Interaction, Function and Regulation of the Tight Junction Protein Tricellulin

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Content

1 List of abbreviations

aa	amino acid
ATP	adenosin-5´-triphosphat
BBB	blood-brain barrier
bp	base pair
BSA	bovine serum albumin
bTJ	bicellular tight junction
CFP	cyan fluorescent protein
CK1	casein kinase 1
CK2	casein kinase 2
Cld	claudin
CRFR1	corticotropin-releasing factor receptor
DAPI	4′,6-diamidin-2-phenylindol
DMEM	Dulbecco's Modified Eagle Medium
DNA	desoxyribonucleic acid
dNTP	desoxyribonucleotid-5'-triphosphat
E.coli	*Escherichia coli*
EDTA	ethylendiamintetraacetat
ER	endoplasmatic reticulum
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FRET	fluorescence resonance energy transfer
FRAP	fluorescence recovery after photobleaching
G418	geneticin
GST	gluthathion-S-transferase
HA	Human influenza hemagglutinin
HEK	human embryonic kidney cells
IB	immunblot
IgG	immunglobulin G
IP	co-Immunpräzipitation
JAM	junctional adhesion molecule
MDCK	madin-darby canine kidney cells
MD3	marevelD3
MEM	minimum essential medium
Occl	occludin
PAGE	polyacrylamidgel- electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
RT	room temperature
SDS	sodiumdodecylsulfate
TEMED	N, N, N', N'-Tetramethyethylendiamin
TER	transepithelial electric resistance
TJ	tight junctions
Tric	tricellulin
Tris	Tris (hydrocymethyl) aminoethan

Triton-X100	t-Oktylphenoxypolyethoxyethanol
tTJ	tricellular tight junctions
Tween20	Polyoxyethylensorbitanmonolaurat
U	units
UV	ultraviolet
YFP	yellow fluorescent protein
ZO	*zonula occludens*
ZO-1	*zonula occludens* protein 1

2 Introduction

Multicellular organs rely on compartmentalization, to protect the body and to maintain homeostasis of the compartments, which is guaranteed by epithelial or endothelial cells. Organs, such as the uterus, ovaries, the brain and epidermis are all barrier containing tissues primarily intended to separate organ compartments (1). However, tissue barriers do not simply separate two distinct compartments or fluids; they provide protection against potentially harmful external agents and notably also arrange communication of neighboring cells.

An essential feature of such barriers is represented by tissue-specific cellular junctions and cell-cell contacts, maintained and regulated by various multi protein complexes such as adherens junctions, gap junctions and tight junctions (TJs). A broad range of distinct disease conditions in humans is directly connected to alterations in epithelial or endothelial barrier properties. This may lead to a loss of stromal homeostasis and thereby affect fluid and electrolyte balance between compartments, within tumor microenvironment or generation of inflammatory states.

Tumor progression or bacterial invasion through tissue barriers is accompanied by epithelial/endothelial barrier dysfunction or tightness modulation, often reflecting tumor- or pathogen-induced alterations of specific structures of these tissues, particularly of the TJs (1, 2). In these cases as in many other, pathological conditions, direct/indirect damage of tissue and TJs are found to relevantly contribute to disease progression.

2.1 Organization and function of epithelium and endothelium

The four major tissue types are muscle, nerve, connective and epithelial tissues. The latter can be classified as simple epithelium, composed of a single layer of cells and covering internal and external surfaces of the body or as stratified epithelium, composed of one or more layers of cells and forming the covering of most internal and external surfaces of the body and its organs. Epithelial cells are polarized and therefore represent a spatial asymmetry of the plasma membrane, with apical surfaces exposed to the lumen and basolateral surfaces in contact with the laterally adjacent epithelial cells and the basement membrane. Consequently, they incorporate different protein compositions at the apical and basal side (3).

The endothelium is a specialized type of epithelial tissue, lining blood vessels lymphatic vessels, and the myocardium. The endothelium is built by a very flat single monolayer of

cells (4). In addition to the bare regulation of the exchange of solutes from the bloodstream and the surrounding tissues, the cells overtake regulatory functions, such as regulating the blood pressure by producing nitric oxide (NO) (5).

A broad variety of epithelial cell-cell contacts are located at the lateral plasma membrane of epithelial cells. The different types of cell junctions and cell-cell contacts within an epithelial tissue are illustrated in Fig.1, which shows adherens junctions (6), gap junctions (7), TJs (8), desmosomes (9) and hemidesmosomes (10). They guarantee tissue mechanical stability, by linking two cells together (desmosomes) and attaching one cell to the extracellular matrix (hemidesmosomes) as well as through the communication of neighboring cells (gap junctions), connection of cells of the corresponding tissues with the actin cytoskeleton (adherens junctions) and the establishment of barriers to separate extracellular regions of distinct fluid compositions (TJs).

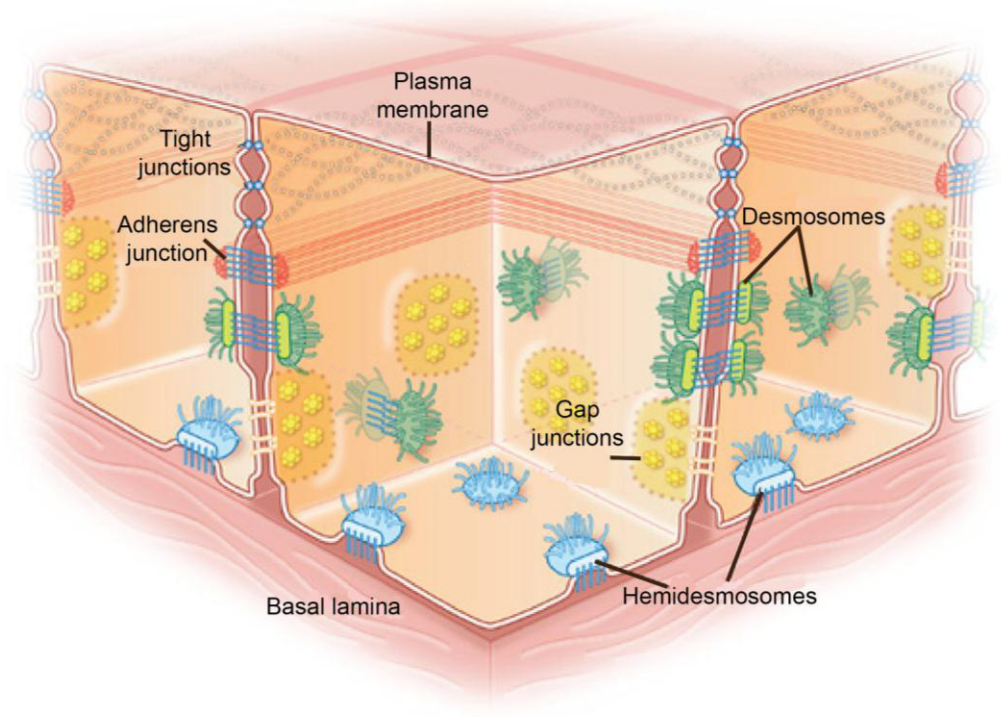

Figure 1: Schematic illustration of an epithelial cell layer and cell contacts
Tight junctions (blue dots) represent the most apical cell-cell contacts. They seal the paracellular cleft against ions and solutes or may build paracellular pores. TJs connect the plasma membranes of adjacent cells. Adherens junctions (red dots) connect the actin cytoskeleton of neighboring cells and provide the cells with mechanic stability. Desmosomes (blue bars) connect the intermediate filaments of adjacent cells, whereas hemidesmosomes (light blue) link the basal lamina with the cytoskeleton. Gap junctions (yellow) are structures forming aqueous channels and thereby regulating communication between neighboring cells. (Modified from Nature Education, 2010).

2.1.1 Tight junctions

Usually TJs are identified as electron microscopically dense protein particles surrounding individual cells and thereby sealing the paracellular cleft between two adjacent cells (11). In freeze-fracture electron microscopy, TJs can be recognized as belt like structures composed of protein particles at the apical side of the lateral plasma membrane (12) (Fig. 2A).

The separation of neighboring tissues is of general importance in higher animals, TJ function is adapted from analogous structures, namely the septate junctions (SJ), as found in invertebrates (13). In contrast to TJs, SJ appear as ladder-like structures under the electron microscope, occupying the whole lateral plasma membrane instead of being restricted to the apical side of the lateral plasma membrane. SJ are formed by proteins analogous to those retrieved in TJs (14).

TJs are composed of a multi protein complex of up to 30 transmembrane proteins and cytosolic scaffolding proteins (reviewed in: (15)), mainly transmembrane proteins such as claudins (Clds) and members of the protein family of TJ-associated MARVEL (MAL and related proteins for vesicle trafficking and membrane link) proteins (TAMPs) (16) and the cytosolic membrane-associated guanylate kinases (MAGUKs) (17, 18). From a functional point of view, the properties of TJs are in principal determined by their composition in terms of different Cld subtypes, although they may be further regulated by the TAMP family (19). Next to the Clds and TAMPs, TJ function is additionally affected by the association with cytosolic scaffolding proteins such as *zonula occludens* protein (ZO)-1 to -3, which belong to the MAGUK family (20).

2.1.1.1 Bicellular and tricellular tight junctions

In an epithelial monolayer, TJs can be found between two cells (bicellular TJs, Fig. 2A) or where three cells join together (tricellular TJs) (21) (Fig. 2B). The typical belt-like structures of bicellular TJs become discontinuous at tricellular contacts, and do not locate exclusively in apical, but extend to the basolateral side. Tricellular TJs were thought to represent the weak points of TJs, until the discovery of tricellulin (Tric) (21). Currently, Tric is considered to be a central sealing element at tricellular contacts (22).

In addition to the presence of Tric, tri- and bicellular TJs differ in their protein composition and the kind of basolateral extension (21). In 1973, Staehelin and colleagues constructed a model of the tricellular cell-cell contact which is valid until today (23). The

TJ-strands where three cells meet stretch from the apical site in the basolateral direction and form a central sealing tube of about 1 µm in length (Fig. 2B).

In general, small ions predominantly pass through the bicellular junctions. The pathway was postulated as high capacity, charge and size-selective pore (22). In contrast, macromolecules pass through TJ-strand discontinuities at the level of bicellular junctions (low capacity, charge and size nonselective leaky) or at the tricellular pore (21, 22), if Tric is not present. It was calculated, that the tricellular central sealing tube exhibits a radius of about 5 nm, which would allow the passage of macromolecules with a molecular weight of up to 10 kDa (22, 23).

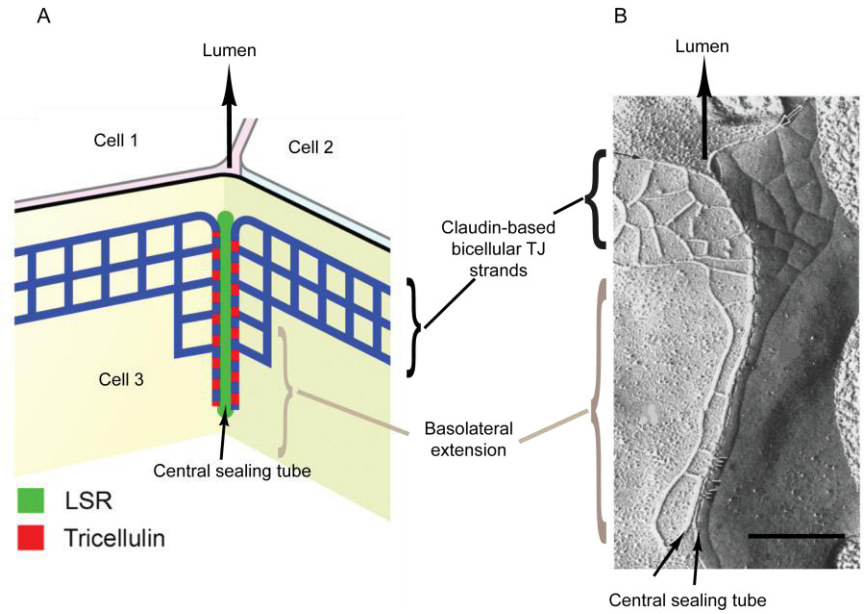

Figure 2: Organization of an epithelial bi- and tricellular contact.
A) Schematic 3D-representation of the TJ-strand network, including the association of the strands within a plasma membrane with those of adjacent cells at the tricellular contact to form paired Cld-based TJ-strands (blue). Tricellulin (Tric, red) may build the linkage between the Cld-based bicellular TJ-strand network and the tricellular contact. Furthermore Tric builds a central sealing tube, which is extended in the basolateral direction and interacts with the landmark for tricellular contacts, the lipolysis stimulated lipoprotein receptor (LSR) (modified according to: (24)).
B) Freeze-fracture replica electron microscopic image of TJs between adjacent epithelial cells from a mouse renal cortical collecting duct. At tricellular contacts, elements of the TJ-strands of the bicellular TJ turn to extend in the basal direction, with an orientation to the bicellular tight junctional belt. Scale bar, 500 nm (modified according to: (23)).

2.2 Molecular composition of tight junctions

The following chapters list and describe the proteins involved in establishing the TJ protein complex.

2.2.1 Claudins

In 1998, the group of S. Tsukita and coworkers isolated the first Cld (protein: Cld, gene: *Cldn*) from purified junctional fractions of chicken livers (25). The overexpression of the integral membrane protein in TJ-free mouse fibroblasts resulted in reconstituted TJ-strands, which led to the assumption that Clds represent the major structural components of TJs (25, 26).

Until now the protein family of Clds has been expanded up to 27 members in mammals and, 26 in humans, with a molecular mass of 20 to 27 kDa (27). Based on their amino acid sequence, the protein family can be subdivided into a group of classic Clds (with high amino acid sequence homology) and a group of non-classic Clds (with a more diverse amino acid sequences) (Fig. 3A) (28). Recently a comparison was made among various phylogenetic analysis proposed for the Cld family (29). The authors suggested a phylogenetic tree that sorts human Clds into eight subgroups, which form four major clusters: cluster I (subgroups A/B) Cld3, 4, 5, 6, 9/Cld8, 17; cluster II (subgroups D/E) Cld1, 7, 19/Cld2, 14, 20; cluster III (subgroups F) Cld10, 11, 15, 18; and cluster IV (subgroups C/G/H) Cld21, 22, 24/Cld12, 16, 25/Cld23, 26, 27 (Fig. 3B) (29).

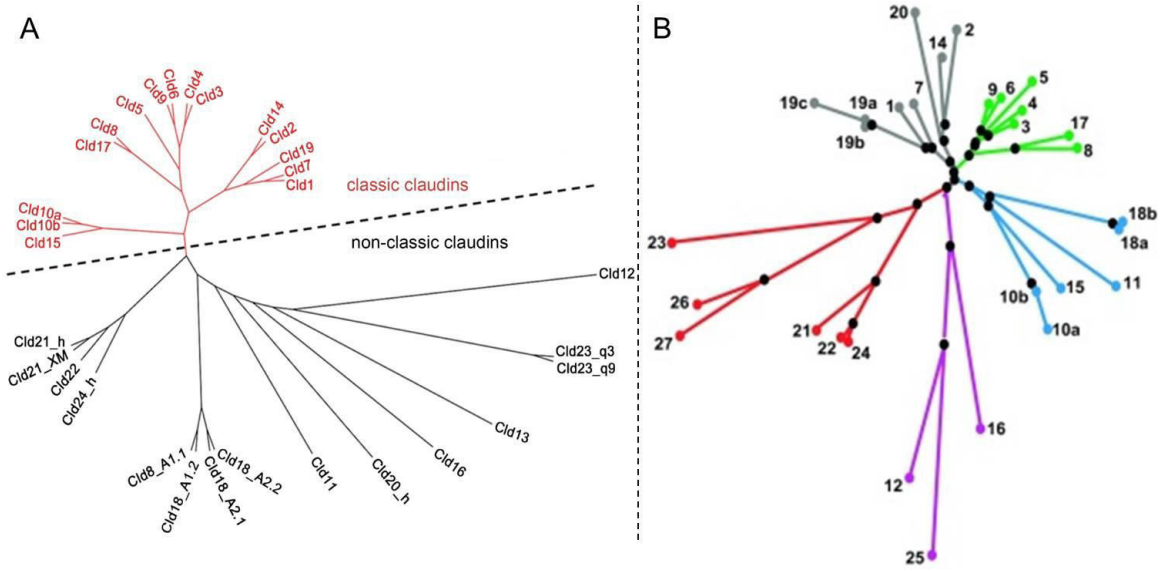

Figure 3: Phylogenetic tree of claudin subtypes.
A) Prior classification of claudin (Cld) subtype classification. Classic Cld1-10, 14, 15, 17, and 19 with high sequence similarity are clustered in red, non-classic Cld11-13, 16, 18, and 20-24 are shown in black. Cld in the mouse sequences were taken as representative. (28).
B) Classification of human Cld subtypes, based on the comparison of various suggested Cld phylogenetic classifications and under consideration of the recently discovered Clds (29).

Despite the shared sequence homology, the expression of different combinations of classic Clds within a given TJ structure leads to different barrier characteristics of the respective tissues (28), Hence it was postulated, that Cld tissue composition determines the permeability characteristics of the corresponding tissue (25, 30).

For instance, a predominant expression of Cld5 is known to tighten the blood-brain barrier (BBB) against small molecules up to 800 Da (31, 32), whereas Cld2 forms pores for cations and water in the intestine (diameter of 3.25 to 4 Å) (33).

In various tissues, Clds exhibit a differential expression pattern (for review see: (30)). Furthermore, Clds are expressed in a specific developmental manner, leading to permeable or tight barrier characteristics during different developmental phases (34, 35). The general topology of a Cld consists of two extracellular loops (ECL1 and ECL2), four transmembrane domains (TMD-I to TMD-IV) and, a short intracellular loop (ICL) connecting TMD-II to TMD-III, a cytosolic N- and C-terminal tail (Fig. 4A) (36). The ECL1 of all Clds contains a disulfide bond (Fig. 4A, yellow), which may be involved in the redox-dependent oligomerization of Clds (37).

The PDZ binding domain is a part of the cytosolic C-terminal tail (expeditions: Cld12, 19a, 21, and 24 to 27) (30). The domain is responsible for the binding of Clds to the PDZ-domains of various scaffolding proteins, such as ZO-1, and thereby their linkage to the actin cytoskeleton (38). These interactions are assumed to be essential for the correct localization and plasma membrane mobility of the corresponding Clds (39, 40).

Importantly, interactions of Clds with other members of the family occur between the plasma membranes of neighboring cells (*trans*-interactions, Fig. 4A), mediated by the ECL2 (26, 31). These interactions have been demonstrated to be mediated by conserved aromatic amino acids within the ECL2, at least for classic Clds (26).

The ECL1 is crucial for the paracellular tightening (41) or paracellular pore formation (42). For Cld2, the size and charge selectivity is realized by non-conserved charged amino acids in the ECL1, whereas the absence of these amino acids implement the paracellular tightening (28). The ECL1 of all Clds contains two highly conserved cysteine residues, that form an intramolecular disulfide bond, being directly involved in the folding of this domain (37).

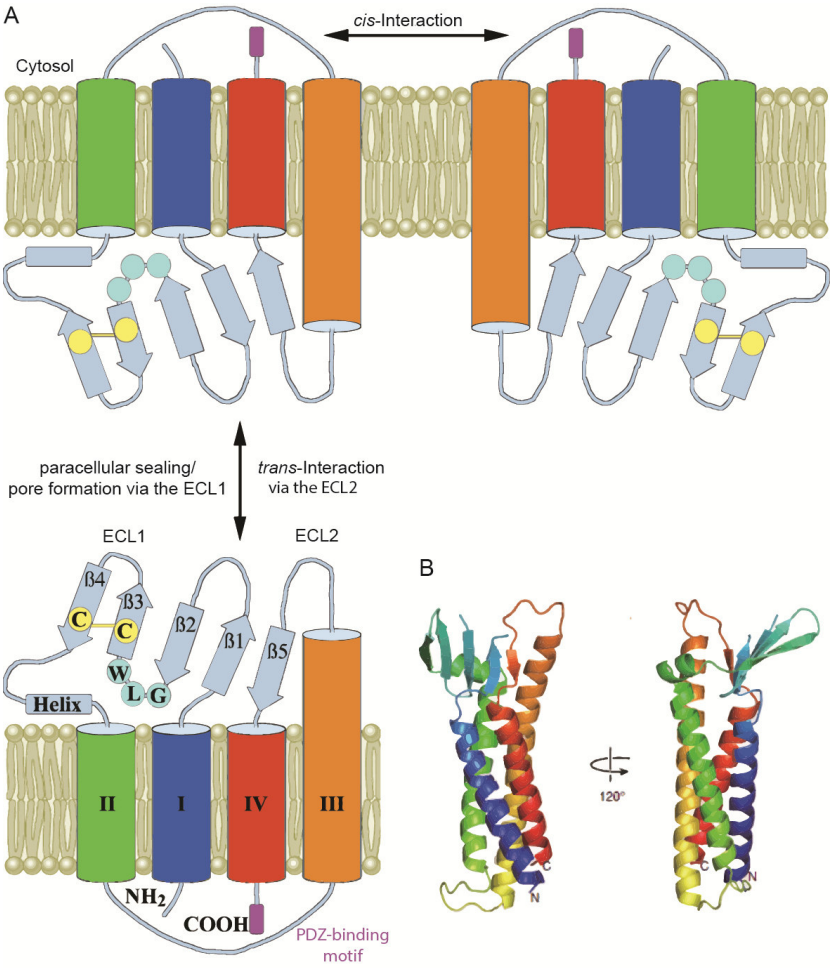

Figure 4: Schematic view of the topology, interaction and structure of classic claudins
(A) Clds consist of four transmembrane domains (TMD-I to TMD-IV), which may be involved in *cis*-interactions (43). Furthermore, the structure bears two extracellular domains (ECL1 and ECL2) as well as cytosolic N- and C-terminal domains. the ECL1 is assumed to form a disulfide bond (37) (yellow) and is thought to be involved in pore formation (42) or in sealing of the paracellular space (41). The ECL2 is known to be involved in maintaining the *trans*-interactions (26) (modified according to (36)).

Moreover, Clds interact with each other also within one plasma membrane (*cis*-interactions, Fig. 4A). For Cld3 this interaction is mainly mediated by TMD-III (43). In TJ-strands Cld interactions occur between different (heterophilic) and same subtypes (homophilic). Although homo- and heterophilic *cis*- and *trans*-interactions have a crucial role in building TJ-strands, the precise mechanism of such assemblies have been unresolved (26, 44).

Very recently, the first crystal structures of Clds were resolved, giving novel insights into the molecular topology and spatial organization of classic Clds15 and 19 (36, 45) (Fig. 4).

2.2.2 Tight junction associated MARVEL proteins

The TAMP family consists of the TJ proteins Occl, Tric and MD3 and is characterized by the shared MARVEL domain (19) (for predicted structure of the MARVEL domain see Fig. 5A). The domain was found in proteins involved in vesicular transport (46) and membrane fusion (47). The TAMPs represent a distinct cluster in the phylogenetic tree of the MARVEL domain containing superfamily (Fig. 5B) (16, 47).

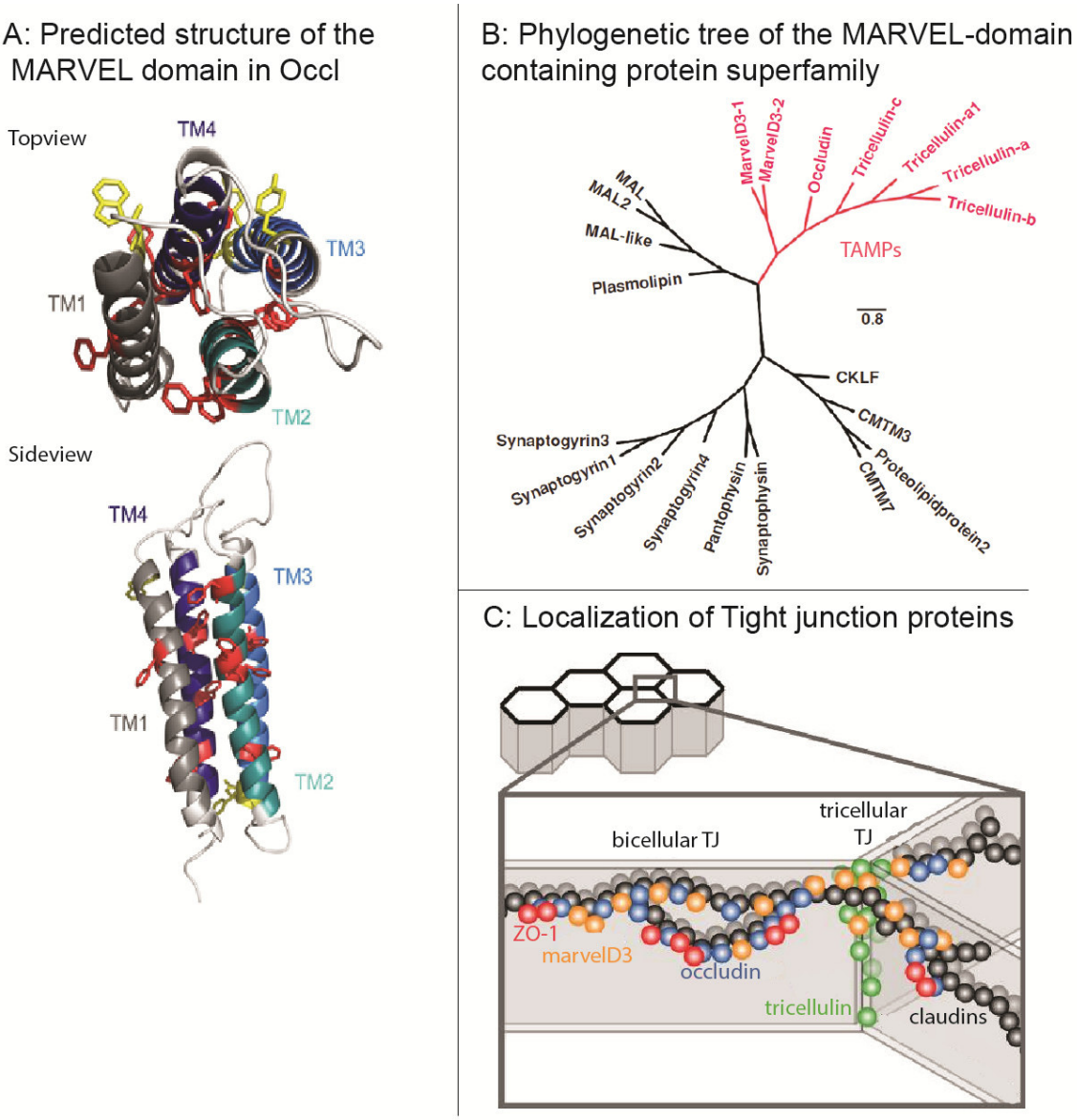

Figure 5: Predicted structure of the MARVEL domain, classification of the TAMPs within the MARVEL protein superfamily and their organization at bi- and tricellular tight junctions

(A) Structure determination for Occl: TM-I, gray; TM-II, cyan; TM-III, blue; TM-IV, dark blue. Phenylalanines are shown in red, tryptophans and tyrosines in yellow. Upper panel is a view of the structural model from outside the cell (48).

(B) Phylogenetic tree of the MARVEL domain containing protein superfamily. Human TAMPs are highlighted in red (16).

(C) The organization of TJ proteins within a small area of a confluent monolayer (see image at the top). The TJ-strands within both bi- and tricellular regions are composed of Clds (black spheres). The TAMPs Occl (blue spheres), Tric (green spheres) and MD3 (orange spheres) are incorporated into Cld-based TJ-strands. Occl is primarily found at bicellular junctions, Tric is localized specifically at tricellular regions, MD3 is present at both sites (19). Occl interacts with the ZO-1 (red spheres) via the Sh3-hinge-GuK-unit, while Cld interacts via the PDZ-1-domain (25). Thus, although there are similarities within the protein family, each TAMP can also be defined by unique characteristics (16, 19). MARVEL, MAL and related proteins for vesicle trafficking and membrane link; TAMPs tight junction associated MARVEL proteins; Occl, occludin; TM, transmembrane domain; Tric, tricellulin; MD3, marvelD3

The MARVEL domain comprises the segments from the 1st to the 4th transmembrane domain of TAMPs, including the two extracellular loops and one intracellular loop (46). It was postulated, that the MARVEL transmembrane motif of Occl mediates oligomerization and targeting to the apicolateral surface in epithelial cells (Fig. 5A) (48).

Moreover, the TAMPs contain cytosolic N- and C-terminal tails of variable length. The homologous MARVEL domain may favor homophilic interactions within the TAMP family and heterophilic interactions with the Cld family (16). Relative localization of the TAMPs to Clds and ZO-1 is schematically illustrated in Fig. 5C and reveals a close arrangement of these distinct proteins within the TJ complex. The data suggest that these proteins should be viewed as interaction partners with both overlapping but also specific contributions to function and regulation of TJs.

2.2.3 Occludin

Occl was the first integral membrane protein to be identified at TJs. Human Occl is characterized by a molecular mass of 65 kDa (49). The C-terminal tail is relatively long (254 amino acids), the N-terminal part appears to be only 66 amino acids (50). Remarkably, a key region of the C-terminal part (~130 amino acids) with presumably high proteolytic stability (51, 52), shows considerable homology to the C-terminal part of Tric (21) and carries an ELL-domain (53) (because of its similarity to the RNA polymerase-II elongation factor ELL (54)). Based on its structure, the domain is termed Occl coiled-coil domain (51). The C-terminal tail is of major importance for the translocation of Occl to the plasma membrane and for endocytotic processes (55). Furthermore, the C-terminal coiled-coil domain mediates redox-dependent homo-oligomerization *in vitro* (56), the direct association to the Sh3-hinge-GuK unit of ZO-1 (53, 57) and F-actin (58) and hence the direct connection of TJs to the actin cytoskeleton.

The crystal structure of the Occl ECLs is unknown but it is assumed, that the ECL2 plays an important role in integrating Occl in the TJ complex and thereby in the barrier characteristics of the respective tissue (59). The ECL2 contains two conserved cysteine residues that are demonstrated to form an intramolecular disulfide bond. Recently, it was demonstrated that the conserved disulfide bond in the ECL2 is involved in the redox-dependent *trans*- and *cis*-oligomerization of Occl (60).

Occl was shown to be multiply phosphorylated at the C-terminal tail (61), underlining the regulatory importance of this segment. Inhibition of casein kinase-2 (CK2)-mediated phosphorylation of Occl S_{408} leads to an elevated trans-epithelial electric resistance (TER) and a remodeling of the molecular interactions to ZO-1 and selected Clds (62). Hence, de-/phosphorylation of Occl lead to the introduction of binding motives or the removal of protein binding sites.

Saitou and colleagues (2000) generated mice lacking the *marvelD1* gene ($Occl^{-/-}$) (63). These mice showed a weak phenotype. Nevertheless, they exhibited postnatal growth retardation, male sterility and abnormalities in various tissues, suggesting that the functions of TJs and Occl are more complex than postulated earlier (49, 63). Furthermore, a mechanism in which other TJ proteins compensate for the Occl loss has been suggested to explain the non-lethality of this knock-out (63). Promising candidates for the compensatory mechanism are other members of the TAMP family, in particular Tric and/or MD3 (19, 64). In experiments with MDCK-II Occl knock-down cells, Tric was redistributed from tricellular contacts to bicellular contacts, in the attempt to compensate for Occl at its usual localization (64).

So far two isoforms, generated by alternative splicing, have been identified: TM4 and 1B. TM4 lacks the TMD-IV and parts of the cytosolic C-terminal end (65). Contrary to the classic model of Occl, the C-terminal domain is located extracellular in these isoform, therefore the plasma membrane-located fraction of Occl is significantly reduced. Although the Occl isoform 1B contains 56 amino acids more at the N-terminus, its subcellular distribution is substantially unaltered compared to full-length Occl (66). Since Occl is expressed in every epi- and endothelial barrier, it is generally accepted as the TJ marker, although its precise function is still controversially discussed (16).

2.2.4 Tricellulin

Tric was described as the first integral TJ protein localized at tricellular TJs (21). Human Tric was characterized as a 558 amino acids polypeptide with a molecular mass of 64 kDa

(67). Here, Tric always corresponds to the longest isoform, Tric-α. In humans, the N-terminal tail contains 187 amino acids, whereas the cytosolic C-terminal tail includes 193 amino acids, with remarkable homology to the Occl coiled-coil domain. The first ECL contains 41 amino acids and has a high number of tyrosine and glycine residues, whereas the second ECL contains 30 amino acids (Fig. 6) (67).

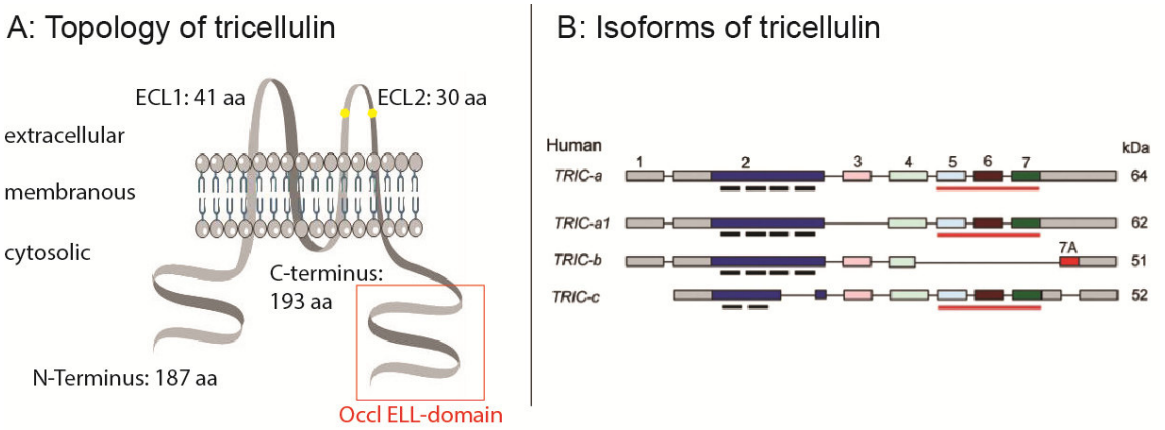

Figure 6: The predicted protein structure of Tricellulin
(A) Topology of human Tric. The last 130 amino acids of the C-terminal end have an identity of 32% identical to Occl (red box). Tric shows a longer N-terminal cytoplasmic domain (187 amino acids) as compared to Occl (61 amino acids). The ECL2 contains two highly conserved cysteine residues (yellow) that may form an intramolecular disulfide bond (60).
(B) Isoforms of human Tric. The black bars underneath exon two mark the predicted transmembrane domains, the red line marks the Occl coiled-coil domain, while the grey areas represent untranslated domains (67).

In mammals, the gene encoding for Tric (and for Occl) is located on chromosome 5. Consequently, it was postulated, that Tric and Occl may have evolved from one common ancestor gene, due to gene duplication events (21), and it is likely that Tric and Occl are able to compensate each other's functions.

The expression of Tric was initially demonstrated in the epithelia of the intestine, gut and kidney (21), later on in other epithelial tissues and the endothelium of the blood-brain barrier (68). Altogether these data suggest an ubiquitous expression of Tric in epithelial and endothelial tissues (68). Furthermore, Tric mRNA was detected in dendritic cells of the immune system (69).

In contrast to Clds, Occl and MD3, Tric is preferentially localized at tricellular contacts in epithelial tissues and cell lines. However, lower amounts of Tric are also detectable at bicellular contacts (19, 25, 47, 64). In TJ-free L-fibroblasts, transfected Tric is equally distributed at the periphery of the lateral plasma membrane, whereas the cotransfection

with Cld1 promotes the localization of Tric at bicellular junctions (19, 64). The Knock-down of Occl in cultured mouse EpH4 mammary epithelial cells leads to predominant localization of Tric at bicellular junctions, resulting in abnormal morphology at tricellular junctions. However, if the expression of Tric is knocked-down, Occl localizes at the tricellular contacts (64). These effects are likely to result from mutual functional regulation of TJ proteins and can be described by the evolutionary similarities with Tric in the specific case of Occl. Although the localization of Tric is compensated by Occl, its unique function, tightening the tricellular junctions against large molecules, is not (21).

The last 130 cytosolic amino acids are highly conserved within Tric and Occl. The domain is involved in the association of Occl to the junction-associated scaffolding protein ZO-1 via its hinge-GuK-domain (51). Based on the evolutionary relationship between Occl and Tric, it is assumed that both proteins associate with ZO-1 via their C-terminal part (19, 67). However, the interaction of Tric with ZO-1 is still controversial discussed. While one group demonstrated binding between GST-labeled Tric C-terminus and recombinant ZO-1 *in vitro* (67), another one showed no binding between GST-Tric C-terminal end and the GuK-domain of ZO-1 (39). However, the association of Tric with ZO-1 is not required for a plasma membrane localization of Tric, since this protein reaches the plasma membrane in ZO-1 free cells (64).

The lipolysis stimulated lipoprotein receptor (LSR) is yet another interaction partner of Tric at tricellular junctions, where it builds a landmark for tricellular contacts and immobilizes Tric at these areas (70-72). The molecular mechanisms guiding Tric to the tricellular junctions are not understood, but it seems likely that a set of different TJ proteins might be involved, since the knock-down of Occl or LSR, or cotransfection with Cld1 alters Tric localization (21, 64, 71).

The localization of Tric at tricellular junctions in epithelial tissues influences the function of the respective barriers, since the stable suppression of Tric showed an inhibition of the TER in mouse Eph4 cells (21). Strong overexpression of Tric results in specific and unspecific localization at tricellular and bicellular junctions, respectively. Conversely, weak overexpression leads to exclusive tricellular localization (22). The properties of epithelial barriers differ, depending on the localization of Tric. Bicellular localization decreases the permeability of ions and solutes, whereas exclusive tricellular localization has no effect on the ion permeability but decreases the permeation of larger solutes (4 to 10 kDa) (22).

Similarly as described for Occl, Tric appears to be multiple phosphorylated in epithelial cells, as suggested by several Western blot bands (MW range of 66-72 kDa) that disappear after phosphatase treatment (21). However, the function of the phosphorylation and the kinases involved are widely unknown (61).

Four splicing variants of the *MarvelD2* gene encoding for Tric are known (Fig. 6B). *Tric-α* represents the longest isoform, consisting of seven exons, while *Tric-α1* lacks exon three and encodes for a 62 kDa protein comprising the Occl coiled-coil domain. *Tric-b* encodes for a 51 kDa isoform which lacks the Occl coiled-coil domain. *Tric-c* is alternatively spliced at exon two, and the derived gene product includes only two predicted TM-domains and represents, with 42 kDa, the shortest Tric isoform (67).

Although the functions of Tric are poorly investigated, Riazuddin and coworkers (2006) already identified a mutation in the *marvelD2* gene that links Tric to the disease of nonsyndromic deafness (DFNB49) in humans (67, 73). The DFNB49 nonsense mutation in the *marvelD2* gene generates a deletion of the last 60 amino acids at the C-terminal cytoplasmic region (67). Very recently, a knock-in mouse which carries the DFNB49 nonsense mutation was generated. In these mice, Tric was not concentrated at tricellular TJ but more broadly distributed. Furthermore, freeze-fracture electron microscopy revealed disruption of the TJ-strands of intramembrane particles connecting bicellular and tricellular junctions (74). Surprisingly, the phenotype of the DFNB49 mutation is limited to the organ of Corti in the inner ear. Here, a strict compartmentalization between areas with high and low ion concentrations is required for the correct function of the organ. In contrast to other human epithelial tissues, where other TJ proteins may compensate for Tric, the compartmentalization is accomplished by a unique tightness of the barrier, in the organ of Corti (67, 74).

In invertebrates, the protein gliotactin from *Drosophila melanogaster* seems to represent the functional counterpart of Tric at SJ (75). It forms protein complexes at tricellular contacts of epithelial cells with discs large, a cytosolic scaffolding protein, belonging to the protein family of MAGUKs (75, 76).

2.2.5 MarvelD3

MD3 was added to the TAMP family in 2010 (19, 47). In contrast to Occl and Tric, the gene encoding for MD3 is located on chromosome 16 in mammals. Alternative splicing of exon three and four leads to MD3 isoform-1 (401 amino acids, 44.9 kDa) and -2 (410 amino acids, 45.9 kDa), with identical N-terminal tail (226 amino acids) but differ in the

MARVEL domain and length of the cytosolic C-terminal tail. This seems to be peculiar for MD3 since the isoforms, described for Occl and Tric, vary exclusively in the length of their cytosolic termini (77, 78) but not in the MARVEL domain. In contrast to Occl and Tric, the C-terminal tail contains no coiled-coil domain, which leads to the assumption that MD3 does not interact with ZO-1 (19, 47).

Physical interactions were demonstrated for MD3 with Occl but not with members of the Cld family, using co-IP assays (19). In contrast to Clds, the transfection of MD3 alone in TJ-lacking cell models was not sufficient to reconstitute TJ-strands (47). However, MD3 localizes together with Cld and Occl at bicellular TJ, and a sub-fraction is also colocalized with Tric at tricellular TJ (19).

Knock-down experiments of MD3 revealed a delayed establishment of TER during culture of Caco-2 cells, but no changes in the expression or localization of other TJ proteins, as it was shown for Occl and Tric (47). This indicates on the one hand, no role of MD3 in regulating TJ composition, and on the other hand, a direct functional influence of the protein on barrier development. The delay in TER formation was further prolonged by a simultaneous siRNA-based down-regulation of Occl or Tric in Caco-2 (19).

Very recent data indicate MD3 as potential regulator of the mitogen-activated protein kinase kinase kinase-1 (MEKK1)-c-Jun NH_2-terminal kinase (JNK) pathway, involved in the regulation of cell proliferation, migration and survival of cells (79). According to this study, plasma membrane localized MD3 recruits MEKK1 to TJs and thereby down-regulates JNK phosphorylation and inhibition of JNK-regulated transcriptional mechanisms. Thus, MD3 might represent a key link between TJ and the MEKK1-JNK pathway to regulate cell behavior and survival (79), indicating the importance of modulating barrier properties and more basic cellular functions, simultaneously.

2.2.6 Tight junction-associated proteins

Besides the integral membrane proteins of TJs described above, a multitude of soluble and membranous proteins are associated with the TJ complex. The ZO proteins represent the most abundant cytosolic protein family associated to cell-cell contacts. ZO-1 was the first protein identified at cell-junctions (20). Three members of ZO proteins have been characterized (ZO-1, ZO-2 (18) and ZO-3 (17)). All members are ubiquitously expressed (80) and are constituted of Sh3-GuK and PDZ-domains, essential for their function as scaffolding proteins (81). The protein family is termed MAGUKs, because of the similarity of the GuK-domain to guanylate kinases (82). In contrast to ZO-2 and ZO-3,

ZO-1 binds the cytoskeletal F-actin with its proline-rich C-terminal half and Occl with its hinge-GuK region, thereby linking the cytoskeleton to TJ (58, 83). Furthermore, ZO-1, -2 and -3 bind with their PDZ-1 domain to the C-terminal part of Clds. This interaction recruits Clds to the TJs, where they stabilize its architecture (38). Hence, the main role of ZO proteins is the scaffolding function between TJ proteins, the linkage of TJ with the actin cytoskeleton and the connection of signaling proteins (e.g., the transcription factor ZONAB) to plasma membrane proteins of TJs (38). Furthermore, ZO-1 and ZO-2 may function as plasma membrane to nucleus shuttle in TJ-free cells or in cells under mechanical stress (84, 85).

The coxsackie virus and adenovirus receptor (CAR), a protein constituted of a extracellular domain, one transmembrane domain and a cytosolic C-terminus (86) belongs to the immunoglobulin superfamily (IgSF), based on structure similarity. CAR colocalizes with ZO-1 in epithelial cells and is thought to be of tightening relevance, since overexpression in cell culture experiments increases the TER in (87).

Further transmembrane proteins of TJs are represented by the protein family of junctional adhesion molecules (JAMs) (JAM-A-C, JAM-like and IgSF5), all with a molecular mass of ~40 kDa (88, 89). JAMs consist of one extracellular domain, one transmembrane domain and an intracellular domain and belonging to the family of IgSF (90). Various protein binding domains are located at the cytosolic C-terminal part, promoting association of JAMs with the SH3-domain of ZO-1 (91, 92), the cell polarity protein Par-3 (91), afadin (93), cingulin and Occl (94).

2.2.7 The Angulin-protein family

The protein family consists of the lipolysis stimulated lipoprotein receptor (LSR), immunoglobulin-like domain-containing receptor (ILDR)-1 and ILDR-2 (95, 96). The first member of this TJ protein family was identified as LSR, by using retrovirus-based cDNA-GFP (green fluorescent protein) fusion libraries (97). Masuda and colleagues (2010) found the receptor to be colocalized with Tric at tricellular TJs (96), where its C-terminal cytosolic domain associates to the cytosolic C-terminal domain of Tric. Knock-down of LSR lacks epithelial barrier integrity, formation of tricellular contacts and, peculiarly, the linkage between bicellular and tricellular contacts (96).

In general, the expression of LSR, ILDR1 and ILDR2 is complementary to each other in most of the tissues, but LSR and ILDR1 are coexpressed in some compartments. Specifically, LSR, similarly to ILDR1, is expressed in tissues forming a strong barrier, e.g.

the bladder epithelium. ILDR2 is expressed at tricellular contacts of weaker epithelial barriers, e.g. epithelia covering neural tissues such as the retinal pigment epithelium (95). Recent data demonstrate the expression of LSR to be specific for the brain endothelium, rather than in other endothelial tissues. The expression correlates with the embryogenetic development of the blood-brain barrier. The knock-out of LSR in mice led to a leaky blood-brain barrier (for small molecules) during embryogenesis (98).

Mutations in the *ILDR1* gene cause nonsyndromic deafness DFNB42 (99), since the recruitment of Tric to tricellular contacts was found to be impaired. Hence LSR, ILDR1 and ILDR2 build landmarks for tricellular TJs and recruit Tric to ensure correct barrier integrity (95).

2.3 Tight junction role in pathological events

Various diseases are directly or indirectly linked to alterations in the barrier properties of epithelial or endothelial tissues and of the TJs. Examples are inflammatory bowel and kidney diseases, cancer of epithelial tissues or vulnerability to microorganism invasion (reviewed in: (1)). The reasons for the alteration of barrier properties are very diverse and range from mutations in a TJ protein gene and changes in the expression or localization of TJ proteins or the specific binding/targeting of TJ proteins by infectious agents. The effects of such modifications can be divided into three main groups (100):

1) Reduction in the paracellular flux of solutes: in kidney diseases such as familial hypomagnesemia, hypercalciuria and nephrocalcinosis are caused by genetic deficits in the genes *Cldn16* (originally named: Paracellin-1) and *Cldn19*, and result in a decreased reabsorption of Mg^{2+} and Ca^{2+} in the nephron (101, 102).

2) Increased permeation of solutes and water: typical examples are inflammatory intestinal diseases, such as *Crohn's* disease. The cytokine-induced upregulation of Cld2 results in an increased cation flux and thereby leads to leak flux diarrhea (103).

3) Increased permeation of large molecules: this kind of events also take place in intestinal inflammation and food poisoning. The pathogen *Clostridium perfringens* binds specifically with its enterotoxin to the extracellular domains of Cld3 and 4 in the intestine and causes damage of the epithelium (104).

The tricellular TJ represents a potential pathway for macromolecules (22). Some pathogens were reported to directly influence the expression or function of Tric. The pathogen *Shigella* targets Tric and penetrates epithelial cells via the tricellular junctions (105).

The various disease states linked directly or indirectly to TJ underlines the importance of these protein complexes.

2.3.1 Tight junctions during alterations of the Redox-state

Many diseases are closely related to alterations in the redox-status of the organism. Ischemia refers to a lack of oxygen and substrate supply to a certain tissue or organ (106), resulting in a breakdown of the cellular metabolism which can severely impact the future functionality of the affected tissue or organ or might even lead to its´ death. The cause of ischemic damage might be a thrombus, an embolus, a constriction or progressive narrowing of an artery (e.g. atherosclerosis), but also traumatic or inflammatory processes. A thrombus or blood clot leads to an occlusion of the respective vessel (micro vessel) and thereby to a lack of oxygen supply to the tissue distal to the thrombus.

However, the origin of ischemia is directly linked to abnormalities of the blood vessels and/or of the constituent endothelial cells (107). In highly aerobic tissues, such as the brain, heart or kidney, insufficient oxygen supply leads to irreversible tissue damages. For the brain, three to five minutes ischemia cause tissue damage in the brain, whereas kidney damage appears after 30 minutes of ischemic injury (108).

The brain endothelium, forming the blood-brain barrier, requires 20% of the arterial O_2 in the brain (109) and ~20% of the total daily absorption of glucose as energy supply (110). During perfusion with O_2 and glucose, mitochondria generate adenosine triphosphate (ATP) under oxygen consumption and contribute to a balanced generation and scavenging of reactive oxygen species (111). In ischemic events, the electron flow is inhibited due to O_2 deprivation and the mitochondria consumes ATP to pump protons from the matrix into the intermembrane space (111). During mitochondrial respiration and reoxygenation, the reactive oxygen species $O_2^{\cdot -}$ (superoxide) and H_2O_2 (hydrogen peroxide) are produced in complex I (NOX, NADPH-dehydrogenase) and in complex III (ubiquinone-cytochrome b-C1) (112, 113). Therefore, metabolic alterations and inflammatory mediators may lead to an enhanced existence of reactive oxygen species (ROS) targeting also the TJs and leading to a leaky barrier (114).

The production of reactive oxygen species during acute ischemia is linked to alterations in TJ protein localization and function (115) and an increased permeation of, as shown for the blood-brain barrier (116).

The direct influence of reactive oxygen species on TJ protein functions is as yet poorly investigated. It was shown, that highly conserved cysteine residues in the ECL1 of Cld1

are involved in the redox-dependent oligomerization of the protein (37). Furthermore, the TAMP Occl *cis*- and *trans*-oligomerizes in a redox-dependent manner via the ECL2. Within the Occl ECL2, two highly conserved cysteine residues determine the structure and function of this domain. Hence, Occl is sensitive to hypoxia and can be viewed as a sensor for ischemic/hypoxic conditions at bicellular TJ (60).

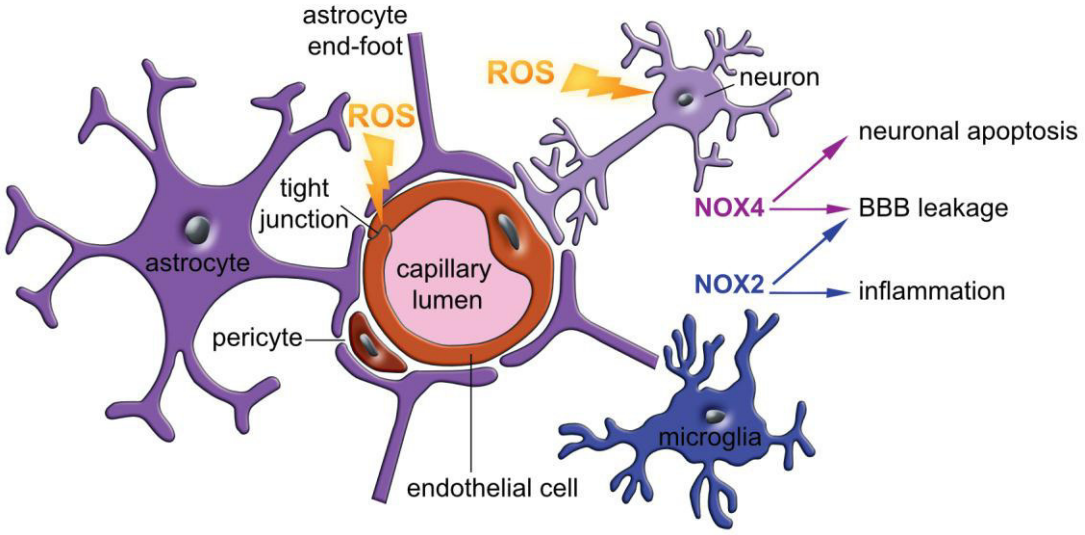

Figure 7: Schematic overview of reactive oxygen species in ischemic events at brain microvessels
The blood-brain barrier (BBB) is formed by endothelial cells at the level of the cerebral capillaries. The figure shows a brain capillary in cross section, with endothelial tight junctions and end-feet of astrocytes covering these capillaries. The figure also depicts pericytes, neurons and microglial cells. Cerebral NADPH oxidase (NOX) activation and subsequent reactive oxygen species (ROS) generation contributes to BBB disruption, inflammation and postischemic neuronal injury (117).

The involvement of Tric in ischemic events was not investigated so far, but based on the knowledge about the protein function and its relationship with Occl, it could represent an important candidate for the increased permeation of macromolecules taking place in hypoxic events (16).

3 Purpose and objectives

Tight junctions are essential for correct compartmentalization of the body and thereby for the function of vital organs. TJ are composed of a protein complex of more than 30 different proteins, which differ in functions and localization. The majority of published cell biological studies investigate functions of single TJ proteins, without considering heterophilic interactions with other TJ proteins. The aim of the thesis is to elucidate heterophilic TJ protein interactions occurring in epi- and endothelial cells, under special consideration of the tricellular TJ protein Tric. Therefore, the thesis is structured in four major parts:

1) Systematic investigations of the Cld subset, expressed in cerebral tissue barriers

To gain insights into the molecular organization of Cld containing barriers, we investigated Cld interactions outside of the TJ environment, to be able to detect the specific characteristics of a Cld-Cld interaction in living cells. The data should improve the understanding of the oligomerization of TJ proteins to establish functional barriers.

2) Homo- and heteromeric complexes formed by Tric

Tric was discovered as a novel TJ protein localized at tricellular TJ. Therefore no information is available concerning structure or interactions of Tric with other members of the TJ protein family. In this project we aimed to understand how the specific tricellular localization is realized and which domains of Tric are involved in directing Tric to tricellular junctions. Furthermore, Occl is involved in formation and organization of TJ-strands. Since Tric and Occl are closely related, interplay of both proteins is suggested. Hence, we investigated the homomeric interactions of Tric and the heteromeric interactions between Tric and Occl.

3) Interplay of tight junction associated MARVEL proteins with claudins

Cld interact in a homo- and heterophilic manner to establish the corresponding barrier characteristic, in epithelial and endothelial tissues. It was demonstrated for the MARVEL domain-containing protein Occl that heterophilic interactions to the Cld family occur. Occl together with the novel MARVEL domain containing proteins MD3 and Tric form the so-called TJ-associated MARVEL protein family (TAMPs). For Tric and MD3 no information regarding the interplay with Cld is available.

In this subproject, we want to identify the role of TAMPs within the TJ complex. Therefore we measured the *cis*- and *trans*-interactions within the TAMP family and

between TAMPs and the Cld family. Furthermore, we analyze the modulation of the Cld-based TJ-network by Occl, Tric and MD3.

4) Influence of hypoxic/ ischemic conditions on functions and interactions of Tric

Recently, it was shown that the disulfide bonds in the Occl ECL2 are hypoxia sensitive and thereby its plasma membrane fraction was reduced in epithelial cells. The protein family of TAMPs is thought to be relevant for the regulation of assembly and maintenance of cellular barriers. Under physiological conditions, Tric tightens tricellular junctions against macromolecules. As pathological disturbances have not been analyzed so far, the structure, function and molecular interactions of human Tric, including potentially redox-sensitive Cys sites, were investigated under reducing/oxidizing conditions at tri- and bicellular contacts. The study help to gain knowledge about the molecular alterations occurring in pathological disturbances targeting TJ and on the TJ role in Pathobiology.

4 Manuscript 1 (Ms1)

Elucidating the principles of the molecular organization of heteropolymeric tight junction strands

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4.1 Synopsis

The overexpression of a single Cld in TJ free cell culture systems leads to reconstituted TJ-strands, therefore the Cld protein family is considered to form the backbone of TJ. Cld subtypes function as a sealing element of the paracellular cleft against ions and solutes (e.g., Cld5 seals the blood-brain barrier for ions and small molecules up to 800 Da). However, other Cld subtypes also build molecular pores (e.g., Cld2 - cationic pore or Cld10b - anionic pore). Typically, the paracellular barrier of a respective tissue is not formed by a single Cld subtype alone, but by a tissue-specific set of Cld subtypes, that determine the properties of the corresponding tissue permeability. In addition, the protein family can be subdivided into a group of classic Clds (with a high amino acid sequence homology) and a group of non-classic Clds (with a highly diverse amino acid sequence).

In the present study we analyzed the molecular organization of heteromeric TJ-strands, occurring in cerebral barriers, composed out of the Cld1, 2, 3, 5 and 12. We attempted to understand, how and whether the various Cld subtypes build up paracellular barriers. Furthermore, we aimed to identify the molecular determinants of proteins promoting the interactions between the Cld subtypes. TJ proteins interact between neighboring epi- and endothelial cells to seal the paracellular cleft (*trans*-interaction). In addition, Clds may oligomerize within one plasma membrane (*cis*-interaction). To analyze the ability of a single Cld subtype to form homo- and heteromeric complexes, we used TJ-free HEK cells. The properties of a Cld subtype and the interactions taking place between different Cld subtypes were determined by measuring the ability to lateral oligomerization (*cis*-interaction, FRET), transcellular association (*trans*-interaction, cell-scan), the plasma membrane mobility (FRAP) and the ability to form TJ-strands (freeze-fracture electron microscopy).

To analyze whether Cld oligomerization occurs outside of cell-cell contacts in the *endoplasmic reticulum* (ER) or intracellular transport vesicles, we established a FRET-measurement in single cell suspensions via flow cytometry. With this method it was possible to investigate thousands of cells of one individual sample (in cell suspensions via photometry and in single cell measurements via flow cytometry).

Trans-interactions: The classic Cld1, 3 and 5, but not the non-classic Cld12, were capable of homophilic *trans*-interactions. These interactions occur via the ECL2 of the respective Cld and led to association of neighboring cells, while the ECL1 is then sealing the paracellular cleft. Previously, we showed that defined residues (e.g. Y148) in the ECL2 of

Cld5 are conserved among classic Clds and are involved in homophilic *trans*-interactions. Determinants of *trans*-interactions in the ECL2 are conserved between Cld5 and Cld3 (26). Therefore, we proposed that homologous residues are involved in homophilic *trans*-interaction of other classic Clds. Despite homologous determinants of *trans*-interactions, Cld3/Cld5 *trans*-interactions were weaker than those for Cld3/Cld1, Cld5/Cld1 and the respective homophilic *trans*-interactions.

Moreover, transiently coexpressed Clds colocalized only partly in a subtype specific manner. Strong colocalization in intracellular compartments was found for Cld3/Cld1 and partially for Cld5/Cld1 but not for Cld3/Cld5 and Cld3/Cld2. At contacts of transfected HEK cells, colocalization of all Cld combinations measured (Cld3/Cld1, Cld5/Cld1, Cld5/Cld3 and Cld3/Cld2), was observed. The differences in subcellular distribution indicated Cld subtype specific differences in their capacity for heterophilic *cis*-interactions, targeting to the plasma membrane and/or internalization of the Cld.

Cis-interactions: The analysis of *cis*-interactions at cell-cell contacts indicated Cld subtype specific differences in the strength of interaction, at which the degree of interaction decreased as followed: Cld5/Cld5, Cld5/Cld1, Cld3/Cld1>Cld3/Cld3>Cld3/Cld5. Cld2 was incapable of heterophilic *cis*-interactions with Cld3. The *cis*-interactions measured within intracellular compartments in single cells differed in a subtype specific manner. For cells expressing different Cld-combinations, the FRET-efficiency decreased in the order: Cld5/Cld5>Cld5/Cld1>Cld3/Cld3>Cld3/Cld5. Again Cld2 exhibited no heterophilic binding to Cld3 in intracellular compartments, suggesting a different binding mechanism compared to other classic Cld.

TJ-strands: To get insights into the molecular organization of the intramembranous TJ-strands we analyzed the TJ-strand network of Cld-transfected HEK cells by freeze-fracture electron microscopy. Cld5 was found to form discontinuous chains of intramembranous particles that are associated with the exoplasmic face (E-face) of the plasma membrane. In contrast, Cld3 formed continuous strands with intramembranous particles associated with the protoplasmic face (P-face) of the plasma membrane.

The cotransfection of Cld3 with Cld5 led to a mixed phenotype. Substitution of a *trans*-interaction determinant in the ECL2 of Cld3 (Y147), that strongly inhibits *trans*-interaction, abolished the formation of TJ-strands. The non-classic Cld12 was incapable of TJ-strand formation. These data support the assumption that *trans*-interactions are a prerequisite for strand formation. All together, the results demonstrated Cld subtype specific characteristics of TJ-strand formation.

Plasma membrane mobility*: Trans*-interactions of Cld subtypes led to an immobilization of the respective Cld at cell-cell contacts, depicted by FRAP. Cld5 exhibited a relative low mobile fraction when compared to that of Cld3 or to the loss of *trans*-interaction mutant $Cld5_{Y148A}$ in the ECL2. An explanation for this result could be a higher amount of unpolymerized Cld3 in the plasma membrane or a higher disassembly/assembly rate of the TJ-strands for Cld3.

Proof of paracellular sealing: We established a novel experimental approach to analyze the barrier function of reconstituted TJ-strands in Cld expressing HEK cells. The cells were incubated with the plasma membrane marker CellMask and analyzed by life cell imaging. Incubation with 0.25 µg/ml CellMask for up to 20 min resulted in a strong labeling of contacts without Cld enrichment but not of contacts with Cld enrichment. This demonstrated that the TJ-strands that are reconstituted by expression of Cld3 and/or Cld5 also form a functional diffusion barrier.

In summary, we created a novel model for formation and molecular organization of heteropolymeric Cld strands. After a Cld was translated in the ER, the proteins polymerize in the ER membrane in a subtype specific manner (1). The Cld oligomers are transported to the plasma membrane (2), where the proteins are immobilized via *trans*-interactions to the neighboring cells (3) and via *cis*-interactions to other Cld along one plasma membrane (4). The four steps finally lead to TJ-strand formation and thereby paracellular sealing.

4.2 Experimental contribution

For this work I performed and analyzed major parts of the experiments including FRET measurements, live cell imaging, transfection of eukaryotic cells. I was involved in establishing the novel method of analyzing *cis*-interactions in single cell suspensions. Furthermore, I participated in writing and the discussion of this study.

Contributions of co-authors:

Conception: I. E. Blasig and J. Piontek

Performed the experiments: S. Fritzsche, J. Cording, J. Piontek, S. Richter, M. Walter, Dan Yu, C. Gehring and H. Wolburg

FRET measurement via flow cytometry: J. Cording, J. Hartwig, H.P. Rahn and J.Piontek

Freeze-fracture electron microscopy: H. Wolburg (in cooperation)

Fluorescence recovery after photobleaching: D. Yu and Jerrold R. Turner (in cooperation)

Wrote the paper: J. Piontek and I.E. Blasig

4.3 Manuscript 1

4.4 Supplemental material

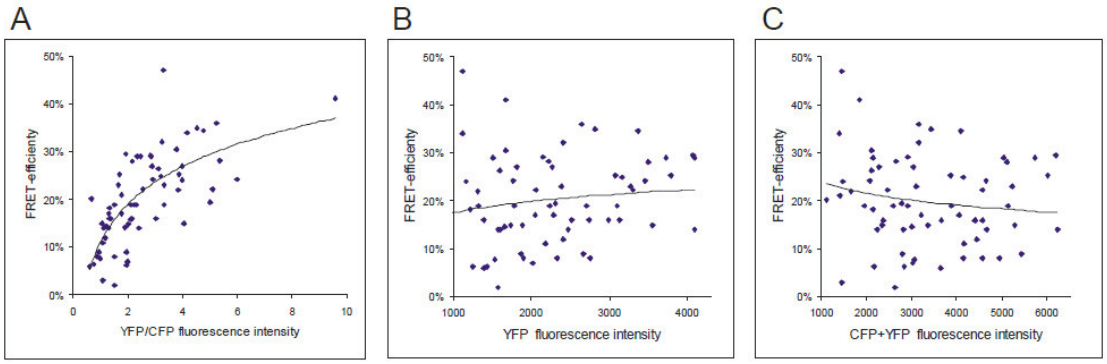

Figure S1:
The FRET-efficiency is strongly dependent on the ratio of acceptor/donor (YFP/CFP) fluorescence intensity (A, R^2=0.51) but not on the YFP fluorescence intensity (B, R^2=0.02) or the CFP+YFP fluorescence intensity (C, R^2=0.02) as a measure of the expression level. Individual data points and non-linear regression curve for Cld5-CFP/Cld5-YFP.

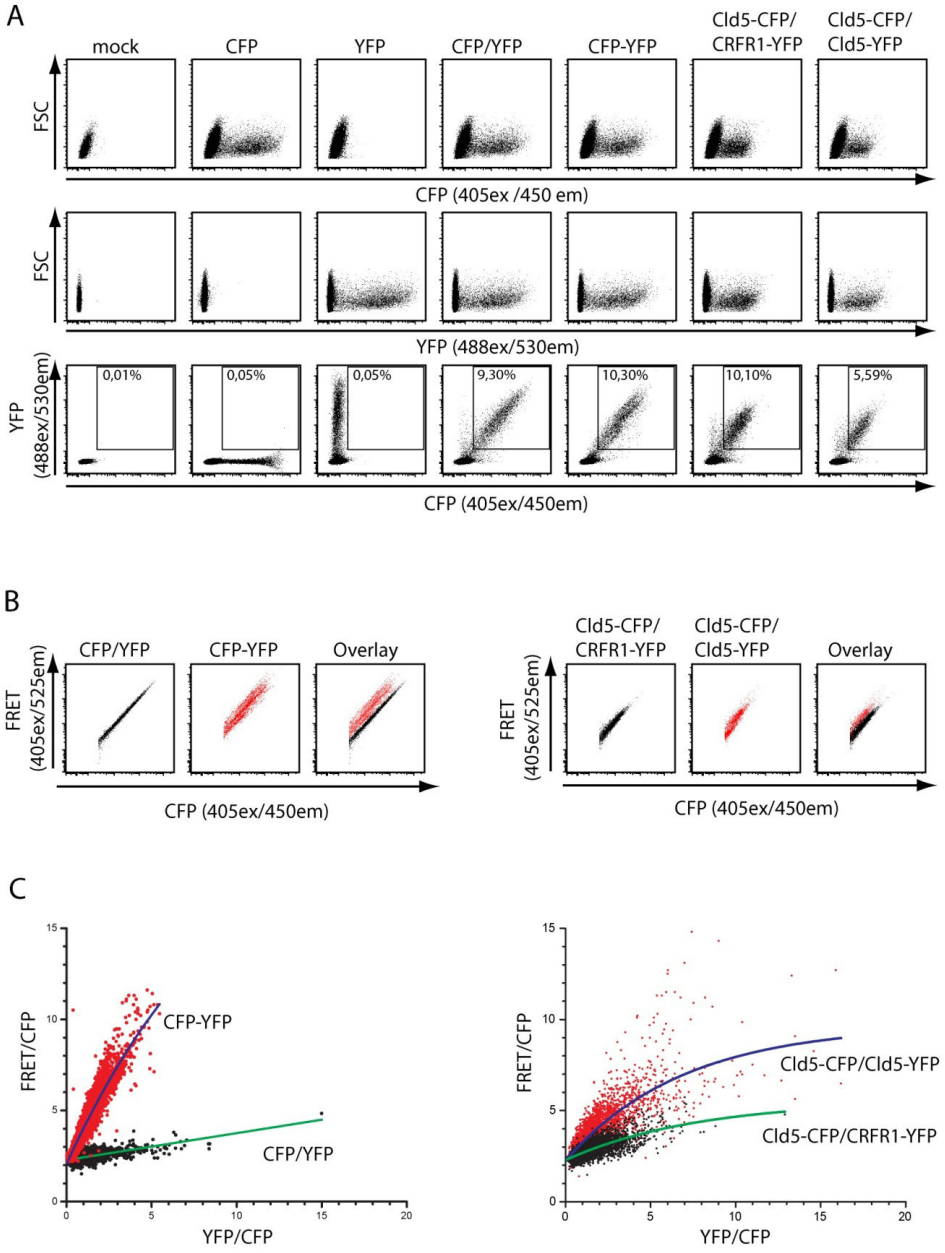

Figure S2:
(A) Flow cytometric expression analysis of HEK cells transfected with different constructs. Non- (mock), CFP- or YFP-transfection were used to account for auto fluorescence. CFP/YFP cotransfection and CFP-YFP tandem were used as FRET negative or positive control, respectively. Cdl5-CFP/CRFR1-YFP and Cld5-CFP/Cld5-YFP were used as pairs of transmembrane proteins shown to be FRET-negative or -positive, respectively, by conventional method. On the axes the channels are given. FSC, forward scatter. (B) Visualization of FRET signal by comparison of CFP/YFP - FRET negative -, CFP-YFP tandem - FRET positive - (*left panel*), Cld5-CFP/CRFR1-YFP - FRET negative - and Cld5-CFP/Cld5-YFP - FRET positive - (*right panel*). FRET-signal is indicated by left shift. (C) Normalization of FRET and YFP values relative to CFP intensity with the corresponding exponential decay fits (left: CFP/YFP (*black dots*, *green line*) and CFP-YFP (*red dots*, *blue line*); right: Cld5-CFP/CRFR1-YFP (*black dots*, *green line*) and Cld5-CFP/Cld5-YFP (*red dots*, *blue line*). The fits were used to calculate the FRET-ratio$_{FC}$ as a measure of FRET-efficiency (see Methods).

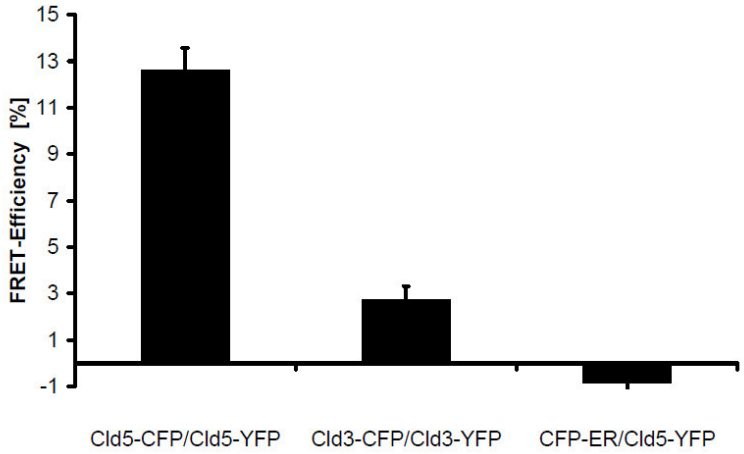

Figure S3: FRET-analysis in intracellular compartments by confocal microscopy. For Cld5-CFP/Cld5-YFP, FRET was significant higher than for Cld3-CFP/Cld3-YFP for which FRET was still higher than for the negative control (CFP-tagged endoplasmic reticulum marker, CFP-ER (BD Biosciences) coexpressed with Cld5-YFP, CFP-ER/Cld5-YFP). 36, 34, and 19 cell–cell contacts were analyzed, respectively; $p<0.001$.

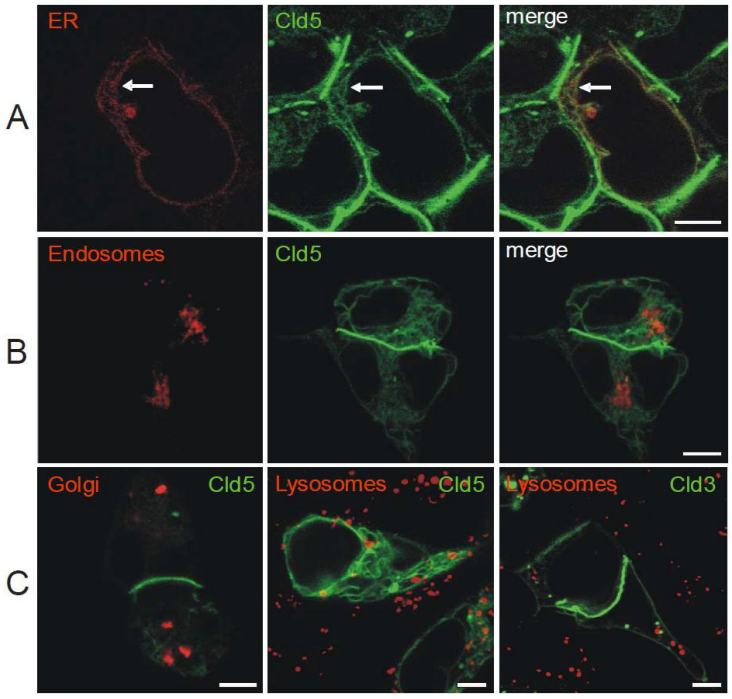

Figure S4:
In intracellular compartments claudins did not considerably colocalize with markers for endosomes, Golgi apparatus or lysosomes but partly with that for the endoplasmic reticulum (A, *arrow*). HEK cells were cotransfected with Cld5-YFP and (A) CFP-ER (marker for endoplasmic reticulum, Clontech), (B) CFP-Endo (marker for endosomes, Clontech) or (C, left) CFP-Golgi (marker for Golgi apparatus, Clontech) and 3 days later analyzed by confocal microscopy. In addition, cells were transfected with Cld5 (C, *middle*) or Cld3 (C, *right*) and 3 days later incubated with 50 nM LysoTracker (Invitrogen) in growth medium for 30 min at 37°C, the medium exchanged by DMEM with 10 mM N-(2-hydroxyethyl) piperazine-N'(2-ethanesulfonic acid) pH 7.5 without phenol red and analyzed by confocal microscopy. *Bar*, 5 µm.

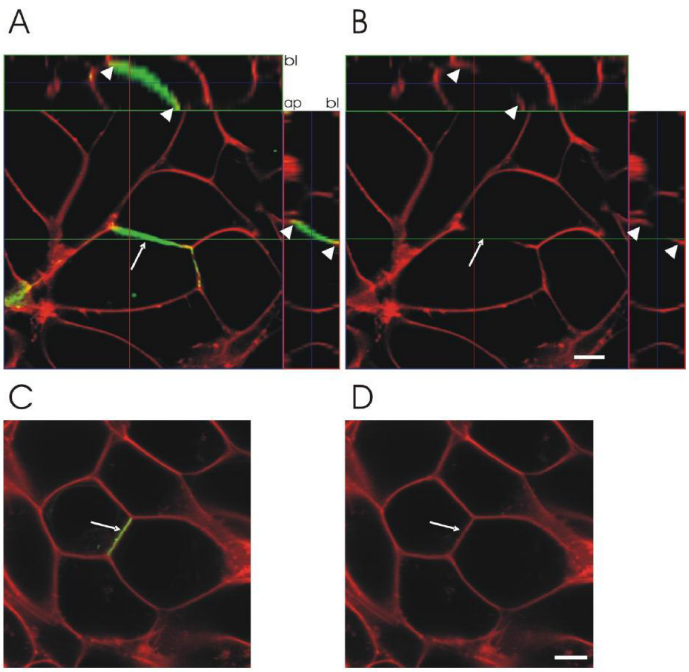

Figure S5:
CellMask labels the apical and basolateral plasma membrane; reconstituted TJs delay the labeling. HEK cells stably transfected with Cld5 were labeled with 0.25 µg/ml CellMask for 5 min (A, B) or 20 min (C, D). After 5 min, CellMask labeling (*red*) is detected in the apical (ap) and basal (bl) part of the lateral plasma membrane but not at the Cld5-positive (green) area at cell-cell contacts (A, B). In contrast, after 20 min, CellMask (*red*) colocalizes with claudin-5 (green) at cell-cell contacts (C, D). Merge (A, C); CellMalsk (B, D); *arrow*, Cld5-enrichment at cell-cell contact; *arrowhead*, apical and basolateral plasma membrane adjacent to claudin-5; *bar*, 5 µm.

5 Manuscript 2 (Ms2)

Tricellulin forms homomeric and heteromeric tight junctional complexes

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5.1 Synopsis

Occl and Clds represent the major TJ components involved in establishing a paracellular barrier of bicellular junctions in epithelia and endothelia to function as efficient barriers between the external and the inner compartments of an organ. A special situation emerges at sites where three cells join together. Tric, a recently identified tetraspan protein concentrated at tricellular contacts, was reported to organize tricellular as well as bicellular TJ (21). Similar to the Occl cytosolic C-terminal part, Tric bears extended cytoplasmic tails and a MARVEL domain, while the cytosolic C-terminus exhibits a homology to the coiled-coil domain within the Occl C-terminal segment.

Since little is known about the mechanisms responsible for this tricellular localization and how Tric affects overall TJ structure, this study aimed to investigate Tric with respect to its translocation to TJ and its ability to form homomeric Tric complexes. Based on the evolutionary relationship between Occl and Tric, a heterophilic Tric-Occl oligomerization was proposed.

To address the question, which domains in Tric are responsible for the tricellular localization, we constructed N- and C-terminal truncation mutants (ΔN-Tric and Tric-ΔC respectively). $Tric_{wt}$ was predominantly located at tricellular contacts in transfected MDCK-C11 cells, while ΔN-Tric revealed continuous distribution all over the plasma membranes at bicellular and tricellular contacts. Tric-ΔC was transported to the cell contact sites less efficiently. These observations led to the conclusion, that the cytosolic N-terminal domain is responsible for the tricellular localization, while the C-terminal domain is involved in efficient transport of Tric to the plasma membrane.

Since tricellular regions do not contain Occl or Cld, a homophilic oligomerization of Tric at these sites was assumed. To test this hypothesis, we performed co-IP experiments with Tric transfected HEK cell lysates. Surprisingly, both N- and C-terminally deleted Tric variants associated with full-length Tric. The double Tric truncation mutant ΔN-Tric-ΔC also associated with full-length Tric, suggesting that the MARVEL domain is sufficient to mediate homophilic Tric-Tric association.

The Tric oligomerization was further confirmed by a FRET-assay in living MDCK-II cells. In epithelial cell lines Tric is located at tricellular TJs, in contrast, if Tric is highly overexpressed it was also detectable at bicellular contact sites. We measured the homophilic association of full-length YFP-Tric and CFP-Tric via FRET at bicellular cell-cell contacts. The interaction appeared in almost the same manner as the interaction of

full-length, N- and C-terminal deleted Tric, determined by co-IP, suggesting that the cytosolic termini are not involved in the homophilic interaction of Tric. It was reported that knock-down of Tric affects the overall TJ organization and Occl localization. Hence a direct heteromeric interaction of both proteins was assumed. We performed co-IP experiments with lysates of cotransfected HEK cells. Full-length Tric, and N- and C-terminal deletion constructs were found to associate with full-length Occl and N- and C-terminal deletion constructs of Occl, respectively. In addition, endogenous Occl was found to associate with full-length FLAG-Tric transfected MDCK-C11 cells, indicating no participation of the cytosolic termini in the heterophilic *cis*-interaction either.

These Tric-Tric and Tric-Occl complexes would occur in one plasma membrane (*cis*-oligomers) or between the plasma membranes of two neighboring cells (*trans*-oligomers). To distinguish between those two, we performed co-IP experiments with lysates of cocultured MDCK-C11 cells stably expressing either Tric-FLAG or HA-(Human influenza hemagglutinin)-Tric or with FLAG-Occl and HA-Tric, respectively. Since no homo- or heterophilic *trans*-oligomers were detectable, the results suggest the presence of homophilic *cis*-oligomeric Tric-Tric and heterophilic Tric-Occl complexes. Both cytosolic segments are not involved in homo- or heterophilic complex formation.

In summary, we clearly demonstrate that the cytosolic N-terminal tail of Tric is essential for the tricellular localization of the protein, while the cytosolic C-terminal tail is involved in efficient transport of Tric to the plasma membrane. Furthermore we were able to detect heteromeric *cis*-complexes with Occl. However, we cannot distinguish between the complexes at the plasma membrane and the complexes formed within transport vesicles.

5.2 Experimental contribution

For this work I established, performed and analyzed FRET-assays to detect homomeric complexes of Tric. Furthermore, I participated in the discussion of this study and wrote parts of the manuscript.

Contributions of co-authors:

Conception: Julie K. Westphal, Ingolf E. Blasig, Michael Fromm and Otmar Huber

Performed the experiments: Julie K. Westphal, Max J. Dörfel, Susanne M. Krug and Jimmi Cording

Wrote the paper: Julie K. Westphal and Otmar Huber

5.3 Manuscript 2

5.4 Supplemental material

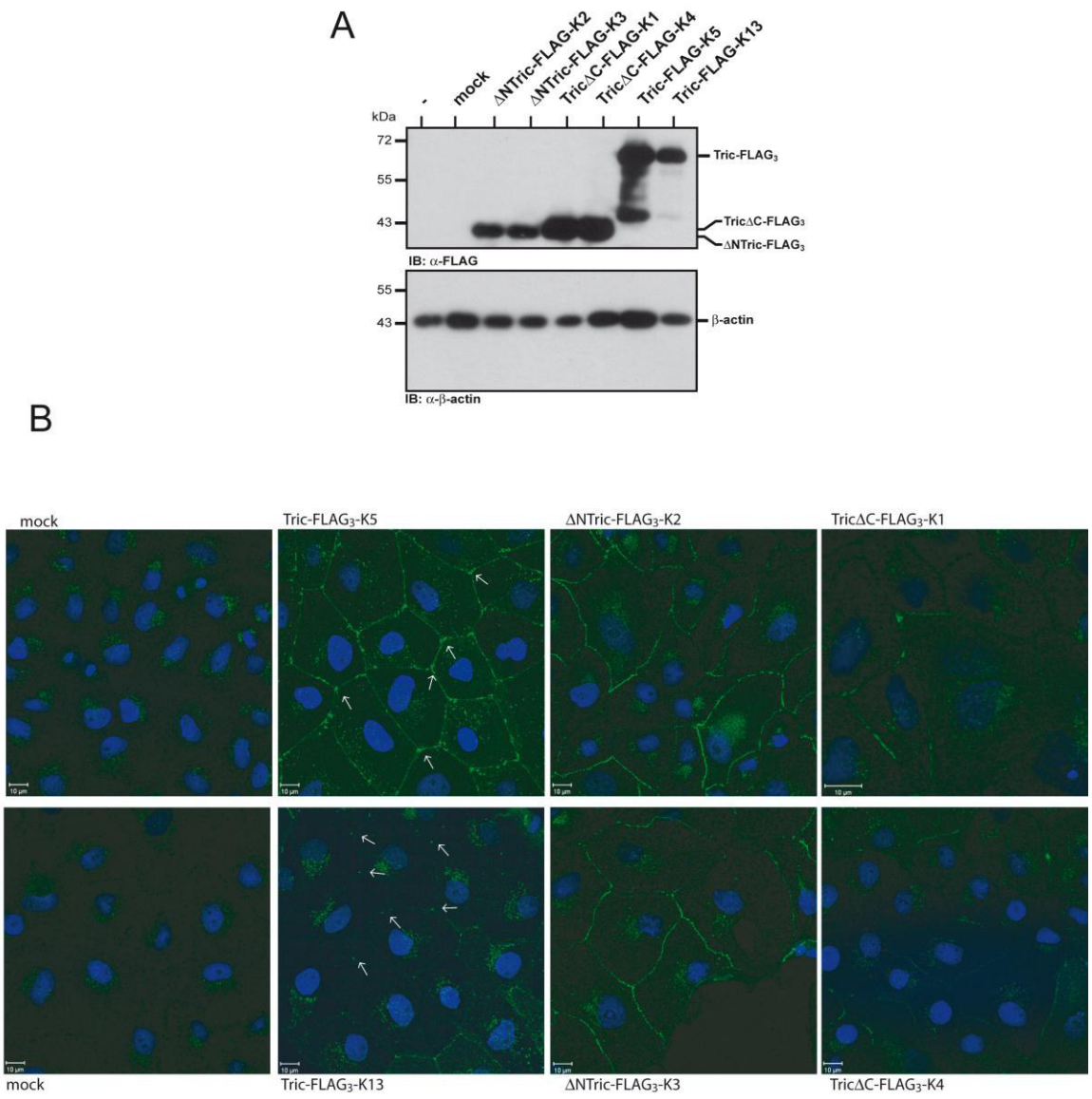

Figure S1:
(A) Western blot analysis of the expression of the FLAG-tagged tricellulin constructs in stably transfected MDCK C11 cell clones. Please note that clone K5 expresses higher levels of the full-length tricellulin construct compared to clone K13. (B) Localization of the tricellulin constructs in the different clones by confocal immunofluorescence microscopy after PFA fixation. In clone K5 localization of Tric-$FLAG_3$ is not restricted to tricellular contacts but extends into the bicellular tight junctions as previously reported.

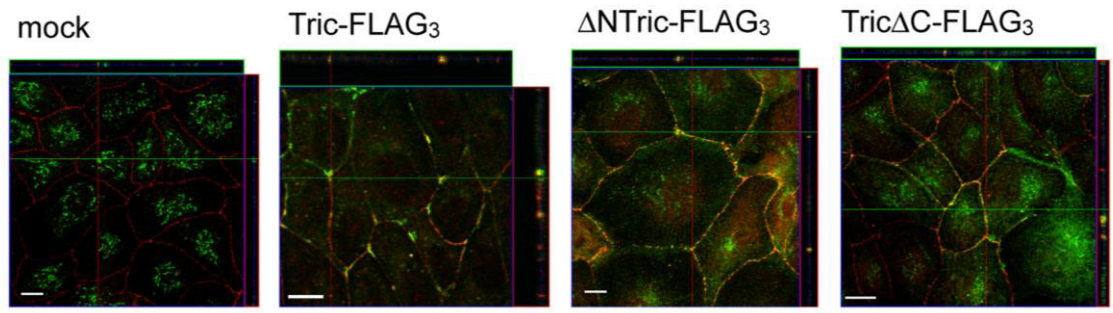

Figure S2: Confocal immunofluorescence images (XYZ scans) reveals co-localization of the tricellulin constructs with ZO-1 at tight junctions.
Please note that the TricΔC-$FLAG_3$ construct is less efficiently translocated to the tight junctions. For the image presented here, a cell that shows relatively strong membrane staining of TricΔC-$FLAG_3$ was chosen.

6 Manuscript 3 (Ms3)

In tight junctions, claudins regulate the interactions between occludin, tricellulin and marvelD3, which inversely modulate claudin oligomerization

Jimmi Cording, Johanna Berg, Nadja Käding, Christian Bellmann, Christian Tscheik, Julie K. Westphal, Susanne Milatz, Dorothee Günzel, Hartwig Wolburg, Jörg Piontek, Otmar Huber and Ingolf E. Blasig

Journal of Cell Science

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http://www.ncbi.nlm.nih.gov/pubmed/23203797

6.1 Synopsis

In the present study we aimed to characterize the recently described TJ-protein family of TAMPs to gain insight into the heterophilic oligomerization between TAMPs and Clds as well as into the modulation of Clds by TAMPs (Occl, Tric and MD3). Furthermore we analyzed the ability of TAMPs to modulate Cld-build TJ-strands via freeze-fracture electron microscopy (FF-EM). Similar as described previously for Clds (MS1), we investigated the homophilic binding for Occl, Tric and MD3, and heterophilic binding within the TAMP family in transfected TJ-free HEK cells.

To determine whether Occl, Tric or MD3 are capable for homophilic or heterophilic *trans*-interactions we measured the protein enrichment of the respective TAMPs at cell-cell contacts via trans-cell scans. Homo- and heterophilic *cis*-complex formation was measured as FRET, while membrane mobility was determined by FRAP. The ability of Occl, Tric and MD3 to form TJ-strands was analyzed via FF-EM.

Occl was the only TAMP being capable to homophilically *trans*-interact, indicating paracellular sealing function of the protein. Tric and MD3 were not enriched between two transfected cells, which exclude *trans*-interactions at bicellular contacts. All TAMPs exhibited homophilic *cis*-interactions, however to different extents. Heterophilic *cis*-interaction was not observed between Occl and Tric, demonstrating that these proteins do not physically interact with each other at the plasma membrane. In contrast, strong colocalization and *cis*-interactions of Occl and Tric within intracellular compartments suggested an intracellular interaction in HEK cells probably during the transport to the cell surface, as suggested earlier (Ms2).

Then, we analyzed the interplay of TAMPs with Clds in HEK cells. The coexpression of Cld1 or Cld5 with Occl enhanced the amount of Occl localized at bicellular contacts. In contrast, a Cld coexpression with Tric initiated a de-novo contact enrichment of the protein, which was absent when Tric was expressed without a Cld. Coexpression of Cld1 or Cld5 revealed no effect on the enrichment factor of MD3.

We tested the heterophilic *cis*-interactions of Occl, Tric and MD3 with the classic Cld1-5 and the non-classic Cld11 at cell-cell contacts of cotransfected HEK cells via FRET. Cld1, which is an ubiquitously expressed Cld, showed a high binding capacity to Occl, Tric and MD3. The latter TAMP additionally *cis*-interacted with Cld3 in a comparable manner. In contrast, the classic Cld2-5 exhibited a relatively low binding capacity to the TAMPs. The non-classic Cld11 exhibited no interaction with any of the TAMPs.

In epithelial cell-lines, such as MDCK-II or Caco-2, Tric is preferentially localized at tricellular contacts. HEK cells lack the regulatory proteins that determine the tricellular localization of Tric; thereby Tric localized at bicellular contacts only. If Tric or Occl are coexpressed with Cld1, the respective TAMP was enriched at the bicellular contacts. Since Tric localizes at tricellular junctions in epithelial cells, there should be a mechanism by which Tric is pushed away from bicellular junctions and then immobilized at tricellular junctions. To gain insights into the mechanism and the participation of Cld1 and Occl, we triple transfected Tric, Occl and Cld1 in HEK cells. In these transfectants Cld1 and Occl were enriched at bicellular contacts between transfected HEK cells, whereas Tric was pushed aside to the non-contact plasma membrane without being enriched at bicellular contacts.

Due to the strong *trans*-interactions of Cld1, the protein is immobilized at cell-cell contacts. When coexpressed with Occl, Tric or MD3, Cld1 was determining the plasma membrane mobility of Occl and Tric, but not of MD3. The plasma membrane mobility of Cld1 measured by FRAP was unaltered due to the coexpressions with the respective TAMPs. In contrast, the plasma membrane mobility of Tric or Occl was significantly reduced in the presence of Cld1. On the other hand, when we treated Caco-2 cells with siRNA targeted against Cld1, we observed an outspread of Tric from tricellular to bicellular contacts. Hence, the plasma membrane mobility of Tric was enhanced due to the missing immobilization of Tric by Cld1.

The transfection of a single classic Cld in HEK cells leads to reconstituted TJ-strands; however, these strands are less complex than endogenous TJ-strands of MDCK-II or Caco-2 cells. To investigate the effect of Cld1/TAMP interactions on the formation of TJ-strands, we analyzed the TJ-strand morphology in HEK cells transfected with Cld1 alone or together with Occl, Tric or MD3, using FF-EM. We calculated the meshes/area as well as the amount of parallel TJ-strands to characterize the contribution of the expressed proteins on the TJ-strand network. Cld1 transfected HEK cells revealed large round TJ-strand meshes, with few branching points. Rarely, TJ-strands were arranged in a parallel manner. We observed striking morphological modifications due to the coexpression of Cld1 with Tric. The TJ-strand network changed from round-shaped strands to more rectangular meshes with reduced mesh size and a negligible amount of parallel TJ-strands. The coexpression of Cld1 with MD3 leads to a mesh shape comparable to HEK cells expressing Cld1 only, but revealed smaller meshes with a higher amount of branching points and more membrane areas with parallel TJ-strands. The Occl/Cld1 coexpression

leads to a TJ-strand network similar to the network only containing Cld1, but frequently short pieces of bundled parallel strands were observed. More strikingly, the protoplasmic fracture face was interrupted without an increase of particles at the exoplasmic fracture face due to the coexpression of Cld1 with Occl. Tric alone formed short, discontinuous up to continuous and seldom branched TJ-strands on the protoplasmic fracture face whereas mono-transfected Occl or MD3 did not.

In this study we provide evidence for an alternating modification of TAMPs by Cld and vice versa. We were able to show that the Occl/Tric interaction published earlier (Ms2), occurs in intracellular compartments only. Occl, in contrast to Tric and MD3, is capable of homophilic intracellular adhesion. All TAMPs are interacting with members of the Cld family to a different extent and are increasing the complexity of the TJ-strand network, at least for Cld1. This interplay seems to be essential for the physiological function of cellular barriers.

6.2 Experimental contribution

For this work I established, performed and analyzed major parts of the experimental work, like FRET-assays and live cell imaging to detect TAMP-TAMP and TAMP-Cld complexes in HEK cells. I established knock-down experiments and tripple transfections. Moreover, I participated in the discussion, conception and I wrote major parts of the manuscript.

Contributions of co-authors:
Conception: Ingolf E. Blasig and Jimmi Cording
Performed the experiments: Jimmi Cording, Johanna Berg, Nadja Käding, Jörg Piontek
Freeze-fracture electron microscopy: H. Wolburg (in cooperation)
Cloning of CFP-Cld11 and homologous FRET Cld11 measurements: Susanne Milatz and Dorothee Günzel (in cooperation)
Wrote the paper: Jimmi Cording and Ingolf Ernst Blasig

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6.4 Supplemental material

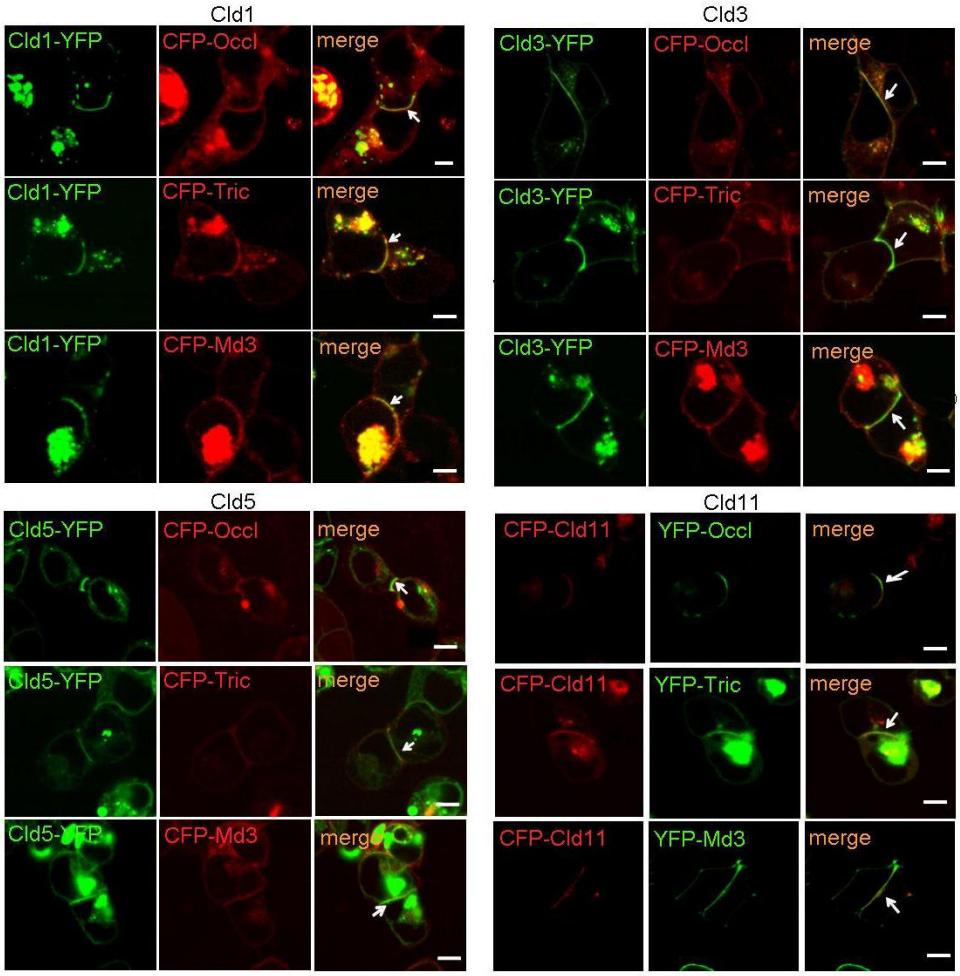

Figure S1: HEK cells were co-transfected with a Cld and a TAMP as indicated.
YFP is pictured in green, CFP in red, colocalization is represented in yellowish (see merge). FRET measurements were performed at cell-cell contacts (arrows). The coexpression of Cld1 or Cld5 with TAMPs led to contact enrichment for Occl and Tric (see Fig. 2A). This effect could not be observed at coexpressions with other Clds. Scale bars, 5 µm. Abbreviations see Fig.1.

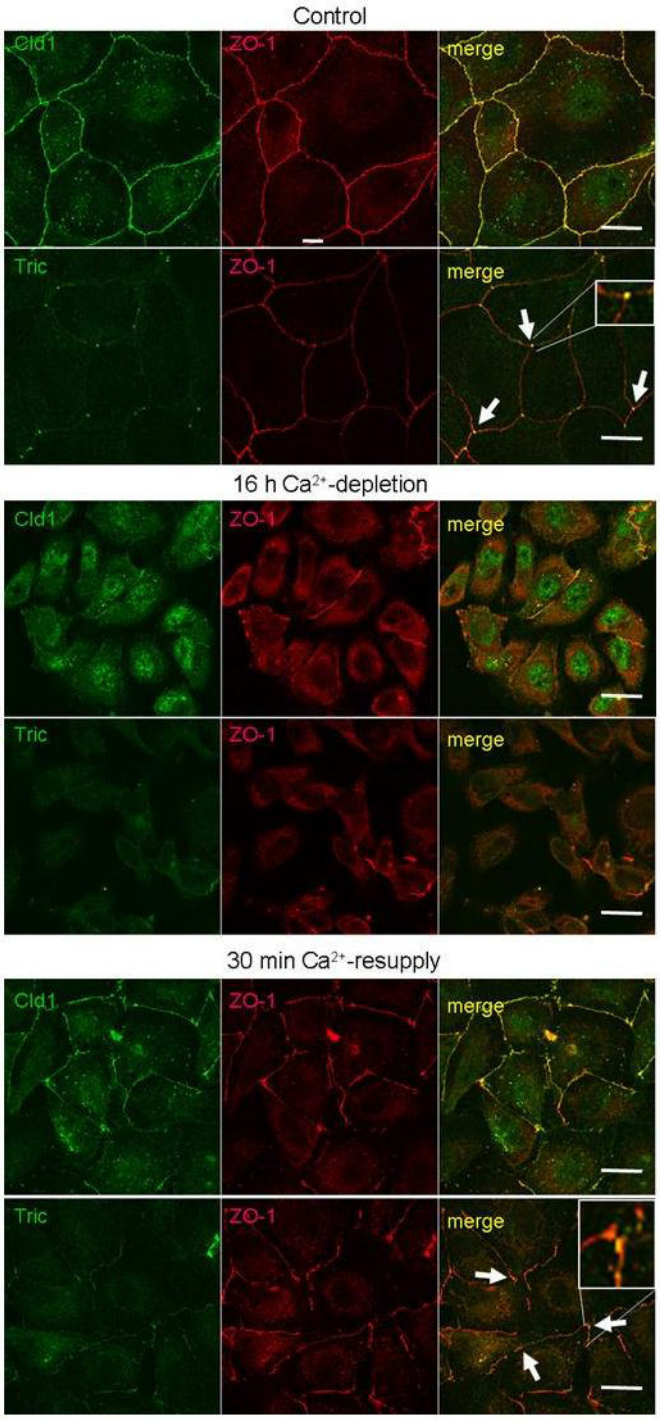

Figure S2: Tricellulin, like *zonula occludens* protein 1 (ZO-1) and claudin-1, is first transported to bicellular cell-cell contacts before it is distributed to tricellular contacts. Coimmunofluorescence staining of Cld1 (green) or Tric (green) together with ZO-1 (red) in MDCK-II cells. In the control, Cld1 and ZO-1 are localized at bicellular, Tric at tricellular TJ (arrows in the lower control panel). After 16 h of Ca^{2+}-depletion, the TJ proteins are localized in intracellular compartments. After 30 min of Ca^{2+}-readdition, Tric like Cld1 and ZO-1 are initially localized at the bicellular TJ (arrows in lower panel after readdition of Ca^{2+}). Scale bars, 10 µm; abbreviations see Fig. 1.

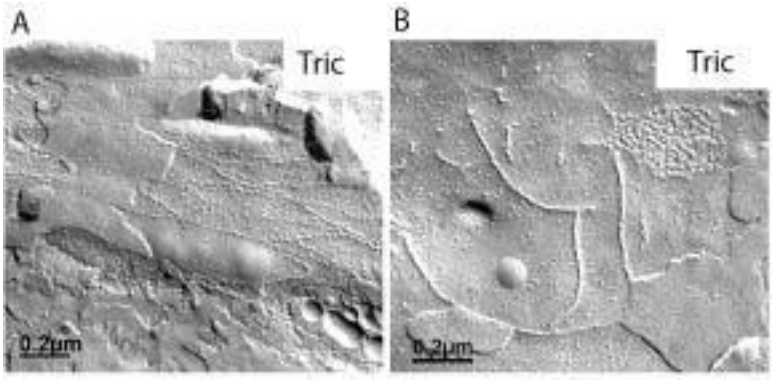

Figure S3: Tricellulin transfected alone into tight junction-free HEK-cells formed short, discontinuous up to continuous and seldom branched tight junction strands on the protoplasmic fracture face.
HEK cell transiently transfected with CFP-Tric were analyzed by freeze-fracture electron microscopy. Further description, see text; scale bars, 0.2 µm; abbreviations as in Fig. 1.

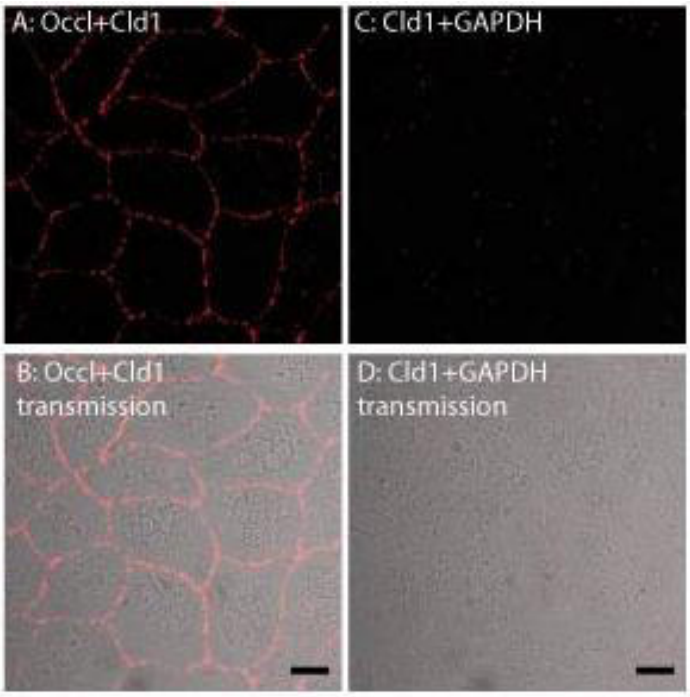

Figure S4: Proximity ligation assay indicated interaction of endogenous claudin-1 with endogenous occludin in MDCK-II cells.
Cells were fixed and incubated with anti-Cld1 and anti-Occl (A) or anti-Cld1 and anti-glyceraldehyde-3-phosphate, GAPDH (C) antibodies. Subsequently, duolink® II proximity ligation assay was performed. Lines of red dots indicating close proximity between Cld1 and Occl were found frequently at cell-cell contacts (A). In contrast, for Cld1/GAPDH red dots are found only rarely and show no clear association with cell-cell contacts (C). In the lower panel overlay of A and C with the respective transmission images is shown (B and D). Scale bars, 10 µm; abbreviations as in Fig. 1.

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7 Manuscript 4 (Ms4)

Redox regulation of cell contacts by tricellulin and occludin: Redox-sensitive cysteine sites in tricellulin regulate both tri- and bicellular junctions in tissue barriers as shown in hypoxia and ischemia

Jimmi Cording, Ramona Günther, Emilia Vigolo, Christian Tscheik, Lars Winkler, Isabella Schlattner, Dorothea Lorenz, Reiner F. Haseloff, Kai M. Schmidt-Ott, Hartwig Wolburg and Ingolf E. Blasig

Antioxidants & Redox Signaling
2015. Epub 2015/04/29
http://www.ncbi.nlm.nih.gov/pubmed/25919114

7.1 Synopsis

Hypoxic tissue injury is the major effect of blood flow disturbances in an organ, as in brain tissue after or in kidney transplantation. Recently, it was shown that the TAMP Occl is hypoxia-sensitive and thereby the plasma membrane fraction, in particular in the junctions between two opposing cells, was reduced in epithelial cells (60). The aim of this study was to explore the possible response of the tricellular contact under pathological circumstances, accompanied by reducing conditions, such as hypoxia. We used MDCK-II cells, expressing TJ proteins endogenously, to characterize the behavior of Tric during hypoxia or reducing conditions such as β-mercaptoethanol (β-ME) or dithiothreitol (DTT) treatment.

Due to oxygen deprivation of confluent MDCK-II monolayers, Tric moved from the tricellular to the bicellular junctions. The data obtained in kidney epithelial cell cultures (MDCK-II) *in vitro* were confirmed *in vivo* where similar redistribution appeared in the tubular epithelium of mice kidneys after renal ischemia: bicellular and cytosolic localization of Tric, Occl loss from bicellular junctions. The same effects were observed when the cells were treated with reducing agents.

Human Tric contains 8 cysteine residues which could form inter- or intramolecular disulfide bonds under oxidizing conditions which potentially could be affected by hypoxia or reductants. To study the molecular mechanisms being responsible for hypoxia-induced alterations in Tric localization function, we generated cysteine to alanine substitution mutants of Tric ($Tric_{CA}$) and transfected these constructs in MDCK-II cells. The relevance of the cysteine residues was analyzed by localization studies in MDCK-II cells, measurements of the transepithelial resistance, passage of small (Lucifer Yellow, 4 kDa) and large tracers (FITC-Dextran 10 kDa) as well as interaction assays to MD3 and Cld1 (FRET), that were found to interact with Tric previously (Ms3).

The majority of the Tric mutants caused no alterations on the tricellular localization. Interestingly, the two ECL2 cysteine mutants $Tric_{C321A}$ and $Tric_{C335A}$ were significantly redistributed to bicellular contacts. The overall results suggested that these cysteine residues may form a disulfide bond within the ECL2, which is essential for correct folding and function of this domain. To further characterize the molecular interactions of the Tric mutants and their functional role in TJs, we investigated the *cis*-interactions of Tric with Cld1 or MD3 at bicellular contacts in TJ-free HEK cells.

The various $Tric_{CA}$ constructs appeared to have interaction values with Cld1 or MD3 similar to the wild type, except for $Tric_{C362A}$, which failed in heterophilic *cis*-interactions. Since HEK cells do not build confluent monolayers and thereby tricellular contacts, the localization of Tric appeared at bicellular contacts. In addition, the cell line does not express TJ proteins, hence, the regulation of Tric is incomplete and this could contribute to the interactions of the protein. We therefore measured the homophilic *cis*-interactions of Tric as FRET at tricellular contacts in transfected MDCK-II cells. The values were considerably higher at tricellular contacts (HEK cells bicellular=4% (Ms2); MDCK-II tricellular=16%), indicating a close self-association of Tric in tricellular contacts. Except for $Tric_{C395A}$, the cysteine substitutions did not significantly disrupt the *cis*-interactions. However, $cysteine_{395}$ in the C-terminal tail is involved in homophilic Tric association at tricellular contacts, while $cysteine_{362}$ in the 4^{th} transmembrane domain is involved in heterophilic *cis*-interactions at bicellular junctions.

To determine whether the cysteine substitutions bear any functional consequences, we measured the barrier integrity in stably transfected MDCK-II cells. MDCK-II cells expressing $Tric_{C321A}$, $Tric_{C335A}$, $Tric_{C362A}$ or $Tric_{C395A}$ failed to establish a remarkable barrier against ions (TER), small and large tracers, indicating a functional role of the respective segments of Tric.

To analyze the impact of the various Tric constructs on the cellular assembly of the TJ proteins ZO-1, Occl and Cld1, we performed co-immuofluorescence stainings. In the MDCK-II cell lines expressing $Tric_{C321A}$, $Tric_{C335A}$, $Tric_{C362A}$ or $Tric_{C395A}$, the plasma membrane localization of ZO-1, Occl and Cld1 was reduced, while the cytosolic fraction was enhanced. Consequently, the findings support the idea of Tric as TJ regulator and thus highlight the importance of the protein for the assembly of the functional barrier integrity.

Furthermore we inspected the TJ-strand assembly on a molecular scale, using freeze-fracture electron microscopy (FF-EM). The transfection of $Tric_{wt}$ led to decreased E-face association resulting in more P-face association, and induced more compact and smaller meshes with a more rectangular shape, including more branching points, compared to the mock transfection. Interestingly, transfection of $Tric_{C321A}$, $Tric_{C335A}$ or $Tric_{C362A}$ led to a parallel strand arrangement with particle association at the P-face only. $Tric_{C395A}$ in the cytosolic C-terminal tail exhibited more parallel TJ-strands and exclusively P-face association. The FF-EM results indicated a superordinate function of Tric in the establishment and maintenance of a compact and functionally effective TJ-strand network, which is mainly build by Clds.

Finally we compared MDCK-II cells expressing $Tric_{wt}$ or $Tric_{C335A}$, respectively, concerning the tricellular enrichment using ultrathin section electron microscopy. Here, $Tric_{wt}$ exhibited dense material between adjacent cells at tricellular junctions, indicating intact TJ-assembly. In contrast, $Tric_{C335A}$ overexpression caused rare cell contacts at bi- and tricellular junctions. The cells expressing $Tric_{C335A}$ exhibited large holes at bi- and tricellular contacts, indicating the essential role of the two cysteine residues in the ECL2 for the structure of the ECL2 to develop functional tricellular junctions.

Based on the data, we created an i-Tasser model of the Tric-ECL2 ($Tric^{314-341}$). In the structure prediction $cysteine_{321}$ and $cysteine_{335}$ are forming an intramolecular disulfide bond, Tric specific prolines 324 and 329 establishes two turns and the cysteine flanking tyrosine 322 and 323 stabilizes the disulfide bond.

The study provides novel insights in the response of Tric to hypoxia or reducing agents and this hypoxia sensitivity is mainly determined by the structure and function of Tric ECL2. The results were obtained *in vitro* in cell culture systems as well *in vivo* in acute kidney ischemia.

Therefore, Tric should be counted as redox-dependent regulator of tricellular contacts, while Occl overtakes these functions at bicellular contacts. In addition, by means of cysteine to alanine substitutions, we were able to identify homo- and heterophilic binding sites in Tric. Since loss of the binding capacity affects the correct organization of an epithelial monolayer, we demonstrated the overall impact of Tric in the assembly and maintenance of cellular barriers.

7.2 Experimental contribution

For this work I established, performed and analyzed most parts of the experimental work, such as hypoxic treatment of MDCK-II, mutagenesis of Tric, FRET assays in MDCK-II and HEK cells, TER and permeation measurements, immunofluorescence staining and live-cell imaging. I contributed to the *in vivo* ischemia experiments (collection of tissue and the immunefluorescence staining). I participated in the discussion and conception, and I wrote major parts of manuscript.

Contributions of co-authors:

Conception: I. E. Blasig and J. Cording

Performed the experiments and experimental setup: J. Cording, R. Günther, E. Vigolo, C. Tscheik, I. Schlattner, L. Winkler, R. Haseloff, D. Lorenz and H. Wolburg

Freeze-fracture electron microscopy: H. Wolburg (in cooperation)

In vivo ischemia: J. Cording, E. Vigolo and K. S. Ott (in cooperation)

Transmission electron microscopy: D. Lorenz (in cooperation)

Wrote the paper: J. Cording and I. E. Blasig

7.3 Manuscript 4

7.4 Supplemental material

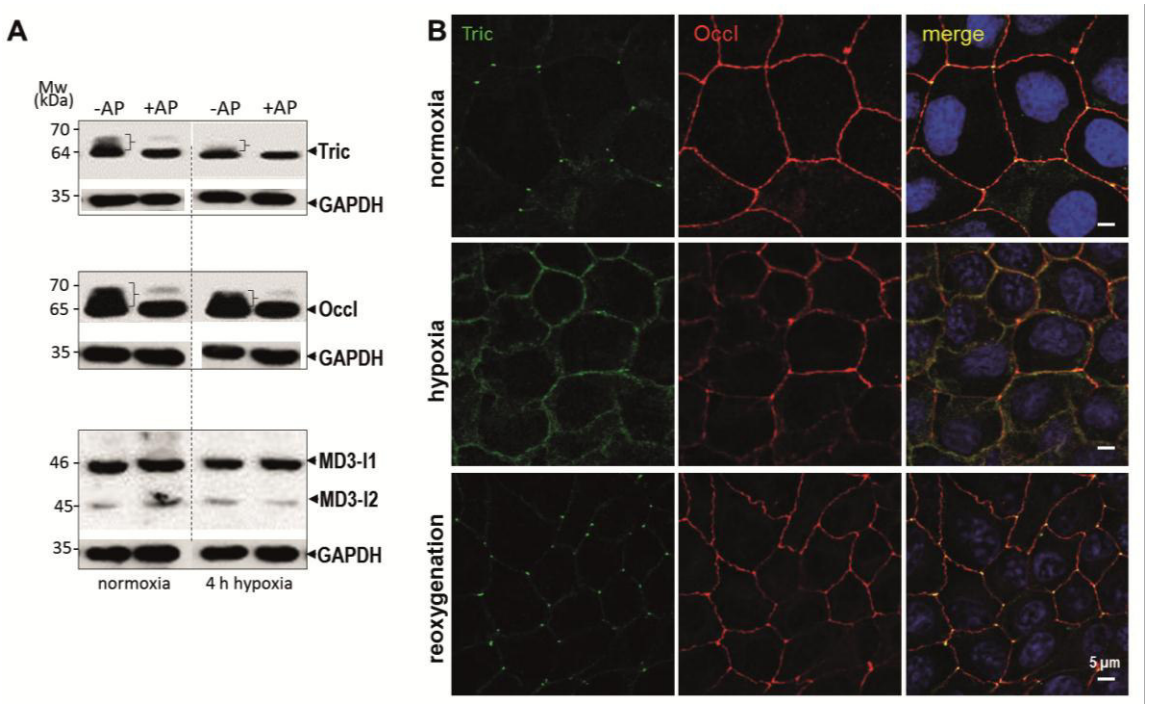

Figure S1: 4 h hypoxia affect phosphorylation and redistribute tricellulin (Tric) and occludin (Occl) which is reversed by reoxygenation, in Madin-Darby canine kidney II cells
(A) Molecular weight shift of Tric and Occl bands after immunoblotting (}) depicts phosphorylation in the triton X-100 insoluble fraction of the proteins, which was reduced 4 h after hypoxic incubation. The shift was alkaline phosphatase (AP)-sensitive confirming its phosphorylation nature. The triton X-100 insoluble fraction was prepared as described. MD3, marvel D3 isoform 1 (MD3-I1) and 2 (MD3-I2); GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (B) The hypoxia-induced redistribution of Tric and Occl was normalized after reoxygenation. Confocal images show that, under normoxic conditions, Tric localized at 3-cell contacts and Occl at 2-cell contacts. After 6 h of hypoxia, Tric redistributed to 2-cell contacts where Occl was partially lost from the 2-cell contact; both were reversed after 16 h posthypoxic reoxygenation.

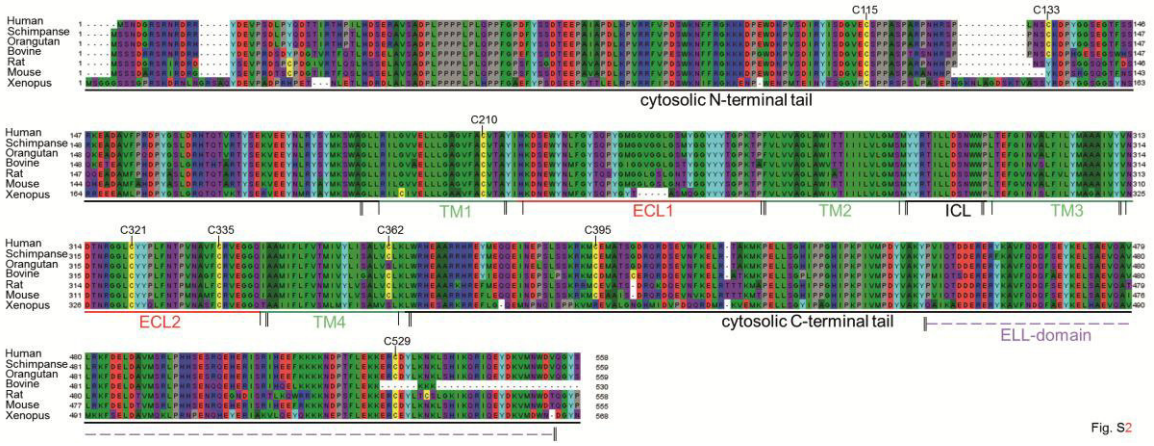

Figure S2: Alignment of human tricellulin isoform a (Tric)
The protein sequences of Tric were taken from the UniProtKB server (10/01/2014). The alignment was calculated with ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and edited by Jalview (http://www.jalview.org/). The underlined sequences illustrate the domains of Tric: Cytosolic parts (black); TM, transmembrane domains (green); ECL, extracellular loops (red); ICL, intracellular loop (black); ELL-domain, an RNA polymerase II elongation factor encoded by the human ELL gene (pink). |, ‖, domain prediction by Raleigh et al. 2010 and Riazuddin et al. 2006, respectively; Cys residues highlighted in yellow.

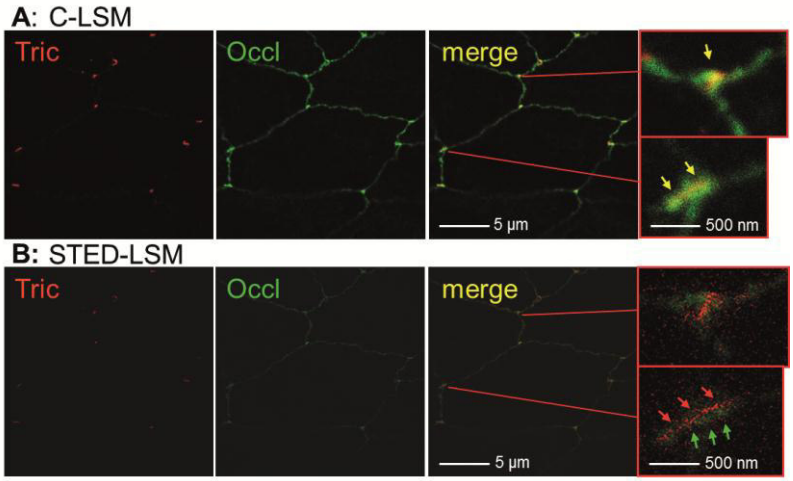

Figure S3: Super-resolution STED-microscopy reveals absence of colocalization between tricellulin (Tric) and occludin (Occl) at 3-cell contacts in contrast to normal LSM
Confluent Madin-Darby canine kidney (MDCK-II) cells were stained against Tric and Occl. (A) Conventional confocal image exhibited strong colocalization of Occl and Tric at the tricellular contacts (yellow in merge). (B) Leica super-resolution STED image exhibited no colocalization of Occl and Tric at the tricellular contacts; see merge and magnifications in red box. Occl surrounded Tric at the tricellular contact.
C-LSM, confocal laser scanning microscopy; STED-LSM, stimulated emission depletion laser scanning microscopy.

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8 Discussion

Tight junctions represent cell-cell contacts located at the apicolateral site of epithelial and endothelial cells. The TJ structures are intermembranous protein particles (11) and are involved in the regulation of paracellular tightness against solutes. More than 40 different proteins are involved in the TJ complexes (122). TJ formation is realized by intercellular interactions of transmembrane TJ proteins (*trans*-interactions) and by interactions along the plasma membrane of one cell (*cis*-interactions), both leading to paracellular sealing.

A distinction is made between the areas where two cells join together (bicellular TJ) and areas where three cells join together (tricellular TJ) (21). Tricellular TJs were first observed in 1973 and thought to represent weak points in paracellular sealing (23). In 2005, the group of M. Furuse discovered Tric as specific component of tricellular TJs, tightening epithelial barriers against macromolecules (21). Bi- and tricellular TJs consist of a different composition of transmembranous proteins, leading to varying morphology (19, 21, 23). Bicellular TJs are composed of a tissue specific set of Clds (25), of Occl (49) and of MD3 (19, 47), whereas tricellular TJs of Tric and proteins belonging to the Angulin family, such as LSR (21, 62, 96). Ions and water may pass epithelial barriers through bicellular TJs, if pore forming Cld2 are present (size- and charge selective, high capacity pathway) (123), whereas only a small amount of ions passes through the tricellular pore (124). The detailed molecular mechanisms by which TJ-strands are formed and how the paracellular sealing is realized are poorly understood. Furthermore, the formation of tricellular TJs is far from being elucidated and is of potential relevance to improve drug delivery.

Based on the open questions mentioned above, I aimed to understand the molecular mechanisms underlying the formation of bi- and tricellular TJs, the dualism of Occl and Tric in localization and function and how TJs are modified and regulated in pathological events. To analyze function and regulation of TJ proteins I used different image based, biochemical and molecular biological methods. Depending on the analyzed proteins, we used TJ-free human embryonic kidney (HEK) cells or cell lines with endogenously expressed TJs (MDCK-II, Caco-2). In HEK cells we were able to characterize the functions of TJ proteins without influence of endogenous TJ proteins. The tricellular localization of Tric is highly dependent on the regulation of other TJ proteins; therefore we used MDCK-II or Caco-2.

Bi- and tricellular *trans*-Interactions of TJ proteins were determined by cell profiling at cell-cell contacts. While *cis*-interactions of TJ proteins were identified as FRET. Both techniques have been established earlier (125). FRET represents a powerful tool to verify distance dependent interactions. However, FRET signals at cell-cell contacts are unlikely to be due to direct energy transfer between *trans*-interacting TJ proteins on opposing plasma membranes because the distance between the respective intracellular CFP and YFP-tags is too big (<8 nm) for efficient FRET. The distance through two cell membranes is much bigger. A major disadvantage of the technique is the dependency on overexpressed fluorescence tagged proteins. Therefore we applied independent methods like co-immunoprecipitation (Co-IP) assays or proximity ligation assays to confirm the interactions obtained by FRET. In turn, the latter methods have the disadvantage, that the interactions cannot be distinguished between *cis*- and *trans*-interactions. To measure *cis*-interactions outside of cell-cell contacts we established a FRET measurement by FACS. The technique has the advantage, that thousands of cells of one sample can be analyzed individually. Previous studies already indicated the feasibility of this technique (126, 127). However, we improved and optimized the method, especially, with consideration of the dependence of the FRET efficiency on the acceptor to donor ratio and the characteristics of junctional proteins (MS1).

Physiological epithelial barrier characteristics were determined by standard transwell assays to analyze paracellular permeability with MDCK-II or Caco-2. However, reconstituted TJ-strands in HEK cells do not form a continuous sealing belt around the cells as in epi- or endothelial cells. Hence, standard transwell assays are hindered in such reconstitution systems. To be able to obtain physiological parameters, we established and applied a novel assay to determine the ability of an individual Cld to arrange a functional barrier at one bicellular contact (MS1).

Ms1) Claudin oligomerization

Initially, I focused on the molecular mechanisms by which TJ-strands are formed in cerebral barriers. In such barriers Cld1, 2, 3, 5, and 12 are preferentially expressed (128-131). By selecting HEK cells, we were able to analyze individual properties and functions of single Cld subtypes outside of a functional TJ environment (26, 44, 132).

The classic Cld1, 3 and 5 (28) showed similar strength in homophilic *trans*-interaction, which is consistent with earlier findings (133). In contrast, the non-classic Cld12 was incapable of homophilic *trans*-interactions.

Previously, we identified conserved aromatic amino acids in Cld5 (Tyr_{148}), which are essential for homophilic *trans*-interactions (125). The conserved Tyr-residue (Tyr_{147} in Cld3) was also essential for heterophilic *trans*-interactions between Cld3 and 5. The amino acid is highly conserved among classic Clds and may explain the heterophilic *trans*-interactions. The specificity of interactions between Cld subtypes was further analyzed by heterophilic *trans*-interaction measurements. We found the heterophilic *trans*-interactions for Cld5/Cld1 and Cld3/Cld1 to be as pronounced as that for homophilic Cld5/Cld5 and Cld3/Cld3 interactions. In contrast, Cld3/Cld5 *trans*-interactions were much less pronounced, although still significantly stronger than the negative control. The association of Cld1 with Cld3 in *trans* is consistent with earlier studies (133-135). However, Cld1/Cld5 *trans*-oligomers were not detectable in other studies. In contrast to Daugherty *et al*., 2007 we used a live-cell approach and our Cld constructs were unable to bind ZO-1 due to the C-terminal fluorescence tag. Both differences could alter the subcellular distribution of a Cld (26, MS1).

Since *trans*-interactions are a precondition for TJ-strand formation and thereby paracellular sealing, we analyzed homomeric TJ-strands build by individual Cld subtypes. Cld3 as well as Cld5 were able to reconstitute TJ-strands in TJ-free cells with subtype specific differences. HEK cells cotransfected with Cld3 and 5 exhibited a mixed phenotype concerning the TJ ultrastructure, with P-face associated particles (derived from Cld3) and E-face association (derived from Cld5). This resembles TJ in the endothelium of the blood-brain barrier *in vivo*, where coexpression of Cld3 with Cld5 was reported to correlate with mixed P-/E-face association and unique tightness of the endothelial junctions (136-138).

Consistent with *trans*-interaction studies, the non-classic Cld12 that failed to establish homophilic *trans*-interactions, also lacked the ability to form TJ-strands (MS1). Non-classic Clds reveal strong differences in the amino acid sequence, compared to classic Clds (28), therefore determinants for *trans*-interactions are less pronounced. However, the non-classic Cld11, in contrast to Cld12, is capable of homophilic *cis*- and *trans*-interactions (unpublished data), demonstrating that these interactions also occur in the protein subgroup. The expression of Cld12 is critical for vitamin D-dependent Ca^{2+} absorption between enterocytes (139). Therefore, the function of Cld12 may be not in the sealing, hence interactions in the TJs do not occur.

Suzuki and colleagues (2015) resolved the first crystal structure of a Cld, the classic Cld15 (36) and later of Cld19 (45). The structure revealed that the ECL2, involved in *trans*-

interactions (28, 125), forms a beta strand, bringing conserved aromatic residues in a position available for homo- and heterophilic *trans*-interactions (36). In the structure, lateral polymerization (*cis*-interaction) is realized by specific regions in the ECL2 and the TM3, forming complementary electrostatic potential surface. In Cld15 Met_{68} in the ECL2 of one monomer fits into a hydrophobic pocket formed by Phe_{146}, Phe_{146} and Leu_{158} in the TM3 and ECL2 of another Cld15 monomer. TJ-strand formation was not observed if one of these amino acids was exchanged to smaller or charged amino acids (36). The Cld15 structure further supports our results concerning *cis*-/*trans*-interactions and TJ-strand formation. Exchange of one of these aromatic residues to Ala in the ECL2 of Cld3 or 5 led to loss of TJ-strand formation (Tyr_{148} in Cld5 (26) and Tyr_{147} in Cld3 (MS1).

The immobilization of Cld subtypes, measured as *trans*-interactions, was further characterized by determining the plasma membrane mobility using FRAP. $Cld5_{Y148A}$ revealed an increased mobile fraction when compared to $Cld5_{wt}$, reflecting diminished immobilization by *trans*-interactions and the importance of aromatic residues in the ECL2 (26, Ms1).

Following our established imaging-based permeation measurements, we were able to demonstrate and quantify barrier function of a single Cld subtype in HEK cells (e.g. Cld3 or Cld5), being sufficient to form a diffusion barrier (MS1).

Clds interact in *cis* and *trans* to form TJ-strands, therefore we systematically investigated the colocalization, homo- and heterophilic *cis*-interactions of Cld subtypes. At cell-cell contacts of Cld-expressing HEK cells we found colocalization for Cld3/Cld5, Cld5/Cld1, Cld3/Cld1 and only partly for Cld3/Cld2. In intracellular compartments the degree of colocalizing Cld subtypes strikingly differed. Intracellular colocalization was commonly found for Cld1/Cld3, partially for Cld1/Cld5, seldom for Cld3/Cld5, and very infrequently for Cld2/Cld3. The subtype-specific differences in colocalization suggest that the subcellular distribution is caused by subtype-specific interactions rather than by undirected aggregation.

The Cld-containing intracellular compartments did not considerably colocalize with markers for endosomes or lysosomes but partly with those for the ER. Because of the different extent of colocalization at cell-cell contacts and at intracellular compartments, we performed FRET analysis at and outside of cell-cell contacts. At cell-cell contacts and therefore presumably in TJ-strands, *cis*- and *trans*-interactions are likely formed, and FRET-efficiencies decreased in the order Cld5/Cld5=Cld5/Cld1=Cld3/Cld1>Cld3/Cld3>Cld3/Cld5 and no FRET was detectable for

Cld3/Cld2. To investigate the *cis*-interaction of Clds outside of cell-cell contacts, FRET was analyzed in single cell suspensions by flow cytometry.

In single cell suspensions, cell-cell contacts and *trans*-interactions are assumed to be absent. Those FRET efficiencies decreased in the order Cld5/Cld5>Cld5/Cld1>Cld3/Cld3>Cld3/Cld5, with no FRET found for Cld3/Cld2. This indicates that *cis*-interactions between Cld pairs are similar outside and inside TJ-strands, independently of *trans*-interactions.

To further test the interaction of Cld-subtypes with absent *trans*-interactions, we measured the *cis*-interactions between $Cld3_{wt}$ and $Cld5_{Y148A}$ (Cld5 mutant with abolished *trans*-interactions) at cell-cell contacts and intracellular compartments. For Cld3/Cld5 FRET, but not Cld5/Cld5 (125), is abolished by Y148A substitution in Cld5 at cell-cell contacts. However, this substitution has no effect on *cis*-interactions measured in suspension. Hence, the data suggest that the spatial proximity between Cld3 and Cld5 in one plasma membrane at cell-cell contacts is promoted by homo- and/or heterophilic *trans*-interactions rather than by direct *cis*-interactions. Based on the results achieved in our group (26, Ms1), we generated a five step model for the formation of heteromeric TJ-strands, for Cld subtypes expressed in cerebral barriers:

1) Two to six Cld molecules form oligomers mediated by *cis*-interactions in intracellular compartments.

2) The varying strength in *cis*-interactions suggests that interactions in intracellular compartments are differentially established in a Cld subtype specific order, and that these interactions do not occur between Cld3/Cld2. The higher FRET-efficiency for homooligomers of Cld5 compared to Cld3 supports the assumption that Cld5 *cis*-oligomers are sterically preferred, when compared to Cld3 *cis*-oligomers. This is consistent with differences in their oligomer size (135). Recent data support the existence of homophilic Cld1 *cis*-oligomers (140). Cld2 is also assumed to form *cis*-oligomers (141), demonstrating, that these processes are common within the Cld protein family.

3) The oligomers are transported to the plasma membrane outside of TJs and then laterally diffuse to cell-cell contacts.

4) At the cell-cell contacts, homo- and heterophilic *trans*-interactions of the oligomers immobilize the Cld oligomers and trigger the TJ-strand formation. The classic Clds 1, 2, 3 and 5 are qualified for homo- and heterophilic *trans*-interactions, whereas the non-classic Cld12 is incapable of homo- and heterophilic *cis*- and *trans*-interactions.

Aromatic residues in the ECL2, conserved between classic Clds (e.g., Y148 in Cld5, Y147 in Cld3), are essential for homo- as well as heterophilic *trans*-interactions but not for the assembly of *cis*-oligomers. The idea of aromatic amino acids being involved in the formation of *trans*-oligomers was further confirmed by the crystallization of the first classic Cld (36).

5) In TJ-strands, spatial proximity of Clds in one membrane due to *cis*-interactions and/or *trans*-interactions occurs in a subtype specific preference. In contrast to the classic Clds, the non-classic Cld12 is not able to form homophilic *trans*-interactions or homopolymeric TJ-strands. Probably, heterophilic *cis*- and/or *trans*-interactions with another TJ protein are necessary for the incorporation of Cld12 in TJ-strands.

In summary, we provide novel mechanistic insights into the principles that define molecular organization of TJ-strands. This improves the understanding of paracellular barriers and might be used to develop new strategies to improve drug delivery across cerebral barriers, e.g., by modulation of the Cld3/Cld5 interaction.

B) Determinants for the tricellular localization of Tricellulin

Next we investigated how the exclusive tricellular localization of Tric in epithelial cells is realized (Ms2). Initially, the group of M. Furuse suggested two independent mechanisms guiding Tric to tricellular TJs. One is the exclusion of Tric from bicellular TJs by Occl. A second one is based on the observation, that Tric is targeted to the edges of the bicellular TJs (21, 64). In order to evaluate both mechanisms, we attempted to expand the knowledge about homophilic Tric interactions and heterophilic interactions with Occl, occurring within epithelial cell sheets. We analyzed the formation of homophilic Tric and heterophilic Tric/Occl complexes by co-IP and FRET in TJ containing MDCK cells. Interaction and localization studies with C- or N-terminal deletion constructs (ΔC and ΔN) of Occl and Tric, were performed to determine the impact of the cytosolic termini.

Full-length Tric localized at tricellular TJ, depending relative to the expression levels Tric was also localized at bicellular TJs, as reported previously (21, 22, 64). In contrary, ΔC-Tric was insufficiently transported to the plasma membrane and at the same time the amount of intracellular vesicles was enhanced. The ΔN-Tric construct was transported to the plasma membrane similar to full-length Tric, but the protein was not accumulated at tricellular TJs. The finding demonstrated the impact of the cytosolic tails in Tric for the correct transport to the plasma membrane and immobilization at tricellular TJs.

In the study of Ikenoushi and coworkers (2008), the authors present contradictory results to ours. ΔN-Tric and ΔC-Tric are both transported to the plasma membrane of bicellular

contacts in TJ-free L-fibroblasts, if cotransfected with Cld1 or -3 (64). The latter Clds are also expressed in the MDCK cells used in our study (142, 143) but in considerable lower amounts than in the L-fibroblast overexpression system. It seems likely, that certain Clds could promote the transport of Tric to the plasma membrane (64), potentially by interactions with the cytosolic tails of Tric (Ms4). Additionally, we analyzed the localization of ΔN-Tric-ΔC in MDCK cells. This double truncation mutant was not reaching the plasma membrane, indicating an essential role of the cytosolic tails in the efficient transport and incorporation of Tric in TJs (Ms2).

As described earlier, the DFNB49 nonsense mutation in the *marvelD2* gene, encoding for Tric, generates a deletion of the last 60 amino acids at the C-terminal cytoplasmic region and leads to a loss of plasma membrane-bound Tric (67). The authors suggested a mechanism, in which the binding of Tric to ZO-1 is prevented by the DFNB49 truncation, resulting in the loss of Tric at tricellular TJs (67, 144). However, the binding of Tric to ZO-1 is controversially discussed in the literature. Raleigh and coworkers (2010) reported that Tric does not bind to the recombinant GuK-domain of ZO-1 (19). Hence, the binding mechanism either differs from the binding of the Occl C-terminal coiled-coil domain to the hinge GuK-domain of ZO-1, or Tric is not binding at all (19). However, our study suggests the cytosolic C-terminal part is essential for the transport of Tric to the plasma membrane, while the cytosolic N-terminal part is essential for the distribution of the protein between bicellular and tricellular TJs (Ms2).

Previous studies demonstrated homophilic association of Occl (145, 146), via its C-terminal coiled-coil domain (51, 147). Based on the evolutionary relationship between Occl and Tric, a homophilic association of Tric is also assumed. Surprisingly, Tric self-association was still observed, when we performed the interaction measurements with the double deletion mutant ΔN-Tric-ΔC, suggesting that Tric oligomerization occurs independently of the cytosolic termini (Ms2).

In order To be able to distinguish between *cis*-oligomers (within one cell) or *trans*-oligomers (between two cells), we performed co-IP experiments using co-cultures of cells expressing full-length HA- or Flag-Tric. In this assay, we were unable to detect any self-association of Tric. Consequently, we concluded that the self-association is not occurring via the extracellular domains. Instead, oligomerization must occur between the transmembrane domains and/or the intracellular loops (Ms2).

In addition, we detected heterophilic Tric-Occl complexes. As observed for the homophilic Tric interaction, deletion of neither the N- nor C-terminal tail in Tric affected

binding to full-length Occl. Although both proteins do not colocalize at the plasma membrane, an interaction of Tric and Occl within a shared transport pathway to the plasma membrane (64) cannot be excluded. Taken together, here we present evidence for the importance of the cytosolic tails in Tric for the tricellular localization of the protein and first proof for homophilic self-association.

C) Claudin-TAMP interactions

As discussed earlier, the localization of Tric in epithelial cells is determined by a set of TJ proteins, such as Clds and Occl (19, 64, Ms2). While direct interactions between Occl and Clds were demonstrated in the literature (140), similar data are not been available for the interactions between Clds and Tric or MD3. In order to gain knowledge about the dynamic interactions within the TJ complex we systematically investigated mutual interactions of both protein families occurring in epithelial cells.

We found Occl to interact homophilically in *cis* and *trans* (Ms3). Homophilic interactions of Occl have been demonstrated by us (44) and others (19) before, using a co-IP assay, a procedure which does not allow to distinguish between *trans*- and *cis*-interactions. In contrast, Tric and MD3 were incapable of homophilic *trans*-interactions. All TAMPs showed homophilic oligomerization within one plasma membrane, but less efficient than classic Clds do (26, 140, Ms1). Furthermore, MD3 is *cis*-interacting with Occl or Tric, but Tric and Occl do not interact with each other. This result is in agreement with data from the literature (19), but it contradicts to our earlier findings (Ms2). In that work, we suggested, that Occl and Tric may interact in shared transport pathways (Ms2), like it was shown for other TJ proteins, like Occl and ZO-1 (148, 149). We verified this hypothesis by using a FRET assay in intracellular compartments, which demonstrated binding between Occl and Tric. Taken together with literature data (19, 64), the result support the assumption that Occl and Tric may be transported together to the bicellular plasma membrane and, in a second step, are separated to bi- or tricellular TJs (Ms3).

Moreover, we provide strong evidence for the oligomerization between members of the Cld family and members of the TAMP family. So far, interactions were suggested based on colocalization (21, 64) or co-IP assays (19), but the exact binding properties have not been analyzed in the living cell so far. The heterophilic *cis*-interaction of Cld1 to all individual TAMPs was remarkable. Cld1 is widely expressed in a multitude of different cellular barriers (150), such as Occl, Tric and MD3 (19, 47). Thus, these interactions might be of general importance in epithelial barriers. This leads to the assumption that the interplay of TAMPs with Cld1 is of general relevance as an organizing protein for the

assembly of TAMPs in TJs. Heterophilic *cis*-interactions of TAMPs and other Cld subtypes was less pronounced (Ms3).

The interaction of Cld1 and -3 to Tric was assumed before, on the basis of colocalization studies (64). Other studies demonstrated FRET between Occl and Clds (140) or between the recombinant Occl coiled-coil domain with Cld1 and -2 (62). The authors of the latter study suggested that the interaction could also be mediated by ZO-1 bound to the Occl coiled-coil domain (151) and the C-terminal PDZ-binding motif of Cld1 (38, 62). However, in our experimental setup the cytosolic C-terminal PDZ-binding motif of Clds is blocked by recombinant fluorescence tags, thereby a ZO-1-mediated interaction between Clds and TAMPs can be excluded.

In addition, cotransfection of Occl, Tric or MD3 with Cld1 or -5 led to an increased localization of Occl at cell-cell contacts, a significant establishment of cell-cell contact localization of Tric but no effect on MD3. In the same manner, the plasma membrane mobility of Occl or Tric but not of MD3 was drastically reduced after coexpression with Cld1 or -5. This influence further underlines the existence of a direct association of Clds with Occl or Tric, which, in consequence, recruits more TAMP molecules to the cell-cell contacts resulting in enhanced co-enrichment (Ms3). An effect for Cld1 on TAMPs was reported earlier (19, 25, 64) demonstrating a striking redistribution of a TAMP from the periphery of the cell membrane to cell-cell contacts due to the coexpression with Cld1 in L-fibroblasts.

To further evaluate the interaction of Cld1 with TAMPs, we performed freeze-fracture electron microscopic analyses after co-transfection of Cld1 with Occl, Tric or Md3 in TJ-free cells. It was demonstrated previously that Occl alone forms small round TJ-strand meshes (49), while it was proposed for Tric (21) and MD3 (19, 47), that these TJ proteins are incapable of forming TJ-strands individually. We were able to reproduce the findings concerning Occl and we detected similar structures for Tric, but not for MD3 (Ms3). Cld1 alone is forming large round TJ-strand meshes rarely in parallel orientation (26) which are specifically altered in shape and/or size due to coexpression with Occl, Tric or MD3. Occl caused longitudinal meshes of Cld1 with more parallel and less continuous strands. Tric altered the shape of the TJ-strand meshes to a more rectangular shape and reduced the mesh size, which is in agreement to earlier observations (64). MD3 has similar but less pronounced effects on the Cld1-based TJ-network. In general, Tric, Md3 and to a lesser extent, Occl enable a more compact and integrated Cld1 strand morphology similar to what occurs in epi- and endothelial cells *in vivo*. In fact, small and rectangular TJ-strands

are well known from epithelial cells expressing Clds and TAMPs endogenously (12, 23, 152).

The interplay of Cld1 with TAMPs seems to be of general importance in the antagonistic behavior of Occl and Tric at bicellular contacts. We elaborated Ca^{2+}-switch experiments to further analyze the transport of Tric to the specific regions of the plasma membrane. Ca^{2+}-switch represents a well-established procedure which allows monitoring both, the internalization of TJ proteins, following a Ca^{2+}-deprivation phase, and TJ formation/assembly after Ca^{2+} has been replenished (153, 154). The typical epithelial cell polarity starts after 30 min Ca^{2+}-repletion, and at the same time Tric and Cld1 colocalized at bicellular junctions of MDCK cells (Ms3). The finding was reproduced by others, linking the Ca^{2+}-deprivation to a breakdown in epithelial permeability (155). As hypothesized, Clds and TAMPs are transported together to the bicellular plasma membrane, as indicated by FRET of intracellular vesicles and Ca^{2+}-switch experiments (Ms3).

To enlighten the mechanism controlling the differential distribution of Tric and Occl in the bi- and tricellular TJs, we performed triple-transfectants of Occl, Tric and Cld1 in TJ-free cells. At bicellular junctions, Occl and Tric compete for the binding to Cld1. The interaction of Cld1 with Occl is stronger or more stable than then the one with Tric, probably because in addition to its affinity to Cld1, Occl is immobilized at the plasma membrane by its relatively strong homophilic *trans*-interactions. In contrast, Tric, unable to *trans*-interact homophilically, is translocated to the tricellular junctions (Ms3) where LSR builds a landmark for tricellular junctions and interacts with Tric (96, 156-158). In agreement with these findings, a knock-down of Cld1 in epithelial cells results in a fragmented appearance of Tric also at the bicellular TJs, demonstrating the impact of Cld1 on the separation of Occl and Tric into bi- and tricellular TJs.

d) Redox-regulation of bi- and tricellular TJs

Except the DFNB49 mutation in *marvelD2* in humans (67, 74), the function of Tric has been investigated so far under normal conditions only. No information concerning its alterations in molecular interaction mechanisms under pathological conditions is available. We therefore addressed the question whether and how Tric and the tricellular TJs are altered in pathological events related to hypoxia (Ms4). It is generally accepted, that alterations in the redox-state affects barrier integrity, resulting in organ injury. The TAMP Occl was shown to be sensitive to hypoxic injuries (60), contributing to the breakdown of the blood-brain barrier (116). More specifically, Occl behaves as a redox-regulator at

bicellular junctions and due to hypoxic stress, the protein translocate from the plasma membrane, thereby losing its regulatory function at the TJs. In particular, two Cys residues in the ECL2 of Occl forming a disulfide bond and thereby stabilize the loop structure. Reactive oxygen species (ROS) occurring in hypoxic events lead to reduction of the disulfide bond, and, thereby, the ECL2 loses its function (60). These ECL2 Cys residues are highly conserved among TAMPs and in the ECL1 of Clds (16, 28), indicating a general structural feature in extracellular domains. This context has prompted us to focus on the interaction sites of Tric under hypoxic and reducing conditions and by means of Cys-to-Ala substitution ($Tric_{CA}$) at bi- and tricellular contacts, similar as done for Occl. We found, that Tric is redistributed from tricellular to bicellular junctions after 4 h of hypoxic stress *in vitro* and after 20 min of ischemic kidney injury *in vivo.* As discussed earlier, Occl may compensates for the localization of Tric and *vice versa* (64). However, during hypoxic injury both proteins are affected, possibly hindering such compensatory mechanism.

In MDCK-II cells the majority of Tric constructs remained localized at tricellular contacts, while the two ECL2 Cys mutants ($Tric_{C321A}$ and $Tric_{C335A}$) localize at bicellular contacts. Based on our findings regarding Occl, we concluded that those Cys residues form a disulfide bond, stabilizing the structure of the ECL2. Consequently, the Cys mutations cause an unstructured loop, which is then unable to form homophilic *trans*-interactions which are essential for the immobilization of Tric at tricellular contacts. Due to the oxidizing milieu in the extracellular space, disulfide bonds are a common element supporting the structure in extracellular domains (159, 160).

Based on our experimental results, we generated the first molecular model of the Tric ECL2 which carries very similar functional elements when compared to the Occl ECL2: redox-sensitive, solvent-inaccessible disulfide bridge and two highly conserved aromatic residues (Phe_{326}, Phe_{334}) on the ECL2 surface. However, in Tric ECL2, the Cys residues arrange two short amino acid chains in parallel without forming strand-like or helical structures. The similar structural principles support the hypothesis that the redox- and disulfide-dependent tricellular *trans*-interaction of Tric is caused similar as proposed for Occl (48) or Clds (28, 36, Ms1). On the other hand in Tric, the Cys residues are surrounded by two turn-forming Pro (Pro_{324}, Pro_{329}), which are Tric-specific and singular in TAMPs. Consequently, the Tric ECL2 structure is neither comparable with the Occl-ECL2 (60) nor with the two Cys residues containing ECL1 of Clds (36).

Next, we investigated the heterophilic *cis*-interactions of Tric to Cld1 or MD3 in at bicellular contacts in HEK cells and in the homophilic Tric association at tricellular contacts in MDCK-II. In the latter cell line, Tric is localized at tricellular junctions, which allows strong *cis*-interactions of the majority of measured $Tric_{wt}$-$Tric_{CA}$ combinations, except for $Tric_{C395A}$. At bicellular contacts of MDCK-II or HEK cells, the *cis*-interaction was relatively low (HEK: 4.6%, MDCK-II: 3.6%), while at tricellular contacts of MDCK-II cells we measured FRET values up to 16%. Hence, this variation in the degree of *cis*-interaction supports the assumption that *trans*-interactions (here: tricellular *trans*-interactions) promote a close proximity of the interaction partners, similar as for Clds (Ms1).

The homophilic *cis*-interactions promoting Cys_{395} is located in the cytosolic C-terminal tail of Tric. For Occl this domain was demonstrated to be essential for the association with ZO-1 and -2 (161) and for the redox dependent self-association (146). The C-terminal domain of Tric shows high homology to the corresponding Occl C-terminal part, therefore a similar function is assumed. But still the association of Tric with ZO-1 is under debate in the literature (19, 67) and has to be proven experimentally.

The interaction measurements of Tric to Cld1 or MD3 were performed at bicellular contacts in TJ-free HEK cells, because of not sufficient colocalization at tricellular TJs in MDCK-II. Among the Tric mutants, we identified Cys_{362} in the 4th TM domain to mediate heterophilic association to Cld1 and MD3. This suggests that the 4th TM domain Cys is outwardly directed from the TM segment and is on the binding surface and only accessible for heterophilic association partners. A disulfide bond, contributing to heterophilic binding, is unlikely as a redox status is undefined within a lipid membrane. Therefore, an intramembrane hydrogen bond between Tric 4th TM Cys residue and a Cys residue is assumed at the 4th TM of Cld1, 3, 4, and 5. The latter contains a highly conserved Cys or another H-bond binding partner, forming a polar residue in the position equivalent to C362 at Tric 4th TM domain. Other Clds, such as Cld2 and 11, which do not bind to Tric (Ms3), contain aromatic or nonpolar amino acids in the corresponding region (28, 29).

The exchange of Cys_{115} to Ala in the N-terminal segment leads to an incomplete tricellular localization. Previously, we showed that the N-terminal domain is essential for targeting Tric to the tricellular contacts (Ms2). The neighboring Ser_{116} is potentially phosphorylated (162), and phosphorylation offers a possible additional pathway to locate Tric at bi- or tricellular contacts. This possibility fits well to our findings, that in hypoxic disturbances Tric is less phosphorylated (Ms4).

In MDCK-II cells, expression of Tric mutant constructs led to a loss in homo- and heterophilic *cis*- and *trans*-interactions and also to leaky epithelial barriers. Interestingly, despite the notion that Tric tightens barriers against macromolecules (22), the above mentioned barrier loss affected also ions, small and large molecules (Ms4). Therefore, our results are not in agreement with published data (22). In MDCK-II cells expressing one of these Tric mutants, also other TJ-proteins like Occl, Cld1 and ZO-1 were affected in their localization at the plasma membrane. Hence, the alterations in Tric function and interaction ability also affects the properties of the TJ proteins analyzed. Furthermore, the molecular organization of TJ-strand networks, as invested by freeze-fracture electron microscopy, was significantly altered. Consequently, we postulate that Tric functions as a superordinate TJ regulator.

The homo- and heterophilic interactions contribute to the physiological organization of the TJ-network. In pathological disturbances, Tric plays a similar role in tricellular interactions as Occl does in bicellular interactions (60), by acting as a reduction sensor between three cells. By increasing the reducing conditions, Occl is internalized (60) and Tric compensates its loss at bicellular junctions (64). The apparent colocalization of Occl and Tric seen by conventional microscopy mistakenly suggests that Occl also regulate the redox state of tricellular junctions. However, super resolution STED microscopy clearly demonstrates that both proteins localize separately (Ms4). In our view both proteins are present in all epi- and endothelial barriers (72, Ms1), potentially sensitive for reducing conditions occurring in e.g. inflammation or ischemia (16).

8.1 Conclusion and outlook

In this thesis, dynamic and mutual interactions of TJ proteins are unveiled. We provided novel insights into the oligomerization mechanisms and thereby TJ-strand formation as well as paracellular sealing of tissue barriers. We identified determinants for homo- and heterophilic Cld/Cld, TAMP/TAMP and Cld/TAMP interactions. Concerning Clds, our findings were largely confirmed by novel structure information from Cld15 and 19, published after our publication. Our data improves the molecular understanding of paracellular barrier tightening and leads to novel strategies to improve drug delivery across cerebral and other tissue barriers.

Also for the TJ protein Tric, we found first determinants for how the tricellular localization is realized, which is of general functional importance. Moreover, the interplay of Occl, Tric and MD3 with members of the Cld family determines the physiological

structure of the TJ-strand network. These findings underline the regulatory impact of the TAMP family, which was assumed but not analysed in detail before. Finally, we were able to demonstrate, that Tric and Occl are primary targets in pathologies linked to alterations in the redox state, taking place in inflammation, cancer or hypoxic events. These findings offer novel perspectives for the diagnosis and treatment of such diseases.

Given that Tric carries functions to organize the TJ-network and thereby the paracellular permeability of a respective tissue barrier, the regulation of the protein by posttranslational modifications is poorly understood. Within eukaryotic cells, phosphorylation represents a powerful tool to regulate functions and/or localization of proteins. In future studies, the regulation by phosphorylation should be enlightened.

Peptidomimetics present a tool to pharmacologically manipulate protein functions. By emerging peptides, derived from the Tric-ECL2, one could propose opening of cellular barriers specifically and transiently for the passage of macromolecules, which is of general interest, for instance in enzyme replacement or antibody therapies.

9 Summary

The paracellular barrier characteristics of epi- and endothelial layers relay on the properties of tight junctions (TJs). Based on the localization, morphology and protein composition, TJs can be classified into areas where two (bicellular TJs) or three cells (tricellular TJs) join together. Tricellulin (Tric) specifically localizes and tightens tricellular TJs while claudins (Clds), occludin (Occl) and marvelD3 (MD3) localize at bicellular TJs. Clds form the backbone of bicellular TJs. TJ-associated MARVEL proteins (TAMPs; Occl, MD3 and also Tric) are assumed to be of regulatory relevance of TJs. Interplay of Clds and TAMPs in the assembly of a functional barrier and in pathological alterations of these interactions have not yet been analyzed. The cumulative thesis consists of four publications, aiming to understand how TJ proteins are regulated, interact with each other and maintain/ affect functions of tissue barriers.

(Ms1) The results led to the generation of a model for heterophilic TJ-strand formation: two to six Cld molecules form oligomers within intracellular membranes. The strength of *cis*-interactions along the cell membrane is Cld subtype specific. The *cis*-oligomers reach the plasma membrane and diffuse to the TJs. Then, *trans*-interactions between Clds of opposing cells enable the formation of TJ-strands. With this study we established two novel methods to measure FRET in intracellular compartments (FRET-FACS) and to determine paracellular permeability by an image-based technique.

(Ms2) We identified the cytosolic C-terminal tail in Tric being essential for the transport to the plasma membrane and the enrichment at its tricellular TJs (N-terminal tail).

(Ms3) Within the TAMPs, MD3 interacts with Occl and Tric at bicellular contacts, whereas Tric and Occl interact during the transport to the plasma membrane only. We demonstrate, for the first time heterophilic interactions between TAMPs and Clds. The interactions are essential for a physiological TJ-strand network, reflected by the immobilization of Tric and Occl at the plasma membrane by Cld1 and by an improvement of the Cld1-formed TJ-strand network by Occl, Tric or MD3. These findings show a novel regulatory function of TAMPs at TJs.

(Ms4) We demonstrated, *in vitro* and *in vivo*, that Tric and Occl are primary targets in pathologies linked to alterations in the redox state. We showed that the ECL2 in Tric and Occl is redox-sensitive and redox changes cause alterations in their structure, function and localization. Therefore, Occl and Tric are redox-regulators of TJs; Tric acts as superordinate TJ regulator not only at the tri- but also at bicellular TJs. Alterations in the

Tric-ECL2 structure alter the localization of Tric and also of other TJ proteins such as Cld1, MD3 and ZO-1. Tric-mediated alterations in the TJ-strand network led to a breakdown of the epithelial barrier properties.

In summary, the findings offer novel perspectives for potential diagnosis and treatment of diseases associated with alteration in redox-state, like inflammation, stroke or cancer. The studies provided substantial contribution for better analysis and understanding of structure and function of TJ proteins under normal and pathological conditions.

9.1 Zusammenfassung

Die parazellulären Barriereeigenschaften von Epi- und Endothelien werden hauptsächlich von *Tight Junctions* (TJs) bestimmt. Hinsichtlich Lokalisation, Morphologie und Proteinzusammensetzung lassen sich TJs in Zell-Zell Kontakte zwischen zwei Zellen (bizelluläre TJs) bzw. drei Zellen (trizelluläre TJs) einteilen. Trizellulin (Tric) dichtet spezifisch trizelluläre TJs ab; der Mechanismus ist unbekannt. Claudin (Cld), Occludin (Occl) und MarvelD3 (MD3) lokalisieren an bizellulären TJs. Cld tragen die Hauptabdichtungsfunktion der bizellulären TJs. Für TJ-assoziierte MARVEL Proteine (TAMPs; Occl, Tric, MD3) wird eine regulierende Funktion angenommen wird. Neben dem trizellulären Abdichtungsmechanismus sollen mögliche Wechselwirkungen zwischen TAMPs und Claudinen herausgefunden werden, die die Bildung und Aufrechterhaltung des TJ Netzwerkes beeinflussen. Gefundene Interaktionen zwischen TJ-Proteinen und Tric sollen unter pathologischen Bedingungen charakterisiert werden. Der kumulativen Arbeit liegen vier eigene Publikationen zugrunde.

(Ms1) Aus den Cld-Interaktionsstudien wurde ein Modell abgeleitet, das die Bildung von heterophilen TJ-Strängen erklärt: zwei bis sechs Cld-Moleküle oligomerisieren in *cis* entlang intrazellulärer Membranen. Die Stärke der *cis*-Interaktionen ist Cld-subtypspezifisch. Die Cld *cis*-Oligomere werden zur Plasmamembran transportiert, wo sie zu den TJs diffundieren. Hier werden die Oligomere durch zusätzliche *trans*-Interaktionen zwischen benachbarten Zellen an den TJs immobilisiert. Für die Arbeit wurde ein neues Verfahren zur parazellulären Permeationsmessung entwickelt sowie eine Methode entwickelt, um mit FACS Lebendzell-FRET Messungen durchführen zu können.

(Ms2) Weiterhin wurde die Bedeutung der zytoplasmatischen Domänen von Tric aufgeklärt. Dabei ist die C-terminale zytosolische Domäne an der Membranlokalisation beteiligt; die N-terminale zytosolische Domäne ist essentiell für die Lokalisation in trizellulären TJs.

(Ms3) Bei den Untersuchungen von Wechselwirkungen zwischen TAMPs sowie zwischen Claudinen und TAMPs wurde gezeigt, dass TAMPs homo- und heterophil interagieren, abgesehen von Tric und Occl Assoziationen. Occl, Tric und MD3 zeigten starke Bindungen zu Cld1 und schwächere zu anderen Claudinen. Weiterhin wurde eine regulatorische Rolle der TAMPs nachgewiesen, wobei Occl, Tric und MD3 die von Cld1 gebildeten TJ-Netzwerke engmaschiger gestalteten.

(Ms4) *In vitro* und *in vivo* fanden wir, dass Tric und Occl spezifische Redoxregulatoren sind. Wir konnten zeigen, dass strukturelle Veränderungen im Tric-ECL2 die Lokalisation von Tric ändert, mit Auswirkungen auf andere TJ Proteine wie Cld1, MD3 und ZO-1. Die durch Tric vermittelten Veränderungen im TJ Netzwerk bewirken einen Zusammenbruch epithelialer Barrieren. Daher muss Tric als übergeordneter Redox-Regulator der TJs angesehen werden.

Insgesamt sind die gewonnenen Erkenntnisse von Relevanz für künftige Untersuchungen zur Diagnostik und Therapie von Erkrankungen die mit Veränderungen des Redoxpotentials einhergehen, wie z.B. Entzündungen, Schlaganfall oder Tumoren. Damit liefert die Arbeit einen substantiellen Beitrag zum besseren Verständnis von strukturellen und funktionellen Eigenschaften von TJ Proteinen unter normalen und pathologischen Bedingungen.

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11 List of publications

11.1 Enclosed in this thesis

1) Elucidating the principles of the molecular organization of heteropolymeric tight junction strands
J. Piontek, S. Fritzsche, J. Cording, S. Richter, J. Hartwig, M. Walter, D. Yu, J. R. Turner, C. Gehring, H. P. Rahn, H. Wolburg and I. E. Blasig
Cellular and Molecular Life Sciences
December 2011, Volume 68, Issue 23, pp 3903-3918

2) Tricellulin forms homomeric and heteromeric tight junctional complexes
J. K. Westphal, M. J. Dörfel, S. M. Krug, J. D. Cording, J. Piontek, I. E. Blasig, R. Tauber, M. Fromm and O. Huber
Cellular and Molecular Life Sciences
June 2010, Volume 67, Issue 12, pp 2057-2068

3) In tight junctions, claudins regulate the interactions between occludin, tricellulin and marvelD3, which, inversely, modulate claudin oligomerization
J. Cording, J. Berg, N. Käding, C. Bellmann, C. Tscheik, J. K. Westphal, S. Milatz, D. Günzel, H. Wolburg, J. Piontek, O. Huber and I. E. Blasig
Journal of Cell Science
15 January 2013, 126, 554-564

4) Redox regulation of cell contacts by tricellulin and occludin: Redox-sensitive cysteine sites in tricellulin regulate both tri- and bicellular junctions in tissue barriers as shown in hypoxia and ischemia
J. Cording, R. Günther, E. Vigolo, C. Tscheik, L. Winkler, I. Schlattner, D. Lorenz, R. F. Haseloff, K. M. Schmidt-Ott, H. Wolburg and I. E. Blasig
Antioxidants & Redox Signaling
2015. Epub April 2015, June 2015, ahead of print

11.2 Other publications

5) Participation of the second extracellular loop of claudin-5 in paracellular tightening against ions, small and large molecules
C. Piehl, J. Piontek, J. Cording, H. Wolburg and I. E. Blasig
Cellular and Molecular Life Sciences; June 2010, Volume 67, Issue 12, pp 2131-2140

6) Occludin Protein Family: Oxidative Stress and Reducing Conditions
I. E. Blasig, C. Bellmann, J. Cording, G. del Vecchio, D. Zwanziger, O. Huber, and R. F. Haseloff
Antioxidants & Redox Signaling; September 1, 2011, 15(5): 1195-1219

7) CK2-dependent phosphorylation of occludin regulates the interaction with ZO-proteins and tight junction integrity
M. J. Dörfel, J. K. Westphal, C. Bellmann, S. M. Krug, J. Cording, S. Mittag, R. Tauber, M. Fromm, I. E. Blasig and O. Huber
Cell Communication and Signaling; 10 June 2013, 11:40

8) A quantitative proteomics approach directed at low-abundant proteins differentially expressed in hypoxia-challenged cells of the blood-brain barrier
K. Mikoteit, M. Schümann, E. Krause, J. Cording, L. Winkler, D.B. Stanimirovic, H.E. deVries, I.E. Blasig and R.F. Haseloff
Antioxidants & Redox Signaling, to be submitted (July, 2015)

11.3 Presentations at international meetings

1) Analysis of interactions between heterophilic tight junction proteins'
J. Cording, C. Piehl, J. Piontek, H. Wolburg and I.E. Blasig
12th International Symposium of the Blood-Brain Barrier, 2009, London, UK

2) Claudins modulate the oligomerization properties of tight junction associated MARVEL proteins
J. Cording, N. Kädig, J. Berg, O. Huber, J. Piontek and I. E. Blasig
14th International Symposium of Signal Transduction in the Blood-Brain Barrier, 2011, Istanbul, Turkey

3) Detection of tight junction strand morphologies of claudins and tight junction associated MARVEL-proteins
J. Cording, N. Kädig, J. Berg, J. Piontek, H. Wolburg and I.E. Blasig
15th International Symposium of signal transduction in the Blood-Brain Barrier, 2012, Potsdam, Germany

4) Tricellulin is sensitive to pathological conditions such as hypoxia and related conditions.
J. Cording, R. Günther, C. Tscheik, L. Winkler, I. Schlattner, H. Wolburg and I. E. Blasig
16th International symposium on signal transduction in the blood-brain barrier, 2013, Sümeg, Hungary

12 Appendix

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12.2 Selbständigkeitserklärung

Hiermit erkläre ich, dass ich diese Arbeit selbständig verfasst habe und keine anderen als die angegebenen Quellen und Hilfsmittel in Anspruch genommen habe. Ich versichere, dass diese Arbeit in dieser oder anderer Form keiner anderen Prüfungsbehörde vorgelegt wurde.

Jimmi Cording Berlin, den