Differential pathways of claudin oligomerization and integration into tight junctions

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Tight junctions are the critical intercellular structure required to establish an epithelial barrier. Among the several classes of proteins required to form tight junctions are the tetraspan transmembrane proteins known as claudins that directly determine paracellular permeability. Considerable progress has been made in understanding how incorporation of different claudins into tight junctions increase or decrease paracellular permeability and ion selectivity. However, it has proven difficult to identify discrete steps in claudin assembly and whether claudins exist in distinct oligomerization states prior to tight junction assembly. Studies of homomeric and heteromeric claudin-claudin interactions using complementary techniques suggest a diversity of pathways used by different claudins to oligomerize and integrate into tight junctions.

Introduction

Tight junctions regulate the fluid flow between cells across the apical/basolateral axis. Several classes of proteins are required to assemble tight junctions, including single and multi-pass transmembrane proteins, cytosolic scaffold proteins and cytoskeletal associated proteins.[1] These proteins all coordinate to produce a functional paracellular barrier, however, it is claudin family proteins that are central to the regulation of tight junctional permeability.[2-5] This is due to two functional classes of claudins, pore forming claudins which create paracellular ion channels and sealing claudins which more generally restrict paracellular flux.

Although there is considerable literature demonstrating specific roles for different claudins in regulating epithelial and endothelial barrier function, how these proteins are specifically assembled into tight junction strands is less well understood. The scope of this article is to summarize current progress in understanding how claudins become integrated into tight junction strands with particular emphasis on what is known about stable higher order claudin intermediates which form prior to incorporation into tight junctions.

Claudins and Tetraspanins

Claudins have four transmembrane domains with the N- and C-terminus oriented toward the cytoplasm (**Fig. 1**). The N-terminus is short containing roughly 7 amino acids and is readily modified with epitope tags or fluorescent proteins which do not interfere with their assembly or function. The C-terminus is considerably more variable, ranging from 20 to over 60 amino acids. The extreme C-terminus of virtually all claudins has the capacity to bind to the cytosolic scaffold proteins zonula occludens-1 (ZO-1) and ZO-2 via a Post-synaptic density 95, Discs large, ZO-1 (PDZ) binding motif.[6] ZO-1 and/or ZO-2 binding promotes tight junction assembly by tethering claudins to the actin cytoskeleton.[7] However, other classes of scaffold proteins, such as PALS-1-associated tight junction protein (PATJ), have also been found to bind to claudins which further regulate junction assembly.[8,9]

Claudins contain two extracellular loop domains which mediate intercellular interactions and are required for control of paracellular permeability.[10,11] The first extracellular loop domain contains cysteines which likely stabilize its secondary structure, since mutating either or both of these cysteines disrupts the ability of cldn-5 to form functional tight junctions.[11] The second extracellular loop is smaller (~16 amino acids) and has been modeled to form a helix-turn-helix structure.[12] Claudin selectivity is mainly controlled by motifs in the first extracellular loop domain, however, claudin-claudin interactions are determined by both extracellular loop domains.[5,13]

Claudins are tetraspan proteins, however, they are not directly homologous with the proteins directly classified as tetraspanins (e.g., CD9, CD81, CD151).[14,15] In particular, claudins have considerably more protein extending outside either the extracellular or cytoplasmic faces of the membrane as opposed to tetraspanins which are almost entirely membrane embedded.[16] However, claudins and tetraspanins both preferentially partition into cholesterol- and sphingolipid-enriched membrane microdomains (also referred to as lipid rafts), suggesting that they interact with membrane lipids in a comparable manner.[14,15,17-19] This is due in part to the fact that both claudins and tetraspanins are palmitoylated, which promotes their partitioning into membrane microdomains and assembly into higher order structures.[14,15,19]

Tetraspanins have been found to promote the trafficking and assembly of several classes of proteins, most notably integrins and MHC class II proteins.[14,15] Tetraspanin-directed membrane
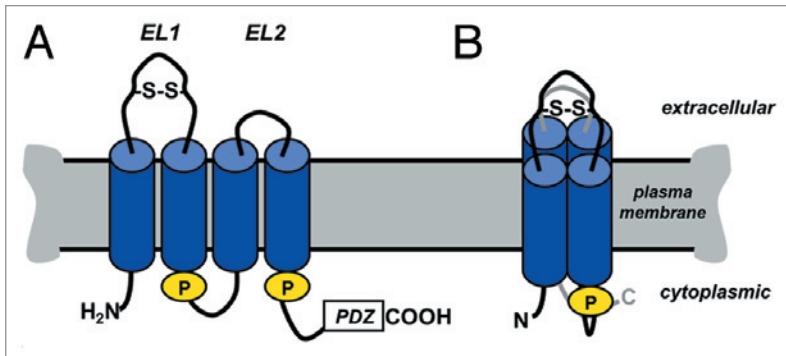
Figure 1. Claudin secondary structure. (**A**) Claudin line diagram showing key features including the two extracellular loop (EL) domains, where EL1 contains a putative di-sulfide bond (S-S). Cylinders represent predicted transmembrane α-helical domains. Also highlighted are two palmitoylation motifs ("P") and the PDZ binding motif at the extreme C-terminus of the protein. (**B**) Claudin conformation in the plane of the membrane, showing the four transmembrane α-helical domains as a tightly packed complex. Adapted from.[4]

assembly is complex, since these proteins interact weakly with each other and with other substrates. Also, tetraspanin-protein interactions are initiated in several distinct intracellular organelles, including the endoplasmic reticulum (ER) and Golgi apparatus. Tetraspanins have also been associated with large-scale
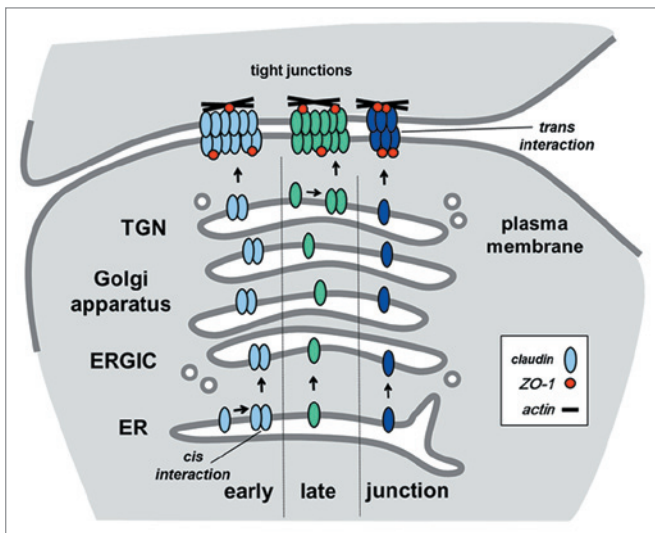
Figure 2. Pathways for claudin oligomerization. In this diagram, the early pathway depicts claudin oligomerization (*cis* interactions) occurring in the endoplasmic reticulum (ER), the late pathway shows oligomerization at the trans Golgi network (TGN). These represent two extremes since oligomerization could also occur in other compartments, such as the ER-Golgi intermediate compartment (ERGIC) or the Golgi apparatus itself. Alternatively, oligomerization could be driven by processes associated with tight junction assembly, including scaffold protein/cytoskeletal tethering and *trans* interactions between claudins on adjacent cells. For simplicity, scaffold proteins are represented by ZO-1, other scaffold proteins also contribute to stabilize claudins by linking them to the cytoskeleton. Also, this diagram only shows homomeric/homotypic interactions, heteromeric and heterotypic claudin-claudin interactions or between claudins and Marvel proteins are expected to follow comparable pathways.

heterogeneous membrane lipid–protein complexes,[14] which are less tractable to biochemical analysis than more classical membrane microdomains.[20-22]

Given roles for tetraspanins in organizing membrane structure, it seems likely that these proteins will participate in tight junction formation as well. Consistent with this hypothesis, proteomic analysis of isolated liver junctions demonstrates that they are enriched for claudin-1, claudin-3 and several tetraspanins including CD81.[23] Consistent with a more direct interaction, claudin-1 associates with the tetraspanin CD81 as part of the hepatitis C virus receptor complex.[24] Moreover, claudin-1 and CD9 partition into comparable membrane microdomains and can be chemically crosslinked, suggesting a direct interaction.[25] Other claudins have been found to weakly associate with CD9.[25]

Another pathway linking claudins to tetraspanins is via the protein Epithelial Cell Adhesion Molecule (EpCAM). EpCAM has several functions and has the capacity to promote cell adhesion, however it does not show homology to Ig-family CAMs and instead has an extracellular domain more closely related to nidogens.[26] There are several lines of evidence that claudin-7 interacts directly with EpCAM and forms a complex with the tetraspanins CD9, CD44v6 and CO-029.[27,28] This complex can also include some claudins (e.g., claudin-1) but not others (e.g., claudin-2, claudin-4) suggesting specific heteromeric interactions with claudin-7 (see below).[29]

EpCAM and claudin-7 tend to be overexpressed by some highly metastatic tumors,[28,30,31] however, EpCAM also has a role in regulating tight junction assembly, since EpCAM deficient mice have decreased incorporation of several claudins into junctions.[32] In fact, EpCAM depleted intestinal epithelial cells show slower rates of barrier formation than cells with normal EpCAM expression.[29] Interestingly, claudin-7/EpCAM complexes tend to be more closely associated with non junctional lateral aspects of epithelial cells although there is some EpCAM associated with tight junctions as well.[29,32] This non-junctional pool of claudin-7 requires EpCAM and has an as yet unknown function. These links between tetraspanins and claudins are intriguing and in need of further study, particularly given evidence that there are distinct pools of membrane microdomains enriched for different tetraspanins that regulate claudin trafficking and assembly.

Heteromeric Claudin Interactions

Claudins on adjacent cells can bind to each other via the extracellular domains across tight junctions, via *trans* interactions (**Fig. 2**). Virtually all claudins have been found to homotypically interact.[33] Heterotypic *trans* interactions have been most conclusively demonstrated to involve claudin-3, which binds to claudin-1, claudin-2 and claudin-5 as well as homotypically to claudin-3 (33–35). There is also some evidence that claudin-1 and claudin-5 may heterotypically interact,[36] although this expected to be weak compared with claudin-3/claudin-5 *trans* binding.[33]

Table 1. Homomeric and heteromeric claudin-claudin interactions

cldn	1	2	3	4	5	6	7	8	9	10a	10b	11	12	14	15	16	17	18	19
1	Y		Y	Y	Y/N*		Y								N				
2		Y	N		Y		N	N											
3	Y	N	Y/N*	Y	w		N*	Y											
4	Y		Y	N*			N*	Y											
5	Y/N*	Y	w		Y													Y	
6														N					
7	Y	N	N*	N*			Y	Y											
8		N	Y	Y			Y	Y											
9														N					
10a										w	w					N*		Y	Y
10b										w	w					N*		w	Y
11												Y							
12													N						
14						N			N										
15	N																		
16										N*	N*					Y/N*		N*	Y
17																			
18					Y					Y	w					N*		w	
19										Y	Y					Y		Y	Y

Summary of cis claudin-claudin interactions using FRET, yeast 2 hybrid assays and co-immunopurification.[29,33,35,36,42,44,47,59,60,75] Y, strong interaction; w, weak interaction; N, no interaction; N*, no interaction by yeast 2 hybrid assay; Y/N*, interaction by FRET, no interaction by yeast two hybrid.

On the other hand, there is ample evidence that several claudins are heterotypically incompatible.[37-39]

Claudins expressed by the same cell also have the capacity to interact in the plane of the membrane via *cis* interactions, which is also known as oligomerization (**Fig. 2**). Heteromeric claudin oligomerization is much more frequent than heterotypic binding. Moreover, not all claudins form homomeric oligomers (**Table 1**). Heteromer specificity has the capacity to create subdomains enriched for specific claudins which, in turn, have the potential to modulate tight junction permeability. Tight junction subdomains can be further stabilized by homotypic interactions across junctions, e.g., through the formation of homotypic heteromers. As a specific example, cells in the reticular lamina of the inner ear have two classes of junctional subdomains, one class enriched for heteromeric claudin-6 and claudin-9 and another enriched for claudin-14, which serve distinct functions.[39] Although tight junctions do not usually have such extreme differences in claudin localization, it is likely that most have fine structure driven by claudin heterogeneity which helps regulate barrier function.

The coordinate interaction between claudin-16 and claudin-19 provides another example of heteromer driven functionality, since mutations in either of these two claudins cause the renal disease Familial Hypomagnesemia with Hypercalciuria and NephroCalcinosis (FHHNC).[40,41] Claudin-16 and claudin-19 hetero-oligomerize to form sodium-selective tight junction pores which provides an indirect mechanism for regulation of magnesium resorption.[42,43] Critically, transport of claudin-16 and claudin-19 are mutually dependent. In the absence of claudin-16, claudin-19 is retained in the perinuclear region of the cell, suggesting Golgi apparatus retention, although this region of the cell may also reflect endosome localization.[44,45] Coordinate trafficking of claudin-16 and claudin-19 is also observed in the intact kidney, indicating that these two claudins form obligate heteromers in order to function.[42] Interestingly, claudin-19 can also hetero-oligomerize with claudin-10a and claudin-18, however, claudin-16 does not.[42] Since claudin-10a and claudin-18 form tight junctions in FHHNC, co-chaperone function of claudin-19 is not required for these claudins to integrate into tight junctions. In addition, claudin-18 forms functional tight junctions in the lung and stomach, which do not express claudin-19.[46] Obligate heteromer formation has also been implicated for trafficking of claudin-4 and claudin-8 in the kidney.[47] However, claudin-4 properly traffics and forms tight junctions in transfected claudin null HeLa cells,[33] suggesting that this may be a unique feature of claudin-4 transport in the kidney. For instance, renal epithelial cells may lack chaperones present in other cell types that are compensated for by claudin-8. Considering that claudin-4 expression is much more prevalent than claudin-8, multiple mechanisms to regulate claudin-4 assembly into tight junctions are likely.

Other examples of claudin heteromers are shown in **Table 1**. Two in situ techniques have been primarily used to measure heteromeric interactions: Fluorescence Resonance Energy Transfer (FRET) and yeast two hybrid assays. FRET has been used to study both homomeric and heteromeric interactions, with particular emphasis on claudins expressed by endothelial cells.[12,36] C-terminal Cyan Fluorescent Protein (CFP) or Yellow

Fluorescent Protein (YFP) tagged claudins used as donor and acceptor fluorophore block binding to ZO-1 and ZO-2, which helps ensure that a direct heteromer interaction is being measured. Also, subconfluent cells or claudin point mutants incapable of *trans* binding enable heteromeric interactions to be distinguished from heterotypic interactions. Using this approach, Piontek and coworkers[36] have found that FRET efficiency differed for different pairs of claudins, ranging from claudin-5 homomers which had a high FRET signal to claudin-3/claudin-5 heteromers which had lower, but detectable FRET. Other pairs, such as claudin-2 and claudin-3 showed no detectable interaction. Moreover, claudin-3/claudin-5 FRET was enhanced as a result of tight junction formation. These data support the model where homotypic heteromers are a major component of fully formed tight junctions.

Hou et al. pioneered the use of a yeast two hybrid assay to assess homomer and heteromer compatibility since yeast do not form tight junctions and thus do not allow heterotypic interactions between transfected mammalian claudins.[42,44,47] This yeast two-hybrid assay was used to show that claudin-16 and claudin-19 hetero-oligomerize.[42] Interestingly, several classes of claudins have been found to be heteromerically incompatible by yeast two hybrid analysis (**Table 1**). These findings are not always concordant with results obtained by mammalian expression systems. For instance, claudin-3 homomers were detected by FRET,[36] yet claudin-3 did not produce a detectable homo-oligmerization signature by yeast two hybrid analysis.[47] The mechanistic explanation for this difference is not known at present. Since membrane microdomains are critical for claudin assembly into tight junctions, one intriguing possibility is that claudin-claudin interactions are sensitive to membrane microenvironment which significantly differs between yeast and mammalian cells.[48,49] In this case, a lack of *cis* interaction in yeasts may reflect claudin specific differences in membrane partitioning and/or conformation. If this is the case, perturbation of lipid composition is likely to have differential effects on different claudins. Consistent with this possibility, cholesterol depletion of Caco2 cells using methyl β-cyclodextrin preferentially inhibited partitioning of claudin-3 and claudin-4 into detergent resistant membrane microdomains as opposed to claudin-1 which was resistant to methyl β-cyclodextrin.[18]

Interactions of Claudins with Marvel Proteins

The Marvel proteins occludin, tricellulin and MarvelD3 represent the other major class of tetraspan proteins associated with tight junctions.[50-52] Several lines of evidence support interactions between occludin and claudins. For example, occludin co-purifies with claudins directly binding to *Clostridium Perfringens* Enterotoxin (CPE).[53,54] Since CPE cannot directly bind occludin,[55,56] a claudin-occludin interaction is required for incorporation of occludin in the complex.[57] Occludin also directly binds to claudin-1 extracellular loop mimetic peptides, suggesting the potential for heteromeric and/or heterotypic interactions between occludin and claudins.[58] In addition, occludin is present in a discrete high molecular weight (mega Dalton) complex

isolated from dodecylmaltoside solubilized liver which also contains claudin-1 and claudin-2.[59]

FRET based assays have also been used to examine *cis* interactions between occludin and different claudins in binary mixtures using transfected HEK cells.[60] In this system, occludin interacts with several claudins to varying degrees where occludin showed a strong *cis* interaction with claudin-1 and a weaker interaction with claudin-2 and claudin-4. Claudin-3, claudin-5 and claudin-11 interacted poorly or not at all with occludin. By contrast, Marvel D3 interacts with claudin-3 to a level comparable with claudin-4.[60] Both tricellulin and Marvel D3 interact with claudin-5 and not with claudin-2 or claudin-11. Interestingly, in a trinary transfected HEK cell system, occludin effectively competes for tricellulin in interacting with claudin-1 which may play a role in directing tricellulin to tricellular contacts.[61,62] Claudin expression also promoted the localization of Marvel proteins to intercellular contacts, consistent with a dual feedback mechanism where these proteins co-assemble into tight junctions.

Taken together these results suggest a role for Marvel proteins in organizing the fine structure of tight junction claudin composition. However the relative stability of these interactions remains to be determined. In particular, studies using Fluorescence Recovery After Photobleaching (FRAP) have shown that claudins tend to be stable components of tight junctions, whereas occludin is significantly more mobile.[63] Thus, occludin/claudin interactions are likely to be more transient than claudin/claudin interactions, particularly in a fully assembled tight junction. This leads to a model where occludin and other Marvel proteins may act as co-chaperones to promote claudin transport through the cell and vice versa which then changes once the claudins are assembled into fully formed tight junctions.

Size of Claudin Oligomers

Biochemical analysis of solubilized claudins suggests that claudins form stable intermediates. Claudin complexes as large as hexamers have been detected by native gel electrophoresis of samples solubilized by perfluorooctanoic acid (PFO).[35,64] Size analysis of solubilized tight junction protein complexes is clearly informative. However, using this approach to analyze claudin oligomerization is confounded by the potential for heterotypic binding and/or stabilization by a shared interaction with another protein, such as occludin or ZO-1. These factors can be partially controlled by using transfected claudin null cell lines, such as fibroblasts, HEK and HeLa cells.[12,33-35]

Critically, oligomer size differs for different claudins and solubilization conditions. For instance, claudin-4 expressed by transfected Sf9 insect cells resolved as stable hexamers when solubilized in PFO,[64] whereas PFO-solubilized claudins from transfected NIH 3T3 cells resolved as either monomers (in the cases of claudin-1 and claudin-4) or trimers/tetramers (for claudin-5).[35] For claudin-1 and claudin-5, 3T3 cells solubilized in 0.1% Triton X-100 and then resolved by native gel electrophoresis produced comparable results. However, claudin-3 expressed by 3T3 cells and solubilized in Triton X-100 appeared to remain as stable tetramers.[35] Moreover, dimers of claudin-1, claudin-2 and

claudin-7 were stable in dodecylmaltoside treated MDCK cells, whereas claudin-4 was completely solubilized under comparable conditions.[59] In this study, claudin-2 was demonstrated to form homodimers, whether the other claudins were homo or heterodimers has not been determined.

Sites of Claudin Oligomerization

Figure 2 represents three potential models for claudin oligomerization, early oligomerization, late oligomerization or oligomerization driven following assembly into tight junctions. The early pathway corresponds to the classical ER quality control pathway for multimeric transmembrane protein oligomerization.[65,66] Consistent with a requirement for claudin quality control early in the secretory pathway, mutant claudins which are misfolded accumulate in the ER, although misfolded claudins more typically are retained in the perinuclear region of the cell, e.g., the Golgi apparatus.[12,45]

Does retention of misfolded claudins reflect control of oligomerization in the ER? This is not likely to be the case. For example, a mutant form of claudin-16 associated with FHHNC (claudin-16-R149L) is retained in the ER but lacks the ability to exert a dominant negative effect on claudin-19 which instead accumulates in the perinuclear region of the cell.[44] This is despite the normal co-trafficking of these two claudins in the healthy kidney epithelium. Another line of evidence comes from an analysis of claudins tagged with an ER retention signal, His-Lys-Lys-Ser-Leu (HKKSL).[4,67,68] Claudin-3-HKKSL and claudin-4-HKKSL are retained in the ER, however neither of these claudins has a dominant negative effect on untagged claudin-3 or claudin-4.[4] By contrast wild type claudin-3 and claudin-4 readily form heteromeric oligomers and tight junctions, indicating that claudin-3/claudin-4 hetero-oligomerization must occur beyond the ER.[33,47]

Given the potential for claudin-claudin interactions in the late secretory pathway, it is useful to compare claudins with another class of tetraspan junction proteins, connexins, which form gap junction channels. Interestingly, connexin43 normally oligomerizes in the *trans* Golgi network (TGN),[69,70] corresponding to the late oligomerization pathway shown in **Figure 2**. Mechanistically, connexin43 monomers are stabilized through an interaction with an ER chaperone protein ERp29.[68] The connexin43/ERp29 complex exits the ER and is transported to the Golgi apparatus where ERp29 dissociates and enables connexin oligomerization to proceed. Although data suggests that claudins do not interact with ERp29,[68] there may be other chaperones which perform a comparable function to stabilize monomeric claudins.

However, in contrast to HKKSL-tagged claudins, connexin43-HKKSL shows a clear, specific dominant negative effect on the trafficking of connexin46 to the plasma membrane.[4,67,71] In this case, connexin43-HKKSL mimics a frameshift mutant connexin43 associated with oculodentodigital dysplasia (fs260) which is retained in the ER and prevents exit of wild type connexin43 from the ER due to a dominant negative effect.[72] Why do HKKSL-tagged and mutant connexin43 act as dominant negatives, while comparable claudins do not? In part, this is due to the fact that the connexin quality control pathway is easily saturated.[73] Thus, ER retained connexins act as dominant negatives by overwhelming ER resident chaperones which enables premature oligomerization to occur.

Moreover, it is well established that connexins form stable hexamers which have been structurally resolved at high resolution.[74] Thus, the inability of ER retained claudins to act as dominant negatives suggests several possibilities. First, it seems likely that claudin oligomers are relatively unstable in situ; this is underscored by the relative difficulty of biochemically isolating claudin complexes due to their instability under a wide array of detergent solubilization conditions.[64] Second, a lack of claudin oligomerization in the ER also indicates that they either do not require chaperones to be stabilized as monomers or do not reach levels of expression that saturate normal ER quality control. Possible control points for claudins which would not be saturable in the ER include a requirement for specific membrane microdomains to support oligomerization or redox control (e.g., regulated formation of extracellular loop disulfide bonds).

Nonetheless, some claudins have been found to stably interact inside the secretory pathway since cells which lack tight junctions or are plated at subconfluent density exhibit formation of claudin oligomers.[47,64,75] However, it seems unlikely that this would occur in the ER. Instead, claudin oligomerization is more likely to happen in the TGN or another late secretory pathway. As described above, given the role of claudin partitioning into membrane microdomains in tight junction assembly and that this largely occurs in the Golgi apparatus, claudin oligomerization via the late secretory pathway seems like the most likely possibility for formation of stable claudin-claudin intermediates. Given the structural diversity of different claudin family members, it is unlikely that all claudins will oligomerize via the same pathway. In fact, different connexins oligomerize in different intracellular compartments depending on their structure,[68,71,76] suggesting the potential for a similar model where different claudins oligomerize via different intracellular pathways.

Summary and Perspective

Claudins play a unique role in regulating paracellular permeability. In order for claudins to effectively function, they need to be properly synthesized, transported to the cell surface and integrated into tight junctions. This includes a requirement for specific interactions with other claudins and other classes of transmembrane and cytosolic tight junction proteins. Unlike many other classes of junction proteins, claudins have proven difficult to analyze via traditional methods. In particular, they are limited in the ability to form highly stable, well defined assembly intermediates tractable to biochemical analysis. This is likely due to the inherent properties of claudins as highly hydrophobic proteins which form large complexes. *Cis* interactions within the complex are likely to be low affinity and dependent on local membrane composition, comparable to tetraspanins. However, once assembled into tight junctions, higher affinity *trans* interactions and scaffold protein binding predominate. Given this, in situ methods have proven useful to identify steps in claudin

processing, with the caveat that they sometimes yield conflicting results. Any discordant results most likely reflect the roles that claudin heterogeneity and cell architecture have in regulating how tight junctions form and function and underscore the need for new approaches to study how claudins interact. For example, super resolution fluorescence microscopy was recently used to demonstrate that claudin-3 and claudin-5 assemble into tight junctions that have fundamentally distinct architecture at 20 nm resolution.[77] Using this approach applied to double or triple labeled samples would help measure claudin fine structure in intracellular organelles and tight junctions.

Moreover, as new claudin mutations associated with human disease are discovered, it is likely that there will be more instances where there will be an induced dominant negative effect as a result of interference with normal claudin transport and assembly. This could either be due to a lack of co-chaperone activity, as is the case in FHHNC or perhaps due to an induced novel interaction, as is the case in other classes of junction-associated disease. Defining stages of claudin olgomerization will likely provide control points with the potential for pharmacologic manipulation which either strengthen tight junction barriers to prevent tissue injury or to temporarily disrupt barriers as a route to drug delivery.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

Acknowledgments

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