Tricellular junctions: how to build junctions at the TRICkiest points of epithelial cells

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ABSTRACT **Tricellular contacts are the places where three cells meet. In vertebrate epithelial cells, specialized structures called tricellular tight junctions (tTJs) and tricellular adherens junctions (tAJs) have been identified. tTJs are important for the maintenance of barrier function, and disruption of tTJ proteins contributes to familial deafness. tAJs have recently been attracting the attention of mechanobiologists because these sites are hot spots of epithelial tension. Although the molecular components, regulation, and function of tTJs and tAJs, as well as of invertebrate tricellular junctions, are beginning to be characterized, many questions remain. Here we broadly cover what is known about tricellular junctions, propose a new model for tension transmission at tAJs, and discuss key open questions.**

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INTRODUCTION

Epithelial sheets cover the surfaces of many organs in the bodies of multicellular organisms and function as a barrier by regulating translocation of fluids, solutes, and cells between compartments. Epithelial cells are polarized and form apical cell–cell junctions between adjacent cells, which are composed of tight junctions (TJs), adherens junctions (AJs), and desmosomes in vertebrates and AJs and septate junctions (SJs) in most invertebrates. In epithelial sheets, where cells are packed two dimensionally, there are many points where three cells meet (Figure 1, A and B). These points are called tricellular contacts. At tricellular contacts, cell–cell junctions take on specialized organizations generally referred to as tricellular junctions, including tricellular TJs (tTJs), tricellular AJs (tAJs), and tricellular SJs (tSJs). Here we discuss what is known about tricellular junctions and the mechanisms that regulate their formation and maintenance, the functional importance of tricellular junctions in development and disease, and unanswered questions.

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Abbreviations used: AJ, adherens junction; SJ, septate junction; tAJ, tricellular adherens junction; TER, transepithelial electrical resistance; TJ, tight junction; tSJ, tricellular septate junction; tTJ, tricellular tight junction.

TRICELLULAR TIGHT JUNCTIONS

Structure of tTJs

TJs, or zonula occludens (ZO), were originally observed by transmission electron microscopy (TEM) as apical structures in epithelial cells where the plasma membranes are tightly apposed, thus serving as a paracellular barrier (Farquhar and Palade, 1963; for review, see Krug *et al.*, 2014; Van Itallie and Anderson, 2014; Zihni *et al.*, 2016). By freeze-fracture replica electron microscopy, TJs appear as multiple sealing strands (Staehelin *et al.*, 1969), which are primarily composed of claudins (Furuse *et al.*, 1998). At tricellular contacts, the apical-most TJ strands from two neighboring cells meet at tricellular contacts and turn basally (Figure 1C). These TJ strands are closely attached, forming a structure called the central sealing element (Staehelin, 1973), which seals the space among the three cells. Other TJ strands from the two neighboring bicellular junctions make many connections with the central sealing element at roughly right angles (Friend and Gilula, 1972; Staehelin, 1973; Wade and Karnovsky, 1974; Walker *et al.*, 1985; Figure 1C). This specialized TJ structure at tricellular contacts, including the central sealing elements and connected TJ strands, is referred to as the tTJ (Ikenouchi *et al.*, 2005). Because the three paired strands of central sealing elements cannot eliminate the space among three cells, it has been speculated that there is a narrow channel, called the central tube (Staehelin, 1973; Walker *et al.*, 1985; Ikenouchi *et al.*, 2005).

Molecular components of tTJs: tricellulin and angulins

Two protein components are known to specifically localize to vertebrate tTJs—tricellulin and angulins (Figure 1D). Tricellulin was the first molecular component of tTJs to be identified (Ikenouchi *et al.*, 2005). Tricellulin (also known as marveld2), occludin, and marveld3 form the TJ-associated MARVEL protein (TAMP) family, which is conserved

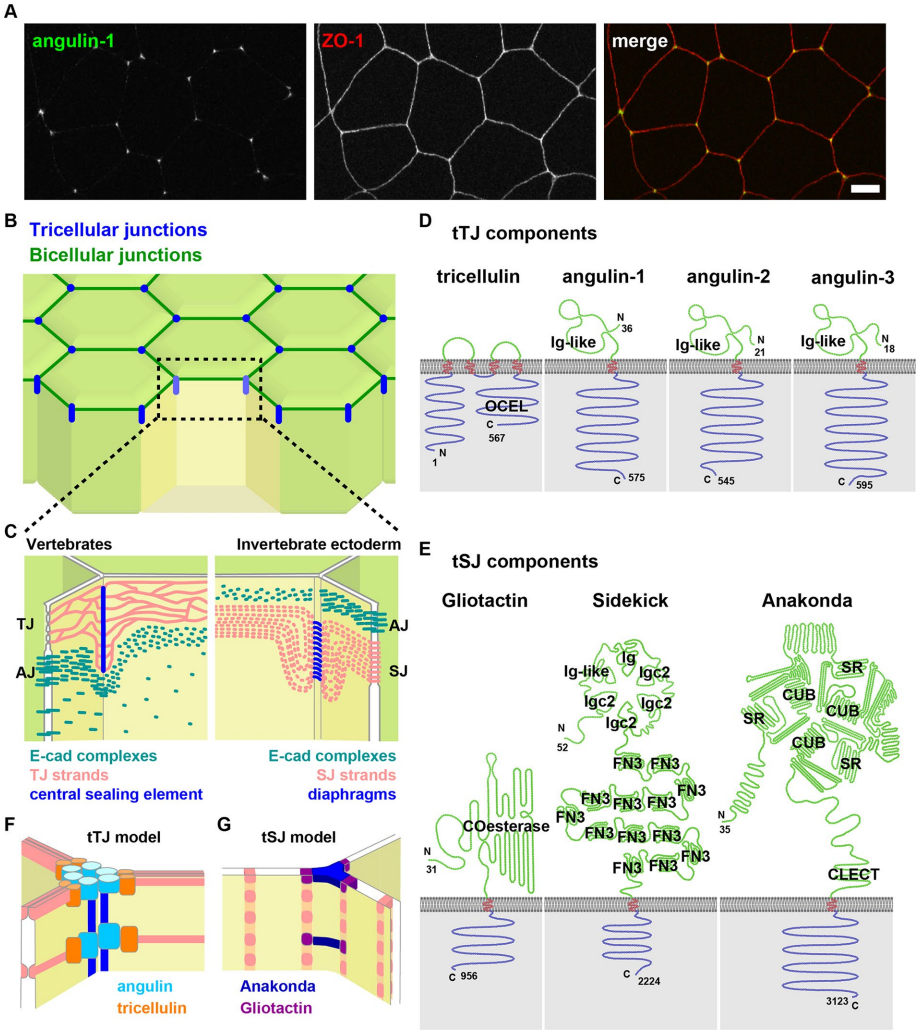

FIGURE 1: Tricellular tight junctions and tricellular septate junctions. (A) Immunofluorescence staining of *X. laevis* gastrula-stage embryo using anti–angulin-1 (tTJ marker; green) and anti–ZO-1 (bicellular TJ marker; red). Bar, 10 μm. (B) Epithelial organization. Tricellular junctions (blue) and bicellular cell–cell junctions (dark green). (C) Structure of tricellular junctions. Vertebrate epithelium (left) has TJs, AJs, and desmosomes (not depicted here). TJ strands (pink) turn in the basolateral direction and make connections to central sealing elements (blue). AJs, consisting of E-cadherin complexes (green), are deepened at the tricellular region. Invertebrate ectoderm epithelium (right) has AJs and SJs. SJ strands (pink) run parallel to the *Z*-axis at the tricellular region and make lateral connections to the diaphragms (blue). (D) Molecular components of tTJs. Top, extracellular; bottom, intracellular. OCEL, occludin ELL-like domain; Ig-like, Ig-like domain. (E) Molecular components of tSJs. COesterase, carboxylesterase domain; Igc2, Ig domain C2-set type; Ig-like, Ig-like domain; Ig, Ig domain; FN3, fibronectin type III domain; SR, scavenger receptor cysteine-rich domain; CUB, complement C1r/C1s/Uegf/Bmp1 domain; CLECT, C-type lectin domain. (F) Model of tTJ organization. Angulins (cyan) help make central sealing elements (blue) and recruit tricellulin (orange)-associated TJ strands (pink). (G) Model of tSJ organization. Anakonda (dark blue) makes diaphragms and recruits Gliotactin (purple)-associated SJ strands (pink).

among vertebrates (Steed *et al.*, 2009; Raleigh *et al.*, 2010). Tricellulin is localized strongly at tTJs and very weakly at bicellular junctions in almost all types of epithelial cells (Ikenouchi *et al.*, 2005). In immunoreplica electron microscopy, tricellulin signal was detected along the central sealing element (Ikenouchi *et al.*, 2005). Tricellulin has four transmembrane domains and large N- and C-terminal cytoplasmic tails. In spite of sequence homology with occludin, the extracellular region of tricellulin does not support homophilic trans-interaction between tricellulin-transfected HEK293 cells (Cording *et al.*, 2013). The latter half of the C-terminal tail is highly homologous to the occludin ELL domain, which binds to ZO-1 (Riazuddin *et al.*, 2006), a TJ plaque protein. Tricellulin knockdown causes altered TJ morphology and decreased transepithelial electrical resistance (TER) in the mammary epithelial cell line EpH4 (Ikenouchi *et al.*, 2005), indicating that tricellulin is required for the maintenance of morphology and barrier function of TJs. In addition, removal of tricellulin disrupts the characteristic structure of TJ strands connected to the central sealing element at tTJs in the inner ear (Nayak *et al.*, 2013), suggesting that tricellulin is required for the formation of proper tTJ structure.

Named for the Latin word *angulus*, meaning corner, the angulins are the other proteins known to localize at vertebrate tTJs. The angulin family proteins, composed of angulin-1 (also known as lipolysis-stimulated lipoprotein receptor [LSR]), angulin-2 (also known as immunoglobulin [Ig]-like domain–containing receptor [ILDR] 1), and angulin-3 (also known as ILDR2, LISCH-like, or C1orf32), are single-pass transmembrane proteins (Masuda *et al.*, 2011; Higashi *et al.*, 2013). Angulin family proteins have an extracellular Ig-like domain, which in other proteins, such as junctional adhesion molecule (JAM), is known to mediate cell recognition and adhesion, although the adhesion activity of this domain has not been directly tested in angulins. The angulins are differentially expressed in various epithelial tissues (Higashi *et al.*, 2013). All angulin proteins have multiple splice isoforms (Higashi *et al.*, 2013; Reaves *et al.*, 2017), and at least one isoform of each angulin is localized at tricellular junctions and weakly at bicellular junctions in each epithelial tissue examined (Masuda *et al.*, 2011; Higashi *et al.*, 2013). Angulin-1 is detected at central sealing elements of tTJs by immunoreplica electron microscopy (Masuda *et al.*, 2011). EpH4 cells express primarily angulin-1 (vs. the other angulins), and angulin-1–knockdown EpH4 cells exhibit reduced TER and increased macromolecule flux, indicating that angulin-1 is required for maintenance of barrier function (Masuda *et al.*, 2011; Higashi *et al.*, 2013). Expression of angulin-2 in angulin-1–knockdown EpH4 cells rescued the TER and macromolecule flux phenotypes, suggesting that angulin-2 has barrier-supporting properties similar to angulin-1. In contrast, angulin-3 expression in the angulin-1–knockdown cells resulted in limited restoration of barrier function (Higashi *et al.*, 2013).

Angulins recruit tricellulin to tTJs

In angulin-1–knockdown cells, tricellulin does not show specific localization at tTJs and is instead uniformly localized at the bicellular membrane (Masuda *et al.*, 2011). Conversely, angulin-1 is still clearly

localized at tTJs in tricellulin-knockdown cells (Masuda *et al.*, 2011). Similarly, in angulin binding–deficient tricellulin–knock-in mice, angulin-2 is still localized at tricellular contacts of hair cells in the inner ear, whereas mutant tricellulin is absent from tTJs (Nayak *et al.*, 2013). Based on these results, it was proposed that angulins establish primitive tTJs at tricellular contacts and recruit tricellulin to help these structures mature. Although conserved regions in the cytoplasmic tail of angulins and the C-terminal cytoplasmic tail of tricellulin are required for the recruitment of tricellulin (Masuda *et al.*, 2011; Higashi *et al.*, 2013), whether the interaction between angulins and tricellulin is direct or indirect is unclear.

Model of tTJ architecture

Neither superresolution nor electron microscopy data are available to distinguish the localization of angulins and tricellulin at tTJs. However, it is possible to speculate about the molecular organization of tTJs, and a model of tTJ architecture was proposed in which angulins comprise the central sealing element of tTJs to form landmarks for nascent tTJs and then recruit tricellulin together with claudin-based TJ strands (Masuda *et al.*, 2011; Figure 1F). This model is supported by the following experimental data: 1) angulins can form a molecular complex without tricellulin (Masuda *et al.*, 2011), 2) angulins can interact with tricellulin (Masuda *et al.*, 2011; Higashi *et al.*, 2013), and 3) freeze-fracture electron microscopy of claudin/tricellulin double-transfected HEK293 cells shows that tricellulin can interact with claudin-based TJ strands and alter their morphology from a rounded shape to more-rectangular meshes (Ikenouchi *et al.*, 2008; Cording *et al.*, 2013). Furthermore, TJ strands in these modified HEK293 cells intersect at roughly right angles in a way that closely resembles the structure of tTJs observed in freeze-fracture replica electron microscopy where TJ strands make end-on connections to central sealing elements (Ikenouchi *et al.*, 2008; Cording *et al.*, 2013). Recently it was found, using superresolution microscopy, that another TAMP family protein, occludin, is concentrated at the ends and intersections of TJ strands (Van Itallie *et al.*, 2017), which suggests that the TAMP family member tricellulin may also be integrated at the intersections of TJ strands, particularly those TJ strands intersecting with the central sealing element.

tTJ biogenesis

To maintain epithelial order, epithelial cells must be continuously renewed (Ragkousi and Gibson, 2014). Old cells undergo apoptosis and are extruded from the epithelial sheet (Eisenhoffer and Rosenblatt, 2013), and new cells are added by cell division. In some situations, specific cell types need to be inserted into or removed from the epithelial sheet (Sedzinski *et al.*, 2016). In addition, cell intercalation within the epithelial plane is important for many developmental processes, such as convergent extension (Walck-Shannon and Hardin, 2014). In all of these processes, cell–cell junctions—including tTJs—are reorganized, while at the same time, the epithelial barrier must be maintained. Recent studies are beginning to shed light on tTJ biogenesis and remodeling during these dynamic processes.

Recently we reported how tTJs are formed during epithelial cytokinesis in *Xenopus* embryos (Higashi *et al.*, 2016). In the *Xenopus* gastrula-stage epithelium, most daughter cells are separated by neighboring cells after cytokinesis, and each daughter cell makes a new tricellular contact with two neighboring cells (Figure 2A). This is in clear contrast with the *Drosophila* epithelium, where daughter cells maintain contact after cytokinesis (Gibson *et al.*, 2006; Founounou *et al.*, 2013; Herszterg *et al.*, 2013; Guillot and Lecuit, 2013a). In the *Xenopus* epithelium, at the two newly formed tricellular contacts, angulin and then tricellulin are recruited to establish mature tTJs (Figure 2A). In almost half of the divisions observed, the two nascent tricellular junctions merge and then redistribute over the course of 1 h to form two tTJs among two daughter cells and one neighboring cell (Figure 2A).

Another example of tTJ biogenesis occurs in the cornified stratified epithelia of the mammalian epidermis, where cells proliferate in the basal layer and then differentiate and move up to the surface to sequentially form three distinct layers of the stratified epithelia, including the stratum granulosum. Of interest, TJs and tTJs are formed only between cells in the second cell layer of the stratum granulosum, where they serve as a barrier (Furuse *et al.*, 2002; Kubo *et al.*, 2009). A recent study beautifully described how TJs and tTJs are maintained during continuous turnover of cells in the second layer of the stratum granulosum (Yokouchi *et al.*, 2016; Figure 2B). When an epidermal cell sporadically translocates from a lower layer to the second layer of the stratum granulosum, the preexisting upper cell forms additional TJs at its basal side to make a double-edged polygon, and the preexisting TJs at the apical side disappear over time. By transiently making TJs at both the apical and basal sides of cells, this specialized epithelium can maintain barrier function during the continuous turnover of cells. Of note, angulin-1 and tricellulin localize all along the newly forming cell–cell junctions at the basal side, whereas they localize specifically at tricellular vertices at the apical side. As the preexisting TJs disappear at the apical side, both angulin-1 and tricellulin become focused at tricellular contacts in the newly formed junctions. Yokouchi *et al.* (2016) speculated that newly formed basal junctions are "tricellular junctions" among the preexisting cell, the translocating cell, and surrounding cells, which is consistent with the their cell-packing model, in which the epidermal cells have a flattened tetradecahedron shape (polygon with 14 faces; also known as Kelvin's tetrakaidecahedron).

A third example of tTJ biogenesis is associated with the penetration of keratinocyte TJs by the dendrites of Langerhans cells—major immune cells responsible for uptake and presentation of antigens in the skin (Kubo *et al.*, 2009). When activated, Langerhans cells extend their dendrites between keratinocytes, and the tips of dendrites penetrate keratinocyte TJs to take up antigens from the extra-TJ environment above the TJs. Intriguingly, when dendrites are inserted between keratinocytes, tricellulin is recruited to the newly formed tricellular contacts among the dendrites and keratinocytes. Similar transepithelial projections from basal cells were observed in the epididymides (Shum *et al.*, 2008). Furthermore, cell insertion into epithelial sheets is observed in many developmental and regenerative processes, including emergence of multiciliated cells in the *Xenopus laevis* epidermis (Sedzinski *et al.*, 2016) and insertion of olfactory neurons in the olfactory epithelium (Leung *et al.*, 2007; Higashi *et al.*, 2013). Future studies are required to further investigate how new tTJs are established when cells or cell processes penetrate TJs.

Signaling at tTJs

Given the unique structure and position of tTJs in the cell, one would predict that specific signaling pathways might regulate tTJ formation and maintenance and that specific signals might be relayed from tTJs to other parts of the cell (i.e., to other junctions or the nucleus). However, very limited information is available about signaling related to tTJs.

Tricellulin and angulins may be regulated by phosphorylation. In Western blots of epithelial cell lysates, tricellulin is detected as multiple bands, which are phosphorylation dependent (Ikenouchi *et al.*, 2005). Because the function of occludin, another TAMP family protein, is regulated by casein kinase 2–dependent phosphorylation

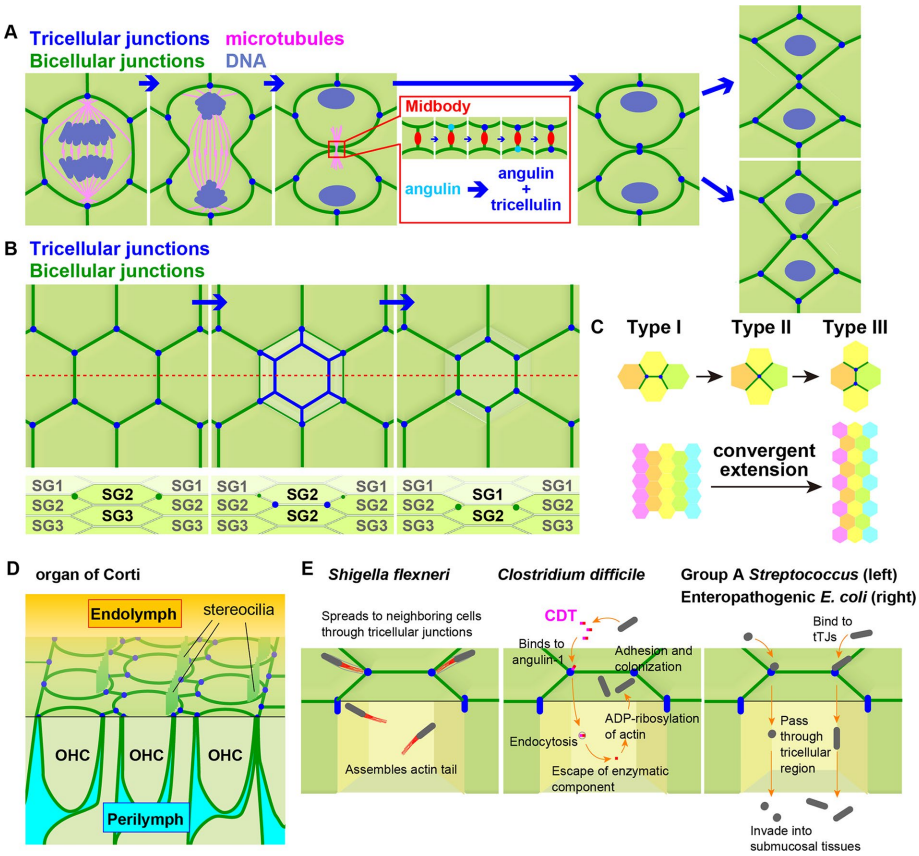

FIGURE 2: New tricellular junction formation and functional importance of tricellular junctions. (A) Nascent tTJ formation after cytokinesis. When the cleavage furrow ingresses and the bicellular cell–cell junctions (green) from each side of the furrow meet, a new cell–cell interface between neighboring cells and two new vertices are formed. Angulin-1 (cyan) and then tricellulin are recruited to the newly formed vertices and build mature tTJs (blue). Formation of one tTJ is soon followed by the other. After cytokinesis, new tTJs either separate as the bicellular junctions between them elongate (top) or fuse and reorganize to make tTJs among a different combination of the cells (bottom). (B) tTJ formation during turnover of epidermal cells. TJs exist only at the second layer of stratum granulosum (SG2). The red dotted line in the top, en face view indicates the position of the cross-section view (bottom). The TJ-bearing cells at SG2 turn over sporadically. When a cell at SG2 is going lose its apical cell–cell junctions, new cell–cell junctions are formed at the basal side of the cell. These new cell–cell junctions are formed among three cells, including a neighboring cell in the SG2 layer and an underlying cell in the SG3 layer, and the new junctions are enriched with tTJ components such as angulin-1 and tricellulin. As the upper cell–cell junctions disappear, tTJ components of the new, lower cell–cell junctions gradually become focused at cell vertices. (C) Cell intercalation during convergent extension. In type I/II/III transition, shortening of cell–cell junctions perpendicular to the tissue elongation axis results in fusion of two tricellular junctions and formation of a four-way junction. Then the four-way junction becomes two tricellular junctions along the elongation axis. In some cases, instead of four-way junctions, more than four cells make a multiway junction (also called a rosette; not depicted here). (D) tTJs (blue) are important for barrier function of the sensory epithelium and viability of hair cells in the inner ear. OHC, outer hair cells. (E) Tricellular junctions are involved in pathogenesis of versatile species of bacteria. *S. flexneri* spreads to neighboring cells via the tricellular junctions by utilizing host actin and penetrating through the corners of infected cells. *C. difficile* secretes the binary toxin CDT, which binds to angulin-1, a tTJ component. CDT is then incorporated into the cell by endocytosis and modulates actin and microtubules, which induces cell protrusions at the cell surface and contributes to adherence and colonization of the bacteria. Group A *Streptococcus* and enteropathogenic *E. coli* preferentially bind to tricellular junctions and invade into submucosal tissues through tricellular junctions.

(Raleigh *et al.*, 2011), it is plausible that phosphorylation of tricellulin also modulates its function. The kinase responsible for tricellulin phosphorylation has not been identified, although c-Jun N-terminal kinase (JNK) is implicated in the regulation of tricellulin expression (Kojima *et al.*, 2010). Angulin-1 is also observed as multiple bands in Western blots of epithelial cell lysates (Masuda *et al.*, 2011), which is apparently due to phosphorylation or might be caused by additional posttranslational modifications. It has been shown that JNK1-dependent phosphorylation of angulin-1 at Ser 288 is important for exclusive localization of angulin-1 at tTJs (Nakatsu *et al.*, 2014).

tTJs may be localized sites of Rho family GTPase signaling. The Cdc42 GEF Tuba is reported to bind the N-terminal cytoplasmic tail of tricellulin (Oda *et al.*, 2014). Tuba is localized at bicellular junctions through its binding to the TJ plaque protein ZO-1, and Tuba regulates junctional curvature of epithelial cells by activating Cdc42 and its effector N-WASP (Otani *et al.*, 2006). It is not known whether Tuba localizes to tTJs in epithelial cells and regulates Cdc42 activation at these sites. Given that the effect of tricellulin knockdown on epithelial configuration was limited to the phase of cell–cell junction formation (Oda *et al.*, 2014) and that tricellulin-knockout mice do not exhibit global epithelial defects (Kamitani *et al.*, 2015), the contribution of tricellulin to Tuba- and Cdc42-dependent regulation of epithelial order remains elusive.

TRICELLULAR ADHERENS JUNCTIONS

Structure and molecular composition of tAJs

The structure and core molecular components of AJs have been identified; however, the composition and organization of tAJs is still emerging. The zonula adherens was originally characterized by transmission electron microscopy as a cell–cell junction structure located just basal to the TJ with a characteristic ~20 nm intercellular space between parallel cell membranes filled with low-density material and accompanied by high-density material in the cytoplasm adjacent to the junctions (Farquhar and Palade, 1963). Later it was appreciated that AJs can be divided into two categories based on their morphology: apical belt-like AJs of the zonula adherens and spot-like AJs (also called punctum adherens; Yonemura, 2011b). The key molecular components of both types of AJs are the cadherin/catenin complex, composed of the transmembrane protein E-cadherin and cytoplasmic catenins. Cadherin/catenin complexes are directly or indirectly linked with the actin cytoskeleton, which was visible in the original electron microscopy images as the *high-density* cytoplasmic material associated with the junctions. β-Catenin binds to the cytoplasmic tail of E-cadherin and recruits α-catenin, which binds to F-actin. α-Catenin forms a stable interaction with F-actin only under

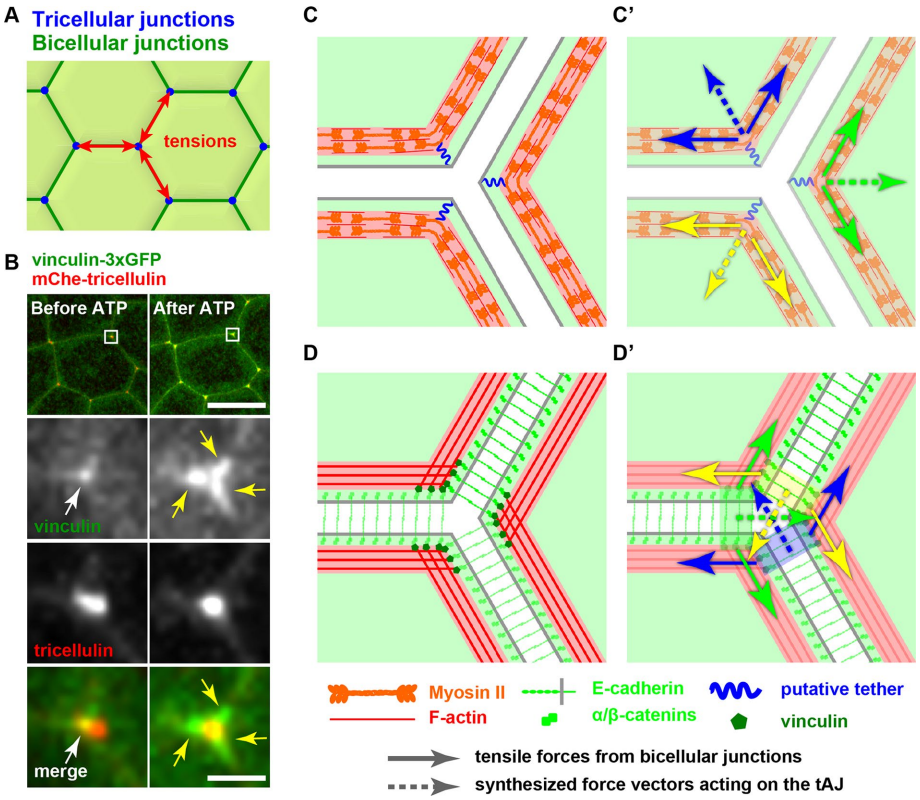

FIGURE 3: Tension transmission at tAJs. (A) Line tension (red arrows) is applied on the tricellular junctions where three cells meet. (B) Vinculin-3xGFP is localized strongly at tAJs and weakly at bicellular AJs. At 30 min after addition of 50 μM ATP to increase tension, vinculin signal is separated into three spots, suggesting that line tension along cell–cell junctions is transmitted at bicellular AJs adjacent to tAJs. Scale bar, 20 μm (top), 2 μm (bottom). (C) Previously proposed myosin II organization model at tAJs (Ebrahim *et al.*, 2013). Myosin II and F-actin make a quasisarcomeric structure along bicellular cell–cell junctions, and at tAJs, the rod region of a myosin II filament is tethered to the membrane by an unknown molecule, which makes myosin II filaments bend to fit the curvature of tricellular vertices of the cells. Tensile forces (arrows) from bicellular junctions are synthesized (dotted arrows) and applied on the putative tether, generating a "separation force" (C'). (D) Tension transmission model at tAJs based on TEM images from Yonemura (2011a) and Choi *et al.* (2016). Actomyosin bundles make end-on connections with cadherin–catenin complexes at tAJs, and the force vectors applied on trans-E-cadherin complexes from two adjacent cells (arrows) are synthesized toward the center of the vertex (dotted arrows), generating a "tightening force," which could help to tighten tricellular junctions (D').

actomyosin-generated tension (Buckley *et al.*, 2014). Of interest, the orientation of the F-actin linkage is different at the belt-like AJs, where actin is aligned parallel to the cell–cell junctions versus the spot-like AJs, where actin makes end-on connections with cell–cell junctions (Yonemura, 2011b). Recent studies using superresolution microscopy showed that the belt-like AJ is composed of clusters of spot-like AJs (Wu *et al.*, 2015). Furthermore, F-actin was implicated in corralling the spot-like E-cadherin clusters (Wu *et al.*, 2015), and actomyosin drives their coalescence and stabilization to form the apical belt-like AJ (Ratheesh and Yap, 2012).

In a review article, Yonemura (2011a) was the first (to our knowledge) to introduce the term tAJ. Many questions remain about the structure and molecular composition of tAJs. The cadherin/catenin complex is present at tAJs, and its localization often appears to be deeper (localized more basally) compared with its localization at belt-like AJs of bicellular junctions. However, the nature of E-cadherin interactions, the cytoplasmic scaffold proteins involved, and the orientation of the actin linkages at tAJs have not been well defined. In addition, we do not know whether there are specific molecular components that are localized strongly to tAJs, as is the case for tTJs. Finally, it has not been determined whether interactions between tTJ and tAJ components are important for proper organization of tricellular contacts.

tAJs are tension hot spots

AJs sense and transmit force generated by actomyosin from one epithelial cell to another. This ability to detect and respond to mechanical stimuli from their neighbors is essential for epithelial cells to maintain and remodel adhesions during cell shape change events such as cell division, cell extrusion, apical constriction, and intercalation. When the actomyosin-mediated tugging force between the two cells is increased, the area of cadherin-mediated adhesion between two cells increases (Liu *et al.*, 2010). Similarly, in focal adhesions, increased mechanical tugging force results in focal adhesion growth. Mechanotransduction—the process by which mechanical forces are converted into biochemical signals—has been well documented for focal adhesions (e.g., force-dependent recruitment of adaptor proteins, kinases, and actin to focal adhesions; Bershadsky *et al.*, 2003). Emerging evidence indicates that AJs can also serve as sites of mechanotransduction. Recent work shows that AJs become reinforced under increased force, and α-catenin plays a central role in mechanotransduction at AJs. α-Catenin senses increased force and responds by changing conformation, revealing a binding site for vinculin (le Duc *et al.*, 2010; Yonemura *et al.*, 2010). Vinculin is also an actin-binding protein; therefore, recruitment of vinculin promotes an increase in junctional F-actin, resulting in strengthening of the AJ (Yonemura, 2011b). In addition, vinculin can recruit Ena/VASP proteins, leading to new linear actin polymerization in response to increased junctional tension (Leerberg *et al.*, 2014).

tAJs are potentially important sites for mechanotransduction. Mathematical modeling of cells in epithelial tissues using a vertex model approach in which cells are represented in edges and vertices (Trichas *et al.*, 2012) suggests that tricellular contacts are tension hot spots where increased force may be applied to junctional proteins. Vertices experience high tension due to outward forces generated by actomyosin-dependent line tension acting along each of the cell edges connected to a vertex (Figure 3A). Both in silico and in vivo data show that cell edges and vertices are highly dynamic, as cell rearrangements take place during cell division and morphogenesis, causing changes in actomyosin-dependent line tension along the cell edges (Farhadifar *et al.*, 2007; Rauzi *et al.*, 2008) and putting additional stress on the vertices. Recent experimental data support the conclusion that tAJs are tension hot spots and show that in response to this increased tension, specific proteins are recruited to tAJs. First, vinculin accumulates strongly at tricellular junctions in

comparison to bicellular junctions (Higashi *et al.*, 2016). Fluorescently tagged vinculin is recruited to AJs in response to increased tension and can be used to approximate junctional tension in vivo (Hara *et al.*, 2016; Higashi *et al.*, 2016). When junctional tension is acutely increased globally, there is a significant increase in recruitment of vinculin–triple green fluorescent protein (3xGFP) or the tension-reporting anti–α-catenin α18 antibody to tricellular and bicellular junctions, but the signal is particularly elevated at tricellular junctions (Yonemura *et al.*, 2010; Higashi *et al.*, 2016). Of interest, under increased tension, the tricellular vinculin signal is very intense and is observed as three spots (Figure 3B), which may indicate a mechanosensitive response to strengthen the connection to F-actin specifically at tAJs, as we discuss later.

Second, myosin II may be enriched at tAJs in a tension-sensitive manner. In the *Xenopus* embryo epithelium, signal for phospho–myosin II light chain is weakly localized along bicellular junctions and intensely localized at tricellular junctions (Reyes *et al.*, 2014). When junctional tension is reduced by knocking down the actomyosin scaffolding protein anillin, junctional phospho–myosin II signal is reduced and no longer enriched at tricellular junctions (Reyes *et al.*, 2014). In addition, Ebrahim *et al.* (2013) reported periodic assemblies of bipolar myosin II and actin forming a sarcomere-like belt around each epithelial cell; intriguingly, myosin II was localized at the corner of each cell. Furthermore, the fluorescence intensity of myosin II was higher at those sites (Ebrahim *et al.*, 2013). It has been demonstrated that increased tension recruits and stabilizes myosin II along bicellular junctions in intercalating cells of the *Drosophila* embryo (Fernandez-Gonzalez *et al.*, 2009), which raises the possibility that tricellular recruitment of myosin II could be tension dependent as well.

Third, Canoe/afadin, a scaffolding protein that cross-links AJs and the actin cytoskeleton, appears to play an important role in strengthening the AJ–actin link at tAJs. In *Drosophila*, Canoe is essential for linking the actin cytoskeleton to AJs during the mechanical stresses involved in morphogenetic movements during development (Choi *et al.*, 2011; Sawyer *et al.*, 2011). In addition, Choi *et al.* (2016) showed that in ZO-1/ZO-2 double-knockdown Madin–Darby canine kidney cells, which exhibit elevated junctional tension via a RhoA/Shroom3/ROCK pathway, cells respond to the increased junctional tension by recruiting additional afadin to both bicellular and tricellular junctions. Furthermore, they showed that afadin is necessary for maintaining tissue integrity and barrier function under high tension and is particularly important for maintaining adhesion and actomyosin architecture at tAJs (Choi *et al.*, 2016). Afadin is also known to mediate interactions between AJs and TJs, which could be important at tricellular junctions (Ooshio *et al.*, 2010).

Model for tension transmission at tAJ

Recently, conflicting models representing how actin is organized at the tAJ have been proposed. One model, proposed by Ebrahim *et al.* (2013), is based on their data observing the organization of myosin II, which was double tagged so that they could visualize the head and tail of myosin II, in explant cultures of organ of Corti from P2 mice. Strikingly, they found that myosin II and actin were organized in a periodic sarcomere-like belt around each epithelial cell. Further, they reported that myosin II appeared to be physically linked to the tAJ, and, due to this physical interaction, the myosin II filaments were bent inward at the cell corners (Figure 3C). The protein responsible for this potential linkage of myosin II to the tAJ was not identified. An alternate explanation consistent with the data is that two overlapping orientations of myosin II make up the myosin II accumulation observed at cell corners. This model will require additional superresolution microscopy and/or electron microscopy (EM) to confirm the orientation of F-actin and myosin II. A second group of models is based on EM images of F-actin orientation at tAJs (Gomez *et al.*, 2011; Yonemura, 2011a; Choi *et al.*, 2016). Yonemura (2011a) showed, by EM of MTD-1A cultured epithelial cells, that F-actin is connected to tAJs in a perpendicular end-on manner. Choi *et al.* (2016) showed similar organization of F-actin at tAJs in MDCK cells exhibiting increased tension due to ZO knockdown. They proposed that each cell edge acts as an independent contractile unit, with F-actin bundles anchored end-on to cadherin/catenin complexes at tAJs (Figure 3D). Therefore, building on these models, we propose that actomyosin bundles make end-on connections with cadherin–catenin complexes at tAJs. Under increased tensile force along cell–cell junctions, it is predicted that "separation forces," that is, forces that promote separation of cells at the tricellular junctions, are applied (Figure 3C′; Jarvis, 1998). This could lead to disengagement of tricellular junctions and opening of the central tube. However, because additional proteins, including vinculin, are recruited to strengthen the connection to the tAJ under high tension and E-cadherins make extracellular transdimers, the force vectors applied on trans–E-cadherin complexes from two adjacent cells can be synthesized and generate a "tightening force" toward the center of the vertex, which could help maintain tricellular junctions in dynamic epithelial tissues (Figure 3D′).

TRICELLULAR JUNCTIONS IN INVERTEBRATES

Most invertebrates have SJs, which are located basal to AJs and are made up of multiple belt-like strands of septa spanning the intercellular space (Izumi and Furuse, 2014). SJs are regarded as functionally analogous to TJs and form a paracellular permeability barrier, although the protein components of SJs are distinct from those of TJs. At invertebrate tricellular contacts, the intercellular space is connected by regularly spaced, vertically stacked triangular structures called diaphragms (Figure 1, C and G). Each bicellular SJ strand turns either apically or basally near tricellular junctions and makes a loop to become parallel to the axis of vertical tricellular junctions, forming the limiting septa (also called limiting strands). The sides of limiting septa are connected to the diaphragms (Graf *et al.*, 1982; Noirot-Timothee *et al.*, 1982; Figure 1, C and G).

Three protein components of tricellular septate junctions (tSJs) have been identified (Figure 1E). First, Gliotactin, a cholinesterase-like transmembrane protein localized exclusively at tSJs, is required for establishment of proper SJ structure and function (Schulte *et al.*, 2003). Of interest, Gliotactin expression is lost from the midgut of aged flies, which exhibit aging-associated phenotypes, including increased intestinal stem cell proliferation and blocked terminal differentiation of enterocytes (Resnik-Docampo *et al.*, 2017). Loss-of-function analysis showed that Gliotactin maintains barrier function of intestinal cells and contributes to maintaining stem cell homeostasis in a non–cell-autonomous manner (Resnik-Docampo *et al.*, 2017). Second, Sidekick, a transmembrane protein with Ig domains, is also localized at tricellular contacts (Lye *et al.*, 2014) and is required for pattern formation in the eye (Nguyen *et al.*, 1997). The role of Sidekick in SJ assembly and barrier function has not been determined. Third, Anakonda (also known as Bark beetle), a transmembrane protein with a unique tripartite extracellular domain and a C-terminal PDZ-binding motif, is exclusively localized at tSJs and is required for proper assembly and function of tSJs (Byri *et al.*, 2015). In addition, Anakonda is required for the localization of Gliotactin at tSJs. It has been suggested that Gliotactin is integrated with the limiting septa on each side of the tSJ (Schulte *et al.*, 2003) and that the large tripartite

extracellular domain of Anakonda self-organizes in the triangular extracellular space and helps make the diaphragms there (Byri *et al.*, 2015; Figure 1G). Gliotactin, Sidekick, and Anakonda have no sequence similarity to tricellulin or angulins, suggesting that the molecular mechanisms regulating the assembly of the tSJ and tTJ may be distinct.

FUNCTIONAL IMPORTANCE OF TRICELLULAR JUNCTIONS

Failure in tTJs causes nonsyndromic familial deafness

Mutations in TJ genes—including tTJ genes—can cause deafness. The organ of Corti, located in the cochlea of the inner ear, is a specialized epithelial tissue (Figure 2D). Mechanosensory hair cells are embedded in the epithelial cells of the organ of Corti and are bathed in endolymph (high electric potential and unique ion composition: high potassium and low sodium) at the apical side and in perilymph (normal ion composition) at the basal side. Hair cells convert the vibration signal detected by stereocilia at the apical side into electric action potential and relay it to auditory neurons. Failure in barrier function of the organ of Corti epithelium results in cell death of hair cells and causes deafness. For example, mutation of claudin-14 causes nonsyndromic familial deafness, DFNB29 (OMIM 614035; Wilcox *et al.*, 2001), and claudin-14–knockout mice exhibit deafness, with severe loss of hair cells (Ben-Yosef *et al.*, 2003).

Truncation of the C-terminal cytoplasmic tail of tricellulin has also been reported as a cause of nonsyndromic familial deafness, DFNB49 (OMIM 610153; Riazuddin *et al.*, 2006). There was no obvious phenotype other than deafness, which is probably because the inner ear epithelium is most susceptible to barrier dysfunction, whereas other proteins may play compensatory functions in other organs. Knock-in mice harboring a tricellulin R497* mutation, which is equivalent to one of the DFNB49 mutations (R500*), also exhibit severe deafness (Nayak *et al.*, 2013). The C-terminus of tricellulin is sufficient for recruitment of tricellulin by angulin-1 (Masuda *et al.*, 2011) and is required for the recruitment by angulin-2 (Higashi *et al.*, 2013). The tricellulin R497* mutation prematurely terminates the protein, resulting in loss of the C-terminal occludin-ELL domain. In the tTJs of tricellulin R497*–knock-in mice, tricellulin R497* was mislocalized, and the linkage between bicellular TJ strands and the central sealing elements was completely lost (Nayak *et al.*, 2013), suggesting that tricellulin serves as a connector of claudin-based TJ strands with tricellular central sealing elements (Figure 1F). The phenotypes of tricellulin-knockout mice and R497* knock-in mice were in fact very similar (Kamitani *et al.*, 2015), indicating that mislocalization of tricellulin caused by C-terminal truncation abolishes the entire function of tricellulin.

Occludin loss-of-function studies also support the conclusion that tricellulin is important for proper hearing. Occludin is an integral membrane protein in bicellular TJs and belongs to the TAMP family along with tricellulin. It has been reported that loss of occludin causes redistribution of tricellulin from tTJs to bicellular TJs in MDCK II cells (Ikenouchi *et al.*, 2008). Occludin-knockout mice exhibit a complex phenotype, including growth retardation and chronic inflammation (Saitou *et al.*, 2000). Occludin-knockout mice also exhibit deafness caused by apoptotic cell death of hair cells (Kitajiri *et al.*, 2014). The localization of tricellulin was perturbed in the organ of Corti, which leads to loss of barrier function (Kitajiri *et al.*, 2014), and may explain the cause of hair cell loss.

Among the three angulins, only angulin-2 is expressed in the organ of Corti (Higashi *et al.*, 2013). Mutation of angulin-2 was reported as a cause of another nonsyndromic familial deafness, DFNB42 (OMIM 609646; Borck *et al.*, 2011). Angulin-2–knockout mice exhibit deafness with postnatal loss of hair cells (Higashi *et al.*, 2015; Morozko *et al.*, 2015; Sang *et al.*, 2015). In angulin-2–knockout mice, tricellulin localization was not lost from tTJs, and the connection between bicellular TJ strands with the central sealing element is maintained (Morozko *et al.*, 2015). This could be explained by compensatory localization of angulin-1 at tTJs of the knockout mice. In contrast, angulin-1 was negligibly detected in wild-type mice (Higashi *et al.*, 2015). In these mice, angulin-1-based tTJs show less prominent central sealing elements compared with angulin-2–based tTJs in freeze-fracture replica EM (Morozko *et al.*, 2015), and the localization of tricellulin is slightly shifted to the basolateral side (Higashi *et al.*, 2015; Morozko *et al.*, 2015), which may result in the loss of strong barrier function required for proper organ of Corti function and maintenance of hair cells.

tTJs in blood–brain and blood–retinal barrier

Although tricellular junctions in endothelial cells are less well characterized than those in epithelial cells, recent studies shed light on their importance in blood–brain barrier (BBB) and blood–retinal barrier (BRB). Maintenance of BBB and BRB is essential for homeostasis of neurons, and extrapolating from epithelial barrier function, it seems likely that not only bicellular TJs but also tTJs are required for endothelial barrier function in the brain and retina. Indeed, tricellulin (Mariano *et al.*, 2013) and angulin-1 (Iwamoto *et al.*, 2014) are expressed and localized at tricellular contacts in brain and retinal endothelial cells but not in peripheral endothelial cells. Of interest, there is no BBB in particular regions of the brain, such as the choroid plexus and circumventricular organs. Of note, tricellulin and angulin-1 were not observed at endothelial tricellular contacts in these tissues (Iwamoto *et al.*, 2014). Recently the functional importance of tTJs in brain endothelium was reported. The expression of angulin-1 in brain endothelial cells correlates with the onset of BBB formation, and the brain endothelial cells in angulin-1–knockout mice become more permeable to small molecules, which may explain the embryonic lethality of angulin-1–knockout mice (Sohet *et al.*, 2015). Furthermore, angulin-1 is down-regulated in experimental models of multiple sclerosis and stroke, which implicates a role for angulin-1 in pathological BBB leakage (Sohet *et al.*, 2015). In the future, freeze-fracture replica EM of tTJs in brain or retinal endothelium will be necessary to provide solid evidence of tTJs in blood vessels in vivo.

tTJs implicated in type 2 diabetes

Little is known about potential connections between tTJ proteins and type 2 diabetes, but several studies provide intriguing clues. Angulin-3 was identified as a candidate modifier of susceptibility to type 2 diabetes in mice (Dokmanovic-Chouinard *et al.*, 2008). Furthermore, human angulin-3 is located in Chr1q23, which has been repeatedly associated with type 2 diabetes (Dokmanovic-Chouinard *et al.*, 2008). In addition, angulin-1 was originally cloned as a lipoprotein receptor (Yen *et al.*, 1999), and liver-specific knockdown was reported to cause hypertriglyceridemia (Narvekar *et al.*, 2009), which is one of major risk factors for type 2 diabetes. In the future, it will be interesting to determine how tricellular junction proteins are mechanistically involved in the pathogenesis of type 2 diabetes.

Tricellular junctions in bacterial pathogenesis

Several pathogenic bacteria exploit tricellular junctions to effectively infect epithelial cells (Figure 2E). For example, *Shigella*, the cause of bacterial dysentery, invades and proliferates in the colonic epithelium. *Shigella flexneri* hijacks the cellular actin assembly machinery to generate an actin tail, allowing it to move around within infected cells. Then *S. flexneri* forms protrusive pseudopodia at tricellular junctions in a tricellulin-dependent manner (Fukumatsu *et al.*, 2012).

Neighboring cells engulf the bacterium-containing pseudopodia, which results in spreading of *S. flexneri* from infected cells to surrounding healthy cells.

Clostridium difficile, a cause of antibiotic-associated diarrhea and pseudomembranous colitis, secretes the binary toxin *C. difficile* transferase (CDT), which consists of a binding component and an actin-ADP ribosylating enzymatic component. The binding component binds to the cell surface, induces endocytosis, and allows the enzymatic component to translocate into the cytosol. Then actin restructuring within the cell causes cell death and at lower toxin concentration induces formation of microtubule-based protrusions, which enable adherence and colonization of the bacteria. Of interest, the CDT-binding component uses angulin-1 as a receptor (Papatheodorou *et al.*, 2011). Related toxins from *Clostridium perfringens* and *Clostridium spiroforme* also use angulin-1 as a receptor (Papatheodorou *et al.*, 2011, 2012). It has not been investigated whether the binding of the toxins occurs at epithelial tricellular junctions.

Group A *Streptococcus* (GAS) is a cause of various human diseases called GAS infections, ranging from mild, superficial infections, such as strep pharyngitis (strep throat), to life-threatening systemic infection. Recently it was shown that GAS targets tricellular junctions through an interaction with tricellulin using host-derived plasminogen as a ligand to breach the epithelial barrier, which may explain the mechanism of GAS invasion into submucosal tissues (Sumitomo *et al.*, 2016). Similarly, enteropathogenic *Escherichia coli* was also reported to target tTJs using the bacterial effector protein EspG1 (Morampudi *et al.*, 2017). If novel molecular components of tricellular junctions are identified, it will be interesting to test whether they, like tricellulin and angulin-1, are also exploited by bacterial pathogens.

Tricellular junctions in cell division

Recently it was reported that tricellular junctions regulate the orientation of cell division in *Drosophila* epithelial cells (Bosveld *et al.*, 2016). During mitosis, when cells round up, the interphase cell shape is no longer maintained, but Gliotactin-based tricellular junctions maintain information regarding the anisotropy of the interphase cell shape. The tricellular junctions recruit the dynein-associated protein Mud. In this way, the distribution of tricellular junctions orchestrates dynein- and Mud-dependent astral microtubule pulling forces, which orient the mitotic spindle along the long axis of the interphase cell, thus assuring generation of equal-sized daughter cells. Mitotic spindle dynamics of vertebrate epithelial cells have recently been analyzed using automated tracking of live images (Larson and Bement, 2017). In this study, the mitotic spindle exhibits rapid oscillatory rotation during late metaphase, and the spindle poles frequently approach and nearly contact to the cell cortex and then rapidly move away from it. Of interest, the cell cortex seems to have specific contact targets for spindle poles. Tricellular junctions could be potential candidates for such cortical targets because tTJs contain specific components including tricellulin and angulins. In addition, NuMA, the vertebrate homologue of Mud, appears to be concentrated at tricellular junctions in vertebrate epithelial cells (see Figure 5b in Gloerich *et al.*, 2017). It would be interesting to test directly whether vertebrate tricellular junctions serve as a spatial landmark to orient cell division.

Tricellular junctions in development and morphogenesis

Epithelial cells play major roles in morphogenetic movements during development. One such morphogenetic movement that occurs as epithelial tissues elongate is cell intercalation (Guillot and Lecuit, 2013b). During intercalation, the cells that formerly resided next to each other become separated by neighboring cells, which squeeze in between and form new cell–cell contacts (Figure 2C). In this situation, two tricellular junctions first fuse to make one four-way junction, which then separates into two new tricellular junctions formed among a different combination of cells. It would be interesting to investigate how this exchange of tricellular junctions is regulated and whether tTJ proteins are involved in this process. After cytokinesis in the *Xenopus* epithelium, the two daughter cells are separated by neighbors, but they frequently reorganize their tTJs and make a daughter–daughter interface (Higashi *et al.*, 2016; Figure 2A), making this a potential model for studying the cell intercalation process.

OPEN QUESTIONS

How are new tTJs specified?

Although it is likely that angulins and Anakonda make up the primary structure of tTJs and tSJs, respectively, how they recognize the tricellular contact points is unclear. For Anakonda, an attractive model has been proposed in which it makes equal contacts with the surfaces of the three cells at tSJs using extracellular triple repeat regions and, for steric reasons, becomes selectively stabilized by three-way contacts at cell vertices (Byri *et al.*, 2015). For angulin-2, a similar model has been presented (Kim *et al.*, 2015). By constructing a computational three-dimensional structure model, it was suggested that three angulin-2 proteins from each cell make a complex at tTJs where the Ig-like extracellular domains form a trimer.

However, at least for tTJs, angulin trimer formation might not be the main driving force of tTJ specification because angulins are also preferentially localized at four-way junctions, which may not stabilize trimers (Masuda *et al.*, 2011; Figure 1C). In addition, angulin-2 is localized at tTJs between olfactory neurons and supporting cells in the olfactory epithelium, in which three cells meet at angles of 90, 90, and 180° (Higashi *et al.*, 2013). Furthermore, angulin-1 makes a trans-complex at bicellular contacts and is not preferentially localized at tricellular contacts when expressed in L fibroblasts, suggesting that angulin-1 alone cannot recognize tricellular contacts (Masuda *et al.*, 2011).

Another speculative model is that angulins or Anakonda accumulate where the TJ or SJ barrier is not sufficient. Because the components of bicellular TJs cannot efficiently seal the tricellular contacts, the barrier is breached at tricellular regions. If one assumes that angulins or Anakonda are excluded from the region where the barrier is established or that they are preferentially localized at a breach area, then they are necessarily localized at tricellular contacts. This model could explain why angulin-1 is localized at newly forming cell–cell junctions at the basal side in an epidermal cell sheet (Yokouchi *et al.*, 2016). As the old TJ gradually loses its barrier function, angulin-1 accumulates along the entire cell–cell junction in the lower layer. As the barrier function of the new bicellular TJ become mature, angulin-1 is excluded from bicellular TJs. Because there is no direct evidence proving this "leak-detection" model, future studies will be required to test this attractive hypothesis.

Other possibilities for how proteins may recognize the tricellular contact points invite further investigation. One possibility is that angulins or Anakonda can sense a specific feature of the tricellular region, such as negative membrane curvature of cell corners or specific lipid composition. Another possibility is that the molecular machinery of abscission (e.g., endosomal sorting complex required for transport) or the midbody may trigger the formation of tTJs or help transport tTJ components to the cleavage site after cell division. Curiously, when nascent tTJs are formed after cytokinesis, the

timing of tTJ establishment is slightly different between the two tTJs (Figure 2A). Because the abscission event also occurs first on one side of the midbody and then later at the other side, it is possible that the abscission machinery and/or midbody are involved in tTJ specification.

Are there other molecular components of tricellular junctions or binding partners for them?

No tAJ-specific component has been identified. In addition, it is unknown whether tAJs undergo tension-dependent changes. For bicellular AJs, loading of a mechanical tugging force positively regulates the size of cell–cell junctions (Liu *et al.*, 2010) and causes recruitment of vinculin (Yonemura *et al.*, 2010). The size and molecular composition of the tAJ are also likely to be regulated by applied tension (Figure 3B). It will be interesting to test whether tension-induced changes at the tAJ are accompanied by recruitment of additional scaffold or signaling molecules (e.g., kinases) that are specific to tAJs or similar to the proteins recruited to bicellular AJs under tension.

For tTJs and tSJs, although specific transmembrane components have been identified, binding partners are still largely unknown. ZO-1 (Riazuddin *et al.*, 2006) and Tuba (Oda *et al.*, 2014) have been reported as tricellulin-binding proteins, and Tjp2iso3 (splicing isoform of ZO-2) colocalizes with tricellulin in Sertoli cells (Chakraborty *et al.*, 2014). Future studies using new techniques such as the BioID approach may reveal additional interacting partners for tTJ and tSJ components.

Are tricellular junctions and bicellular junctions mutually dependent?

tTJ formation is dependent on bicellular TJs. For example, tricellulin is not localized at tricellular contacts in ZO-1/2 double-negative cells, which lack TJs (Ikenouchi *et al.*, 2007), and loss of occludin causes mislocalization of tricellulin (Ikenouchi *et al.*, 2008; Kitajiri *et al.*, 2014). Whether tTJs affect bicellular TJs is controversial. Several groups have reported phenotypes of tricellulin loss, which vary from no obvious change (Van Itallie *et al.*, 2010; Nayak *et al.*, 2013; Kamitani *et al.*, 2015), to abnormal occludin localization near tricellular contacts (Ikenouchi *et al.*, 2005), to disruption of apical actin structure (Oda *et al.*, 2014; Salomon *et al.*, 2017). Because tTJ formation depends on bicellular TJs, and TJ formation is believed to depend on AJs, one would expect that tTJ formation is also affected by AJs. Recently it was reported that knockdown of EpCAM causes unusual expansion of the apical domain at tricellular contacts and basal displacement of tricellulin through abnormal accumulation of myosin II at tricellular contacts (Salomon *et al.*, 2017), suggesting that bicellular AJs may influence tTJ positioning.

Do tTJs have unique selective permeability?

Epithelial tissues have TJs with specialized selectivity for fluid and solutes. For example, the proximal tubules of kidney have high permeability for cations and contribute to reabsorption of sodium ions from primitive urine. It is unclear whether tTJs have specific unique selectivity. There are two distinguishable pathways for paracellular transport (Watson *et al.*, 2001; Guo *et al.*, 2003; Van Itallie *et al.*, 2008), called the pore pathway and the leak pathway (Anderson and Van Itallie, 2009; Shen *et al.*, 2011). The pore pathway allows ions and water to pass through with high charge and size selectivity and is probably formed by intercellular, channel-like structures made up of claudins (Yu *et al.*, 2009; Krug *et al.*, 2014; Suzuki *et al.*, 2015). In contrast, the leak pathway is not size selective and has relatively low capacity compared with the pore pathway. The physical basis of the leak pathway is unclear, but the idea that local transient discontinuities of TJ strands correspond to the leak pathway is widely accepted (Shen *et al.*, 2011). Given that there is a 10-nm-wide "tube"-like central sealing element at the tTJ, tTJs are another candidate for the leak pathway. Overexpression of tricellulin at tTJs in MDCK II cells strengthened the barrier for macromolecules without affecting ion permeability (Krug *et al.*, 2009), which supports the idea that tTJs constitute the leak pathway. Knockdown of tricellulin (Ikenouchi *et al.*, 2005) or angulin-1 (Masuda *et al.*, 2011; Higashi *et al.*, 2013) in EpH4 cells, which normally have high resistance for ions, causes increased permeability of macromolecules as well as ions. It is intriguing that angulin-1–knockdown cells did not show changes in charge and size selectivity, although they exhibit ~10-times-higher ion permeability (Higashi *et al.*, 2013), which might suggest that there is no "leak" at tTJs. Such a property of selective permeability is reminiscent of the nuclear pore, which is filled with disordered proteins enriched with Phe and Gly residues and allows the passage of macromolecules such as mRNAs, at the same time restricting permeation of other molecules. Of interest, the first extracellular loop of tricellulin (and occludin) contains a Tyr- and Gly-rich sequence. An alternate possibility is that the knockdown of tTJ proteins changes the structure of bicellular TJs, which affects the continuity of TJ strands and opens up the leak pathway.

SUMMARY

The molecular machinery comprising tricellular junctions is starting to be uncovered. Because tricellular junctions are critical for barrier function, mechanohomeostasis of epithelial tissues, development, and several diseases, it will be exciting to further investigate tricellular junction components and their functional roles in both cultured epithelial cells and in vivo models.

Note added in proof. During the review process, a new report about angulin-2 was published (Gong *et al.*, 2017). The authors showed that angulin-2, which was known to be expressed in the kidney, regulates paracellular water transport and urine concentration.

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