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Review

Structural and functional associations of apical junctions with cytoskeleton

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Abstract

Actin dynamics play multiple roles in promoting cell movement, changing cell shapes, and establishing intercellular adhesion. Cell contact events are involved in tissue morphogenesis, immune responses, and cancer cell invasion. In epithelial cells, cell–cell contacts mature to form apical junctions with which the actin cytoskeleton physically associates. Living cell imaging shows, however, that the apical junctional complex is less dynamically regulated than the actin cytoskeleton, indicating that their interaction does not remain stable. Given that several cell adhesion modules are clustered at apical junctions, the sum of weak or transient interactions may create linkages that can be strong yet easily remodeled. Here we describe how subcellular protein interactions are coordinated to induce changes in actin organization and dynamics, in response to the status of apical junctions.

Keywords: Actin cytoskeleton; Cadherin; Epithelial cell polarity; Nectin; Small G protein

Contents

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1. Introduction

Apical junctions are a hallmark of polarized epithelial cells. They play a role in providing the paracellular barrier required to separate tissue spaces [1], partly contributing to maintenance of the apical–basal cell polarity and providing a site for signaling [2]. At the ultrastructural level, tight junctions and adherens junctions appear as highly organized structures that encircle the apical–lateral boundary of cells. These structures are composed of transmembrane proteins such as claudins, cadherins, and nectins. These proteins are associated with cytoplasmic proteins that are directly or indirectly connected to the actin cytoskeleton. A transmembrane protein and its cytoplasmic adaptor proteins may thus form a functional module that works both individually and in combination in order to establish and maintain apical junctions.

In many morphogenetic processes and cellular functions, actin filaments are required to be reorganized rapidly in response to changing conditions and signals. Actin remodeling is accomplished by assembling multi-molecular complexes of structural and regulatory proteins at specific cell contact sites. Then, actin filaments undergo elongation, branching, bundling, and depolymerization, leading to assembled superstructures.

Previous studies have contributed to define components required for the formation of apical junctions and the establishment of epithelial cell polarity and have revealed the elaborate biochemical interactions among more than 40 transmembrane and cytoplasmic proteins, defining their functional role in animals and cells in culture. Much information has been obtained by gain-of-function and loss-of-function approaches. In addition, the photobleaching technique using green fluorescence protein (GFP)-fusion proteins and three-dimensional analyses using Madin–Darby canine kidney (MDCK) cells have provided us with significant insights into the formation of apical junctions and cell polarity. Here, we summarize these recent advances and discuss their significance.

2. Structural organization of tight and adherens junctions

Apical junctions, comprising tight junctions and adherens junctions, are sites of mechanical attachment regulated by dynamic changes in the actin cytoskeleton. Adherens junctions have denser actin-filament networks than those of tight junctions in polarized epithelial cells as clearly distinguished by electron microscopy. Tight junctions act as a seal that prevents the paracellular transport of large molecules. Due to their location at the apical–lateral border of cells, they also act as a fence that physically separates the plasma membrane into the apical and basolateral membrane domains. The “fence” function of tight junctions, however, is not directly related to epithelial polarity [3]. Immediate to the basal aspect of the tight junctions, adherens junctions encircle the apical portion of adjacent epithelial cells; however, in nonepithelial tissues, they form punctate or streak-like attachments. Adherens junctions have prototypic roles in stabilizing the epithelium by promoting tight-junction formation and mechanically supporting its function [4]. They further participate in establishing epithelial cell polarity and facilitate cell–cell communication that regulates cell proliferation and movement [5]. In mammalian epithelial cells, tight and adherens junctions are typically aligned from the apical side to the basal side [6], while the septate junctions of epithelial cells in *Drosophila*—which function as a seal similar to the tight junctions of mammalian cells—are localized in the middle of the lateral membrane domains [7]. The apical–basal alignment of tight and adherens junctions may thus not be a prerequisite for the survival of living organisms.

The strength of the extracellular interactions of cell adhesion molecules and that of the intracellular linkages to the actin cytoskeleton are two major factors that determine how effectively cell adhesion functions. Cell adhesion is mediated by transmembrane proteins that are specifically located at each junction. Claudins and occludin are located in tight junctions [4,5]. Junction adhesion molecules (JAMs) are located in the apical part of tight junctions [5,8]. Both cadherins and nectins are located in adherens junctions and play major roles in linkages to the actin cytoskeleton [9–12]. The extracellular domain of cadherins forms homophilic *trans*-dimers in a Ca^{2+}-dependent manner whereas that of nectins forms homophilic or heterophilic *trans*-dimers in a Ca^{2+}-independent manner. It remains controversial whether or not cadherins first bind to form *cis*-dimers on the surface of the same cells and then promote cell–cell contacts by forming homophilic *trans*-dimers [13]. In mammals, the cadherin family is composed of over 70 members and exclusively mediates cell–cell adhesion of the same cell type, whereas the nectin family is composed of 4 members and further mediates cell–cell adhesion of different cell types. Heterophilic *trans*-interactions have been detected between nectin-2 and nectin-3, between nectin-1 and nectin-3, and between nectin-1 and nectin-4. Importantly, heterophilic *trans*-dimers of nectins form stronger cell–cell attachment than homophilic *trans*-dimers [14–16], indicating that heterophilic engagement of nectins may play key roles during cell recognition and sorting. Adherens junctions provide flexible and dynamic cell adhesion through cadherins and nectins in many tissues. The strength of the cell contact is measured with optical tweezers [17] and by the force that is required to separate paired cells expressing cadherins [18]. These studies indicate that only a part of E-cadherin is associated with the cytoskeleton in cultured cells and that the maturation of cadherin-mediated cell contacts strongly depends upon Rac and Cdc42 small G protein activities. Intracellular signalings and scaffold remodeling may thus regulate cooperative *trans*-interactions of cadherins and nectins as well as their association with the actin cytoskeleton at adherens junctions.

A common feature of cell adhesion molecules is that their cytoplasmic tails are associated with adaptor proteins (also called anchoring proteins) such as zonula occludens (ZO) proteins, catenins, and afadin (Table 1) (Fig. 1). ZO proteins specifically bind cell adhesion molecules at tight junctions including claudins, occludin, and JAMs [2], whereas catenins and afadin bind cadherins and nectins, respectively [19,20]. Adaptor proteins capture cell adhesion molecules and influence their location on the cell surface although adaptor proteins are not necessarily the primary cue for the localization of cell adhesion molecules [21]. The interaction between cell adhesion molecules

Table 1
Molecules linking apical junctions to the actin cytoskeleton

Adaptor proteins associated with adhesion molecules:	
Zonula occludens	
ZO-1	ZO-1/2 heterodimer
ZO-2	Formation of tight junctions
ZO-3	ZO-1/3 heterodimer
Afadin	Adaptor protein for nectins
	Interaction with ZO-1 and α-catenin
Catenins	Adaptor proteins for cadherins
α-Catenin	Putative allosteric molecular switch between the cadherin–catenin module and actin filaments
	Inhibiting the Arp2/3 complex for binding actin filaments
β-Catenin	Binds cytoplasmic tails of cadherins
	Transducer of Wnt signaling
$p120^{ctn}$	Upregulation of Rac and Cdc42
	Downregulation of RhoA and NF-kB signaling
Actin-binding proteins associated with conjunctional complexes:	
Arp2/3	Y-branched actin filaments
	Lamellipodia formation
Cortactin	Arp2/3-mediated actin assembly
	Cadherin-based contact zone extension
WASP/WAVE	Nucleation-promoting factor of Arp2/3 complex
N-WASP	Activated by Cdc42 and PI(4,5)P2
WAVE 2	Activated by Rac
Formins	Recruited by α-catenin to adherens junctions
	Linear actin filament/radial actin cable
	Stabilization of adherens junctions
	Filopodia formation
Ena/VASP	Actin filament elongation at barbed end by antagonizing with capping proteins
	Extension of filopodia
Annexin 2	Assembled by PI(4,5)P2 and Cdc42
	Adluminal formation in three-dimensional MDCK cysts
ADF/cofilin	Actin depolymerization and debranching
	Severing actin filaments to form new barbed ends and elongation
Ezrin	Associates with membrane and actin filaments in epithelial cells
Myosin VI	Associates with E-cadherin, vinculin, and actin filaments

and adaptor proteins is essential for cell adhesion. For instance, the mutant E-cadherin lacking the binding domain for adaptor proteins does not promote cell adhesion [22]. Mice lacking α-catenin, β-catenin, or afadin show embryonic lethality although mice lacking β-catenin are likely to die of defective roles in the Wnt signaling [23–25]. In *Drosophila*, the single ZO-1 homolog, polychaetoid, is required for junction remodeling during both tracheal development [26] and dorsal closure [27]. Cell adhesion molecules and adaptor proteins may thus form functional modules that mediate distinct functions at apical junctions, thereby recruiting cytoplasmic components in order to induce the further maturation of cell–cell contacts and the subsequent formation of apical junctions and cell polarity.

The idea that cell adhesion molecules are directly linked to the actin cytoskeleton via adaptor proteins at apical junctions is based on biochemical studies showing that adaptor proteins have a domain for binding actin filaments (Fig. 1). Claudins, occludin, and JAMs interact with ZO proteins that have a domain for binding actin filaments [5,8]. Cadherins directly bind β-catenin, which in turn binds α-catenin that contains a domain for binding actin filaments. α-Catenin then binds vinculin and α-actinin that have a domain each for binding actin filaments [28–30]. Cadherins are thus connected in parallel to the actin cytoskeleton via these proteins [29]. Nectins directly bind afadin that has a domain for binding actin filaments. The idea has generally been accepted for years as evidenced by naming the catenins after "catena," which means a chain in Latin. However, the idea is not entirely correct. The adaptor proteins, ZO-1 and afadin, act as scaffolds that interact simultaneously with several cytoplasmic proteins. For instance, the nectin–afadin module is anchored to the actin cytoskeleton via at least 3 routes: the ponsin–vinculin unit [31], the afadin DIL-domain-interacting protein (ADIP)–α-actinin unit [32], and the LIM-domain-only 7 (LMO7)–α-actinin unit [33]. In this article, we focus on the "adaptor" proteins connecting 2 different molecules [34], i.e., cell adhesion molecules and actin filaments. To understand the relationship between apical junctions and the actin cytoskeleton, the adaptor proteins for cell adhesion molecules should be correctly recognized as scaffold proteins.

Specificities of tight and adherens junctions are less clear in the linkage to the actin cytoskeleton than they are in cell surface structures and functions. First, there is little difference in the cytoplasmic region of cell adhesion molecules, when comparing the overall structures between their subtypes. For example, the cadherin subtypes show the highest homology in the cytoplasmic region [35,36], suggesting that the cytoplasmic specificity of cadherins is not as prominent as the extracellular specificity. Second, the adaptor proteins are not always aligned to tight junctions or adherens junctions. Although ZO proteins are major scaffolds at tight junctions, they are located in adherens junctions in tissues lacking tight junctions. In the broader context, ZO-2 and β-catenin can be located in the nucleus. α-Catenin is suggested to interact with the components of gap junctions and desmosomes [37,38]. Although afadin acts as a cue that locates nectin-2 to the adherens junctions of colon epithelia in mice, afadin is found at the leading edges of migrating cells [our unpublished data]. Third, the adaptor proteins interact with each other to organize the relationship between cell adhesion modules. Direct interactions are detected between ZO-1 and α-catenin, between ZO-1 and afadin, and between α-catenin and afadin [39–41] (Fig. 1). Claudins, cadherins, and nectins may thus act independently at the cell surface but act cooperatively via cytoplasmic connections between their adaptor proteins. Furthermore, afadin binds Rap1 and induces signalings to regulate other cytoplasmic protein functions implicated in the maturation of cell–cell contacts and the establishment of epithelial cell polarity [11,42]. Finally, the molecular structures of adaptor proteins and other intracellular proteins associated with cell adhesion molecules or the actin cytoskeleton are evolutionally well-conserved [43]. α-Catenin is a distant relative of vinculin. β-Catenin and p120 catenin ($p120^{ctn}$) are distantly related molecules, comprising the Armadillo-repeat superfamily, and are also structurally related to the components of desmosomes [43]. The catenins act as the machinery that specifically binds cadherins at adherens junctions, but they may also play roles in regulating the microtubule cytoskeleton. For instance, $p120^{ctn}$ regulates the nocodazol sensitivity of microtubules and affects cell motility in cadherin-deficient cells [44]. Additionally, ZO-1 binds many different connexins that are structural components of gap

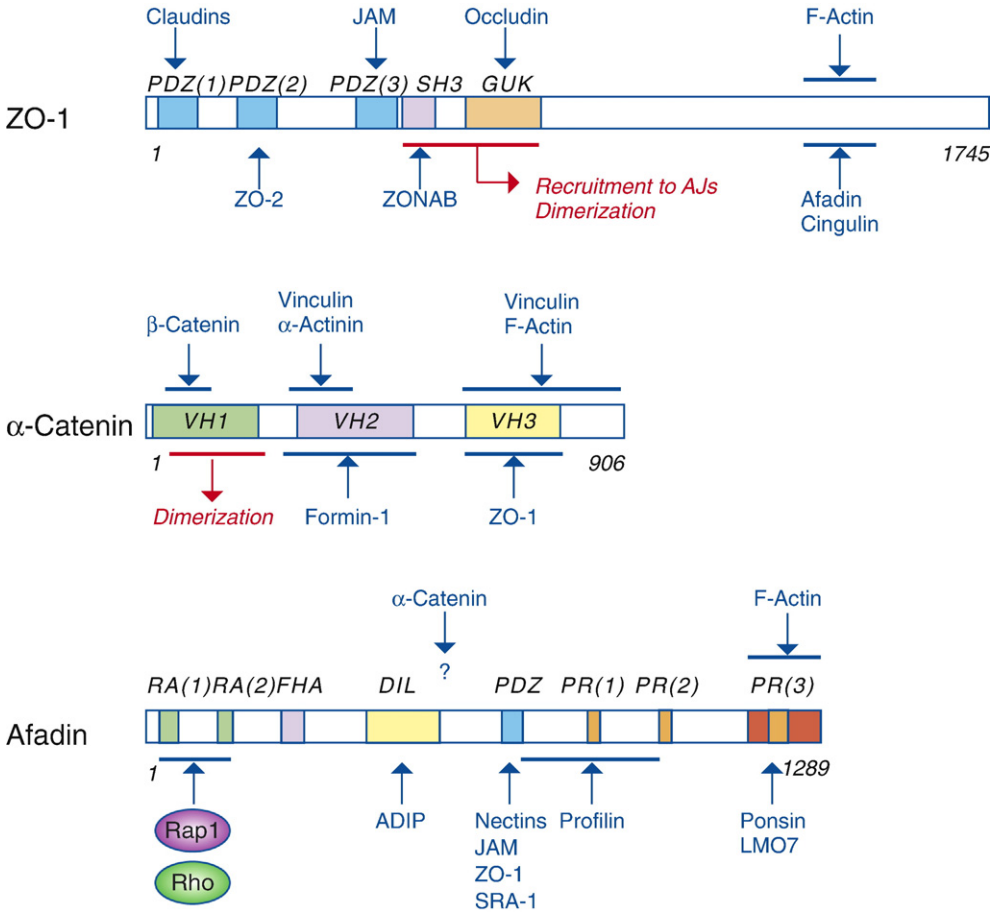

Fig. 1. Adaptor proteins containing an actin-binding domain. Cell adhesion molecule-associated proteins including ZO-1 [207,208], α-catenin [43,209], and afadin [11,20] have an actin-binding domain at the C-terminus. However, it is not yet clear whether the primary function of these proteins at the apical junctions is to bind actin as adaptors or other proteins as scaffolds. They actually mediate a variety of protein interactions. Notably, they interact with each other. The domains implicated in ZO1–α-catenin and ZO-1–afadin interactions are shown while those in α-catenin–afadin have not yet been determined. Abbreviations: PDZ, the PDZ domain; SH3, the Src-homology 3 domain; GUK, the guanylate kinase domain; VH 1–3, the vinculin-homology 1–3 domain; RA, the Ras-associated domain; FHA, the forkhead associated domain; DIL, the dilute domain; and PR, the proline-rich domain.

junctions [45]. Notably, many proteins containing PDZ (postsynaptic density protein (PSD)-95, discs-large, and ZO-1) domain(s) are distributed in apical junctions. Cell adhesion complexes including tight junctions, adherens junctions, desmosomes, gap junctions, and focal contacts may thus depend upon the functions of intracellular proteins structurally related to the adaptor proteins.

The efficient formation and maintenance of apical junctions appear to require the force generated by actin-dependent movement. However, the dynamics of the linkage between apical junctions and the actin cytoskeleton have not fully been addressed. No researcher has detected the formation of a ternary complex composed of a cell adhesion molecule, an adaptor protein, and actin filaments. The role of adaptor proteins to physically link cell adhesion molecules to the actin cytoskeleton does not explain the involvement of small G proteins; neither is this role sufficient to account for the dynamic reorganization of the actin cytoskeleton that occurs during the assembly of apical junctions and the formation of epithelial sheets. Importantly, there are close subcellular networks among ZO proteins, catenins, and afadin (Fig. 1). Because of the networks among the adaptor proteins, cell adhesion modules may play mutually dependent roles at tight junctions and adherens junctions, resulting in the formation of linkages that are strong and yet easily remodeled by extracellular signaling.

3. Involvement of the actin cytoskeleton in cell adhesion

Actin filaments mechanistically confer a driving force to cell movement, and they are involved in functions of individual cells and morphogenetic processes in animals. In polarized epithelial cells, actin filaments form the cortical belt encircling the most apical end of the lateral membrane domain and support apical junctions; however, at the basement of the cell, they form stress fibers and support focal contacts. There is a “cross-talk” between the cell–cell and cell–matrix junctions and the formation of both types of junctions is mutually regulated [46,47]. This regulation plays a critical role in morphogenesis and cytokinesis during which the actin cytoskeleton is vigorously reorganized whereas the apical junctions remain intact. Dynamic actin remodeling is observed in a variety of biological events, such as gastrulation [48], the formation of the neural tube, closure of the dorsal cleft, closure of wounds, invagination of epithelial cells or the mesoderm, and changes in cell shape during tube formation [49]. Mechanical links between apical

junctions and the actin cytoskeleton may be required to generate a tensile force because epithelial invagination is caused by the constriction of cells on their apical sides. Furthermore, actin filaments are required to remodel rapidly in response to changing extracellular conditions and signals. In *Drosophila*, the transmembrane protein T48 recruits adherens junctions and Rho guanine nucleotide exchange factor (GEF)-2 to the site of apical constriction and induces rapid and intense changes in cell shape [50]. During the invagination of the mesoderm in *Drosophila*, the adherens junction shifts in response to the apical constriction by the actomyosin cytoskeleton [51]. Therefore, cell adhesion and movement are not mutually exclusive but sometimes-coupled events, even though the movement of the junctions is not simply mediated by a tensile force that arises from the constriction of the actin cytoskeleton.

Apical junctions appear to be physically linked to the actin cytoskeleton because altering the actin cytoskeleton destabilizes the junctional complex and vice versa. When cells are treated with agents disrupting actin filaments such as latrunculin A or cytochalasin D, tight junctions and adherens junctions eventually disappear [52–55]. The disruption of the cortical actin cytoskeleton interferes with the localization of cadherin–catenin complexes, leading to an abolishment of adherens junctions [56]. On the other hand, the disruption of β-catenin in *Drosophila* leads to a loss of characteristic cell shapes, disorganized actin cytoskeletons [57], and widespread defects in the polarity of the actin cytoskeleton [58]. When the function of adherens junctions is downregulated by the depletion of β-catenin, the actomyosin ring constricts without causing a change in the cell shape [51]. Therefore, apical junctions and the cytoskeleton may play cooperative roles in the maintenance of epithelial cell structures.

Actin filament organization and dynamics have been imaged in living cells using the fluorescence recovery after photobleaching (FRAP) technique. It has been observed that the fluorescent intensity of green fluorescent protein (GFP)-tagged actin does not correlate with the state of actin polymerization. In order to analyze the dynamic changes in actin filaments, FRAP was introduced to decipher the turnover of actin molecules by analyzing the recovery of fluorescence after photobleaching, a method to erase fluorescence by the laser at the defined area. Using FRAP, the cortical actin was shown to be much more dynamic than the cadherin–catenin complex [59]. This finding indicates the lack of a highly stable linkage between adherens junctions and the actin cytoskeleton. However, the finding would not explain why altering the actin cytoskeleton destabilizes adherens junctions. It appears unlikely that there is no physical linkage or that some specific proteins are concentrated at the apical junctions to regulate transient linkages. The FRAP data would suggest that neither the cadherin–catenin nor the nectin–afadin modules participate in stable linkages. Nevertheless, the apical junctions are required for epithelial cells to establish a strong and polarized intercellular adhesion [60–62]. Therefore, cadherin- and nectin-based cell adhesions should induce changes in actin organization and dynamics using indirect and flexible machinery.

Although there exist multiple cell adhesion modules in epithelial cells, none of them appear to have decisive roles in the linkage to the actin cytoskeleton. Then, what are these redundant modules required for? It may be of note that the linkage between apical junctions and the actin cytoskeleton is neither solid nor stable but rapidly and easily remodeled. In the RNA interference studies using MDCK or EpH4 cells, it was observed that the reduced levels of E-cadherin and afadin do not abolish the establishment of the apical junctions [63,64]. Commonly observed results demonstrated the delay in forming mature junctions. A similar observation has been definitely provided by the loss-of-function experiments with ZO-1. Experimental evidence so far suggests that ZO-1 alone is not required for cells to form tight junctions. When ZO-1 is deleted from cultured epithelial cells by either homologous recombination [3] or RNA interference [65], tight junction assembly is markedly slowed, but normal barrier properties are ultimately achieved. These studies suggest that ZO-1 is required for the normal kinetics of tight junction assembly. Taken together, the redundancy of cell adhesion modules could closely correlate with the normal kinetics that ensure the rapid remodeling of the actin cytoskeleton. Cell adhesion may be the sum of weak linkages, which may provide cells with the machinery to undergo rapid reorganization of actin filaments. A local assembly of regulators for actin filaments is critical to perform these cellular changes efficiently. For this purpose, it seems practical that both tight junctions and adherens junctions are located side by side at the apical region in mammalian epithelial cells. Conceivably, signaling pathways involving small G proteins and phosphoinositides are necessary for cells to spatially and temporally recruit relevant molecules.

Despite considerable efforts by many research groups, the precise mechanism of dynamics between apical junctions and the actin cytoskeleton has not been fully addressed. Although the adaptor proteins are capable of directly binding actin filaments, they mainly regulate actin dynamics via several actin-binding proteins. The linkage between apical junctions and the actin cytoskeleton appears to be formed by the sum of several weak bindings although such weak and transient interactions cannot be detected individually [66,67]. The primary significance of multiple adhesion modules appears to be to combine and correlate each module's role in actin dynamics for the rapid remodeling of the epithelial sheet, maintaining some part of cell adhesion. Compensatory modules could probably back up the role if one module is compromised. Evidently, the cadherin–catenin and nectin–afadin modules play dynamic roles by exchanging the cycle of assembly and disassembly at the cell surface and the cytoplasm. The common goal of such adhesion modules would be to enable individual cells to rearrange intercellular contacts during cell division or migration whereas the epithelial sheet maintains its integrity on the whole.

4. Molecular basis of the linkage between apical junctions and the actin cytoskeleton

4.1. Actin activities and structures

Modes of actin organization at the cell cortex are briefly summarized in Fig. 2. Earlier excellent reviews provide detailed information on actin dynamics [68,69]. The actin filaments are

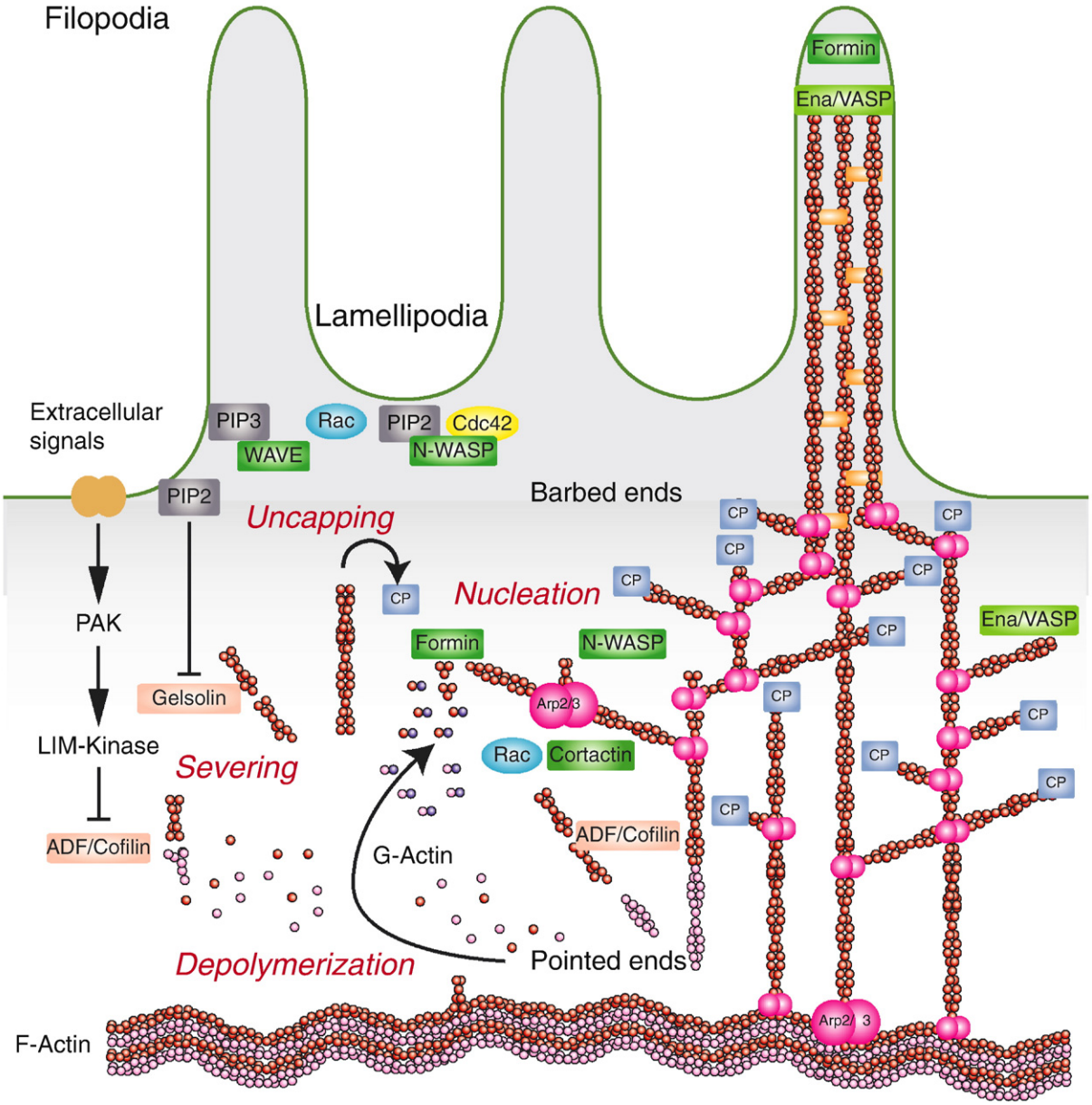

Fig. 2. Modes of actin dynamics during filopodia and lamellipodia formation. Actin filaments spread the barbed ends toward the cell margin to form filopodia and lamellipodia. While the barbed ends are sealed with capping proteins (CP), the pointed ends are severed and depolymerized by ADF/cofilin and gelsolin. Monomeric actin is coupled with profilin, converted to its ATP-binding form, and then recruited to the barbed ends for recycling. Lamellipodia contain branched actin networks formed by nucleators such as the Arp2/3 complex, cortactin, and N-WASP whereas filopodia contain elongated actin bundles formed by formins and Ena/VASP. Fascin and other crosslinking proteins indicated by yellow boxes mediate the formation of the actin bundles. Ena/VASP protein modulates distinct modes of actin organization possibly at adherens junctions [132] although they are not involved in PKA-mediated localization of claudin-16 to tight junctions [133]. Rho family small G proteins and phosphoinositides regulate actin dynamics. Activated Rho family proteins mediate signalings through N-WASP, WAVE, cortactin, and formins as their effectors. Activated Rho family proteins also activate PAK and LIM kinase [185] that regulate actin depolymerization by LIM-kinase-mediated inhibition of ADF/cofilin activity. Notably, PI(4,5)P2 has an important role in coupling actin assembly to plasma membrane extension [210] and may enhance the formation of intercellular projections and contact zone extension [23]. Rac1 binds to PIP5 kinases [211,212] and facilitates localized PI(4,5)P2 production at *N*-cadherin-based cell–cell contacts [76]. Locally generated PI(4,5)P2 inhibits gelsolin severing and dissociates gelsolin from actin filaments, thereby facilitating barbed-end uncapping, which favors growth of actin networks necessary for adhesion zone extension and intercellular contact strengthening. Other cadherin-associated, actin-binding proteins such as vinculin, Arp 2/3, and cortactin may be directly or indirectly regulated by PIP5 kinase-mediated PI(4,5)P2 synthesis [213–216].

double helical polymers of globular subunits with a molecular polarity that is arranged in a head-to-tail fashion. The barbed end is favored for growth while the pointed end is catalytically less active. Actin filaments in cells are oriented perpendicularly with respect to the cell surface. The ATP–actin monomer forms a complex with profilin that catalyzes the exchange of ADP for ATP [70]. Profilin maintains a pool of actin monomers ready to elongate barbed ends. A set of actin regulators initiates the formation of new actin filaments by a process called nucleation. In order to start filament growth, actin monomers first form trimers

that act as a "nucleus" or "seed" for the subsequent elongation of actin filaments. However, the spontaneous assembly of actin monomers is unfavorable because actin dimers or trimers are very unstable. Therefore, nucleation-promoting proteins (nucleators) such as the actin-related protein-2/3 (Arp2/3) complex [71] and formins [72,73] are required to efficiently promote de novo actin polymerization (Table 1). Once elongation is initiated, the filaments grow rapidly until capping proteins block the barbed ends [74]. After the actin filaments have aged by the hydrolysis of ATP, the actin-depolymerizing factor (ADF)/cofilin proteins promote debranching and depolymerization to disassemble the actin filaments in cells [75]. Locally generated PI(4,5)P2 inhibits gelsolin severing and facilitates barbed-end uncapping, which favors the growth of actin networks necessary for *N*-cadherin-mediated adhesion zone extension and intercellular contact strengthening [76]. Thus, actin recycles the processes of polymerization and depolymerization.

Actin filaments are formed in 2 ways: the elongation of pre-existing actin filaments by uncapping the barbed end or generating a new barbed end by severing the filaments and the de novo actin polymerization through nucleation events (Fig. 2) [77]. The forces driving membrane protrusion are generated by monomeric actin polymerizing onto actin filaments at the cell front. The actin networks, which are composed of branched actin filaments, result in ruffle formation when the adhesion strength is low. Two populations of actin filaments with distinct dynamics, the lamellipodium and the lamellum, are colocalized at the migrating edge of epithelial cells. The lamellipodium is a dynamic actin network and overlaps with a less dynamic actin network of the lamellum [78]. The regenerating lamellipodium forms a cohesive and separable layer of actin above the lamellum. When a cell is stationary, however, actin filaments assemble at the margin of the cell and move away from the edge. This system is described as the actin treadmill because the entire network slowly flows back and depolymerizes while actin polymerizes at the front [79].

To initiate the formation of junctions, cells accumulate actin filaments and protrude structures that resemble lamellipodia and filopodia in nascent adhesion (Fig. 3). There exist junctional actin bundles and peripheral thin actin bundles that are spatially and functionally distinct actin populations [80]. Whereas junctional actin stabilizes clustered cell adhesion molecules at cell–cell contacts, thin actin bundles contract to increase the lateral cell height. Following cadherin-dependent adhesion in human keratinocytes, actin polymerization occurs at cell–cell contacts as well as at flanking bundles with different dynamics. Junctional actin bundles are regulated more dynamically than peripheral thin bundles [80]. These 2 populations change and become indistinguishable to form a cortical actin ring that is characteristic of mature and fully polarized epithelial cells [80]. Nascent adhesions are converted into stable zones of contact, which indicates a morphological process where the limited initial points of cell–cell contacts progressively extend into the stable zones of adhesion [60–62,81]. This entails active cooperation between cadherin adhesion and the force-generating capacity of the actin cytoskeleton. An inhibitor of barbed-end growth, cytochalasin D, disrupts lamellipodia formation as well as the formation of the cortical actin ring in cells, suggesting that the actin cytoskeleton is maintained by a dynamic equilibrium between actin assembly and disassembly even after the apical junctions have become fully developed in cells.

In MDCK cells, cell contact formation by nectins precedes the clustering of E-cadherins (Fig. 3). Nectins first initiate cell adhesion at protruded margins and then recruit cadherins to the nectin-based cell adhesion sites to establish adherens junctions [12]. Multiple domains of nectins act uncooperatively to promote cell adhesion whereas those of cadherins act cooperatively to form a cluster, which may partly support the "fork initiation and zipper" hypothesis for adherens junction formation [82]. Cadherins exclusively promote adhesion between homotypic cells whereas nectins have a dual role in promoting adhesion between homotypic cells and between heterotypic cells. Such differential properties suggest that nectins may be more effective than cadherins in cell recognition and sorting during tissue morphogenesis.

4.2. Regulatory mechanisms of the linkage between cell adhesion molecules and actin filaments

Cell adhesion molecules are essential for anchoring the ends of the actin filaments to the apical junctions. However, the accumulation of actin filaments at cell–cell contacts does not always discriminate between de novo actin assembly and passive redistribution of pre-existing actin filaments. Cell adhesion molecules are connected to the actin cytoskeleton through several mechanisms including the capture of actin filaments, nucleation of new filaments, and modifications of particular actin structures [83]. The adaptor proteins containing an actin-binding domain may simply capture actin filaments in epithelial cells. In contrast, the formation of lamellipodia and filopodia is based on the reorganization of actin filaments including the nucleation of new actin filaments, elongation and depolymerization of the existing actin filaments, and bundling the actin filaments by crosslinking. At the leading edges of migrating cells, many barbed ends of actin filaments are generated by the nucleation of new filaments rather than by the cleavage of the existing filaments or decapping of barbed ends [84,85]. Therefore, scaffold remodeling may as well require the nucleation of actin filaments for the accumulation of peripheral thin actin bundles during the initiation and maturation of apical junctions.

Cadherin-based cell adhesions induce a rapid turn over of actin filaments. Actin remodeling is accomplished on a time scale of tens of seconds in the leading edge of migrating keratinocytes [86] and of several minutes in stress fibers by assembling multi-molecular complexes of structural and regulatory proteins [87–89]. α-Catenin recruits actin-nucleating proteins and regulates their activities to modify actin dynamics at the adherens junctions. For example, the localization of both formin-1 and enabled (Ena)/vasodilator-stimulated phosphoprotein (VASP) proteins to adherens junctions is dependent on α-catenin [23,90]. Furthermore, both cortactin [91] and a component of the Arp2/3 complex, p34 [92], coimmunoprecipitate with E-cadherin. The cadherin–catenin complex could thus trigger the new synthesis of actin filaments by the recruitment of actin-

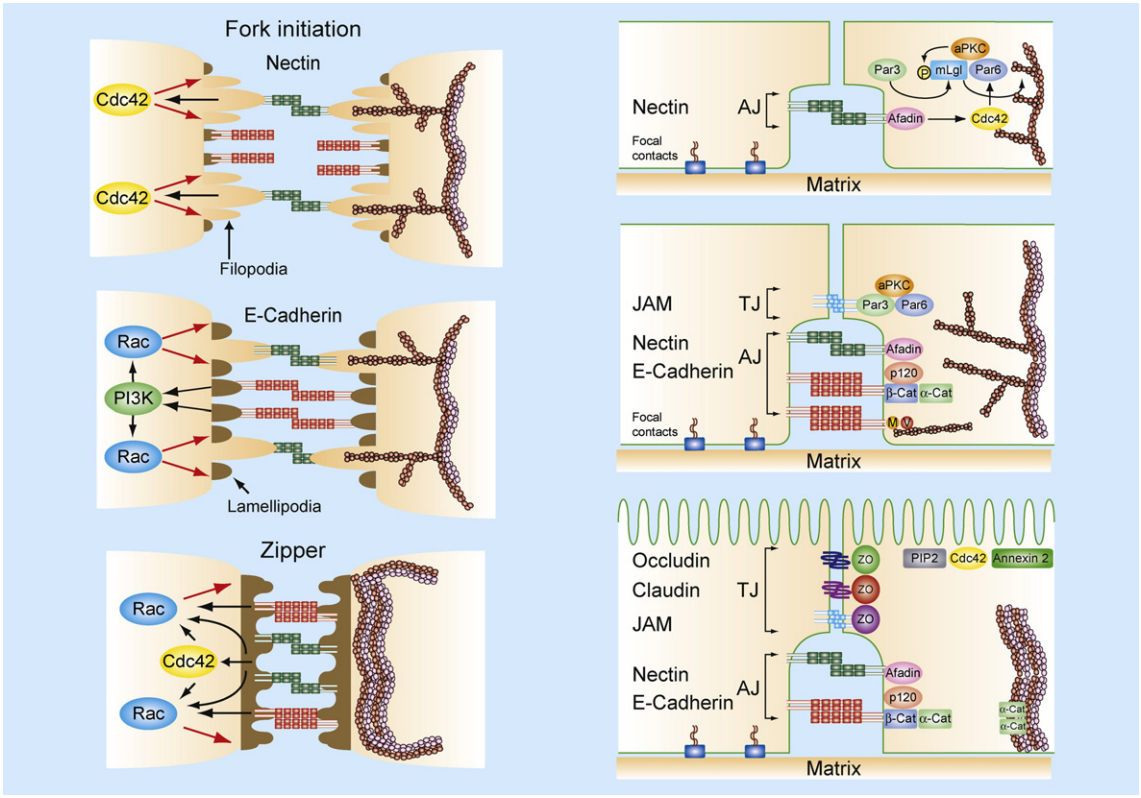

Fig. 3. Maturation of cell contacts, formation of apical junctions, and establishment of apical–basal polarity in mammalian epithelial cells. Initial cell–cell contacts involve the formation of lamellipodia and filopodia. Left panels show contact formation by nectins that precedes the clustering of E-cadherins in MDCK cells. Nectins may play more versatile roles than E-cadherins especially in the initial cell–cell contacts. Conceivably, nectins may play physiological roles in cell recognition and sorting in vivo because nectins have a dual role in promoting heterophilic and homophilic engagement in contrast to cadherins that mediate solely homophilic engagement. Engagement of nectins leads to activation of Cdc42 and Rac [160,162] while that of E-cadherins mainly induces Rac activation via PI3K rather than Cdc42 activation [151]. The panels show how cells form filopodia when Cdc42 is activated by nectin engagement, as well as lamellipodia when Rac is activated by nectin and E-cadherin engagement in the horizontal plane. Filopodia and lamellipodia coalesce as zippers close, leading to the established epithelial structure. Right panels show the formation of new cell–cell contacts by nectins, stabilization of these new contacts, and establishment of apical junctions and cell polarity. Thin actin bundles are formed at the flanking regions of nectin- and E-cadherin-based cell contacts, and then the actin bundles promote the establishment of the vertical plane of cuboidal cells. As the apical junctions mature, the tight junction modules are recruited to the nascent nectin-based cell contacts by afadin and the subsequent activation of Cdc42. Par-3 is necessary to form the tight junction structure at the apical side of the nectin-based cell–cell adhesion. Par-3 also promotes the association of nectin and afadin although Par-3 is not essentially required to form the nectin-based cell–cell adhesion [42]. The cell polarity complex composed of Par-3/Par-6/aPKC and mLgl plays a role in recruiting tight junction components just on the apical side of adherens junctions [192]. Components such as PI(4,5)P2, activated Cdc42, and annexin 2 are localized to the adluminal surface of polarized MDCK cells. Myosin VI (as shown by M) associates with E-cadherin and recruits vinculin (as shown by V) at the cell–cell contacts in a late stage of junction maturation [137].

nucleating proteins to cell–cell contacts, leading to the maturation of adherens junctions.

Conceivably, adaptor proteins may play additional roles except for capturing pre-existing actin filaments. Interestingly, α-catenin may act as a molecular switch that regulates actin dynamics at adherens junctions. Although the cadherin–catenin complex coordinates actin dynamics to assemble and stabilize adherens junctions, the quaternary E-cadherin/β-catenin/α-catenin/actin complex has not been identified so far. On the contrary, α-catenin does not provide a stable linkage to the actin cytoskeleton, but its binding abilities to E-cadherin/β-catenin and to actin are mutually exclusive. α-Catenin assembled into the cadherin–catenin complex does not bind to actin [59]. α-Catenin exists both as monomers binding to E-cadherin/β-catenin and as dimers interacting with actin [93]. Thus, the emerging new insight is that α-catenin may continuously shuttle between an "inactive" cadherin-bound pool and an "active" actin-bound pool regulating the cytoskeleton. Reportedly, the fusion protein of E-cadherin and α-catenin is able to link adherens junctions to the actin cytoskeleton [94]. However, cells expressing the fusion protein do not change shape during mitosis and show impairment in motility within the epithelial sheet [94], indicating that adherens junctions formed by the fusion protein are not committed to rapid remodeling. α-Catenin could thus act as an allosteric regulator of the cytoskeleton when recruited to adherens junctions (Fig. 4).

4.3. Relevant proteins in remodeling the actin cytoskeleton

More than 60 actin-binding proteins comprise several classes: actin nucleators, actin bundling/crosslinking proteins, capping proteins at both ends, and actin-depolymerizing/severing proteins [95,96] (Table 1). Some novel actin-binding proteins including afadin have been identified by a blot overlay assay

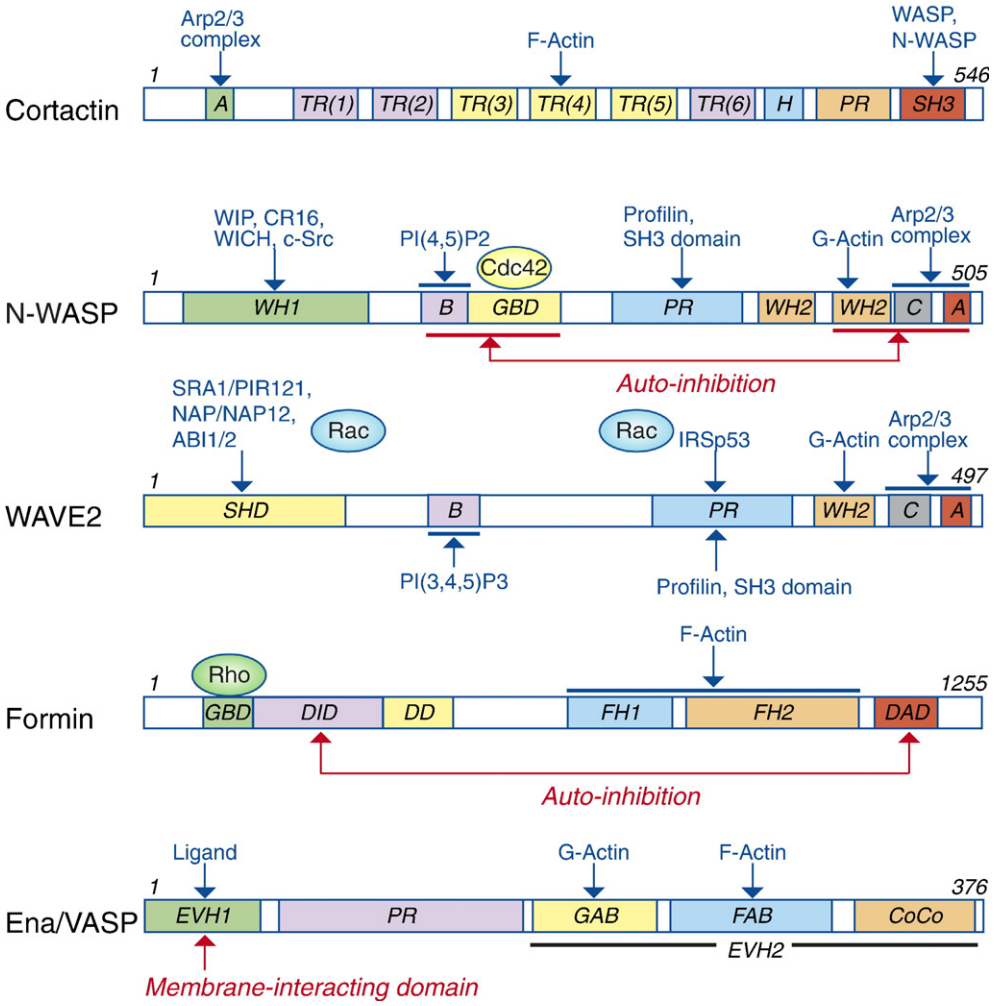

Fig. 4. Actin-binding proteins localized to apical junctions. The structures of actin-binding proteins recruited to cell adhesion sites are schematically shown. Cortactin interacts biochemically with the Arp2/3 complex [105,110] and is colocalized with Arp2/3 at sites of homophilic cadherin adhesion [91]. Cortactin coimmunoprecipitates E-cadherin from MDCK cells whereas cortactin mutants lacking the binding site for F-actin or Arp2/3 inhibit circumferential actin accumulation [91]. N-WASP and WAVE2 regulate Arp2/3 activities and the branching of actin filaments in lamellipodia [77,217]. The VCA domain at the C-termini of N-WASP and WAVE2—composed of a G-actin-binding domain (WH2), a cofilin-homology domain (C), and an acidic domain (A)—is critical for Arp2/3 activation [217]. N-WASP and WAVE2 are proposed to shuttle G-actin to Arp2/3 [218]. While N-WASP associates directly with the active form of Cdc42 at the Cdc42/Rac-interactive binding (CRIB) region, WAVE2 associates indirectly with the active form of Rac, cooperating with SRA1, NAP/NAP12, and ABI1/2 or with IRSp53 [118,219,220]. Rac signaling is important for WAVE2 localization at cell–cell contacts [55]. Formin homology 1 and 2 (FH1 and FH2) domains are involved in nucleating the polymerization of linear actin filaments and promoting their elongation [121]. Formin-1 is localized to cadherin-mediated cell–cell contacts in an α-catenin-dependent manner; its association with the cadherin–catenin complex is necessary for the formation and maintenance of adherens junctions in keratinocytes [90]. A constitutively active Dia1 (Diaphanous-related formin-1) mutant lacking the N-terminal portion is unable to localize to cell–cell junctions and does not show any junction-strengthening effect [221]. Dia1 is one of the Rho targets; RhoA binding triggers the transition of Dia1 from a closed and inactive conformation to an open and active conformation [123,222,223]. Ena/VASP promotes the elongation of actin filaments at the barbed ends, implicated mainly in filopodia formation. All members of the Ena/VASP family have a tripartite structure, consisting of an Ena-VASP-homology 1 (EVH1) domain, a central proline-rich domain (PR), and a terminal EVH2 domain [224]. The EVH1 domain binds to FP4 motifs found in proteins such as vinculin, ActA, and zyxin. The central proline-rich (PR) region binds SH3 domains and the actin-binding protein profilin, whereas the terminal EVH2 domain supports tetramerization and F-actin binding. Although the evidence for an in vivo nucleating activity of Ena/VASP is still missing, homophilic engagements of E-cadherins are sufficient to recruit Ena/VASP to the cell surface [131]. Other abbreviations: TR, tandem repeats; H, helical region; WH1–2, WASP-homology 1–2 domain; B, basic domain; V, Verprolin-homology domain; SHD (WHD), SCAR/WAVE-homology domain; DID, diaphanous inhibitory domain; DD, dimerization domain; DAD, diaphanous autoregulatory domain; GAB, G-actin-binding domain; FAB, F-actin-binding domain; and CoCo, coiled–coil domain.

using ^{125}I-labeled filamentous actin (F-actin) [20,97]. Among them, several actin regulators are localized to either established or nascent apical junctions (Fig. 4). The Arp2/3 complex [92] and its activator cortactin [91]; formins [90]; Ena/VASP proteins [98]; the ezrin, radixin, and moesin (ERM) proteins [99]; and myosins appear to have critical roles in forming linkages between cell adhesion molecules and the actin cytoskeleton.

The Arp2/3 complex nucleates branched actin filaments and plays a central role in actin assembly and branching in lamellipodia [77] but not in filopodia [100]. Two of the subunits are actin-related proteins of the Arp2 and Arp3, and the remaining five subunits are referred to as actin-related protein complex (ARPC) 1–5 [101]. ARPC subunits bind to actin filaments whereas Arp2/3 is thought to mimic an actin dimer or trimer and to function as a template for

the initiation of branched actin filaments [101]. The Arp2/3 complex is localized to sites of initiating cell contact [102]. Since α-catenin has been suggested to play an inhibitory role on the Arp2/3 complex [93], α-catenin dimers may regulate the transition from cortical branched filaments at nascent contacts to actin bundles in mature contacts by competing with the Arp2/3 complex for binding to actin filaments [93]. In contrast, the dysfunction of the Arp2/3 complex might be associated with cancer metastasis [103]. Notably, the Arp2/3 complex itself is catalytically inactive and requires 2 classes of nucleation-promoting factors, namely cortactin and Wiskott–Aldrich syndrome proteins (WASP) along with WASP-family verprolin-homologous proteins (WAVE). Cortactin binding triggers conformational changes in the Arp2/3 complex, which brings its Arp2 or Arp3 subunits closer together, possibly mimicking the barbed end of a filament. On the other hand, WASP-related proteins deliver the first actin monomer to Arp2 and/or Arp3 [104].

Cortactin is a potent regulator essentially required for Arp2/3-dependent actin polymerization [91,105,106], playing a role in the conversion of nascent adhesions into stable zones of contact. Cortactin is a multidomain actin-binding protein [107,108] that can interact directly with both Arp2/3 and actin filaments via different domains [109]. Although cortactin interacts with ZO-1 and some members of the $p120^{ctn}$ family [110], a major target of cortactin is the Arp2/3 complex [105,111] whose activity is necessary for cadherin-based actin accumulation. Cortactin inhibits the debranching of Arp2/3-generated actin filaments, which can potentially stabilize the cortical actin networks [105]. Cortactin and the Arp2/3 complex accumulate in cell–cell contacts in response to the engagement of E-cadherins, notably at the outer margins of contact zones that are actively extended and remodeled. However, the coimmunoprecipitation of cortactin with E-cadherin does not depend on actin filament integrity because the efficiency of coimmunoprecipitation is unaffected by latrunculin A [91]. Cortactin may thus act as one of the signal integrators in cadherin-based contacts, coupling adhesion to actin dynamics and junctional assembly.

WASP or neuronal WASP (N-WASP) and related WAVE family proteins are other regulators for the Arp2/3-dependent actin assembly that occurs in response to E-cadherin engagement. While the expression of WASP is restricted to hematopoietic cell lineages, N-WASP is expressed in epithelial cells. The direct binding of cortactin activates Arp2/3-driven actin nucleation [105,111,112], and this event is enhanced on the association of N-WASP protein [113]. Cortactin and N-WASP can bind simultaneously to the Arp2/3 complex [112]. While cortactin associates with the Arp2/3 complex and inhibits branch dissociation, N-WASP transiently binds the Arp2/3 complex and is released from the complex after branching. The Arp2/3 complex is activated by the C-terminal VCA domain (V, the verprolin-homology domain or WASP-homology 2 (WH2) domain; C, the cofilin-homology domain or central domain; A, the acidic domain) of N-WASP. The VCA domain of N-WASP is sufficient for the activation of the Arp2/3 complex in vitro to polymerize branched actin filaments. The VCA domain is folded intramolecularly but activated when N-WASP binds to activated Cdc42 and phosphatidylinositol-(4,5)-bisphosphate PI(4,5)P2 (Fig. 4; Fig. 6) and/or when phosphorylated by c-Src [114,115]. WAVE has originally been identified as a protein family containing the VCA domain, and WAVE binds activated Rac via SRA1 or IRSp53 to promote the formation of lamellipodia [116,117]. It is still unclear whether WAVE activity is suppressed by the intramolecular interaction, as is the case for N-WASP [118].

Formins are localized to nascent adherens junctions at developing cell–cell contacts. Although formins do not contain actin-like domains as opposed to the Arp2/3 complex, formins nucleate linear unbranched actin filaments in filopodia and facilitate the elongation of actin filaments at the barbed end [72,73,119] (Fig. 2). Unbranched actin filaments are essential for the formation of radial actin cables and the stabilization of adherens junctions in epithelial cells [43]. There are 15 formins in mammals; the best-studied formins are the diaphanous-related formins such as mDia that are regulated by autoinhibitory intramolecular interactions between the N- and C-termini (Figs. 4 and 6). The formin homology (FH)1 domain of formins recruits profilin–actin complexes whereas the FH2 domain mediates actin assembly. Formins are also direct effectors of Rho family G proteins [120]. The N-terminal G protein binding domain (GBD) domain of mDia binds RhoA and RhoC in the GTP-bound state. Notably, Rho engagement is shown to displace the autoinhibitory DAD domain and activate formins [121–124]. Formins have the ability to increase the rate of filament elongation at the barbed end by 2–15-fold over the rate of diffusion-limited elongation at the barbed ends [120,125]. Furthermore, some formins associate with microtubules, indicating a role for formins in mediating the active interplay between actin filaments and microtubules [120].

Ena/VASP may participate in apical junction assembly. Ena/VASP elongates pre-existing actin filaments by competing with inhibitory capping proteins for barbed ends although it does not nucleate actin filaments [126–128]. Ena/VASP has a much stronger activity in protecting growing bared ends than do formins [129] and further mediates filament bundling [130,131]. Ena/VASP is localized to regions near the ends of actin filament bundles where filopodia embed and radial actin fibers emanate (Fig. 2). Such actin cables then provide the force necessary to actively bring epithelial cells together and seal them into continuous sheets [23]. Since E-cadherin engagement recruits Ena/VASP to the cell surface, Ena/VASP appears to be involved in the formation of adherens junctions [131,132] although Ena/VASP is not essentially required for the protein kinase A-mediated localization of claudin-16 to the tight junctions [133].

ERM proteins may also participate in apical junction assembly because they are localized to the inner surface of plasma membranes and act as an adaptor for the linkage between transmembrane proteins and F-actin. ERM proteins appear to simply capture actin filaments because they do not act as actin nucleators for remodeling F-actin. Rho and PI(4,5)P2 activate ERM proteins, while protein kinase (PKC)α, Rho kinase, and Nck-interacting kinase phosphorylate ERM proteins [134]. Among ERM proteins, ezrin is the only protein expressed in polarized epithelial cells and could thus be involved in apical junction stability. However, the junctional membrane proteins with which ezrin associates are not yet identified [99].

There is increasing evidence that myosins are also involved in forming apical junctions. Nonmuscle myosin II has commonly been observed at cell–cell contacts [135] where it serves to promote adhesion and the local accumulation of cadherins [136]. Furthermore, the actomyosin ring and its activator Rho kinase are colocalized to the apical junctions during epithelial wound healing [137]. Myosin light chain accumulation is colocalized with afadin but not with E-cadherin or α-catenin signals at the apical–lateral border, suggesting that a complex including ZO-1 and afadin serves as a scaffold for the assembly and localization of the myosin ring adjacent to the wound [137]. Claudin–ZO-1 and nectin–afadin modules may thus anchor the actomyosin cables to cell–cell adhesion sites around the wound to form a continuous ring. A recent study shows myosin VI is recruited to E-cadherin adhesions at a late stage in the maturation of cultured epithelial monolayers of MCF7 and MDCK cells (Fig. 3) [138]. A biochemical complex comprising myosin VI, E-cadherin, and vinculin is detected when these cells form extensive and continuous contacts. Therefore, myosin VI appears to regulate the recruitment of vinculin to cadherin adhesions in order to reorganize the perijunctional actin cytoskeleton, leading to the generation of cohesive and linear cadherin contacts. These studies suggest that myosin II and VI isoforms may serve as an actin-based anchor in order to link claudin, nectin, and cadherin complexes onto perijunctional actin filaments.

These subsets of actin regulatory proteins can dynamically alter the state of actin at apical junctions, which may account for the very rapid polymerization of actin induced by cadherin clustering. However, lamellipodia and filopodia have extensively been studied in nonepithelial cells constitutively activated by Rac and Cdc42. Earlier studies on lamellipodia and filopodia cannot readily be extrapolated to explain the remodeling of the actin cytoskeleton during the cell contact maturation of epithelial cells. The engagement of cadherins and nectins does induce the local activation of Rac and Cds42, but it remains unknown how and when these signals subside. Furthermore, the formation of the apical junctions and their maintenance might be mechanistically different events although such a possibility has not been fully examined. Alternatively, regulators of the microtubule cytoskeleton could be localized to adherens junctions. Actually, a number of proteins have been identified that interact with formins and the Arp2/3 complex regulatory machinery, and these proteins make strong candidates for mediating cross-talk between the actin and microtubule cytoskeleton [120,139]. Further studies may thus provide the molecular link among actin polymerization, microtubule-force generation, and additional adhesion-site formation.

5. Role of small G proteins in regulating apical junction dynamics

5.1. Rho family small G proteins and remodeling of cortical actin filaments

The cadherin–catenin, nectin–afadin, and claudin–ZO modules act not only as a mechanical complex connecting to the actin cytoskeleton but also as a scaffold for signalings (Fig. 5). Most importantly, Rho family and Rap1 small G proteins play coordinate roles during actin remodeling. The roles of Rho family proteins have been well documented: Rho induces stress fibers, Rac promotes lamellipodia, and Cdc42 promotes filopodia in cells in culture. The upregulation of Rho activity occurs during junction assembly in primary keratinocytes [52]. In tumorigenic and nontumorigenic cell lines, however, Rho is not activated after cadherin adhesion [140], and instead increased contractility may disrupt cell–cell contacts [141,142]. MDCK or keratinocyte cells expressing dominant active Rac1 or Cdc42 show enhanced staining for the cortical actin belts and E-cadherin signals at the cell adhesion borders [143–145]. In contrast, a dominant negative mutant of Rac1 reduces the levels of actin filaments at microvilli and those of claudins 1, and 2, JAM-1, and E-cadherin but not occludin and ZO-1 [18,61]. MDCK cells overexpressing the dominant active or negative mutants of Rho, Rac, and Cdc42 show structural changes of actin filaments and dissociation of tight junction proteins associated with the increase in paracellular permeability [146]. In addition, there is increasing evidence that the engagement of cadherins and nectins induces the Rho family protein-mediated signalings during cell contact maturation (Fig. 5). Since the downregulation of either module does not hinder the formation of the adherens junction structure [147,148], cadherins and nectins play cooperative roles by sharing signaling mechanisms that are required to determine the precise size and localization of the apical junction structure. These studies suggest that a temporal and spatial regulation of contractility via Rho family protein activities is necessary for epithelial homeostasis and morphogenesis.

5.2. E-cadherin-mediated signalings

Homophilic *trans*-interaction of E-cadherins eventually leads to the activation of Rac and Cdc42 [149], which are essential for cells to form cadherin-based lamellipodia and filopodia [150]. Rac appears to play more important roles than Cdc42 in cadherin localization or junctional assembly [149]. Previous studies have revealed the activation of several signaling pathways. Recruitment of the tyrosine kinase c-Src and subsequent Rap1 activation through the phosphorylation of Crk and C3G trigger the initial signaling process [151]. Rap1-mediated phosphatidylinositol-3 kinase (PI3K) activation then promotes Rac activation via Vav2 [152]. Additionally, Tiam1, a Rac GEF, is also localized to adherens junctions to play a role in activating Rac [152]; further, $p120^{ctn}$ may act as a negative regulator of Rho and a positive regulator of Rac and Cdc42 [153]. Rac is thus recruited to nascent contacts at the leading edges of E-cadherin-based lamellipodia. The engagement of E-cadherins defines sites for cell surface extension by locally activating Rac, which in turn stimulates E-cadherin-associated Arp2/3 to drive actin assembly [154]. Consistently, Arp2/3 requires signal-mediated stimulation to achieve full activity [155]. A recent study using live-cell imaging shows that Rac1 and RhoA activities and their downstream effectors, Arp2/3 and actomyosin, are restricted to zones at the edges of the expanding cell contact initiated by E-cadherins [81]. Rac may

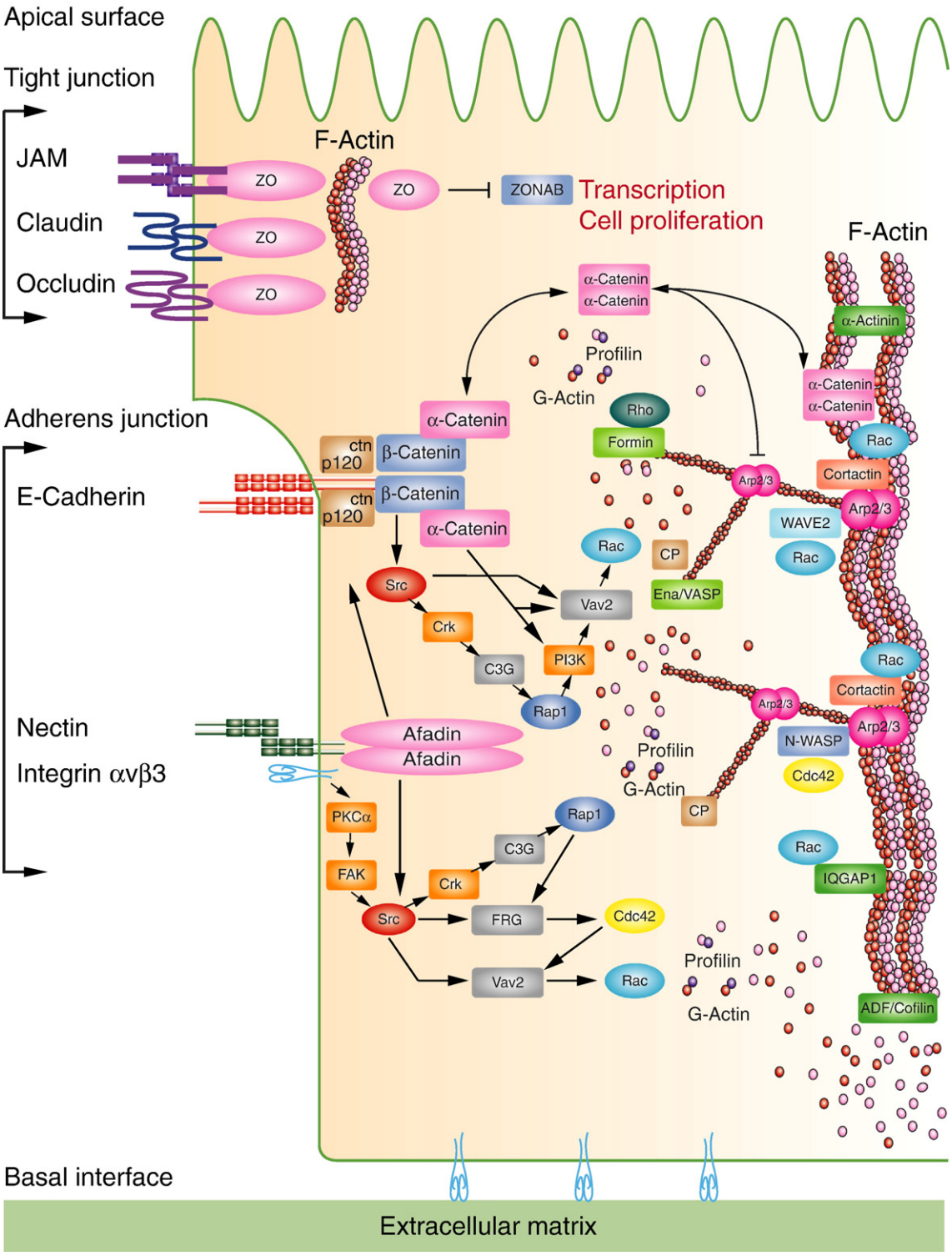

Fig. 5. Signaling pathways leading to formation of apical junctions. Signalings from engagement of cadherins and nectins play primary roles in regulating actin dynamics at adherens junctions. Those from tight junction modules appear to be less significant probably because cadherins and nectins play major roles at nascent cell–cell contacts and because tight junction modules are recruited to the adhesion sites at the later stage during the maturation of junctions. E-cadherin and nectin engagements share the same signaling pathway comprising c-Src and subsequent Rac activation [81,161,225,226]. The activation of Cdc42 via FRG, however, is specifically detected in nectin-mediated signalings [151,160,162]. In addition, nectins associate with the activated form of αVβ3 integrin through the interaction of extracellular domains [227,228]. Integrins associate with the extra cellular matrix at the basal membrane domain. These signalings may subside as apical junction matures. In the steady state, however, cells are ready to initiate signalings for actin remodeling in response to extracellular conditions. ZO-1 controls the activity of ZONAB that is a transcription factor promoting cell proliferation and gene expression. When ZO-1 concentration reaches its maximum level, ZONAB becomes restricted to the cytoskeleton and inhibited by binding the SH3 domain of ZO-1 [205,206].

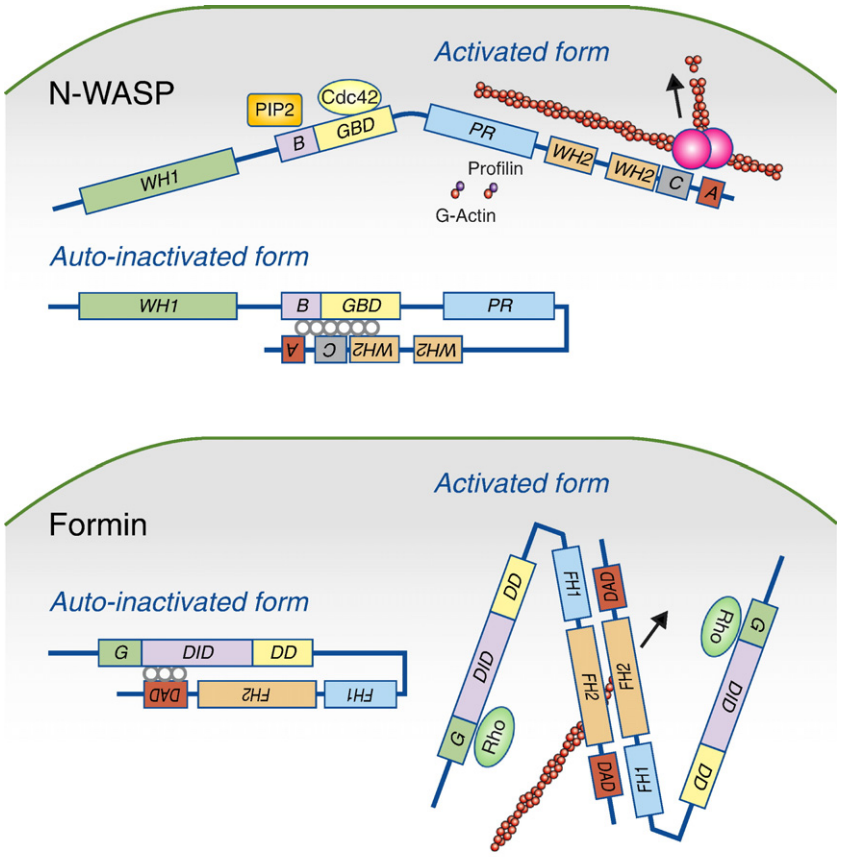

Fig. 6. Actin-binding proteins as effectors for Rho family small G proteins. N-WASP and formin-1 share common features: auto-inhibition by intramolecular folding and activation by binding to the GTP-bound form of Cdc42 or Rho. The roles of Cdc42 and Rac can be related to the regulation of Arp2/3-mediated actin polymerization. N-WASP is a prototypic protein with biding domains for Cdc42 and actin. Cdc42 binding triggers the transition of N-WASP from a closed inactive conformation to an open active conformation. In contrast, the mechanism underlying the apparent Rho dependence of adherens junctions [141,144] is still poorly understood. Formin-1 (Dia1) is one of the Rho target proteins. RhoA binding triggers the transition of formin-1 from an inactive conformation to an active conformation [123,222,223] and promotes formin-1 to form dimers. Thus, it may be of note that N-WASP and formin-1 participates in the Rho family protein-dependent regulation of adherens junctions in a similar manner.

thus play a key role in the initiation and expansion of E-cadherin-mediated cell–cell adhesion.

Another mechanism to make E-cadherin-based cell adhesion solid is to inhibit the endocytosis of E-cadherins. E-Cadherins are incorporated from the cell surface when they are not engaged in cell adhesion, or when adherens junctions are disrupted by the action of hepatocyte growth factor/scatter factor. $p120^{ctn}$ that binds the juxtamembrane domain of E-cadherin plays an additional role in inhibiting the endocytosis of E-cadherins. On the other hand, activated c-Src enhances the endocytosis of E-cadherins by inducing the tyrosine phosphorylation and ubiquitylation of the E-cadherin complex [156,157]. Thus, the endocytosis of E-cadherins is likely to depend on the elaborate balance of signalings. Nectins also appear to regulate the endocytosis of E-cadherins as follows.

5.3. Nectin-mediated signalings

Heterophilic and homophilic *trans*-interactions of nectins activate Rac and Cdc42 [12,158]. When nectins are engaged, c-Src is recruited and activated at the defined cell–cell contacts (Fig. 5). Recently, the integrin αVβ3 that is colocalized with nectin-3 has been shown to mediate c-Src activation through PKCα and focal adhesion kinase (FAK) phosphorylation, as well as the recruitment of E-cadherin to the nectin-based adhesion sites in Ca^{2+}-switch experiments of MDCK cells [159]. c-Src recruits C3G to nectin-based adhesion sites through Crk, leading to the activation of Rap1 [160]. Rap1 then activates FRG, a GEF for Cdc42, causing the activation of Cdc42 [151]. Rap1 activation is thus necessary for the recruitment of Rac and Cdc42 GEFs to the site of the initial nectin-based cell adhesion in order to provide a link with the actin cytoskeleton. On the other hand, c-Src phosphorylates FRG to activate Cdc42 [161] as well as Vav2, a GEF for Rac1, so as to activate Rac1 [162]. Cdc42 moreover activates Vav2, which in turn causes the activation of Rac1. These are the current understandings of signaling pathways initiated by nectin engagement. Nectins engaged in *trans*-interaction further recruit IQGAP to the initial cell contact site [163] and then activate Rac to inhibit the endocytosis of E-cadherins through the IQGAP-mediated reorganization of the actin cytoskeleton [164]. These events stabilize non-*trans*-interacting E-cadherins

on the cell surface, promoting further E-cadherin engagement for cell adhesion. Notably, the signaling events from nectins partly overlap those from E-cadherins (Fig. 5).

Rap1 is involved in the recruitment of afadin that binds not only nectins but also other junctional components such as Par-3, ZO-1 and JAM-A [42,165–167]. Afadin has 2 Ras-association (RA) domains, which can bind Rap1 (Fig. 1). Since afadin acts as a Rap1 effector and a regulator of $p120^{ctn}$, afadin can explain Rap1-dependent effects on E-cadherin [165,168,169]. Afadin prompts $p120^{ctn}$ to inhibit the endocytosis of E-cadherins in the presence of Rap1. A mutant of afadin lacking the RA domain, but not wild-type afadin, can inhibit E-cadherin endocytosis in the absence of Rap1 [170]. Furthermore, afadin forms a complex with $p120^{ctn}$, which enhances the interaction between $p120^{ctn}$ and E-cadherin [171]. RNA interference of afadin in MDCK cells disrupts the recruitment of the tight junction proteins claudin-1, occludin, JAM-A, and ZO-1 to cell–cell contacts but not E-cadherin, α-catenin, β-catenin, and $p120^{ctn}$. Interestingly, the effect of knocking down afadin on E-cadherin can be rescued by a mutant $p120^{ctn}$ that is constitutively active but not by full-length $p120^{ctn}$ [169]. Therefore, it appears likely that nectins activate Rap1 that then binds and activates afadin to induce the subsequent events. Active afadin interacts with $p120^{ctn}$, forming a ternary complex comprising Rap1, afadin, and $p120^{ctn}$ and recruiting α-catenin, α-actinin, and vinculin to further strengthen E-cadherin-based cell adhesion. This complex also strengthens the binding between $p120^{ctn}$ and E-cadherin that inhibits the endocytosis of E-cadherins and further promotes *trans*-interactions of E-cadherins. The engagement of E-cadherins and nectins thus induces elaborate interactions between their adaptor proteins to establish mature adherens junctions.

Both activities of Rac and Cdc42 are necessary for cell contact maturation (Fig. 3). Rac induces the formation of lamellipodia and efficiently mediates the coalescence of the cell–cell adhesion between the filopodia like a "zipper," whereas Cdc42 increases the number of filopodia and cell–cell contacts. E-cadherin also activates Rac in a PI3K-dependent or -independent manner in epithelial cells [149]. Rac activation by newly engaged E-cadherin further contributes to inhibiting the endocytosis of E-cadherins through the IQGAP-mediated actin remodeling. Thus, nectins can accelerate the formation of adherens junctions through the activation of these small G proteins.

5.4. Role of Rap1 small G protein in regulating apical junction dynamics

The Rap1 signaling has been implicated in the regulation of the actin cytoskeleton and recruitment of E-cadherin. Rap1 activation rescues a transformed or spindle-cell-like phenotype induced by oncogenic Ras in fibroblasts and MDCK cells [170,172]. Inhibition of Rap1 using Rap GTPase activating protein (GAP) restores mature E-cadherin-based cell–cell junctions and does not affect the localization of the tight junction marker ZO-1, but shows the delayed reformation of adherens junctions upon a Ca^{2+} switch in MCF7 cells [174]. Thus, Rap1 has a role in the maturation of junctions but not in the maintenance of junctions. One of the critical roles of Rap1, however, is to activate Rac and Cdc42 via Vav2 and FRG, respectively. Both pathways are triggered by the engagement of E-cadherins as well as nectins. Another important role of Rap1 is to recruit afadin and regulate its function.

Rap1 accumulates and functions at the local cell adhesion sites. Rap1 is activated after restoration of the cell–cell junctions following a Ca^{2+} switch [174,175]. Rap1 activity is temporally and spatially regulated by 3 GEF molecules: C3G, PDZ-GEF1, and DOCK4. C3G interacts with E-cadherin and competes with β-catenin for the binding when cell adhesion is induced after a Ca^{2+} switch. C3G disappears as the intercellular junctions mature [173]. C3G also mediates the nectin-induced activation of Rap1 [160]. PDZ-GEF1 binds β-catenin and the scaffold proteins MAGI-1 or MAGI-2 [174,176,177]. DOCK4 specifically activates Rap1, enhancing the formation of adherens junctions; further, it undergoes mutation during tumorigenesis [178]. Interestingly, the tumor-associated mutant of DOCK4 shows the activation of Rac and Cdc42 rather than Rap1 [179]. Localized activation of GEFs for Rap1 may thus play a role in the activation of Rap1 that is required during the initial phase of intercellular junction formation and is downregulated when the junctions mature.

Rap1 regulates apical junctions as follows. The engagement of E-cadherins and nectins triggers c-Src activation and subsequent Rap1 activation, which are necessary for the recruitment of afadin as well as Rac and Cdc42 GEFs to the initial cell–cell contacts in order to provide a link with the actin cytoskeleton (Fig. 5) [152,179]. The inhibition of the endocytosis of E-cadherins and the remodeling of the actin cytoskeleton is further required for the maturation of apical junctions, probably involving extracellular stimuli that activate Rap1 and PDZ-GEF. However, additional observations indicate the complex role of Rap1 signaling in cell adhesion. For instance, Rap1 promotes integrin-mediated cell adhesion and secretion [180], interaction with the Cdc42–Par3–Par6–aPKC complex [181,182], maintenance of cell–cell adhesion in endothelial cells [183], and induction of cortical actin possibly through the inhibition of Rho [184]. These studies suggest that Rap1 must respond to multiple temporal and spatial cues at the plasma membrane, and its roles appear to be more complex and not restricted to the regulation of the actin cytoskeleton.

5.5. Actin-binding proteins as effectors for Rac and Cdc42

The actin cytoskeleton is regulated by extracellular signalings as shown by a prominent example of cell migration caused by chemoattractants. During the cell adhesion process, Rho family small G proteins regulate actin dynamics by 2 biochemical pathways: the activation of actin polymerization and inhibition of actin depolymerization. Whereas nucleators such as formins and the Arp2/3 complex activate actin polymerization, ADF/cofilin inhibits actin depolymerization. LIM kinase phosphorylates the serine residue at the N-terminal region of ADF/cofilin, and this serine-phosphorylation disables ADF/cofilin from interacting with actin (Fig. 2) [185]. Since Rho proteins activate LIM kinase via p21-activated kinase activation, Rho

proteins eventually inhibit actin depolymerization, causing cells to maintain the actin cytoskeleton. Attempts to identify the Rac- and Cdc42-dependent effectors have been successful in elucidating the relevant proteins modulating actin dynamics.

Rac is directly activated by E-cadherin engagement [140, 144,154,186,187] and is likely to be spatially confined to the outer margins in extending contact zones [61,154,186]. Local activation of the Rho family proteins is necessary for initial cell–cell contacts to be driven by overlapping membrane lamellipodia from contacting cells that are directly regulated by actin polymerization and branching induced by the Arp2/3 complex [187]. As cell adhesion proceeds, lamellipodial activity is reduced over the contacting area, and there is a concomitant reorganization of actin filaments [60–62,98,188]. Interestingly, Rho family proteins regulate both cortactin and N-WASP proteins, which are the major nucleation-promoting factors for Arp2/3 activity. Although a direct interaction between cortactin and Rac has not been detected, Rac stimulates cortactin recruitment in motile cells [189]. WASP and N-WASP are normally allosterically regulated, but they stimulate Arp2/3 complex-mediated actin polymerization when bound to activated Cdc42 via the CRIB/GBD (Cdc42- and Rac-interacting domain/G protein binding) domain and PI(4,5)P2 via the B (basic) domain [34] (Fig. 6). Furthermore, formins possess a domain for binding RhoA and RhoC and form dimers to promote the elongation of actin filaments (Fig. 6). N-WASP and formins could be prototypic proteins with domains that directly bind Rho family proteins as well as actin filaments. By analogy to these proteins, other complicated protein interactions may converge with simple molecular mechanism(s). Thus, it is of note that the actin nucleators and their regulators reorganize the actin cytoskeleton in response to the Rho family protein-mediated signaling.

Molecular tools for remodeling the actin cytoskeleton appear to be the endpoints of the Rac, Cdc42, and Rap signalings and phosphoinositide-mediated regulation that are initiated by the engagement of E-cadherins and nectins. This hypothesis is attractive because the mechanisms are able to explain some of the indirect binding reactions, easy remodeling, and the rapid response of actin dynamics that have been observed by experimental studies. Moreover, it satisfactorily explains the recent findings of FRAP analysis on cell adhesion in living cells. In contrast to the cadherin–catenin and nectin–afadin modules, it remains unclear how the claudins–ZO module regulates the actin cytoskeleton. Although the basic mechanisms modifying actin filaments such as elongation, branching, and depolymerization have been extensively studied, the precise mechanisms regulating actin dynamics at cell adhesion sites remain largely unknown mostly because of the presence of numerous actin-binding proteins and a variety of possible biochemical combinations of their interactions.

6. Functional association of the actin cytoskeleton with epithelial cell polarity

The actin cytoskeleton is required for epithelial cell polarization and maintaining tall and cuboidal cell shape. Cells remodel the cytoskeleton to become epithelial cells by several processes, such as the formation of new cell–cell contacts, stabilization of these contacts, maturation of junctions, and acquisition of a cuboidal cell morphology [90]. Epithelial cell polarity primarily depends upon the dissociation of the plasma membrane into the apical and basolateral domains. In the absence of ZO proteins, claudins are unable to polymerize; moreover, tight junction formation is completely abolished [3]. However, the cells continue to demonstrate apical–basal polarity as judged by marker proteins. The apical and basolateral proteins appear to be properly delivered by Rab-mediated specialized trafficking, probably independent of ZO protein functions. Therefore, this allocation is responsible for the asymmetric distribution of membrane-associated proteins. On the other hand, the polarity protein complex may select membrane proteins by recognizing their phosphorylation state, thereby excluding phosphorylated proteins from some specific membrane domains. These events may initiate the promotion of epithelial cell polarity.

A proper asymmetric distribution of cytoskeletal elements is necessary in order to organize cells into structures such as tissues and organs. A polarity protein Par-3 recruits PTEN to the cell–cell junctions in *Drosophila* photoreceptor epithelial cells, thereby spatially restricting PI(3,4,5)P3 to the apical domain [190]. Par-3 is localized to tight junctions in MDCK cells, where it interacts with Rich1 (a GAP for Cdc42) and Amot (a scaffolding protein) and regulates the Cdc42-dependent maintenance of tight junction integrity [191]. In addition, JAMs directly interact with Par-3 that forms a ternary complex with Par-6 and atypical protein kinase C (aPKC), suggesting that JAMs are involved in the formation of cell polarity through these proteins [192]. The Par-3/Par-6/aPKC complex regulates the association of afadin with nectins in MDCK cells, and it could play cooperative roles with afadin in the formation of adherens junctions as well as tight junctions (Fig. 3) [42]. Nectins initiate cell–cell adhesion by recruiting cadherins to establish mature adherens junctions [12], and further recruit JAMs, claudins, and occludin to the apical side of adherens junctions, eventually forming tight junctions in epithelial cells (Fig. 3) [193,194]. In contrast to cadherins, nectins are likely to participate in establishing epithelial cell polarity during this process not only because nectin-1 and nectin-3 bind Par-3 but also because nectin engagement activates Cdc42 that further binds Par-6 [192]. Junctional components are thus involved in establishing epithelial cell polarity although it remains largely unknown how these biochemical interactions are connected to actin filament dynamics.

A proper asymmetric distribution of internal signaling components is also essential to establishing and maintaining cell polarity. As locally activated Rac1 is necessary for epithelial tight junction formation [195], Cdc42 is also activated upon cell–cell contacts and plays a role in epithelial morphogenesis [196, 197]. FDG1, a GEF for Cdc42, is necessary for lumen formation in vivo [198], and Cdc42 recruits Par-6/aPKC to establish apical–basal polarity. In addition, phosphoinositides act as key regulators of cellular polarity. The cellular distribution of PI(3,4,5)P3 and PI(4,5)P2 is regulated during epithelial cell polarization in addition to chemotaxis, cytokinesis, growth cone formation, and cyst formation. PI3K and PTEN are the key

enzymes that catalyze their interconversion. During cyst formation of MDCK cells, PTEN segregates PI(4,5)P2 to the apical surface, recruiting annexin 2 and Cdc42, which spatially regulate actin assembly [199]. The apical localization of PI(4,5)P2 is sufficient to recruit polarity protein complexes of Par-6 and aPKC, Cdc42, and annexin 2 to the apical surface (Fig. 3) [199]. When PI(4,5)P2 is introduced into the basolateral membrane domain, the marker proteins for apical lumen and tight junctions, ZO-1, gp135, and ezrin, make a shift to the basolateral domain. PTEN and PI(4,5)P2 may thus play a critical role in the regulation of the asymmetric distribution of cytoplasmic components as well as the formation of the central canal and adluminal plasma membrane domain.

Notably, annexin 2 regulates the actin cytoskeleton to establish epithelial cell polarity in the adluminal surface of cells (Fig. 3). MDCK cells expressing reduced levels of annexin 2 exhibit abnormal lumen formation and lower actin filament levels [199]. On the other hand, annexin 2 is implicated in the assembly of E-cadherin to nectin-based cell adhesion in MDCK cells [200]. E-cadherin does not accumulate at the nectin-based cell contacts when levels of annexin 2 are reduced by depletion or RNA interference during Ca^{2+} switch experiments. These studies suggest that annexin 2 may act as one of the key regulators of the actin cytoskeleton establishing both apical junctions and cell polarity, and that the establishment of cell polarity and the formation of apical junctions and epithelial lumen are closely linked events involving the rearrangement of the actin cytoskeleton.

7. Conclusions

Cell adhesion molecules and adaptor proteins form modules that function both individually and in combination at apical junctions. Apical junctions exist in dynamic equilibrium of assembly and disassembly between these modules and the actin cytoskeleton. Adaptor proteins not only link cell adhesion molecules to the actin cytoskeleton but also act as scaffolds that interact with a variety of cytoplasmic proteins. Claudins, cadherins, and nectins act cooperatively through cytoplasmic connections between their adaptor proteins. Such dynamics provide cells with linkages between cell adhesion molecules and the actin cytoskeleton that are strong and yet easily remodeled by extracellular signaling.

Adherens junctions have prototypic roles in stabilizing the epithelium by promoting tight-junction formation and epithelial cell polarity. Both cadherins and nectins are located in adherens junctions and play major roles in linkages to the actin cytoskeleton. Cadherins exclusively promote adhesion between homotypic cells whereas nectins further promote adhesion between heterotypic cells. Heterophilic engagement of nectins may play key roles during cell recognition and sorting in vivo. In MDCK cells, cell contact formation by nectins precedes the clustering of E-cadherins. Furthermore, nectins are likely to participate in establishing epithelial cell polarity through binding Par-3 and activating Cdc42. In addition, annexin 2 may act as one of the key regulators of the actin cytoskeleton establishing both apical junctions and cell polarity. The establishment of cell polarity and formation of apical junctions and epithelial lumen are thus closely linked events involving the rearrangement of the actin cytoskeleton.

Cells accumulate actin filaments and protrude structures that resemble lamellipodia and filopodia in nascent adhesion. Lamellipodia and filopodia have been well studied in nonepithelial cells expressing activated forms of Rac and Cdc42. In epithelial cells, the maturation of cadherin- and nectin-based cell contacts strongly depends on Rac and Cdc42 activities, suggesting that both cadherins and nectins induce changes in actin organization and dynamics using signaling pathways. It remains unanswered, however, how and when localized activations of Rac and Cdc42 are downregulated and whether or not these signals are necessary for the maintenance of matured apical junctions. In addition, Rap1 has a role in the maturation of junctions. The critical roles of Rap1 are to activate Rac and Cdc42 and to recruit afadin and regulate its functions. A ternary complex comprising Rap1, afadin, and $p120^{ctn}$ recruits α-catenin, α-actinin, and vinculin in order to strengthen E-cadherin-based cell adhesion. However, the roles of Rap1 appear to be more complex and not restricted to the regulation of the actin cytoskeleton.

There exist more than 60 actin-binding proteins, and the combination of interactions of these proteins with more than 40 transmembrane and cytoplasmic proteins appears extremely variable. However, it is almost certain that the formation of cell–cell contacts and apical junctions requires the nucleation of new actin filaments and elongation of the existing actin filaments. Actin nucleators play major roles in promoting de novo actin polymerization. Notably, N-WASP and formin-1 are prototypic proteins characterized by the molecular structures with binding domains for Rho family proteins and PI(4,5)P2. These proteins may represent the elaborate biochemical interactions between adaptor proteins and actin-binding proteins as well as the important roles of signaling pathways involving Rho family proteins and phosphoinositides.

Active interplay between actin filaments and microtubules may participate in stabilizing apical junctions. The catenins play roles in regulating the microtubule cytoskeleton. $p120^{ctn}$ regulates the nocodazol sensitivity of microtubules and affects cell motility in cadherin-deficient cells. Furthermore, some formins associate with microtubules. In fact, a number of proteins have been identified that interact with formins and the Arp2/3 complex, and these proteins make strong candidates for mediating cross-talk between the actin and microtubule cytoskeleton. Further studies may thus provide the molecular link among actin polymerization, microtubule-force generation, and additional adhesion-site formation.

Apical junctions act as not only the sites of mechanical attachment regulated by actin dynamics but also the sites of signalings required for cell–cell communication. Components of apical junctions actually have roles in signaling. α-Catenin regulates cell proliferation and the organization of the brain [201]. β-Catenin mediates the Wnt signaling pathway and associates with the lymphoid enhancer factor–T-cell factor (LEF/TCF) to regulate transcription [202,203]. β-Catenin also prevents epidermal growth factor receptor-mediated proliferation signaling [204]. In addition, afadin acts as a scaffold that promotes anti-apoptotic signals

and inhibits PDGF-mediated proliferation signaling [our unpublished data]. Furthermore, ZO proteins are localized to the nucleus and play unidentified roles, cooperating with the ZO-1 associated nucleic acid binding protein (ZONAB) that controls the expression of genes involved in cell cycle progression (Fig. 5) [205,206]. Although the entire range of functions of these proteins remains to be determined, apical junctions directly coordinate the morphological aspects of cells and spatiotemporal signaling pathways. Establishing how these events are linked to actin dynamics and the organization of epithelial structures will be the most important challenge of the coming years.

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