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Rho GTPases and Actomyosin: partners in regulating epithelial cell-cell junction structure and function

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Abstract

Epithelial tissues are defined by polarized epithelial cells that are integrated into tissues and exhibit barrier function in order to regulate what is allowed to pass between cells. Cell-cell junctions must be stable enough to promote barrier function and tissue integrity, yet plastic enough to remodel when necessary. This remarkable ability to dynamically sense and respond to changes in cell shape and tissue tension allows cell-cell junctions to remain functional during events that disrupt epithelial homeostasis including morphogenesis, wound healing, and cell division. In order to achieve this plasticity, both tight junctions and adherens junctions are coupled to the underlying actomyosin cytoskeleton. Here, we discuss the importance of the junctional linkage to actomyosin and how a localized zone of active RhoA along with other Rho GTPases work together to orchestrate junctional actomyosin dynamics. We focus on how scaffold proteins help coordinate Rho GTPases, their upstream regulators, and their downstream effectors for efficient, localized Rho GTPase signaling output. Additionally, we highlight important roles junctional actin-binding proteins play in addition to their traditional roles in organizing actin. Together, Rho GTPases, their regulators, and effectors form compartmentalized signaling modules that regulate actomyosin structure and contractility to achieve proper cell-cell adhesion and tissue barriers.

Keywords

adherens junction; tight junction; F-actin; Myosin II; formin; Arp2/3

Introduction

Epithelial tissues act as selective, regulated barriers that separate different compartments in the body. This compartmentalization allows distinct tissues and organs to perform their specialized functions while receiving appropriate signals from the external environment. The epithelial barrier regulates the movement of solutes, fluids, macromolecules, and even immune cells or pathogens through the space between epithelial cells. The apical junctional

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complex (AJC) in vertebrates, which is composed of tight junctions (TJs) and adherens junctions (AJs), plays the critical role of sealing the paracellular space and adhering epithelial cells to one another (Hartsock and Nelson, 2008; Van Itallie and Anderson, 2014). Both TJs and AJs are connected to the actomyosin cytoskeleton, and tightly regulated actomyosin dynamics are critical not only for junction formation, but also for structure and function of TJs and AJs during epithelial tissue homeostasis, and morphogenesis (Lecuit and Yap, 2015; Rodgers and Fanning, 2011; Takeichi, 2014). Rho GTPases are critical for proper dynamic organization and contractility of junctional actomyosin. When misregulated, abnormal junctional F-actin polymerization or Myosin II contraction can drive pathological conditions (Ivanov et al., 2010; Marchiando et al., 2010).

In this review we first examine the linkage of the core TJ and AJ proteins to the actomyosin cytoskeleton and mechanisms by which this linkage can be dynamically regulated in space and time. We then discuss critical roles Rho GTPases play in regulating the structure of junctional actin and Myosin II. We propose that scaffold proteins are a commonly used organization system to bring Rho regulators, GTPases, and effectors together for proper localized Rho GTPase signaling output. We discuss how actin-binding proteins not only organize junctional actin but also play additional functional roles at cell-cell junctions. We conclude by highlighting some fascinating topics that require additional studies in the future.

Linkage of core TJ and AJ proteins to the actomyosin cytoskeleton

The ultrastructure of cell-cell junctions in a variety of epithelial tissues was first identified in a seminal electron microscopy study by Farquhar and Palade (Farquhar and Palade, 1963). The authors described TJs (or *zonula occludens*), where the space between epithelial cells is almost completely obliterated, AJs (or *zonula adherens*), located just basal to the TJ where the cell membranes are brought in close proximity (~20 nm apart), as well as “conspicuous bands of dense material located in the subjacent cytoplasmic matrix”, which we now know to be junctional actomyosin (Farquhar and Palade, 1963).

TJs are an important determinant of epithelial barrier function. They seal the intercellular spaces between adjacent epithelial cells and form regulated, selective (size- and ion-specific) barriers. Barrier function can be acutely regulated in epithelial tissues by signaling mechanisms – notably by changes in actomyosin contractility (Shen et al., 2011). To achieve these functions, the TJ transmembrane proteins (Claudins, Occludin, immunoglobulin-like JAMs) for TJ strands, which are linked to the underlying actomyosin cytoskeleton via cytoplasmic plaque proteins (Zonula Occludens (ZO) proteins, Cingulin, Afadin, etc.) (Van Itallie and Anderson, 2014) (Figure 1). The ZO proteins (ZO-1, ZO-2, and ZO-3) bind to the cytoplasmic tail of Claudins and Occludin with their N-termini (Itoh et al., 1999; Li et al., 2005). ZO-1 interacts with F-actin through its C-terminus; ZO-2 and ZO-3 also interact with F-actin, although the binding sites have not been determined (Fanning et al., 1998; Wittchen et al., 1999). ZO proteins are proposed to initiate the polymerization of Claudins into TJ strands (Umeda et al., 2006), and ZO-1 has the ability to stabilize Claudin strands (Van Itallie et al., 2016).

AJs, located just basal to the TJ, mediate cell-cell adhesion and mechanically integrate epithelial cells into tissues. AJs are functionally important for epithelial homeostasis and morphogenesis. In addition to resisting mechanical forces from neighboring cells, the contractile actomyosin network associated with AJs can also transmit tension across cell-cell junctions to neighboring cells, actively shaping tissues during development. For example, pulsatile contraction of actomyosin coupled to AJs promotes apical constriction of individual cells, which collectively leads to tissue folding (Coravos et al., 2016; Takeichi, 2014), and promotes intercalation, during which cells remodel their cell-cell contacts through neighbor exchange (Lecuit and Yap, 2015). Each of these functional roles of AJs is dependent on regulated linkage of the core molecular components of AJs to the actomyosin cytoskeleton.

The core AJ components include the transmembrane proteins (E-cadherin and Nectins) and cytoplasmic plaque proteins (β -catenin, α -catenin, p120-catenin, Vinculin, Afadin, etc.) (Quiros and Nusrat, 2014; Ratheesh and Yap, 2012) (Figure 1). E-cadherin forms both small spot-like clusters along the lateral membrane as well as an apical belt-like structure, the “zonula adherens” (ZA), which is located just basal to the TJ. F-actin plays an important role in corralling the small E-cadherin clusters (Wu et al., 2015), and actomyosin drives their coalescence and stabilization at the apical ZA (Ratheesh and Yap, 2012). The linkage of E-cadherin to F-actin is achieved via catenin proteins. β -catenin binds to the cytoplasmic tail of E-cadherin, and α -catenin binds to β -catenin. Although α -catenin can bind F-actin, there were controversies about whether α -catenin can simultaneously bind to both the cadherin/catenin complex and F-actin (Yamada et al., 2005). Recent work demonstrated that α -catenin can indeed bind both, but only under actomyosin-generated force (Buckley et al., 2014; Nelson and Weis, 2016). This work showed that under tension, the cadherin/catenin complex forms a stable bond with F-actin (Buckley et al., 2014). Furthermore, actomyosin-mediated tension promotes a conformational change in α -catenin, which reveals a binding site for Vinculin (Yonemura et al., 2010). In this way, Vinculin is only recruited to cell-cell junctions under mechanical tension, thus reinforcing cell adhesion and the linkage to F-actin in the face of mechanical force (le Duc et al., 2010; Yonemura et al., 2010).

Dynamic nature of cell-cell junctions and their linkage to actomyosin

Static electron microscope or immunofluorescence images of cell-cell junction proteins, combined with the sheer number of proteins that localize to junction, might lead one to believe that cell-cell junctions are stable, heavily crosslinked structures. However, fluorescence recovery after photobleaching (FRAP) experiments overturned this model and demonstrated junctions are continuously remodeled by rapid protein turnover (Huang et al., 2011; Shen et al., 2008; Yamada et al., 2005). FRAP studies have proven to be a powerful tool to tease apart junction protein complexes, their interactions with the cytoskeleton, and how junctions remodel in response to stimuli such as changes in tension (Priya et al., 2013; Ratheesh et al., 2012; Van Itallie et al., 2016; Yonemura et al., 2010). For example, FRAP was recently used to demonstrate that plasticity of cell-cell junction structure and the linkage to actin is important for maintenance of cell-cell adhesion and barrier function when cells undergo cell-cell junction remodeling during cell division in *Xenopus* embryos (Higashi et al., 2016). This study showed that tension generated by the cytokinetic

contractile ring is transmitted to the adherens junction, recruiting Vinculin, and stabilizing the dynamics of adherens junction proteins specifically at the division site (Higashi et al., 2016).

Cells in developing epithelial tissues undergo rearrangements and shape changes, such as cell division, apical constriction, and cell intercalation, which drive the dramatic events of embryonic morphogenesis. Likewise, in adult epithelial tissues, there are multiple cell shape change events that challenge tissue integrity and require cell-cell junction remodeling including cell division, cell extrusion, and wound healing (Guillot and Lecuit, 2013; Lecuit and Yap, 2015). So how are cell-cell junctions stable enough to promote barrier function and tissue integrity, but plastic enough to remodel when necessary? We argue that carefully orchestrated control of Rho GTPases is critical for regulating junctional actomyosin dynamics underlying junction formation, maturation, homeostasis, and morphogenesis. Rho GTPases regulate the polymerization and organization of actin and the activation of the motor protein Myosin II. The activation of specific Rho GTPases is precisely regulated in space and time. In this way, Rho GTPases can provide both basal, steady state activity levels and also can be activated acutely in response to specific signals – both chemical and mechanical.

Rho family GTPases are critical regulators of cell-cell junctions

Rho GTPases are a conserved family of 20 small GTPases that regulate cytoskeletal dynamics in a variety of contexts (Heasman and Ridley, 2008). Most Rho GTPases, including the prototypical family members – RhoA, Rac1, and Cdc42 – cycle between an active, GTP-bound state, and an inactive, GDP-bound state (Figure 2). When in their active GTP-bound conformation, Rho GTPases associate with cellular membranes and can interact with and activate specific effector proteins, resulting in localized effects on the cytoskeleton. For example, active RhoA promotes formation of actomyosin contractile arrays via its key effector proteins: formin, which nucleates unbranched actin filaments, and Rho-associated coiled-coil kinase (ROCK), which phosphorylates the regulatory light chain of Myosin II to increase contractility. Rho GTPases are activated by guanine nucleotide exchange factors (GEFs) and inactivated by GTPase activating proteins (GAPs). Additionally, Rho guanine nucleotide dissociation inhibitors (GDIs) contribute to inactivation by extracting GTPases from the plasma membrane, binding inactive GDP-bound GTPases, and preventing them from re-activation or degradation (Boulter et al., 2010). Therefore, the location and extent of Rho GTPase activity and thus GTPase signaling output is strongly dependent on the localization and net activity of GEFs, GAPs, and GDI, along with availability of effectors.

A role for Rho GTPases in regulating cell-cell junctions was first established by studies showing that manipulating GTPase function with constitutively active or dominant negative Rho GTPases or with inhibitors disrupted junctional integrity (Citi et al., 2014; Quiros and Nusrat, 2014; Ratheesh et al., 2013). These studies demonstrated that Rho GTPases are important regulators of both the AJ (Braga et al., 2000; Braga et al., 1999; Braga et al., 1997; Takaishi et al., 1997) as well as the TJ (Jou et al., 1998; Nusrat et al., 1995). The level of Rho GTPase activation must be precisely balanced; either too much or too little could disrupt junction integrity.

In order to determine whether active Rho GTPases localize to cell-cell junctions, thus implying a functional role at junctions, several types of molecular probes have been employed for static and live imaging studies (Stephenson and Miller, 2017). Each of these approaches takes advantage of the GTPase binding domain (GBD) of a Rho effector protein, which is specific for the Rho GTPase of interest and binds only the active, GTP-bound conformation of the protein. Together, these studies demonstrate that zones of active Rho GTPases are localized specifically at cell-cell junctions – active Rac1 during cell-cell junction expansion (Yamada and Nelson, 2007), active RhoA and active Rac1 at steady state (Brezna et al., 2015; Priya et al., 2015; Ratheesh et al., 2012; Reyes et al., 2014; Terry et al., 2011). Work in cultured mammalian epithelial cells suggested that the junctional Rho activity zone is quite stable (Priya et al., 2015). However, studies in a more dynamic developing epithelium (gastrula-stage *Xenopus* embryos) revealed that in addition to stable, steady-state RhoA activity zones encircling the cell perimeter, transient, localized accumulations or “flares” of active RhoA were observed. These junctional Rho flares were observed in response to: 1) disruption of cell-cell junction integrity by genetic manipulation of a junctional scaffold protein (Reyes et al., 2014), 2) disruption of the GAP activity of a Rho GAP protein (Brezna et al., 2015), and 3) mechanical challenges to cell-cell junction integrity (Clark et al., 2009) (Stephenson and Miller, unpublished). How these junctional Rho activity zones and Rho flares are spatially and temporally regulated by specific GEFs that determine where Rho is activated, specific GAPs that limit the spread of GTPase activity, and effectors that carry out the cytoskeletal response is currently a topic of intense study.

Rho GTPases and the organization of junctional actomyosin

The organization of junctional actin evolves as junctions mature (Figure 3). In cultured epithelial cells, initial cell-cell junction formation occurs after migrating cells make contact with one another (Yonemura et al., 1995). Following this initial contact, active Rac1 and Cdc42 promote a branched actin network that extends the interface between the two cells and stimulates cadherin ligation (Samarin and Nusrat, 2009; Vasioukhin et al., 2000; Verma et al., 2004; Yamazaki et al., 2007). Rac1 activity and Arp2/3 are similarly needed to form a properly elongated junction between daughter cells following cytokinesis in the *Drosophila* dorsal thorax epithelium (Herszterg et al., 2013). As junctions mature, the branched actin network is converted into linear actin filaments that are organized into bundles decorated with Myosin II, creating a functional contractile array (Choi et al., 2016; Drenckhahn and Dermietzel, 1988; Ebrahim et al., 2013; Takeichi, 2014). It is currently debated whether these bundles form a ring or terminate at tricellular junctions, making each bicellular junction a singular contractile unit (Choi et al., 2016). Additionally, junctions are linked to medial-apical actomyosin, which spans the apical surface of epithelial cells and drives pulsatile contractions that cause cell-scale apical constriction and tissue-scale invagination (Coravos et al., 2016; Lecuit and Yap, 2015).

RhoA establishes a contractile actomyosin array at the AJC by stimulating actin polymerization through formins and activating Myosin II through ROCK. For example, if Dia1 is knocked down, robust cell-cell adhesion is not maintained in cultured epithelial (MCF-7) cells, and this is dependent on Myosin II activity (Carramusa et al., 2007). Several

other members of the formin family (15 members in mammals and other higher vertebrates) have recently been implicated at cell-cell junctions (for more detail see (Grikscheit and Grosse, 2016)). Interestingly, both Rac1 and Cdc42 can also bind and trigger formin activity (Grikscheit and Grosse, 2016). For example Formin-like 2 helps maintain normal junctional actin downstream of Rac1 activity in human epithelial cells (Grikscheit et al., 2015). Additionally, during the junction maturation process in cultured epithelial (MTD-1A and Caco-2) cells, Cdc42 establishes F-actin cables that span cellular junctions by localizing to tricellular contacts via Tricellulin and the Cdc42 GEF Tuba (Oda et al., 2014). Although these F-actin cables appear reminiscent of formin-polymerized F-actin, the mechanism by which Cdc42 establishes these F-actin structures is unknown.

Despite the lack of an observable branched F-actin network at mature junctions, Rac1- and Cdc42-dependent Arp2/3 activity is also required to maintain mature apical junctions. Arp2/3 has an established role in generating a dynamic population of actin at junctions (Brieher and Yap, 2013). It is possible that Rac1 functions via Arp2/3 to nucleate many nascent branched actin filaments, which are then converted into a contractile network through RhoA activity. In fact, branched F-actin networks can be transformed into contractile F-actin networks in cells (Hotulainen and Lappalainen, 2006) and *in silico* in the presence of bundling proteins, motor proteins, and anchor points (Letort et al., 2015). Later in this Review, we will discuss actin-binding proteins that help organize actin structure at cell-cell junctions.

Junctional roles for Myosin II

Myosin II can use its motor activity to slide actin filaments connected to the AJC and to apply tension on the junction and neighboring cells. This tension is important for normal junction establishment, maintenance, and disassembly as well as generating cell-scale and tissue-scale shape changes that drive morphogenesis. The biological significance Myosin II plays in epithelial barrier function *in vivo* was recently demonstrated in mice. A conditional knockout of Myosin IIA in mouse intestinal epithelium resulted in altered expression and localization of AJC proteins, resulting in a compromised intestinal barrier (Naydenov et al., 2016). These animals also showed low levels of inflammation in the intestine, and animals with experimental colitis exhibited more tissues damage compared to controls (Naydenov et al., 2016).

Several recent studies have underscored Myosin II's mechanical contributions to maintaining cell-cell junctions by generating tension at the AJC. In a recent study in *Drosophila*, expression of the transcription factor Snail was associated with junction disassembly and epithelial to mesenchymal transition; however, Myosin II-driven contractility alone prevented Snail-induced junction disassembly and promoted junction maturation (Weng and Wieschaus, 2016). Several studies have provided clues about molecular mechanisms that may underlie such functional results. First, Myosin II-mediated mechanical force regulates AJ size and junction protein turnover; AJs are stabilized under high tension and more dynamic under reduced tension (Liu et al., 2010; Priya et al., 2013; Ratheesh and Yap, 2012; Yonemura et al., 2010). Second, when the mechanosensitive protein α -catenin is under high tension, it recruits Vinculin to AJs (Yonemura et al., 2010).

Vinculin then can bind actin directly and also can recruit the Ena/VASP complex to promote actin polymerization to strengthen the connection between the AJ and the actin cytoskeleton (Leerberg et al., 2014). Indeed, a recent study in *Xenopus* epithelial cells, demonstrates implementation of both of these mechanisms. When increased tension was generated by a physiological mechanical input – tension generated by the contractile ring during cell division – embryos exhibited a stabilization of AJ proteins and recruited Vinculin to the division site to strengthen the AJ in response to locally applied tension (Higashi et al., 2016).

The three Myosin II isoforms, Myosin IIA-C exhibit tissue-specific expression patterns as well as unique and overlapping properties. Myosin IIA localization and activity is Rho-dependent, and knocking down Myosin IIA severely disrupts TJs and AJs (Ivanov et al., 2007; Smutny et al., 2010). In cultured epithelial cell lines, Myosin IIA regulates assembly and disassembly of both AJs and TJs, whereas Myosin IIB and IIC isoforms were not essential for these processes (Ivanov et al., 2007). In contrast to Myosin IIA, junctional localization of Myosin IIB is Rap1-dependent, and although knocking down Myosin IIB in MCF-7 cultured epithelial cells affects actin dynamics at the AJC, there is little effect on the AJ itself (Smutny et al., 2010). In further support of Myosin IIA's unique role at cell-cell junctions, in well-differentiated epithelial cells, Myosin IIA is the predominantly expressed isoform, whereas Myosin IIB is not expressed or is only found in trace amounts (Babbitt et al., 2009). Myosin IIC's function at cell-cell junctions is currently less clear. Myosin IIC and IIA, but not IIB, are expressed in the mouse intestinal epithelium (Naydenov et al., 2016). Myosin IIC also selectively accumulates at the AJC in some epithelia (Ebrahim et al., 2013; Ivanov et al., 2007). However, knockdown of Myosin IIC in intestinal epithelial cells revealed no gross defects in cell morphology or junction structure (Ivanov et al., 2007). Interestingly, in mouse mammary gland tissue, an epithelial to mesenchymal transition coincides with reduced expression of Myosin IIC and concomitant increased expression of Myosin IIB as well as increased Myosin IIA phosphorylation (Beach et al., 2011). Notably, of the three Myosin isoforms, Myosin IIB has the highest duty ratio (remains strongly bound to actin longer) (De La Cruz and Ostap, 2004; Wang et al., 2003), a property which the authors suggest would allow Myosin IIB to generate sustained contractile force required for specific developmental events in the mammary gland epithelium (Beach et al., 2011). Therefore, it seems likely that Myosin IIB may also be important for prolonged force production at cell-cell junctions, whereas Myosin IIA and IIC may serve other essential roles in facilitating junction homeostasis.

In addition to the essential function of Myosin II motor activity in generating actomyosin contractile force, recent work suggests that Myosin II also plays important motor-independent functional roles including bundling actin and scaffolding proteins at the AJC. For example, Myosin IIA functions as a scaffold that promotes RhoA-GTP output at junctions; Myosin IIA scaffolds ROCK1 at the ZA, and ROCK1 works through Rnd3 to prevent the accumulation of the Rho GAP p190RhoGAP-B, which would negatively regulate RhoA signaling at the ZA (Priya et al., 2015; Priya et al., 2017). Additionally, Myosin II filaments can serve as an F-actin bundling proteins. This ability to bundle F-actin is likely important for structuring the apical actomyosin bundles at the AJC. Moreover, recent work has shown that Myosin IIA filaments can form large lateral stacks of motor groups, which may be important for organizing actin structure at junctions (Fenix et al.,

2016). Interestingly, these stacks of Myosin IIA filaments can be assembled, although to a lesser extent, from mutants of Myosin IIA that have reduced motor activity (Fenix et al., 2016). Future work is required to further distinguish how Myosin IIA-C contribute to establishing and organizing a functional contractile F-actin network at junctions through both motor-dependent and motor-independent mechanisms.

Scaffold proteins regulate Rho GTPase output at cell-cell junctions

To establish the contractile actomyosin network at the AJC, Rho GTPase activity has to be tightly regulated spatially and temporally. It is well accepted that the placement and extent of GTPase activity is the result of the net activity of GEFs and GAPs. The current list of GEFs and GAPs known to regulate Rho family GTPases at cell-cell junctions is long, and includes GEFs and GAPs important for junction assembly, maturation, homeostasis, and disassembly (for more detail see (Citi et al., 2014; Quiros and Nusrat, 2014; Zihni and Terry, 2015)). Unsurprisingly, the list of GEFs and GAPs that governs these processes appears to differ by cell type and continues to grow as proteomic studies identify novel potential regulators (Guo et al., 2014; Van Itallie and Anderson, 2014; Van Itallie et al., 2013). As the list of GEFs, GAPs, and even GTPases at the AJC grows, it becomes apparent that cells must have mechanisms to organize GTPase signaling. Scaffold proteins can fulfill this role by bringing regulators, GTPases, and effectors together to efficiently couple GTPase activation with output (Garcia-Mata and Burridge, 2007; Marinissen and Gutkind, 2005) (Figure 4).

Scaffold proteins are key components of cell signaling pathways that tether signaling components to a discrete cellular location, promoting efficient signaling by limiting the diffusion of components and ensuring that the correct targets become activated. Furthermore, scaffold proteins may mediate the response to stimuli by participating in negative or positive feedback loops to carefully control the signaling output. Proteins that scaffold RhoA signaling at the AJC may dictate the outcome of RhoA activation in several ways including: 1) by controlling whether specific targets (e.g. ROCK, formins, or transcription factors) become activated; 2) by anchoring signaling output to specific structures (e.g. TJ, ZA, or spot-like AJs); 3) by modulating whether and how much contractile force results from signaling (e.g. enough to generate tension on the junction, change cell shape, or completely disengage the junctions); and 4) by coordinating tension between neighboring cells (e.g. via mechanosensitive junctional proteins that can promote signaling in response to tension changes in neighboring cells). Similarly, scaffolds for Rac1 or Cdc42 signaling could dictate whether Arp2/3 activation generates protrusive structures at the membrane or simply contributes to a pool of F-actin that is incorporated into the contractile array. Below, we present several examples of scaffold proteins important for directing Rho family signaling output at the AJC.

Cingulin and Paracingulin

Cingulin and Paracingulin have similar structures, as well as overlapping and distinct binding partners. Cingulin is localized at the TJ in a ZO-1-dependent manner (Umeda et al., 2004), whereas Paracingulin localizes to both the TJ and AJ (Citi et al., 2012). Both form

parallel homodimers, with a globular head domain, a long coiled-coil rod domain, and a small globular tail (Citi et al., 2012; Cordenonsi et al., 1999). Cingulin can directly bind to F-actin, Myosin II, and microtubules through its head domain (Cordenonsi et al., 1999; D'Atri and Citi, 2001; Yano et al., 2013). Paracingulin also associates with the actin and microtubule cytoskeleton, and disruption of either interferes with its junctional targeting. In contrast, only disruption of the actin cytoskeleton disrupts Cingulin's junctional targeting (Paschoud et al., 2011).

Cingulin and Paracingulin can both bind several Rho family GEFs and GAPs. Both proteins can bind MgcRacGAP, a RhoA GAP protein, and GEF-H1, a RhoA GEF that is inactivated by Cingulin/Paracingulin binding (Guillemot et al., 2014; Guillemot et al., 2008). A complex of Cingulin, p114RhoGEF, ROCK2, and Myosin IIA is important for confining RhoA signaling to the apical junctions in Caco-2 and Human Corneal Epithelial (HCE) cells, and for normal tight junction maturation in HCE cells (Terry et al., 2011). Paracingulin recruits the GEF Tiam1 to junctions where it activates Rac1 during junction assembly following calcium switch (Guillemot et al., 2008). Paracingulin also forms a complex with the Rac1, SH3BP1 (a Cdc42 GAP), CD2AP, and the actin capping protein CapZ α . SH3BP1 knockdown results in filopodial structures at junctions, mislocalized F-actin and Cdc42 activity, and aberrant junction formation (Elbediwy et al., 2012).

With so much potential for interaction with GEFs, GAPs, and cytoskeletal components, what remains unclear is how Cingulin and Paracingulin regulate which GEFs and GAPs are “on” at a given time. A simple interpretation is that they simply act as targeting molecules, recruiting GEFs and GAPs to the junctions where they are then activated or inactivated by other proteins (e.g. by kinases). Another possibility is that the extended rod domain functions as a molecular ruler, as was proposed for ROCK2 (Truebestein et al., 2015). Cingulin binds actin, Myosin II, microtubules, and the C-terminus of ZO-1 with its head domain, whereas many GEFs and GAPs can bind to its rod domain. Perhaps the head domain anchors Cingulin to the cytoskeleton while the rod domain acts to position GEFs and GAPs near membrane-bound GTPases in certain contexts. Interestingly, Yano et al. found a potential phosphorylation-dependent conformational change which enhances Cingulin-microtubule binding (Yano et al., 2013), so it is possible that this or other modifications govern the orientation or conformation of Cingulin to give GEFs and GAPs access to GTPases or couple it to the relevant cytoskeletal structures (Figure 4A).

p120 catenin

p120 catenin is a member of the armadillo repeat (ARM) family of proteins along with β -catenin, and it binds to the juxtamembrane domain of cadherins, promoting their stability by preventing Cadherin endocytosis (Kourtidis et al., 2013). p120 can influence RhoA, Rac1, and Cdc42 signaling via direct interactions and recruitment of GEFs, GAPs, and effectors to junctions (Kourtidis et al., 2013). p120 appears to coordinate RhoA/Rac1 antagonism as it can inactivate RhoA through p190RhoGAP-B recruitment and stimulate Rac1 and Cdc42 activation through the GEF Vav2 (Noren et al., 2000; Ponik et al., 2013; Valls et al., 2012; Wildenberg et al., 2006; Zebda et al., 2013). Interestingly, downregulation of RhoA through p120 catenin requires cadherin association (Yu et al., 2016), whereas upregulation of Rac1

and Cdc42 requires unbound p120 catenin (Noren et al., 2000; Valls et al., 2012). Additionally, p120 can bind directly to RhoA-GDP *in vitro* and suppress GTP exchange, functioning similarly to Rho GDI (Anastasiadis et al., 2000). However, p120 also functions as a positive regulator of contractility by localizing ROCK1 and Shroom3 to the cadherin/catenin complex (Lang et al., 2014; Smith et al., 2012). Thus, p120 catenin can take on many roles as a regulator of GTPase signaling depending on the cell context, and these are likely controlled in part by cadherin binding, alternative splicing, and phosphorylation, all of which can change the affinity of p120 for RhoA (Castano et al., 2007; Yanagisawa et al., 2008).

A recent study has identified a novel role for p120 catenin in regulating RhoA activation during cytokinesis. This study found that p120 catenin binds to MKLP1 and MP-GAP and constrains RhoA activity to the furrow (van de Ven et al., 2016). Anillin was also identified as a p120-interactor in this study. Interestingly, Anillin and MKLP-1, along with other prominent cytokinesis regulators MgcRacGAP and Ect2, have roles in RhoA regulation at junctions (Ratheesh et al., 2012; Reyes et al., 2014), so it would be interesting to further investigate ties between these proteins and p120 catenin at junctions (Figure 4B).

Afadin is a scaffolding protein that can bind actin and several junctional proteins. Afadin links the AJ transmembrane protein Nectin to the actin cytoskeleton and also helps crosslink TJ transmembrane proteins with F-actin (Quiros and Nusrat, 2014; Van Itallie and Anderson, 2014).

Afadin is necessary for maintaining tissue integrity and barrier function under high tension and is particularly important for maintaining adhesion at tricellular junctions (Choi et al., 2016; Tian et al., 2016). The *Drosophila* Afadin ortholog, Canoe, is critical for linking the actin cytoskeleton to the AJ during the mechanical stresses of development (Choi et al., 2011; Sawyer et al., 2011). Additionally, Afadin appears to play a role in regulating actomyosin-mediated paracellular permeability through its interactions with the TJ transmembrane protein JAM-A, ZO-2 and PDZ-GEF1; this complex regulates apical actomyosin contraction via the small GTPases Rap2 and RhoA (Monteiro et al., 2013). In addition to its role in organizing F-actin, Afadin associates with the small GTPase Rap1 and p120 catenin to stabilize E-cadherin (Hoshino et al., 2005; Sato et al., 2006). Specifically, Afadin interacts with p120 catenin associated with non-*trans* interacting E-cadherin, preventing E-cadherin's endocytic removal from the junction and allowing it to make *trans* interactions, thus promoting junction maturation (Hoshino et al., 2005; Sato et al., 2006).

Anillin

Anillin itself is a well-known scaffolding protein. It ensures successful cytokinesis by bundling F-actin, linking F-actin and Myosin II to the membrane, and regulating RhoA activity at the contractile ring (Piekny and Maddox, 2010). The N-terminal domains of Anillin participate in actomyosin binding/assembly, while the C-terminal domains include PH and C2 domains, which anchor it to the membrane, a RhoA binding domain, which allows it to interact with active RhoA, and binding sites for interacting with the GEF Ect2 and the GAPs MgcRacGAP and p190RhoGAP-A (Frenette et al., 2012; Manukyan et al., 2015; Piekny and Maddox, 2010; Sun et al., 2015). Early in cytokinesis, Anillin participates

in a positive feedback loop in which its accumulation at the contractile ring is both dependent on and enhances Rho activation (Piekny and Glotzer, 2008). Later in cytokinesis, it interacts with p190RhoGAP-A in a tension-sensitive manner, inactivating RhoA in response to excessive force (Manukyan et al., 2015). Thus, Anillin helps to fine-tune RhoA signaling by coupling GEFs, GAPs, and RhoA with cytoskeletal components like F-actin, Myosin II, and formins.

We recently reported a population of Anillin at cell-cell junctions that is important for proper accumulation of junctional actomyosin and organization of TJs and AJs in *Xenopus laevis* embryos (Reyes et al., 2014). Interestingly, the GEFs and GAPs known to interact with Anillin at the contractile ring are also found at cell-cell junctions (Priya et al., 2015; Ratheesh et al., 2012). Anillin depletion results in ectopic “flares” of active RhoA and F-actin, suggesting that Anillin helps to spatially confine RhoA signaling to the AJC (Reyes et al., 2014). However, it has yet to be determined whether Anillin can fine-tune actomyosin tension at cell-cell junctions like it can at the cytokinetic contractile ring. While Anillin has no known junction-specific interactors, its interactions with the actomyosin cytoskeleton or RhoA signaling proteins may be sufficient to recruit it to junctions. Anillin may also regulate junctions through an indirect pathway. In mammalian epithelial cells (DU145 and SK-CO15), depletion of Anillin disrupted junctions despite the fact that Anillin localization could not be detected at junctions. Instead, loss of Anillin misregulated junctions through the JNK pathway (Wang et al., 2015) (Figure 4C).

Other scaffolds for Rho GTPase regulation at cell-cell junctions

Additional scaffold proteins may be important for directing Rho family GTPase signaling at the AJC as well. α -catenin is a mechanosensitive protein that can bind to Vinculin under tension, reinforcing AJ connection to the cytoskeleton (Yonemura et al., 2010). Together with dynamic microtubules, α -catenin stabilizes the Rho GEF Ect2 and MgcRacGAP at the ZA, promoting RhoA signaling (Ratheesh et al., 2012). p190RhoGAP-B is excluded from junctions under these conditions, either indirectly through Rac1 inactivation or through ROCK1 phosphorylation of Rnd3 (Priya et al., 2015; Ratheesh et al., 2012). ZO-1 can interact with many of the core TJ components, including Claudins, Occludin, other ZO proteins, Cingulin, and Paracingulin. ZO-1 can bind to GEFs for Cdc42 (Tuba) and RhoA (PDZRhoGEF), and these interactions appear to be important for junction assembly (Itoh, 2013; Otani et al., 2006). Many GEFs themselves may serve as scaffolds to help make the connection between a GTPase and its effectors. Interestingly, 37% of GEFs in the human Dbl homology family of GEFs contain putative PDZ-binding motifs at their C-termini (Garcia-Mata and Burridge, 2007); these motifs can interact with PDZ domain-containing proteins, which are abundant at cell-cell junctions. Finally, several actin-binding proteins discussed below can also act as scaffolds to help orchestrate Rho GTPase signaling at junctions.

Actin-binding proteins at cell-cell junctions: organizing F-actin and more

Similar to the roles scaffold proteins play to facilitate proper Rho GTPase signaling at the AJC, actin-binding proteins can work independently or with Myosin II to organize actin

filaments, link them to the membrane, promote their stability or depolymerization, and act as scaffolds for signaling. Despite their importance in organizing and regulating the actin cytoskeleton, relatively little is known about the functional role of actin-binding proteins at the AJC. For example, the previously mentioned scaffold Anillin is known to perform all of the above mentioned potential functions of actin-binding proteins in the context of cytokinesis; however, whether Anillin is serving some or all of those functions at the AJC is not known. Here, we will focus on several examples of actin-binding proteins that have been shown to play important roles at the AJC.

Coronin 1B is an actin-binding protein known to be important for cell motility and cytokinesis (Chan et al., 2011). Coronin functions to de-branch F-actin networks by displacing the Arp2/3 complex, creating more flexible hinge points (Chan et al., 2011). Recent work has positioned Coronin 1B as the critical actin-binding protein for restructuring perijunctional actin from a branched F-actin network to contractile bundles (Michael et al., 2016). Furthermore, Coronin is required for stabilizing Myosin II at the AJC, which can positively regulate Rho signaling (Michael et al., 2016; Priya et al., 2016).

α -actinin is a F-actin bundling protein in the calponin homology family that, together with Myosin II, can build contractile arrays of actomyosin (Otey and Carpen, 2004). α -actinin localizes to cell-cell junctions (Craig and Pardo, 1979) and bundles F-actin at the AJC (Tang and Brieher, 2012). The major isoform of α -actinin in the kidney, α -actinin-4 (a.k.a. FSGS1), was recently shown to promote Arp2/3-dependent actin assembly at AJs (Tang and Brieher, 2012). Knock down of α -actinin-4 disrupted actin assembly at AJs. Moreover, expression of a single point mutant α -actinin-4, which was associated with patients suffering from the human renal disease focal segmental glomerulosclerosis (FSGS), inhibited actin assembly at junctions in a dominant negative fashion. Interestingly, this α -actinin mutant was still able to localize to junctions and bundle F-actin, indicating that α -actinin-4 must contribute to Arp2/3 actin assembly in ways outside of its function in bundling F-actin.

Filamin, an actin bundling protein that also belongs to the calponin homology family, crosslinks actin filaments orthogonally (Nakamura et al., 2011). In addition to this role in organizing actin, Filamin scaffolds a quaternary complex with the calcium channel CaR, RhoA, and the Rho GEF Trio to signal for cell-cell adhesion formation (Tu and You, 2014). Disrupting this Filamin scaffold inhibits junction formation and keratinocyte differentiation (Tu and You, 2014). This study provides an example of a protein classically known to organize F-actin that can also act as a scaffold for Rho GTPase signaling.

Spectrin & Adducin are actin-binding proteins that connect the actin cytoskeleton to the plasma membrane. While the cadherin/catenin complex connects to the actin cytoskeleton through α -catenin, this interaction is tension-sensitive: weak under low tension and strong under high tension (Buckley et al., 2014; Yamada et al., 2005). Therefore, particularly low-tension states may require additional membrane-actin crosslinkers to organize and position actin bundles at the AJC. Underlying the plasma membrane is a lattice of Spectrin, which can link the actin cytoskeleton to the plasma membrane through Adducin. Both Spectrin and Adducin are enriched at the AJC (Naydenov and Ivanov, 2011). Knockdown of Adducin results in Spectrin depletion and a reduction in F-actin cables at the AJC (Naydenov and

Ivanov, 2010), disrupting both early junction formation and destabilizing mature junctions, making the junctional Spectrin-Adducin-F-actin linkage an interesting target for future studies.

Future Perspectives

The linkage of TJs and AJs to the actomyosin cytoskeleton is important for junction formation, maturation, and maintenance as well as events that require junction remodeling or reinforcement. Here, we have discussed the central role that Rho GTPases play in regulating junctional actomyosin structure, dynamics, and contractility. Fluorescent biosensors for active Rho GTPases have been useful tools to identify junctional Rho GTPase activity zones. An important goal for the future will be to expand the repertoire of Rho GTPase biosensors available to include other Rho GTPases that may play important junctional roles in specific tissues or biological processes. Additionally, building on cell-scale studies in cultured epithelial cells, it will be important to study active Rho GTPase dynamics during morphogenetic processes at the tissue scale. For example, in *Xenopus laevis* embryos, in addition to baseline junctional RhoA activity zones, we have identified several conditions where increased localized, transient accumulations or “flares” of active RhoA are observed. We propose that junctional Rho flares may help allow the flexibility for epithelial cells to change shape during processes like cytokinesis and tissue morphogenesis by repairing local breaks in junctions that occur as cells change shape. Notably, increased epithelial permeability is commonly seen during inflammation. Inflammatory stimuli are known to promote changes in junctional actomyosin organization and contractility that lead to junctional disassembly and barrier function defects in various inflammatory conditions (Ivanov et al., 2010). Continued study of the mechanisms by which Rho GTPases and their regulators are involved may help reveal new therapeutic targets to attenuate defects in barrier function in these pathological conditions.

Since the biological output of Rho GTPase activity is strongly dependent on the GEFs, GAPs, GDI, and effectors, identifying Rho GTPase signaling modules that act in specific cellular functions is key. To that end, it may be useful to examine scaffold proteins that are known to act in other biological functions, but may play analogous roles at junctions. For example, there are striking similarities between the structure and protein players of the cytokinetic contractile ring and the apical actomyosin bundles at cell-cell junctions (Padmanabhan et al., 2015). However, there are also notable differences such as the highly organized structure of the microtubules of the mitotic spindle, which deliver key Rho GTPase regulators during cytokinesis, and the fact that junctional actomyosin can contract, expand, and provide steady state tension whereas as the cytokinetic contractile ring constricts. In any case, the proteins and mechanisms that regulate cytokinesis may provide a rich source of potential players at junctions.

Despite a long history of ultrastructural studies of cell-cell junctions, key unanswered questions remain about actin’s organization at junctions. For example, both Arp2/3 and formins clearly play roles at cell-cell junctions, but how they coordinate to form the junctional actin bundles observed at the ultrastructural level and generate actin structures required during junction remodeling events is unclear. Recent studies have shed light on how

Arp2/3-dependent branched actin can be converted to actin bundles. An important challenge will be to define which formins are involved in specific epithelial tissues and biological processes. To date, only a handful of the 15 vertebrate formins have been assessed at cell-cell junctions. Furthermore, how junctional and medial-apical actomyosin is organized and attached to junctions is debated. Whether actin makes end-on or side-on attachments at junctions could vary in different tissues depending upon expression of specific cytoplasmic plaque proteins that link the transmembrane proteins to actin, and could have important implications for mechanotransduction, junctional remodeling, and barrier and tissue integrity at these sites. Employing new superresolution imaging approaches in different epithelial tissues should help answer these outstanding questions.

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Figure 1. The apical junctional complex (AJC) establishes epithelial barrier function, cell adhesion, and integrity of epithelial tissues

A) A side view schematic of epithelial cells showing the two types of junctions in the AJC, the tight junction (TJ), which establishes the barrier function, and the adherens junction (AJ), which regulates cell-cell adhesion. In the enlarged view below, the architecture of core TJ and AJ proteins is shown (see legend identifying key proteins). Transmembrane proteins facilitate interaction between cells, and scaffolding proteins connect the transmembrane proteins to bundles of contractile F-actin and Myosin II. Note that in this view the bundles of actomyosin are oriented perpendicular to the plane of the cross-section. B) A top view schematic of epithelial cells. In the enlarged views below, one model for the organization of actomyosin is depicted with bundled antiparallel F-actin, crosslinked by α -actinin, and decorated with Myosin II motors.

Figure 2. Rho GTPase cycle, key effectors, and resulting actin organization

A) Typical Rho family GTPases cycle between an active, GTP-bound state and an inactive, GDP-bound state. GEFs activate GTPases by promoting the exchange of GDP for GTP, while GAPs inactivate GTPases by stimulating GTP hydrolysis. Rho GDI sequesters Rho-GDP in the cytoplasm, protecting it from degradation and preventing its activation. In the active conformation, Rho GTPases activate effector proteins leading to the biological output, which depending on the Rho GTPase involved, results in specific, localized effects on the cytoskeleton. B) RhoA-GTP signals through its effectors, formins and ROCK, to promote

the formation of actomyosin contractile arrays. C) Rac1-GTP and Cdc42-GTP signal through their effectors – WAVE and N-WASP, respectively – to promote Arp2/3-mediated branched actin structures. In some cases, Rac1 and Cdc42 can also trigger formin activity to promote unbranched actin polymerization.

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Figure 3. Possible models and key players in junction formation, maturation, and stability

A) Cells migrate towards each other in a Rac1- and Cdc42-dependent manner. Activity of these GTPases establishes an Arp2/3 branched F-actin network that pushes cell membranes together to establish cell-cell contacts. Cdc42-GTP also generates protrusive filopodia that are important for cell-cell contact formation (not shown). B) Junction elongation and early junction tension are promoted by Rac1 and Cdc42. The branched F-actin network is converted into a linear contractile actomyosin array through RhoA activation and by Coronin1B displacing Arp2/3 complexes, creating more flexible hinge points to linearize F-actin. C) Stable junctions are maintained through proper RhoA activity, which promotes contractile F-actin through stimulating F-actin polymerization via formins and Myosin II

activity via ROCK. A population of Arp2/3-dependent F-actin that is rapidly turning over also contributes to stable junctions (not shown).

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Figure 4. Scaffolding proteins dictate Rho GTPase signaling outcomes

A-C) Speculative models of how three scaffold proteins may facilitate proper Rho GTPase signaling output. In each case, the scaffold protein is shown in turquoise on the left, and key binding partners of the scaffold protein are listed. A) A speculative model of how Cingulin might regulate Rho signaling output. The head domain of Cingulin can bind F-actin and Myosin II, anchoring it to the cytoskeleton. Under certain conditions (e.g. as a result of phosphorylation or ZO-1 binding, shown here), Cingulin may reorient, giving GEFs (e.g. p114RhoGEF, shown here) and GAPs that are bound to Cingulin's rod domain access to

their target GTPases. B) p120 catenin is a hub for RhoA/Rac1 signaling. When bound to E-cadherin, p120 catenin prevents E-cadherin endocytosis, contributing to junction maturation and stability, and promotes RhoA inactivation by recruiting p190RhoGAP-B. In its cytoplasmic form, p120 catenin promotes Rac1 activation (and Cdc42 activation, not shown here) by recruiting the GEF Vav2. p120 also performs GDI-like RhoA inactivation by binding to RhoA-GDP, preventing its activation. C) Junctional Anillin may act to coordinate RhoA signaling in a tension-sensitive manner, as it does during cytokinesis. Under low or moderate tension, Anillin acts to scaffold active RhoA with formins and junctional actomyosin. We speculate that a tension-sensitive conformation of Anillin, may recruit p190RhoGAP-A to promote RhoA inactivation in order to fine-tune RhoA signaling and maintain balanced tension at the AJC.