Claudin-3 and Claudin-5 Protein Folding and Assembly into the Tight Junction Are Controlled by Non-conserved Residues in the Transmembrane 3 (TM3) and Extracellular Loop 2 (ECL2) Segments∗§

Received for publication, November 11, 2013, and in revised form, January 28, 2014 Published, JBC Papers in Press, January 29, 2014, DOI 10.1074/jbc.M113.531012

Jan Rossa†, Carolin Ploeger†∥, Franze Vorreiter‡∥, Tarek Saleh§¶, Jonas Protze§, Dorothee Günzel§, Hartwig Wolburg§, Gerd Krause§, and Jörg Piontek‡§

From the †Leibniz-Institut für Molekulare Pharmakologie, Department of Structural Biology, 13125 Berlin, Germany, the ‡Institute of Clinical Physiology, Charité - Universitätsmedizin Berlin, 12203 Berlin, Germany, and the §Institute of Pathology and Neuropathology, Department of General Pathology, University of Tübingen, 72076 Tübingen, Germany

Background: The transmembrane claudins assemble into polymeric tight junction strands.
Results: Residues involved in differential folding and assembly of claudin-3 and claudin-5 were identified.
Conclusion: Subtype-specific cis-dimerization contributes to the differing ultrastructure of tight junction strands.
Significance: The molecular insights improve the understanding of the formation of paracellular barriers to molecules.

The mechanism of tight junction (TJ) assembly and the structure of claudins (Cldn) that form the TJ strands are unclear. This limits the molecular understanding of paracellular barriers and strategies for drug delivery across tissue barriers. Cldn3 and Cldn5 are both common in the blood-brain barrier but form TJ strands with different ultrastructures. To identify the molecular determinants of folding and assembly of these classic claudins, Cldn3/Cldn5 chimeric mutants were generated and analyzed by cellular reconstitution of TJ strands, live cell confocal imaging, and freeze-fracture electron microscopy. A comprehensive screening was performed on the basis of the rescue of mutants deficient for strand formation. Cldn3/Cldn5 residues in transmembrane segment 3, TM3 (Ala-127/Cys-128, Ser-136/Cys-137, Ser-138/Phe-139), and the transition of TM3 to extracellular loop 2, ECL2 (Thr-141/Ile-142) and ECL2 (Asn-148/Asp-149, Leu-150/Thr-151, Arg-157/Tyr-158), were identified to be involved in claudin folding and/or assembly. Blue native PAGE and FRET assays revealed 1% n-dodecyl β-D-maltoside-resistant cis-dimerization for Cldn5 but not for Cldn3. This homophilic interaction was found to be stabilized by residues in TM3. The resulting subtype-specific cis-dimer is suggested to be a subunit of polymeric TJ strands and contributes to the specific ultrastructure of the TJ detected by freeze-fracture electron microscopy. In particular, the Cldn5-like exoplasmic face-associated and particle-type strands were found to be related to cis-dimerization. These results provide new insight into the mechanisms of paracellular barrier formation by demonstrating that defined non-conserved residues in TM3 and ECL2 of classic claudins contribute to the formation of TJ strands with differing ultrastructures.

The paracellular barrier in epithelia and endothelia is formed by tight junctions (TJ).4 Freeze-fracture electron microscopy (FFEM) revealed TJ as an Anastomosing network of strands composed of transmembrane particles (1). The tetraspan membrane proteins of the claudin (Cldn) family constitute the backbone of TJ (2, 3). On the basis of their barrier properties, claudins can be functionally grouped in barrier-forming or channel-forming claudins (4, 5). The ability of claudins to form paracellular ion channels is mainly determined by their first extracellular loop (ECL1) (4, 6, 7). The crystal structure of claudins is unknown, and the mechanism of TJ assembly is unclear. Assembly and disassembly of TJ is regulated by a panel of TJ-associated proteins controlling, e.g., the transport of claudins or their linkage to the cytoskeleton. However, the assembly of claudins into TJ strands probably depends directly on the following claudin-claudin interactions: (I) intramolecular folding and intermolecular assembly by (Ia) co- or posttranslational cis-oligomerization (within one membrane) and (Ib) trans-interaction (between opposing plasma membranes) that mediates the formation of polymeric strands (2, 8). Hereafter, (I) is mentioned as “folding” and (II) as “assembly.”

It is assumed that heteropolymeric TJ strands are formed by interactions between the same claudin family members (homophilic) and between different ones (heterophilic) at cell-cell contacts (3, 9, 10). However, the biochemical and biophysical analysis of the assembly of TJ strands is limited by their sensitivity to detergents. This, so far, prevented solubilization or in

* This work was supported by Deutsche Forschungsgemeinschaft (DFG) Grant PI837/2-1, by DFG Research Unit Grant FOR 721/2 TP6 and TP7, and by the Forschungsbund Berlin e.V.

† This article contains supplemental Table S1.

‡ Present address: Institute of Pathology, University Hospital Heidelberg, Heidelberg, Germany.
‡‡ Present address: Helmholtz Centre for Environmental Research, Leipzig, Germany.
‡§ To whom correspondence should be addressed: Institute of Clinical Physiology, Charité Berlin, Hindenburgdamm 30, 12203 Berlin, Germany. Tel.: 49-308445456; Fax: 49-3084454239; E-mail: joerg.piontek@charite.de.

4 The abbreviations used are: TJ, tight junction; CFP, cyan fluorescent protein; FFEM, freeze-fracture EM; Cldn, claudin; ECL, extracellular loop; P-face, protoplasmic face; E-face, exoplasmic face; TM, transmembrane; ICL, intracellular loop; Ch, chimera; EF, enrichment factor; BN-PAGE, blue native PAGE; DDM, n-dodecyl-β-D-maltoside; PM, plasma membrane.
vitro reconstitution of intact TJ strands. To circumvent this limitation, we analyzed claudin assembly in a native cellular environment.

TJ strands were reconstituted in HEK293 cells by transfection with claudin constructs C-terminally tagged with YFP. In this system, strand formation can be analyzed independently of endogenous claudins and TJ-regulating proteins containing a PDZ domain (2). TJ strands were detected by FFEM or single molecule-based nanoscopy (11). Trans-interaction between claudins was detected by enrichment of claudins at contacts between claudin-expressing cells (hereafter designated “contact enrichment”). Cis-interaction was analyzed by FRET assays. This reconstitution approach and mutagenesis were used to identify the determinants for the trans-interaction of Cldn5 (2), heterophilic compatibility of claudins, and TJ-associated MARVEL proteins (9, 12) together with a bioinformatics analysis to distinguish between classic and non-classic claudins and modeling of the ECL2 of Cldn3 and Cldn5 (4, 13). Residues identified as relevant for claudin assembly were verified as contributing to paracellular barriers in vitro and in vivo (14, 15).

In this study, we focused on two prototypes of barrier-forming claudins, Cldn3 and Cldn5, both of which are relevant to the blood-brain barrier. Cldn5 tightens the blood-brain barrier for molecules smaller than 800 Da (16). Hints of Cldn3 expression in the brain, loss of Cldn3 under pathologic conditions, and transcriptional regulation of Cldn3 in brain endothelial cells suggest that Cldn3 contributes to blood-brain barrier tightness (17, 18).

Cldn3 and Cldn5 are both capable of sealing the TJ (19, 20), but they form TJ strands with different ultrastructures (9): continuous-type strands associated with the protoplasmic (P) face of the membrane (Cldn3) or particle-type strands with spaced intramembranous particles associated with the exoplasmic (E) face (Cldn5). To identify claudin segments and residues involved in this difference, a panel of Cldn3/Cldn5 chimeras (ChA-ChG) was created by mixing the different predicted segments (transmembrane segments (TM1–4), intracellular loops (ICLs), and ECLs). The chimeras were expressed in HEK293 cells and screened for their capability to form TJ strands (2) with microscopic and biochemical analyses. The results provide novel insights into the molecular mechanism of paracellular barrier formation.

**EXPERIMENTAL PROCEDURES**

*Plasmids—Expression vectors for fusion proteins of CFP/GFP/YFP with murine Cldn3, Cldn5, Cldn5-F147A, and Cldn4 were based on pECFP-N1/pEFYP-N1/pEGFP (Clontech, Mountain View, CA) and have been described previously (2, 21). Chimeras (ChA-ChG) on the basis of Cldn3 and Cldn5 were generated with restriction-free cloning (22). ChA, Cldn3 M1-R144/Cldn5 E146-V218; ChB, Cldn3 M1-V99/Cldn5 G101-V218; ChC, Cldn5 M1-V77/Cldn3 Q77-V219; ChD, Cldn5 M1-G101/Cldn3 A101-V219; ChE, Cldn5 M1-L160/Cldn3 G160-V219; ChF, Cldn3 M1-R144/Cldn5 E146-L160/Cldn3 G160-V219; and ChG, Cldn3 M1-P27/Cldn5 M29-V77/Cldn3 Q77-R144/Cldn5 E146-V218. Site-directed mutagenesis of Cldn3, Cldn5, and the chimeras was performed similarly as described previously (2). The generated constructs are summarized in supplemental Table S1.*

**Cell Culture and Transfection**—HEK293 (9) cells were cultured in DMEM (Invitrogen) containing 10% (v/v) fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Transient transfections were performed using PEI (Polysciences, Eppelheim, Germany) or Lipofectamine 2000 (Invitrogen) according to the recommendations of the supplier.

**Live-cell Imaging**—For live-cell imaging, 2 days after transfection, cells were transferred to 1 ml Hanks’ balanced salt solution with Ca²⁺, Mg²⁺, glucose, and sodium bicarbonate and without phenol red (Invitrogen). The plasma membrane was visualized by addition of 20 μl of trypsin blue (Sigma-Aldrich, Hamburg, Germany), 0.05% in PBS. Cells were examined with a LSM 510 META system containing an Axiovert 135 microscope equipped with a PlanNeofluar ×100/1.3 objective (Carl Zeiss, Jena, Germany), (2).

To quantify claudin enrichment at contacts between two claudin-expressing cells, confocal images of living cells were analyzed using the LSM 510 software (Carl Zeiss). Two different methods were used.

The enrichment factor (EF) was measured using intensity profiles of confocal images. Contacts between two cells were identified by the trypsin blue fluorescence peaks indicating the plasma membrane. For each cell pair, three fluorescence intensity profiles were quantified. For cultures of Cldn/chimera-YFP-expressing cells, the EF was calculated as (intensity at contact between two Cldn/chimera-expressing cells)/(sum of intensities at contact between the two expressing cells and non-expressing cells) (2).

The EF could not be usefully determined for constructs that showed a saturation of the signal at contacts between two Cldn/chimera-expressing cells but no clear signal between Cldn/chimera-expressing and non-expressing cells. For these constructs, the percentage of enrichment-positive contacts was quantified as contacts between two Cldn/chimera-YFP-expressing cells with a strong YFP signal.

To quantify the ratio of claudin signals in the plasma membrane versus the nuclear membrane, images of single Cldn/chimera-expressing cells were taken. Either the plasma membrane identified by trypsin blue or the nuclear membrane identified morphologically were detected close to the saturation of the signals (depending on which signal was stronger). Five fluorescence intensity profiles per cell were quantified and averaged.

**FRET experiments at cell-cell contacts were performed using Cldn-CFP/Cldn-YFP-cotransfected HEK293 cells, as described previously (2).**

**Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE)**—Pellets of 10 cm² of transiently transfected HEK293 cells were resuspended in sample buffer (1× NativePAGE™ sample buffer, 1% DDM (Invitrogen), and EDTA-free protease inhibitor mixture (Roche Applied Science)). Lysates were centrifuged for 30 min at 15,000 × g and 4 °C. To 5 μl of supernatant, 5 μl of sample buffer including 0.5% NativePAGE™ G-250 sample additive (Invitrogen) was added. As a marker, we used NativeMark™ unstained protein standard (Invitrogen) and applied a correction factor of 0.6 similar to that used for claudins, as described previously (23). BN-PAGE was performed according to the
instructions of the manufacturer (Invitrogen) on the basis of Ref. 24. Gels were transferred to PVDF membranes. Membranes were fixed in 8% acetic acid for 15 min, destained with 62.5 mM Tris/HCl, 2% SDS for 15 min, washed with H2O, and blocked with 3% milk powder for 1 h. Western blotting was performed with mouse anti-GFP (catalog no. IL-8, Clontech) and HRP-conjugated goat anti-mouse (Invitrogen).

FRET Analysis by Spectrofluorometry/FRET Ratio—A close proximity between claudin molecules was detected by FRET between CFP and YFP tags. Cldn-CFP/Cldn-YFP cotransfected HEK293 cells were lysed in PBS containing 1% DDM (Sigma-Aldrich) and EDTA-free protease inhibitor mixture. After centrifugation (30 min, 15,000 × g, 4 °C), the supernatant was analyzed in a FP-6500 spectrofluorometer (Jasco, Gross-Umstadt, Germany). CFP fluorescence was detected with λexcitation of 425 ± 5 nm and λemission of 475 ± 5 nm; YFP with λexcitation of 490 ± 5 nm and λemission of 525 ± 5 nm; and the FRET signal with λexcitation of 425 ± 5 nm and λemission of 525 ± 5 nm. Cross-talk of YFP fluorescence to the CFP and FRET signal was determined with samples containing either YFP or CFP only; for λexcitation of 425 ± 5 nm and λemission of 475 ± 5 nm, the signal was 2% of the YFP signal (ε1 = 0.02), and for λemission of 525 ± 5 nm, the signal was 5% of the YFP signal (ε2 = 0.05). As a measure of the FRET efficiency, the FRET ratio was calculated as (FRET signal – ε2 × YFP fluorescence)/(CFP fluorescence – ε1 × YFP fluorescence). For negative controls, a FRET ratio of about 0.4 was obtained (9).

Freeze-fracture Electron Microscopy—HEK293 cells were transfected with Cldn/chimera-YFP constructs. Three days later, they were washed with PBS with Ca2+/Mg2+, fixed with 2.5% glutaraldehyde (electron microscopy-grade, Sigma-Aldrich) in PBS Ca2+/Mg2+ for 2 h, washed, and processed for freeze-fracture electron microscopy as reported previously (25). For quantification of the continuity and the P-/E-face association of the TJ strands, the particle coverage along strand axis was determined as follows. FFEM images were analyzed with ImageJ by measuring the length of the strands and counting the intramembranous particles at a given position along the strand axis in 10-nm steps. The particle coverage was defined as the number of particles per 10 nm/total length of analyzed strands. This was done for the P- and E-faces. For each construct, more than seven strand network areas were analyzed.

Bioinformatics—Sequence alignments for murine Cldn3 and Cldn5 were created with the GCG program package (GCG Wisconsin package, Accelrys Inc., San Diego, CA) and visualized with Geneious Pro 5.4.4 (Geneious Pro 5.4.4 created by Biomatters). Transmembrane helix predictions were performed with the GCG program package and TMHMM (26).

Statistics—Unless stated otherwise, results are shown as mean ± S.E. Statistical analyses were performed using Prism version 5.0 (GraphPad, San Diego, CA). First, normality tests were performed (D’Agostino and Pearson omnibus and Shapiro-Wilk and Kolmogorov-Smirnov test). Data sets exhibiting normal distribution were analyzed using unpaired, two-tailed Student’s t test. Data sets not showing normal distribution were analyzed using Mann-Whitney U test.

RESULTS

Subcellular Distribution of Cldn3/5 Chimeras Indicates Hampered Folding or Trans-interaction—Seven chimeras (ChA-ChG, Fig. 1, A and B) with a C-terminal YFP tag were expressed by transfection in the TJ-free cell line HEK293. Similarly to Cldn3 and Cldn5, the chimeras ChA, ChF, and ChG colocalized with the plasma membrane. However, in contrast with Cldn3 and Cldn5, the chimeras ChA, ChF, and ChG were not enriched at contacts between claudin-expressing cells (Fig. 2, A, B, D, E, G, and H). This indicates inhibition of the trans-interaction. FFEM was performed to analyze whether this inhibition affects strand formation. Although extended networks of TJ strands were easily detected for Cldn3 and Cldn5 (Refs. 2, 9 and Fig. 2C), almost no strands were detected for ChA, ChF, and ChG (Figs. 2F and 10, A–C). These results show that ChA, ChF, and ChG are strongly impeded in trans-interaction and strand formation.

In contrast with Cldn3, Cldn5, ChA, ChF, and ChG, the chimeras ChB, ChC, ChD, and ChE were restricted to intracellular compartments and did not localize within the plasma membrane (Fig. 2, I–L). This suggests that the folds of ChB, ChC, ChD, and ChE do not pass the intracellular quality control necessary for efficient plasma membrane targeting.

Although the cells showed different expression levels and, partly, a pronounced intracellular localization of the respective claudin construct, the enrichment of the construct between cells that express the construct depended on the molecular properties of the construct and not on whether it was expressed at low or high levels. In addition, stable expression of these constructs also led to similar subcellular distribution and contact enrichment, as did the transient expression (data not shown).

Taken together, all the chimeras showed an impediment to form TJ strands. This indicates that the different segments (TMs, ECLs, and ICL) of Cldn3 and Cldn5, which were mixed in the chimeras (Fig. 1), do not match structurally.

Identification of ECL2 Residues Involved in Trans-interaction by Rescue of Contact Enrichment for ChF—ChF represents Cldn3 with the putative ECL2 of Cldn5 (Figs. 1 and 3A). The lack of contact enrichment of ChF (Fig. 2G) suggests that the exchange of ECL2 of Cldn3 by that of Cldn5 was sufficient to diminish trans-interaction. To identify residues involved in this inhibition, Cldn3-like residues were reintroduced into ECL2 of ChF. Because the transition between ECL2 and TM3 could not be predicted exactly, the ECL2 sequence of Cldn5 in ChF was also N-terminally expanded (Fig. 3A).

First, multiple substitutions of neighboring residues in the ECL2 region differing between Cldn3 and Cldn5 were analyzed. T141I/I142V/I143V and Y157R/L159M, but not N148D/L150T or V153E/S154A, strongly increased contact enrichment (Fig. 3B). Analysis of single substitutions revealed that T141I and Y157R were responsible for increased contact enrichment (Fig. 3, B and C). Combining T141I/I142V/I143V with Y157R/L159M resulted in an additive increase in contact enrichment (Fig. 3B). A similar additive increase was obtained by combining T141I with Y157R (Fig. 3C). These results indicate that the residues differing between Cldn3 and Cldn5 at
positions 141 (in Cldn3 = 142 in Cldn5) and 157 (in Cldn3 = 158 in Cldn5) are involved in trans-interaction.

To investigate the role of ECL2 residues in contact enrichment in more detail, Cldn3-mimicking residues were introduced into Cldn5. At transition to TM3 I142T, but not V143I or V144I, strongly inhibited contact enrichment (Fig. 3D). V154E/S155A only led to a minor decrease. D149N/T151L double substitution inhibited contact enrichment for the Cldn5 construct, whereas neither D149N nor T151L single substitutions exhibited any inhibition (Fig. 3D). Taken together, the data obtained with ChF and Cldn5 demonstrate that ECL2 residues differing between Cldn3 and Cldn5 are involved in contact enrichment.

Identification of Incompatible Residues Responsible for the Different Subcellular Localization of Chimeras—The finding that ChA but not ChB was detected in the plasma membrane (Fig. 2, E and I) suggests that residues differing between these chimeras (TM3 and ICL) are structurally incompatible with other residues in ChB, resulting in misassembly and intracellular retention of ChB. A comparison of TM3 of Cldn3 and Cldn5 revealed the presence of two Cldn5-specific cysteines (Fig. 4A, top panel). Because the sulfhydryl group of Cys possesses special biophysical properties (e.g. electrostatic potential), its contribution to the different phenotypes of ChA and ChB was analyzed by Ser substitutions. Strikingly, C127S, but not C136S, in ChB enabled contact enrichment, and C136S inhibited contact enrichment induced by C127S in ChB (Figs. 4, A and B, and 10, I–L). This indicates that, in ChB, the sulfhydryl group of Cys-127, but not of Cys-136, in the Cldn5-like TM3, prevents contact enrichment, presumably by disturbing folding/assembly of the chimera. In addition, the corresponding C137S, but not C128S, in Cldn5 inhibited contact enrichment (Fig. 4B), indicating that the sulfhydryl group of Cys-137, but not that of Cys-128, is necessary for the correct folding and assembly of Cldn5.

Substitution of the Incompatible C127 in TM3 Is Sufficient to Rescue Strand Formation for ChB—Consistently, with contact enrichment, FFEM showed that ChB-C127S is able to form TJ strands (Fig. 4C). The strands formed by ChB-C127S showed a particle-type morphology with intramembranous strand particles on the E-face as well as on the P-face of the membrane (particle coverage along strand axis of 65.8 ± 2.2% on the E-face and 37.0 ± 1.5% on the P-face). Interestingly, this mixed P/E-face association was similar to that found for Cldn3/Cldn5 copolymers (9) and was in between the strand morphology found for Cldn5 (particle coverage of 61.9 ± 1.2% on the E-face and 53.2 ± 1.3% on the P-face, particle type) and the one for Cldn3 (particle coverage of 4.0 ± 1.0% on the E-face and 94.7 ± 1.9% on the P-face, continuous type). C128S substitution in
Cldn5 (corresponding to C127S in ChB) did not change E-face association nor the particle-type appearance of the strands (Fig. 4D, particle coverage of 64.0 ± 4.0% on the E-face and 5.8 ± 1.8% on the P-face). The very few strands found for ChA showed Cld3-like continuous (> 90% particle coverage) and P-face-associated strands (Fig. 10A). Together, these FFEM data and the sequence differences between ChA and ChB (Fig. 1) indicate that residues in TM3/ICL of Cldn5 but not C128 alone are strongly involved in the E-face association and particle-type appearance of strands.

Coexpression of ChB-C127S with Cldn5 increased, whereas coexpression with Cldn3 decreased, E-face association of the resulting strands (Fig. 10, F and G). FRET at cell-cell contacts demonstrated a close proximity of ChB-C127S with the coexpressed Cldn3 or Cldn5 (Fig. 10H). These data suggest that ChB-C127S is able to copolymerize with Cldn3 and Cldn5 and that this copolymerization results in a shift of P-/E-face association.

The Block of Contact Enrichment of ChA and ChF Is Rescued by Substitutions in TM3—ChB-C127S showed contact enrichment in the plasma membrane and many intracellular signals (Fig. 4A). In contrast, ChA was homogenously distributed in the plasma membrane (Fig. 2, D and E). To identify residues involved in this different subcellular localization, ChA was expanded stepwise toward ChB-C127S by introducing Cldn5-like residues N-terminally of ECL2 (Fig. 5A). The substitution T141I/I142V/I143V did not lead to contact enrichment (Fig. 5B). In contrast, S136C/S138F drastically increased contact

FIGURE 2. Segment incompatibility in chimeras results in two different deficiency phenotypes. A and B, contact enrichment. Cldn3 and Cldn5 (green) are enriched at contacts between two claudin-expressing HEK293 cells (arrows). C and F, representative FFEM images showing a network of TJ strands for Cldn5 but no TJ strands for ChA. I–L, ChB, ChC, ChD, and ChE (green) are restricted to intracellular compartments and do not colocalize with the plasma membrane (red). A, B, E, G, and I–L, living HEK293 cells expressing claudin-YFP constructs were stained with trypan blue to visualize the plasma membrane (red) and analyzed by confocal microscopy. Scale bars = 10 μm. C and F, for FFEM, HEK293 cells were fixed with glutaraldehyde. Scale bars = 0.2 μm.
enrichment. A similar increase was obtained with S138F, but not S136C, in Cha. T141/I1142/V1143 reduced the increase induced by S136C/S138F (Fig. 5B, Cha-S136C/S138F+141TI/IVV143 (Cha CFIVV)), and T141/I1142/V1143 reduced the increase induced by S138F (Fig. 5B, Cha S138F/T141I). The data show that S138F is sufficient to rescue contact enrichment of Cha and that T141/I1142/V1143 counteracts this rescue effect.

To analyze the influence of TM4 on S138F-mediated contact enrichment of Cha, similar substitutions were introduced in ChaF, which differs from Cha in TM4 (Fig. 5, C and E, pictograms). Similar as in Cha, in ChaF, substitutions S136C/S138F and S138F, but not S136C, strongly increased contact enrichment (Fig. 5, B and E). In contrast to Cha, in ChaF, the substitutions T141I/I1142/V1143 and T141/I1142/V1143 did not reduce the increase induced by S136C/S138F (Fig. 5B, ChaF-S136C/S138F+141TI/IVV143 (ChaF CFIVV)). Taken together, these data indicate that S138F in TM3 is sufficient to rescue contact enrichment independently of the differences between Cldn3 and Cldn5 in TM4, whereas the effect of T141/I1142/V1143 depends on TM4.

Substitutions in Cha and ChaF Leading to Contact Enrichment Enable Strand Formation—To verify that chimeras showing contact enrichment are able to form TJ strands, FFEM was performed. ChaF-S136C/S138F, ChaF-S136C/S138F+T141/I1142/V1143 (ChaF CFIVV), and ChaA-S136C/S138F formed many strands (Fig. 5, H, J, and K). For these constructs, extensive networks of branched strands similar to Cldn3 and Cldn5 were found. In addition, the strongest tendency to form multiple parallel bundles was observed for ChaA-S136C/S138F (Fig. 5K). The strands formed by these chimeras were rather continuous with particles associated with the P-face. In summary, continuity and P-/E-face association of the strand particles decreased in the following order: Cldn3 ≥ ChaF-S136C/S138F ≥ ChaF-S136C/S138F+T141/I1142/V1143 (ChaF CFIVV), and ChaA-S136C/S138F formed many strands (Fig. 5, H, J, and K).

Further Analysis of Substitutions in Cldn3 and Cldn5—Because residues differing in Cldn3 and Cldn5 strongly affected strand formation of chimeric constructs, the role of differing residues was analyzed further by introducing Cldn5-like residues into Cldn3 and Cldn3-like residues into Cldn5. In Cldn3, S138F and T141/I1142/V1143 increased contact enrichment (Fig. 6A), whereas the reciprocal substitutions in Cldn5, I142/T (Fig. 3D) and F139/S (Fig. 6B), decreased contact enrichment. However, F139/S and I142/T in Cldn5 did not prevent formation or change P-/E-face association or the particle-type appearance of strands (Figs. 5I and 10, D and E). Taking wild types and chimeras together, the presence of Phe at position 138/139 and Ile at 141/142 correlate with strong contact enrichment.

Because C128 and C137 of Cldn5 were found to be relevant for contact enrichment (Fig. 4B), Cys was introduced at the corresponding position in TM3 of Cldn3 (A127C, S136C). However, contact enrichment was not changed (Fig. 6A). Similarly, C14A in TM1 of Cldn5 did not affect contact enrichment. These results underline that the effect of a sulfhydryl group depends on the protein context.

Further substitutions found to affect contact enrichment were A132T in TM3 of Cldn5 (Fig. 6B) and exchanging residues in the ICL that differ greatly between Cldn3 and Cldn5 (Fig. 6, A and B, 108QDET111/109APGP112). However, these effects were weaker than those from other substitutions in TM3 or ECL2.
Effect of the Substitutions on the Amount of Claudin Constructs in the Plasma Membrane—The claudin constructs colocalized differentially with the plasma membrane (summarized in supplemental Table S1). Cldn3 and Cldn5 both exhibited contact enrichment but differed in the amount detected in the plasma membrane outside contacts between claudin-expressing cells. To investigate plasma membrane localization independently of trans-interaction, single claudin-expressing cells without contact to another claudin-expressing cell were analyzed (Fig. 7, A and B). The ratio of the claudin signals in the plasma membrane versus the nuclear membrane (as an intracellular reference membrane) was quantified using confocal intensity profiles (Fig. 7 C, PM/NM ratio). The PM/NM ratio of Cldn3 was much higher than that of Cldn5. Removal of the Cldn5-specific sulfhydryl group by C128S and Cldn3-mimicking I142T increased the PM/NM ratio in Cldn5. ChB-C127S and ChB-C127S/I141T exhibited similar PM/NM ratios as Cldn5-I142T. For ChA, the PM/NM ratio was similar to that of Cldn3wt and was unchanged by S138F or S138F/T141I in ChA (Fig. 7C). These results indicate that the amount of claudin constructs in the plasma membrane is affected by residues in TM3.

Biochemical Analysis of Claudin Oligomerization—For biochemical analysis of claudin oligomerization, BN-PAGE of claudins solubilized with 1% DDM was used as described previously (23). Cldn5 migrated consistently with being a dimer (Fig. 8A). Only a faint band consistent with a monomer was detectable. In contrast, Cldn3 was detected as a monomer, whereas a dimer was hardly detectable. Because Cldn4 has been described as migrating as a monomer on BN-PAGE (23), it was used as control. As expected, Cldn4 migrated consistently with being a monomer, similarly to Cldn3 (Fig. 8A). Furthermore, Cldn5-F147A, which has been shown previously to lack the ability for trans-interaction (2), was detected as a dimer, similarly to Cldn5. This indicates that the detected Cldn5 dimer is formed by cis-interaction.

To verify the different extents of dimerization for Cldn5 versus Cldn3, FRET assays (for detection of close proximity) were performed. HEK293 cells were cotransfected with Cldn-CFP/Cldn-YFP, the cells were lysed as for BN-PAGE, and the FRET signals were analyzed by spectrofluorometry. A strong FRET signal was obtained for Cldn5 but not for Cldn3 (Fig. 8B). In addition, the FRET signal for Cldn5 was strongly inhibited by the addition of 0.5% SDS. Together, the BN-PAGE and FRET data strongly suggest that Cldn5, but not Cldn3, forms a stable non-covalent cis-homodimer that is DDM-resistant but SDS-sensitive.
FIGURE 5. S138F substitution is sufficient to rescue contact enrichment and strand formation of ChA and ChF. A, schematic highlighting the sequence differences between ChA, ChB, and ChF. Alignment of Cldn3 and Cldn5 sequences and substitutions that were analyzed are indicated (boxes). B, quantification of contact enrichment of ChA and ChF constructs. S138F substitution is sufficient to rescue contact enrichment in ChA and ChF. The effect of 141TII/IVV143 but not of S138F depends on TM4. CFIVV, S136C/S138F/H14111/143. Data are mean ± S.E., n = 39. For ChA-T141I, n = 22. *, p < 0.001 versus ChA (dark blue columns) or versus ChF (light blue or red columns) or as indicated. C–F, representative images for subcellular distribution of claudin-YFP constructs (green) in living HEK293 cells. The plasma membrane was stained with trypan blue (red). Contact enrichment is indicated by arrows. Scale bars = 5 μm. G–K, FFEM analysis. Similar to Cldn3wt (G), ChF-S136C/S138F (H) and ChF-CFIVV (I) form huge networks of rather continuous-type strands with a strong P-face association. K, ChA-S136C/S138F forms many bundles of continuous or fragmented strands with more particles on the P-face than on the E-face. J, Similar to Cldn5wt, Cldn5-I142T forms networks of particle-type strands with a very high E-face association. Scale bar = 0.2 μm.
dimer/monomer ratio (Fig. 8A). A low dimer/monomer ratio, similar to Cldn3, was found for ChF, ChF-S138F, ChA, ChA-S138F, and ChA-S136C/S138F/H11001 T141I/I142V/I143V (ChA-CFIVV). In contrast, ChB-C127S showed a clearly higher dimer/monomer ratio. Because ChB-C127S and ChA-CFIVV differ in the N-terminal half of TM3 and ICL, this region appears to be strongly involved in differential dimerization. However, substitution of an ICL motif strongly differing between Cldn3 and Cldn5 (108QDET111/109APGP112) did not change the dimer/monomer ratio (Fig. 8B). In contrast, for ChB-C127S/T141T, the dimer was even more pronounced than for ChB-C127S. In addition, Cldn5-I142T, but not Cldn5-F139S, exhibited more monomers than Cldn5wt. Together, the data indicate that position 141 (Cldn3)/142 (Cldn5), but not 138 (Cldn3)/139 (Cldn3), and also residues in the N-terminal half of TM3 are involved in dimerization.

**TJ Strands Formed by Chimeras Because of the S138F Substitution Represent a Diffusion Barrier for a 870-Da Marker—**The TJ strands reconstituted by claudin expression in HEK293 cells do not form a continuous belt-like paracellular barrier in the monolayer (2). Hence, standard permeability assays using a filter insert, as performed for polarized epithelial cells with endogenous TJs (14), are inapplicable. To demonstrate that the constructs showing contact enrichment and strands in freeze-fracture replica constitute a functional diffusion barrier, a tracer exclusion assay was performed (9). Standard incubation of cells with trypan blue labels the whole plasma membrane (Figs. 2, 4, 5, and 7). In contrast, after a short incubation with trypan blue (873 Da) or Cellmask™ (≈2 kDa) in a lower concentration, the tracer also labels the plasma membrane but is excluded from cell-cell contacts with enriched claudin signals (9). This tracer exclusion was found for different chimeras showing contact enrichment and strands but not for chimeras lacking both. Representative images for the different constructs are given for ChA-S138F and ChA (Fig. 9). This indicates that ChA-S138F, but not ChA, forms a diffusion barrier at cell-cell contacts for markers of ≈1 kDa.

**DISCUSSION**

In this study, a panel of Cldn3/Cldn5 chimeras was screened to identify protein segments and residues involved in folding and assembly of classic claudins such as Cldn3 and Cldn5. Experimental reconstitution of TJ strands, mutagenesis, native gel electrophoresis, and confocal and electron microscopy revealed novel insights into the molecular determinants of claudin folding and assembly and the formation of paracellular barriers.

**Mislocalization of Cldn3/5 Chimeras Revealed Segments of Cldn3 and Cldn5 That Do Not Match—**Analysis of subcellular localization of ChA to ChG showed that, in contrast to wild-
Residues Contributing to Claudin Assembly

FIGURE 8. Analysis of claudin dimerization by blue native PAGE and FRET. A, cldns from transfected HEK293 cells were solubilized with 1% DDM and analyzed by blue native PAGE. The claudin constructs differ in their dimer/monomer ratios. Bands corresponding to cis-dimers were detected for Cldn3 but hardly at all for Cldn5. I142 and other residues in TM3 of Cldn5 stabilize the corresponding Y158 in Cldn5 is necessary for homophilic trans-interaction (2), but Cldn3-Cldn5 trans-interaction was found to be relatively weak (9). These findings indicate that the R157/Y158 difference in the ECL2 contributes to claudin subtype-specific trans-interaction.

Identification of ECL2 Residues Involved in Trans-interaction—The block of trans-interaction by exchanging the ECL2 of Cldn3 by that of Cldn5 (Fig. 2G) enabled us to search for non-conserved residues involved in trans-interaction. Interestingly, Y157R strongly increased contact enrichment in ChF (Fig. 3C). The corresponding Y158 in Cldn5 is necessary for homophilic trans-interaction (2), but Cldn3-Cldn5 trans-interaction was found to be relatively weak (9). These findings indicate that the R157/Y158 difference in the ECL2 contributes to claudin subtype-specific trans-interaction.

Furthermore, T141I in ChF increased, whereas the inverse I142T in Cldn5 decreased contact enrichment (Fig. 3, B and D). In Cldn5, D149N/T151L diminished contact enrichment (Fig. 3C). The corresponding Y158 in Cldn5 is necessary for homophilic trans-interaction (2), but Cldn3-Cldn5 trans-interaction was found to be relatively weak (9). These findings indicate that the R157/Y158 difference in the ECL2 contributes to claudin subtype-specific trans-interaction.

Identification of Incompatible/Mismatched Residues by Comparison of Different Chimera Phenotypes—Differences between chimeras concerning their subcellular localization had to be caused by sequence differences. In particular, the different phenotypes for ChA (plasma membrane type) and ChB (intracellular type) had to be due to the different residues in TM3 or ICL (Fig. 1). Consequently, substitutions in this region were generated and analyzed by confocal and electron microscopy. Residues that influence claudin membrane localization, contact enrichment, and strand formation. Two points were revealed as discussed in the following paragraphs.

1) The sulfhydryl group of Cys-128 but not that of Cys-137 in TM3 of Cldn5 prevented contact enrichment of ChB (Fig. 4). In Cldn5, Cys-128 was not necessary for contact enrichment. This indicates that, in a putative TM helix bundle, Cys-127 in Cldn5 does not fit with TM1 and TM2 of Cldn3, which are present in ChB.

In contrast, the sulfhydryl group of Cys-136/137 in TM3 was necessary for strong contact enrichment of ChB and Cldn5 (Fig.

type claudins, none of the chimeras exhibited contact enrichment as an indicator for trans-interaction (Fig. 2). In addition, the lack of efficient strand formation was verified by FFEM. This indicates that, for all chimeras, the mixed segments of Cldn3 and Cldn5 do not match structurally.

Two different phenotypes were obtained for the chimeras: the "plasma membrane type," with a presence in the plasma membrane but lack of contact enrichment indicating direct or indirect inhibition of trans-interaction, and the "intracellular type," an intracellular accumulation indicating misfolding or misoligomerization that does not pass intracellular protein quality control (8, 27). Chimeras with at least two TMs of Cldn5 or TM1 and ECL1 of Cldn5 belong to the intracellular type. Regarding the plasma membrane type, exchanging ECL2 of Cldn3 with that of Cldn5 (ChF) was sufficient to strongly inhibit trans-interaction and strand formation. Even the presence of ECL1 and ECL2 of Cldn5 in ChG was not sufficient to enable strong contact enrichment. These results indicate that residues in the ECL2 that differ between Cldn3 and Cldn5 and the transition between the ECLs and the TMs contribute to the ability to interact in trans. To identify mismatching residues in chimeras and, thus, residues involved in claudin folding or assembly, we subsequently tried to rescue the deficiency phenotypes of the chimeras by mutagenesis.

2) Identification of ECL2 Residues Involved in Trans-interaction—The block of trans-interaction by exchanging the ECL2 of Cldn3 by that of Cldn5 (Fig. 2G) enabled us to search for non-conserved residues involved in trans-interaction. Interestingly, Y157R strongly increased contact enrichment in ChF (Fig. 3C). The corresponding Y158 in Cldn5 is necessary for homophilic trans-interaction (2), but Cldn3-Cldn5 trans-interaction was found to be relatively weak (9). These findings indicate that the R157/Y158 difference in the ECL2 contributes to claudin subtype-specific trans-interaction.

Furthermore, T141I in ChF increased, whereas the inverse I142T in Cldn5 decreased contact enrichment (Fig. 3, B and D). In Cldn5, D149N/T151L diminished contact enrichment (Fig. 3C). The corresponding Y158 in Cldn5 is necessary for homophilic trans-interaction (2), but Cldn3-Cldn5 trans-interaction was found to be relatively weak (9). These findings indicate that the R157/Y158 difference in the ECL2 contributes to claudin subtype-specific trans-interaction.

Identification of Incompatible/Mismatched Residues by Comparison of Different Chimera Phenotypes—Differences between chimeras concerning their subcellular localization had to be caused by sequence differences. In particular, the different phenotypes for ChA (plasma membrane type) and ChB (intracellular type) had to be due to the different residues in TM3 or ICL (Fig. 1). Consequently, substitutions in this region were generated and analyzed by confocal and electron microscopy. Residues that influence claudin membrane localization, contact enrichment, and strand formation. Two points were revealed as discussed in the following paragraphs.

1) The sulfhydryl group of Cys-128 but not that of Cys-137 in TM3 of Cldn5 prevented contact enrichment of ChB (Fig. 4). In Cldn5, Cys-128 was not necessary for contact enrichment. This indicates that, in a putative TM helix bundle, Cys-127 in Cldn5 does not fit with TM1 and TM2 of Cldn3, which are present in ChB.

In contrast, the sulfhydryl group of Cys-136/137 in TM3 was necessary for strong contact enrichment of ChB and Cldn5 (Fig.
The corresponding S136C substitution increased contact enrichment in ChA-S138F (generating ChA-S136C/S138F) and ChF-S138F but not in ChA, ChF, and Cldn3 (Figs. 5E and 6A). These data indicate that a sulfhydryl group at position 136/137 in TM3 matches Cldn5-like TM3 and TM4, e.g. to F139. Whether the sulfhydryl groups of residues 128 and 137 influence intramolecular interactions or cis-interaction between the TMs is unclear. However, as a result, trans-interaction and strand formation are affected indirectly.

2) S138F in TM3 is sufficient to rescue contact enrichment, and strand and diffusion barrier formation of ChA and T141I counteracts this (Figs. 5B and 9). S138F rescued contact enrichment in ChA (Fig. 5B) without changing the ChA amount in the plasma membrane (Fig. 7C). A similar rescue was obtained in ChF. Because ChA and ChF differ in TM4, the effect of S138F in TM3 is independent of sequence differences between Cldn3 and Cldn5 in TM4. In contrast, the effect of T141I depends on TM4.

In Cldn3, S138F and T141I increased contact enrichment (Fig. 6A), whereas the reciprocal substitutions in Cldn5, I142T (Fig. 3D) and F139S (Fig. 6B), decreased contact enrichment. I141/142T differentially affected Cldn5 and ChB-C127S.
Residues Contributing to Claudin Assembly

respectively, regarding contact enrichment (Figs. 3D and 4B), the amount in the plasma membrane (Fig. 7C), and dimerization (Fig. 8A). These results and sequence comparisons suggest that the I141/I142T-mediated changes depend on differences in TM1 and TM2.

Taking wild types and chimeras together, the presence of Phe at position 138/139 and Ile at position 141/142 correlate with strong contact enrichment. The molecular mechanism by which these residues affect claudin folding and assembly is further addressed by a modeling approach. 

BN-PAGE and FRET Reveal TM3-mediated Cis-dimerization for Cldn5 but Not for Cldn3—Several gel electrophoresis systems have been used to analyze oligomerization of solubilized claudins. We obtained the most consistent results with the DDM/BN-PAGE system (23). Cldn5 migrated consistently with being a dimer (Fig. 8A), similar to Cldn2 (23). In contrast, Cldn3 was found mainly as a monomer, similar to Cldn4 (Fig. 8A and Ref. 23). Cldn5-F147A, which is deficient for trans-interaction (2), was detected as a dimer. This suggests that the Cldn5 dimer is formed by cis-interaction. FRET supported non-covalent homodimerization of Cldn5 but not of Cldn3 (Fig. 8B).

DDM-resistant claudin dimers have been suggested to be a fundamental structural unit of larger detergent-sensitive complexes that might represent the 10-nm particles of TJ strands seen in FFEM (23). Our findings support this idea and indicate that channel-forming claudins (e.g. Cldn2) as well as barrier-forming claudins (e.g. Cldn5) are able to form these dimers. It has been assumed that the lack of dimerization found for Cldn4 could be due to the lack of the ability to self-organize into strands (23). Here we show that Cldn3, which forms strands, does not form stable dimers. Hence, DDM-resistant cis-dimerization is not a precondition for the ability to form TJ strands but reflects claudin subtype-specific assembly properties.

BN-PAGE analysis of Cldn3/Cldn5 chimeras showed that Cldn5-specific residues in TM3 critically contribute to cis-dimerization. This is consistent with cross-linking experiments with Cldn2/Cldn4 chimeras, suggesting dimerization via TMs and proximity of TM2 (23).

TM3 Contributes to the Ultrastructure of TJ Strands—Paracellular tightness correlates with the number of strands and the extent of their cross-linking detected with FFEM after aldehyde fixation (28, 29). Breaks of >20 nm in the strand network are often found under pathological conditions with increased permeability. In contrast, gaps <20 nm define the “particle type” of strands formed by Cldn2, Cldn5, or Cldn10b (30–32), whereas Cldn1 and Cldn3 form a continuous “strand type” without gaps (3, 33). In addition, these continuous strands are found on the P-face of the membrane, whereas, for the particle-type, particles are found on the E-face (Cldn5) or E- and P-faces (Cldn2, Cldn3/5 copolymers). Importantly, mixed P-/E-face association correlates with blood-brain barrier-specific tightness (9, 17, 34). However, the molecular base for these ultrastructural differences remained unclear.

Here we reveal a relationship between this ultrastructural appearance and cis-dimerization of claudins. For Cldn3, Cldn5, and chimeras of these, E-face association and particle type (Figs. 2G; 4C and D; 5, G–K; and 10, A–F) correlated with cis-dimerization (Fig. 8A). A similar correlation is found for Cldn2 and Cldn4 by comparison of literature (7, 23).

The most striking difference was found between ChA-S136C/S138F and ChB-C127S (Figs. 4C and 5K). This shows that the C-terminal half of Cldn5, including TM3 but not C128, is essential for stable cis-dimerization and, thereby, for strands with particles on the E-face (Fig. 11).

In summary, we revealed subtype-specific cis-dimers as subunits of claudin polymers that contribute to the distinct ultrastructure of TJ strands. We identified non-conserved residues in TM3 and the ECL2 of classic claudins, affecting cis- and/or trans-interaction, thereby influencing the morphology and barrier function of TJ strands (Fig. 11). These mechanistic insights advance the molecular understanding of paracellular barrier formation and could facilitate the identification of key interactions for specific TJ modulation for the improvement of drug delivery or for therapeutic barrier protection.

Acknowledgments—We thank Ria Knittel for help with freeze-fracturing and Michael Fromm for critical reading of the manuscript.

REFERENCES
1. Staehelin, L. A. (1974) Structure and function of intercellular junctions. Int. Rev. Cytol. 39, 191–283
2. Piontek, J., Winkler, L., Wolburg, H., Müller, S. L., Zuleger, N., Piehl, C., Wiesner, B., Krause, G., and Blasig, I. E. (2008) Formation of tight junction. Determinants of homophilic interaction between classic claudins. FASEB J. 22, 146–158
3. Furuse, M., Sasaki, H., and Tsukita, S. (1999) Manner of interaction of heterogeneous claudin species within and between tight junction strands. J. Cell Biol. 147, 891–903
4. Krause, G., Winkler, L., Mueller, S. L., Haseloff, R. F., Piontek, J., and Blasig, I. E. (2008) Structure and function of claudins. Biochim. Biophys. Acta
1. Czupalla, K., and Yu, A. S. (2013) Claudins and the modulation of tight junction permeability. *Physiol. Rev.* **93**, 525–569
2. Yu, A. S., Cheng, M. H., Angelow, S., Günzel, D., Kanzawa, S. A., Schneeberger, E. E., Fromm, M., andCoalson, R. D. (2009) Molecular basis for cation selectivity in claudin-2-based paracellular pores. Identification of an electrostatic interaction site. *J. Gen. Physiol.* **133**, 111–127
3. Colegio, O. R., Van Itallie, C., Rahner, C., and Anderson, J. M. (2003) Claudins and the modulation of tight junctions. *Tissue Barriers* **1**, e24518
4. Piontek, J., Fritzsch, S., Cording, J., Richter, S., Hartwig, J., Walter, M., Yu, D., Turner, J. R., Gehring, C., Rahn, H. P., Wolburg, H., and Blasig, I. E. (2011) Elucidating the principles of the molecular organization of heteropolymic tight junction strands. *Cell. Mol. Life Sci.* **68**, 3903–3918
5. Daugherty, B. L., Ward, C., Smith, T., Ritzenhaller, J. D., and Koval, M. (2013) Regulation of heterotypic claudin compatibility. *J. Biol. Chem.* **282**, 30005–30013
6. Kaufmann, R., Piontek, J., Gröll, F., Kirchgessner, M., Rossa, J., Wolburg, H., Blasig, I. E., and Cremer, C. (2012) Visualization and quantitative analysis of reconstituted tight junctions using localization microscopy. *PloS ONE* **7**, e31128
7. Cording, I., Berg, J., Küding, N., Bellmann, C., Tscheik, C., Westphal, J. K., Milatz, S., Günzel, D., Wolburg, H., Piontek, J., Huber, O., and Blasig, I. E. (2013) In tight junctions, claudins regulate the interactions between occludin, tricelulin and marvedlD3, which, inversely, modulate claudin oligomerization. *J. Cell Sci.* **126**, 554–564
8. Winkler, L., Gehring, C., Wenzel, A., Müller, S. L., Piehl, C., Krause, G., Blasig, I. E., and Piontek, J. (2009) Molecular determinants of the interaction between *Clostridium perfringens* enterotoxin fragments and Claudin-3. *J. Biol. Chem.* **284**, 18863–18872
9. Piehl, C., Piontek, J., Cording, J., Wolburg, H., and Blasig, I. E. (2010) Participation of the second extracellular loop of claudin-5 in paracellular tightening against ions, small and large molecules. *Cell. Mol. Life Sci.* **67**, 2131–2140
10. Zhang, J., Piontek, J., Wolburg, H., Piehl, C., Liss, M., Otten, C., Christ, A., Willnow, T. E., Blasig, I. E., and Abdelilah-Seyyfried, S. (2010) Establishment of a neuroepithelial barrier by Claudin5a is essential for zebrafish neural tube closure and ventricular lumen expansion. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 1425–1430
11. Nitta, T., Hata, M., Gotoh, S., Seo, Y., Sasaki, H., Hashimoto, N., Furuse, M., and Tsukita, S. (2003) Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. *J. Cell Biol.* **161**, 653–660
12. Wolburg, H., Wolburg-Buchholz, K., Kraus, J., Rascher-Eggstein, G., Liebner, S., Hamm, S., Duffner, F., Grote, E. H., Risau, W., and Engelhardt, B. (2003) Localization of claudin-3 in tight junctions of the blood-brain barrier is selectively lost during experimental autoimmune encephalomyelitis and human glioblastoma multiforme. *Acta Neuropathol.* **105**, 586–592
13. Liebner, S., Corada, M., Bangsow, T., Babbage, J., Taddei, A., Czupalla, C. J., Reis, M., Felici, A., Wolburg, H., Fruttiger, M., Taketo, M. M., von Melchner, H., Plate, K. H., Gerhardt, H., and Dejana, E. (2008) Wnt/b-catenin signaling controls development of the blood-brain barrier. *J. Cell Biol.* **183**, 409–417
14. Milatz, S., Krug, S. M., Rosenthal, R., Günzel, D., Müller, D., Schulzke, J. D., Amasheh, S., and Fromm, M. (2010) Claudin-3 acts as a sealing component of the tight junction for ions of either charge and uncharged solutes. *Biochim. Biophys. Acta* **1798**, 2048–2057
15. Amasheh, S., Schmidt, T., Mahn, M., Florian, P., Mankertz, J., Tavalali, S., Gitter, A. H., Schulzke, J. D., and Fromm, M. (2005) Contribution of claudin-5 to barrier properties in tight junctions of epithelial cells. *Cell Tiss. Res.* **321**, 89–96
16. Veshnyakova, A., Piontek, J., Protze, J., Waziri, N., Heise, I., and Krause, G. (2012) Mechanism of *Clostridium perfringens* enterotoxin interaction with claudin-3/4 protein suggests structural modifications of the toxin to target specific claudins. *J. Biol. Chem.* **287**, 1698–1708
17. van den Ent, F., and Löwe, J. (2006) RF cloning. A restriction-free method for inserting target genes into plasmids. *J. Biochem. Biophys. Methods* **67**, 67–74
18. Van Itallie, C. M., Mitic, L. L., and Anderson, J. M. (2011) Claudin-2 forms homodimers and is a component of a high molecular weight protein complex. *J. Biol. Chem.* **286**, 3442–3450
19. Schägger, H., and von Jagow, G. (1991) Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Annu. Biochem.* **199**, 223–231
20. Wolburg, H., Liebner, S., and Lippoldt, A. (2003) Cerebral endothelial tight junctional structure as revealed by freeze-fracture. *Meth. Mol. Biol.* **89**, 51–66
21. Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer, E. L. (2001) Predicting transmembrane protein topology with a hidden Markov model. Application to complete genomes. *J. Mol. Biol.* **305**, 567–580
22. Claessen, J. H., Kundrat, L., and Ploegh, H. L. (2012) Protein quality control in the ER. Balancing the ubiquitin checkbook. *Trends Cell Biol.* **22**, 22–32
23. Claude, P. (1978) Morphological factors influencing transepithelial permeability. A model for the resistance of the zona occludens. *J. Membr. Biol.* **39**, 219–232
24. Claude, P., and Goodenough, D. A. (1973) Fracture faces of zonae occludentes from "tight" and "leaky" epithelia. *J. Cell Biol.* **58**, 390–400
25. Furuse, M., Sasaki, H., Fujimoto, K., and Tsukita, S. (1998) A single gene product, claudin-1 or -2, reconstitutes tight junction strands and recruits occludin in fibroblasts. *J. Cell Biol.* **143**, 391–401
26. Morita, K., Sasaki, H., Furuse, M., and Tsukita, S. (1999) Endothelial claudin. Claudin-5/TMVCF constitutes tight junction strands in endothelial cells. *J. Cell Biol.* **147**, 185–194
27. Inai, T., Kaminura, T., Hirose, E., Iida, H., and Shibaya, Y. (2010) The protoplasmic or exoplasmic face association of tight junction particles cannot predict paracellular permeability or heterotypic claudin compatibility. *Ear. J. Cell Biol.* **89**, 547–556
28. Inai, T., Sengoku, A., Hirose, E., Iida, H., and Shibaya, Y. (2009) Freeze-fracture electron microscopic study of tight junction strands in HEK293 cells and MDCK II cells expressing claudin-1 mutants in the second extracellular loop. *Histochem. Cell Biol.* **131**, 681–690
29. Liebner, S., Fischmann, A., Rascher, G., Duffner, F., Grote, E. H., Kallbacher, H., and Wolburg, H. (2000) Claudin-1 and claudin-5 expression and tight junction morphology are altered in blood vessels of human glioblastoma multiforme. *Acta Neuropathol.* **100**, 323–331