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Review

Tight Junction Structure and Function Revisited

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Tight junctions (TJs) are intercellular junctions critical for building the epithelial barrier and maintaining epithelial polarity. The claudin family of membrane proteins play central roles in TJ structure and function. However, recent findings have uncovered claudin-independent aspects of TJ structure and function, and additional players including junctional adhesion molecules (JAMs), membrane lipids, phase separation of the zonula occludens (ZO) family of scaffolding proteins, and mechanical force have been shown to play important roles in TJ structure and function. In this review, we discuss how these new findings have the potential to transform our understanding of TJ structure and function, and how the intricate network of TJ proteins and membrane lipids dynamically interact to drive TJ assembly.

Highlights

Tight junction strand formation and membrane apposition formation are differentially regulated.

Claudins form charge-selective small pores, while junctional adhesion molecules regulate the formation of size-selective large pores.

Tight junction proteins regulate epithelial polarity, although how tight junctions form a membrane fence remains unclear.

Tight junction associated membrane proteins regulate tight junction assembly in conjunction with zonula occludens protein phase separation, membrane lipids, mechanical force, and polarity signaling proteins.

Tight Junctions Regulate Epithelial Barrier and Polarity

Tight junctions (TJs) are epithelial intercellular junctions located at the most apical region of cell–cell contacts. TJs are structurally defined by electron microscopy. On ultrathin sections, TJs appear as a region with close apposition of adjacent plasma membranes where adjacent plasma membranes appear to partially fuse with each other [1], and on freeze-fracture replicas, they appear as a network of fibrils (TJ strands) [2,3]. The membrane hemifusion sites on ultrathin sections correspond to the TJ strands on freeze-fracture replicas [3]. An important function of TJs is to form a permeability barrier that restricts free diffusion of molecules across the intercellular space (gate function) and to act as a membrane fence that restricts intermixing of apical and basolateral plasma membrane domains (fence function) [4–6].

The molecular composition of TJs has been extensively investigated. A family of integral membrane proteins, **claudins** (see Glossary), comprise TJ strands and play pivotal roles in regulating paracellular permeability [4–8]. Claudins interact with the **zonula occludens (ZO) family** of scaffolding proteins via their cytoplasmic region [9], which in turn are essential for TJ assembly [10–12]. Besides claudins, membrane proteins like TJ-associated MARVEL domain-containing proteins (TAMPs: **occludin**, **tricellulin**, MarvelD3) and **junctional adhesion molecules (JAMs)** within the immunoglobulin superfamily localize to TJs (Figure 1C) [13–16]. Although the identification of the protein components of TJs largely contributed to the protein-centric view of TJ organization, how the TJ-associated membrane proteins and scaffolding proteins work together with the membrane lipids to create the permeability barrier or membrane fence has been debated. Recent findings have suggested that, in addition to claudins, JAMs and membrane **cholesterol** have important roles in TJ structure and function [12,17]. Meanwhile, new concepts for TJ assembly, including roles of ZO protein phase separation and mechanosensation, are emerging [18–20]. In this review, we discuss how the understanding of TJ structure and function should be revisited in light of these new findings. TJs are also known to play important roles in cell signaling, which cannot be covered in this review. We refer the readers to other reviews on this topic [4,21,22].

TJ Structure

The landmark work by Farquhar and Palade [1] defined the morphological hallmarks of TJs: close apposition of neighboring plasma membranes accompanied by membrane kissing points where

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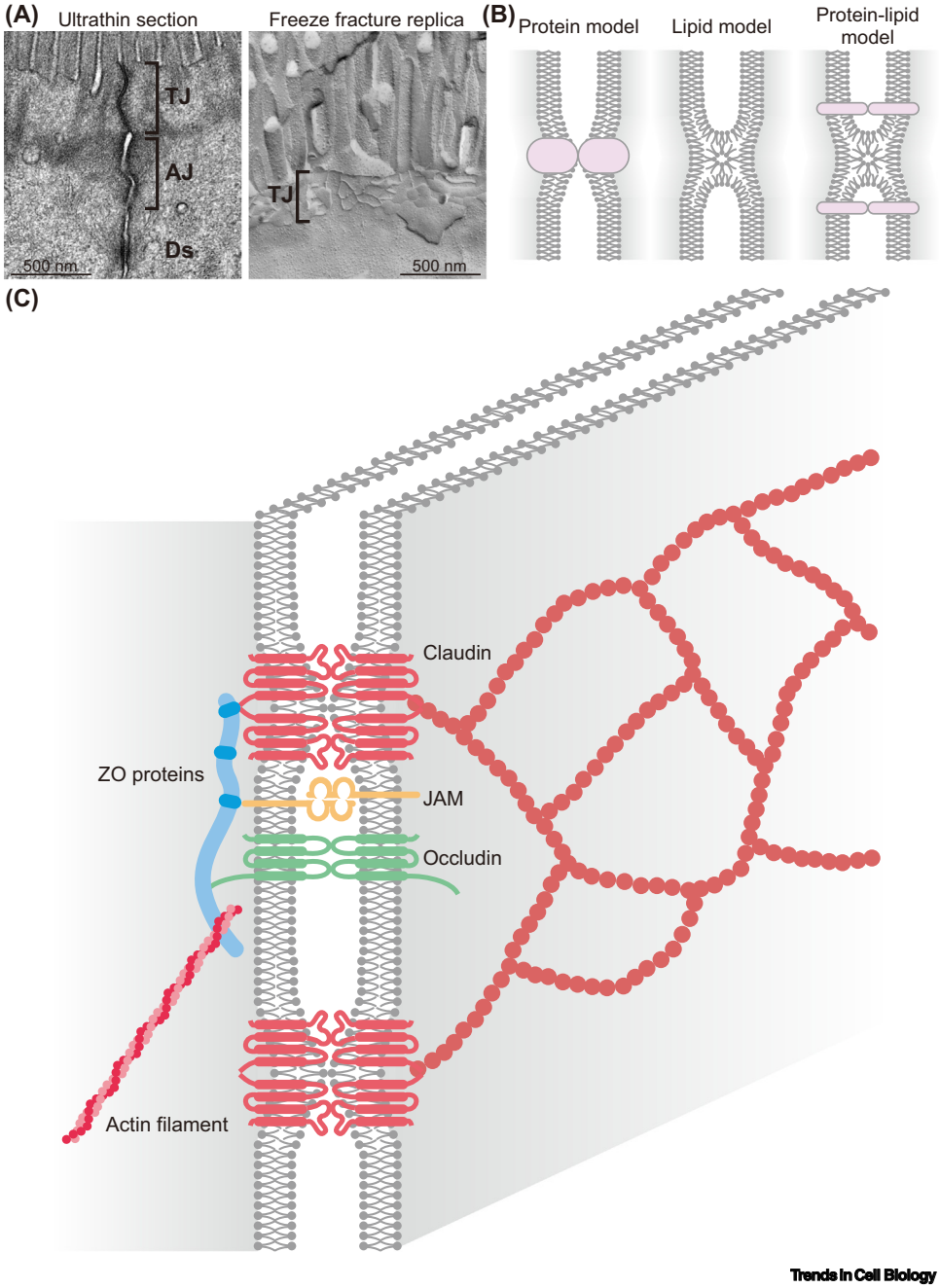

Figure 1. Structure and Molecular Composition of TJs. (A) Morphology of TJs observed by electron microscopy. On ultrathin sections, TJs are found at the most apical region of the junctional complex, where the two neighboring plasma membranes are closely apposed to one another. On freeze-fracture replicas, TJs are observed as anastomosing strands at the most apical region of cell–cell contacts. Scale bars, 500 nm. (B) Models for the structure of TJs. Two models have been proposed to explain the electron microscopy observations. In the protein model, it is proposed that TJs are formed by transmembrane proteins. In the lipid model, it is proposed that TJ strands represent an inverted lipid cylinder. In the protein–lipid hybrid model, it is proposed that TJ-associated membrane proteins function to stabilize and organize the inverted lipid cylinder. (C) Molecular organization of TJs. Claudins, occludin, and JAMs are the major integral membrane proteins of TJs. Claudins form TJ strands, corresponding to membrane kissing points. TJ-associated membrane proteins are localized at apical cell–cell junctions by interacting with the ZO family of scaffolding proteins, serving as links between TJs and the actin cytoskeleton. Abbreviations: AJ, adherens junction; Ds, desmosome; JAM, junctional adhesion molecule; TJ, tight junction; ZO, zonula occludens.

Glossary

Cholesterol: a sterol type of lipid residing in the membranes of animal cells. Cholesterol is thought to alter membrane fluidity and contribute to the formation of membrane microdomains.

Claudin: a four-pass transmembrane protein that constitutes TJ strands. The charged residues in the extracellular region are thought to determine the charge selectivity of the paracellular channel. Some claudins have neutral extracellular regions and act to form a barrier.

Crumbs/Pals1/PATJ complex: a protein complex containing a transmembrane protein of Crumbs. Crumbs interacts with Pals1, while Pals1 further interacts with PATJ. The complex localizes apical of TJs and plays an important role in epithelial polarity.

Junctional adhesion molecules (JAMs): a class of cell–cell adhesion molecules with two Ig repeats that localize to TJs. JAM-A, JAM-B, and JAM-C are known, and play important roles in regulating the epithelial barrier and polarity.

Occludin: a four-pass transmembrane protein localized at TJs. Although the precise function of occludin remains elusive, phosphorylation of occludin has been linked to dynamic regulation of TJ function.

Par-3/Par-6/aPKC complex: an evolutionarily conserved protein complex that plays pivotal roles in regulating cell polarity. aPKC (atypical PKC) is a protein kinase and forms a complex with PDZ domain containing proteins Par-3 and Par-6.

Phase condensates: membraneless subdomains formed by phase separation of biomolecules, which is characterized by a local increase in the concentration of a phase-separated biomolecule.

TJ proteins: TJ-localized proteins, including TJ-associated membrane proteins, TJ-undercoating scaffolding proteins, and TJ-associated signaling proteins.

Tricellulin: a tetraspanning protein that is a member of the TJ-associated MARVEL domain-containing proteins. Tricellulin is concentrated at tricellular TJs.

ZO of family proteins: TJ-undercoating scaffolding proteins belonging to the MAGUK (membrane-associated guanylate kinase) family. ZO-1, ZO-2, and ZO-3 are known, and have

adjacent plasma membranes appear to fuse with one another (Figure 1A). In subsequent studies, TJs appeared as anastomosing strand structures on freeze-fracture replicas (Figure 1A), corresponding to the membrane kissing points on ultrathin sections [2,3]. Based on these observations, three models were proposed to explain TJ structure. The protein model was initially proposed based on the sensitivity of TJ strand morphology to glutaraldehyde fixation, and assumes that membrane proteins mediate the formation of TJ strands (Figure 1B) [2–4]. The lipid micelle model was subsequently proposed based on the morphological similarity between inverted cylindrical lipid micelles (hexagonal H_{II} phase) and TJ strands on freeze-fracture replicas, and assumes that TJ strands are inverted lipid cylinders wherein the exoplasmic leaflets of neighboring plasma membranes are continuous (Figure 1B) [4,23,24]. The protein–lipid hybrid model proposes that the inverted lipid micelle structure is organized by the concerted actions of TJ membrane proteins and lipids (Figure 1B) [25,26].

similar structure: N-terminal PDZ, SH3, GUK domains, and C-terminal actin-binding region. The ZO of family proteins proteins scaffold TJ-associated transmembrane proteins and various cytoplasmic proteins, and ZO-1 and ZO-2 play crucial roles in TJ formation.

The identification of TJ-associated proteins has greatly advanced our understanding of TJ structure and function (Figure 1C). Claudins were identified in 1998, and overexpressed claudins were shown to reconstitute TJ strands in fibroblasts [7,8]. Subsequently, removal of claudins by genetic knockout (KO) or treatment with claudin-binding fragment of *Clostridium perfringens* enterotoxin was demonstrated to reduce TJ strand formation, suggesting that claudins constitute TJ strands [27–29]. Although complete loss-of-function analyses of claudins in typical epithelial cells are challenging because of the coexistence of multiple claudin subtypes in most epithelial cells, it was recently shown in MDCK II cells (derived from canine kidney) that claudin-1/2/3/4/7 quintuple-KO (claudin quintuple-KO) cells lacked TJ strands, demonstrating that claudins are essential for TJ strand formation in epithelial cells [12]. Based on the claudin crystal structure, it has been proposed that claudins assemble in an antiparallel double-row manner, consistent with the observed TJ strand structure [30–32]. These findings provide strong evidence that claudin polymers form TJ strands, in support of the protein model. Nevertheless, cholesterol and very long-chain ceramides were shown to play important roles in TJ biogenesis [17,33–36], and many **TJ proteins** are palmitoylated and partition into lipid raft fractions [37–39], suggesting that lipid microdomains may be involved in TJ formation.

Despite the lack of TJ strands and membrane kissing points, adjacent plasma membranes were closely apposed to one another in claudin quintuple-KO MDCK II cells [12]. Additional removal of JAM-A from claudin quintuple-KO MDCK II cells resulted in widening of the intercellular space [12]. These findings suggest that TJ strands and membrane apposition are distinct structures, and that while TJ strand formation strictly requires the presence of claudins, membrane apposition formation does not. Based on these results, we favor the view that claudins constitute TJ strands, while JAMs in addition to claudins are involved in membrane apposition, and that the ZO family of proteins act as scaffolds to bring the membrane proteins together for assembly. In contrast to claudins and JAMs, the role of occludin in TJ biogenesis remains unclear, although phosphorylation of occludin has been linked to dynamic regulation of TJ function [40–49].

TJ Functions

Gate Function

Epithelia act as barriers to compartmentalize the body. However, the permeability of epithelia varies depending on their functions. A major function of TJs is to form a permeability barrier in tight epithelia and to determine the selective permeability in leaky epithelia [4–6]. Physiological studies have suggested that paracellular permeability is determined by at least two pathways: the charge-selective small-pore pathway (Figure 2A,B) with estimated diameter of ~4Å, and the size-selective pathway permitting permeation of molecules up to ~60Å [4–6,50].

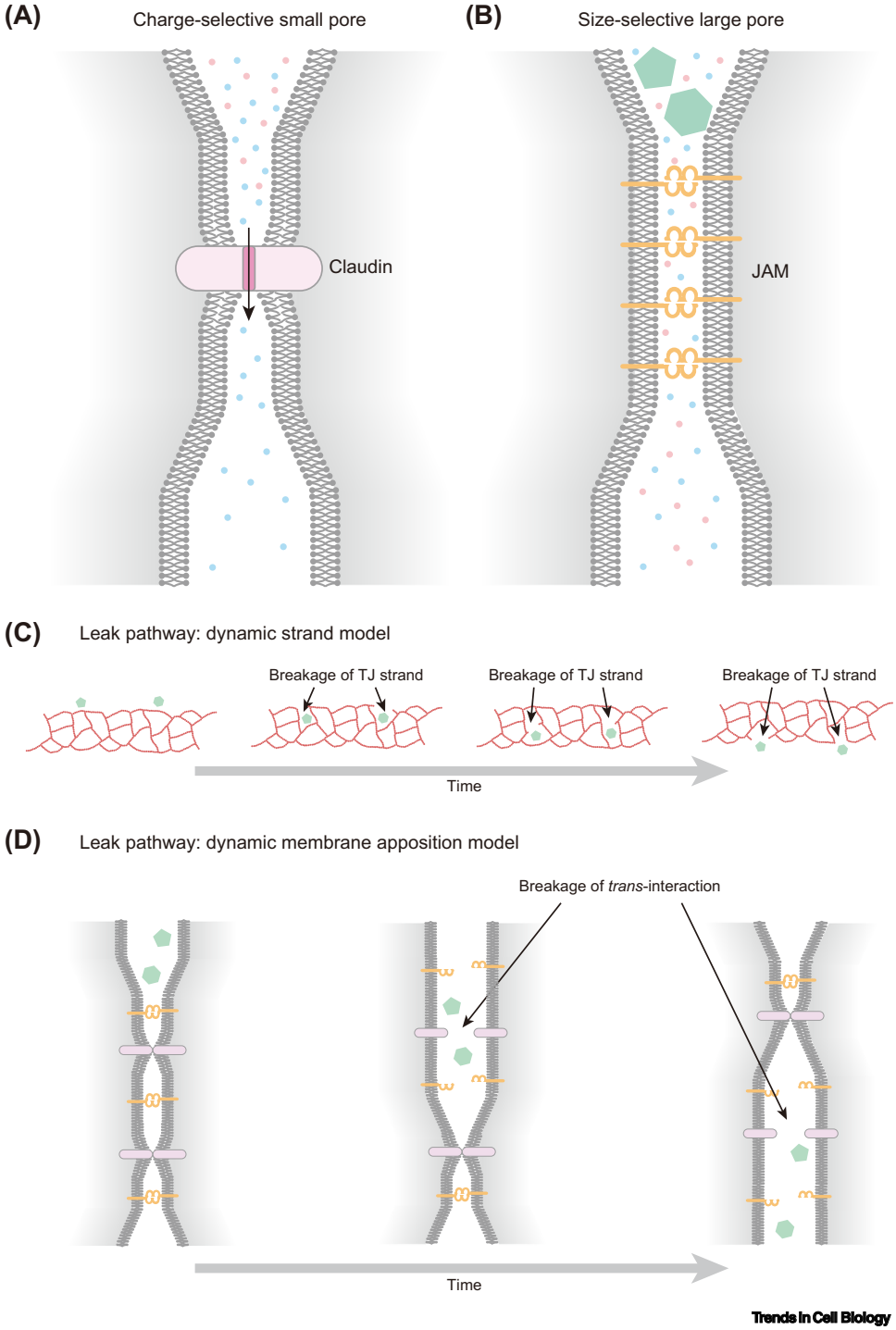

Figure 2. Gate Function of TJs. Paracellular permeability is determined by at least two pathways: charge-selective small-pore pathway with estimated diameter of ~4Å (A) and size-selective pathway permitting permeation of molecules up to ~60Å (B). Pink and blue circles represent electrolytes with negative/positive charge, while green polygons represent macromolecules, such as proteins. Claudins play pivotal roles in the formation of charge-selective small pores, while JAM-

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The discovery of claudins paved the way toward understanding the molecular basis of TJ permselectivity. Claudin-16/paracellin was identified in 1999 as a gene responsible for familial renal hypomagnesemia, suggesting that claudins may control the paracellular conductance of ions [51]. In support of this idea, another study demonstrated that overexpression of claudin-2 markedly increased the ion conductance of epithelia, converting a tight epithelium into a leaky epithelium [52]. Subsequent studies demonstrated that claudin-2 forms cation-selective paracellular channels [53,54]. Site-directed mutagenesis and claudin crystal structure elucidation suggested that the permselectivity is determined by charged residues in the first extracellular domain of claudins [30,31,54,55], and a recent patch-clamp study showed that claudin-2-dependent paracellular channels are dynamically gated [56]. These findings demonstrate that claudins are responsible for forming the charge-selective small-pore pathway that regulates ion conductance (Figure 2A).

Recently, it was reported that claudin quintuple-KO MDCK II cells exhibit disruption of the permeability barrier against ions and small molecules up to 4 kD, but retain the macromolecule permeability barrier [12], suggesting that the macromolecule permeability barrier can form in the absence of claudin-based TJ strands. Similar barrier defects were reported in claudin-5 KO mouse endothelial cells with size-selective loosening of the barrier [57], although the impact of claudin-5 KO on TJ strand formation remains to be characterized.

The question arises as to how the macromolecule permeability barrier can form in the absence of claudin-based TJ strands. JAMs have been implicated in epithelial barrier function [58–61]. Claudin quintuple-KO MDCK II cells and claudin-5 KO endothelial cells retain close membrane apposition [12,57]. When JAM-A was further deleted from claudin quintuple-KO MDCK II cells, the intercellular space was widened and the macromolecule permeability barrier for molecules larger than 4 kD was disrupted [12], demonstrating that JAMs have a critical function in macromolecule permeability barrier formation. Considering the intriguing similarity between the intermembrane distance in claudin quintuple-KO MDCK II cells (6–7 nm) and the pore size of the size-selective large-pore pathway (~60Å), it is tempting to speculate that close membrane apposition acts as a molecular sieve that physically occludes the passage of larger macromolecules (Figure 2B). Consistent with this idea, there are some examples of macromolecule permeability barrier formation in the absence of TJ strands [62–64]. Although adherens junctions (AJs) have been thought to be able to act as a macromolecule permeability barrier [62,64], it is of interest whether JAMs also play some roles. As the intermembrane distance of JAM-mediated contacts and AJs are different, the size-dependency of the macromolecule permeability barrier might be tuned by the adhesion molecules. In addition to JAMs and AJs, occludin and tricellulin have been implicated in macromolecule permeability barrier formation, although the role of occludin is under debate [65–69]. Whether JAM-A directly controls the macromolecule permeability barrier or whether it acts through occludin or tricellulin requires further clarification.

Under inflammatory conditions, actomyosin reorganization occurs and macromolecule permeability increases, and is termed the leak pathway [70–72]. The leak pathway has been proposed to be regulated by transient breakage of TJ strands, designated the dynamic strand model (Figure 2C), emphasizing the importance of claudin *cis*-interactions [4–6]. This idea was

dependent membrane apposition may act as a molecular sieve in the formation of size-selective large pores. Macromolecule permeability increases under inflammatory conditions, known as the leak pathway. The leak pathway may be regulated by transient breakage of TJ strands (dynamic strand model) (C) or transient breakage of *trans*-interactions between TJ-associated membrane proteins, resulting in opening of the intermembrane space (dynamic membrane apposition model) (D). Green polygons represent the macromolecules. Abbreviations: JAM, junctional adhesion molecule; TJ, tight junction.

supported by time-lapse imaging of GFP–claudins expressed in fibroblasts, wherein TJ strand-like structures underwent breakage and reannealing [73,74]. Furthermore, fluorescence recovery after photobleaching analyses showed that ZO-1 dynamically associates with TJs and that myosin activation increases ZO-1 exchange [74–76]. Because ZO proteins are required for TJ strand assembly [10–12], these findings imply that TJ strands are destabilized under inflammatory conditions, and support the concept that the leak pathway could be regulated by actomyosin-dependent changes in the dynamics of ZO proteins, inducing remodeling of TJ strands. However, a recent study showed that although TJ strand formation does not occur, the macromolecule permeability barrier is retained in claudin quintuple-KO MDCK II cells [12], suggesting that other factors besides dynamic remodeling of TJ strands contribute to the leak pathway. Considering the potential importance of membrane apposition in the macromolecule permeability barrier, it could be that the leak pathway reflects transient breakage of the *trans*-interaction sites between neighboring plasma membranes, designated the dynamic membrane apposition model (Figure 2D). In this case, actomyosin contraction may apply contractile force to TJs and induce transient focal widening of the intercellular space, resulting in leakage of macromolecules along the paracellular space.

Fence Function and Epithelial Polarity

As well as the barrier function, another important function of epithelia is transport of substances between the external environment and the internal body. Epithelial transport is driven by an electrochemical gradient generated by transporter proteins localized in a polarized manner. Epithelial polarity is required to generate the electrochemical gradient, while the epithelial barrier is essential for its maintenance. For efficient epithelial transport, it is essential that epithelial polarity and the epithelial barrier are coupled to one another [77]. Classic experiments showed that TJ assembly is closely correlated with epithelial polarity establishment, exemplified by the asymmetric localization of membrane proteins [78–80]. Subsequent demonstration that the membrane lipids in the exoplasmic leaflet cannot freely diffuse across TJs led to the idea that TJs act as a membrane fence that segregates the apical and basolateral plasma membranes (Box 1) [81]. TJs also play important roles in epithelial polarity by acting as scaffolds for polarity signaling proteins, including

Box 1. TJ and Fence Function

Classic studies have demonstrated that some endogenous lipids (Forssman antigen etc.) are restricted to the apical plasma membrane [91,120,121]. Importantly, when fluorescent lipids (N-Rh-PE) are incorporated into the exoplasmic leaflet of the apical plasma membrane, they do not diffuse into the basolateral plasma membrane, depending on the integrity of TJs as shown by low Ca^{2+} treatment [81]. However, when fluorescent lipids are fused into both exoplasmic and cytoplasmic leaflets of the bilayer, diffusion into the basolateral plasma membrane occurs [81]. By contrast, it has been reported that the lack of redistribution of fluorescent lipids may depend on the properties of the probes used [81]. These results led to the idea that TJs act as a membrane fence to restrict diffusion of lipids residing in the exoplasmic leaflet [81].

In EpH4 cells (derived from mouse mammary gland), sphingomyelin clusters, corresponding to the sphingomyelin/cholesterol-rich ordered-phase membranes, are specifically formed in the apical plasma membrane [122]. In ZO-1 KO/ZO-2 KD EpH4 cells which were shown to lack TJs, sphingomyelin cluster formation is not affected, and octadecylrhodamine B added to the apical plasma membrane does not diffuse to the basolateral plasma membrane, suggesting that TJs are dispensable for membrane fence formation [122]. However, in ZO-1/ZO-2 double KO MDCK II cells, Forssman antigen is ectopically found in the basolateral plasma membrane, corroborating the importance of TJs in lipid polarity [12]. By contrast, Forssman antigen is not mislocalized to the basolateral plasma membrane in claudin quintuple-KO MDCK II cells, suggesting that membrane kissing points and TJ strands are dispensable for fence function [12]. The diffusion of fluorescent lipids has not been examined in claudin quintuple-KO MDCK II cells.

Taken together, although the roles of TJs in membrane fence formation have been debated for decades, definite evidence has not been obtained. This is largely due to inconsistencies in the experimental approach – the readout of the fence function, and the method to perturb TJ function. Compared with the classic studies utilizing low Ca^{2+} treatment, it is now possible to specifically interrogate the function of TJs using genome editing, and the importance of TJs in membrane fence formation can be tested by

the **Par-3/Par-6/aPKC complex** and **Crumbs/Pals1/PATJ complex**, while the polarity signaling proteins play crucial roles in TJ assembly, suggesting a reciprocal relationship between TJs and polarity signaling proteins [82–90].

The question of how TJs form a membrane fence also needs to be addressed. Considering the tight apposition of neighboring plasma membranes at TJs and the selectivity of the membrane fence against lipids in the exoplasmic leaflet, an attractive hypothesis is the lipid micelle model (Figure 3A) [23,24]. In this model, it is assumed that the exoplasmic leaflets of neighboring plasma membranes partially fuse with one another, acting as a membrane fence. However, it was shown that apical plasma membrane lipids cannot diffuse across cell boundaries into neighboring cells, thus refuting this model [91].

An alternative model is the protein-based membrane fence. In this model, local clustering of TJ membrane proteins results in membrane fence formation. This model was recently tested by examining the epithelial polarity phenotypes in claudin quintuple-KO MDCK II cells. In these cells, epithelial polarity was not disorganized and an endogenous apical lipid (Forsmann antigen) did not mislocalize to the basolateral side despite the absence of TJ strands [12], suggesting that TJ strands *per se* are not essential for membrane fence formation. This implies that TJ strand breakage and reannealing implicated in the leak pathway do not perturb epithelial polarity. JAMs have been previously implicated in epithelial polarity [92,93], and further removal of JAM-A from claudin quintuple-KO MDCK II cells results in sporadic epithelial polarity defects [12]. Although it remains to be clarified whether membrane fence disruption occurs in these cells, the findings are consistent with the importance of TJ membrane proteins for epithelial polarity. Clustering of TJ membrane proteins can potentially regulate membrane fence formation by several mechanisms, including size-dependent exclusion, molecular crowding, or local assembly of lipid microdomains, which are not mutually exclusive with each other (Figure 3B–D) [94].

The idea that size-dependent exclusion may regulate membrane fence formation is supported by a recent *in vitro* reconstitution study, in which claudin-4 reconstituted unilamellar vesicles formed homotypic adhesive interfaces that excluded membrane proteins with large extracellular domains [95]. It is likely that the close membrane apposition induced by claudin-4 mediates sorting of membrane proteins based on the extracellular domain size [96], suggesting that TJs with close membrane apposition may form a membrane fence against membrane proteins with bulky extracellular domains (Figure 3B). However, size-dependent protein sorting and exclusion cannot explain the membrane fence against lipids in the exoplasmic leaflet. Another possibility is that clustering of TJ-associated membrane proteins induces local molecular crowding, resulting in exclusion of other molecules, including membrane proteins and lipids, from the local membrane domain (Figure 3C). Given the strong accumulation of TJ membrane proteins at TJs, it is possible that TJ membrane proteins induce molecular crowding. However, whether molecular crowding of TJ membrane proteins can efficiently exclude lipid molecules remains to be demonstrated, and it is difficult to explain how molecular crowding can selectively affect the lipids residing in the exoplasmic leaflet. It is also possible that local clustering of TJ-associated membrane proteins induce the formation of a lipid microdomain wherein specialized proteins and lipids accumulate, while excluding other molecules (Figure 3D). Indeed, TJ components were shown to partition into detergent-insoluble raft-like fractions [37], supporting the idea that TJs are specialized membrane domains. However, to explain the selectivity of the membrane fence against the exoplasmic leaflet by the lipid microdomain model, one needs to assume that the lipid microdomain organization is asymmetric between the exoplasmic and inner leaflets, a possibility yet to be experimentally tested.

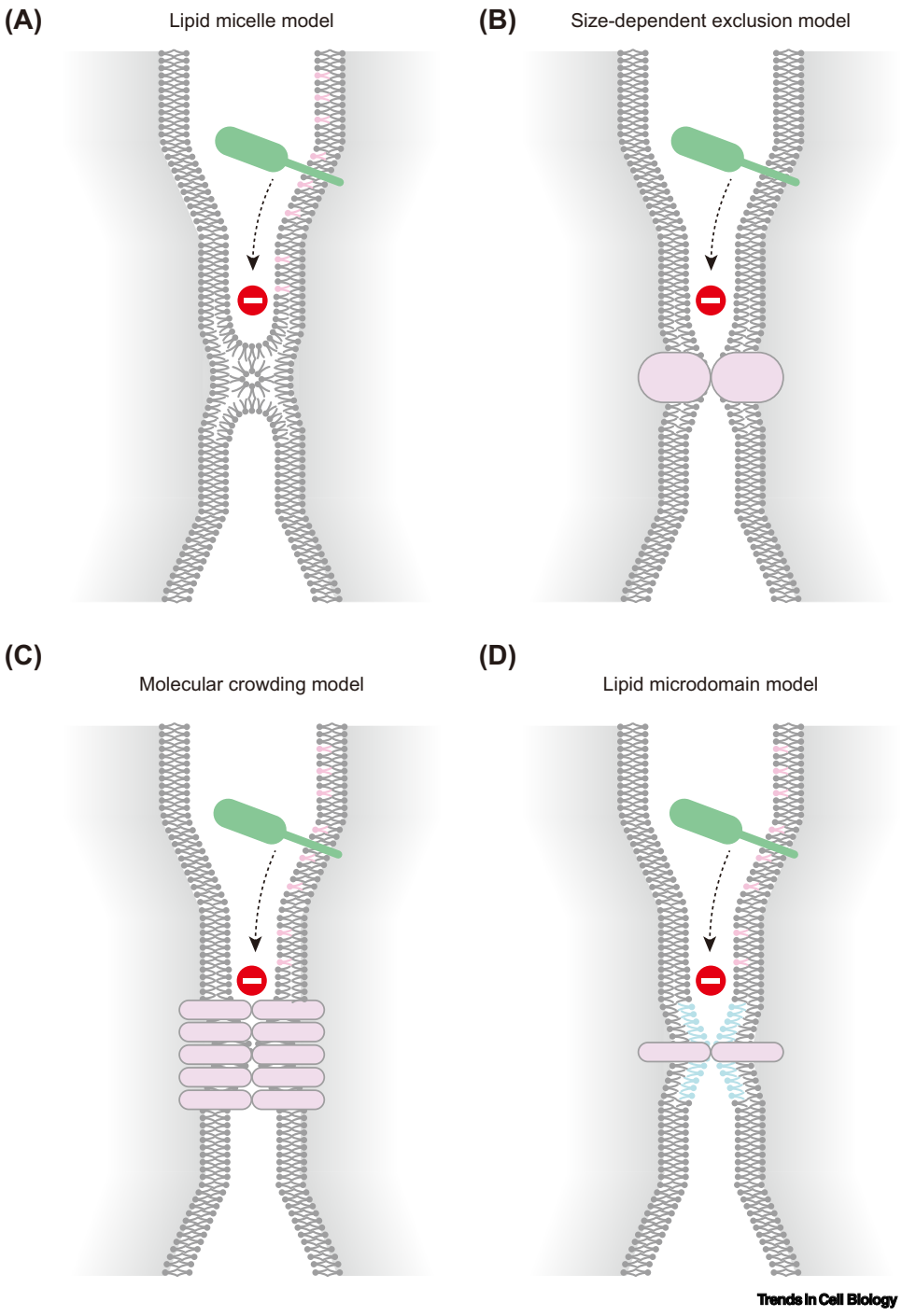

Figure 3. Models for the Fence Function of TJs. (A) In the lipid micelle model, it is assumed that the exoplasmic leaflets of the neighboring plasma membranes partially fuse with one another at TJs. This model can explain the selectivity of the membrane fence toward lipids in the exoplasmic leaflet. Although this model assumes the continuity of the exoplasmic leaflet between neighboring cells, evidence suggests that lipids do not diffuse to neighboring cells, refuting this model. (B) In the size-dependent exclusion model, the close membrane apposition formed at TJs excludes membrane proteins based on the size of the extracellular domain. Due to spatial constraint, membrane proteins with bulky extracellular

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In light of the available data, it is likely that TJ-associated membrane proteins contribute to membrane fence formation. However, whether TJ-associated membrane proteins can form a diffusion barrier against lipids has not been demonstrated, and how the membrane fence can selectively act on lipids in the exoplasmic leaflet remains unclear.

TJ Assembly

TJ assembly has been extensively investigated using the calcium switch method, wherein cells are grown in low-calcium medium and cell–cell junction formation is initiated by increasing the extracellular calcium concentration. In this model, primordial junctions containing AJ markers and ZO-1 [97] are formed after initiation of junction formation. Subsequently, occludin and JAM-A are recruited to the primordial junctions, followed by accumulation of claudin and Par-3/aPKC relatively late during junction assembly [84,97].

ZO-1 and ZO-2 are essential in TJ assembly, because ZO-1/2-deficient cells fail to form TJs [10–12]. The ZO family of proteins are multidomain scaffolding proteins that can form oligomers and interact with TJ-associated membrane proteins, actin cytoskeleton, AJ proteins including α-catenin and afadin, and signaling proteins [9,98–104]. Recent findings have suggested that the ZO family of proteins can form phase-separated droplets that recruit multiple TJ components including claudin and occludin [18,19]. These findings led to the proposal that recruitment of the ZO protein family to primordial AJs may trigger phase separation of these proteins, leading to further recruitment of TJ-associated membrane proteins to drive TJ assembly [18].

TJs are reported to form membrane microdomains [37], and cholesterol and very-long-chain ceramides have important roles in TJ formation [33–36]. It was recently shown that loss of AJs in α-catenin KO EpH4 cells was accompanied by alterations in plasma membrane lipid composition, resulting in endocytosis of claudins [17]. Supplementation of cholesterol to α-catenin KO cells partially restored TJ formation, while depletion of cholesterol in normal cells disrupted TJ formation [17], suggesting that membrane lipids are important for TJ assembly.

Many TJ proteins are palmitoylated and partitioned into lipid raft fractions [37–39]. Clustering of lipid-raft-associated membrane proteins can induce coalescence and stabilization of lipid rafts [105]. Given that ZO **phase condensates** can recruit multiple TJ components [18], ZO protein phase separation might trigger clustering of TJ-associated membrane proteins, which in turn promotes lipid microdomain formation at TJs. Although this is an attractive hypothesis, many questions remain unanswered. For example, although ZO protein phase separation can be regulated by self-inhibition and phosphorylation [18], it remains unclear how the phase separation of ZO proteins is regulated during junction assembly. Furthermore, TJ assembly is a multistep process, given that JAM-A/occludin assembly precedes claudin accumulation during junction assembly [84,97], and it remains to be clarified when and where the ZO protein phase separation occurs

domains are excluded from the TJ region, forming a diffusion barrier. The model can explain the fence function toward membrane proteins with large extracellular domains, but has difficulty in explaining the fence function towards lipids. (C) In the molecular crowding model, clustering of TJ-associated membrane proteins induces local molecular crowding, resulting in exclusion of other molecules, including membrane proteins and lipids, from the TJ region. However, it is not clear how this model can explain the selectivity of the membrane fence towards lipids residing in the exoplasmic leaflet. (D) In the lipid microdomain model, local clustering of TJ-associated membrane proteins induces the formation of a lipid microdomain wherein specialized proteins and lipids accumulate, excluding other molecules. The lipid microdomain may coincide with the TJ strands, or may lie adjacent to the TJ strands. In this model, to explain the selectivity of the membrane fence towards lipids residing in the exoplasmic leaflet, it is required that the lipid microdomain organization be asymmetric between the exoplasmic and inner leaflets, although this possibility has not been tested. Apical transmembrane proteins are indicated in green, while TJ-associated membrane proteins are indicated in pink. Apical lipids are colored in pink, while lipid microdomains are colored in blue. Abbreviation: TJ, tight junction.

and what impact it has on TJ assembly. Moreover, it has been shown that ZO phase condensates can recruit claudin and occludin, but not JAM-A [18], raising the question of how JAM-A is incorporated into TJs.

TJ assembly is regulated by the actomyosin cytoskeleton [104,106,107]. Recently, it has been reported that ZO-1 self-inhibition can be regulated by mechanical force [20] and that ZO protein phase separation is regulated by self-inhibition [18], suggesting that mechanical force plays an important role in TJ assembly. Meanwhile, ZO-1 droplets have been shown to be associated with actomyosin retrograde flow and transported to TJs in zebrafish embryos [19]. Because a basal-to-apical actomyosin retrograde flow has been observed in cell–cell junctions [108], it is possible that ZO phase condensates associate with retrograde flow at the lateral membrane and become transported to TJs. In support of this idea, a recent pulse-chase study suggested that newly synthesized claudins are added to the basal side of TJs [109]. Meanwhile, recent findings have suggested that TJs and the actin cytoskeleton may be loosely coupled [74,110], and further analyses are warranted to examine how TJs and the actin cytoskeleton interact with one another.

While mechanical force could be important for TJ assembly, excess tension can disrupt the epithelial barrier [70–72], suggesting that the degree of tension applied to TJs must be finely tuned. Because various signaling molecules associated with TJs can regulate the actomyosin cytoskeleton [4,21,22,103,104], there could be a homeostatic system that optimizes the level and orientation of the tension applied to TJs. Consistent with this notion, recent studies have indicated that junctional actomyosin belt assembly is augmented when TJs are disorganized [12,102,111–115] and that Rho-dependent actomyosin contraction can repair small breaks in TJs [116].

Polarity signaling complexes also play important roles in TJ assembly [82–90]. aPKC can phosphorylate claudin-4 and JAM-A, and this phosphorylation is required for apical junction localization [117,118]. Given that Par-3/aPKC and claudin accumulation occur relatively late during cell junction assembly [84], the molecular events following ZO-1 accumulation at primordial junctions might result in aPKC-dependent phosphorylation of claudins, triggering TJ strand assembly.

Concluding Remarks

More than 20 years after the discovery of claudins, our understanding of TJ structure and function is still rapidly evolving. A picture is emerging that TJs are macromolecular complexes wherein claudins and other TJ-associated membrane proteins coassemble with the help of ZO proteins, membrane lipids, and mechanical force. In the future, we expect that application of super-resolution microscopy, which has already yielded insights into the organization of reconstituted TJ strand-like structures [74,119], and correlative light–electron microscopy in conjunction with structural biology studies will allow integration of the molecular understanding of TJs with the classic electron microscopy findings to generate a more complete structural model of TJs (see Outstanding Questions). Observation of native TJ strands in epithelial cells, and determining the localization of individual TJ components with spatial precision will deepen our understanding of TJ structural organization. In addition, new insights on ZO protein phase separation and membrane microdomain formation will not only advance our understanding of TJ organization and assembly, but also yield clues for how TJs can generate a membrane fence, how TJ subdomains are formed (Box 2), how TJs and other intercellular junctions separate from one another, and how TJs can be dynamically regulated. Development of new techniques to measure the barrier function with increased solute repertoires and spatiotemporal resolution is highly anticipated for deeper understanding of how the epithelial barrier is dynamically regulated. Moreover, because the electrolyte barrier and macromolecule barrier can be molecularly uncoupled, we expect to

Outstanding Questions

How do TJ-associated membrane proteins and the associated protein network assemble the TJ structure?

How are TJs located at the most apical region of intercellular junctions?

What is the mechanism for TJ subdomain formation?

What roles do claudin-independent macromolecule barriers play *in vivo*?

How is barrier function dynamically regulated?

What is the nature of the membrane fence?

What is the nature of TJ-associated membrane microdomains?

What roles do lipids and mechanical force play in TJ formation?

Box 2. TJ Subdomains

Accumulating evidence suggests that subdomains can form within TJs. For example, claudin-3/16/19 and claudin-10b segregate from one another and form a TJ mosaic pattern in the thick ascending limb of Henle's loop in rodent kidney, wherein individual TJs possess either claudin-3/16/19 or claudin-10b in a mutually exclusive manner [123]. Similarly, in intercellular junctions between sensory and non-sensory cells of rodent inner ear, claudin-14 and claudin-9/6 segregate to form subdomains, with claudin-14 occupying the most apical parallel TJ strands and claudin-9/6 forming the anastomosing TJ strand network at the more lateral side [124]. Recent studies have also suggested that Crumbs/Pals1/PATJ complex also suggested that the Crumbs/Pals1/PATJ complex, which had been thought to localize to TJs, defines a unique apical domain of TJs [125,126].

gain a more nuanced understanding of how the epithelial barrier is regulated in diverse tissues in accordance with their physiology. Finally, further molecular dissection and reconstitution studies combined with biophysical approaches will advance our understanding of how TJ molecules collaborate with the actin cytoskeleton, mechanical force, polarity signaling, and membrane lipids to regulate epithelial barrier function, polarity, and signaling.

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