

Regulating cell–cell junctions from A to Z

Jeff Hardin

Department of Zoology, University of Wisconsin-Madison, Madison, WI 53706

Epithelial sheets often present a “cobblestone” appearance, but the mechanisms underlying the dynamics of this arrangement are unclear. In this issue, Choi et al. (2016. *J. Cell Biol.* <http://dx.doi.org/10.1083/jcb.201506115>) show that afadin and ZO-1 regulate tension and maintain zonula adherens architecture in response to changes in contractility.

The textbook view of epithelial cells is that once such cells adopt a close, hexagonal packing, their “honeycomb” or “cobblestone” arrangement is static. This fixed appearance is misleading, as these cells are more like players in a rugby scrum, locked in a tussle in which the forces exerted by each of the players on the others maintains their seemingly static arrangement, but by a very dynamic force balance. How such balance is maintained in epithelia is a subject of substantial interest. A crucial role is played by F-actin and nonmuscle myosin II isoforms, which are deployed in contractile networks that transiently attach to cell–cell junctions to generate tensile forces along cell–cell boundaries (Lecuit and Yap, 2015). Contractile arrays of actomyosin are regulated by the monomeric G protein Rho, its upstream regulators, including Rho guanine nucleotide exchange factors (Quiros and Nusrat, 2014), and its effectors ROCK/Rho kinase and Shroom3 (Nishimura and Takeichi, 2008), but also by tension-mediated feedback between the myosin network and the junction (Lecuit and Yap, 2015). Cell–cell adhesion, including cadherin-dependent adhesion, also plays a crucial role in this process. As cells engage with one another via interactions of the extracellular domains of their cadherin complexes, they transduce forces to the actomyosin cytoskeleton through catenins. β -Catenin binds to the cytoplasmic domain of classical cadherins and recruits α -catenin, which binds F-actin.

Given the dynamic nature of epithelia, the attachment of contractile actomyosin networks to junctions are also subject to regulation. One aspect of epithelial architecture that has received relatively little attention is that a typical epithelial monolayer (Fig. 1 A) displays two main types of cell–cell interfaces: bilateral junctions (BCJs), in which two cells establish a relatively long stretch of contact, and cellular vertices, which represent a confluence of three or more cell edges to form tricellular junctions (TCJs) or multicellular junctions. TCJs are not well understood, but are known to contain unique molecular components (Furuse et al., 2014; Flores-Benitez and Knust, 2015). In this issue, Choi et al. show that the multivalent scaffolding proteins afadin and ZO-1/2 regulate the spacing of and tension along lateral contacts in cultured cells, thereby shedding light

on how contractile arrays containing bilateral and tri- or multicellular contact points are regulated in epithelia.

Afadin and ZO-1/2 are far from new players at junctions. Afadin binds α -catenin, actin, and other cytoskeletal and junctional proteins and associates with the transmembrane protein nectin, which appears to form an alternative adhesion system at adherens junctions (Mandai et al., 2013). The zonula occludens proteins ZO-1 and ZO-2 are tight junction proteins that bind claudins and are required for tight junction formation (Itoh et al., 1999; Balda and Matter, 2008). In addition, ZO proteins also bind to α -catenin (Itoh et al., 1997), are involved in establishing the zonula adherens (ZA; Ikenouchi et al., 2007), and potentiate cadherin-dependent adhesion in *Caenorhabditis elegans* (Lockwood et al., 2008) and *Drosophila melanogaster* (Choi et al., 2011). Knockdown of ZO-1 and ZO-2 (ZO KD) in MDCK cells has previously been shown (Fanning et al., 2012) to lead to dramatic alterations of the ZA: F-actin and myosin IIs assemble into striking apical arrays at the ZA, spaced at regular intervals. In addition, the normally sinuous boundaries between cells give way to very straight borders (Fig. 1 A).

Using superresolution microscopy, diffraction-limited junctional laser ablation, cell morphometry, kinetic analysis, and a whole-monolayer approach to contractility, Choi et al. (2016) now extend this story. To test whether contractility is increased after ZO KD, the authors first measured the recoil after laser ablation of ZO KD cells; an increase in recoil velocity indicated that the straight junctional boundaries between ZO-depleted cells are under tension. Imaging analysis of BCJs showed that the increase in contractility in ZO KD cells is associated with a strikingly dynamic behavior of the BCJs. Individual BCJs were found to undergo periods of shortening and elongation, whereas neighboring BCJs underwent compensatory, opposite changes in length. These changes in contractility have effects on the entire tissue sheet as well: whereas control cell sheets remained flat when detached from the substratum, ZO KD cells contracted into a cup-like shape. This constriction was blocked by the myosin inhibitor blebbistatin. Overall, these experiments indicated that ZO proteins regulate myosin assembly and contractility across the cellular sheet.

To dissect the protein network mediating increased contractility in ZO KD cells, Choi et al. (2016) examined the role of ROCK and found that ROCK inhibitors abolished the straight BCJs, which became curvilinear. Additionally, Shroom3, which is known to recruit ROCK (Nishimura and Takeichi, 2008), was cytoplasmic in control cells but junctional in ZO KD cells. Transient Shroom3 overexpression led to ROCK recruitment to the

Correspondence to Jeff Hardin: jhardin@wisc.edu

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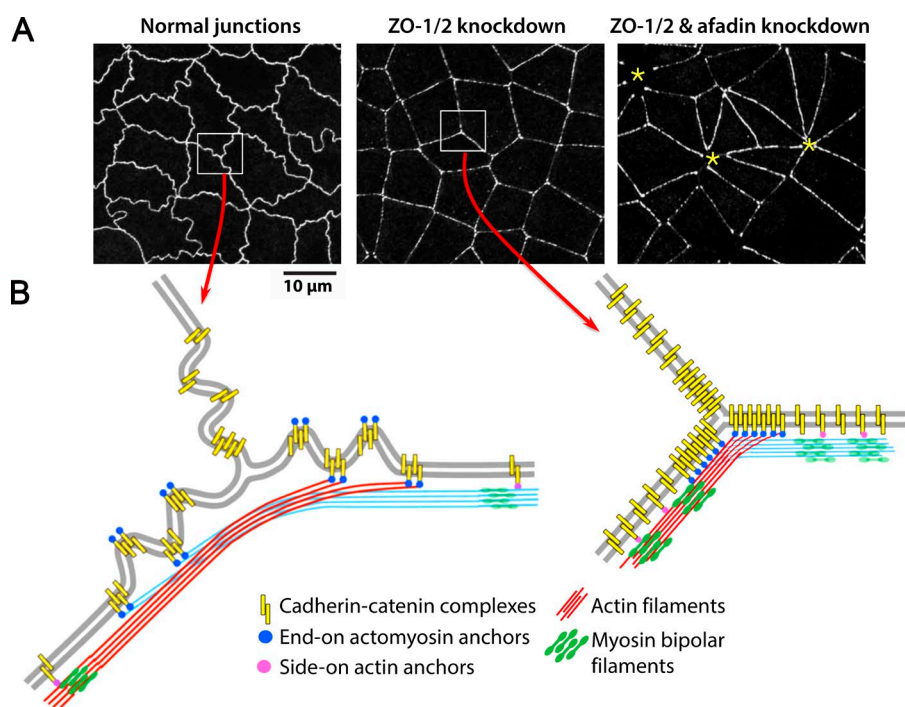


Figure 1. ZO proteins and afadin regulate junctional tension and organization in cultured cells. (A) Untreated MDCK cells have sinuous cell boundaries, whereas ZO KD cells show extremely straight boundaries. When ZO proteins and afadin are knocked down, cells adopt contact zones of irregular length with other cells, sometimes clustering into foci (asterisks). Images courtesy of Mark Peifer (Choi et al., 2016). (B) A model for actomyosin organization at adherens junctions (adapted from Choi et al., 2016). Contractile actomyosin arrays run parallel to bicellular junctions and are anchored by side-on attachments (pink circles). At TCJs, end-on binding of actin, likely stabilized by afadin, anchors actomyosin filaments. In ZO KD cells, contractile elements and cadherin complexes collapse toward TCJs, and myosin minifilaments adopt a regularly spaced arrangement.

ZA and drove formation of an actomyosin network similar to that in ZO KD cells. Conversely, Shroom3 knockdown resulted in loss of the actomyosin arrays in ZO KD cells. Collectively, these data indicated that Shroom3 is an effector of increased apical contractility in ZO KD cells.

The researchers used ZO KD cells to test how tissue integrity is maintained despite elevated contractility and how junctions are remodeled to maintain integrity when increased tension is present. Afadin is a good candidate: the *Drosophila* homologue of afadin, Canoe, plays roles in convergent extension and collective cell migration; in its absence, actomyosin networks at the apex of constricting epithelial cells in the embryo contract in a catastrophic, uncontrolled fashion (Sawyer et al., 2009), suggesting a potential role for afadin in the maintenance of tissue integrity during morphogenetic movements. Choi et al. (2016) therefore turned their attention to afadin. ZO KD cells have significantly more afadin at their adherens junctions and TCJs, a pattern reminiscent of the normal distribution of Canoe in *Drosophila* (Sawyer et al., 2009). Knocking down afadin by shRNA in ZO KD cells led to further defects in cell–cell boundary maintenance. In addition to the taut appearance of bicellular borders, cell boundary length became much more irregular, with occasional foci of highly contracted cells (Fig. 1 A). Velocimetry analysis and live-cell imaging indicated that loss of both ZO proteins and afadin led to large-scale cell movements within the monolayer not seen after ZO KD alone.

New imaging techniques used by Choi et al. (2016) revealed further details about the changes in actomyosin arrays in ZO KD cells. Superresolution imaging of myosin light chain kinase staining via structured illumination showed that myosin II assembles into arrays of myosin minifilaments spaced ~415 nm apart along bicellular contacts. Superresolution and transmission electron microscopy also revealed reorganization of F-actin and E-cadherin at TCJs in ZO KD cells. Lateral F-actin bundles appeared to terminate end-on at TCJs at sites where E-cadherin was present. ZO KD therefore induces assembly of a remarkably ordered actomyosin array along BCJs, and these

arrays appear to be separate contractile units that anchor end-on at the ZA. Moreover, based on staining for vinculin and a specific epitope in α E-catenin that serve as markers for regions under high tension (Yonemura et al., 2010), the end-on attachments of actin cables to the ZA at TCJs experience significant tensile stress. Strikingly, although vinculin and α E-catenin accumulation at TCJs was relatively uniform after ZO KD, their distribution was more heterogeneous after ZO/afadin KD. Differences in staining paralleled differences in cell border length and correlated with the level of tension measured at BCJs after laser cutting, suggesting that afadin contributes to the ability of cells to distribute forces at TCJ/multicellular junctions throughout the monolayer. Lastly, the researchers investigated whether internal cues downstream of ZO KD are sufficient for myosin recruitment or whether such recruitment depends on mechanical cues exerted by neighboring cells. They designed an assay mixing small islands of wild-type cells surrounded by ZO KD cells (or vice versa) and found that the development of the contractile array at the ZA depends on the contractility of neighboring cells; however, afadin recruitment to the ZA was less dependent on the sustained contractility of neighboring cells. Taking these data together, Choi et al. (2016) propose that cells respond to elevated contractility by increasing junctional afadin; because combined ZO/afadin knockdown dramatically alters cell shape and barrier function in response to elevated contractility, afadin acts as a robust scaffold that maintains ZA architecture most crucially at TCJs.

Although many aspects of the model proposed by Choi et al. (2016) remain to be tested, their data suggest new features regarding the detailed assembly of actomyosin contractile arrays in confluent cells (Fig. 1 B). In control cells, actomyosin arrays presumably extend parallel to individual BCJs. Choi et al. (2016) propose that these actomyosin bundles act as separate contractile units that terminate near TCJs, allowing the generation of tension along BCJs. In ZO KD cells, excessive assembly of actomyosin filaments, perhaps exacerbated by the tendency of F-actin/myosin minifilament arrays to self-assemble, somehow

leads to regularly spaced actomyosin arrays, and perhaps collapse of cadherin complexes and other components toward TCJs. There is a precedent for such lateral collapse of cadherin-dependent attachments: it is a prominent feature of cadherin complexes at sites of high tension in the epidermis of the *C. elegans* embryo (Choi et al., 2015). If the new model of Choi et al. (2016) is correct, then the foci seen in ZO KD/afadin KD cells may be similar to what happens in a game of tug of war when one team stops pulling. If some end-on attachments (assisted by afadin) fail, filaments might be expected to collapse along BCJs as the other, still tethered end of a set of filaments contracts toward the remaining attachment at the opposite cell vertex.

Several other interesting questions remain. First, what is the relationship of the striking, regularly spaced bipolar myosin II minifilaments that form in ZO KD cells to myosin arrays in normal cells? It is clear that untreated cells have junctional actomyosin networks, but not with this strict periodicity. One possibility is that this spacing is simply an epiphenomenon; when not appropriately anchored along junctions, actomyosin networks may self-organize as they are known to do in other systems, such as in the contractile ring and in migrating cells (Srivastava et al., 2015; Fenix et al., 2016). More optimistically, the spacing may represent an intensified version of processes that operate in normal cells at bicellular and multicellular contact sites. If so, components of the model of Choi et al. (2016) will require further investigation. For example, the organization of F-actin along BCJs remains unclear, as are the proteins that mediate the side-on binding envisioned in this model. It is also uncertain whether proteins assist bundling of filaments and what role dynamic growth and shrinkage of actin filaments plays in end-on binding. In some contexts, junctions are capable of seeding polymerization of F-actin (Briehner and Yap, 2013), and it may be that actin dynamics are important in the processes studied here.

A second question has to do with the community events within monolayers that Choi et al. (2016) describe. The neighbor effects on ZA morphology that they document are intriguing, as are the long-range accelerated movements of cells lacking both ZO proteins and afadin. Collective properties of monolayers are only beginning to be explored; connecting these properties with subcellular events is an exciting future challenge. Whatever the answers to these new questions, the work of Choi et al. (2016) refines our understanding of the roles of key scaffolding proteins in organizing and anchoring junctions in epithelia.

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