

Claudin-Claudin Interactions, Practical Examples

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ABSTRACT

Tight junctions (TJs) are composed of three cell-adhesion molecules (CAMs) which regulate paracellular permeability in epithelial and endothelial cells. Claudins (CLDNs), occludin (OCLN), and junctional adhesion molecules (JAMs). Currently, the comparative strength of the interactions between different CAMs are unknown, and no easily replicable model of a TJ has been created. CLDN-CLDN interactions are responsible for barrier properties of tissues. To address this gap, we resorted to bacterial expression of TJ proteins. These quantitative techniques developed in our laboratory were applied as a proof of concept to the case of the Atlantic salmon (*Salmo salar* or SALSA). During migration between fresh and salt water (smoltification), the TJs in the SALSA skin change CLDN composition-upregulating claudins 3 and 10. Through our measurements, we determined that these two claudins are more capable of maintaining their cell-adhesion strengths in the increased salinity of ocean water. We present other examples where TJ proteins strength can be measured to answer other relevant questions. Our results suggest that quantification of the TJ cell-adhesion is possible and can be used to interpret or further studies the complexity of the TJ.

Keywords: Claudins; Tight Junctions; Blood-Brain Barrier; Atlantic Salmon; Claudin Shifting; JAMs; Occludin

Abbreviations: AJS: Adherens Junctions; TJS: Tight Junctions; JAMS: Junctional Adhesion Molecules; CLDNS: Claudins; OCLN: Occluding; CAMS: Cell-Adhesion Molecules; BBB: Blood-Brain Barrier; CNS: Central Nervous System

Introduction

In living organisms, cell-cell interactions are achieved through proteins with adhesive properties interact via junctions that maintain homeostasis [1]. There are two main cell junctions, those requisite for mechanical properties--adherens junctions (AJs)--and adhesive properties--tight junctions (TJs) [2]. Localized at the apical region of cells, TJs control and modulate paracellular transport and provide adhesive properties [3-5]. TJs are composed of junctional adhesion molecules (JAMs), claudins (CLDNs), and occludin (OCLN). JAMs belong to the immuno-globulin superfamily of proteins and contribute homophilic cell-adhesion properties, and heterophilic interactions with integrin in the leukocyte adhesion cascade [2,6]. JAMs are suspected to act as fail-safe when CLDNs and OCLN interactions have been disrupted, though they contribute little to barrier integrity in the presence of both these proteins [7,8]. CLDNs contain four α -helix transmembrane domains and two extracellular loops [9,10]. CLDNs are responsible for assembly of the TJ as well as permeability properties [11,12]. Like CLDNs, OCLN is a tetraspanin, it is not required for TJ forma-

tion, though knockout in mice yielded several physiological deficits indicating the role of OCLN in stabilization and optimization of barrier function [11,13]. These three cell-adhesion molecules (CAMs) are bound directly to the actin skeleton through the proteins ZO-1 and ZO-2 [7,14]. TJs have been shown to undergo constant remodeling, with OCLN being the most mobile among the three integral proteins [15].

Although all three CAMs play vital roles in the function of TJs, changes in CLDN expression at the TJ have been shown to modulate barrier efficacy most dramatically through direct changes to interactions with the actin cytoskeleton [14,16]. In an organism such as *Salmo salar* (Atlantic salmon), endothelial and epithelial CLDN shifting [17] occurs during smoltification to adjust paracellular permeability as the fish migrates from freshwater to saltwater. This migratory process revealed increased expression of CLDN3 in the kidney [18], and a four-fold increase in CLDN10 in various organs [19]-suggesting that remodeling of TJs occurs throughout the entirety of the salmon to maintain homeostasis in its novel environment. Milatz et. al demonstrated that CLDN3 acts as a paracellular pathway barrier against

small ions of positive or negative charge as well as other uncharged solutes [20], indicating that in the increased concentration of sodium and chloride ions CLDN3 maintains interactions well, maintaining its minimal barrier integrity. CLDN10 has been shown to mediate paracellular reabsorption of Na⁺ and Cl⁻ in kidney proximal tubules [21,22]. Beyond these studies, CLDN interaction characterization in the presence of solutes has yet to be investigated. Perhaps the most important example of a TJ in humans is the blood-brain barrier (BBB). Composed of mostly CLDN5 [23] with minute levels of CLDN1, 3, and 12 [4] the BBB is tasked with preventing large (>400 Da) or charged molecules from crossing through the paracellular space and disrupting the homeostasis of the brain [24].

It has been suggested that peptides, proteins, or immune cells that enter the central nervous system (CNS) through the BBB can cause ischemic stroke, Alzheimer's disease, schizophrenia, and multiple sclerosis among other diseases and disorders [25,26]. In the context of stroke, it has been shown that CLDN1 upregulation and CLDN5 downregulation / redistribution [27] leads to increased leakiness of the BBB. Targeting claudin 1 with siRNA in the BBB post-stroke improved barrier function in mice [16]. Understanding how combinations of CAMs interact could prove crucial to developing therapies to preventing or counteracting the above listed ailments by strengthening the connections in the TJ via JAMs, OCLN, and CLDNs, or loosening the TJs to allow paracellular transport of drugs through the BBB to combat tumors or disorders of the CNS. Creating an effective model of the TJ has been difficult. Contemporaneously, TJ models have been limited to attempts in recreating conditions of the BBB. As stated above, the BBB complicates drug delivery, blocking large/ionized molecules from crossing into the cerebral space. Development of an effective TJ model for the interaction of various proteins could allow for the design of pharmaceutical molecules to efficiently cross the BBB and effectively treat numerous conditions. Perhaps the most popular of these mammalian cell BBB models utilizes a semi-permeable membrane within which cells may be cultured in medium. It has been shown thus far to be one of the cheapest and fastest methods to obtain a model sufficient to test drug-delivery methods to CNS [26].

We propose that the study of TJ interactions may be modeled by *E. coli* engineered to express TJ proteins on their outer membranes. *E. coli* proliferates rapidly compared to mammalian cells, are easier to transform, and are cheaper to obtain. Our *E. coli* method has been shown to be consistent with current literature concerning methods of BBB drug delivery [28].

Materials and Methods

Reagents and Genes

All gene sequences were obtained from <https://www.uniprot.org> and Plasmids were synthesized by TWIST Biosciences (San Francisco, Ca, USA) and cloned in plasmid pET28a (Kanamycin resistance) between NcoI and XhoI, leaving the 6xHis tag to the C-terminus. Salmo salar protein accession numbers: CLDN3 (B5XBK9), CLDN25b (B1H3P1), CLDN10 (B1H3N2), CLDN30 (B1H3P7) and OCLN (A0A1S-3KL48). Human proteins accession number: CLDN1 (O95832), CLDN2 (P57739), CLDN3 (O15551), CLDN5 (O00501), CLDN10 (P78369), OCLN (Q16625), JAM-A (Q9Y624), JAM-B (P57087), JAM-C (Q9BX67), and JAM4 (Q9NSI5). Mus musculus CLDN1 (O88551), and Sus scrofa CLDN1 (A0A287A1F1).

Cellular Transformations

Bacterial strains included BL21 DE3 (New England Biolabs) and MG1655 *E. coli* (<https://www.addgene.org/61440/>). Cell transformations are conducted according to heat-shock protocols. A single colony is taken from the plate and grown overnight in 5mL LB. In the morning a 1:1000 dilution is prepared and grown until OD₆₀₀ = 1.0. IPTG (1mM) is then added to induce overexpression of proteins at room temperature (typically 22 C) [28].

Qualitative Analysis of CLDN Strength

MG1655 cells were transformed to express TJ proteins using the above method and plated on 0.2% agar solution then placed in an incubator at 37 C overnight, allowing the cells to proliferate and migrate across the plate. When mixing combinations of CLDN expressing *E. coli*, 25 µL of each cell type (OD₆₀₀ = 1) were vortexed together and plated on the 0.2% agar plate.

Flow Cytometry Data and Analysis

The iCLASP methodology was performed according to protocols provided by Rollins, et al [28]. Flow Cytometry data (Experimental Slope) were analyzed using SAS software version 9 (SAS Institute Inc., Cary, NC, USA) and the Mixed Procedure method to generate p-values, standard deviation, and standard error and to determine statistical significance. For all experiments, $\alpha = 0.05$. Data was collected for each sample in four different experiments (n = 4). Each condition was measured in 12-replicates. Thus, for each, data points correspond to the average of 12-replicates and n = 4, or 48 data points. Statistical differences were identified for all samples in each graph. All samples are run in 12 replicates the day of the experiment, and each experiment was performed at least three times. In each graph (Figures 1-4) all values were different from each other, with $p < 0.03$.

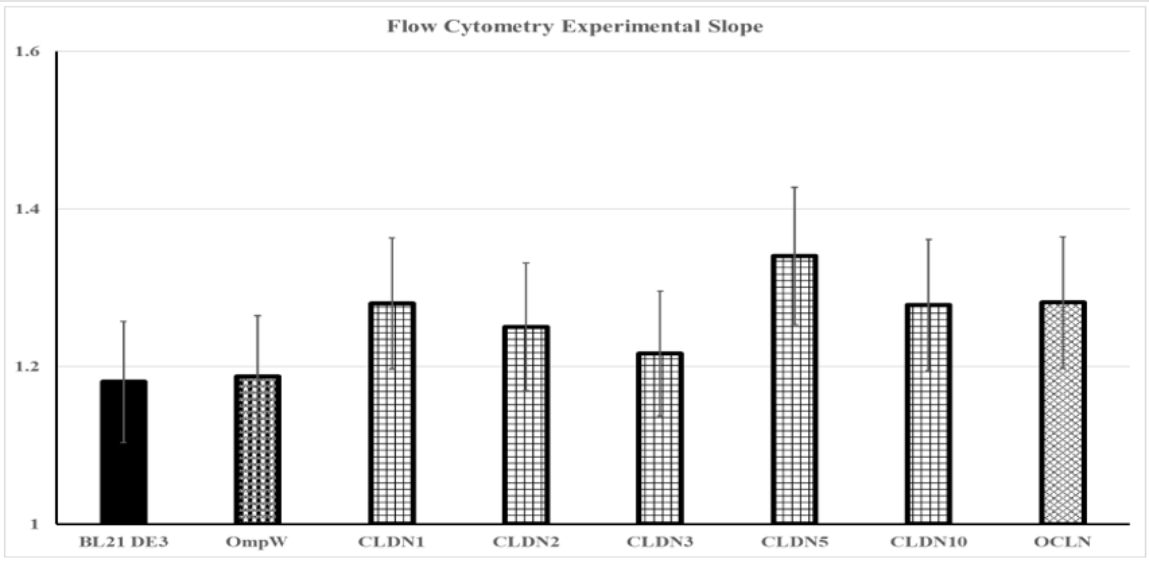


Figure 1: BL21DE3 cells expressing OmpW (control), or a TJ protein (CLDN1, 2, 3, 5, 10, OCLN) were subjected to Flow Cytometry and experimental slopes representing levels of cell aggregation was measured (larger slopes indicating greater levels of aggregation).

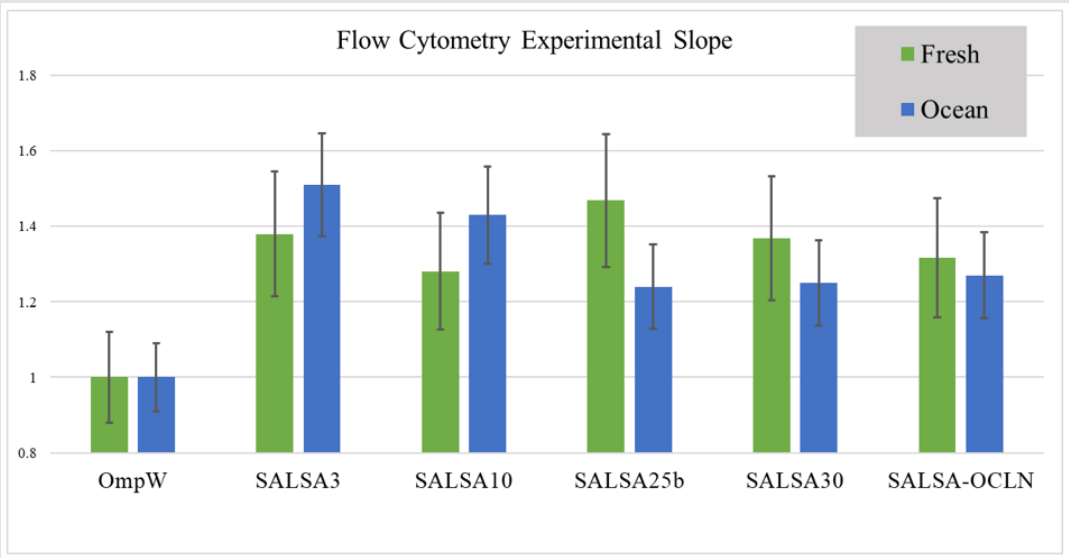


Figure 2: Flow cytometry results of CLDNs in both Fresh (green) and Ocean (blue, 0.48 M NaCl) PBS buffer solution. The experimental slope of BL21 DE3 cells expressing OmpW fused to the corresponding Atlantic Salmon claudin protein or occludin. This graph shows how sensitive these proteins are to a higher salt concentration as well as their strength under each environment.

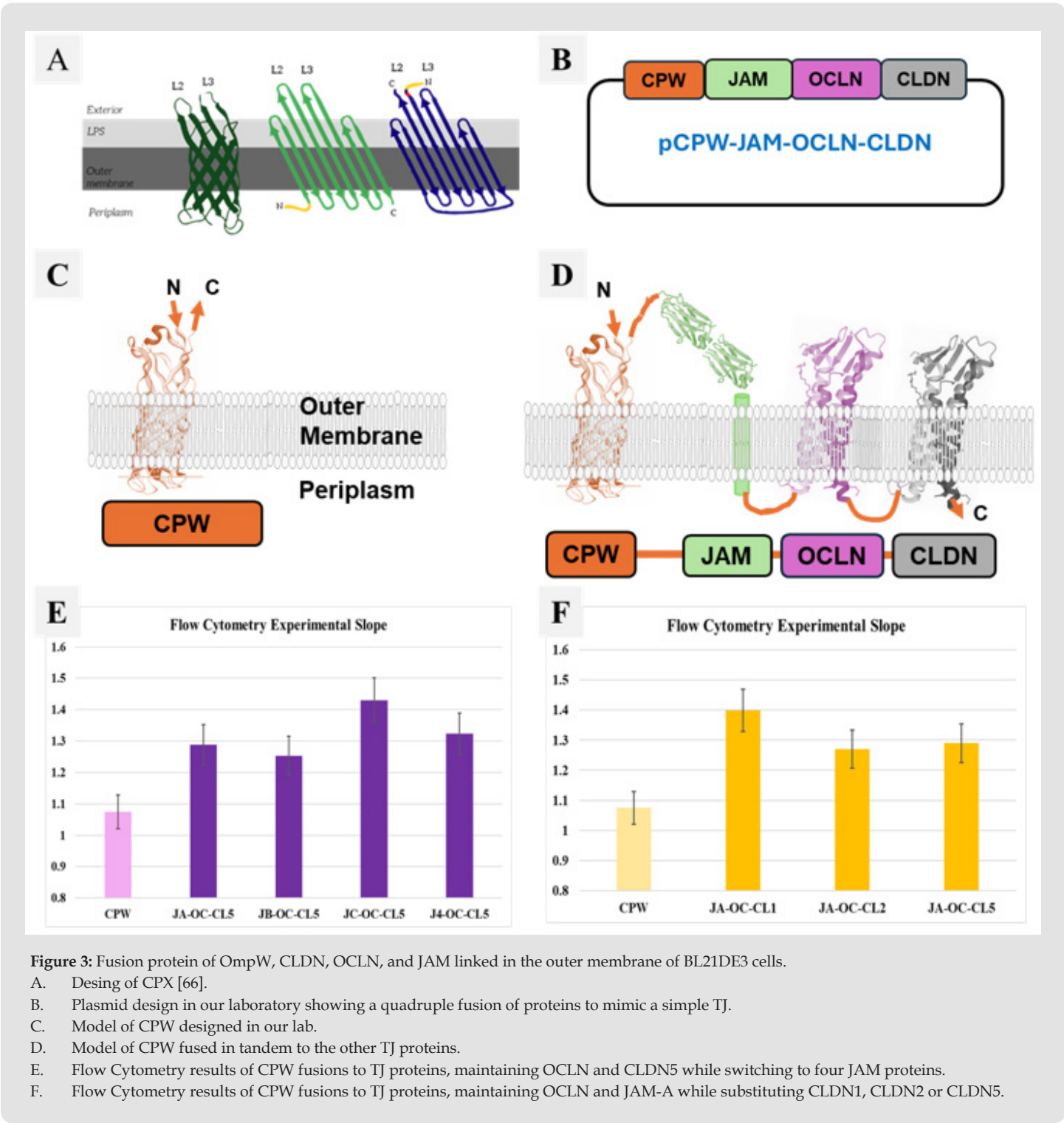


Figure 3: Fusion protein of OmpW, CLDN, OCN, and JAM linked in the outer membrane of BL21DE3 cells.

A. Design of CPX [66].

B. Plasmid design in our laboratory showing a quadruple fusion of proteins to mimic a simple TJ.

C. Model of CPW designed in our lab.

D. Model of CPW fused in tandem to the other TJ proteins.

E. Flow Cytometry results of CPW fusions to TJ proteins, maintaining OCN and CLDN5 while switching to four JAM proteins.

F. Flow Cytometry results of CPW fusions to TJ proteins, maintaining OCN and JAM-A while substituting CLDN1, CLDN2 or CLDN5.

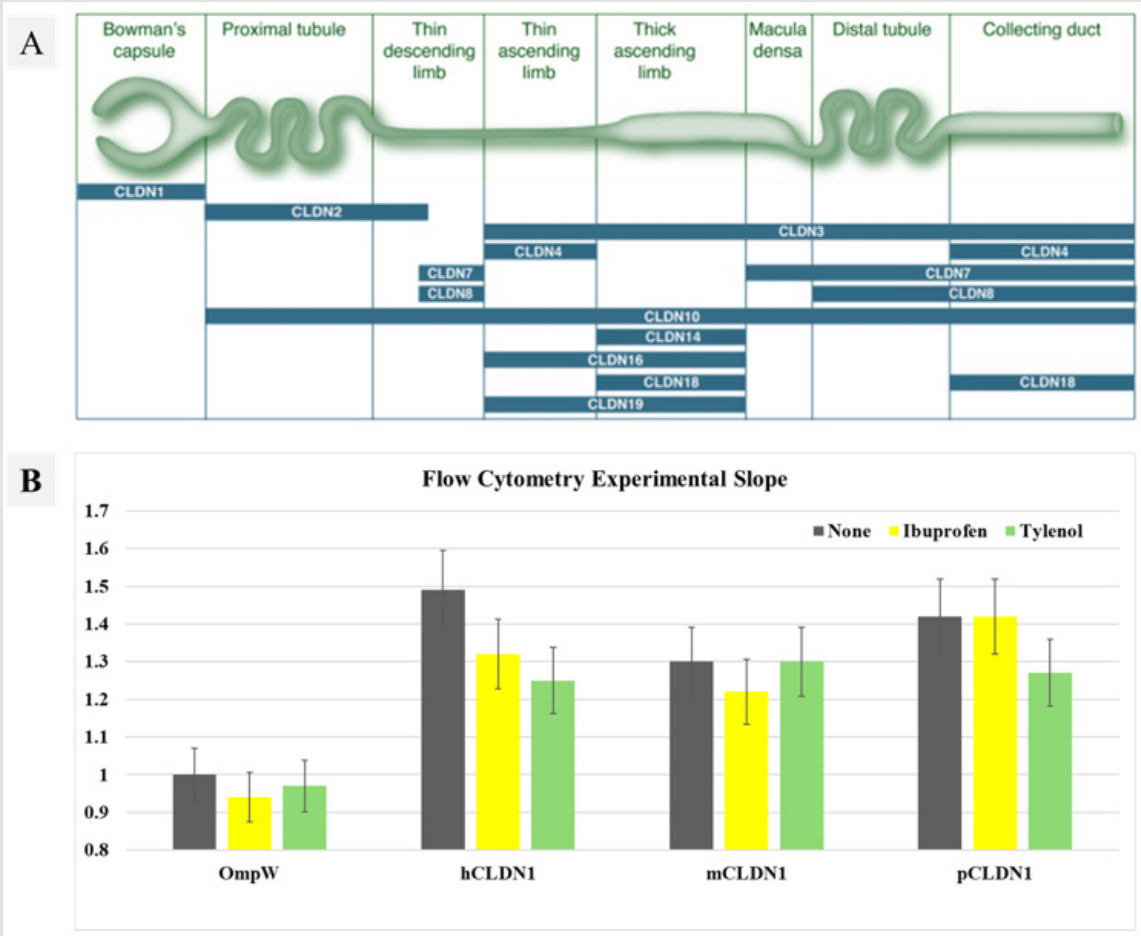


Figure 4: Fusion protein of OmpW and CLDN homologs in BL21 DE3 *E. coli* cells.
A. Distribution of CLDNs in the main segments of the nephron.
B. Flow Cytometry results of OmpW fusions to CLDN1 from human, pig or mice. Experimental slopes are calculated when cells are exposed to Ibuprofen and Tylenol.

Results and Discussion

Expression of TJ Proteins in *E. coli* Outer Membrane

As performed by Rollins et. al, we sought to express TJ proteins on the outer surface of *E. coli* cells, endowing them with TJ properties and aggregation abilities [28]. We utilized OmpW, an outer membrane protein of *E. coli*, as our fusion point for addition of the TJ proteins. OmpW, like claudins and occludin, involves four extracellular loops.

These loops form a β -barrel with both the N and the C termini located in the periplasm [28,29]. This beta-barrel formation is implicated in virulence of active strains of *E. coli* [29,30]. As TJ protein N and C termini also end in the cytosol of mammalian cells [28], we engineered a hybrid protein linking the C terminus of OmpW to the N terminus of either claudins, occludin, or JAMS. This method would expose the adhesive domains of these TJ proteins to the environment outside the cell, allowing aggregates with neighboring cells to form as seen in Figure 5 [31,32].

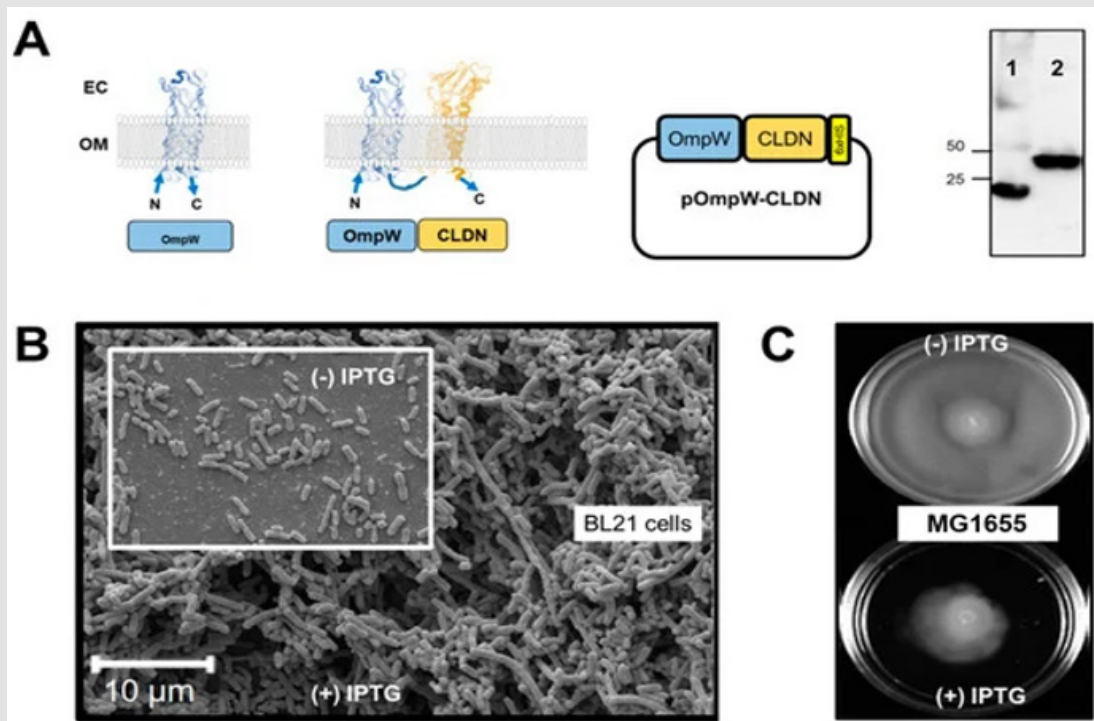


Figure 5: CLDN expression in BL-21 *E. coli*

A. OmpW has both N and C terminus in the periplasm, making it a suitable option for combination with TJ proteins such as JAMs, OCLN, and CLDNs. Plasmids were synthesized with a c-terminus his tag. Anti-his antibody western blot of 1-OmpW and 2-OmpW-CLDN.

B. The background Electron micrograph of BL-21 *E. coli* overexpressing the OmpW-CLDN fusion protein in the presence of IPTG. The forward photograph is *E. coli* with the hybrid vector sans IPTG presence. Below is a photograph of wildtype *E. coli* cells. Wildtype *E. coli* is capable of forming aggregates of 2-20 cells during transfer of DNA between cells, though These aggregates disperse after mating has occurred [31,32]. Comparison with the micrographs of cells expressing the OmpW-CLDN vector demonstrate its efficacy in creating multicellular globular aggregates.

C. MG1655, a motile variant of *E. coli*, growing on plates of LB + 0.25% agar. The top image contains MG1655 cells after 24 h of growth, from a single drop of cells at OD₆₀₀ = 1, placed at the center of the plate. In contrast, and under identical conditions, when protein expression (OmpW-CLDN1) is induced (1 mM IPTG in the LB+ agar plate), the cells' horizontal expansion is reduced in a 24-h period. The plate seems to indicate that aggregation of the cells, induced by expression of OmpW-CLDN1, prevents them from displaying their full mobility, and thus cannot advance beyond a small radius from the center. (Reproduced with permission from Rollins et al [28]).

Qualitative Analysis of CLDN-CLDN Interaction Strengths

As a preliminary analysis of CLDN-CLDN interaction strength, CLDN proteins were expressed on the outer membranes of MG1655 *E. coli* cells—a motile variant of *E. coli* [33]. After 24 hours of growth from a single drop of cells at OD₆₀₀ = 1, 1 mM IPTG was added to induce overexpression of the CLDN proteins, and 5 μ L of cells were placed in the center of a plate composed of LB + 0.2% agar solution. The plates were then incubated at 37 C, over a 24 h period. TJ-like interactions between cells stimulated the formation of aggregates in the center of the plates and prevented cell motility. This procedure was performed with a wildtype MG1655 cell control, OmpW, OCLN, and CLDN1, 2, 3, 5, and 10. Results may be seen in Figure 6. As expected, lack of a

CLDN or OCLN protein expressed on the outer membrane of *E. coli* cells allowed for maximum cell motility. CLDN3 expressions yielded a similar result as the controls. The complete spread of cells throughout the plates with no grouping of cells in the center would indicate that CLDN3 likely does not possess strong cell-cell adhesion properties. This explanation is supported by the findings of Agarwal et al, that ovarian cancer cell motility is increased with overexpression of CLDN3[34]. The lack of adhesive properties of overexpressed CLDN3 would increase cell motility and allow for metastasis. According to CLDN3 and 4, although both containing highly conserved extracellular loops show no homophilic interaction capability, further indicating that CLDN3 likely does not serve as strong cell-adhesive role in the TJ [35].

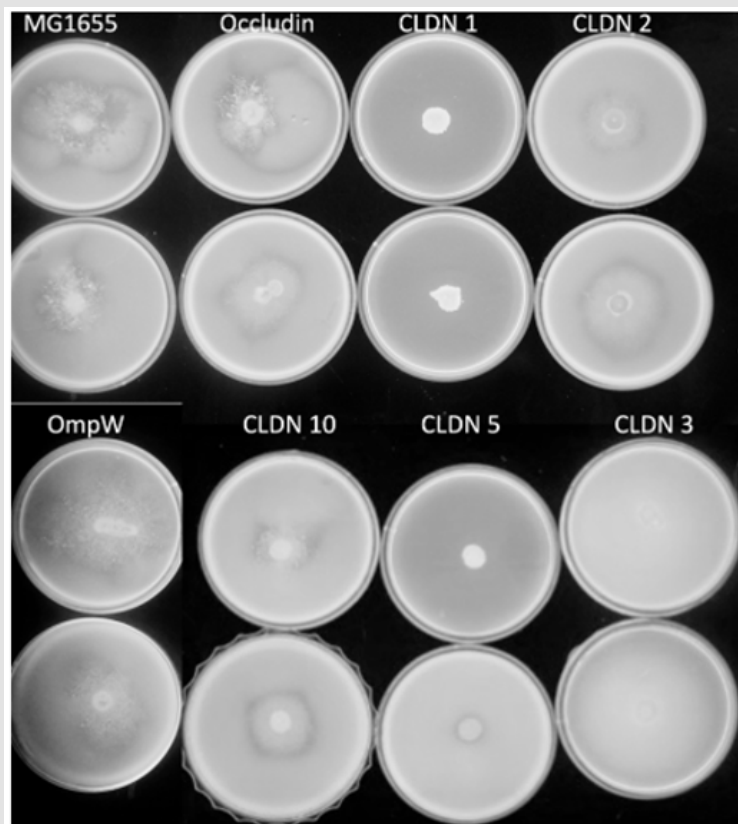


Figure 6: Overexpression of OmpW-fusion proteins in MG1655 *E. coli* cells. Controls were wildtype MG1655 cells, and cells expressing only OmpW. CLDNs 1, 2, 3, 5, and 10 as well as OCLN were analyzed according to the method described in paragraph 2.2.

CLDN2 appears to have greater barrier-formation properties than CLDN3, but not on a comparable scale to CLDN1 or 5. CLDN2 functions in leaky epithelial cells and contributes to positively charged ion and water channels to the epithelium [36-38]. CLDN2 deficient mice have decreased bile flow from the removal of paracellular water channels provided by CLDN2, leading to the formation of gallstones [39]. CLDN10 appears to have moderately adhesive properties. Because this protein functions largely in regulating ion permeability via highly specific channels [40,41] it is intuitive that cell-adhesion would not be its main function, though it still provides more barrier integrity than both CLDNs 2 and 3. The four-fold increase in expression of this protein during smoltification of the Atlantic Salmon would indicate the need for more ion channels in various organs, and likely not for any barrier purposes. CLDNs 1 and 5 have very similar strengths, as suggested by virtually no cells visible outside of the center aggregate. CLDN1 is found in various integral barriers of mammalian organs such as the skin, the lungs, the duodenum, and the BBB [42-44], demonstrating its important role in the maintenance of several epithelial TJ barriers. CLDN5 is mostly known for its involvement as the main proponent in the BBB of both barrier formation and integrity [23,45,46]. The strength of the CLDN5 interaction suggests that adhesion and barrier integrity are likely a central purpose of the protein.

Qualitative Analysis of Inter-Claudin Interactions

CLDN2 + CLDN10: Based on the results of the figure above, CLDNs 2 and 10 interact favorably, allowing the cells to form an aggregate in the center of the plate. This combination of CLDNs is found in the proximal tubules of the kidney [47,48]. CLDN2 primarily functions to form cation and size selective pores of 6.5-7.0 angstroms in diameter [49]. CLDN10 has two splice-variants in the proximal tubule, 10a and 10b. CLDN10a regulates anion selectivity while 10b regulates cation selectivity [22]. Curry et. al demonstrated via a thiol-reactive pore blocker, that inhibition of cation permeability through CLDN2 channels did not affect anion permeability via CLDN10 pores, showing that these channels operate independently and in parallel of one another [50]. It is evident that although these proteins do not explicitly affect one another's function, they are able to interact well enough with each other to prevent cell motility in MG1655 *E. coli* cells.

CLDN3 Combinations: The addition of cells expressing CLDN3 to cells expressing other CLDN proteins showed similar results across each trial. No significant interactions were present, either with other TJ proteins or with itself. CLDN3 is co-expressed with CLDN2 in the proximal tubule of the kidney, and individually expressed in separate portions of the descending thin limbs [50]. CLDN3 is also located in

alveolar epithelial tissue. Upregulation of this protein has shown to decrease adhesive-barrier function [51,52]. By these studies and our results, we may conclude that CLDN3 likely does not hold a barrier role and decreases barrier efficacy in conjunction with other CLDN proteins, which conclusion was made by Furuse, et al. [53].

CLDN1 and CLDN2 Combinations: This combination of CLDNs is found in several locations. As seen in Figure 7, the combination appears to have favorable cell-adhesive properties. The literature supports this conclusion. Found in the epithelial cells of the Choroid plexus, these proteins assist in forming the blood-cerebrospinal fluid (CSF) barrier [54]. Re-arrangement of the tight junction in autoimmune

encephalitis does not occur, indicating a regulatory role for barrier function. CLDN1 and 2 combinations are also found in Bowman's capsule, with CLDN1 being integral in forming crescentic lesions in glomerulonephritis. It is thought that these strong TJs form to prevent interstitial damage caused by the penetration of filtrates through Bowman's space [47]. Upregulation of CLDNs 1 and 2 in the bowels has shown to be indicative of ulcerative colitis [55], though it is not known if this upregulation is a potential cause of the disease [56], or if it serves as a compensatory mechanism to combat the chronic inflammation [57]. We suggest that CLDNs 1 and 2 interact favorably with each other in the context of cell-adhesion and will likely continue to be localized to adjacent tight junctions in the body.

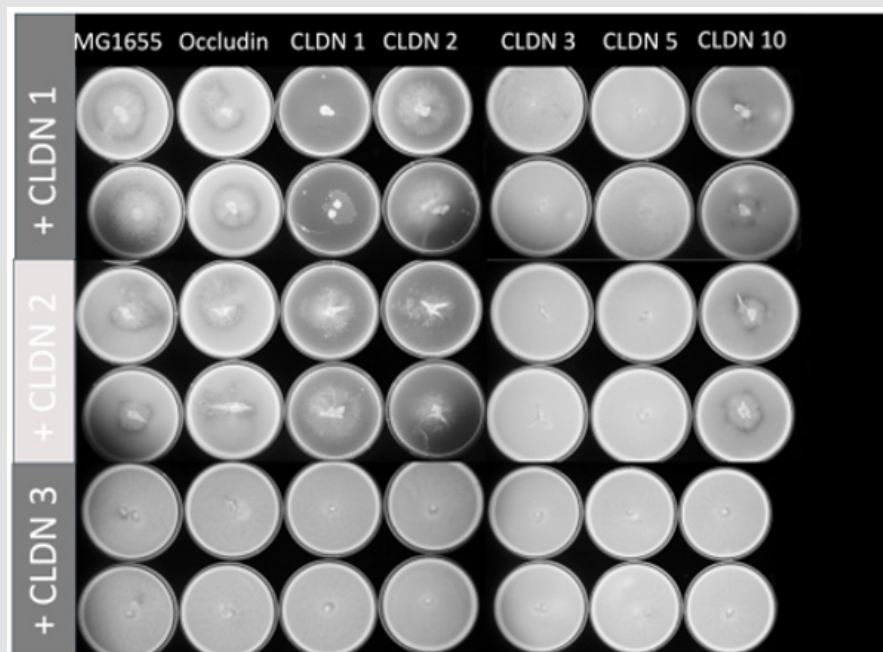


Figure 7: Mixtures of MG1655 *E. coli* cells expressing different fusion proteins OmpW-CLDN proteins. The presence of large aggregates in the center of the plate indicates strong intercellular protein-protein interactions.

Human CLDN1 and CLDN5 Combinations: The BBB in humans is composed of TJs involving CLDNs 1, 3, 5, and 12, –CLDN5 being the most expressed [4,23]. During various CNS pathologies CLDN1 is upregulated, and CLDN5 is downregulated [16,25], leading to BBB chronic leakiness. Targeting CLDN1 with siRNA has proven effective in reducing leakiness [16]. From the results obtained in the figure above, combinations of CLDN1 and 5 reveal no visible interactions. Our results support the finding that upregulation of CLDN1 in a predominantly CLDN5 domain leads to chronic barrier leakiness.

CLDN2 + CLDN5: CLDN2 and CLDN5 hold vastly different roles, with CLDN2 being a pore-forming protein while CLDN5 is a sealing protein. Our results show little to no interaction between the two proteins, seen by the complete covering of the agar plate by MG1655

cells. CLDN2 and CLDN5 are often found expressed in similar locations such as in the gut-vascular barrier. Upregulation of CLDN2 and downregulation of CLDN5, however, is implicated in the decrease of barrier integrity of Crohn's Disease [58,59].

CLDN1 + CLDN10: CLDN1, like CLDN5, functions as a sealant against paracellular transport [59]. CLDN10 functions as an ion channel [60]. These two TJ proteins are found in the biliary tract, although no studies have been done regarding their interactions, and their expression does not seem to affect one another [61]. Our results indicate independence between the two proteins, with a grouping of CLDN1 *E. coli* in the center of the plate with cells expressing CLDN10 spread along the plate periphery.

Flow Cytometry of CLDNs

For a more quantitative analysis of CLDN strength, we used the flow cytometry method as developed by Rollins, et al. [28]. BL21DE3 *E. coli* cells were transformed to express OCLN or a CLDN protein on the outer membrane, then run through the flow cytometer. Results are shown in Figure 4. The findings from this trial confirmed the indications of the qualitative trials performed with MG1655 cells in Figures 6 & 7. Homophilic CLDN interaction strength is as ordered: 5>1>10>2>3. OCLN displayed a strength similar to that of CLDN1. Furthermore, the Atlantic Salmon smoldering was used as an example to determine how adhesion would be affected by salt concentrations similar to ocean salinity. PBS buffer was mixed with 0.48 M NaCl and the experiment was performed again with this new solution. All aggregate sizes were significantly reduced except for claudins 3 and 10 (Figure 2). This finding is supported by the literature, again indicating that CLDNs 3 and 10 function as ion channels, and are often found in locations where ions must be absorbed into the body or excreted. Further studies must be done to determine why CLDN2 was affected the way that it was, as it and CLDN10 function together as ion channels in the proximal tubule of the kidney [62]. We prepared the samples in PBS to represent Fresh water, where the salt concentration was low (125 mM NaCl), while the Ocean water was represented by PBS with 0.48 M NaCl added. Only CLDN3 and CLDN10 were insignificantly affected by the presence of increased salt concentration, resulting in increased strength of CLDN-CLDN interactions.

Our results correspond to the previously reported changes observed in the Atlantic Salmon during smoltification [18,19]. The physiologic TJ plasticity reminds both of the adaptability of claudin expression and its gene specific retention in the TJ composition; this process has been described as claudin switching [63,64], and it has been observed in inflammatory bowel disease (IBD) [65]. In an effort to understand the complexity of TJs, where all three CLDN, OCLN and JAM proteins are expressed at the same time, we resourced to a previously engineered outer membrane protein. Circularly permuted OmpX (CPX) is a membrane protein derived from the outer membrane protein OmpX of *E. coli*. CPX is created by circular permutation of OmpX, which places the N and C termini on the outside of the bacterial cell (Figure 3) [66]. We modeled OmpW after CPX to produce CPW. To this protein we fused a JAM protein, followed by OCLN, followed by a CLDN protein (Figure 3). To further establish the significance of these results we transformed the BL21DE3 *E. coli* cells to express CLDNs, occludin, and JAMs of the TJ simultaneously on the outer membrane of the cells. As all three of these proteins contain intracellular loops terminating in the periplasm [28], we engineered a hybrid protein connecting all three proteins, as shown in Figure 3, and anchored them to the outer membrane of the *E. coli* cells by the C-terminus of the OMP-W protein. Flow cytometry was performed (Figure 3) and compared to the CLDN alone results. JAM A is localized to the BBB while JAMs B and C have been found in endothelial cells [67,68].

Breakdown of the BBB integrity has been shown to be correlated with a decreased expression of JAMs with the greatest impact being JAM-C in the endothelial cells of the BBB [67,68] (Figure 3E), suggesting that the strongest TJ combination consists of JAM C, CLDN5, and occludin. Finally, we maintained JAM-A and OCLN as a constant and switched the CLDN protein to be 1, 2 or 5. These results (Figure 3F) hinted at the idea that although CLDN5 is estimated to be the strongest CLDN of the family [69], its strength might be modulated by the presence of the other TJ proteins, a fact that has not been studied well due to the lack of experimental tools. A final example afforded by iCLASP is the opportunity to examine CLDN homologs among different species. The expression of CLDN proteins varies (another example of CLDN switching) among the different segments of the nephron [49,70]. It is believed that each CLDN may enable the permeability or reabsorption of water and ions as needed by that segment. Recently biotechnology and medicine have made giant leaps toward the use of pig kidneys for human transplantation [71,72]. One question that may arise soon is whether the homologs of the CLDNs in the kidney behave similarly in a way that they can match the physiological functions of the human kidney. Another area where homologs of CLDNs may play a role is in the use of animal models for pharmaceutical trials [73].

The concerning issue with using mice and rats in clinical trials is the mismatch of their physiology and biology compared to humans, reducing considerably the translation of those results into human reactions, leading to inaccuracies in predicting how a drug or treatment will work in humans, side effects, etc. causing drugs to fail in human clinical trials. TJs in the kidney vary in complexity based on the segment's function. The composition of CLDNs in the different segments have been studied in the human kidney (Figure 4A) [70]. A recent publication describes the burden on animal trials and the fact they contribute to over 90% of clinical drug development failure [74]. In Figure 4 we explored incubating homologs of CLDN1, the main CLDN related to filtration of blood at the Bowman's capsule, with 1 μ M Ibuprofen or Acetaminophen (Tylenol). Acetaminophen (Tylenol) and Ibuprofen may have toxic effects in the kidney under certain overdosing events [75] and may serve well here to illustrate the point of molecular differences in the nephron based on CLDN1 homologs. Figure 4B shows that in the absence of any compounds the strength of these CLDNs is ordered as Human>Pig>Mouse. Figure 4B also hints that sensitivities are different among the homologs. Mouse CLDN1 is insensitive to Tylenol. Pig CLDN1 is insensitive to Ibuprofen.

Human CLDN1 is sensitive to both Tylenol and Ibuprofen. Some of the differences in strength might be expected if we consider that in mice the average hourly mean arterial blood pressure ranges from 105 to 115 mmHg and that average daily heart rate ranges from 594 to 665 beats per minute [76]. Having stronger CLDN1-CLDN1 interactions could result in hypertension in mice. In the pig for example, direct systolic pressures between 73 and 230 mmHg and diastolic pressures between 52 and 165 mmHg were measured [77]. Such variability could also be regulated at the molecular level by the pig's

physiology at the kidney, heart, and lungs. That knowledge is currently unavailable. The differences in behavior of the CLDN1 homologs when incubated with Ibuprofen or Tylenol is also indicative of physiological differences that may not be manifest during clinical trials but only after, when the drugs tested reach their intended target. In the event pig kidneys become a reality for xenotransplantation it would be advisable to test toxicity for this newly acquired organ and the host's physiological consequences.

Conclusion

TJ protein-protein interactions currently have not been experimentally analyzed but have been inferred based on in vivo experimentation and observations. We propose that the qualitative MG1655 *E. coli* method and the quantitative BL21DE3 *E. coli* method iCLASP may be used as inexpensive and high throughput methods to easily analyze interactions between TJ proteins under various stimuli. Endowed with these tools we found that CLDNs 1 and 5 appear not to interact well with each other, compromising barrier integrity of the BBB under pathologic circumstances as described in the literature. We found that in the Atlantic Slamon the barrier integrity of CLDNs 3 and 10 is unaffected by the ocean water's salt concentration, giving an explanation of the selection of these CLDNs during smoltification. Finally, we shed light on the use of iCLASP to study homologs of TJ proteins and explore consequences of disregarding these potential differences during drug discovery clinical trials or future xenotransplantation. These methods proved effective in the study of TJ protein-protein interactions and grant further effort in their study.

Availability of Data and Materials

All data is included as part of the article. Materials may be found as part of the materials and methods portion. Any further questions may be referred to the corresponding author JH or DM.

Author Contributions

JH and DM designed the research study. JH, KW, TL, and DM performed the research. DM and JH interpreted the data. JH wrote the manuscript. All authors made editorial changes and approved the final manuscript. All authors participated sufficiently in the work and have agreed to be accountable for all aspects of the work.

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Conflicts of Interest

The authors declare no conflicts of interest.

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