecule), which are involved in TJ barrier regulation [24,35], and in adhesion between epithelial cells [12]. Tricellular TJ contain tricellulin and angulins and represent important sites for permeability of large molecules through the TJ barrier [36,37]. The second canonical function of TJ is to form an intramembrane fence to prevent the free diffusion of transmembrane proteins and exoplasmic membrane leaflet lipids between apical and lateral compartments, and thus maintain membrane polarity [28]. Although claudin-4 can partially reconstitute the TJ intramembrane fence in artificial membrane vesicles in vitro [38], the membrane polarity is maintained in epithelial cells lacking claudins [24,39], indicating redundant functions with additional transmembrane components. In contrast, disrupting the ZO protein-dependent connection between TJ transmembrane proteins and the cytoskeleton disrupts the localization of polarity complex proteins [24], pointing to a critical role of cytoskeletal organization and force in regulating membrane domain segregation. Finally, an important function of TJ is to participate in the homeostatic mechano-regulation of cellular functions, for example to maintain barrier function under tensile stress. Several lines of evidence support the notion that the cytoplasmic TJ plaque functions as an insulator, to protect the claudin strands from the forces generated at the neighboring ZA [21,22,40–43].

The *zonula adhaerens* (ZA) is the epithelial-specific form of cadherin-based AJ. The ZA is not described in endothelial cells, since TJ and AJ are molecularly intermixed in endothelia, and circumferentially organized, distinct TJ and ZA are not detected [44]. Cadherin-based adhesions are present in epithelial and non-epithelial cells, but in polarized epithelial cells cadherin-based adhesions are present both at the ZA, and all along lateral contacts. Lateral contacts are underlain by loosely organized actin and myosin filaments [1,45], and whether lateral contacts can be broadly classified as AJ remains to be determined [1]. What distinguishes ZA and AJ from cadherin-based lateral contacts is that lateral contacts do not show clustering of junctional components: transmembrane adhesion molecules, underlying cytoplasmic scaffolding proteins, and cytoskeletal filaments. Moreover, what distinguishes ZA from lateral contacts molecularly and functionally in epithelial cells are at least two features. The first is the accumulation of afadin and PLEKHA7 at the ZA, which are undetectable, thus absent or at very low levels, along lateral contacts [46,47]. Additional proteins that are exclusively detected at the ZA include ADIP, vinculin, α-actinin and ponsin [48]. The second feature is the presence at the ZA and not lateral contacts of a highly organized circumferential (peri-junctional) contractile belt of actomyosin, which comprises cytoplasmic nonmuscle myosin-2A and β-actin filaments. This belt is tethered to the juxtamembrane area by a branched network of cytoplasmic nonmuscle myosin-2B and γ-actin filaments [22,49]. Lateral contacts have loosely organized cortical actin and myosins, and although clustered actin and myosin filaments are associated with AJ in non-epithelial cells, their spatial arrangement is different from the ZA [1]. The sarcomere-like circumferential arrays of myosin and actin filaments occur only at the ZA, and they form a transcellular cable system that links together neighboring cells [50]. The main canonical function of the ZA is in fact to maintain cell-cell adhesion and integrity of epithelial tissues, through the actomyosin belt, which is tethered to cadherin complexes to provide a strong mechanical resistance against stresses applied to cells and the tissue. It is important to note, however, that both afadin and PLEKHA7, as well as other components of the ZA, and ZO-1, are detected at AJ of non-epithelial cells, which are spot-like and not circumferential, though still associated with bundled cytoskeletal filaments [51], indicating that clustered, force-regulated assemblies of these proteins are used in a different spatial configuration in non-epithelial cells.

In addition to these canonical functions of barrier, adhesion and mechano-regulation, TJ and ZA fulfill major roles in signaling, to control the organization of the cytoskeleton, the maintenance of apicobasal polarity, and the regulation of gene expression, cell proliferation, differentiation and morphogenesis. Molecular mechanisms involved in these functions include the recruitment, regulation and sequestration of transcription factors, RNA binding proteins, and GEFs (Guanine nucleotide Exhange Factors) and GAPs (GTPase Activating Proteins) that fine-tune the activities of Rho family GTPases, and thus orchestrate local and global cytoskeletal organization (reviewed in [35,52–54]). Furthermore, proteomic analysis shows that TJ contain a number of molecules, such as synaptotagmin-VII, HOMER, and glutamate and NMDA (N-Methyl-D-Aspartate) receptors, which are typical of presynaptic and postsynaptic contacts, and others that indicate roles in vesicular trafficking, membrane rheology, and translation [55]. In essence, together with other types of junctions, TJ and ZA/AJ function as major structural and signaling platforms to regulate the shape and activities of individual cells, and their collective behavior as a tissue.

3. ZO proteins: scaffolds for the assembly of the claudin-based paracellular TJ barrier

ZO (*Zonula Occludens*) proteins comprise ZO-1, ZO-2 and ZO-3. ZO-1 (220 kDa) was identified both as a TJ and AJ protein by raising monoclonal antibodies against junctional membrane fractions from liver [56–58]. ZO-2 and ZO-3 were identified as 160 kDa and 130 kDa proteins present in ZO-1 immunoprecipitates [59,60]. ZO proteins are expressed in epithelial, endothelial and non-epithelial cells, and most cells express two ZO proteins, typically ZO-1 and ZO-2 [61–65], whereas ZO-3 expression is less widespread [66,67]. Invertebrate organisms only express one ZO protein. The *D. melanogaster* homolog of ZO-1, *Polychaetoid*, is localized at AJ, and is required for correct epithelial morphogenesis [68–70]. In *C. elegans*, the ortholog ZOO-1

cooperates with the cadherin-catenin complex in the anchorage of AJ to the actin cytoskeleton during morphogenesis [71].

ZO proteins are the most important scaffolding proteins for transmembrane TJ proteins [32,72–78]. Specifically, they are required for the assembly of claudin-based TJ strands, and thus for the establishment, maintenance and regulation of TJ barrier function [32,79]. Depletion of ZO-1 in epithelial cells causes delayed recruitment of claudins and occludin to TJ, and delayed barrier establishment [80,81]. The depletion of ZO-2 has no discernible effects on TJ structure, but decreases claudin expression, cell aggregation and the recruitment of ZO-1, occludin and cadherin during junction assembly [82]. On the other hand, ZO-3 is not required for TJ assembly [83], and its expression does not rescue the phenotype of cells depleted of ZO-1 and ZO-2 [32]. Importantly, only cells that are double KO/KD for ZO-1 and ZO-2 show a complete loss of TJ strands, demonstrating that ZO-1 and ZO-2 have redundant functions in claudin scaffolding [32]. However, non-redundant functions of ZO-1 and ZO-2 during early vertebrate development are indicated by the observation that the single KO of either ZO-1 or ZO-2 in mice leads to an early embryonic lethal phenotype, with a failure in junction formation, and a disruption of the paracellular barrier [84,85]. In contrast, ZO-3 is dispensable for viability, although KO male mice are sterile, due to changes in Sertoli cell junctions [83,85]. In embryoid bodies, ZO-1 and ZO-2 are required for extra-embryonic endoderm integrity, primitive ectoderm survival and normal cavitation [86]. In this model system, double KO of ZO-1 and ZO-2 causes epithelium disorganization and loss of TJ barrier function, whereas KO of ZO-2 alone has no phenotypic effect, and KO of ZO-1 alone has an intermediate phenotype [86]. Intestinal epithelial-specific ZO-1-KO mice show epithelial cells with normal polarity, but an excessive contraction of subapical actomyosin, the formation of junctional actin rings, and a disrupted brush border structure [41].

What is the structural basis for the scaffolding of transmembrane proteins by ZO proteins? As shown in Fig. 1, ZO proteins comprise multiple structural domains, and their N-terminal PDZ (Psd95-Dlg-ZO-1) domains interact with the C-terminal PDZ-binding motifs of claudins [78]. The PDZ1 and PDZ2 domains of ZO-1 also interact with the C-termini of connexins, implicating a role of ZO-1 in the organization and regulation of gap junctions [87–94]. In agreement, inducible cardiomyocyte-specific ZO-1 deletion in mice alters atrio-ventricular node conduction by disrupting the accumulation of connexin-40 (Cx40) at gap junctions [95]. ZO-1 also interacts with cystic fibrosis transmembrane conductance regulator (CFTR) [96] (Fig. 1) and is detected at slit diaphragms in the kidney glomerular epithelium [97,98]. In fact, podocyte-specific depletion of ZO-1 results in glomerulosclerosis, with aberrant distribution of the slit diaphragm components [98,99]. Other domains of ZO proteins, distinct from the PDZ1 domain, scaffold additional transmembrane proteins. For example, JAM-A binds to the PDZ2/3 domain of ZO-2 and ZO-1, respectively [72], and occludin to the GUK (guanylate kinase) domain [75–77]. In summary, ZO-1 is a multifunctional scaffolding protein involved in diverse junctional and non-junctional complexes.

The claudin scaffolding function of ZO proteins is regulated. Specifically, scaffolding requires ZO protein dimerization, since the phenotype of cells depleted of ZO-1 and ZO-2 cannot be rescued by a N-terminal fragment of ZO-1 containing the PDZ domains but lacking the SH3-U5-GUK-U6 region (SG module), but is rescued by a dimerizable, truncated form of ZO-1 that contains SH (Src Homology 3) and GUK domains [32]. Dimerization of ZO proteins occurs through PDZ2-PDZ2 [100,101], and SG-SG interactions [32,102,103]. However, this latter region is also crucial for the indirect interaction of ZO proteins with the actomyosin cytoskeleton: the SG module binds to α-catenin [18,77,104], vinculin [105], afadin [20,106], the spectrin-binding protein protein 4.1R [107], and the myosinVII- and actin-binding protein shroom2 [108] (Fig. 1). Furthermore, intramolecular interactions

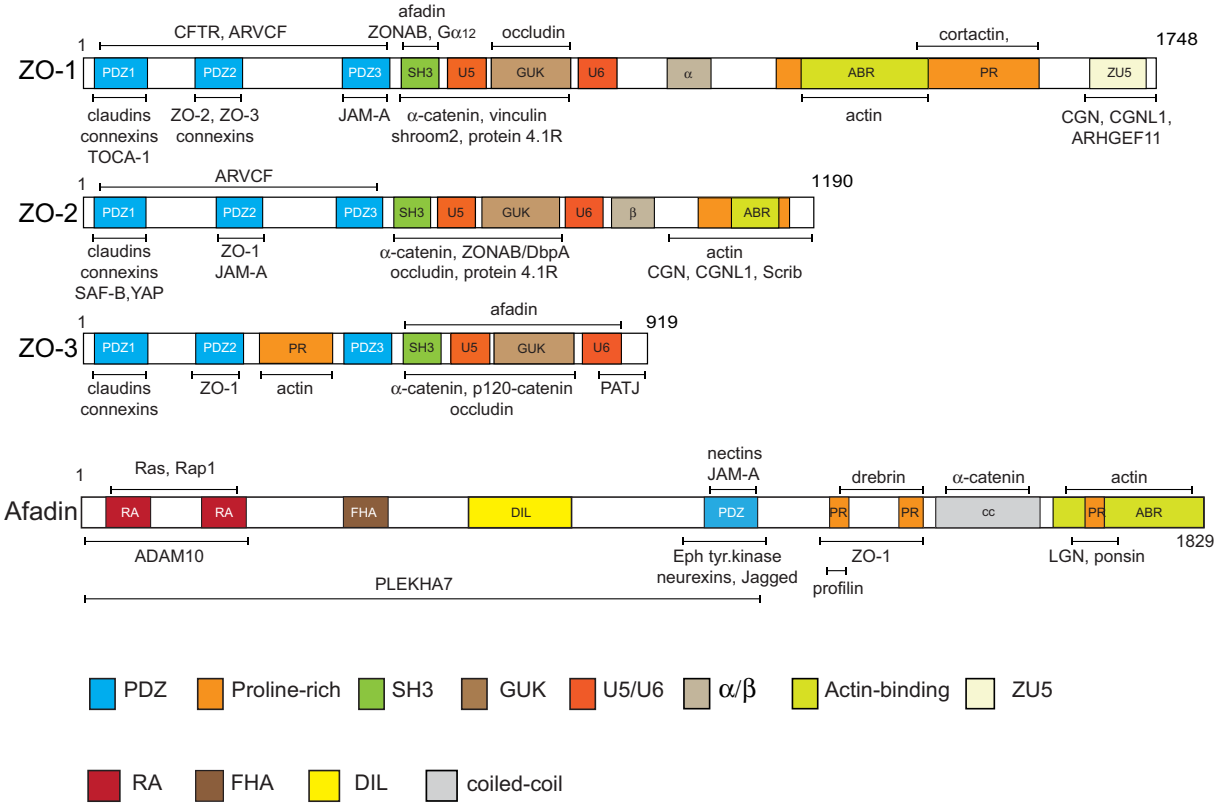

Fig. 1. The domain organization of ZO proteins (ZO-1, ZO-2, ZO-3) and afadin. Domains are color-coded and indicated below the graphs. Interacting partners are indicated above or below each protein/domain, with a line that delineates the approximate localization of the binding sites(s). Interacting proteins for which the precise binding site(s) have not been defined have been omitted from the schemes.

occur between the SH3-GUK, U5 and U6 domains [109], which control ZO protein targeting to junctions and coordinate its activities [16,32,75,110–112]. Importantly, the PDZ3-SH3-GUK supramodule of ZO-1 (ZPSG) also undergoes a mechano-sensitive intramolecular interaction with the C-terminal ZU5 domain (Fig. 1), which controls the scaffolding of its interactors occludin and DbpA/ZONAB [21], this latter a transcription factor that binds to ZO-1, as described below. In summary, the PDZ and ZPSG regions are critically involved in scaffolding functions of ZO proteins, through both intra- and intermolecular interactions, and these interactions are subjected to regulation by actomyosin-dependent force [21].

4. ZO proteins: major players in the cross-talk between TJ and the actin cytoskeleton

The interaction of TJ with the actin cytoskeleton is crucially important for TJ assembly and dynamics, for the regulation of TJ barrier function, and for the organization of the apical actomyosin cytoskeleton. Indeed, although giant unilamellar vesicles (GUVs) where claudin-4 has been exogenously reconstituted show limited intramembrane diffusion of proteins [38], there is no evidence that a functional paracellular barrier can either be generated or regulated in the absence of cytoskeletal anchorage.

ZO proteins play a central role in the cross-talk of TJ transmembrane proteins with the actin cytoskeleton. ZO proteins co-pellet with actin filaments, and exogenous truncated constructs comprising the C-terminal domains of ZO-1 and ZO-2 are targeted to actin filaments in transfected cells [75,101], whereas ZO-3 binds to actin filaments through its N-terminal region [113] (Fig. 1). Some observations indicate that ZO-1 and actin filaments may be loosely coupled directly [114,115], and the ZO-1 actin-binding region (ABR) is not required for cytoskeleton-associated ZO-1 functions [41]. This suggests that ZO-1 structural/functional interactions with actin filaments might be either indirect, or regulated by multivalent interactions with other proteins. These may include cingulin and paracingulin [116–119] and ARHGEF-11, which bind to the ZU5 domain [22,120], and cortactin, which interacts with the C-terminal proline-rich region in *Drosophila* ZO-1 [121] (Fig. 1). For example, unfolding of the ZU5 domain of ZO-1 by cingulin has been proposed to modulate the interaction of ZO-1 with phalloidin-labelled actin filaments [22]. Furthermore, as noted above, the ZPSG region interacts with several additional actin-binding proteins, such as afadin, vinculin and α-catenin (Fig. 1). Thus, both direct and indirect interactions are involved in ZO protein cross-talk with the actomyosin cytoskeleton. In addition, a third mechanism is the direct or indirect interaction of ZO-1 with signaling proteins that control Rho family GTPases, as discussed below.

Through their different interactions, ZO proteins organize actomyosin filaments at the AJC. For example, ZO proteins are required for the integration of cytoplasmic nonmuscle myosin-2 into the apical junctional belt and for correct actomyosin organization at cell-cell contacts [15,16,24,41,42,79,120,122,123]. The ability of ZO-1 to modulate the organization of cytoplasmic nonmuscle myosin-2B (NM2B) depends on cingulin, since membrane tortuosity is lost and NM2B accumulation at junction is reduced in cells KO for cingulin, when ZO-1 is still present [22]. Depletion of both ZO-1 and ZO-2 induces a dramatic expansion in the thickness of the AJ-associated actomyosin belt and the accumulation of myosin-2 at the cell apex [41,124], thereby increasing apical and junctional tension [125]. This and other evidence support the hypothesis that ZO proteins together with cingulin act as dissipators of mechanical force generated by the actomyosin cytoskeleton at junctional contacts [22,43] (reviewed in [12]). The ability of ZO-1 to organize the cytoskeleton may be modulated by alternative splicing of the α domain (Fig. 1), which distinguishes two isoforms for ZO-1, α(+) and α(−) [126–128], which are differentially expressed during early mouse development [129]. It was recently shown that the α(−) isoform is associated with enhanced assembly of actin stress fibers and is expressed in higher levels in cancer cells and in cells that have undergone epithelial-to-mesenchymal transition [130].

Through their bidirectional interaction with actomyosin, ZO proteins regulate TJ barrier function [131]. Claudins form pores and barriers with size and charge-selectivity for ions, whereas larger molecules permeate through transient breaks in claudin strands, and through tricellular junctions [30]. ZO protein dynamics and exchange between cytoplasmic and junction-associated pools is regulated by myosin light chain kinase (MLCK)-dependent activation of actomyosin contractility [132], and the dynamic behavior of claudins is regulated by their intermittent tethering to the ZO protein scaffold [114]. Rho-dependent activation of MLCK also promotes increased permeability to solutes in physiological and pathological conditions by remodeling TJ structure [133–135].

5. Scaffolding of signaling proteins by ZO proteins

ZO proteins act as direct and indirect scaffolds for regulators of Rho family GTPases, which in turn are required to organize the cytoskeleton during junction assembly and disassembly, and to modulate TJ barrier function. For example, ZO proteins are required for Rac activation to drive junction formation [15], and ZO-1 recruits to junctions the BAR (Bin/Amphiphysin/Rvs) protein Toca-1 (Transducer Of Cdc42-dependent Actin assembly), which can recruit the Cdc42 effector N-WASP (Neural Wiskott-Adlrich syndrome protein) [136]. The activation of Cdc42 requires the specific GEF Tuba, which is also enriched at TJs through its association with ZO-1 [137]. In addition, the ZU5 domain of ZO-1 binds directly to the RhoA activator ARHGEF11 [120] (Fig. 1). Other mechanisms through which ZO proteins regulate TJ barrier function and actomyosin tension involve the interaction of ZO-2 with JAM-A, which in turn recruits the afadin-PDZ-GEF complex to regulate Rap2c [74], the interaction of ZO-1 with cingulin and paracingulin, which recruit p114RhoGEF to epithelial and endothelial junctions [22,123,138], and the interaction of ZO-1 with Shroom3 [42].

ZO proteins also act as scaffolds for transcription factors. ZO-1 and ZO-2 interact directly, through the SH3 domain, with the Y-box transcription factor DbpA/ZONAB (DNA-binding protein A/ZO-1-associated Nucleic Acid Binding protein, YBX3) [139,140] (Fig. 1). When epithelial cells form a confluent monolayer and are fully polarized, DbpA/ZONAB is sequestered by ZO proteins at TJ, whereas at low confluence ZONAB translocates to the cytoplasm and the nucleus, where it regulates genes to promote cell proliferation and inhibit differentiation [139,140]. The region of ZO-1 that binds to ZONAB/DbpaA also recruits the regulator of TJ assembly $G\alpha_{12}$ [141] (Fig. 1). ARVCF (Armadillo Repeat protein deleted in VeloCardioFacial syndrome) is an armadillo-repeat protein of the p120(ctn) family, which can be localized both at junctions and in the nucleus and interacts with the PDZ domains of both ZO-1 and ZO-2 [142]. Finally, ZO-2 also interacts with the transcription factors YAP (Yes-Associated-Protein) and SAF-B (Scaffold Attachment Factor B) [143–147] (Fig. 1).

6. Afadin: central scaffolding protein for epithelial morphogenesis

Afadin was originally purified as a F-actin-binding protein from the cytosol fraction of rat embryonic brain and was localized at cadherin-based ZA and AJ [148]. Truncated afadin sequences were also identified in AF-6, a pathological gene resulting from fusion of afadin sequences with the acute lymphoblastic leukemia-1 (ALL-1) gene [149]. However, the protein product of AF-6 is not considered to exist in nature [150]. Afadin exists in long and short differentially spliced isoforms (l-afadin and s-afadin) [148] and is also a Ras target [151]. Afadin is evolutionarily conserved and is expressed in insects and worms. Canoe, the *D. melanogaster* homolog of l-afadin, was isolated by virtue of its severe rough eye phenotype [151,152], and is involved in adhesion and morphogenesis [153,154].

Afadin comprises N-terminal Ras-associating (RA) domains, followed by a forkhead-associated (FHA) phosphopeptide recognition domain, a dilute (DIL) domain homologous to the cargo-binding domain of myosin-V, a central PDZ domain, three proline-rich regions, a coiled-coil region, and an actin-binding C-terminal domain (Fig. 1) [155]. The N-terminal domains of afadin interact with several transmembrane adhesion and signaling proteins, such as the Ras-like GTPase Rap1 [151], Eph-related receptor tyrosine kinases [156], neurexins [157], nectins [158], and ADAM10 [51]. In neurons, the RA domain of afadin binds to active R-Ras, and induces axon branching through F-actin reorganization [159]. The C-terminal half of afadin interacts with actin filaments and several actin-binding proteins, such as ponsin [160], α-catenin [155,161,162], profilin [163], and drebrin [164] (Fig. 1). It also binds to LGN, a regulator of heterotrimeric G proteins that also interacts with NuMA [165].

In epithelial cells, afadin is a major scaffolding protein of the ZA, and is critical for the biogenesis of the AJC, the regulation of cell adhesion, cytoskeletal organization, asymmetric division, planar cell polarity, cell differentiation, and migration [154]. The formation of the ZA begins with an initial cell-cell contact mediated by homophilic and/or heterophilic interaction of nectins, which recruit afadin and cadherin-catenin complexes, through the interaction between afadin and α-catenin [161,162,166]. Clusters of E-cadherin interact by trans-dimerization to reinforce cell-cell adhesion contacts, connect to bundles of actin filaments, and expand to form a dynamic spot-like AJ [167]. Nectin engagement ignites a positive feedback loop which promotes AJ assembly, by activating the tyrosine kinase Src, which promotes the recruitment of the Rap1 GEF C3G to nectins, which results in the activation of Rap1 [168]. Binding of active Rap1 to the RA domain of afadin directs afadin membrane localization in endothelial cells, and in a positive feedback afadin regulates the activation of Rap1 to promote pro-angiogenic activities [169]. In epithelial cells the Rap1-afadin interaction promotes junction stabilization by binding to p120-catenin and inhibiting endocytosis of the pool of cadherin that is not yet engaged in trans-interactions [170,171]. Afadin and ZO-1 cooperate in the regulation of junctional actomyosin organization and tension, as indicated by the observation that cells depleted of ZO-1, which display an elevated contractility of the junctional actomyosin array, respond by increasing junctional afadin [42]. The depletion of both ZO-1 and afadin leads to altered cell shape and barrier function in response to elevated contractility, suggesting that afadin acts as a robust protein scaffold that maintains ZA architecture at tricellular junctions [42]. Importantly, afadin binds to α-catenin complexed with β-catenin and enhances its F-actin–binding activity, thus promoting the correct actomyosin organization at AJ/ZA [155].

The formation of AJ is required for TJ formation. ZO-1 is recruited to nascent AJ by interaction with α-catenin-cadherin and afadin-nectin complexes [17–20,172,173]. Afadin binds directly to the SH3 domain of ZO-1 through a region comprising two proline-rich stretches (Fig. 1), and this interaction is important for TJ assembly [20]. The C-terminus of l-afadin, but not s-afadin, binds actin filaments, and the nectin-afadin complex is formed even when the actin cytoskeleton is disrupted, whereas recruitment of cadherin-associated proteins and ZO-1 to nectin-based adhesion sites requires the integrity of actin filaments [174]. This reinforces the notion that mechanical stretching of ZO-1 through multivalent interactions with actin-binding proteins is critically involved in ZO-1 multimerization and phase separation [175]. The Ig-like adhesion molecule JAM is also clustered to AJ through afadin, and to TJ by ZO proteins [72,74,172,176]. Nectin and JAM-A are critical for the apico-basal alignment of AJ and TJ in epithelial cells [23,24]. Maturation of the apical junctional complex results in the apical segregation of ZO-1 and its association with occludin [17].

The central role of afadin in the biogenesis of AJ and TJ is demonstrated by the observation that its KO is embryonically lethal in mice, with epithelial cells showing defective cell adhesion and polarization, leading to ectoderm disorganization and loss of somites [177,178]. On the other hand, conditional KO of afadin in the vascular endothelium impairs angiogenesis [169]; in intestinal epithelia it compromises TJ barrier function and organization of the stem cell compartment [179]; in the brain results in hydrocephalus and loss of ependymal cells [180]; and in the kidney it triggers defective lumen morphogenesis [181]. In embryoid bodies, afadin KO results in altered localization of nectin and other junctional molecules, in impaired accumulation of the Par complex proteins, and in reduced activation of Cdc42 and aPKC (atypical protein kinase C) [182]. In breast cancer cells, afadin negatively regulates collective and individual cell migration, invasion, and activation of Src kinase and Ras/MAPK [183]. Afadin has also been implicated in planar spindle orientation, by acting as a molecular bridge between the mitotic spindle and cortical F-actin [165].

7. Apical polarity complex scaffolding proteins: Par proteins, PALS1, PATJ, MUPP1, MAGIs and Amot

The Par3-Par6-aPKC apical polarity complex was originally discovered through screens in *C. elegans* [184], and has been studied extensively in *Drosophila*, where the Par3 homolog is Bazooka [5,6]. In vertebrate epithelial cells Par3 is localized at TJ [185,186], and in coordination with the Crumbs polarity complex it organizes the asymmetric organization of epithelial cells by antagonizing the activity of basolateral polarity proteins [187–189].

Par3 exists in two isoforms (PARD3, 180 kDa, and PARD3B, 140 kDa). The larger isoform comprises a N-terminal CR1 (Conserved Region 1) domain, involved in homo-oligomerization and clustering [6,190,191], three PDZ domains, an aPKC binding domain, and a C-terminal domain (Fig. 2). The first PDZ domain (PDZ1) of Par3 has been involved in Par3 recruitment to TJ, through interaction with JAM-A [192–194] and in binding to Par6 [195,196], nectin [197], and the adaptor protein GAB1 (Grb2 Associated Binding protein 1) [198]. Par3 facilitates AJ and TJ formation by promoting the association of afadin with nectin [199]. The second PDZ domain (PDZ2) of Par-3 binds to phosphatidylinositol (PI) lipid membranes [200], whereas the PDZ3 domain interacts with the phosphoinositide phosphatase PTEN (Phosphatase and TENsin homolog), whose junctional localization is required for epithelial polarization [201]. In fact, the distribution of phosphoinositide lipids is crucial for epithelial polarization [202] and Par3 could maintain the phosphoinositide concentration gradient by bringing PTEN to the apical side of the membrane [201]. The PDZ3 domain of Par3 also binds to the Hippo pathway transcription factor YAP, resulting in the dephosphorylation of LATS-1/2 (LArge Tumor Suppressor kinase 1/2) and YAP through its association with the protein phosphatase PP1α (Protein Phosphatase 1α). This leads to enhanced YAP activation and cell proliferation at low density [203]. A central region of Par3 binds to aPKC, and the association of Par3 with either 14-3-3 or aPKC and the clustering and localization of Par3 are regulated by phosphorylation [204–207]. Finally, the C-terminus of Par3 binds to the Rac GEF Tiam1 (T-lymphoma Invasion And Metastasis inducing protein 1) to control TJ assembly [208,209].

Par6 comprises a N-terminal PB1 (Phox and Bem1p) domain, a semi-CRIB (Cdc42 and Rac Interactive Binding) domain and a single PDZ-domain (Fig. 2). Par-6 has three isoforms encoded by different genes: PARD6A (37 kDa), PARD6B (41 kDa) and PARD6G (41 kDa). The N-terminal domain of Par6 interacts directly with aPKC [186], with the TGF-β receptor TβR1 [210] and with the regulatory domain of the GEF ECT2 (Epithelial Cell Transforming 2), which activates both Cdc42 and aPKC activity [211]. Through its semi-CRIB domain, Par6 binds the active GTP-bound form of Cdc42 [195], thereby spatially confining its activity to TJ [212]. The PDZ domain of Par6 not only interacts with Par3, but also links the core complex to other polarity complexes, via its interaction with Pals1 [213], Crumbs-3 [214], and the lateral polarity complex protein LGL (Lethal Giant Larvae) [215,216].

The Crumbs polarity complex is composed of a transmembrane

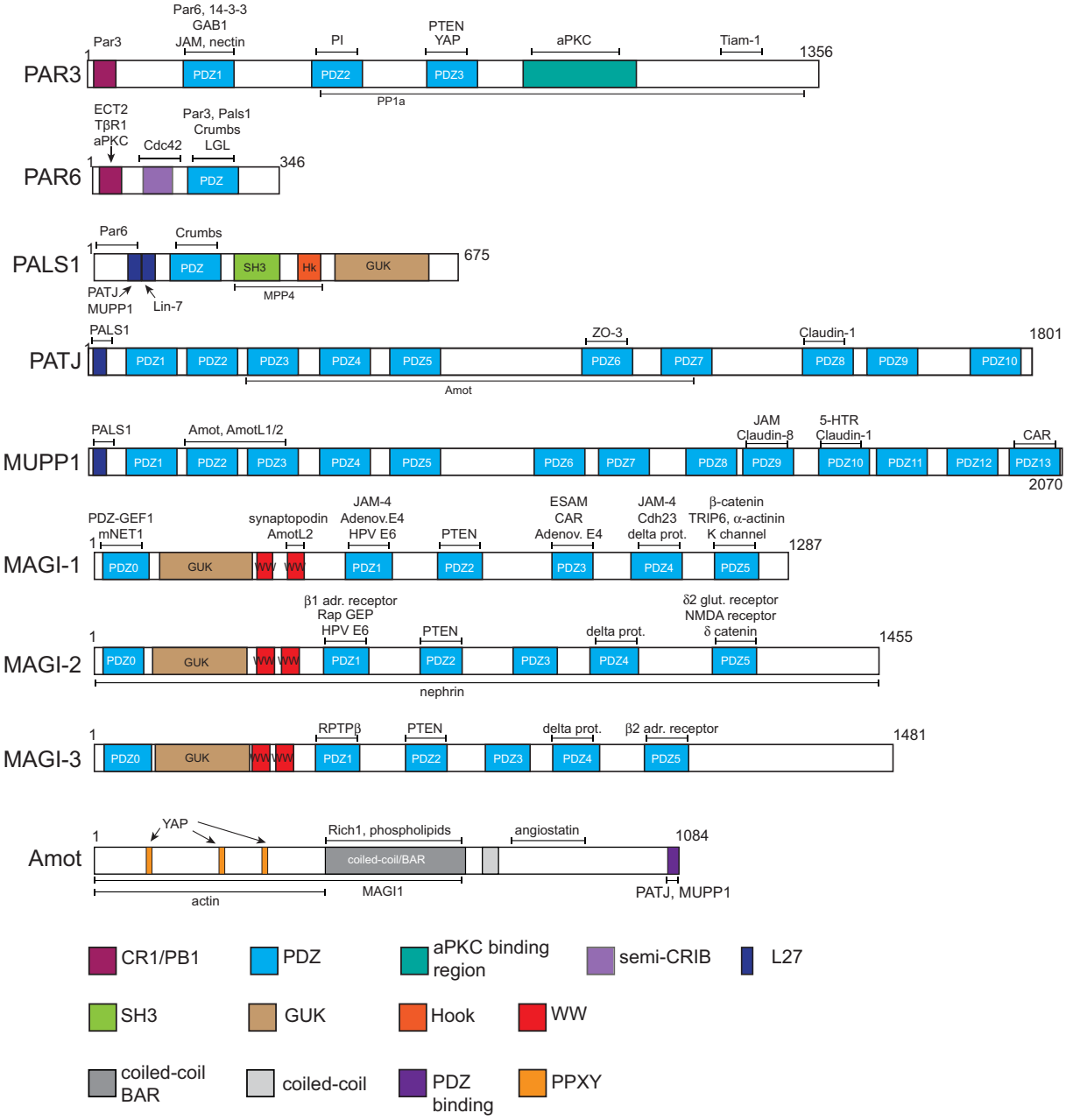

Fig. 2. The domain organization of apical polarity complex scaffolding proteins (Par3, Par6, PALS1, PATJ, MUPP1) MAGI proteins (MAGI-1, MAGI-2, MAGI-3) and Angiomotin (Amot). Domains are color-coded and indicated below the graphs. Interacting partners are indicated above or below each protein/domain, with a line that delineates the approximate localization of the binding sites(s). PATJ and MUPP1 are drawn to smaller scale. Interacting proteins for which the precise binding site(s) have not been defined have been omitted from the scheme.

protein (Crumbs) and two cytoplasmic scaffolding proteins, PATJ and PALS1.

PALS1 (Protein Associated with Lin-7; 77 kDa) is the mammalian homolog of the *D. melanogaster* Stardust protein and belongs to the MAGUK (membrane-associated guanylate kinase) family. PALS1 is targeted to the apical side of the cell through its association with Crumbs, and interacts with PATJ and MUPP1 [217,218]. The N-terminus of PALS1 binds to Par6 and is followed by two N-terminal L27 domains followed by a PDZ-domain, an SH3 domain, a hook and finally a guanylate kinase (GUK) domain [219] (Fig. 2). The first L27 domain interacts with the N-terminal L27 domain of PATJ and MUPP1 [217], and the second L27 domain interacts with Lin-7 [219]. The PDZ-domain of PALS1 interacts with the cytoplasmic ERL1 region of Crumbs-1 and -3 [217,220]. The crystal structure of the PALS1-Crumbs complex shows that the PDZ-SH3-GUK region of PALS1 forms a structural supramodule that contributes to the tight binding to Crumbs [221]. Recent quantitative proximity proteomics results indicate that Pals1 defines the vertebrate equivalent of the invertebrate marginal zone [222].

PATJ (Pals1 Associated Tight Junction protein) (195 kDa) and MUPP1 (Multi-PDZ Domain protein 1) (220 kDa) are the two mammalian homologs of Dpatj/DiscsLost (Dlt) in *Drosophila*. Both PATJ and MUPP1 are multi-PDZ scaffolding proteins, since they contain 10 and 13 PDZ domains, respectively (Fig. 2), and they function as scaffolds for both transmembrane and cytoplasmic TJ proteins. PATJ binds to ZO-3 via its 6th PDZ domain and claudin-1 via its 8th PDZ domain [223]. PATJ recruits angiomotin (Amot) to the plasma membrane, together with its interacting partners merlin and YAP, preventing transcriptional activation of YAP target genes [224,225]. MUPP1, first identified as an

interactor of the serotonin receptor 5-HT_{2C}R [226], binds claudin-8 and JAM with its 9th PDZ domain, and claudin-1 with its 10th PDZ domain [227,228] (Fig. 2). MUPP1 is recruited to TJ through the interaction of its PDZ13 domain with the coxsackievirus and adenovirus receptor (CAR), an Ig-like adhesion protein of TJ [229]. PATJ and MUPP1 are required for TJ formation [230] and for the localization of occludin and ZO-3 at TJ [231]. At the organism level, Par3 has been implicated in mammary gland morphogenesis [232], PALS1 in retinal histogenesis [233,234], and MUPP1 mutations are involved in hydrocephalus [235], through abnormally high permeability of the choroid plexus [236].

Polarity proteins together with Ig-like adhesion proteins establish and maintain polarity independently of the assembly of claudin-based TJ strands, as indicated by the observation that in Eph4 cells lacking both ZO-1 and ZO-2 TJ are disrupted and claudin strands are undetectable, but polarity is still present [32,39]. Similarly, MDCK lacking all claudins maintain polarity and, interestingly, also sites of membrane apposition which form barrier to macromolecules [24]. However, in MDCK cells depletion of both ZO-1 and ZO-2 results in disorganized polarity, and simultaneous deletion of claudins and JAM-1 results in complete loss of barrier function and sporadic epithelial polarity defects [24]. This indicates that JAM-A, which is scaffolded by ZO proteins, is important both for TJ barrier function (see also [24,74,237]) and polarity.

MAGI (membrane-associated guanylate kinase (MAGUK) with inverted orientation) proteins (MAGI-1, MAGI-2 and MAGI-3) have been localized at epithelial TJ and at AJ [238–245]. However, they are also expressed in nervous, muscle and other non-epithelial tissues, where they form scaffolds for different transmembrane proteins, including glutamate and β-adrenergic receptors and potassium membrane channels [246–250] (Fig. 2). For example, MAGI2 is also known as S-SCAM (Synaptic Scaffolding Molecule), a synaptic scaffolding protein that interacts with neuronal receptors and ∂-catenin [251,252]. The *C. elegans* homolog of MAGI-1 functions as a stabilizer of apical junctions and interacts with afadin, indicating that MAGI proteins are evolutionarily conserved junctional scaffolding proteins [253]. Structurally, MAGI proteins are characterized by a N-terminal GUK domain, two tandem WW (Trp-Trp) domains (instead of a SH3 domain that is present in most other MAGUK family proteins), and six PDZ domains, five of which are located C-terminal to the GUK-WW domains (Fig. 2) [238]. At epithelial and endothelial junctions, MAGI-1 interacts with Ig-like adhesion molecules such as JAM-4 [254] and ESAM [255], and with β-catenin [239], whereas MAGI-3 interacts with the receptor tyrosine protein phosphatase RPTP-β [245]. Through these interactions, and also by recruiting the GEFs PDZGEF-1/RapGEP and mNET1 [256,257], MAGI-family proteins help stabilize epithelial and endothelial cell-cell junctions. In fact, MAGI protein cleavage by caspases is an important step for cell-cell detachment in apoptosis [258]. Both MAGI-1 and MAGI-2 also play an important scaffolding role at the glomerular slit diaphragms between podocytes, where they bind to the Ig-like transmembrane protein nephrin [259,260], and the actin-binding proteins synaptopodin and α-actinin-4 [261]. In agreement, studies on KO mice show that MAGI-2 is critical for the formation and maintenance of the glomerular filtration barrier [262]. Finally, MAGI proteins interact with the C-terminal PDZ binding motif of PTEN, and stabilize it in a complex with vinculin and β-catenin at AJ, by facilitating its phosphorylation by microtubule-associated kinases [241,243,263,264]. By stabilizing PTEN, and sequestering the zyxin family protein TRIP6 (Thyroid Receptor Interacting protein 6), MAGI proteins act as tumor suppressors, and inhibit cell invasion, migration and anchorage-independent growth [265]. MAGI proteins, as many other PDZ proteins [266], are also targeted by viruses. Human papilloma virus (HPV) and adenovirus oncoproteins E4 and E6 bind to the PDZ1 and PDZ3 domains of MAGI-1 and MAGI-3, and induce their cytoplasmic degradation [267,268] (Fig. 2).

Angiomotin (Amot-p130) (Fig. 2) [269,270] belongs to the differentially spliced motin family of proteins, which also comprises Amot-p80, angiomotin-like protein 1 (AmotL1), also known as junction-enriched and -associated protein (JEAP) [271] and angiomotin-like protein 2 (AmotL2), also known as MAGI-1 associated coiled-coil tight junction protein (MASCOT) [272]. Amot proteins are localized at TJ/AJ of epithelial and endothelial cells, where they play a role in junctional organization and signaling [224,269,272–274]. Structurally, motin family proteins are characterized by a N-terminal region which contains three highly conserved glutamine-rich L/P-PXY motifs, central coiled-coil/BAR and coiled-coil domains, and a C-terminal PDZ-binding motif. The L/P-PXY motifs are absent from the Amot-p80 isoform, and mediate direct interaction with the transcription factor YAP [225,275–277] (Fig. 2). Through interaction with YAP, with Hippo pathway kinases and with additional signaling pathways (AMPK, mTOR, Wnt and MAPK/ERK), Amot family proteins have been implicated in cancer, either as tumor suppressor or oncogenes (reviewed in [278,279]). The coiled-coil region of Amot mediates the formation of either homo- or hetero-polymers [272,280], and also interacts with lipid- and cholesterol-containing membranes [281,282]. PATJ and MUPP1 bind to the C-terminal PDZ-binding motifs of Amot family proteins, but this interaction is not required for their localization at TJ [224,283]. Amot-p130 is recruited to junctions through its extended N-terminal domain, which associates with MAGI-1 [284] and F-actin [285]. Amot forms a complex with the Rac/Cdc42 GAP Rich1 to inhibit its activity at junctions, and thus promotes internalization of polarity complex proteins [224]. Merlin competitively binds to Amot and thus releases Rich1 from the Amot-inhibitory complex, allowing Rich1 to inactivate Rac1 [286]. Amot was first identified as a protein that interacts with angiostatin, a potent and specific inhibitor of angiogenesis [270], and Amot family proteins are indeed involved in vasculature development and endothelial junction dynamics [287–290]. In fact, PATJ/MUPP1, Amot-p80 and the Rho GEF Syx form a ternary complex that regulates the directional migration of endothelial cells [273], by trafficking from the junctions to the leading edge of migrating cells [281,282]. During the early stages of vascularization, Amot-p80 is highly expressed in the endothelial cells, which promotes endothelial cell migration and blood vessel formation, whereas Amot-p130 is highly expressed at later stages, where it inhibits angiogenesis through stabilization of junctions [280].

8. Cingulin and paracingulin: actomyosin and microtubule-associated scaffolds for GEFs and GAPs

Cingulin (CGN) was identified by raising monoclonal antibodies against a protein that co-purified with cytoplasmic nonmuscle myosin-2 from intestinal epithelial cells [291,292]. Paracingulin (JACOP, CGNL1) was identified by raising monoclonal antibodies against a junction-enriched fraction isolated from liver [293] and by sequence homology to cingulin [294]. No invertebrate homologs for either cingulin or paracingulin have been described. Cingulin and paracingulin differ in their localization and mode of recruitment to junctions. While cingulin localization is restricted to TJ, where it is recruited by ZO-1 [80,291,295], paracingulin has been localized both at TJ and ZA, depending on the tissue, and is recruited to junctions either by ZO-1, PLEKHA7 or both, depending on cell type [123,293,296].

Both cingulin and paracingulin are parallel homodimers, each subunit consisting of a globular “head” domain, a coiled-coil “rod” domain, and a small globular “tail” domain [116,293,297] (Fig. 3). A conserved sequence (ZO-1-Interaction-Motif (ZIM)) at the N-terminus of the head domain is required for their interaction with ZO-1 and for recruitment to junctions, both in epithelial [22,295,296] and endothelial [123] cells. The head domain of cingulin also interacts with actin [298] microtubules [118,119], ZO-2 and ZO-3, whereas myosin-2 interacts in vitro with both head and rod domains of cingulin [116,295]. The coiled-coil domains of cingulin and paracingulin also interact with GEFs and GAPs, such as GEF-H1, p114RhoGEF, MgcRacGAP, and Tiam1 [123,138,299–301], and the endosomal traffic

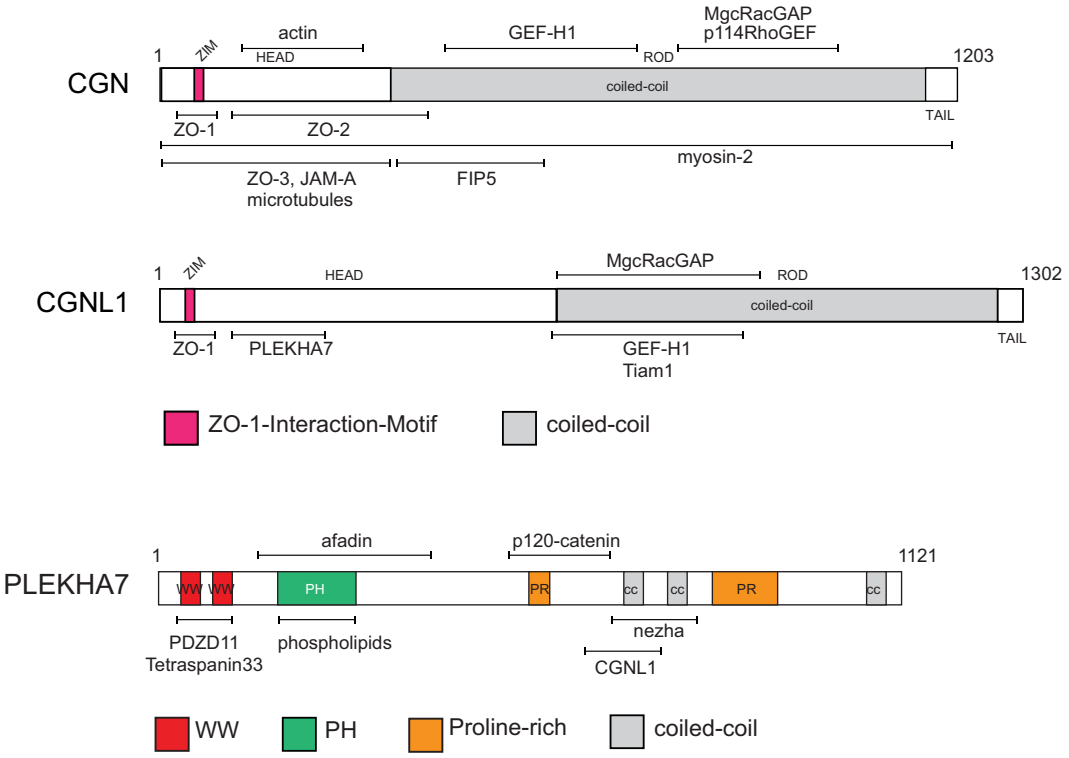

Fig. 3. The domain organization of cingulin, paracingulin and PLEKHA7. Domains are color-coded and indicated below the graphs. Interacting partners are indicated above or below each protein/domain, with a line that delineates the approximate localization of the binding sites(s). Interacting proteins for which the precise binding site(s) have not been defined have been omitted from the scheme.

protein FIP5 [119]. Opening of the rod domain of cingulin is stimulated by AMPK phosphorylation [118,302], and presumably renders binding sites for the above proteins on the rod domain available.

Cingulin and paracingulin are localized in the distal layer of the TJ plaque, with respect to the membrane, as demonstrated by electron microscopy analysis of immunogold-labelled tissue sections [291,292,303] and super-resolution light microscopy of cultured cells [304]. The measured distance of immunogold label for cingulin was 40–65 nm in chicken intestinal epithelial cells [291,292,303], and of 51 nm in rat kidney [303]. For ZO-1, the measured distance of the gold particles was 23 nm and 14 nm in chicken intestine and rat kidney, respectively [303]. Thus, the membrane-proximal cytoplasmic layer contains ZO proteins, consistent with their scaffolding of TJ membrane proteins, and the distal layer contains cingulin and paracingulin, indicating a close structural and functional association with the cytoskeleton. This respective positioning is confirmed by super-resolution microscopy [304]. However, these distance values may be affected by the fixation, the size of primary/secondary antibody complexes and the position of epitopes, and additional experiments are required to establish the position of 130 nm-long rod domain of cingulin [291] with respect to the membrane.

There are different lines of evidence indicating a functional link of cingulin and paracingulin with actomyosin. First, cingulin co-pellets with and bundles actin filaments in vitro [298,302], and paracingulin is colocalized with actin filaments in tissues, and when exogenously expressed in fibroblasts [293,296]. Second, the FRAP dynamics of cingulin and paracingulin are very similar to those of actin, since they almost completely dynamically exchange between the junctional and cytoplasmic pools [305]. Finally, recent experiments indicate two important aspects of the role of cingulin in actomyosin organization and interaction with the TJ. First, by binding to the ZU5 domain of ZO-1, cingulin unfolds ZO-1 and promotes the interaction of the ABR domain of ZO-1 with actin filaments [22], in agreement with the observed stabilizing function of the ZU5 domain [81,306]. Second, cingulin tethers the juxtamembrane network of NM2B and γ-actin to ZO-1 to maintain apical stiffness and down-regulate apicolateral membrane tension [22]. Nonmuscle myosin isoforms have distinct localizations, regulations and functions at the ZA: NM2A is assembled at the perijunctional actin belt and provides the force necessary for reinforcement and maintenance of E-cadherin-based cell-cell adhesion, whereas NM2B organizes the juxta-membrane branched network of actin filaments that controls the translational movement of the ZA [49,307,308]. Thus, we proposed that cingulin, by promoting the tethering of the γ-actin-NM2B branched network to TJ contributes to dampening the tensile forces generated at the ZA [22].

Cingulin also binds to microtubules [118,119,302], through a patch of basic residues in the head domain, which bind to acidic residues in the C-terminal tail of tubulin [119]. Binding of cingulin to microtubules is promoted by phosphorylation of the head domain by AMPK kinase [118]. This phosphorylation controls the conformation of cingulin but is not required for the interaction with actin filaments [302]. Through its interaction with microtubules, cingulin organizes the planar apical network of microtubules [118], and allows the docking of FIP5-containing endosomes to the apical membrane initiation site (AMIS), thereby controlling lumen formation in epithelial cysts [119]. Paracingulin also co-pellets with microtubules in vitro [309], and nocodazole treatment leads to fragmented and reduced junctional labelling for paracingulin [305]. In summary, the present evidence indicates that cingulin and paracingulin could be central players in the cross-talk between actin and microtubule cytoskeletons [22].

Cingulin and paracingulin also function as scaffolds/adaptors for GEFs and GAPs (Fig. 3) [52]. On the one hand, cingulin sequesters GEF-H1 at mature junctions of confluent epithelial cells to down-regulate cytoplasmic RhoA activity and stress fiber formation [299], as well as regulate through RhoA downstream expression of claudin-2 and many additional genes [310–312]. On the other hand, binding of p114RhoGEF to cingulin and paracingulin spatially restricts RhoA activation at junctions, promoting accumulation of actin and myosin, and

junction assembly [123,138]. p114RhoGEF is also regulated by the FERM domain protein Lulu2 [313]. Depletion studies also show that paracingulin regulates Rac1 and Cdc42 activities during junction formation, by modulating both the Rac GEF Tiam1 and the Rac/Cdc42 GAP SH3BP1 [301,314]. In agreement, in endothelial (HUVEC) cells depletion of paracingulin inhibits Rac1 activity and increases RhoA activity, thus disrupting VE-cadherin association with the actin cytoskeleton and destabilizing AJ [315].

Depletion of either cingulin or paracingulin in cellular model systems does not produce striking changes in the architecture and function of TJ [301,310,311], suggesting some redundancy. However, the KO of cingulin in cultured epithelial cells results in a decrease in the TJ accumulation of ZO-1 [22], suggesting that although barrier function is not significantly affected by cingulin depletion in MDCK cells [301,310,311], the complete KO may have different consequences. However, the dynamics of barrier development and gene expression are altered in cingulin and paracingulin-depleted cells, due to altered activation of RhoA and Rac1 [299,301,310–312]. Cingulin-KO mice are viable and fertile, and mucosal damage repair is impaired, possibly through altered RhoA activity [316]. Although the assembly of claudin-based TJ fibrils or the establishment of a normal epithelial barrier function does not appear affected in cingulin-KO embryoid bodies and mouse epithelial tissues [311,316], endothelial barrier function is decreased in the brain of cingulin-KO mice [317]. Furthermore, cingulin overexpression reduces proliferation and improves barrier function of endothelial cells, by down-regulating RhoA activity through the sequestration of GEF-H1 [317,318]. Finally, cingulin may also be involved in neural development in the chick, since either depletion or overexpression of cingulin expands the migratory neural crest population, correlating with altered RhoA expression [319]. Depletion of paracingulin inhibits retinal vascular development, indicating that paracingulin stabilizes the interaction of VE-cadherin with the actin cytoskeleton through Rac1-dependent signaling [315]. In summary, cingulin and paracingulin are TJ and ZA adaptor proteins that play an important role in the organization of the cytoskeleton, in part through local and global modulation of Rho family GTPases, which also impacts on gene expression.

Cytoskeletal organization is relevant for cancer and other diseases. Indeed, in pleural mesothelioma cingulin is downregulated through translational repression by the microRNA miR-24-3p, correlating with increased RhoA activity and migration, and poor survival [320]. The expression of cingulin is lost in squamous carcinomas [321,322], whereas in endometrial and other cancers higher paracingulin levels positively correlate with better prognosis and survival [323]. In contrast, cingulin is upregulated by miR-125b in patients with irritable bowel syndrome with diarrhea, suggesting either an involvement in pathogenesis of altered TJ barrier function [324], or an attempt of the cell to compensate a dysregulated system. Cingulin is also a target of Raptor, a key component of the Target-of-Rapamycin (TORC) complex. In mice with Sertoli cell (SC)-specific KO for Raptor, cingulin is downregulated, correlating with changes in Rac1 activity and cytoskeletal organization, leading to impairment of testis development, and infertility [325]. Additional studies indicate an involvement of paracingulin in schizophrenia [326] and type 2 diabetes mellitus [327], through unknown mechanisms.

9. PLEKHA7: microtubule-associated scaffold for AJ/ZA transmembrane proteins

PLEKHA7 (Pleckstrin Homology Domain-Containing Family A Member 7) (145 kDa) was discovered independently as an interactor of either p120-catenin [47] or paracingulin [46,296,328]. No invertebrate homologs for PLEKHA7 have been described. PLEKHA7 is localized at the ZA of epithelial and endothelial cells [46,47] and at spot-like AJ of non-epithelial cells [51].

PLEKHA7 comprises two N-terminal WW domains, the first one (WW1) being crucial to form a scaffold for nectins [329] and the Tspan33-ADAM10 complex [51,330], either through a direct interaction, in the case of Tspan33, or indirectly, by scaffolding the small adaptor protein PDZD11 (Fig. 3). PDZD11 together with the second WW domain of PLEKHA7 is also required cooperatively for efficient binding of PLEKHA7 to Tspan33 [51,330]. Specifically, communication between the tandem WW domains of PLEKHA7 and the PLEKHA7–PDZD11 interaction cooperatively promote the formation of a hydrophobic patch in the WW1 domain that fits the C-terminal residues of Tspan33 [330]. After docking to the PLEKHA7 complex by the Tspan33-PLEKHA7-PDZD11 interaction, ADAM10 is locked at AJ by binding directly to the N-terminus of afadin [51]. The PH (Pleckstrin Homology) domain of PLEKHA7 mediates interactions with phospholipids [331] and afadin [332], whereas the central region comprising, the two coiled coil domains (Fig. 3), binds to the E-cadherin-associated protein p120-catenin, the microtubule minus end-binding protein nezha/CAMSAP3 (calmodulin-regulated spectrin-associated protein 3) [47], and the TJ/ZA protein paracingulin [46]. The tethering of microtubules through PLEKHA7 stabilizes AJ [47]. Decreased junction stability was proposed to be the mechanisms through which down-regulation of PLEKHA7 expression by the transcription factor Insm1 (Insulinoma-associated protein 1) promotes neural delamination during neocortex development [333]. Another potential link between PLEKHA7 and cytoskeletal regulation is the observation that PLEKHA7 overexpression accelerates scratch wound closure, that it binds to Cdc42 and Rac1, and that it colocalizes with the Rho GTPases at the leading edge of migrating cells, suggesting that it functions as a GTPase Activating Protein (GAP) of Rac1 and Cdc42 [334]. Multiple actin-binding, microtubule-related and intermediate filament proteins are detected in PLEKHA7 immunoprecipitates [335,336].

PLEKHA7 has several roles in regulation of signaling. It is involved in recruiting to junctions key proteins involved in RNA regulation, specifically an active miRNA microprocessor complex, comprising DROSHA and DGCR8 [336], and core components (Ago2, GW182, PABPC1) of the RNA-induced silencing complex (RISC) and related mRNAs and miRNAs [54]. By promoting microRNA-mediated silencing of oncogenic mRNAs and of genes that promote anchorage-independent cell growth, PLEKHA7 links junction integrity to the control of cellular behaviors relevant for tumor progression [54,336]. Importantly, PLEKHA7 expression is reduced in high grade ductal and lobular breast carcinomas [337] and in epithelial ovarian cancers [338]. Additional signaling pathways that are regulated by PLEKHA7 are related to the control of calcium homeostasis. Depletion of the PLEKHA7 homolog Hadp1 (Heart ADaptor Protein 1) in zebrafish decreases cardiac output and leads to bradycardia and hypo-contractility, correlating with increased in the amplitude and duration of the ventricular Ca^{2+} transient [331]. The phospholipid-binding PH domain of Hadp1 is important in its membrane localization, and it was proposed that Hadp1 controls either metabolism of PI4,5,P_2, or IP_3-induced Ca^{2+} release, or plasma membrane Ca^{2+} conductance or reuptake [331]. PLEKHA7-mutant rats exhibit attenuated hypertension in response to a high salt diet, correlating with increased and prolonged Ca^{2+} transient, and increased NO (nitric oxide) production in aortic endothelial cells [339]. Importantly, genome wide association studies (GWAS) implicate PLEKHA7 as a susceptibility locus for human blood pressure and hypertension [340–343], and primary angle closure glaucoma [344–347]. The regulation of calcium homeostasis by PLEKHA7 could be mechanistically involved in these diseases.

10. Biophysics of apical junctions: regulation by force and liquid-liquid phase separation

Although the molecular composition of apical junctions and several of the molecular interactions underlying their assembly and functions have been clarified, the precise mechanisms that drive the assembly, disassembly, positioning and regulation of apical junctions are not

Table 1
PSPredictor scores for proteins predicted to undergo liquid-liquid phase separation (PSP) proteins (> 0.5 threshold score). The list contains apical junction-associated proteins, and other proteins that are either localized at lateral junctions, or directly or indirectly regulate junctional proteins. Scores were calculated using the online tool http://www.pkumdl.cn:8000/PSPredictor/ [360].

Proteins	PSPredictor	Accession numbers	Species
MARK4 (Microtubule Affinity Regulating Kinase 4)	0.9997	NP_001186796.1	*Homo sapiens*
YAP (Yes1 associated transcriptional regulator)	0.9997	NP_001123617.1	*Homo sapiens*
Shroom1	0.9996	NP_001166171.1	*Homo sapiens*
MARK2 (Microtubule Affinity Regulating Kinase 2)	0.9995	NP_001034558.2	*Homo sapiens*
ZO-3 (tight junction protein ZO-3 isoform 1)	0.9991	NP_001254489.1	*Homo sapiens*
CAMSAP3 (calmodulin-regulated spectrin-associated protein 3)	0.9992	NP_065953.1	*Homo sapiens*
AMOT (angiomotin)	0.9985	NP_001106962.1	*Homo sapiens*
TAZ (transcriptional co-activator with PDZ-binding motif)	0.9982	NP_001161750.1	*Homo sapiens*
Rich1 (Rho GTPase Activating Protein 17)	0.9975	NP_001006635.1	*Homo sapiens*
FIP5 (rab11 family-interacting protein 5)	0.9965	NP_056285.1	*Homo sapiens*
AMOTL1 (angiomotin-like protein 1)	0.9962	NP_570899.1	*Homo sapiens*
Shroom2 (Shroom Family Member 2)	0.9962	NP_001640.1	*Homo sapiens*
SH3BP1 (SH3 domain-binding protein 1)	0.9947	NP_061830.3	*Homo sapiens*
AMOTL2 (angiomotin-like protein 2)	0.9937	NP_001265612.1	*Homo sapiens*
MARK1 (Microtubule Affinity Regulating Kinase 1)	0.9927	NP_061120.3	*Homo sapiens*
ZONAB (Y-box-binding protein 3)	0.9926	NP_003642.3	*Homo sapiens*
SIPA1L-3 (Rap1 GAP, signal induced proliferation associated 1 like 3)	0.9886	NM_015073.3	*Homo sapiens*
ARHGEF11 (rho guanine nucleotide exchange factor 11)	0.9879	NP_055599.1	*Homo sapiens*
TARA (TRIO and F-actin-binding protein)	0.9859	NP_001034230.1	*Homo sapiens*
ZO-1 (tight junction protein ZO-1)	0.9846	NP_003248.3	*Homo sapiens*
MAGI1 (membrane-associated guanylate kinase, WW and PDZ domain-containing protein 1)	0.9794	NP_001028229.1	*Homo sapiens*
PAR3 (partitioning defective 3 homolog)	0.9788	NP_062565.2	*Homo sapiens*
SCRIB (scribble planar cell polarity protein)	0.9787	NP_874365.3	*Homo sapiens*
LATS1 (serine/threonine-protein kinase Large Tumor Suppressor Kinase 1)	0.9779	NP_004681.1	*Homo sapiens*
CGNL1 (paracingulin, cingulin-like protein 1)	0.9735	NP_001239264.1	*Homo sapiens*
MARK3 (Microtubule Affinity-Regulating Kinase 3)	0.9693	NP_001122390.2	*Homo sapiens*
p114RhoGEF (rho guanine nucleotide exchange factor 18)	0.9656	NP_001354752.1	*Homo sapiens*
Cortactin (src substrate cortactin)	0.9568	NP_005222.2	*Homo sapiens*
PLEKHA7 (Pleckstrin Homology Domain-Containing Family A Member 7)	0.9499	NP_001254489.1	*Homo sapiens*
MAGI2 (membrane-associated guanylate kinase, WW and PDZ domain-containing protein 2)	0.9496	NP_036433.2	*Homo sapiens*
TUBA (ARHGEF36)	0.9461	NP_056036.1	*Homo sapiens*
NMMHC-IIA (myosin-9)	0.9248	NP_002464.1	*Homo sapiens*
LATS2 (serine/threonine-protein kinase Large Tumor Suppressor Kinase 2)	0.9008	NP_055387.2	*Homo sapiens*
CGN (cingulin)	0.8976	NP_065821.1	*Homo sapiens*
NMMHC-IIB (myosin-10)	0.8956	NP_005955.3	*Homo sapiens*
ZO-2 (tight junction protein 2)	0.8610	NP_004808.2	*Homo sapiens*
Tiam1 (T-lymphoma invasion and metastasis-inducing protein 1)	0.7604	NP_001340617.1	*Homo sapiens*
PATJ (crumbs cell polarity complex component)	0.7537	NP_795352.3	*Homo sapiens*
MUPP1 (multiple PDZ domain crumbs cell polarity complex component)	0.6834	NP_001317566.1	*Homo sapiens*
Trio (triple functional domain protein)	0.6174	NP_009049.2	*Homo sapiens*
Eplin (LIM domain and actin binding 1)	0.5169	NP_057441.1	*Homo sapiens*

completely understood. Recent experiments have highlighted a new role for mechanical force and the formation of biomolecular condensates as potential biophysical mechanisms involved in AJC dynamic remodeling and functions [12,175,348].

As already mentioned, one feature that distinguishes the ZA from lateral contacts is the presence of a tensile circumferential actomyosin belt in the submembrane cortical region. This belt links together all cells of the epithelial sheet into a coherent unit, thus providing resistance to mechanical stresses applied to epithelial and endothelial tissues, through the mechano-response of the cadherin-α-catenin-vinculin complex [10,349,350]. However, a second important function of the ZA-associated actomyosin belt is to regulate TJ assembly and barrier function [12,102,133]. Different experiments show that the formation of the tensile actomyosin belt at the ZA is crucial for the assembly of continuous AJ and TJ [173,351,352]. Vinculin is also critical for the robustness of the epithelial sheet TJ barrier to ions, to protect it against mechanical fluctuations [353]. These observations raise an important question: how does force impact on specific TJ constituents? Since ZO proteins, the main scaffolding proteins of TJ, are important targets/regulators of the actomyosin cytoskeleton, it is important to determine how the conformation and function of ZO proteins is regulated by the organization and contractility of the actomyosin cytoskeleton. We found that force regulates the conformation and scaffolding functions of monomeric ZO molecules. By tagging ZO-1 at its N-terminal and C-terminal ends, we showed by structured illumination microscopy that when both ZO-1 and ZO-2 are expressed, ZO-1 is always in a stretched conformation at the TJ. Instead, when ZO-2 is depleted, and tension is decreased, either by disrupting actin filament organization or by inhibiting myosin activity, ZO-1 assumes a folded conformation, which is unable to scaffold the interactors DbpA/ZONAB and occludin through the ZPSG region [21]. These observations indicate that the stretched conformation of ZO proteins is the “active” form, which can multimerize into a submembrane scaffold that efficiently connects claudin strands to the cytoskeleton and bind to signaling and other proteins. In contrast, monomeric folded ZO proteins, which may physiologically exist in the cytoplasmic soluble fraction, are the inactive form. In our model [21], force would promote the stretching of ZO monomers, thus promoting their dimerization and integration into junctions. This model is in agreement with FRAP experiments, showing that ZO-1 is stabilized in *Xenopus* embryos under increased tension [40].

A second, and possibly related mechanism of biophysical regulation is the formation of biomolecular condensates [354,355]. It was recently shown that ZO proteins undergo liquid-liquid phase separation in cells and in vitro, and this is important for developmental mechano-sensing events [103,356]. Beutel et al. showed that ZO proteins are distributed both along lateral AJ, at a concentration of 0.5–1 μM, and at TJ, at a concentration of 40–50 μM, and that in vitro phase separation occurs in

vitro only at concentrations above 2.5 μM. Thus, it could be hypothesized that a local increase in ZO protein concentration, caused by ZO-1 binding to its ligands, could induce its stretching, multimerization and phase separation. This hypothesis requires validation by experiments in vitro, with the caveat that actomyosin-generated tension occurs physiologically at the ZA and could affect the conformation and activity of ZO-1 interactors. Since liquid phase separation of ZO molecules requires their unfolding [103], and force unfolds ZO-1 [21] we favor the hypothesis that force generated at the ZA is critical for the formation of biomolecular condensates of ZO proteins, through their tension-dependent stretching [175]. A role of force is consistent with the observation that ZO-1 is localized at discontinuous spots throughout all layers of the stratified epithelium of the epidermis, and is only detected in a continuous circumferential belt, e.g. in putative large condensates, in the outermost layer, where increased junctional tension is demonstrated by the presence of the stretched form of α-catenin [357]. Beutel et also showed that he formation of ZO liquid condensates is inhibited by phosphorylation [103], suggesting that another mechanism of regulation could be differential phosphorylation of the lateral versus zonular pools, which has been shown also for E-cadherin-associated complexes [2,336,358]. Importantly, phosphorylation may also affect junctional protein cross-talk with the cytoskeleton [359], and hence act indirectly to modulate the mechano-response of ZO proteins. Together, these observations indicate that several mechanisms, including phosphorylation, force, inter and intra-molecular interactions, could collectively induce ZO proteins to undergo a conformational switch, leading to stretching, multimerization, and liquid phase separation [175].

11. Biomolecular condensates of junctional proteins: predictions, regulation, and emergent properties

Using a sequence-based machine-learning tool to predict liquid phase separation [360], we examined whether, besides ZO proteins, additional cytoplasmic scaffolding proteins of apical junctions, and associated cytoskeletal and signaling proteins, are predicted to undergo liquid phase separation (Tables 1 and 2 and Fig. 4). Phase separation can either occur through interaction between multiple folded domains or can be mediated by intrinsically disordered regions [360].

Among apical polarity complex proteins, Par3 and PATJ/MUPP1 are predicted phase-separating proteins (PSPs), whereas Par6, aPKC and PALS1 are not, based on a threshold score of 0.5 (Tables 1 and 2 and Fig. 4). Regarding basolateral polarity complex proteins [361,362] Scribble but not Dlg is a predicted PSP (Tables 1 and 2, Fig. 4). The MARK/Par-1 family of Ser/Thr protein kinases shows structural similarities to the AMPK kinase family, and these proteins regulate polarity by phosphorylating microtubule-binding proteins and organizing the microtubule cytoskeleton [363,364]. All the MARK kinases show a high PSP prediction score (Table 1, Fig. 4). In contrast, AMPK kinase and its upstream regulator LKB1, which regulate TJ assembly and cingulin association with microtubules [118,302,365], are not predicted PSPs (Table 2, Fig. 4).

In the Hippo pathway [366], the LATS1/2 kinases and its substrate YAP are predicted PSPs, whereas the upstream MST1 kinase is not (Tables 1 and 2, Fig. 4). Both the YAP and ZONAB/DbpA transcription factors, which are regulated by interactions with junctional proteins [139,140,277,367–370], are predicted PSPs, whereas the RNA-processing TJ-associated protein symplekin [371–373] is not (Tables 1 and 2, Fig. 4).

Among the major scaffolding proteins discussed here, in addition to ZO proteins, predicted PSPs include cingulin and paracingulin, PLEKHA7, Amot and AmotL1/2, and MAGI1 and MAGI2 (Table 1 and Fig. 4). In contrast, afadin, MAGI3, and the PLEKHA7 interactor PDZD11 are not predicted PSPs (Table 2 and Fig. 4).

Among actin-binding proteins, predicted PSPs include the A and B heavy chain isoforms of cytoplasmic myosin-2 (MHC2A, MHC2B), Shroom1/2, cortactin and eplin (Table 1, Fig. 4), whereas the catenins (p120-catenin, α-catenin, β-catenin), vinculin, ezrin and merlin are not predicted PSPs (Table 2, Fig. 4).

With regards to junction-associated microtubule-associated proteins, the minus-end binding proteins CAMSAP3 (nezha [47]) and the Rab11- and kinesin-binding protein FIP5 are also predicted PSPs (Table 1, Fig. 4). Finally, we examined the junction-associated signaling proteins, e.g. GEFs and GAPs, transcription factors and associated

Table 2
PSPredictor scores for proteins predicted not to undergo liquid-liquid phase separation (Non-PSPs) (< 0.5 threshold score). The list contains apical junction-associated proteins, and other proteins that are either localized at lateral junctions, or directly or indirectly regulate junctional proteins. Scores were calculated using the online tool http://www.pkumdl.cn:8000/PSPredictor/ [360].

Proteins	PSPredictor	Accession numbers	Species
Afadin (adherens junction formation factor)	0.4326	NP_001353249.1	*Homo sapiens*
CD2AP (CD2-associated protein)	0.3776	NP_036252.1	*Homo sapiens*
MAGI3 (membrane-associated guanylate kinase, WW and PDZ domain-containing protein 3)	0.2862	NP_001136254.1	*Homo sapiens*
Dlg1 (disks large homolog 1)	0.2516	NP_001091894.1	*Homo sapiens*
p120catenin (catenin delta-1)	0.1466	NP_001078927.1	*Homo sapiens*
MgcRacGAP (Rac GTPase-activating protein 1)	0.1423	NP_001119575.1	*Homo sapiens*
PALS1 (MAGUK p55 subfamily member 5)	0.1372	NP_071919.2	*Homo sapiens*
α-Catenin (catenin alpha-1)	0.1152	NP_001310911.1	*Homo sapiens*
Vinculin	0.0466	NP_003364.1	*Homo sapiens*
Par6 (partitioning defective 6 homolog alpha)	0.0451	NP_058644.1	*Homo sapiens*
p190RhoGAP (rho GTPase-activating protein 35)	0.0433	NP_004482.4	*Homo sapiens*
Symplekin	0.0360	NP_004810.2	*Homo sapiens*
Ezrin	0.0309	NP_001104547.1	*Homo sapiens*
LKB1 (serine/threonine-protein kinase STK11)	0.0280	NP_000446.1	*Homo sapiens*
ARHGAP12 (Rho GTPase-activating protein 12)	0.0258	NM_018287.7	*Homo sapiens*
GEF-H1 (rho guanine nucleotide exchange factor 2)	0.0245	NP_001155855.1	*Homo sapiens*
β-Catenin (catenin beta-1)	0.0217	NP_001091679.1	*Homo sapiens*
MST1 (STE20-Like Kinase Macrophage Stimulating 1)	0.0217	NP_006273.1	*Homo sapiens*
aPKC (atypical protein kinase C zeta)	0.0196	NP_002735.3	*Homo sapiens*
aPKC (atypical protein kinase C iota)	0.0137	NP_002731.4	*Homo sapiens*
AMPK1 (protein kinase AMP-activated catalytic subunit alpha 1)	0.0128	NP_006242.5	*Homo sapiens*
AMPK2 (protein kinase AMP-activated catalytic subunit alpha 2)	0.0050	NP_006243.2	*Homo sapiens*
ECT2 (epithelial cell transforming 2)	0.0038	NP_001245244.1	*Homo sapiens*
Merlin (neurofibromin 2)	0.0023	NP_000259.1	*Homo sapiens*
PDZD11 (PDZ domain-containing protein 11)	0.0004	NP_001357103.1	*Homo sapiens*

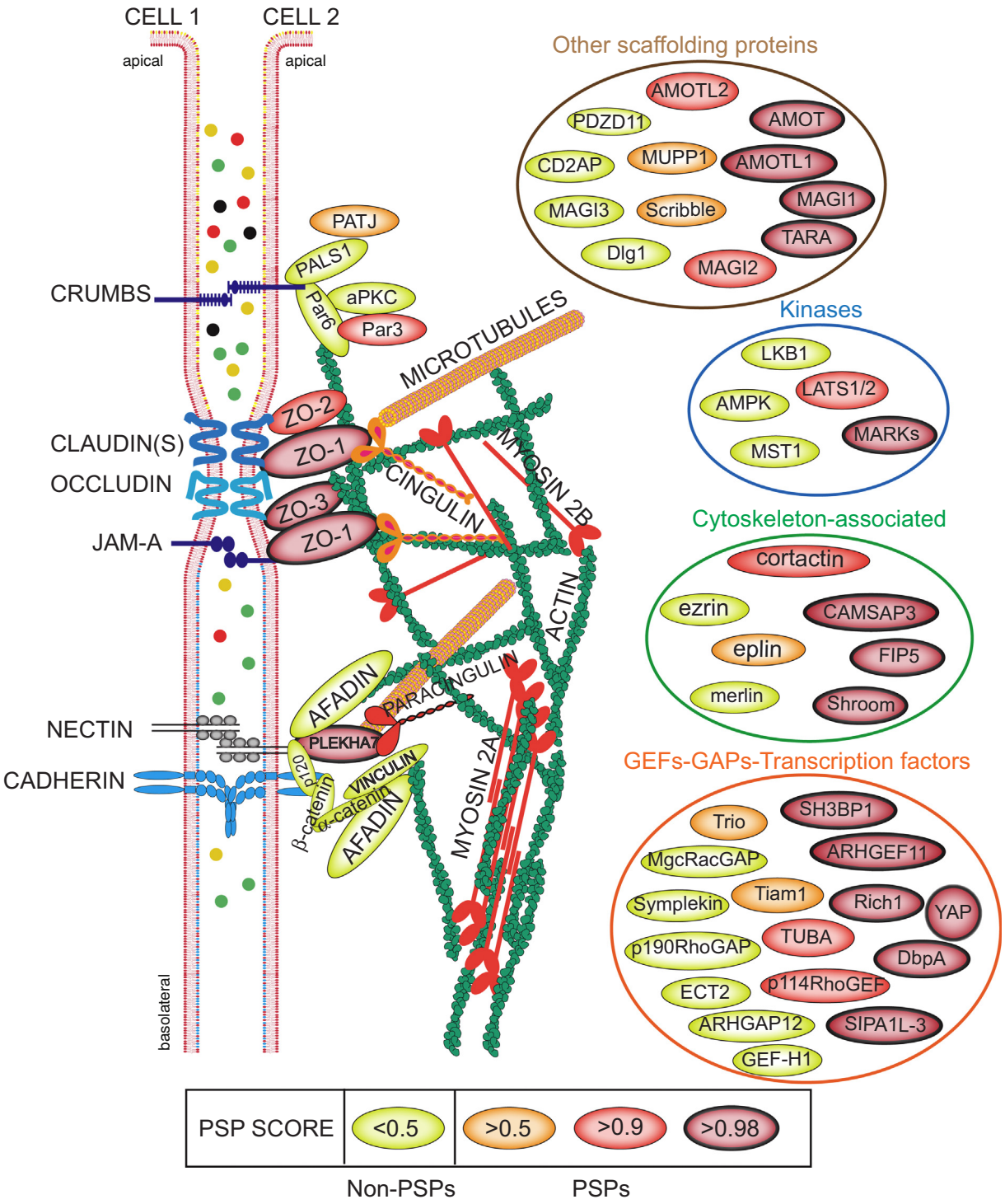

Fig. 4. Simplified schematic diagram of the organization of the apical junctional complex (APC), with predicted phase separating proteins (PSPs) colored in different shades of red/orange, and non PSPS colored in yellow, according to the predicted PSP score (see graphical legend). Major transmembrane proteins of TJ and ZA, as well as Crumbs, and actin and myosin filaments and microtubules are also indicated. The depicted organization of actin filaments (parallel and bundled in the peri-junctional belt, and cross-linked in the juxta-membrane area) and nonmuscle myosin-2A (peri-junctional) and myosin-2B (juxta-membrane) reflects recent data [22,49]. On the right, additional proteins that are not included in the scheme for the sake of clarity are divided into functional groups. Some of these proteins are implicated in mediating the interactions that are schematically represented, for example the PLEKHA7-CAMSAP3 complex mediates the ZA interaction with microtubules. The extracellular round dots are indicated to graphically represent barrier function, and are color-coded as follows: green: solutes which can freely pass through the paracellular channels; orange: solutes that can partially pass through the paracellular channels; red: diffusion prohibited at the TJ but can pass through the leak pathway; black: solutes which cannot pass through the TJs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

scaffolds, for their potential to undergo liquid-liquid phase separation. We found that several GEFs and GAPs, namely Trio and its interactor Tara [374,375], Tiam1 [208,209], p114RhoGEF (ARHGEF18) [123,138], ARHGEF11 [120], Tuba [137], Rich1 [224], SIPA1L-3 [376] and SH3BP1 [314] are predicted PSPs, whereas GEF-H1 [299], ECT2 [211,300,377], ARHGAP12 [376], MgcRacGAP [300] and p190RhoGAP [378] are not predicted PSPs (Tables 1 and 2 and Fig. 4).

Although the accuracy of this prediction method still requires experimental validation, recent publications support the idea that several of the proteins identified here as potential PSPs do in fact form biomolecular condensates [22,379–381]. For cingulin and paracingulin, we previously described a distribution of fluorescently tagged proteins in the form of dynamic cytoplasmic and junction-associated "aggregates" in live isolated MDCK cells [305]. These structures fuse, drip and are dynamically rearranged in time-lapse videos, consistent with the hypothesis that they are biomolecular condensates [22,305]. It is interesting to note that RNAs are essential components of a variety of biomolecular condensates [354,355], and that PLEKHA7 is a predicted PSP, and controls the junctional recruitment of RNAs and associated proteins [54,336].

Based on the predictions above, it can be proposed that the formation not only of TJ, but also ZA and presumably many other types of junctions (gap junction, synapses) is driven by liquid-liquid phase separation of specific, and partially redundant, scaffolding and associated proteins. In agreement with this idea, PSD-95, a PDZ-containing postsynaptic organizer, and associated proteins undergo phase separation [382,383].

Several questions are raised by these observations and deserve further attention. The first concerns the regulation of the phase separation properties of each protein. Although the ZO-1 sequence predicts that it is a PSP, ZO-1 exists also in a non-condensated form [103], consistent with previous FRAP studies, showing junctional and soluble pools [132,384]. As mentioned above, the "switch" to initiate liquid phase separation may be force, clustering, post-translational modifications, multivalent interactions, or a combination of these triggers. Conversely, could force induce a non-PSP protein to phase separate, or to become a client protein for pre-existing condensates? What regulates the shape, scale and size of condensates, which of the junctional PSP first nucleates condensation, and how is coalescence of different condensates regulated? As many junctional proteins interact with each other, it is possible that they co-assemble into the same condensate to form a macromolecular complex, instead of forming separate condensates that coalesce together. Interestingly, Schwayer et al. noted that the size of the non-junctional ZO-1 condensates was affected by the presence or absence of the C-terminal region, suggesting that interactions of this region with additional proteins regulate size of condensates [356]. Similarly, Beutel et al. noticed that the C-terminus of ZO-1 was required to spread the ZO-1 condensates along a continuous junctional belt [103]. Since the organization of actin filaments has been shown to control the size of nuclear condensates [385], it is likely that actomyosin organization at junctions and in the cytoplasm, also controls the size and shape of the biomolecular condensates of junctional proteins. Furthermore, junctional sub-compartments could be generated because of the immiscibility of different liquid-like phases, similarly to what observed for nucleoli [386]. Finally, these observations raise questions about the mechanisms through which the major mechano-sensing proteins of the AJ/ZA, e.g. α-catenin and vinculin, are recruited to junctions. Are they recruited as client proteins into condensates formed by PLEKHA7 and other AJ/ZA-associated proteins, such as ZO proteins, or through other mechanisms?

Another question concerns the emergent properties of junctional and signaling proteins, when present in biomolecular condensates, either as PSPs or client proteins. Several ZA transmembrane and cytoplasmic components, such as E-cadherin, nectins, catenins, and ADAM10 are detectable both at lateral contacts and ZA, but they are specifically clustered at the ZA [51,387]. Clustering of transmembrane proteins by liquid phase separation of their scaffolding proteins may increase the local concentration of transmembrane molecules and allow them to spontaneously form ordered polymers. This could be the case for claudins, which are detectable along the lateral membrane, and also in the nucleus [322,388–390], but are clustered and assemble into the typical TJ strand network only when they are concentrated by binding to ZO proteins. In the case of the ZA-associated complexes, their clustering and linkage to the actomyosin cytoskeleton could render transmembrane complexes, such as ADAM10-nucleated toxin pores, particularly stable and resistant to endocytosis, when compared to lateral contact-associated pores [51]. Another consequence of protein clustering may be altered enzymatic and receptor activity [354]. Zonular junctions are sites of accumulation of growth factor receptors, transmembrane receptor kinases and phosphatases, and other enzymes, such as the metalloprotease ADAM10 [51,391–393]. These enzymes and receptors cross-talk with structural junctional components, thereby influencing junction assembly and dynamics. However, their enzymatic activities and ligand-binding properties could be modified by inside-out signaling via condensation and mechano-regulation of their scaffolds. On the other hand, while sequestration of GEF-H1 by TJ-localized cingulin inhibits its activity [299], how other GEFs and GAPs, either PSPs or clients (Fig. 4), are regulated by liquid phase separation remains to be determined.

12. Conclusions

The spatial organization of cells is crucial for their physiology. It can be argued that localization of proteins at specific sites is the single most important factor that controls their activity. Aberrant localization and biophysical properties of proteins can be either the cause or consequence of pathogenic processes, independently of levels of mRNAs or protein. Thus, understanding the mechanisms through which scaffolding proteins are assembled at specific cellular sites, and how their interaction with transmembrane proteins and other adaptor and signaling proteins is regulated and translates into emergent functions is a central question in cell biology. Cells have developed a flexible molecular toolkit to scaffold a variety of transmembrane proteins, including cell adhesion and barrier/channel forming molecules, receptors for growth hormones and neurotransmitters, and, last but not least, proteins that are used as receptors by bacterial and viral proteins. Each cell type can configure its molecular scaffolding toolkit according to its specific needs and tissue context. Central to the toolkit are structural domains such as PDZ, SH3, WW, GUK, coiled-coil and proline-rich regions, which mediate protein-protein interactions, and allow building ordered scaffolding networks, in part through phase separation. The second central element to the toolkit is the presence, in a subset of scaffolding proteins, of domains that mediate direct or indirect interactions with cytoskeletal proteins and their regulators. This allows specific cell types, for example proliferating cells in developing and adult tissues, and those in organs subjected to mechanical stresses, to adapt to the challenges of continuous junctional remodeling and dynamic interaction with mechanical inputs, in part through proteins that function as mechano-regulated switches. Finally, interaction of junctional scaffolding proteins with transcription factors, RNAs and RNA-binding proteins, and other signaling molecules couples tissue organization to regulation of gene expression and intracellular signaling pathways. Much remains to be learned in this fascinating field of cell biology, but the combination of improved imaging technologies, new biophysical approaches and studies at the structural, cellular and organism level promises to provide exciting new insights.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

influence the work reported in this paper.

Acknowledgements

The Citi Laboratory is funded by the State of Geneva and by the Swiss National Science Foundation (Grant n. 31003A_172809).

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