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## **Tricellular junctions: a hot corner of epithelial biology.**

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### **Abstract**

**As the result of an intricate interplay between mechanical and biochemical cues, coordinated cell dynamics are at the basis of tissue development, homeostasis and repair. Numerous studies have addressed the interplay between these two inputs and their impact on cellular dynamics. These studies largely focus on bicellular junctions (BCJs). Recent works have illuminated that tricellular junctions (TCJs), the junctions where 3 cells contact, play important roles in epithelial tissues beyond their well-known structural function in preserving epithelial barrier integrity. Indeed, TCJs have recently been implicated in the regulation of collective cell migration, division orientation, cell proliferation and cell mechanical properties. More generally, the TCJ distribution aligns with the cell shape and mechanical stress orientation within the tissue, while their positioning encapsulates the packing topology. Importantly, known regulators of growth signalling and of cell mechanical properties are also localized at TCJs. Therefore, TCJs emerge as spatial sites to sense and integrate biochemical and mechanical inputs to guide epithelial tissue dynamics.**

## Introduction

As selective permeability barriers epithelial cell sheets cover organs separating them from the external milieu. To ensure paracellular barrier function cells are tightly packed and form specialized adhesive contacts along their apical-basal axes, at both the bicellular junctions (BCJs) and at the tricellular junctions (TCJs). In vertebrate tissues, cell-cell adhesion is mediated by the apical tight junctions (TJs), basal-lateral adherens junctions (AJs) and the desmosomes, while in invertebrate tissues adhesion is mediated by the apical AJs and the basal-lateral septate junctions (SJs) (Figure 1a, b) (for detailed structural information on AJs, TJs, SJs and TCJs see reviews [1–6]). Thus, BCJs and TCJs are core structures of epithelial tissues. They have been extensively studied from a structural point of view and shown to be critical for tissue barrier functions in numerous tissues.

TCJ function and organization have been mainly studied at the level of TJ and SJ. The organization and structure of the tricellular vertebrate TJs and invertebrate SJs appear largely similar [3,5]. However, the apical-basal organization of the TJs and SJs is different and the TCJ molecular components do not share sequence homology, although they perform functionally analogous tasks (Figure 1a', b'). TCJ barrier integrity in vertebrate systems is mediated by the Tricellulin and the Angulin protein family [7–9], while in *Drosophila*, barrier integrity is provided by Gliotactin (Gli) and Anakonda (Aka) (also known as Bark beetle, Bark) [10,11,12••]. Numerous works have focused on the roles of BCJ mechanical properties, their formation and remodelling in promoting tissue dynamics and regulating epithelial packing (reviews [13–15]). Recent studies now show that epithelial tissue dynamics and mechanics can be understood, if the function of TCJs is taken into account. After a brief description of the TCJ structure in vertebrate and invertebrate systems, we will focus primarily on the emerging roles of TCJs in epithelial cell division, migration, cell mechanics and stem cell maintenance.

## TCJ structure, protein composition and regulation

Vertebrate and invertebrate TCJ structure and formation are best characterized at the level of the TJ and SJ, respectively. In vertebrate and invertebrate model systems TCJ channels, or pores, are present along the apical-basal axis at the contact interfaces between three cells. They are formed by a series of stacked diaphragms, tricellular channel diaphragms (TCD) in invertebrates or a central sealing element (CSE) in vertebrates (Figure 1a,b) (reviewed in [5,6]). In vertebrate systems, the bicellular TJs form strands that attach to the central sealing element

[16], while in invertebrates the bicellular SJ strands connect to the diaphragm along the apical-basal axis forming a lateral limiting strand (LS) at the tricellular contact interface [17–19]. From a molecular point of view, in vertebrates, Tricellulin mediates the connection between the TJ strands and central sealing element via the Angulin proteins (Angulin1-3) [8,9,20]. In invertebrates the molecular link between the lateral limiting strands and the tricellular channel diaphragms has been proposed to be mediated by the Gli protein [10,21], while the connection between the tricellular channel diaphragms and Gli is mediated by Aka [12••]. Aka is a transmembrane domain with a large extracellular domain. Based on experimental data and computer simulations, it is proposed that the geometry of the TCJ facilitates the self-organization of the extracellular domains of Aka proteins from three adjacent cells into a tripartite septum in the TCJ lumen (Figure 1a')[12••].

To understand how TCJs are formed and remodeled, it is essential to determine how proteins are trafficked and targeted to the TCJs. Currently, the cellular and molecular mechanisms regulating protein targeting and localization to the TCJ are still not fully understood. However, the Auld lab has provided insight into this central question in *Drosophila* by analyzing how Gli levels are regulated at the TCJ. The localization and levels of Gli are regulated at the protein level by phosphorylation-dependent endocytosis and degradation facilitated by the SJ protein discs large (Dlg) [22–24] and the C-terminal Src kinase (Csk) [25••]. Moreover, Gli mRNA levels are regulated through a feedback mechanism that utilizes the bone morphogenetic protein (BMP) pathway to upregulate a microRNA, miR-184, which targets the 3'UTR of Gli for degradation [26].

Combined these studies show that TCJs are complex molecular structures, of which the molecular composition needs to be strictly controlled to ensure barrier integrity.

### **TCJ formation during epithelial tissue remodeling and division**

In order to preserve barrier function during homeostasis or morphogenesis, remodelling and *de novo* formation of BCJs and TCJs needs to be tightly coupled with local cellular dynamics such as cell elimination by extrusion, apoptosis, delamination [27] or fusion [28], cell addition upon division [29] or cell insertion [30], or changes in cell positions during cell-cell rearrangements [31]. Two studies have addressed how *de novo* TCJs are formed in the mouse ear skin epithelium [32,33••]. The mouse ear skin is a multi-layered epithelium composed of 1) an outer barrier, the stratum corneum (SC), 2) followed by the TJ barrier, the stratum

granulosum (SG), 3) the stratum spinosum (SS) and 4) the proliferative layer, the stratum basale (SB). Cells from the proliferative layer traverse through the stratum spinosum and stratum granulosum to finally reach the stratum corneum. Yokouchi et al. [33••] demonstrated that during this upward migration the cells form a specialized 3D shape filling structure resembling a Kelvin's tetrakaidecahedron (14-sided solid with six rectangular and eight hexagonal sides) and establish Tricellulin/Angulin-1 positive TCJs with the cells of the stratum granulosum to preserve barrier integrity when entering the stratum granulosum layer. The second study addressed how antigen-presenting dendritic cells of the skin, the Langerhans cells, located between the stratum spinosum and stratum basale layers, generate dendrites that penetrate the stratum granulosum to uptake antigens in the space between the stratum granulosum and stratum corneum layers and within the stratum corneum [32]. When a dendrite penetrates the TJ of the stratum granulosum layer, Tricellulin accumulates at the tricellular contact sites between two stratum granulosum cells and the dendritic cell to establish new TCJs, thus preserving barrier integrity.

In parallel to these studies on the remodelling of TCJ, the *de novo* TCJ formation in monolayered proliferative epithelia is currently emerging as an active and important field of research. *De novo* BCJ formation at the level of the AJs upon cell division within epithelial tissues has been extensively characterized. Combined, these studies demonstrate that the formation of a new BCJ relies on an intricate interplay between the dividing cell and its neighbouring cells [34,35,36••]. One recent study in *Xenopus* provides insights into TCJ formation at the level of the TJs during cell division and how this is coordinated with AJ formation [37••]. In cells of the *Xenopus* gastrula epithelium new TCJs are formed during the final stages of cytokinesis. Upon midbody formation and AJ establishment, the TCJ proteins Angulin-1 and Tricellulin are recruited to the nascent TCJs, which closely appose to the midbody to form two mature TCJs between each daughter cell and its two neighboring cells. This mode of cytokinesis results in the creation of a bicellular neighbor-neighbor interface, which has also been reported in the chick epiblast cells [35]. In many different epithelia and model systems, including *Xenopus*, *Drosophila*, *Hydra*, and the zebrafish cell division results in the formation of a daughter-daughter cell interface [38–44]. This raises the question of whether the creation of a daughter-daughter interface requires a different mode of TCJ establishment. Our recent findings show that within the *Drosophila* notum tissue, TCJ maturation at the level of the SJ initiates after the apical daughter-daughter AJ has been

formed (Wang et al., submitted). Upon AJ formation, the daughter cells and their neighboring cells remain connected to the midbody forming a 4-cell structure. This 4-cell structure resolves as the midbody undergoes a basal movement. This midbody basal movement is concomitant with the formation of a new SJ between the two daughter cells and the establishment of new TCJs between the two daughter cells and one neighboring cell.

### **TCJs as cell shape and stress orientation sensors: Cell division orientation**

Cell division orientation contributes to cell fate specification, tissue organization and morphogenesis [29]. Within tissues the direction of the cell shape anisotropy generally aligns with the tissue stress axis, and, accordingly, divisions tend to be oriented along the global tissue stress axis [28,45–49]. Recently, it was shown that within the *Drosophila* pupal epithelium the TCJ distribution (TCJ bipolarity) aligns with the principal cell shape axis when cells are elongated, and with the direction of the mechanical stress within the tissue [50••]. Importantly, the conserved Dynein-associated protein Mud (NuMA in mammals), which controls the pulling forces on the astral microtubules to orient the spindle during mitosis [51], is recruited to the TCJs. Mud accumulation at TCJs occurs at the level of the SJs, in G2 interphase where it remains during mitosis. The TCJ localization of Mud is independent of the classical Gai/Pins (LGN in mammals) regulators of Mud localization [51]. Instead, Mud TCJ localization depends on the TCJ protein Gli and on the SJ proteinDlg, which regulates Gli localization [22–24,50••]. While the cell shape anisotropy is lost upon mitotic rounding, the anisotropy of TCJ bipolarity remains relatively constant. Therefore, the TCJ distribution of Mud in the rounded mitotic cell encapsulates both the interphase cell geometry, and, possibly, the tissue stress axis ensuring division orientation along the cell shape and global tissue mechanical stress orientations (Figure 2). Importantly, a similar TCJ-based mechanism for division orientation was recently reported in the *Xenopus* animal cap cells [52]. In this context, it was shown that the loss of C-Cadherin abrogated spindle orientation relative to the TCJ distribution and it was proposed that C-Cadherin enrichment at the TCJs recruits the LGN/NuMA complex to orient the spindle.

Independently of the exact mechanisms of Mud TCJ recruitment, these studies establish that the TCJ distribution allows cell to “sense” the orientation of cell shape and mechanical stress anisotropy. As the TCJ distribution controls division orientation and TCJ bipolarity aligns both with the cell shape and tissue stress axes, the information encapsulated

by their position may further promote the dissipation of tissue stress and regulate epithelial cell packing during tissue development [41,46,53].

### **TCJs as tissue planar polarity cues**

While the epithelial cells are polarized along their apical basal axis (apical-basal polarity), a conserved feature of epithelial tissues is that the cells are also polarized within the plane of the tissue (planar cell polarity, PCP), [54,55]. The *Drosophila* wing epithelium has served as a paradigm to study the mechanisms of PCP by the Frizzled (Fz) PCP pathway. Within the *Drosophila* wing, cells form polarized ridges at their cell apical membranes. In addition, each cell produces a distally pointed hair with the hair pedicle located at the peak of the ridge [56]. This planar polarized organization of the cells requires Frizzled PCP signalling and depends on their regular hexagonal packing during pupal stages [47,56,57]. Mutations in *Gli* disrupt the alignment of the hairs independently of classical Frizzled PCP signalling [58]. *Gli* localizes at the TCJs during early pupal wing development and translocates together with the SJ protein Coracle to a more apical position, where they form ribbon structures, which are the presumptive ridges, beneath the prehair base during later stages. Upon loss of *Gli* function, the formation of these ribbons was compromised and cells appear more disorganized. This study demonstrates that TCJs impact on PCP and possibly on cell packing.

PCP signaling has also been implicated in the directional migration of epithelial cell sheets or collective migrations. Collective cell migrations are essential for tissue morphogenesis and wound healing, and their dysregulation can lead to tumorigenesis (reviewed in [59]). Within the *Drosophila* ovary, the follicular epithelium covering the germ cells undergoes a stereotypic collective migration whereby the entire tissue crawls on the basement membrane and rotates around the germ cells to elongate the future egg [60–62]. This rotation is driven by whip-like actin protrusions that emanate from the basal TCJs [63••]. The direction of these protrusions is regulated by a basal planar signalling network involving the Fat2 proto-cadherin, the DLar receptor tyrosine phosphatase and the WAVE regulatory complex protein *Abi*, which are all enriched at the basal TCJs during early stages [63••,64–66]. It was proposed that the TCJ localization of Fat2 promotes the polarization of the actin cytoskeleton through a yet unknown mechanism to initiate the collective migration [63••]. Although compelling evidence indicates that Fat2 and DLar regulate follicle cell migration, the

interplay between these two signalling pathways remains elusive, since Fat2 and DLar do not directly interact [64,67].

Combined, these findings illustrate that TCJs act as signalling hubs for PCP signalling guiding cell polarity independently of the classical Frizzled PCP pathway. It will be interesting to explore whether TCJ-dependent PCP signalling exists in other tissues and contexts.

### **Mechanical regulation at TCJs**

TCJs sustain the junctional tension generated by three or more BCJs during cell rearrangements [68–70]. The forces exerted on the TCJs are not constant, but continuously fluctuate due to the changes in BCJ length and tension associated with morphogenetic processes (reviewed in [31] and [71–78]). Accordingly, several studies have now revealed how the BCJ and TCJ mechanical properties are coordinated and provide feedback for each other.

BCJs are generally under tensile stress and are thus pulling on the TCJs [53]. As TCJs are mechanically coupled to the BCJs, TCJs are likely to respond to these forces generated along the BCJs. Indeed, several studies reported that changes in the BCJ tension or adhesion lead to changes in the molecular composition of TCJs. Increasing junctional tension by adding the drug calyculin A or ATP to embryonic tissues of *Xenopus* causes the accumulation of the F-actin binding protein Vinculin at TCJs. Upon wounding in cultured MDCK monolayers, an actomyosin purse string is formed, which participates in the closing of the wound. The tension generated by the constriction of this purse string induces the localization of  $\alpha$ -Catenin in its stretched conformation and of Vinculin at the TCJs [79]. Similarly, increased BCJ tension after double knock-down of the ZO-1 and ZO-2 TJ proteins in MDCK monolayers increases the TCJ localization of Afadin, E-Cadherin, Vinculin and of  $\alpha$ -Catenin in its stretched conformation [80]. Conversely, reducing BCJ tension by depleting the scaffolding protein Anillin results in an impaired junctional integrity and in a reduced amount of active MyosinII at the TCJs in gastrula-stage *Xenopus* embryos [81].

TCJs not only respond to changes in BCJ tension, but also feedback on the BCJ tension [82]. When Caco2 cells are grown at subconfluent conditions, the TCJ associated Tricellulin recruits the Cdc42 GEF Tuba, which activates Cdc42 to promote the assembly of an actomyosin meshwork at the TCJs as well as BCJs. Accordingly, the loss of the Tricellulin induced BCJ curving and changed tissue topology indicative of defects in BCJ tension [82]. Lastly, in the mammalian gut epithelial tissue, loss of EpCAM dependent cell-cell adhesion at BCJs results in

a hypercontractility at the TCJs [83••], due to the accumulation of myosins IIa and IIb at the TCJs. This increases the contractility along the apical-basal axis of the cells, which leads to the abnormal expansion of the cell apical domain and epithelial dysplasia (Figure 3a,b). Thus, the tight regulation of contractile forces at TCJs is essential to maintain tissue integrity and homeostasis [83••].

### **TCJs as regulators of stem cell homeostasis and tissue growth signaling**

The role of TCJs as regulators of epithelial integrity is not restricted to the mammalian gut. Recently, Resnik-Docampo and colleagues showed that the age-associated loss of the barrier function in the *Drosophila* midgut is associated with an impaired TCJ organization and results in stem cell over-proliferation [84••]. They found that the loss of Gli at the TCJs of the midgut enterocytes promotes such age-associated barrier dysfunction by preventing terminal differentiation and increasing intestinal stem cell proliferation (Figure 4). The intestinal stem cell over-proliferation is due to the loss of Gli function in the differentiated enterocytes, which promotes Jun N-terminal kinase (JNK) signaling pathway in the intestinal stem cell. However, the link between TCJs and JNK signaling appears more complex and could be tissue dependent. Indeed, as opposed to the loss of Gli in the midgut, the over-expression of Gli in the *Drosophila* wing disc induces an increase in cell proliferation by upregulating the JNK pathway [24]. As Gli over-expression induces cell delamination and apoptosis, this increase in proliferation was proposed to reflect a compensatory mechanism necessary to buffer the cell loss. Since the loss of Gli function and the overexpression of Gli trigger JNK pathway activation, one can envision that both conditions cause tissue injury resulting in elevated JNK stress signaling and increased proliferation.

The role of TCJs in regulating growth signaling likely extends beyond the control of the JNK pathway. Indeed, several proteins that have been implicated in the Hippo growth control pathway are enriched at TCJs at the level of the AJs. The actin-associated proteins Zyxin and Enabled, which promote tissue growth through the Hippo pathway were found enriched at the TCJs in different *Drosophila* epithelial tissues [85–87]. Likewise, the Warts kinase, which phosphorylates the transcription factor Yorkie to inhibit growth, and its interaction partner, the actin-associated protein Ajuba, appear enriched at TCJs in the *Drosophila* wing disc epithelium [88,89]. Lastly, Hippo signalling activity at TCJs may be coordinated by Csk. Csk activity controls the levels of Gli at TCJs preventing Gli spreading in BCJs [25••]. Based on

genetic epistasis experiments, it was proposed that Csk regulates tissue growth upstream of Ajuba, Zyxin and Warts [90]. As described above, TCJs have been shown to play a conserved role in the regulation of cell division orientation [50,52]. It is therefore interesting to note that the Warts kinase phosphorylates Mud to promote its cortical localization and planar spindle orientation [91]. Thus, TCJs may integrate growth signalling and geometrical inputs to regulate division.

### **Conclusions and future considerations**

As discussed in this opinion, recent findings clearly demonstrate that TCJs have additional functions well beyond their previously known structural function in preserving epithelial barrier integrity. Rather than behaving as passive structures sitting at the corners of BCJs, TCJs are active structures that respond to changes in BCJ tension and feedback to regulate BCJ mechanical properties. Because, i) the TCJ distribution aligns with the cell geometric and stress patterns, and captures the cell topological information, ii) TCJs respond to changes in BCJ tension and can feedback on BCJ tension, iii) key regulators of cell mechanical properties and growth signalling are localized at TCJs, TCJs may act as signalling hotspots, of which spatial distributions allow cells to integrate mechanical and biochemical inputs to guide local cell dynamics, while preserving tissue barrier function and topology. Perhaps one of the biggest challenges to understand the specific contribution of TCJs versus BCJs on cellular dynamics will be to specifically perturb or modify protein functions at either of these junctions. Furthermore, the localization and activities of proteins at TCJs are regulated along the cell apical-basal axis. Dissecting the interplay between BCJs and TCJs along the apical-basal axis should provide exciting new avenues to better understand cellular dynamics and growth control during tissue development, homeostasis and repair.

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**Figure 1. Cell-cell adhesion structures in *Drosophila* and mammals.**

(a, b) Schematics showing the organization of the invertebrate and vertebrate cell-cell adhesion sites along the apical-basal axis (a,b), and cross sections of the TCJ at the level of the septate junction (SJ) and tight junction (TJ) (a',b'). In invertebrate tissues, adhesion is mediated by the apical adherens junctions (AJs) and the septate junctions (SJs), while, in vertebrate tissues, cell-cell adhesion is mediated by the tight junctions (TJs), AJs and desmosomes (for reviews see [1–6]). In *Drosophila* the AJs are located above the SJs, while the functionally equivalent structures in vertebrates, the TJs, are located above the AJs. In both invertebrates and vertebrates, at the level of the SJ or TJ, respectively, TCJ channels or pores are present along the apical-basal axis formed by a series of stacked diaphragms, tricellular channel diaphragms (TCD) in invertebrates or a central sealing element (CSE) in vertebrates (a,b) (reviewed in [5,6]). In vertebrate systems, the bicellular TJs form strands that attach to the central sealing element [16], while in invertebrates the bicellular SJ strands connect to the diaphragm along the apical-basal axis forming a lateral limiting strand (LS) at the tricellular contact interface [17–19]. At the dihedral angles of 120° at the *Drosophila* TCJ the Aka proteins from three cells form a trimer and recruit Gli [10,12••]. In turn, Gli interacts with the lateral limiting strand (LS) connecting the SJs with the TCDs to form a mature TCJ and to provide a protective barrier (a'). In mammalian cells, the Angulin family of proteins Angulin-1, (also known as lipolysis-stimulated lipoprotein receptor), Angulin-2 (also known as immunoglobulin like domain-containing receptor) and Angulin-3 (also known as immunoglobulin like domain-containing receptor 2, C1orf32 or LISCH-like) likely interact through their extracellular Ig-like domains with the CSE, while their intracellular domains can interact with Tricellulin which in turn connects to the TJ strands to form a mature TCJ and provide barrier function (b') (reviewed in [5,6] and [7–9,20]).

**Figure 2. TCJ distribution controls cell division orientation.**

During G2 phase Mud (red) localizes at TCJs and remains associated with TCJs during mitosis, where it regulates the pulling forces on the astral microtubules to orient the mitotic spindle [50••]. The TCJ distribution (TCJ bipolarity, red bars) aligns on average with the orientation of cell shape (blue bars) and the stress direction (black bars). Upon mitosis cells round up and the cell shape anisotropy is lost, while the TCJ bipolarity is maintained. Thus, the Mud TCJ

distribution controls the division orientation along the interphase cell shape and tissue stress directions.

**Figure 3. Increased contractility at TCJs promotes apical cell area increase and dysplasia.**

(a, b) Schematic showing the effect of the loss of EpCAM mediated cell-cell adhesion in the mammalian gut epithelium on the cellular level (a) and the tissue level (b). Upon EpCAM loss of function actomyosin accumulates at the TCJs resulting in an increase in contractility along the apical-basal cell axis at TCJs (a) [83••]. As a result the apical cell area (blue area) increases (b), triggering epithelial dysplasia and the formation of tufts in the gut epithelium (b, arrowheads).

**Figure 4. TCJ prevent intestinal dysplasia and premature aging.**

Schematic of the *Drosophila* midgut. Intestinal stem cell (ISC), Enteroblast (EB), Enteroendocrine (EE), Enterocyte (EC). Upon loss of TJC integrity (1) the JNK pathway is upregulated in the ECs (2) [84••]. In turn JNK signaling promotes ISCs to proliferate, while terminal differentiation of EBs is prevented resulting in intestinal dysplasia (3). Finally, the paracellular barrier is compromised and flies eventually die prematurely (4).







