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REVIEW

SUBJECT COLLECTION: ADHESION

Regulation of Cdc42 and its effectors in epithelial morphogenesis

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ABSTRACT

Cdc42 – a member of the small Rho GTPase family – regulates cell polarity across organisms from yeast to humans. It is an essential regulator of polarized morphogenesis in epithelial cells, through coordination of apical membrane morphogenesis, lumen formation and junction maturation. In parallel, work in yeast and *Caenorhabditis elegans* has provided important clues as to how this molecular switch can generate and regulate polarity through localized activation or inhibition, and cytoskeleton regulation. Recent studies have revealed how important and complex these regulations can be during epithelial morphogenesis. This complexity is mirrored by the fact that Cdc42 can exert its function through many effector proteins. In epithelial cells, these include atypical PKC (aPKC, also known as PKC-3), the P21-activated kinase (PAK) family, myotonic dystrophy-related Cdc42 binding kinase beta (MRCKβ, also known as CDC42BPB) and neural Wiskott–Aldrich syndrome protein (N-WASp, also known as WASL). Here, we review how the spatial regulation of Cdc42 promotes polarity and polarized morphogenesis of the plasma membrane, with a focus on the epithelial cell type.

KEY WORDS: Cdc42, Epithelia, MRCK, PAK, Par complex, Polarity

Introduction

Cell polarity – the asymmetric distribution of membrane domains, cytoskeletal components and organelles – is a fundamental feature of cells and often underpins cell behavior and function. Most organs contain epithelial cells, and understanding the mechanisms of epithelial cell morphogenesis is a key goal of cell and developmental biology. Regulation of the actomyosin and microtubule cytoskeletons, polarized delivery of proteins and variation in lipid composition all contribute to the polarization and morphogenesis of epithelial cells (Apodaca et al., 2012; Braga, 2016; Crawley et al., 2014; Jewett and Prekeris, 2018; Rodriguez-Boulan and Macara, 2014). The small GTPase cell division control protein 42-homolog (Cdc42) has been shown to influence all of these processes, making this factor an essential regulator of epithelial morphogenesis.

Here, we begin with an overview of Cdc42 and review the mechanisms of Cdc42 function during polarized growth in the budding yeast, and polarity establishment in the *Caenorhabditis elegans* embryo. We then compare these mechanisms to those that drive the polarized morphogenesis of the epithelial plasma membrane, focusing on the role of Cdc42 during apical membrane morphogenesis, lumen formation through hollowing, and lateral junction maturation.

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An overview of Cdc42

Cdc42 was discovered in yeast and belongs to a large family of small (20–30 kDa) GTP-binding proteins (Adams et al., 1990; Johnson and Pringle, 1990). It is part of the Ras-homologous Rho subfamily of GTPases, of which there are 20 members in humans, including the RhoA and Rac GTPases, (Hall, 2012). Rho, Rac and Cdc42 homologues are found in all eukaryotes, except for plants, which do not have a clear homologue for Cdc42. Together, the function of Rho GTPases influences most, if not all, cellular processes.

In the early 1990s, seminal work from Alan Hall and his collaborators identified Rho, Rac and Cdc42 as main regulators of the actomyosin cytoskeleton. These studies showed that while RhoA can promote stress fiber formation in Swiss 3T3 cells (Ridley and Hall, 1992), Rac induces the formation of lamellipodia (Ridley et al., 1992) and Cdc42 promotes filopodia formation in these cells (Nobes and Hall, 1995). The ability of Rho, Rac and Cdc42 to remodel and structure the actomyosin cytoskeleton in such a specific manner has profound implications for cell morphogenesis, as modulation of the cytoskeleton affects many processes, including polarity, cell adhesion, vesicular trafficking, cell migration and cytokinesis. Subsequent work has revealed how these small GTPases can elicit specific cytoskeleton regulations. For example, formation of filopodia downstream of Cdc42 depends on the conserved Cdc42 effector N-WASp (also known as WASL) (Aspenström et al., 1996; Kolluri et al., 1996; Symons et al., 1996) and diaphanous-related formins (Peng et al., 2003). N-WASp promotes branched F-actin organization through the Arp2/3 complex (Machesky and Insall, 1998), and formins promote linear unbranched F-actin (Pruyne et al., 2002; Sagot et al., 2002b; for a recent review see Ridley, 2015). In eukaryotes, most small GTPases can be associated with the plasma membrane upon prenylation of their C-terminal CAAX domain (Roberts et al., 2008). While a significant fraction of Cdc42 is associated with the Golgi complex (Erickson et al., 1996), it is also detected in trafficking vesicles and at the plasma membrane. At these locations, Cdc42 can activate downstream effectors by binding to their Cdc42- and Rac-interactive binding motif (CRIB) domain (Burbelo et al., 1995; Manser et al., 1994; Symons et al., 1996). To date, at least 45 proteins encoded by the human genome have been shown to act as effectors of Cdc42 (Table S1).

An essential feature of a vast majority of Rho GTPases is that they can reversibly switch between an active, GTP-bound state (on) and an inactive, GDP-bound state (off). Consequently, these proteins are viewed as molecular switches whose on/off state can be controlled spatially and temporally in cells (Diekmann et al., 1991; Hart et al., 1991). This property is particularly relevant for Cdc42 function during cell polarity, including in epithelial cells, by allowing the localized activation of this small GTPase and its downstream effectors to promote plasma membrane differentiation, F-actin regulation and to direct trafficking (Etienne-Manneville, 2004). Spatial activation of Rho GTPases is controlled by guanine exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitor (GDIs). GEFs activate

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small GTPases by catalyzing the exchange of GDP to GTP. Conversely, GAPs inactivate small GTPases by enabling their intrinsic GTPase activity. Additionally, in the cytosol, GDIs bind to Rho GTPases to keep them in their inactive, GDP bound state. Up to 82 GEFs and 67 GAPs have been identified in the human genome (Hall, 2012), with 30 GEFs and 20 GAPs thought to regulate Rho GTPases alone, including 22 GEFs and eight GAPs linked to Cdc42 in vertebrates (Table S2). GDIs have been less studied, and three RhoGDIs (RhoGDI 1 to RhoGDI 3) have been linked to Cdc42 localization (Hoffman et al., 2000; Lin et al., 2003).

Mechanism of Cdc42-dependent polarity in yeast

Polarized growth in the budding yeast *Saccharomyces cerevisiae* and division in the fission yeast *S. pombe* is controlled by Cdc42. In the budding yeast, germinating spores initiate polarized growth as a single cluster of Cdc42-GTP determines the nascent bud. Initially, multiple clusters of Cdc42–GTP can be detected at the membrane. However, competition for rapidly diffusing cytoplasmic factors between these initial clusters leads to the elimination of all but one (Bendezú et al., 2015; Goryachev and Pokhilko, 2008; Klünder et al., 2013; Slaughter et al., 2009; Woods and Lew, 2019) (Fig. 1).

Recent elegant optogenetic manipulation of this pathway has illustrated how an initial symmetry breaking event such as localizing of the Cdc42 GEF Cdc24 can trigger polarization (Witte et al., 2017). Once a cluster of Cdc42–GTP forms, it can be amplified through the recruitment of the cytosolic Cdc42 effector P21-activated kinase (PAK) Cla4 (Bose et al., 2001), which can interact with Cdc24 and the adapter molecule Bem1 (Peterson et al., 1994). Recruitment of Cla4–Bem1–Cdc24 feeds into the activation of nearby Cdc42 molecules, thus growing the Cdc42–GTP cluster (Bendezú et al., 2015) (Fig. 1). This step of amplification is favored because Cdc42–GTP is more stable at the membrane than Cdc42–GDP, which is maintained in the cytosol through its interaction with the RhoGDI Rdi1 (Hoffman et al., 2000).

During polarized growth, F-actin-dependent transport of Cdc42 (Wedlich-Soldner et al., 2003, 2004) also contributes to rapid recruitment and accumulation at the bud site. Interaction between Cdc42 and the exocyst component Sec3 promotes polarized secretion (Zhang et al., 2001, 2008). In addition, Cdc42–GTP interacts with the formin Bni1p (Evangelista et al., 1997) to promote the formation of F-actin tracks that are directed toward the bud and support vesicle trafficking (Evangelista et al., 2002; Pruyne et al., 2004; Sagot et al., 2002a). Therefore, in yeast, Cdc42 regulates polarized growth by coupling polarity at the membrane and cargo delivery.

Polarization of the *C. elegans* embryo by localized inhibition of Cdc42

Two developmental contexts in *C. elegans* are particularly relevant to this review: the one-cell embryo (zygote) (Fig. 2) and the four-to-six cell embryo, which undergoes radial polarization (Fig. 3).

Cdc42 in the one-cell *C. elegans* embryo

The *C. elegans* embryo establishes its antero-posterior (A-P) body axis before the first embryonic cleavage, which is asymmetric. This model system was used by Kenneth Kemphues and collaborators in the late 90s to study the mechanisms of A-P polarity. Groundbreaking genetic screens identified the Partitioning-defective (*par*) genes as being required to establish the antero-posterior axis of the cell (Kemphues et al., 1988; Watts et al., 1996). Later, the conserved serine/threonine atypical PKC-3 [PKCζ and PKCι in vertebrates (PKCζ,ι hereafter) and aPKC in *Drosophila*] was added to this list of core regulators of A-P polarity (Tabuse et al., 1998). *par* genes encode adapter proteins (PAR-3, PAR-6 and PAR-5), serine/threonine kinases [PAR-1, PAR-4 (LKB1 in vertebrates, also known as STK11)] and PAR-2. In the zygote, A-P polarity is marked by the anterior segregation of the Par complex which consists of PAR-3, PAR-6 and PKC-3, and posterior accumulation of PAR-1 and PAR-2, which ultimately instruct asymmetric division through regulators of spindle position. The distribution of these proteins along the A-P axis depends on actomyosin flows and requires the reciprocal phosphorylation of PAR-1 by PKC-3 and PAR-3 phosphorylation by PAR-1 (Goehring and Grill, 2013; Motegi and Seydoux, 2013). Importantly, the relationship between Cdc42, PAR-6, PKC-3 and PAR-1, and the inhibition of PAR-3 [Bazooka (Baz) in *Drosophila*] by PAR-1 are both also part of the conserved signaling pathways that operate in epithelial cells to regulate polarized morphogenesis, (reviewed in Rodriguez-Boulan and Macara, 2014; St Johnston and Ahringer, 2010; Tepass, 2012).

In the zygote, PAR complex assembly allows for loading of PAR-6–PKC-3 onto the cortex and displacement of PAR-3–PAR-6–PKC-3 toward the anterior pole of the cell through posterior-to-anterior contractile flows of actomyosin (Goehring et al., 2011) (Fig. 2A). Cdc42 supports this process by promoting the stability of the PAR complex at the cortex, the recruitment of PAR-6-aPKC through direct binding to PAR-6, and by regulating actomyosin flow (Rodriguez et al., 2017; Wang et al., 2017). Importantly, the Cdc42-PAR-6-PKC-3 complex drives A-P polarity as PKC-3 phosphorylates the posterior PAR (pPAR) proteins PAR-1 and

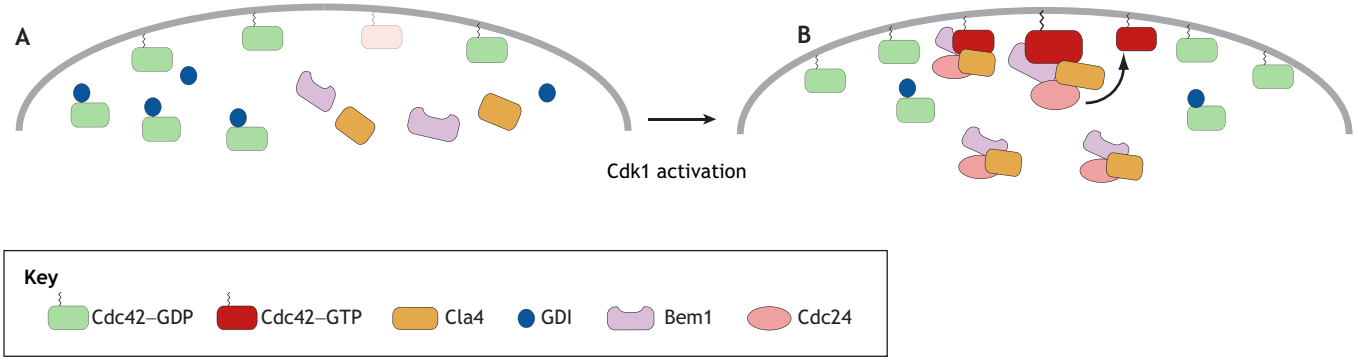

Fig. 1. Mechanism of Cdc42-dependent polarity in budding yeast. Simplified mechanism of Cdc42 polarization of the budding yeast. (A) Prior to entry into S phase, no Cdc42 activation is detectable at the membrane. (B) During S phase, Cyclin dependent kinase 1 (Cdk1) is activated and promotes the binding of Bem1–Cla4 to Cdc42. In turn, this promotes the recruitment of the Cdc42 GEF Cdc24; this contributes to a positive feedback loop through the recruitment of additional Cdc42 molecules.

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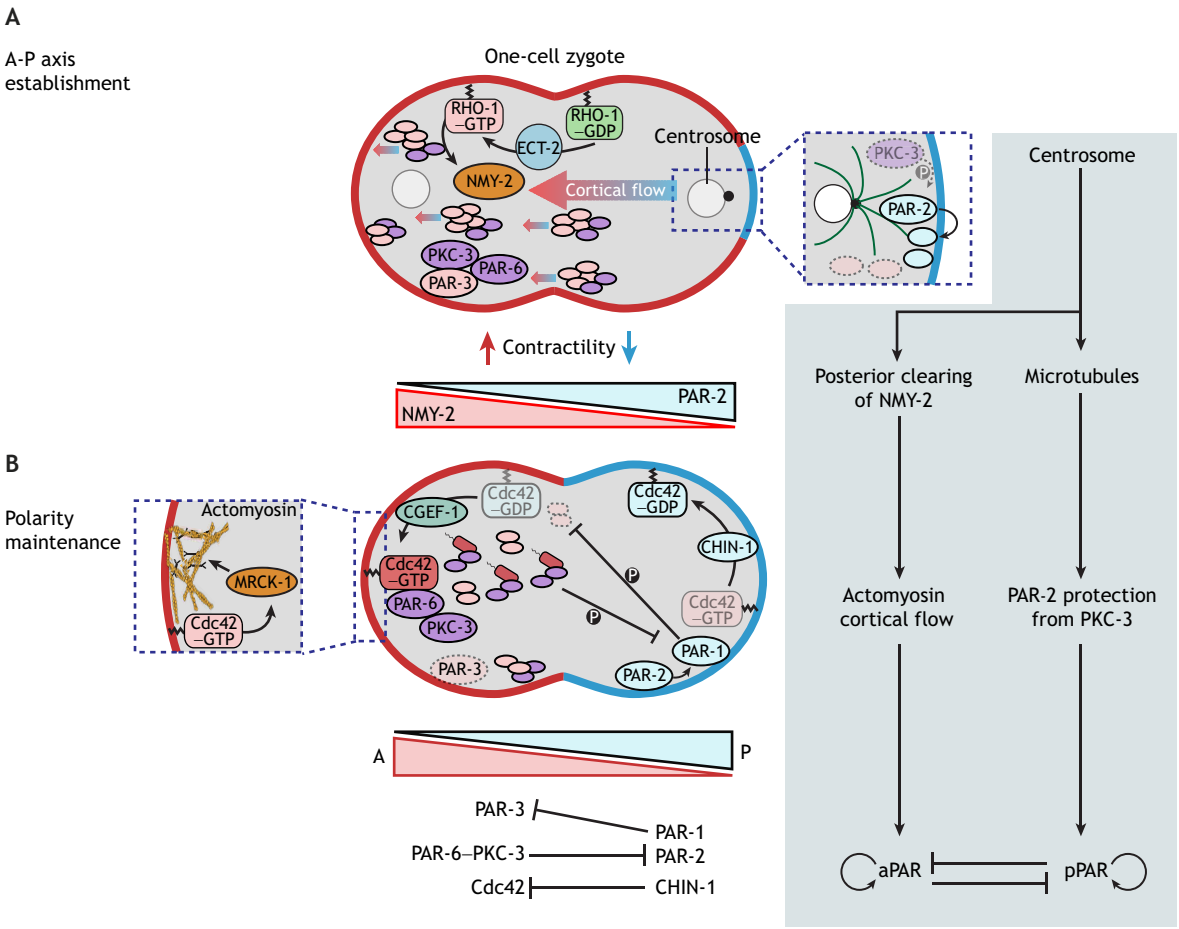

Fig. 2. Polarization of the *C. elegans* zygote. The *C. elegans* embryo establishes its antero-posterior (A-P) body axis before the first division, which is asymmetric. (A) In the early one-cell zygote, A-P axis establishment is achieved through activation of myosin II (NMY-2 in *C. elegans*) by active RHO-1, downstream of the GEF ECT-2. This results in cortical flow towards the anterior pole, which promotes anterior accumulation of the PAR complex (PAR-3–PAR-6–PKC-3). At this stage PAR-2 is loaded onto the membrane at the posterior pole in microtubule-dependent manner (right inset). (B) In the later-stage zygote, polarity is maintained by spatially restricting the activity of Cdc42 to the anterior pole through the GEF CGEF-1 at the anterior pole and the localization of the GAP CHIN-1 at the posterior pole. At the anterior pole, the actomyosin cytoskeleton is regulated by MRCK-1, which acts downstream of Cdc42. Reciprocal antagonism between PAR-1 and PAR-3, and PKC-3 and PAR-1 promotes stable polarity.

PAR-2 to exclude them from the anterior pole of the cells (Aceto et al., 2006; Gotta et al., 2001; Kay and Hunter, 2001; Rodriguez et al., 2017). In addition, Cdc42 regulates actomyosin flow dynamics through PKC-3 (Cheeks et al., 2004; Munro et al., 2004) and, later on, the formation of an actomyosin cap at the anterior pole of the cell through myotonic dystrophy-related Cdc42 binding kinase 1 (MRCK-1) (Kumfer et al., 2010; Munro et al., 2004) (Fig. 2B). This regulation is conserved throughout evolution as MRCKβ [Genghis Khan (Gek) in *Drosophila*] regulates actomyosin at the apical pole of epithelial cells downstream of Cdc42 in mammalian cells and in *Drosophila* (Zihni et al., 2017).

As the zygote polarizes, active Cdc42 accumulates at the anterior pole of the cells, together with PAR-3, PAR-6 and PKC-3 (Kumfer et al., 2010). Anterior activation of Cdc42 results from the posterior accumulation of the RhoGAP Chimaerin homolog (CHIN-1), which inactivates Cdc42 (Beatty et al., 2013; Kumfer et al., 2010) (Fig. 2B). The RhoGEF CGEF-1 contributes to regulating the activation of Cdc42 and its cortical enrichment at the anterior pole of the cell (Kumfer et al., 2010). Therefore, whereas in yeast the localized recruitment of the Cdc42 GEF activates Cdc42 at the incipient bud site, in the *C. elegans* zygote, the localization of a GAP plays an important role in spatially regulating where Cdc42 is active.

Spatial regulation of Cdc42 during radial polarity

In the *C. elegans* blastoderm, radial polarization is regulated by Cdc42, which is activated at the junction-free, outward-facing membranes (Anderson et al., 2008). This is because the Cdc42 GAP PAC-1 is recruited at the lateral membrane that mediates cell–cell junction (Fig. 3). In these cells, activation of Cdc42 at the junction-free membrane drives the selective accumulation of PAR-6–PKC-3 (Marston et al., 2016; Rohrschneider and Nance, 2009). Cell junctions are mediated by the main adherens junction protein E-cadherin homologue HMR-1 and associated catenins HMP-1, HMP-2 and the p120 homolog JAC-1 (Klompstra et al., 2015). Radial symmetry is first established as HMR-1 engages in *trans* to promote lateral junctions between embryonic cells. Formation of lateral junctions then leads to the recruitment of PAC-1 via the linker protein PICC-1, which binds to JAC-1 (Klompstra et al., 2015). As PAC-1 is recruited to the lateral junction, Cdc42 is thus inactivated. Simultaneously, at the junction-free membrane, active GTP-loaded Cdc42 becomes enriched. Here, Cdc42 activity is promoted by two GEFs, ECT-2 and CGEF-1, which function redundantly (Chan and Nance, 2013). As is the case in the zygote, in the blastoderm Cdc42–GTP promotes the localized recruitment of PAR-6–PKC-3 and MRCK-1, and their activation to regulate actomyosin, which

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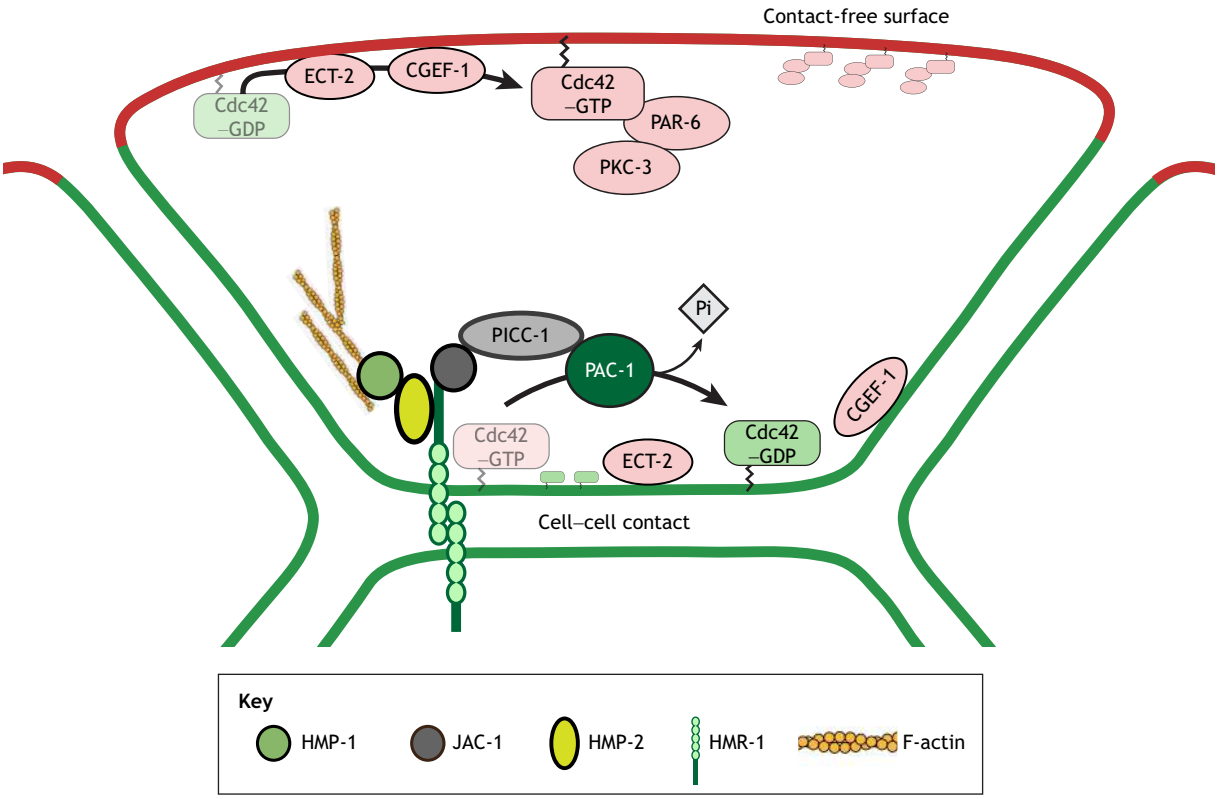

Fig. 3. Cdc42 regulates polarization of the *C. elegans* blastocyte. Cell–cell contacts containing HMR-1 are shown outlined in green and junction-free, outward facing membranes in red. PAC-1 is recruited to the junction through PICC-1, leading to the conversion of Cdc42–GTP into Cdc42–GDP. At the junction-free membrane, ECT-2 and CGEF-1 promote the accumulation of Cdc42–GTP and the associated recruitment of the PAR-6–PKC-3 complex. The polarized distribution of Cdc42–PAR-6–PKC-3 and PAR-3 is required for cell morphogenesis during gastrulation.

promotes cell constriction and internalization during gastrulation (Harrell and Goldstein, 2011).

Role of Cdc42 in epithelial cell types

Our knowledge of the mechanisms of epithelial morphogenesis is mostly based on genetic approaches in relatively simple model organisms such as *Drosophila melanogaster*, and cultured mammalian cells. While there are some differences in the topology of vertebrate cells compared to invertebrate cells, and the mechanisms of polarity establishment, many of the molecular factors that regulate epithelial polarity in invertebrates are conserved in mammals.

Epithelial cell polarity

Epithelial cells can adopt various shapes from flat, or squamous, to tall, or columnar. They can assemble into sheets that can be stratified. They are polarized along the apical (top)–basal (bottom) axis, and this polarity is readily visible at their plasma membrane (Fig. 4A). Typically, the apical membrane faces the luminal space or external milieu and consists of tightly packed microvilli, which contain bundled F-actin. The apical membrane may present a non-motile primary cilium, which is a microtubule-based organelle that acts as a signaling hub (Malicki and Johnson, 2017). Motile cilia may also be present at the apical surface of the cell, where they can promote mucus clearance, as for example in the lung (Mitchison and Valente, 2017). Discrete lateral domains that mediate cell–cell adhesion and can act as paracellular diffusion barriers are found along the lateral surface. The basal domain is in contact with the extracellular matrix (ECM). This polarized regionalization underpins tissue morphogenesis as it allows these cells to assemble into sheets that function as diffusion barriers (Tyler, 2003).

A shared feature between all epithelial cell types is the presence of a cell–cell junction at the apical–lateral border of the plasma membrane. In vertebrates, this junction is the paracellular junction and is called the tight junction. It contains transmembrane molecules that engage in *trans* to seal the epithelium (Fig. 4B). These include occludins, claudins and junctional adhesion molecule A (JAM-A, also known as F11R), which are linked to the cytoskeleton through proteins such as the adaptor proteins zonula occludens (ZO)1, ZO2 and ZO3 (also known as TJP1–TJP3 in vertebrates), and cingulin (Ebnet et al., 2004; Matter and Balda, 2003; Tsukita et al., 2001). Basal to the tight junctions are the adherens junctions, which mediate cell–cell adhesion and signaling (Harris and Tepass, 2010b), and contain E-cadherin (Ecad hereafter) and nectin family proteins. Interaction between Ecad molecules in *trans* promotes intercellular adhesion and coupling to the actomyosin cytoskeleton through the catenin adapter proteins α-catenin and β-catenin (Lecuit and Yap, 2015; Steinbacher and Ebnet, 2018). In addition, some tissues have desmosomes, which contain cadherin-like proteins that are linked to keratin intermediate filaments to form spot-like junctions at the lateral membrane. These contribute to the promotion of mechanical resilience in epithelia (Garrod and Chidgey, 2008). Finally, GAP junctions consist of connexin molecules that assemble into hemichannels and directly connect the cytosol of two neighboring cells to allow the exchange of molecules and ions (Dermietzel et al., 1990).

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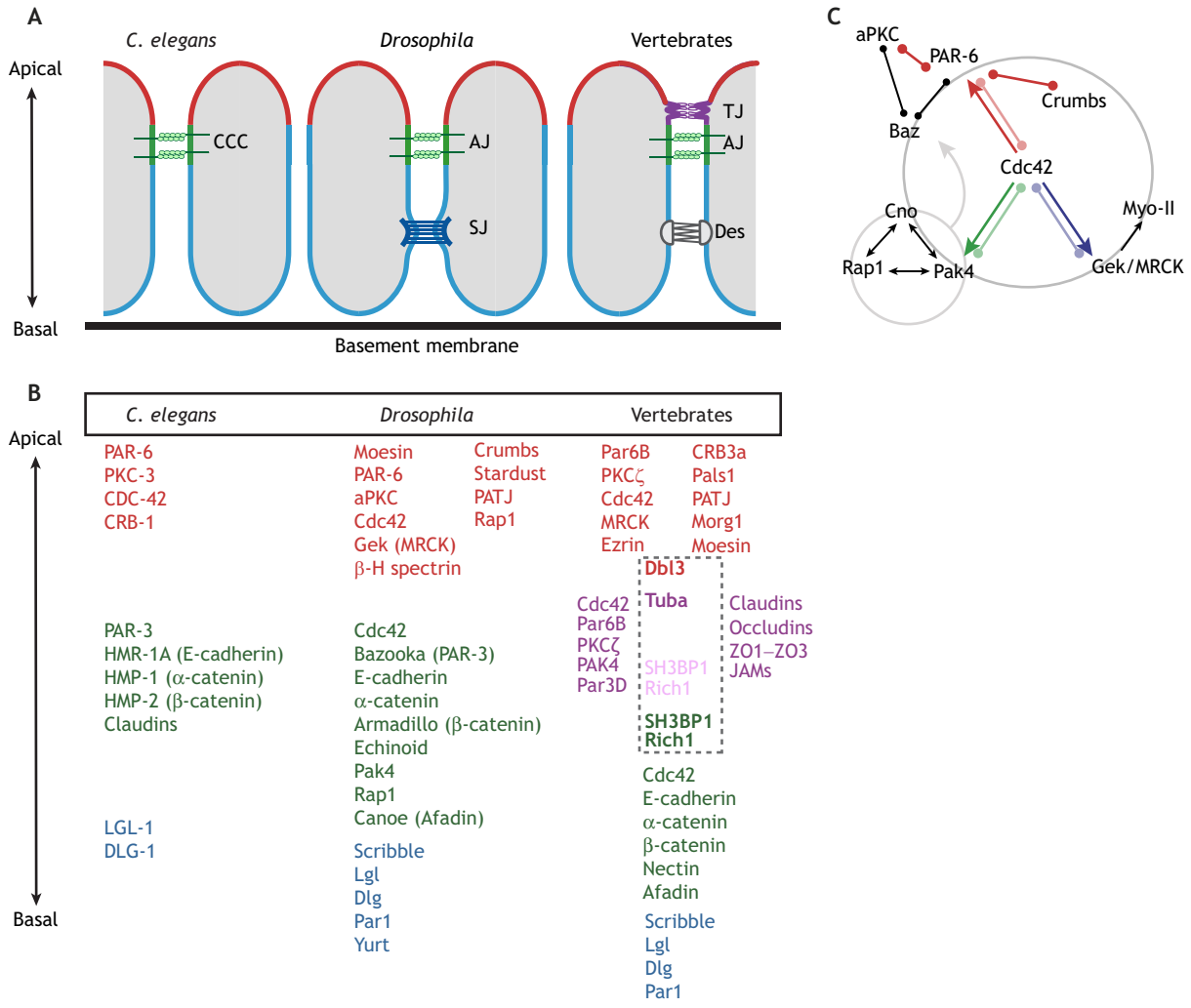

Fig. 4. Epithelial cell junctions and principal regulators of epithelial morphogenesis. (A) Schematic representation of typical epithelial cells in *C. elegans*, *Drosophila* and vertebrates. CCC, cadherin–catenin complex; AJ, adherens junction; SJ, septate junction; TJ, tight junction; Des, desmosomes. Apical and contiguous sub-apical membranes are outlined in red, lateral and basal membranes in blue. AJ and CCC are in green, and TJ in purple. (B) Overview of the principle proteins that have been shown to regulate apical–basal polarity and/or morphogenesis in these three model systems. Color coding as in panel A. (C) Schematic representation of the network of apical proteins that orchestrate apical membrane and apical–lateral junction morphogenesis in *Drosophila* (and, in particular, in the pupal photoreceptor) and vertebrate cells. Arrows indicate activation and connector lines protein interactions. Color coding as in panel A.

The configuration of epithelial cells in invertebrates is similar but not identical to that found in vertebrates (Knust and Bossinger, 2002; Tepass et al., 2001) (Fig. 4A). In *Drosophila* the apical–lateral junction is the adherens junction. The paracellular junction, and equivalent of the tight junction, is called the septate junction. An exception to this organization is found in the *Drosophila* mid-gut, where the septate junction is the apical–lateral junction, and is found apical to the adherens junction (Chen et al., 2018). In *C. elegans,* the apical–lateral junction is called the cadherin–catenin complex (CCC) and is composed of HMR-1A (Ecad), HMP-1 and HMP-2 (catenins) and claudins (Labouesse, 2006) (Fig. 4A,B). Another adhesion complex, consisting of DLG1 and AJM-1 is found immediately basal to the CCC and has been proposed to serve as paracellular barrier (Asano et al., 2003).

Epithelial polarity protein networks

Drosophila and *C. elegans* genetics have been instrumental in identifying genes that regulate cell polarity and epithelial morphogenesis (Fig. 4B,C). In addition, biochemical evidence shows that the apical proteins can assemble into canonical complexes that are conserved through evolution. These complexes include the PAR complex and the Crumbs complex (Crumbs–PALS1–PATJ) (Bulgakova and Knust, 2009; Tepass, 2012). However, these complexes are interlinked because PAR-6 can bind to Crumbs (CRB3 in vertebrates) and Stardust (PALS1 in vertebrates, also known as MPP5) (Hurd et al., 2003; Kempkens et al., 2006; Lemmers et al., 2004; Wang et al., 2004), and Stardust to Bazooka (*Drosophila* homologue of PAR-3) (Krahn et al., 2010). Therefore, the interactions between Crumbs, Stardust/PALS1, PAR-6 and Bazooka/PAR-3 are likely to be dynamic (Fig. 4C). Importantly, Cdc42 regulates how these proteins interact with each other during epithelial morphogenesis. For instance, in *Drosophila*, Cdc42 is required for recruitment of Bazooka and PAR-6–aPKC to the plasma membrane and for the apical recruitment of PAR-6–aPKC and Crumbs. This is in part because Cdc42 binding to PAR-6 promotes the binding of PAR-6 to Crumbs (Nunes de Almeida et al., 2019 preprint; Walther and Pichaud, 2010). Presumably, Cdc42 binding to PAR-6 promotes a conformational rearrangement that potentiates the affinity of PAR-6 for binding to Crumbs (Peterson et al., 2004; Whitney et al., 2011). Via this mechanism,

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Cdc42 coordinates the association of Bazooka, PAR-6 and aPKC, and Crumbs recruitment to promote apical membrane and adherens junction morphogenesis. Similar to *Drosophila*, in vertebrate cells such as Madin–Darby canine kidney cells (MDCK), Cdc42 regulates the localization of PAR-6–PKCι (Martin-Belmonte et al., 2007). In *Drosophila*, the interaction between PAR-6–aPKC and Crumbs promotes the separation of the apical membrane and adherens junction. Crumbs binding to PAR-6 is thought to outcompete PAR-6 binding to Bazooka, leading to the exclusion of Bazooka from the PAR complex (Walther and Pichaud, 2010; Morais-de-Sá et al., 2010; Nunes de Almeida et al., 2019 preprint). Bazooka exclusion also requires Bazooka phosphorylation by aPKC at a conserved serine (S980 in flies; S827 in PAR-3) (Krahn et al., 2010; Morais-de-Sá et al., 2010; Walther and Pichaud, 2010; Hirose et al., 2002). Bazooka exclusion from the PAR complex leads to its localization to the apical–lateral boundary, where it is thought to promote adherens junction morphogenesis. Similarly, in vertebrate cells, PAR-3 localizes at the tight junction, basal to CRB3, PALS1 and PAR-6. Although it is not clear where the interactions between Crumbs/CRB3, Stardust/PALS1, PAR-6–aPKC and Bazooka/PAR-3 take place in cells, one possibility is that they occur where these proteins co-localize, i.e. at the apical tip of the tight junction (Zihni et al., 2014) in vertebrate cells and the apical region of the adherens junction in fly cells (Walther et al., 2016; Walther and Pichaud, 2010).

Cdc42 regulates epithelial morphogenesis

Epithelial cell culture models have provided important insights into the potential mechanisms of junction maturation during epithelial morphogenesis. In 2D epithelial monolayers where cell–cell junctions have been disrupted, either through calcium depletion or scratch assays, and then allowed to reform (Gumbiner and Simons, 1986; Todaro et al., 1965), Ecad-rich spot-like junctions, also referred to as primordial junctions, form as filopodia-like extensions make contact between neighboring cells (Fig. 5A,B). In MDCK cells, the formation and maturation of these spot-like junctions, which also contain the tight junction proteins ZO1 and JAM-A, is regulated by Rac, Rho and Cdc42 (Coopman and Djiane, 2016) (Fig. 5B). Junction maturation in 2D cultures requires Cdc42 and its effectors PAR-6B–PKCζ,ι , and P21-activated kinase 4 (PAK4) (Jin et al., 2015; Wallace et al., 2010) (Fig. 5C).

Role of the Cdc42–PAR-6–aPKC axis

Cdc42 binding to PAR-6 is thought to regulate the localization of PKCζ,ι. Three PAR-6 proteins have been characterized in mammals: PAR-6A, PAR-6B and PAR-6C (also known as PARD6A, PARD6B and PARD6C, respectively) (Gao and Macara, 2004; Noda et al., 2001), and one PAR-6 protein in *Drosophila* (Petronczki and Knoblich, 2001). A feature common to all PAR-6 proteins is the presence of a pseudo-CRIB domain juxtaposed to a PDZ domain, both of which contribute to supporting the binding of Cdc42 (Garrard et al., 2003; Joberty et al., 2000; Ranganathan and Ross, 1997). In addition, the N-terminus of PAR-6 binds to aPKC/PKCζ,ι (Joberty et al., 2000; Lin et al., 2000; Suzuki et al., 2001). PAR-6A, PAR-6B and PAR-6C can all localize to the tight junction (Durgan et al., 2011; Gao and Macara, 2004), while PAR-6B also localizes to the apical membrane (Hayase et al., 2013) in MDCK cells. In 2D cultures of human bronchial 16HBE cells, decreasing the levels of Cdc42, PAR-6B or PKCζ,ι stalls junction maturation, as only spot-like junctions can be detected in

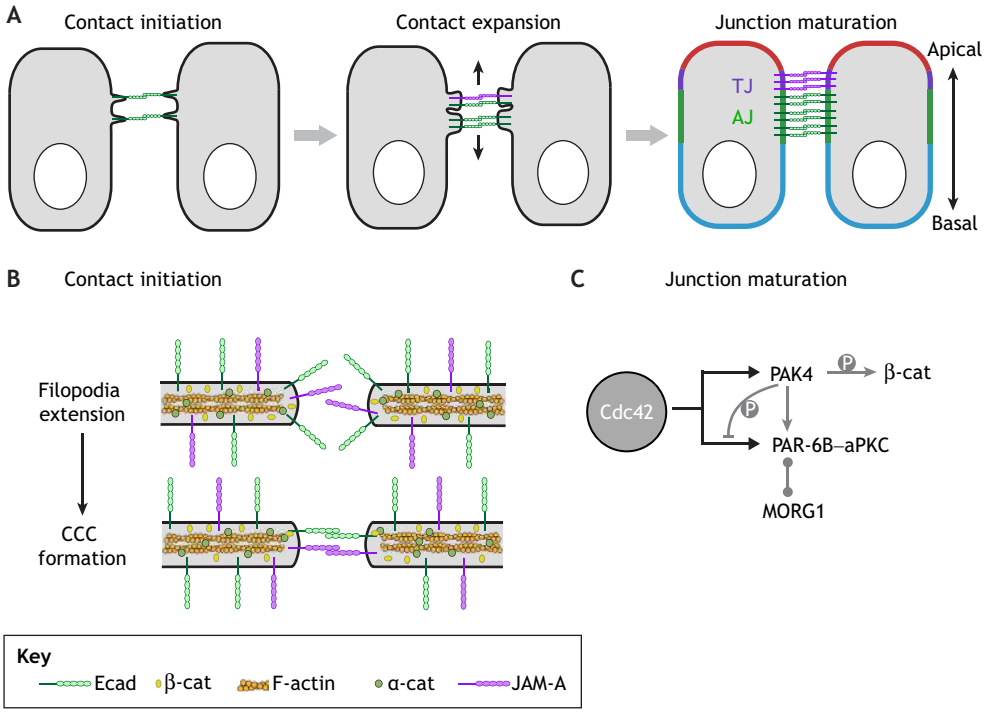

Fig. 5. Cdc42 regulation during epithelial morphogenesis. (A) Schematic representation of how junctions mature during epithelial morphogenesis in epithelial monolayers (such as MDCK and Caco-2 cells). (B) Initial junctional contacts consist of spot junctions mediated by filopodia that extend between cells. These filopodia present adhesion molecules, including Ecad, nectins and JAM-A. As intercellular contacts are made, a cadherin–β-catenin–α-catenin complex assembles (CCC). PAR-3 and associated PAR-6B–PKCζ,ι can be recruited through JAM-A. (C) Following initial spot junction formation, TJ and AJ mature; this is regulated by Cdc42 through PAK4 and PAR-6–aPKC. In vertebrate cells, junction maturation depends on PAK4-mediated phosphorylation of PAR-6B, which excludes it from the AJ and instead favors its localization at the TJ and apical membrane through interacting with MORG1. Junction maturation also depends on PAK4-mediated phosphorylation of β-catenin, which stabilizes Ecad.

these cells (Jin et al., 2015; Wallace et al., 2010). Similarly, inhibiting Cdc42 in 2D cultures of Caco-2 and MDCK cells interferes with adherens junction assembly (Fukuhara et al., 2003; Otani et al., 2006). In addition, manipulation of Cdc42 using dominant-negative or constitutively active transgenes shows that it regulates endocytosis at the apical membrane of MDCK cells, as well as delivery of basolateral cargoes (reviewed in Harris and Tepass, 2010a). Further evidence that Cdc42 regulates apical endocytosis *in vivo* is found in *Drosophila* tissues and in the salivary gland in mice (Georgiou et al., 2008; Harris and Tepass, 2008; Leibfried et al., 2008; Shitara et al., 2019).

How exactly the Cdc42–PAR-6–aPKC complex promotes epithelial morphogenesis is not fully understood. The PAR-6–aPKC complex contributes to the maintenance of epithelial polarity by phosphorylating L(2)gl and PAR-1, which leads to their dissociation from the plasma membrane (Benton and St Johnston, 2003; Böhm et al., 1997; Hurov et al., 2004; Hutterer et al., 2004; Plant et al., 2003; Suzuki et al., 2004). In addition, findings for *Drosophila* and mammalian epithelial cells, combined with biochemical studies, indicate that the functions of PAR-6–aPKC during this process include suppressing the contractility of the actomyosin cytoskeleton (Ishiuchi and Takeichi, 2011; Röper, 2012), promoting Ecad endocytosis (Georgiou et al., 2008; Leibfried et al., 2008), and stabilizing Crumbs at the plasma membrane to maintain the integrity of the apical–lateral junction (Harris and Tepass, 2008). Furthermore, in vertebrate cells, PKCζ,ι phosphorylation of the tight junction components JAM-A, claudin-4 and occludin, (D'Souza et al., 2007; Iden et al., 2012; Jain et al., 2011) promotes junctional integrity.

The Cdc42–PAK4 axis in junction maturation

As noted above, the Cdc42 effector PAK4 [Mushroom bodies tiny (Mbt) in *Drosophila*] is also required to promote junction maturation, both in *Drosophila* and vertebrates (Jin et al., 2015; Schneeberger and Raabe, 2003; Selamat et al., 2015; Wallace et al., 2010; Walther et al., 2016) (Fig. 5C). PAK4 belongs to the Type II PAK family, which comprises PAK4, PAK5 and PAK6 (Bokoch, 2003). Broadly, PAK kinases are required for the regulation of cytoskeletal dynamics, as well as for the localization or turnover of adherens junction components at the plasma membrane (Pirraglia et al., 2010; Tay et al., 2010; Walther et al., 2016). Binding of Cdc42–GTP to the CRIB domain of PAK4 is thought to only marginally increase their kinase activity; however, it has been shown to regulate their localization. For instance, binding of Cdc42 to Mbt/PAK4 localizes it to developing adherens junctions in human cells (Wallace et al., 2010), zebrafish (Selamat et al., 2015) and *Drosophila* epithelial cells (Schneeberger and Raabe, 2003). PAK4 can also promote F-actin morphogenesis through activating LIMK and the actin-severing protein cofilin (Twinstar in *Drosophila*) (reviewed in Rane and Minden, 2014). This could also stimulate junction maturation and apical membrane morphogenesis. Interestingly, in human cells, PAK4 phosphorylates PAR-6B, which promotes its binding to MORG1 and facilitates the recruitment of PAR-6B–aPKC through CRB3 (Hayase et al., 2013; Jin et al., 2015) (Fig. 5C). Phosphorylation of PAR-6 by Mbt is not conserved in flies (Walther et al., 2016); however, there is evidence that Mbt regulates junction maturation by phosphorylating Armadillo (Arm; the fly homolog of β-catenin) and that this regulation is conserved across different species (Menzel et al., 2008; Selamat et al., 2015). In the pupal fly photoreceptor, Mbt-mediated phosphorylation of Arm stabilizes adherens junction components including Bazooka at cell–cell contacts. Retention of Bazooka at the adherens junction plays a role in preventing the ectopic localization of the PAR complex and Ecad at the lateral membrane (Walther et al., 2016). Although the mechanisms underlying Mbt/PAK4 function during junction maturation are not fully understood, recent work in *Drosophila* has linked Mbt function during adherens junction maturation to that of the small GTPase Rap1 and its effector Canoe (Cno; afadin in humans), which binds to F-actin (Walther et al., 2018). The Rap1–Cno pathway also regulates Bazooka localization in the fly embryo during cellularization (Bonello et al., 2018), and Ecad trafficking in MDCK cells (Hogan et al., 2004).

Cdc42 GEFs and GAPs during epithelial morphogenesis

The spatial regulation of Cdc42 has been shown to be essential for the regulation of junction formation and maintenance. A key regulator of Cdc42 in epithelial cells is its GAP SH3BP1, which has been shown to promote junction assembly in 2D cultures of Caco-2 cells and in 3D spheroids (Elbediwy et al., 2012). SH3BP1 forms a complex with the ZO1 binding partner paracingulin (CGNL1) and the scaffold protein CD2AP, a protein that has been shown to regulate F-actin dynamics and endocytosis (Gauthier et al., 2007; Tang and Brieher, 2013). Further, SH3BP1 colocalizes with occludin and β-catenin, and is thus found at both tight junctions and adherens junctions. In human intestinal Caco-2 cells, SH3BP1 is required to limit Cdc42 activity in order to promote assembly of the peri-junctional actin belt that stabilizes the adherens junctions and promotes tight junction formation (Elbediwy et al., 2012). Therefore, junction maturation requires limitation of Cdc42 activity at the junctions. Similarly, in MDCK cells, the Cdc42 GAP Rich1 (also known as ARHGAP17), which is related to SH3BP1, is required for the maturation and maintenance of tight junctions (Wells et al., 2006). In these cells, Rich1 is enriched at the basal part of the tight junctions and at the apical region of adherens junctions (Wells et al., 2006). Binding of Rich1 to Amot, a protein that is found both at tight junctions and adherens junctions, is thought to regulate Rich1 localization to these junctions (Wells et al., 2006). The function of Rich1 in maintaining tight junction integrity has been in part linked to the Rich1–Amot module, which regulates the turnover of tight junction components (Wells et al., 2006). Localization of SH3BP1 and Rich1 bears similarities to that of PAC1 in the *C. elegans* embryo, which raises the possibility that preventing Cdc42 activity at the developing cell–cell junctions is required for junction maturation. Further, the RhoA–Rok pathway promotes adherens junction morphogenesis and can be inhibited by Cdc42 through aPKC phosphorylation of Rok in *Drosophila* (Röper, 2012) and ROCK1 in MDCK cells (Ishiuchi and Takeichi, 2011). It is therefore conceivable that Cdc42 activity needs to be limited at the developing adherens junction as part of a mechanism that controls the balance between the RhoA–Rok and Cdc42–PAR-6–aPKC pathways.

Next to SH3BP1 and Rich1, the Cdc42 GEF Tuba has been found to localize at the apical tip of the tight junction in 2D cultures of Caco-2 cells. In these cells, Tuba has been shown to be required for the normal maturation of adherens junctions, and for their maintenance (Otani et al., 2006). At least part of the function of Cdc42 during these processes was attributed to the Cdc42 effector N-WASp, and thus branched F-actin morphogenesis. Therefore, it is likely that Tuba-mediated activation of Cdc42 leads to the activation of both the N-WASp and PAR-6–aPKC pathways. How exactly Cdc42–GTP might distribute between N-WASp and PAR-6–aPKC is not well understood. One possibility is that clusters of Cdc42–PAR-6–PKCζ,ι exist in close vicinity to Cdc42–N-WASp clusters. In addition, a recent study in MDCK cells has shown that the RhoGEF

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FARP2 also regulates Cdc42 function during tight junction assembly (Elbediwy et al., 2019). However, how FARP2 function relates to that of Tuba during epithelial morphogenesis is not clear.

Role of Cdc42 in regulating lumen formation

3D cultures of epithelial cells (Griffith and Swartz, 2006; Yamada and Cukierman, 2007) have been instrumental in elucidating the mechanisms of epithelial morphogenesis, and in particular luminogenesis (Fig. 6). In MDCK spheroids, apical recruitment of Cdc42 has been proposed to depend on annexin 2 and the lipid phosphatase PTEN, which prevents accumulation of phosphatidylinositide-3,4,5-triphosphate (PIP3) at the apical pole of the cells (Martin-Belmonte et al., 2007). However, Cdc42 localization is not limited to the apical membrane, and it is not well understood where Cdc42 is activated in these cells (Martin-Belmonte et al., 2007). Similarly, in the *Drosophila* photoreceptor, PTEN at the adherens junctions limits levels of PIP3 at the apical membrane, which also contains PIP2 (Pinal et al., 2006) and Cdc42–GTP (Nunes de Almeida et al., 2019 preprint). Regulation of PIP2 and PIP3 levels along the apical–basal axis of epithelial cells is therefore conserved through evolution. In 3D MDCK spheroids, apical activation of Cdc42 appears to depend on the coincidence between PIP2 at the membrane and the presence of Tuba in the apical cytosol.

In addition to regulating trafficking and phosphorylating junctional proteins, the Cdc42–PAR-6B–PKCζ axis also regulates luminogenesis through spindle regulation (Bryant et al., 2010; Jaffe et al., 2008). In 3D MDCK spheroids, apical–basal polarity is already apparent at the two-cell stage as the initial founder cell divides (Fig. 6A). During cell division, the placement of the cleavage furrow is linked to the orientation of the spindle, which depends on Cdc42. The cleavage furrow determines the formation of the midbody during cell division, which, in turn, determines apical identity (Jaffe et al., 2008; Mitsushima et al., 2009; Qin et al., 2010; Rodriguez-Fraticelli et al., 2010). During this process, the transmembrane phosphoglycoprotein podocalyxin, which is localized all around the founder cell, is transcytosed toward the apical membrane initiation site (AMIS), which forms at the midbody and is marked by PAR-3 and components of the exocyst (Bryant et al., 2010; Li et al., 2014; Willenborg et al., 2011). Transcytosis of podocalyxin appears to be particularly important to establish the apical–basal axis and requires the presence of Rab35 (Klinkert et al., 2016; Mrozowska and Fukuda, 2016). Concomitantly, the tight

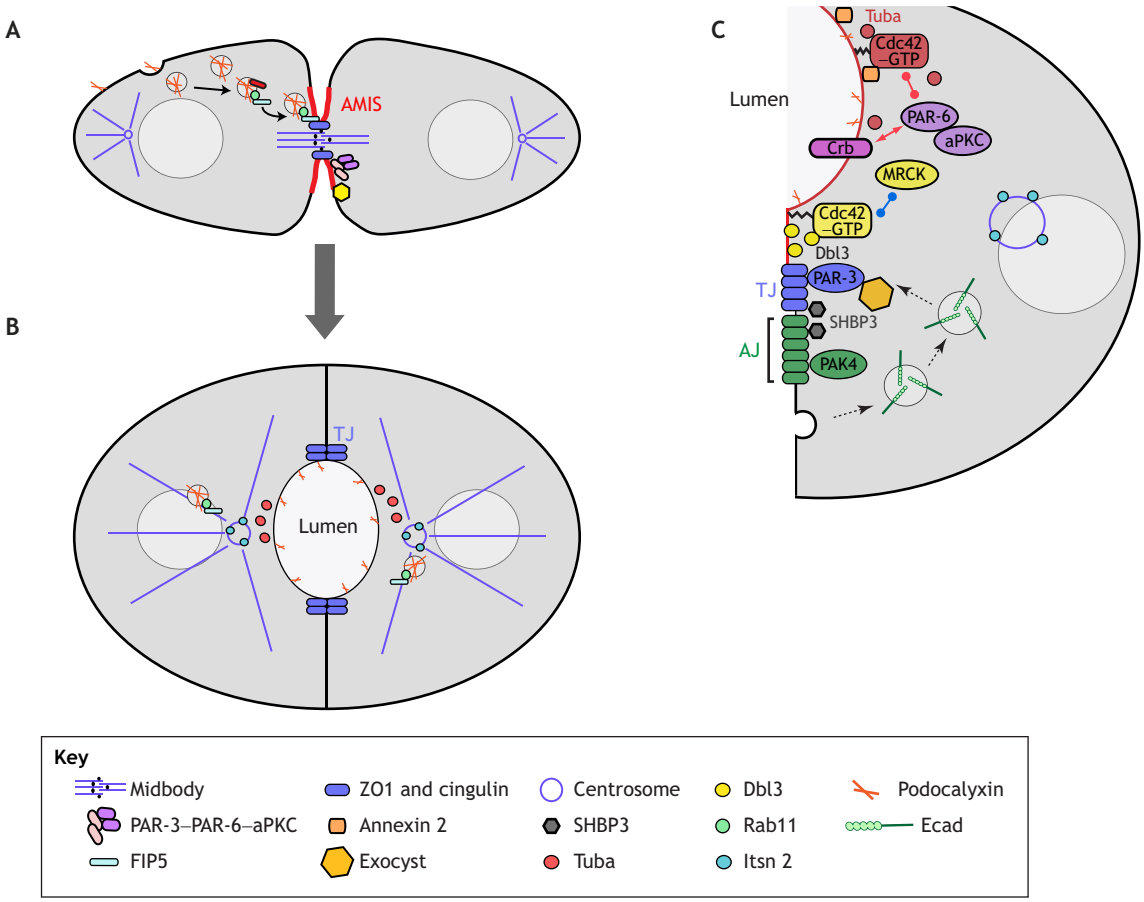

Fig. 6. Epithelial morphogenesis in vertebrate cells in 3D. Illustrated steps of luminogenesis in 3D culture. (A) The midbody serves as a landmark where ZO1 and cingulin mark the apical membrane initiation site (AMIS). The AMIS instructs further steps of lumen formation, including apical cargo delivery. Delivery of apical cargos, including podocalyxin, which is trancytosed from the basal membrane, is directed toward the AMIS. The endocytic pathway promotes the delivery of apical cargo, including Rab11 and FIP5. FIP5 binds to cingulin to promote vesicle docking. (B) As a central lumen forms, the tight junction begins to differentiate laterally to the apical membrane. (C) Activation of Cdc42 at the apical membrane arises from the coincidence of annexin 2-dependent recruitment and immobilization by activation through Tuba proteins. Tuba is found in the cytosol in the vicinity of the apical cortex, while intersectin-2 (Itsn2) is localized at the centrosome. Dbl3 is localized at the TJ to regulate microvilli morphogenesis through MRCK. PAR-3 is localized at the TJ, where it serves as a receptor for the exocyst, which regulates the transcytosis of Ecad to ensure AJ integrity.

junction protein cingulin is recruited to the AMIS, where it interacts with FIP5 (also known as RAB11FIP5) to promote cargo delivery by the Rab8a–Rab11a pathway (Fig. 6B) (Mangan et al., 2016). In addition, Cdc42 ensures that during cell division, the spindle is aligned perpendicular to the plane of the junctions (Jaffe et al., 2008). This regulation during mitosis also depends on aPKC and ensures that only a single central lumen is formed.

In addition to Tuba, another Cdc42 GEF, intersectin-2 (Itsn2), regulates lumen formation (Fig. 6A,B) (Qin et al., 2010; Rodriguez-Fraticelli et al., 2010). Like Tuba, intersectin-2 regulates spindle positioning during mitosis. In Caco-2 cells, intersectin-2 accumulates at the centrosome and around the edge of the spindle poles, suggesting that it regulates Cdc42 activity in the vicinity of these locations (Rodriguez-Fraticelli et al., 2010). At the spindle pole, intersectin-2 might promote Cdc42-dependent interaction between the astral spindle and the cell cortex. Conversely, Tuba, which localize to the tight junctions in Caco-2 cells and the cytoplasm in vicinity of the apical membrane in 3D MDCK spheroids (Otani et al., 2006; Qin et al., 2010; Rodriguez-Fraticelli et al., 2010), is thought to contribute to spindle alignment by preventing the spindle pole from interacting with the apical cortex. The effectors of Cdc42 function at the centrosome remain to be identified, but might include factors, such as PAK2 (Mitsushima et al., 2009) or the formin diaphanous 3 (Yasuda et al., 2004), which have been linked to Cdc42 and are known to regulate spindle orientation.

While Cdc42 likely regulates apical cargo delivery (Musch et al., 2001) and the endocytic pathway (Harris and Tepass, 2008), its apical activation depends on the apical trafficking pathway. This relationship between Cdc42 and apical cargo delivery is well supported by the finding that in 3D MDCK spheroids, Rab11-dependent delivery of apical proteins is required for normal Cdc42 activation (Bryant et al., 2010). Furthermore, reminiscent of the mechanism of Cdc42 polarity in budding yeast, recent work in the Caco-2-derived LS174T-W4 cell line using FRAP experiments has shown that activation of Cdc42 by Tuba leads to a threefold increase in the immobilization of Cdc42 to the apical membrane (Bruurs et al., 2017). This promotes Cdc42 clustering and presumably enables a reaction-diffusion mechanism that is comparable to that operating in budding yeast to determine polarity. In epithelial cells, this mechanism might contribute to ensuring that only one apical site is specified. In addition, there is evidence that in both vertebrate and invertebrate epithelial cells, Cdc42 is present on trafficking vesicles (Harris and Tepass, 2008; Bryant et al., 2010; Willenborg et al., 2011). It is therefore possible that in epithelial cells, Cdc42 is activated by Tuba in the vicinity of the apical membrane. This would promote the accumulation of Cdc42–GTP at the apical membrane that bears annexin 2, and coincide with the delivery of cargos such as CRB3 (Bryant et al., 2010; Willenborg et al., 2011). In this model, PAR-3, which localizes at the AMIS, might serve as a marker for the targeted delivery of apical cargos. A role for PAR-3 in facilitating the delivery of apical cargo, including that of Ecad, is supported by the finding that PAR-3 can interact with Exo70 (also known as EXOC7), a component of the exocyst, which mediates secretory vesicle docking at the plasma membrane (Fig. 6C) (Ahmed and Macara, 2017).

Cdc42 promotes apical membrane morphogenesis through Gek/MRCK

In addition to regulating luminogenesis and junction integrity, Cdc42 also promotes apical membrane morphogenesis. Work in Caco-2 cells has shown that this function is linked to the actomyosin cytoskeleton and the Cdc42 GEF Dbl3 (Zihni et al., 2014). In these cells, Dbl3 is recruited to the apical membrane in part through binding to Ezrin, which cross-links the actomyosin cytoskeleton to the plasma membrane. A main effect downstream of the Dbl3–Cdc42 pathway is the activation of MRCKβ (Gek in *Drosophila*), which regulates the actomyosin cytoskeleton through activation of myosin II (Zihni et al., 2017). Therefore, Cdc42 coordinates the morphogenesis of sub-apical membranes and microvilli through PAR-6–aPKC/PKCζ,ι and MRCK (Zihni et al., 2014, 2017). The architecture of this protein network is very similar to that operating in the *C. elegans* zygote (Fig. 2), which supports the anterior recruitment of PAR-6–PKC-3 and regulates the actomyosin cytoskeleton through MRCK-1. These similarities suggest that coupling of anterior recruitment and cytoskeletal regulation is a conserved feature of the mechanisms through which Cdc42 regulates cell polarity. How Cdc42 distributes between MRCKβ/Gek and PAR-6–aPKC is not clear, and one possibility is that this distribution depends on the GEF that is associated with Cdc42 during epithelial cell morphogenesis.

Conclusion and perspectives

Cdc42 plays an essential role during cell polarity establishment in yeast and animal cells. At the core of this role is its local activation or inactivation by GEFs and GAPs, and its links to the regulation of actomyosin, membrane delivery and endocytosis. Several GEFs and GAPs have been shown to regulate Cdc42 to promote epithelial morphogenesis in vertebrate epithelial cells. However, it is unclear where exactly Cdc42 is activated or inactivated in these cells. It is also unclear how Cdc42 distributes between the different GEFs involved, and how specific responses are achieved downstream of Cdc42. Furthermore, some of the Cdc42 GEFs and one of the GAPs identified to date appear to partially overlap at the lateral junctions. An interesting possibility is that these junctional domains are heterogeneous and consist of a collection of co-existing discrete molecular platforms, including some containing active Cdc42 and others where it is inhibited. These domains might correlate with stages of junction maturation and thus different pools of junctional proteins, or might reflect that their dynamics is linked to endocytosis or membrane delivery. It is also possible that GEFs and GAPs might exchange within these discrete molecular platforms, thus dynamically regulating Cdc42. Super-resolution approaches and single-molecule tracking will help to test these hypotheses and elucidate how exactly Cdc42 activation and inactivation contribute to the morphogenesis and maintenance of epithelial structures. Furthermore, determining the stoichiometry of the canonical epithelial polarity complexes that lie downstream of Cdc42, and the biophysical properties of their constituent proteins, will be required to truly understand the mechanisms of epithelial polarity and morphogenesis.

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Competing interests

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Supplementary information

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