Lorenza González-Mariscal *Editor*

Tight Junctions

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ISBN 978-3-030-97203-5 ISBN 978-3-030-97204-2 (eBook)
https://doi.org/10.1007/978-3-030-97204-2

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

Tight junctions are intercellular seals located at the uppermost portion of the lateral membrane of epithelial cells that regulate the passage of ions and molecules through the paracellular pathway. This book provides an updated view of tight junctions that will be of interest to a broad audience of students and scientists in the fields of Cell Biology, Neuroscience, and Cancer.

The book starts with a historical recount of the discovery of tight junctions by electron microscopy and the evolution of the concept of tight junctions from static seals to dynamic structures challenged by a variety of physiological and pathological conditions. In the second chapter, the reader will discover how the appearance of tight junctions, molecular composition, and barrier function vary at sites where three or more epithelial cells meet in contrast to places where only two cells are in contact. Chapter 3 provides a novel view of tight junctions as mechanosensory, revealing that the interaction of tight junction components with the contractile actomyosin network not only allows the detection of changes in force in the epithelia but also allows the cell to elicit a homeostatic response. Chapter 4 describes signaling between the plasma membrane and the nucleus as the tight junction adaptor protein ZO-2 moves to the nucleus and inhibits gene transcription and cell proliferation.

The diversity of claudins and their function in different tissues and pathological conditions allowed us to devote Chap. 5 to the study of claudin-2 in intestinal disorders and recount in Chap. 10 the role of diverse claudins in the lung. In addition, in Chap. 9, the participation of claudins in cell–matrix interactions and stem cell regulation are addressed. Finally, three more chapters describe how intestinal tight junction proteins are affected by inflammation (Chap. 6), infection with the enteropathogenic *Escherichia coli* (Chap. 7), and microbial-derived molecules (Chap. 8).

The importance and particularities of endothelial tight junctions are analyzed in other two chapters. First, the blood–retinal barrier is reviewed in Chap. 11, describing alterations in blinding eye diseases and addressing potential regenerative therapies that target endothelial tight junctions. Second, Chap. 12 describes the opening of tight junctions in the blood–brain barrier during paracellular extravasation of metastatic cells and how the tight junctions of the blood–tumor barrier restricts the

passage of therapeutic drugs to tumors in the brain. The final chapter of this book (Chap. 13) describes how endocytosis is a fundamental process of tight junction dynamics and reveals how its regulation could be used to enhance drug delivery.

All the topics in this book have been written by experts in tight junctions to whom I express my gratitude for the effort and generosity shown in providing their time and knowledge for the elaboration of their chapters.

Mexico City, Mexico Lorenza González-Mariscal

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A Historical and Evolutionary View of Tight Junctions

Marcelino Cereijido and Jacqueline Martínez Rendón

Abstract The essential functions of a polarized epithelium are to separate the spaces between tissues and regulate the exchange of materials between them, functioning as an interface with the external environment. Tight junctions (TJ) are the anatomical structures responsible for creating this barrier. These cell junctions are regulated and selective, and vary depending on the tissue in which they are found.

However, for many years it was considered that TJ were at the border of cells and their function was limited to blocking the passage of substances between cells, so it is understandable that they received names as "terminal bar." It was not until the arrival of electron microscopy that it was possible to resolve that these "terminal bars" are, in fact, a complex of cell junctions.

In this chapter, we will see the history and how the concept of tight junctions evolved. We will discuss the main functions of this type of cellular contacts and the experiments that allowed to study their structure and biology.

Keywords Transporting epithelium · Cell contacts · Tight junctions · Evolution · Microscopy

Abbreviations

TJ	Tight junctions
TER	Transepithelial electrical resistance
TrEp	Transporting epithelium

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L. González-Mariscal (ed.), *Tight Junctions*,
https://doi.org/10.1007/978-3-030-97204-2_1

A Single Cell in the Ocean

At the beginning of the twentieth century, life was considered a God-given gift to humanity, and biologists were reluctant to analyze it using a thermodynamic basis.

Nevertheless, Erwin Rudolf Josef Alexander Schrödinger, who was earlier awarded the Nobel Prize (1933) for his wave equation, wrote one of the most influential biological books ever named, *What Is Life?* [1], in which he proposed that living organisms are considered to be thermodynamically open systems. This idea would be easily understood by imagining a single cell in the sea exchanging substances with its environment, and this environment behaves as an infinite and constant reservoir that is not exhausted by the removal of nutrients nor spoiled by receiving catabolites and detritus excreted by the cells.

On the other side, if we think in a cell lodged in a recondite fold of the brain, the liver, or any other organ of a mammal, we can note that its surrounding is reduced to an extremely thin film, even though it behaves as an infinite and constant reservoir as if it was an immense ocean.

Consequently, this makes us wonder how evolution could generate metazoans whose cells can be meters away from the outer environment (think of a hepatocyte sunken in the liver of a whale). Evolution coped with this situation by developing transporting epithelia (TrEp), consisting of barriers of tightly packed cells, which generate a fluid compartment named "internal medium" that occupies some 20% of the human body.

Hence our cells do not exchange directly with the sea, but with this internal medium. In spite of being a comparatively small space, this internal medium may act as a constant reservoir because TrEp manage to regulate its composition through a complex mechanism called homeostasis, derived from Greek "homoios" (similar) and "stasis" (stability).

The epithelia acts as an interface with the external environment, withstands strong mechanical or chemical stress, and works as a diffusion barrier between compartments with different compositions, protecting the organism from toxins and microorganisms, extruding metabolic wastes, and taking up nutrients [2–4].

Exchange of substances through transporting epithelia proceeds through a transcellular and a paracellular route. It requires two fundamental features of TrEp: the first one consists of polarized cells that have an apical membrane domain which is structurally, molecularly, and physiologically different than the basolateral one. And the second one, the paracellular route, proceeds instead between the epithelial cells and is limited by cell-cell contacts called tight junctions (TJ) that partially seal the interspace between cells and transform them into selective permeability barriers restraining substances flow through the intercellular space [5, 6].

Evolution of Knowledge on the Tight Junction Structure and Composition

For almost a century, the anatomical formation of TrEp was studied by dissecting a frog skin and gastric or intestinal mucosa and mounting them between two Lucite chambers with saline solutions on both sides. Fluxes through the intercellular spaces were practically negligible, because these spaces were sealed by molecular and anatomical structures, whose details could not be observed with optical microscopes.

This seal was also expected to be impermeable and located at the very limit of cells between the lumen and intercellular space. Therefore, it is understandable that the anatomical formation detected by light microscopy at the outermost edge of intercellular space received names such as *Schlussleisten*, "terminal bars," *bandelettes de fermeture*, "hoops," "occluding junctions," "tight junctions," "gaskets," and "attachment belts" [7–12].

The introduction of electron microscopy from 1950 to 1955 permitted the observation of plasma membranes stained with osmium tetroxide (OsO_4), and the plasma membrane appeared as a sequence of three layers, [dark]-[light]-[dark], which corresponded to the [cytoplasmic polar groups]-[hydrophobic chains]-[external polar groups] of biochemical models. The finer resolution of electron microscopy also revealed that the "terminal bar" is, in fact, a complex of different types of specialized intercellular junctions, which received the names TJ (*zonula occludens*), intermediate junction (*zonula* or *fascia adherens*), and desmosome (*macula adherens*) [6]. In fact, desmosomes were well known from earlier studies, and neighboring cells may also establish gap junctions. In Fig. 1a, the intercellular space shown

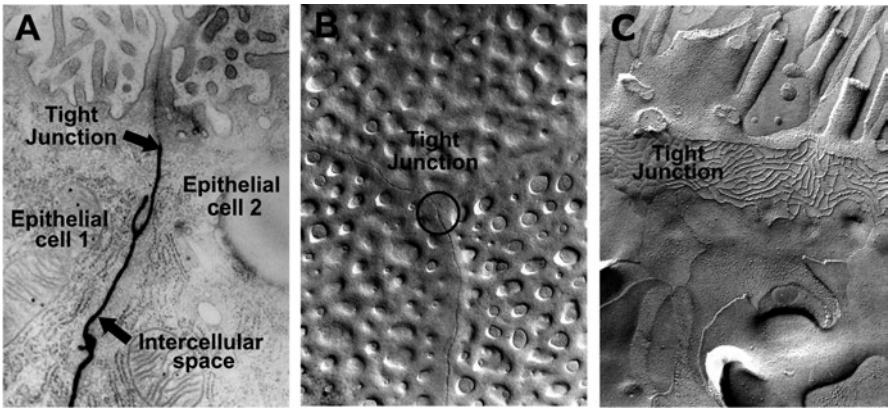

Fig. 1 Structure of tight junctions. (**a**) Transmission electron microscopy of two adjacent cells from the epithelium. Lanthanum hydroxide applied from the lower solution; it cannot diffuse beyond the TJ. (**b**) Freeze fracture showing the meeting point of three epithelial cells, with the corresponding TJ. (**c**) A freeze fracture of the epithelium of the mouse's small intestine that passed exactly at the level of a TJ, showing the belt of junctional strands. In some of them, the cut passed through the membrane of one of the cells and in some other segments through the neighboring one

between two neighboring cells is full of lanthanum hydroxide, this marker was added at the basal side of the epithelium, and it diffused freely between the cells until the TJ stopped it [13–17].

A few years later, freeze-fracture studies of both epithelia and endothelia revealed that TJ consists of a distinctive reticular pattern or meshwork of fibrils embedded in the plane of the membrane. Figure 1b shows the intestinal epithelium as seen from the lumen. The apical domain appears as an archipelago in the upper part of the photo because the microvilli were cut in several transverse angles during the preparation of the specimen, giving the appearance of a cleared woodland. Three neighboring cells contact each other at the TJ, which looks like a piece of needlework. And in Fig. 1c, the TJ appears as if it was made of different strands that came together to form a continuous belt, which forms a seal all around the outermost edge of the intercellular space [18–21].

When studies of membrane permeability to water and solutes were extended from single cells to the epithelia, it seemed natural to assume that in these structures, permeation occurs across cell membranes and not through the intercellular space [12, 22–28].

The suggestion that the occluding junction essentially constitutes a tight seal was supported by a demonstration that the diffusion of macromolecules detectable by transmission electron microscopy, such as hemoglobin and ruthenium red, stops exactly at the locations of these junctions (Fig. 1a) [23, 26, 29].

Principal Functions of Tight Junction

The TJ have two principal functions in the TrEp; they act as gate and fence. The fence function of TJ refers to the maintenance of polarity as mentioned before, and TJ appears as a flat meshwork of anastomosing filaments in freeze-fracture surrounding the basolateral side at the outermost limit of the intercellular space and restricts the movement of the different components of the membrane from the apical to the basolateral domains maintaining the polarity of the plasma membrane, allowing the vectorial transit across TrEp [30].

The gate function refers to the capacity of TJ to regulate the passage of ions, molecules, and water through the paracellular pathway and can be detected by measuring the transepithelial electrical resistance (TER) of the tissue.

There are epithelia with high electrical resistance (i.e., the epithelium of the frog skin, above 1500 $\Omega.cm^2$) and epithelia such as those of the small intestine, the gallbladder, and the proximal segment of a nephron with comparatively low resistance (approximately 20–80 $\Omega.cm^2$). Obviously, the epithelia with high electrical resistance have a very small water flux through the paracellular route, while those with low electrical resistance have large fluxes through the paracellular route [31–34].

Decades ago, Phillippa Claude and Daniel Goodenough posed an obvious assumption, where each strand is an electrical resistor. If so, TER should increase linearly with the number of strands (Fig. 2a, segmented black line) [35]. However,

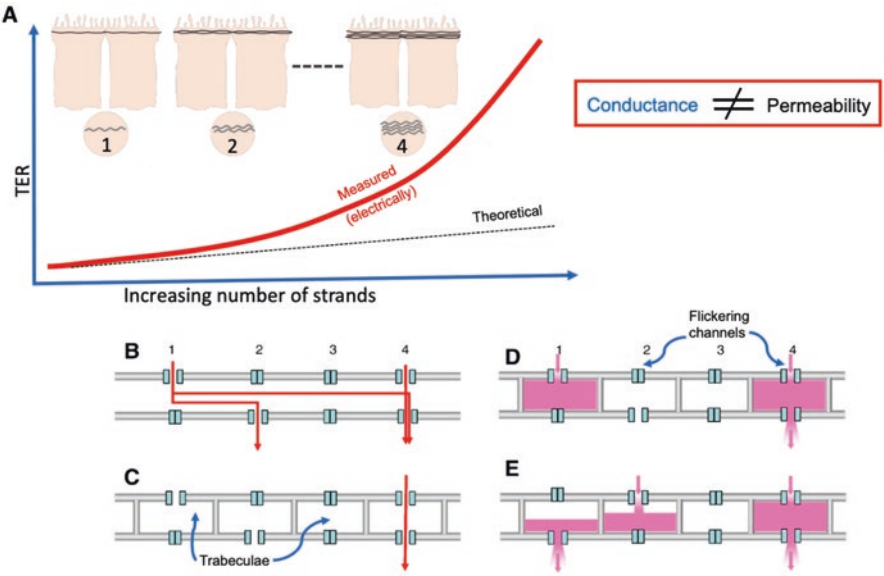

Fig. 2 The role of trabeculae and flickering channels. (**a**) Segments of transporting epithelia with TJ having one, two, and four strands. The dotted black line below shows the theoretical correlation predicted. Redline instead depicts the actual TER found experimentally. (**b**) represents a TJ with two strands and randomly flickering ion channels. (**c**) is a TJ with two strands, randomly flickering channels but with trabeculae. Red lines represent electric currents applied to measure TER. (**d**) and (**e**) are the same TJ but explored through the diffusion of a colored marker. Red rectangle: electrical conductance is not equal to permeability

when the electrical resistance of different epithelia is plotted against the number of strands in their TJ, it is observed that the increase in resistance with each additional strand is not linear [2].

Accordingly, we put forward a theoretical model explaining the relationship because a TJ is by no means a simple series of strands, but they have trabeculae and flickering channels that explain satisfactorily the values of TER found experimentally [5, 36]. Since each strand acts as a resistor, a TJ composed of two strands should in principle have a TER value twice that of a TJ with only one strand, while one with five strands would have a TER value five times higher and so on. Contrary to this theoretical expectation, the actual relationship between the TER and strand number found experimentally is not linear, but rather corresponds to the line shown in Fig. 2a. To account for this peculiarity, Phillippa Claude suggested that the strands possess channels that can be open or closed. TER should be the inverse of conductance (G). In turn, the conductance G and permeability should be directly related, i.e., conductance is the electrical manifestation of ion permeability. However, working with María Susana Balda and Karl Matter [36, 37], we discovered that in some situations in which we experimentally modified TJ, conductance (measured through the passage of current) and diffusion (measured through the flux of radioactive tracers) varied independently.

We produced a different model depicted in Fig. 2b. It represents a segment of a TJ that contains only two horizontal strands with ion channels. An electrical current (red lines) crossing the first strand can immediately cross the second through any of the ion channels that happen to be open at that precise moment (i.e., simultaneously). However, if the TJ has trabeculae (segments of strands going vertically from one strand to the next) (Fig. 2c), a current crossing the first strand through channel 1 can proceed only if the corresponding channel in the lower strand is also in the open configuration, i.e., through the fourth arrangement of channels in Fig. 2c. Figure 2d and e illustrates a situation in which permeability is studied by adding a tracer to the upper compartment. The tracer penetrates the TJ through any of the channels that happen to be open (channels 1 and 4 in the example). While the tracer that has penetrated through channel 1 is not able to keep flowing because the channel in the lower strand is closed, the tracer that penetrated through channel 4 can keep diffusing through the lower strand because this channel is also open. A moment later (Fig. 2e), the channel in the lower strand opens, and the tracer can pursue diffusion. Because the strands in the TJ contain trabeculae, the TJ is "compartmentalized"; thus, the overall increase in the TER is more pronounced with the addition of further strands (Fig. 2a, red curve).

In this description, we refer to a static arrangement. However, it must be taken into account that the structure and degree of tightness depend on the actin cytoskeleton and vary in response to intracellular signals mediated by a large number of protein species, including PKC, PLC, adenylate cyclase, calmodulin, nonreceptor tyrosine kinases, and G protein receptors. Thus, the junctional belt around the cells "is highly dynamic." The TJ may even reversibly disassemble to allow the passage of leukocytes. It may also change as a cell ages or be present only during specific stages of development.

TJ do not communicate with neighboring cells. Cell-cell communication is due to communicating gap junctions [15]. The confusion comes from the fact that gap junctions are often established between the strands of TJ [13, 15–17]; hence one erroneously attributes communication to junctional strands.

The Study of Tight Junction's Assemble

Since the multitude of molecules that constitute the TJ and other cell-cell and cell-substrate junctions, many membrane molecules with a polarized distribution and the highly complex mechanisms responsible for junctions and polarization were perfected along ages of evolution. Thus, it is evident that this large number and variety of molecular species that required millions of years of evolution might not have coincided within minutes in the same multicellular organism.

Of course, in those years, epitheliologists took samples of mature epithelia to study the generation of TJ, but the TrEp was useless because TJ and polarity are synthesized, assembled, and functionally expressed in mature epithelia. To avoid this difficulty, it occurred to us to devise an artificial epithelium by seeding MDCK

cells derived from a dog kidney and cultured them at confluency on an artificial and translucent nylon net, covered with 1 cm in diameter of collagen, and left them overnight. A priori, the probability that the monolayer of the cell would attach to the collagen/Nitex support and have only one layer because no cells would attach to the apical border of the already attached cells was so remote that we almost discarded our plans. However, it is very hard to throw away a cherished idea: we did try to make an artificial transporting epithelium. Our enthusiasm grew as we started to constate that it worked! Twenty hours later, the discovery was delightful due to cells had established TJ, and they had polarized.

However, we realized that those cells, obtained by harvesting with EGTA (a calcium chelator) and trypsin (an enzyme that hydrolyzes peptides into their amino acidic building blocks), were seriously damaged and spent most of the overnight hours repairing their structure and cell membrane. In this case, the cells were seed at confluency; after 30 min, the cells were transferred to a Ca^{2+} and cell-free medium. The next day, the confluent monolayer of cells did not have either TJ or polarity. Nevertheless, upon switching them to media-containing Ca^{2+}, the cells developed TJ and polarized in less than 2 h. We name this technique "calcium switch" and used to investigate how transporting epithelia develops TJ and cell polarity [38–40].

It must be taken into account that this synthesis of junctions and polarity takes place in cells that already have all the mechanisms and molecules involved or can synthesize them de novo if these were destroyed by trypsin. However, it is taken for granted that processes would mimic normal synthesis and assembly of TJ. The assumption is justified by studies on natural preparations, such as synthesis of TJ in the villi of the intestinal mucosa and other instances where cells migrate from the depth of a crypt to the apex of villum, or observed in steps from morula to embryo [41–44].

Tight Junctions Under Special Situations

TJ's proper regulation in transporting epithelia allows the permeation of enormous macrophages while entirely blocking the passage of small molecules of toxins produced by an infection with bacteria [45]. On the other hand, the epithelia that form a nephron are capable of producing TJ with a TER precisely needed to withstand the osmotic gradient between plasma and the filtrated liquid circulating in the lumen of a particular segment. It's not surprising that failures or the absence of TJ expose the organism to grave risks [46].

The relationship between failures of TJ and terrible pathological processes, most of them autoimmune, is when TJ allow the passage of molecules that should not reach the extravascular space, contact the immune system, and trigger the synthesis of antibodies. These risks are mainly prevented by the property of TJ to be established in the mixtures of epithelial cells derived from any organ and even derived from different animal species. This property explains why multiple transitions from

one type of the epithelium to a different one along the digestive tract are perfectly sealed.

TJ are not only found in epithelia but also between endothelial cells of capillary vessels. As in the case of epithelia, the comparison of the relatively low permeability of the plasma membrane with the relatively high one of the capillary wall suggested long ago that most of the transendothelial flux of water and small solutes occurs in the intercellular space. This pathway is also limited by the tight junction, except for the endothelium of microvessels in hemopoietic tissues. The tightness of endothelial TJ may be very low, as in the spleen and endocrine glands, or very high, as in the brain and the retina. The number and arrangement of the strands in endothelia also vary from arteries and veins to small vessels [27, 47].

Additionally, under certain circumstances, TJ can be traversed by whole germ cells. They may also be traversed by leukocytes migrating toward the side of infection. This process seems to be quite delicate, as the seal is reestablished after the leukocyte reaches the opposite side.

TJ may even be found between cells that are neither epithelial nor endothelial, such as those of the glia [48], muscle fibers [49–51], and fibroblast [52], and may even be present between two regions of the same cell.

Remarks

It has been more than a century after TJ have attracted the attention of light microscopists. Furthermore, the TJ is no longer considered a static, almost inert seal, whose only role is a mechanical barrier to the passage of substances. Today the "lip" of the TJ observed by transmission electron microscopy appears to be the tip of an iceberg, where the cytoskeleton, cell-cell contact molecules, scaffolding proteins, calmodulin, protein kinase C, phospholipase C, adenylate cyclase, and G-proteins coordinate to afford a weak sealing of just 10 Ω cm^2, as in the proximal tube of the kidney, or a strong blockade of several thousands of ohms as in the urinary bladder.

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Tricellular Tight Junctions

Tomohito Higashi and Mikio Furuse

Abstract Epithelial barrier must be sealed not only at tight junctions between two cells but also at tricellular contacts where three epithelial cells join. At these points, a specialized structure called tricellular tight junction (tTJ) exist. In this chapter, we will describe the structure and molecular compositions of the tTJ and present a current model for the molecular organization of the tTJ. We also describe how tTJs contribute to the maintenance of barrier function and how they are remodeled and rearranged. tTJs are also important for physiological events. Some of the components of the tTJ are important for hearing. Endothelial tTJs constitute blood-brain and blood-retinal barriers. Finally, we will introduce recent approaches to break through tTJs for efficient drug delivery.

Keywords Central sealing elements · Joint strands · Angulin · Tricellulin

Abbreviations

BBB	Blood-brain barrier
BRB	Blood-retinal barrier
BTJ	Bicellular tight junction
CDT	*Clostridium difficile* transferase
FFEM	Freeze-fracture replica electron microscopy
ILDR1	Immunoglobulin-like domain-containing receptor 1

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L. González-Mariscal (ed.), *Tight Junctions*,
https://doi.org/10.1007/978-3-030-97204-2_2

LSR	Lipolysis-stimulated lipoprotein receptor
SG2	Second layer of the stratum granulosum
TAMP	TJ-associated MAL and related proteins for vesicle trafficking and membrane link protein
TEM	Transmission electron microscopy
TJ	Tight junction
tTJ	Tricellular tight junction

Introduction

Tricellular tight junction (tTJ) is a specialized structure of tight junction (TJ) found at the tricellular contacts of epithelial and part of endothelial cells. Along with TJs, tTJs seal the space between adjacent cells and regulate the paracellular permeation of solutes and fluids, whereby contributing to the maintenance of the epithelial and endothelial barrier. Since the tricellular contacts are structurally unique, tTJs are assembled with a specialized set of proteins distinct from TJs between two cells (bicellular TJs, bTJs). As the epithelial and endothelial barrier is crucial for homeostasis of some tissues, impaired function of tTJs results in diseases including congenital deafness.

Structure of tTJs

The tTJs are located at the apical side of the tricellular (or multicellular) contacts, where three (or more) epithelial or endothelial cells adhere to each other (Fig. 1). In the transmission electron microscopy (TEM) image of the ultrathin section, the plasma membranes of the cells are closely attached at the center of the three cells [1, 2]. This attachment of the plasma membrane at tricellular contacts is also observed in the more basal level [2]. In freeze-fracture replica electron microscopy (FFEM), TJs appear as continuous anastomosed networks of fibril-like structure called TJ strands [3–6]. In contrast to the bTJs, which are composed of TJ strand networks running parallel to the apical surface, tTJs extend in the apico-basal direction. They are made up of the combination of "central sealing elements" [4] and "joint strands" (defined here). The central sealing elements are three TJ strands which are seamlessly connected to the apical-most TJ strand of the bTJ at the apical side. Each strand of the central sealing elements seals the cell boundary of the vertex region and extends to the basal side. The joint strands are "short TJ strands associated with the central sealing elements" [2] and make a perpendicular connection with the central sealing element like branches of a ladder. Multiple joint strands turning to the apical direction are consolidated and connected to the strand networks of bTJs at the apical side of the TJs.

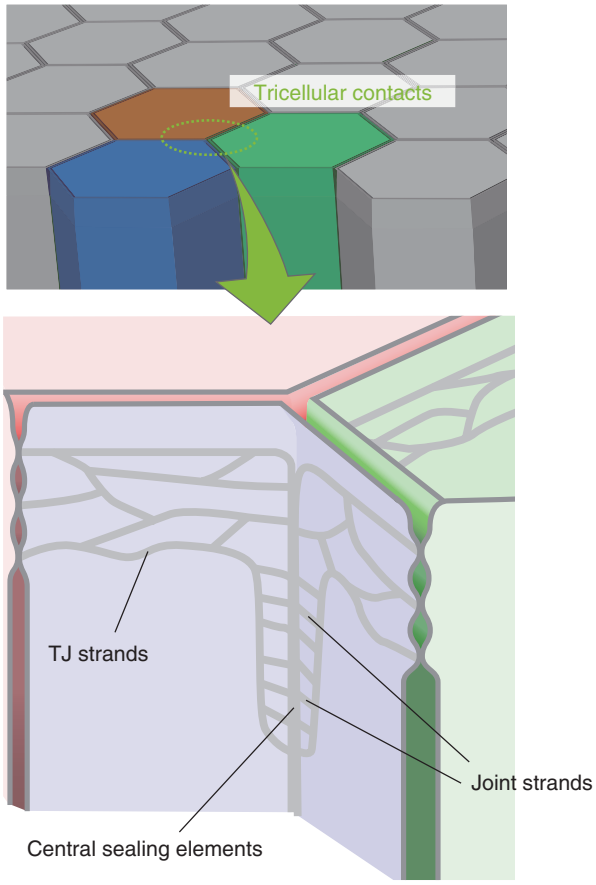

Fig. 1 Schematic illustration of the tricellular tight junctions (tTJs). Most apical TJ strands join at the tricellular contacts. They turn to the basal direction and make the central sealing elements. Other TJ strands of the bTJ make perpendicular connections with the central sealing elements. Some of them occur at the basal side below the TJ level. Here, we call these short TJ strands associated with the central sealing elements "joint strands"

Molecular Constituents of tTJs

Four transmembrane proteins are known to be specifically localized at tTJs, angulin-1 (also known as lipolysis-stimulated lipoprotein receptor (LSR)) [7], angulin-2 (also known as immunoglobulin-like domain-containing receptor 1 (ILDR1)) [8], angulin-3 (also known as ILDR2) [8], and tricellulin (also known as marveld2) [9] (Fig. 2).

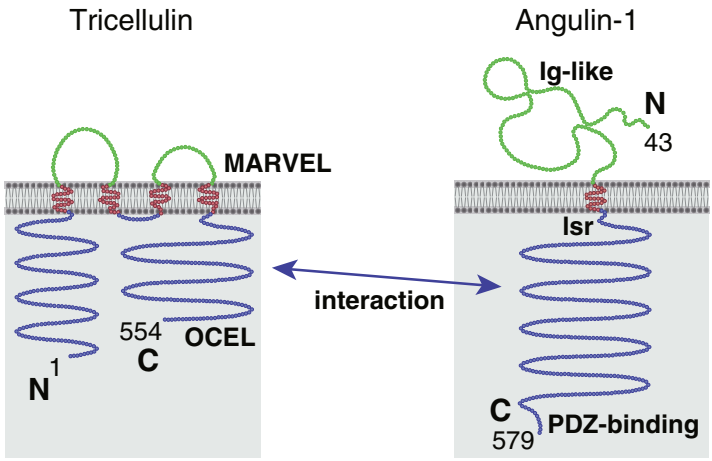

Fig. 2 Molecular components of tTJs. Tricellulin has a four-transmembrane MARVEL domain and a carboxy-terminal OCEL domain. Angulins have an extracellular Ig-like domain and a long cytoplasmic tail with a PDZ-binding motif at the carboxy terminus. The juxtamembrane region of the cytoplasmic tail is called LSR domain, which contains multiple palmitoylation sites. The carboxy-terminal cytoplasmic tail of tricellulin and amino-terminal half of the cytoplasmic tail of angulins are required for the recruitment of tricellulin to tTJ by angulins

Angulins

Angulin-1, angulin-2, and angulin-3 belong to the angulin family and possess an extracellular immunoglobulin-like domain, a single transmembrane domain, and a long cytoplasmic tail [7, 8]. Angulins have several splicing isoforms, and some isoforms of angulin-1 and angulin-3 have an extra exon at the extracellular region near the membrane, which hinders the tricellular localization of angulins [2]. The juxtamembrane domain of the cytoplasmic tail of angulins undergoes palmitoylation by DHHC family proteins, and the palmitoylation also modulates the localization of angulins at tTJs [10]. The carboxy-terminus of angulin-1 ends with a hydrophobic three-amino-acid PDZ-binding motif, L-V-V, which interacts with the second PDZ domain of ZO-1, a TJ plaque protein [2]. Angulins are required for the recruitment of tricellulin at tTJs [2, 7, 8]. On the other hand, tricellulin is not required for the localization of angulins at tricellular contacts [2, 7]. The cytoplasmic tail of angulins is responsible for tricellulin recruitment [7]. Three angulins are differentially expressed in epithelial tissues, and at least one of the angulins is expressed in most epithelial tissues [7, 8]. Angulin-1 is broadly expressed in the epithelial tissues including the small intestine, proximal tubules in the kidney, skin, bottom region of the intestinal glands in the colon, and liver [7, 8]. Angulin-2 is expressed in the tissues including the distal tubules and collecting ducts of the kidney, bladder, upper region of the intestinal glands in the colon, and pancreas [8]. On the other hand, expression of angulin-3 is found in limited tissues such as retinal pigment epithelium, perineurium, the podocytes of the glomeruli in the kidney, and mesothelial cells [8].

Tricellulin

Tricellulin has four transmembrane domains [9] and belongs to the TJ-associated MAL and related proteins for vesicle trafficking and membrane link (MARVEL) protein (TAMP) family [11, 12]. Transmembrane domains share homology with the other TAMP family proteins, occludin and Marveld3. Carboxy-terminal cytoplasmic tail of tricellulin contains an OCEL domain which is highly homologous to that of occludin [9]. Both tricellulin and occludin have been reported to interact with ZO-1 through the cytoplasmic tail [13, 14], although the binding surfaces of occludin and tricellulin are different [15, 16]. Amino-terminal cytoplasmic domain of tricellulin interacts with Cdc42 GEF Tuba and activates Cdc42 [17]. Tricellulin is expressed in almost all of the epithelial cells in the body [9].

Other bTJ Components

The central sealing elements appear as the extension of most apical TJ strands of bTJs, which are composed of claudins, occludin, and JAM-A [18–21], and have an appearance similar to TJ strands in FFEM [3–6]. Indeed, claudins and occludin are also positive at these regions, which are observed as a rod-like structure extended from the most apical elements of TJs at the tricellular vertices of epithelial cells in immunofluorescent staining [2]. Since it is difficult to tease apart whether these proteins are localized at the central sealing elements and/or the joint strands by fluorescence microscopy, future studies using electron or expansion microscopy would reveal whether they are incorporated in the central sealing elements together with angulins.

Molecular Organization of tTJs

The roles of angulins and tricellulin on the establishment of tTJ structure are beginning to be elucidated by gene knockout approaches. MDCK II cells express angulin-1 and tricellulin, and the expression levels of angulin-2 and angulin-3 are negligible [2, 22, 23]. In angulin-1-knockout MDCK II cells, the attachment of plasma membranes at tricellular region was not observed in both apical TJ and basolateral levels, and there were no central sealing elements and associated joint strands in FFEM [2]. Outer hair cells of angulin-2-knockout mice still retain the central sealing elements and joint strands in FFEM [24], which could be explained by the compensatory localization of angulin-1 and tricellulin recruitment at the tricellular contacts in angulin-2-knockout mice [25]. The vestibular hair cells in angulin-2-knockout mice have less prominent central sealing elements with a decreased number of particles and scarce joint strands. Since vestibular cells express angulin-2 [24, 25] and a

much lesser extent of angulin-1 (unpublished data), a trace amount of angulin-1 might not be enough to form normal tTJ structures in these cells of angulin-2-knockout mice.

On the contrary to angulin-1-knockout MDCK II cells, plasma membrane attachment and central tubule elements were retained in tricellulin-knockout MDCK II cells. In these cells, joint strands were missing, and the bicellular TJ strands appeared to fail to make perpendicular connections with the central sealing elements. Instead, they turn around near the central sealing elements and join with each other. Similar structures were observed in the utricular hair cells in the inner ear of the truncated tricellulin-knockin mice [26], which is described below.

It has been shown that tricellulin modulates the morphology of TJ strands. Co-expression of tricellulin and claudin-1 in L fibroblasts [27] or HEK293 cells [28] resulted in the formation of TJ strands enriched with rectangular meshes. Double knockout of occludin and tricellulin resulted in reduction of bifurcations in TJ strand network of bTJs in MDCK II cells [29], suggesting that tricellulin and occludin stabilize the branching points of TJ strands. This view is supported by the observation using super-resolution microscopy showing that Halo-tagged occludin concentrates at the branching points of claudin-2-based TJ strands in Rat-1 fibroblasts [30]. Since the joint strands are missing in the tricellulin-knockout MDCK II cells and animals, tricellulin is likely to be specifically required for connection of joint strands with the central sealing elements, and occludin (and probably another TAMP member Marveld3) may not substitute this function of tricellulin.

Involvement of other bTJ components in tTJ architecture has been also examined. The plasma membrane attachment at tricellular contacts was maintained in claudin-null MDCK II cells and claudin/JAM-A-knockout MDCK II cells [2], indicating that neither claudin nor JAM-A is required for the formation of this structure.

Based on the studies described above, current model of tTJ molecular organization (Fig. 3) is:

1. Angulins are likely to be responsible for mediating plasma membrane contact formation at tricellular vertices and the formation of the central sealing elements.
2. Angulin recruits ZO-1 to the tricellular region independent of tricellulin, and the recruitment of ZO-1 is required for the accumulation of claudins at basolateral tricellular extension.
3. Tricellulin is required for the formation of joint strands and perpendicular connections of bTJ strands and central sealing elements.

It is not understood how angulins are recruited to the epithelial vertices where new tTJs will be established and maintained at these sites. Specific interaction of the extracellular domain of angulins would be required for the recognition of tricellular contacts [10, 31]. The juxtamembrane region of the cytoplasmic tail of angulin-1 is highly palmitoylated, and the tricellular localization of angulin-1 was impaired when the cysteine residues required for palmitoylation were mutated or the expression of four DHHC family palmitoyl transferases was suppressed in EpH4 cells [10], suggesting that lipid modification is involved in the localization or retention of angulins at tTJs. This view was supported by the observation that depletion of

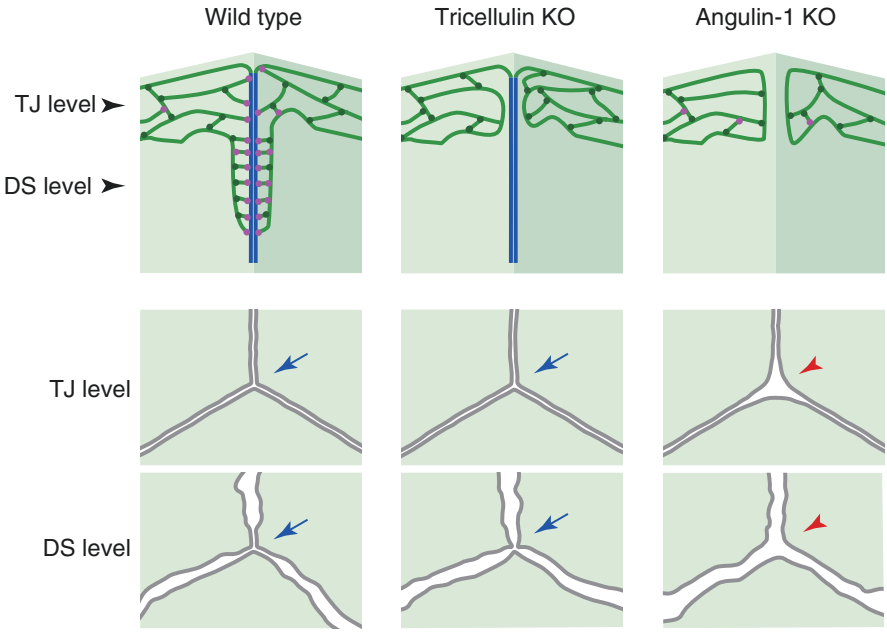

Fig. 3 Current model of the molecular composition of tTJs. In wild-type cells, angulins (blue lines) form the central sealing element. Tricellulin (magenta dots) is associated with the central sealing elements and connects the joint strands to them. Occludin (dark green dots) and tricellulin stabilize the branching points of TJ strands (green lines). At both TJ and desmosomal (DS) levels, plasma membranes of the cells are attached at the tricellular contacts (blue arrows). In tricellulin-KO cells, angulin-based central sealing elements are unaffected, whereas the joint strands are lost. The plasma membrane contacts are in most cases retained. In angulin-KO cells, the central sealing elements (and associated joint strands) are missing, and there are gaps between the plasma membrane of tricellular region at both TJ and DS levels (red arrowheads)

cholesterol diminished the specific localization of angulin-1 at tricellular contacts [10].

Barrier Function of tTJs

It is believed that tTJs constitute paracellular barriers together with TJs.

Angulin-1-knockdown EpH4 cells [7] and angulin-1-knockout MDCK II cells [2] have reduced barriers for ions and macromolecules. However, these angulin-1-deficient cells still maintain barrier compared with claudin-null cells [32] and ZO-1/ZO-2-deficient cells [32, 33], indicating that contribution of tTJ to the epithelial barrier is limited especially in the cells with weak bTJ-based barrier function [34]. Angulin-1-knockout mice exhibited increased permeability for small compounds in the vascular endothelium [35]. Based on these results, the tTJ structure including

central sealing elements and joint strands is essential for the establishment of a strong barrier in epithelial cells. When angulin-2 was exogenously expressed in angulin-1-knockdown EpH4 cells, barrier function was restored for ions and macromolecules [8]. However, angulin-3 has a limited barrier-supporting function [8], suggesting that each angulin has distinct barrier properties.

Effects of tricellulin knockdown on barrier function have been evaluated in cultured epithelial cells, but the results varied depending on experimental setups and cell types. When tricellulin was knocked down, EpH4 cells, MDCK C7 cells, and HT29/B6 cells exhibited increased permeabilities for ions and macromolecules [9, 34, 36], and Caco-2 cells showed modestly delayed development of the barrier for ions [12]. Knockdown of tricellulin in MDCK II cells had no effects [37]. Recent studies using gene knockout approaches showed that loss of tricellulin has no effect, or a modest effect if any, on the permeabilities for ions and macromolecules [2, 29]. Considering that these cells lack the joint strands [2], it is likely that central sealing elements are the main architecture to prevent leakage at tTJs and the contribution of joint strands is modest in the cell culture condition. The joint strands might be required only in the epithelium that requires an extremely tight barrier including a sensory epithelium in the organ of Corti in the inner ear, which will be discussed later.

It has been believed that the central sealing elements form a pore of about 10 nm in diameter [38], which is a good candidate for transport route for water and macromolecules [34, 36, 39–41]. Loss of angulins resulted in an increase in the size of the pores of tTJs [2] and increased paracellular permeabilities for macromolecules [2, 8] and water [42]. One report has shown that angulin-2-knockout mice fail to concentrate urine and exhibit polyurea and polydipsia as well as growth retardation, suggesting that loss of angulin-2 also affects the permeability for water at tTJ [22]. However, these phenotypes were not reproduced by the other group using angulin-2-knockout mice [43].

Remodeling and Rearrangement of tTJ

Epithelial cells in the living tissues undergo dynamic cell rearrangement, which is especially evident during development. Even in adult tissues, cells are constantly renewed. New cells are added by cell division and cell intercalation, and old cells and abnormal cells are removed by cell death and cell extrusion. Migratory cells including germ cells are known to translocate to another compartment by passing through epithelial cell sheets [44]. When these events take place, cell-cell junctions including tTJs must be rearranged, and at the same time, they are required to maintain barrier function. It has been recently beginning to be understood how cells coordinately reorganize tTJs and achieve these mutually opposite tasks.

In most simple epithelia and some stratified epithelia such as the surface epithelium of *Xenopus laevis* gastrula-stage embryos, cell division occurs in the plane of the cell sheets. As a result of cell division, two new cell vertices are created, and nascent tTJs need to be newly established. In *Xenopus* gastrula-stage embryos, new

tTJs emerge one by one at each side of the midbody during 15 min after completion of contractile ring ingression [45]. In each tTJ, angulin-1 is localized first, followed by tricellulin [45], which is consistent with the hypothesis that angulins recruit tricellulin to the tricellular contact sites.

Cell renewal occurs differently in some types of stratified epithelia including the skin. In the skin epidermis of mammalians, stem cells are located at the basal layer (stratum basale) and produce new keratinocytes. Upon differentiation, the keratinocytes move upward from the basal layer to the stratum spinosum, stratum granulosum, and stratum corneum in this order and finally shed off from the top of the stratum corneum. TJs are only found at the apical part of the cells in the second layer of the stratum granulosum (SG2) [46] and limit the paracellular leakage of water and electrolytes. This layer also contains tTJs, which are composed of angulin-1 and tricellulin [47, 48]. When a cell moves to the upper layer from the SG2, the surrounding cells make a new TJ with a cell below the cell leaving the SG2 layer. Since the new TJ is formed among three cells (surrounding cell, basal part of the leaving cell, and apical part of the lower layer cell), it is considered an atypical type of tTJ. Consistently, angulin-1 and tricellulin are also localized at these transient linear tTJs [48]. As the lower layer cell moves up to the SG2, angulin-1 and tricellulin become focused at vertices, suggesting that these tTJ components maintain the continuity of TJ barrier during turnover of keratinocytes.

The skin epidermis is the outermost barrier against foreign materials and pathogens, and it contains Langerhans cells, a specialized form of antigen-presenting dendritic cells, beneath the basal layer. Langerhans cells extend their dendrites between keratinocytes. To access the antigens outside of the TJ barrier, some tips of the dendrites are docked to and penetrate the TJs, reaching the stratum corneum [47]. The integration of the Langerhans cell processes with the TJs is induced by proinflammatory cytokines, such as TNF-α and IL-1β. New TJs and tTJs are formed between the keratinocyte and dendrites of the Langerhans cells at SG2, preventing the breach of the TJ barrier at the penetration site [47].

Hearing Loss and tTJ

Some components of TJs and tTJs are important for hearing [49–52]. CLDN14 is a causative gene of familial non-syndromic hearing loss DFNB29 [53], and claudin-14-deficient mice recapitulate the phenotype [54]. One-amino-acid substitution in the extracellular loop of claudin-9 also causes deafness in mice [55], and a truncation mutation of CLDN9 was identified in human patients with hearing loss [56]. Similarly, truncation mutations of human tricellulin (TRIC) were identified in the familial non-syndromic hearing loss DFNB49 [14]. The knockin mice of tricellulin, which bear a mutant tricellulin mimicking a human mutation [26], and tricellulin-knockout mice exhibit hearing loss [57]. The knockout mice of the bTJ protein occludin also exhibit deafness [58]. In these mice, tricellulin was not confined to the tTJs and mislocalized to the bicellular junctions, which is likely to be

the cause of deafness. Furthermore, angulin-2 is the causative gene of familial non-syndromic hearing loss, DFNB42 [59], and angulin-2-deficient mice also exhibited deafness [24, 25, 60], although angulin-1 compensatively recruits tricellulin to the tTJs [25] and the ultrastructure of tTJ was not disrupted in hair cells of these mice [24].

Hair cells of the organ of Corti in the inner ear converts sound to electrophysiological signal. The hair cells face their apical surface with stereocilia to the scala media (cochlear duct) of the cochlea, which is filled with endolymph. Endolymph has a unique ion composition with high K^+ and low Na^+ concentrations. Scala media has a high electric potential, called endocochlear potential, which is important for hearing. When the sound is transmitted to the inner ear, the vibration of the hair cells results in the opening of K^+ channels on the stereocilia, which causes inward current and action potentials in the hair cells. The basolateral side of the hair cell bathes in perilymph, which has a normal composition of ions, high Na^+ and low K^+. The TJ and tTJ of hair cells and supporting cells are responsible for the separation of the two extracellular fluids with distinct ion compositions, endolymph and perilymph [52]. Failure in the barrier function of these cells might cause leakage of ions and other substances from one compartment to another and induce apoptotic cell death of hair cells. Indeed, the hair cells in the mutant mice described above exhibit apoptotic phenotype and degenerative death [24–26, 54, 55, 57, 60]. The most probable candidate of leaking substance through the TJ or tTJ is K^+ ions. If the K^+ ion concentration is elevated in the basolateral extracellular space, the Na-K ATPase activity is suppressed, and the generation of membrane potential in hair cells is impaired. In line with this, the K^+ ion concentration of perilymph was elevated in claudin-9 mutant mice [26]. There are other hypotheses on the cause of hair cell death, including ATP or Na^+ leakage from the basolateral compartment to the apical one [26].

Endothelial tTJ

The luminal surfaces of blood and lymph vessels are covered by endothelial cells, a specialized type of epithelial cells of the mesodermal origin. Blood vessel endothelial cells in most tissues do not have positive signals for known tricellular components, tricellulin and angulins [61], and intensive analysis by FFEM showed that TJs are not continuous at the tricellular region of endothelial cells in the pulmonary capillary [62], suggesting that there is no tTJ structure in the blood vessel endothelium. This is reasonable because the peripheral blood vessels are leaky and do not require a tTJ-based strong barrier function. However, endothelial cells in the brain and retina have strong barrier properties [63, 64], forming blood-brain barrier (BBB) and blood-retinal barrier (BRB), respectively. In accordance with this, brain and retinal endothelial cells express tricellulin and angulin-1 [61]. The BBB develops between E14.5 and E16.5 in normal mice, which parallels the timing course of angulin-1 expression and localization at the tricellular contacts of endothelial cells [35]. The BBB of angulin-1-deficient mice remains highly permeable to the

low-molecular-weight tracer (Sulfo-NHS-biotin) at E14.5 [35], which may be the cause of the embryonic lethality of angulin-1-kcockout mice before E15.5 [65].

Immune cells are known to transmigrate through the endothelial layer into the perivascular space, a process called diapedesis. It has been known that neutrophils preferentially transmigrate through tricellular junctions of cultured endothelial cells in vitro [66] and peripheral microvessels in vivo [67]. ICAM-1 is preferentially localized at the tricellular corners of endothelial cells and functions as a ligand for LFA-1 integrins on leukocytes. T cells also preferentially utilize tricellular junctions in diapedesis across the BBB [68] in cultured brain microvascular endothelial cells. Although these cells express angulin-1 and tricellulin, it was unclear whether they act as a hallmark for T-cell migration sites because specific localization of these proteins at tTJ was not observed [68].

tTJ-Binding Bacterial Toxins and Application to the Drug Delivery

Transmembrane proteins of TJs are often utilized by viruses and bacterial toxins as receptors [69–71]. Similarly, components of tTJs are also exploited by these pathogens.

Shigella spreads in the epithelial cell sheet using tricellular junctions [72]. In the infected cells, bacteria-containing pseudopodia are formed at tricellular contacts and engulfed by the neighboring cells in a tricellulin-dependent manner.

Group A *Streptococcus* binds to tricellulin using host-derived plasminogen and invade into the submucosal tissues [73], and enteropathogenic *Escherichia coli* is also reported to target tricellulin to disrupt epithelial barrier [74].

Clostridium difficile produces a binary actin-ADP-ribosylating toxin, *Clostridium difficile* transferase (CDT). The binding component of the CDT (CDTb) directly binds to angulin-1 [75], which results in the endocytosis of the toxin. The CDTb creates pores on the endosomal membrane, allow the enzymatic component of the CDT to translocate into the cytosol and induce cytoskeletal reorganization. Related binary toxins, *Clostridium perfringens* iota-toxin (Ib) [75] and *Clostridium spiroforme* binary actin-ADP-ribosylating toxin CST [76], also use angulin-1 as a receptor to enter the epithelial cells.

Since Ib induces endocytosis of angulin-1, a novel mucosal absorption enhancer was developed using the carboxy-terminal fragment of Ib, which was designated angubindin-1 [77]. Angubindin-1 treatment of cultured epithelial cells removed angulin-1 and tricellulin from the tTJ and transiently increased the paracellular permeability for ions and macromolecular solutes [77]. Furthermore, intravenous administration of angubindin-1 induced a transient increase in the permeability of BBB, which enabled the delivery of antisense oligonucleotides to the mouse brain [78].

Closing Remarks

Molecular components of tTJs have been identified in these 17 years, and since then, the molecular mechanisms regulating tTJs have been clarified. In this section, we discussed these mechanisms and the physiological and pathological relevance of tTJs to diseases. Recently, tricellular junctions, including tricellular adherens junctions and tricellular septate junctions, have been gaining increasing attention [79, 80] because they regulate various cellular events including paracellular permeability control [81], junctional tension regulation [82–87], cell division orientation [88, 89], and cell fate determination [90]. How tTJs are related to these events would be an interesting question to be uncovered in future studies.

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Epithelial Mechanosensing at Cell-Cell Contacts and Tight Junctions

John W. Brooks, Robert G. Parton, Alpha S. Yap, and Kinga Duszyc

Abstract This chapter focuses on the mechanosensitive properties of epithelial tissues. Epithelia experience a range of mechanical forces arising both intrinsically from their constituent cells and extrinsically from forces such as touch or alveolar inflation. We discuss how cell-cell junctions, such as adherens junctions and tight junctions, play key roles in the mechanobiology of epithelial tissues. At these sites, forces are generated through contraction of the actomyosin cytoskeleton and transmitted between neighbouring cells and across tissues by adhesion systems within the junctions. We also consider other potential cellular mechanisms that can allow epithelia to respond to mechanical stresses: mechanosensitive ion channels which are implicated in homeostatic control of cell density via modulation of cell proliferation and live-cell extrusion and caveolae, membrane invaginations that can buffer epithelia in response to change in membrane tension.

Keywords Mechanosensing · Mechanotransduction · Tension · Epithelia · Cell-cell junctions · Piezo · Caveolae

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L. González-Mariscal (ed.), *Tight Junctions*,
https://doi.org/10.1007/978-3-030-97204-2_3

Introduction

Mechanical forces are fundamental determinants of tissue integrity, morphogenesis and homeostasis in epithelia and endothelia. Here, we focus on epithelia because, as principal tissue barriers of the body, epithelia experience a diverse range of mechanical stresses that derive both from their constituent cells and from extrinsic forces. Epithelia are capable of detecting such changes in force and can respond in ways that can preserve tissue integrity and homeostasis. Cell-cell junctions play central roles in the mechanobiology of epithelia. They can be sites where active cellular forces are generated by the contractile actomyosin cytoskeleton. The cell-cell adhesion molecules in junctions can serve to transmit forces from cells to cells and/or integrate the transmission of force at the tissue level. As well, epithelial cell-cell junctions possess mechanosensing and signal transduction mechanisms that allow them to detect changes in mechanical force and elicit homeostatic responses. In this chapter, we discuss both well-defined and emerging topics relating to the mechanisms that allow epithelia to use mechanical force as a currency of biological information.

Tissue Forces in Epithelia

Tissues are subjected to constant mechanical forces which have diverse impacts on their integrity and function. These forces can be either compressive or tensile, which we define as forces that may result in tissue compaction or fracture, respectively [1].

Compressive Forces. These are most obviously a consequence of extrinsic physical stimuli, such as the pressure applied to the skin from touch. Although compressive forces are less studied compared to their tensile counterpart, they play important roles in directing, regulating and maintaining various processes during development and adult homeostasis. For example, compressive forces generated by morphogenetic events at the onset of gastrulation in the *Drosophila melanogaster* embryo influence the expression of Twist, a key regulator of epithelial-to-mesenchymal transitions (EMT) during gastrulation. Here, 10% of uniaxial lateral compression was reported to induce ectopic expression of Twist around the entire dorsal-ventral axis of *Drosophila melanogaster*, resulting in ventralisation of the embryo [2]. Furthermore, mechanical compression forces exerted in early *Xenopus laevis* embryos provoke phosphorylation of components of focal adhesions and tight junctions, resulting in tissue strengthening [3]. Additionally, compressive forces increase the abundance of mesenchymal-to-epithelial transition (MET) proteins, while suppressing proteins involved in EMT, resulting in MET-like phenotype [3].

Apart from externally applied stimuli, compressive forces may arise from overcrowding of epithelial tissues due to cell proliferation [4, 5]. This tissue-intrinsic compression provides information to maintain cell density through several mechanisms. For example, overcrowding can halt cell cycle progression at the G1-S

checkpoint [6], though this does not, by itself, correct the elevated cell density. Instead, such compressive forces initiate the process of live-cell extrusion, where surplus cells are physically expelled from the epithelium to moderate cell density [4].

Compressive forces exerted on epithelia can also be a consequence of tensile forces, often occurring tangential to the plane of tension [7]. To simulate the morphological heterogeneity of epithelial tissue in vivo, Gjorevski and Nelson [7] engineered epithelial tissues with various geometric features. They found that with curved, duct-like geometries, regions of compression occurred both tangentially to tensile forces and within the concave regions where the opposing convex region was under tension. Interestingly, these negative forces appeared to be passive and dependent on active generation of cellular tension, as regions of compression did not show elevated cell proliferation, and blocking myosin activity curtailed both tensile and compressive forces within the tissue.

Tensile Forces. These forces have been far more extensively studied compared to compressive forces. Though tensile forces are often considered as *extrinsic* to tissues, such as pulmonary inflation or physiologic gastric distension, *intrinsic* tensile forces are also significant and contribute to homeostasis. These intrinsic forces arise from the contraction of the actomyosin cytoskeleton. In the case of epithelial and endothelial cells, force generation by actomyosin contraction requires a physical connection between the contractile machinery and the cell-cell junctions. This enables transmission of tension between adjacent cells and thus facilitates propagation of the mechanical signal across epithelial tissues. Components of both adherens and tight junction complexes have been shown to experience and respond to changes in cortex-derived tension. Key examples of such proteins include adherens junction (AJ)-based α-catenin and tight junction (TJ)-based ZO-1, both of which were shown to sense increased tension and undergo a conformational change in response to cortical tension [8–14]. Detailed mechanisms of force-sensing across junctions will be discussed later.

A number of studies have revealed the importance of intrinsic forces in the maintenance of epithelial homeostasis and tissue development. For example, Acharya et al. [15] demonstrated that the formin mammalian diaphanous 1 (mDia1) is crucial for contractility at the zonula adherens and stability of tight junctions in epithelial monolayers. Interestingly, as monolayers depleted of mDia1 exhibited increased transepithelial permeability, mDia1-dependent intrinsic tension appeared to be essential for the maintenance of epithelial barrier function. Further, mechanical tension may balance cell proliferation patterns, leading to uniform levels of local cell division, despite inhomogeneous distribution of growth factors across the developing tissues. Using a developing *Drosophila melanogaster* wing as a model for a homogenously growing tissue, Pan et al. [16] demonstrated that local hyperproliferation is accompanied by reduction in junctional tension. This results in increased activity of the Hippo signalling pathway and downregulation of the growth-promoting Yorkie, thus leading to a decrease in local growth rates. These observations, supported by computational simulations, suggested existence of a mechanical feedback mechanism, where the gradual reduction in intrinsic tension increases

Hippo pathway activity to mediate evenly distributed tissue growth [16]. Seminal work by Dupont et al. [17] show that the localisation of the Yes-associated protein (YAP) can be influenced by substrate stiffness. Further, as Irvine and Harvey [18] have reviewed, mechanical signals emanating from cell-cell interactions can modulate the Hippo pathway to control organ growth.

Mechanical Forces as Cellular Information

Importantly, mechanical forces can be transmitted and detected by cells as a mode of communication. This can potentially be elicited in response to both compressive and tensile forces, although here we will focus on mechanotransduction of tensile forces in epithelia. The cellular mechanisms that support communication by mechanical forces involve mechanisms that transmit forces (within and between cells) and mechanisms that sense changes in mechanical forces. Broadly, examples of mechanosensors include proteins which undergo conformational changes in response to tension, ion channels which can be forced open and plasma membrane invaginations which unfold with increased tension. These mechanosensitive responses may elicit recruitment of effector proteins, induction of ion influx/efflux, or induction of gene transcription/silencing due to translocation of proteins to the nucleus. In this chapter, we will discuss a range of various mechanisms that enable cells to respond to mechanical cues, focusing on both junction-based and membrane-based mechanosensitivity, specifically with respect to epithelial tissues.

Mechanosensing at Cell-Cell Junctions

Epithelial cells are joined together by specialised cell-cell junctions: tight junctions (TJs) found at the most apical region of the cell-cell interface; E-cadherin-based AJs, located under TJs; as well as more basally located desmosomes and gap junctions. Cell-cell junctions are fundamental for the development and maintenance of tissues and have a variety of functions. AJs initiate cell-cell contacts and are primary sites of mechanical force sensing; TJs regulate transepithelial permeability; desmosomes provide epithelial tissues with mechanical resistance; and gap junctions permit chemical communication between adjacent cells. Here, we will discuss selected developments in the knowledge of mechanosensing at cell-cell junctions.

AJ-Based Mechanosensing

AJs are characterised by their enrichment of classical cadherins, which can reach concentrations of ~2000 molecules/μm^2 at these cell contacts [19]. Cadherins traverse the plasma membranes (PM) of neighbouring cells, ligating in a homophilic

fashion to bridge across the intercellular space (Fig. 1). Like other classical cadherins, epithelial (E)-cadherin 1 (cadherin 1) comprises three distinct regions: an N-terminal extracellular region, a single transmembrane region and a C-terminal intracellular region. The extracellular region of E-cadherin facilitates the adhesion of adjacent cells, a function reliant on its five extracellular cadherin domains (EC1–5) interspaced by Ca^{2+}-binding motifs. Here, the β-barrel structure of distal EC1 domains from adjacent cells lies antiparallel, exchanging β-strands in *trans* for the primary adhesion site in a Ca^{2+}-dependent manner. Interestingly, the homophilic nature of cadherin ectodomain subtypes can allow selective adhesion between cell types providing a powerful, though not ubiquitous, means of directing morphogenesis [20, 21]. However, such cell sorting can also occur in cell populations expressing quantitative differences of the same cadherin [22]. It has been suggested that the loss of E-cadherin increases the metastatic potential of tumourigenic cells [23, 24] as loss of E-cadherin correlates with increased migration in vitro [25]. However, it has recently become evident that the role of E-cadherin in cancer is much more complicated than previously appreciated. For example, most patients with invasive ductal carcinomas express E-cadherin in both the primary tumour and metastases.

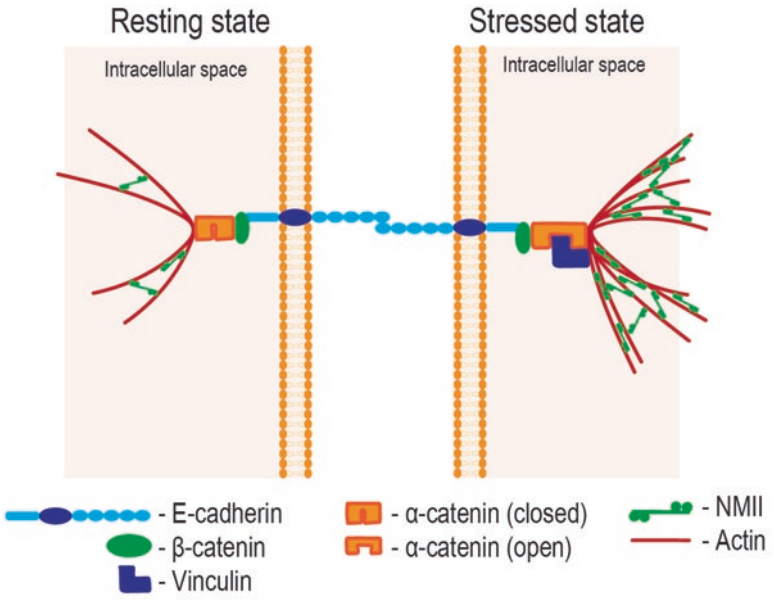

Fig. 1 Adherens junctions (AJs). Schematic representations of the main components of the adherens junction. Cell adhesion is mediated by the EC1 domains of the E-cadherin extracellular regions. Under normal physiological conditions (resting state), the mechanosensitive protein, α-catenin, is in a closed conformation, with a weak affinity for F-actin. Under periods of high tension (>5 pN, stressed state), α-catenin unfolds to expose an F-actin-binding domain, greatly increasing its affinity for F-actin, strengthening the cell's cortex. Additionally, the unfolding of α-catenin exposes a vinculin-binding domain, providing further reinforcement to the cortex by recruiting and binding vinculin

Further, Padamanaban et al. [26] show that the loss of E-cadherin, while increasing invasiveness, decreases metastatic potential, as well as cell proliferation and survival of circulating tumour cells.

AJ Mechanosensing Through α-Catenin and Vinculin. AJs are subjected to constant mechanical stress from both extrinsic and intrinsic sources. Accordingly, AJs have evolved to cope with, and become sensitive to, these stresses. As tensile forces are generated within cells and tissues, E-cadherin molecules work as mechanotransducers to bear and transmit forces to the underlying molecular complex (Fig. 1). In order to propagate tissue tension, E-cadherin adhesions need to be physically coupled to the contractile actomyosin machinery of the cells. Indeed, the intracellular region of E-cadherin binds p120-catenin and β-catenin, with β-catenin associating with the actin-binding protein (ABP) α-catenin, thus bridging the adhesive and force-generating components of the AJs [27]. Importantly, the role of α-catenin is not limited to constituting a passive link between E-cadherin and actin. Instead, molecular properties of α-catenin are altered upon increased tension, making it an active, key AJ-based mechanosensor.

The actin-binding and actin-bundling protein α-catenin contains two distinct mechanosensitive domains for interacting with vinculin and F-actin (Fig. 1). Application of mechanical tension to α-catenin can alter its conformation to expose a cryptic vinculin-binding domain (VBD) and promote vinculin recruitment and binding [8, 10, 11, 13, 14]. The association of vinculin with F-actin strengthens the interactions between cadherin-catenin complexes and the cytoskeleton. During homeostasis, intrinsic tissue tension due to actomyosin results in ~5 pN of force across each E-cadherin molecule [1]; approximately the same level of force is required to unfold α-catenin for recruitment and binding of vinculin [13]. Additionally, the actin-binding domain of α-catenin can also undergo a tension-sensitive conformational change. Here, mechanically induced unfolding of the α1 helix of α-catenin enhances the binding affinity between α-catenin and F-actin (Fig. 1). Under periods of high tension, the ratio of open to closed α-catenin molecules is increased, providing significant reinforcement to the AJ, largely due to this increased association with cortical F-actin. Of note, vinculin and F-actin are not the only binding partners of α-catenin. Other partners include α-actinin [28, 29], afadin [11], EPLIN [30] and ajuba [31, 32]; however their specific contributions to α-catenin-based mechanosensitivity has yet to be well-defined.

In addition to α-catenin, vinculin is itself an important mechanosensitive protein. Cytoplasmic, inactive vinculin exists in an autoinhibited conformation, with the tail domain interacting with the head domain. Abl-dependent phosphorylation at the Tyr822 residue and/or actomyosin contractility, combined with association with α-catenin, leads to unfolding of vinculin [33, 34]. In this active, unfolded state, vinculin strengthens the AJs by binding directly to both α-catenin and β-catenin via the vinculin-head domain (Vh) [35, 36]. Furthermore, vinculin directly associates with F-actin and the actin regulatory protein MENA, an interaction essential for vinculin-dependent F-actin polymerisation in epithelial tissue under mechanical stress [37]. Vinculin at AJs has been shown to potentiate the mechanosensing response of the E-cadherin-catenin complex. le Duc et al. [38] showed that in

vinculin KO cells, cell attachment to E-cadherin-coated coverslips was impaired due to compromised cell spreading. This study also demonstrated that both mechanical force-dependent cell stiffening and reinforcement of adherens junctions were compromised in vinculin KO cells when compared to vinculin-positive cells. Furthermore, vinculin KO cells display perturbed HGF-mediated recruitment of phosphorylated myosin light chain II (pMLCII), suggesting vinculin may play an important role in the recruitment of NMII to AJs following mechanical force [38].

AJ Mechanosensing Through Myosin VI. Recently, the F-actin-binding motor protein Myosin VI was identified as another mechanosensitive protein associated with AJs [39]. Intriguingly, the function of Myosin VI is dependent on the force it is experiencing. Under loads of ~ <2 pN, Myosin VI behaves as a typical motor protein, but under loads exceeding ~2.5 pN, Myosin VI briefly reverses direction along the actin filament and behaves as an anchor [40]. Under mechanical stress in epithelial monolayers, the association between E-cadherin and Myosin VI is rapidly increased [41]. Next, the heterotrimeric Gα12 protein is recruited to the E-cadherin-Myosin VI complex where it activates p114 RhoGEF to enhance RhoA activation, thus reinforcing the cell-cell contact through increasing the pool of cortical actomyosin. However, signalling through Myosin VI also increases epithelial integrity and protects against rupture by increasing the tensile strength of junctions: this is achieved via mDia1-dependent stabilisation of E-cadherin at multicellular vertices [41].

TJ-Based Mechanosensing

While much work has focused on how adherens junctions (AJs) can influence tissue mechanics and mediate mechanotransduction, recent studies have revealed that tight junctions (TJs) can also represent parallel sites of mechanotransduction at cell junctions. TJs are canonically responsible for establishment of a paracellular semipermeable diffusion barrier that limits free passage of ions and solutes through epithelial and endothelial layers [42, 43]. Tight junctions are composed of at least 40 different proteins, including (1) transmembrane proteins (e.g. claudins, occludin and JAM-A), (2) cytoplasmic proteins (e.g. ZO, cingulin) and (3) cytoskeletal filaments (actin, myosin, microtubules) [44] (Fig. 2a).

TJs Regulate Actomyosin Cytoskeleton. Even though TJs and AJs are present in both epithelial and endothelial cells, epithelial junctions are well-defined – TJs are located more apically than AJs. However, in less polarised endothelial cells, AJs and TJs are intermingled, and their spatial separation is less obvious [45, 46]. This different junctional organisation between the cell types may reflect different interplay between tight and adherens junctions. Indeed, downregulation of ZO-1 in epithelial cells results in increased tension on AJs [47–50], suggesting that ZO-1 exerts an inhibitory effect on junctional tension. By contrast, ZO-1 depletion in endothelial cells leads to decreased tension on VE-cadherin [51]. Despite this discrepancy, ZO-1 does not affect cadherin localisation/expression in either epithelial or

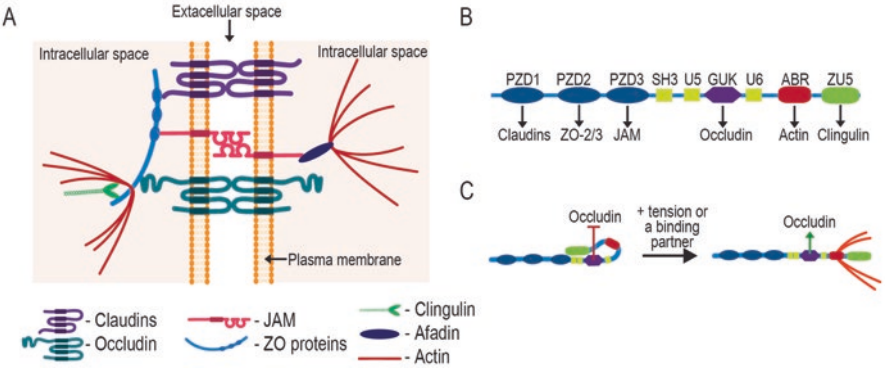

Fig. 2 Tight junctions (TJs). (A) Schematic representation of the basic components of tight junctions. Extracellular part of the TJ transmembrane proteins (occludin, claudins and JAMs) interact at cell-cell contacts existing between the plasma membranes of two neighbouring cells. Cytoplasmic tails of the transmembrane components of TJs connect to actin filaments via scaffolding proteins (ZO proteins, cingulin and afadin). (B) Schematic representation of ZO-1 structure and its interacting partners. (C) ZO-1 conformational change: in the absence of binding partners and/or under low tension, ZO-1 exists in an autoinhibited conformation, preventing its interaction with occludin; upon interaction with a binding partner and/or under high tension, ZO-1 transits to a stretched (active) conformation permitting interactions with occludin and actin

endothelial cells, indicating that changes in tension on cadherins originate from changes in the actomyosin network. Indeed, components of TJs, including ZO proteins, regulate cells' contractile cytoskeleton in various ways. ZO proteins may influence cell contractility by their interactions with actomyosin-associated proteins – α-catenin [52, 53], vinculin [54], shroom2 [55] or cortactin [56]. Furthermore, TJ components regulate multiple aspects of actomyosin by interacting with Rho family GTPases, thus carrying the capacity to support the activation or inactivation of major regulators of actomyosin – primarily RhoA, Rac1 and Cdc42. Among others, ZO-1 interacts with PDZ-RhoGEF [57] and Cdc42 GEF Tuba [58], and cingulin binds p114RhoGEF, GEF-H1 as well as MgcRacGAP [59–61]. Taken together, one way that TJs may influence cell mechanotransduction is by regulating actomyosin contractility, thus modulating AJs and E-cadherin-based mechanotransduction.

Evidence of Mechanotransduction on TJs. As discussed above, for the transduction of mechanical force between cells to mediate cell-cell communication, there must be adhesive junctional proteins which can transmit forces to mechanosensitive proteins. Further, propagation of the intrinsic forces across a tissue requires coupling of junctional mechanotransmitters to the cell contractile cytoskeleton [62, 63]. There are many potential ways for molecular elements of TJs to connect to the contractile apparatus of cells. The N-termini of ZO proteins interact with claudins, occludin and JAM-A, whereas the C-terminal region of ZO-1 and ZO-2 contains an actin-binding region (ABR) [64–66]; afadin links JAM-A and nectins to the actin cytoskeleton [67, 68], and the globular head domain of cingulin binds to ZO-1 and actin, while its coiled-coil region binds to myosin [69, 70] (Fig. 2a). Of note, among

the transmembrane TJ proteins, JAM-A has emerged as a potential mechanotransducer. Using a magnetic tweezers approach, Scott et al. showed that, in single epithelial and endothelial cells, tension imposed on non-junctional JAM-A activates RhoA via GEF-H1 and p115 RhoGEF to increase cell stiffness [71]. There is no evidence, however, that JAM-A works as a mechanotransducer in confluent, junction-forming monolayers. Instead, the search for TJ-based proteins that may participate in mechanotransduction has focused on ZO-1 as a force-bearing protein [9] whose molecular properties may be altered upon increased tension.

ZO-1 as a Mechanosensor. The molecular structure of ZO-1 consists of an N-terminal region containing three PSD-95/DLG/ZO-1 (PDZ) domains (PDZ1–3) that can bind to claudins (PDZ1), ZO-2 or ZO-3 (PDZ2) and JAM (PDZ3). These are followed by Src homology-3 (SH3), unique-5 (U5), occludin-binding guanylate kinase (GUK) and unique-6 (U6) domains. The C-terminal region of ZO-1 consists of actin-binding region (ABR) and ZU5 domain [12, 66, 72] (Fig. 2b). Importantly, Spadaro et al. have recently demonstrated that ZO-1 can exist in either an autoinhibited (folded) or stretched (unfolded) conformation [12]. ZO-1 assumes a folded conformation as a result of intramolecular interactions between the C-terminal ZU5 domain and ZO-1 central region (from PDZ3 to GUK domain). Unfolding of ZO-1 has been attributed to tension generated by actomyosin contractility, as magnetic tweezers experiments showed that physiological pN-scale tension is sufficient to unfold ZO-1 and maintain the 'active' stretched conformation. However, the stretched conformation can also be maintained by heterodimerisation with ZO-2: folding of ZO-1 could only be achieved when inhibition of the contractile network is combined with depletion of ZO-2 [12] (Fig. 2c).

Importantly, this tension-induced change in ZO-1 conformation can affect its intermolecular interactions. The folded, autoinhibited conformation of ZO-1 prevents the GUK domain from binding and recruiting occludin to TJs. It also blocks association of the central domain of ZO-1 with the transcription factor DbpA (Fig. 2c). Thus, tension-induced unfolding of ZO-1 may modulate its molecular interactions and downstream signalling [12]. It remains to be established whether ZO-1 conformational change influences its interactions with other binding partners including α-catenin, afadin and vinculin.

Recent evidence suggests that ZO-1 and many other TJ cytosolic and membrane proteins undergo cytosolic phase separation prior to arrival at the junctional membrane [73, 74]. During early zebrafish development, components of TJs accumulate at the boundary between the yolk syncytial layer (YSL) and the enveloping cell layer (EVL). Interestingly, accumulation of TJ proteins depends on the actomyosin tension within the YSL. Here, cytoplasmic, phase-separated ZO-1 is transported towards junctions by tension-dependent retrograde actomyosin flow, indicating existence of yet another mechanosensitive TJ-based event. Stinkingly, non-junctional ZO-1 must be unfolded and undergo multimerisation to allow phase separation to drive formation of TJ junctions. However, the ABR domain is not required for phase separation, suggesting that cytosolic unfolding of ZO-1 is not induced by tension, but rather by association of another molecule. As a consequence, conformational changes of cytosolic ZO-1 may not be powered by the

actomyosin-generated tension, as phase-separated ZO-1 seems to arrive at the junction in already opened 'active' conformation. Nevertheless, while the ABR domain of ZO-1 may be dispensable for ZO-1 phase separation, it is essential to efficiently integrate ZO-1 into junctions and confer mechanosensitivity upon TJs [74]. What then can facilitate cytoplasmic unfolding of ZO-1? Among many TJ proteins, the ZO-1 binding partner, cingulin, may be present in the highly concentrated phase-separated compartments. Cingulin supports efficient accumulation of ZO-1 at TJs, and its interaction with ZO-1's ZU5 domain may be sufficient to unfold ZO-1. Recent work suggests that ZO-1 exists in a 'double-folded' conformation with its ZU5 domain interacting with the central region of ZO-1 (as described above), as well as with the ABR domain. Binding of ZU5 to the ABR domain is predicted to prevent ZO-1 from forming efficient interactions with F-actin. Binding of cingulin to ZU5 promotes ZO-1 unfolding and may disrupt the interactions between ABR and ZU5, therefore allowing actin binding [75].

Overall, ZO-1 emerges as a possible novel TJ-based mechanosensor; however, further studies will be needed to fully understand the interplay between tension and ZO-1 binding partners in regard to regulation of ZO-1 conformation changes and physiological consequences of these events.

Plasma Membrane-Based Mechanosensing

While we have focused on understanding how cell-cell junctions participate in epithelial mechanobiology, it is important to note that these are not the only components of epithelial cells that display mechanosensitivity. In particular, the plasma membrane (PM) forms the physical boundaries of cells and, thus, is in constant contact with both the external and internal cell environments. As such, the PM constitutes a crucial interface that mediates responses to mechanical stimuli such as external touch, changes in cell curvature or internal osmotic pressure. Here, we will focus on two well-characterised examples of PM-based force-sensing, mechanosensitive ion channels and caveolae membrane invaginations, that have recently begun to be linked to cell-cell interactions.

Piezo1. Unlike other ion channels, which are typically activated/inactivated by interactions with specific ligands or voltage gating, mechanosensitive (MS) ion channels are opened in response to the application of mechanical forces (Fig. 3). MS ion channels are key sensors of mechanical stimuli across a diverse range of living organisms [76] and are some of the fastest signal transducers in cells, translating mechanical information into intracellular signals in the order of milliseconds [77, 78]. Two prevailing mechanisms for such mechanical gating exist. Firstly, the 'force-from-lipids' model, where MS ion channels are opened (activated) by forces from the plasma membrane [79–83] (Fig. 3). Such forces can occur from extrinsic means, such as touch or intrinsically such as osmotic pressure or changes in bilayer curvature induced by factors like local lipid composition [84] or expression of curvature-generating molecules, such as BAR-domain proteins [85]. Secondly, the

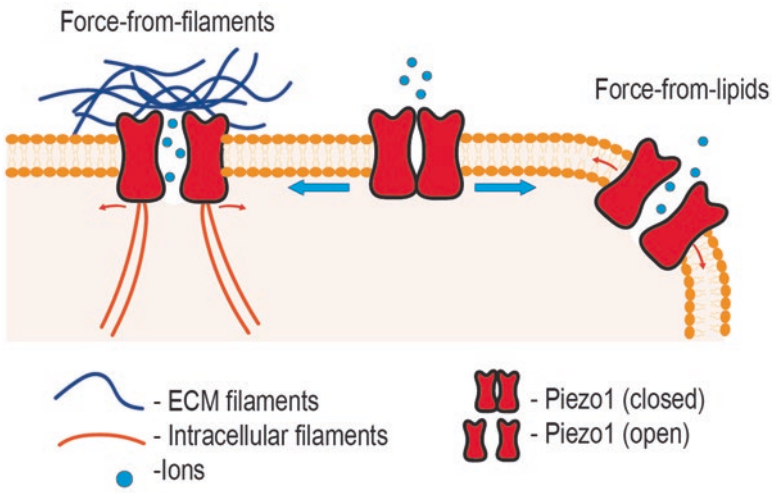

Fig. 3 Mechanosensitive (MS) ion channels. Piezo1 is the most characterised of the MS ion channels. MS ion channels are opened solely by means of mechanical force, rather than by ligand binding or voltage gating. The activation (opening) of MS ion channels occurs via *force-from-filaments* (left) and *force-from-lipids* (right). The former occurs by filament-mediated pulling forces from the extracellular matrix and/or the cytoplasm. The latter occurs during PM deformation, such as occurs through touch, osmotic pressure or curvature via BAR-domain proteins. Opening of Piezo1 channels allows influx/efflux of ions for modulation of processes such as live-cell extrusion and cell proliferation

'force-from-filaments' model (also called the tethered model) posits that MS ion channels can be activated by pulling forces from extracellular and cytoskeletal filaments attached to the channel [86–88].

Here we focus on the MS ion channel Piezo1, which influences a range of epithelial tissue phenomena, such as extrusion and control of population dynamics, that also involve cell-cell junctions. Moreover, Wang et al. [89] reported that Piezo1 can be coupled to E-cadherin, tethering the channels to the actin cytoskeleton via the cadherin-catenin complex, while others have demonstrated that Piezo channels help regulate RhoA signalling and the actin cytoskeleton and that activation of Piezo1 is dependent on integrin-related signalling [90–92]. It should be noted that, although Piezo1 channel is the best characterised example of a MS ion channel, there are other mammalian representants of this group, such as the Piezo isoform, Piezo2 as well as the TREK family of neuronal potassium channels, TREK-1, TREK-2 and TRAAK [93].

Piezo channels are expressed as two isoforms in mammals (Piezo1 and Piezo2), with both being highly expressed in organs where mechanosensation is functionally significant, such as the bladder, colon, kidney, lung, skin and dorsal root ganglia (Piezo2 only) [94]. Piezo channels are the principal means of sensing mechanical stimuli such as touch [94] and vascular blood flow [95]. The Piezo1 channel is a unique protein and does not bear a structural homology to any other channel [96].

Piezo1 has a trimeric, three-bladed propeller shape, which can adopt several conformations, and houses a kinked helical beam and an anchor domain. Each of the three blades contains nine repeat regions, each of which comprises four transmembrane domains, collectively passing the plasma membrane 108 times [97, 98]. This structure was shown to be fundamental in the mechanogating of the Piezo1 channel [99]. Molecular dynamics simulations suggest that increased membrane tension results in flattening and in-plane expansion of the blades, resulting in tilting of helices 37 and 38, which are then pulled away from the channel pore, leading to opening of the Piezo1 channel [100].

Piezo channels have been implicated in a number of physiological processes that also involve cell-cell interactions. Piezo1 is a fundamental ion channel required for live-cell extrusion [4], a process vital for homeostatic control of cell density in epithelia. As noted earlier, a pivotal study by Eisenhoffer et al. [4] demonstrated that live-cell extrusion is triggered by compressive forces due to cell overcrowding. By growing cells to confluence on a silicone membrane stretched to 28% of its original length and then allowing the substrate to recoil, cell density was increased by ~30%, generating compressive forces across the tissue. However, within 6 h the cell density was reduced to homeostatic levels, indicating that cells had been eliminated from the monolayer. Indeed, it was apparent that the tissue was extruding living cells. This live-cell extrusion was mediated by Piezo1, as either inhibition of the channel with gadolinium (Gd^{3+}) or morpholino-mediated knockdown of Piezo1 in zebrafish embryos limited live-cell extrusion and resulted in cell mass formation in the epithelial tissue at sites of high strain.

Further, the Piezo1 channel may promote cell proliferation when cell density is too low and cells are under positive tension [94, 101, 102]. By growing cells to confluence on a flexible substrate and applying a ~ 1.4-fold stretch, Gudipaty et al. [102] demonstrated that the rate of mitosis was increased by approximately fivefold within just 1 h. Under these conditions, treatment with Gd^{3+} or siRNA knockdown of Piezo1 efficiently inhibited stretch-induced proliferation, demonstrating that Piezo1 was at the apex of this phenomenon. These results were also confirmed in vivo in the zebrafish epidermis where both CRISPR-based mosaic knockout and morpholino-mediated knockdown of Piezo1 significantly decreased the number of mitotic cells. Ultimately, the activation of Piezo1 engages intracellular Ca^{2+} signalling to activate the ERK1/2-MEK1/2 pathway and promote the transcription of cyclin B for increased mitosis.

How tensile or compressive forces can elicit either live-cell extrusion or promote cell proliferation through the same Piezo1 channel is a significant question. Gudipaty et al. [102] suggested that the subcellular localisation of Piezo1 may be responsible. At subconfluency, Piezo1 is mainly confined to the nuclear envelope; as confluency is reached but cell density is still low, Piezo1 localises to the endoplasmic reticulum and PM. As cell density continues to increase post-confluency, Piezo1 begins to form large cytoplasmic aggregates. These changes in the subcellular localisation may reflect the adaptive mechanosensitivity of Piezo1, where its presence at the PM is optimal to sense tensile forces and its presence within the cytoplasm is optimal to sense compressive forces.

Caveolae-Based Mechanosensing. Caveolae are 60–80-nm-wide Ω-shaped invaginations of the PM [103, 104] (Fig. 4). They are enriched in cholesterol and sphingolipids, forming specialised lipid rafts that are implicated in numerous biological functions, such as endocytosis, lipid metabolism and mechanosensing. Caveolae may encompass up to 50% of the total PM surface area in cells such as myocytes [105] and their formation and stability largely dependent on two protein families, the caveolins (Cavs) and the cavins.

Caveolae have several characteristics which suit them well for mechanosensing. Their bulb-like morphology constitutes a membrane reservoir which is sensitive to mechanical stimuli and can be flattened by membrane stretch (Fig. 4); they are particularly abundant in tissues which experience significant mechanical forces; they are directly linked to the actin cytoskeleton and are distributed at sites of cell contact [106–109]. Indeed, caveolae have been observed to concentrate at adherens junctions [110]. The mechanical tolerance of the lipid bilayer has been measured at 4–6% areal strain before rupture [111]; thus, the ability of caveolae to passively modulate tension likely provides a significant advantage to cells and tissues affected by intrinsic or extrinsic mechanical forces. In complex living systems, cells must tolerate a range of potentially damaging mechanical stimuli, such as alveolar inflation, muscle stretching, vascular shear stress and volume expansion.

In practice, the ability of caveolae to flatten under mechanical tension has been demonstrated in both isolated cells and multicellular tissues. For example, caveolae were shown to flatten in individual HeLa and mouse lung endothelial cells (MLECs) in response to hypo-osmotic shock [112], as well as primary mouse cardiomyocytes

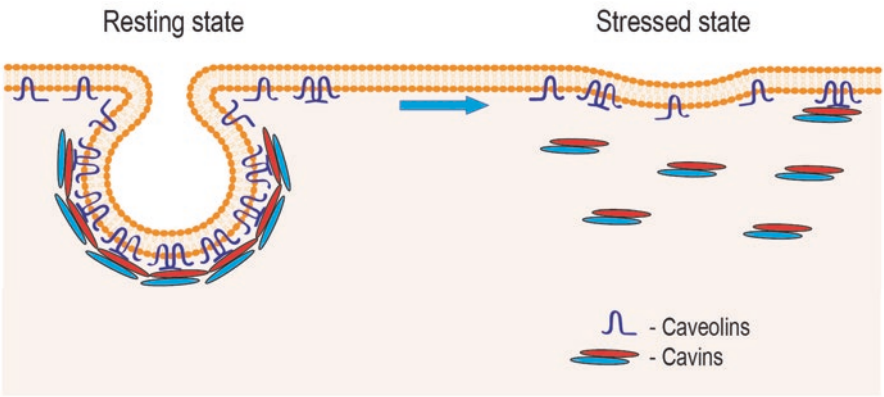

Fig. 4 Caveolae as mechanosensors. Caveolae, small invaginations of the PM, are sensitive to acute increases in membrane tension from both extrinsic and intrinsic stimuli. Under normal physiologic conditions, caveolae exist as bulb-shaped pits, stabilised by a coat of caveolin and cavin proteins (left). Under periods of high tension, caveolae flatten, providing a passive means of protection by releasing a membrane reservoir, thus rapidly increasing the surface area of the cell. When caveolae flatten, cavin proteins are released into the cytosol, leaving the caveolins at the PM. Caveolae flattening also alters the cellular lipid composition, enhancing signalling through the RhoA-ROCK pathway for F-actin modulation and increasing MAPK activity

[113]. This response by caveolae to increased membrane tension has also been demonstrated in multicellular tissues both ex vivo and in vivo. A seminal paper by Dulhunty and Franzini-Armstrong [114] showed that the abundance of caveolae on the surface of frog skeletal muscle was greatly reduced following mechanical stretch and elongation past this point resulted in rupture of the tissue. Soon after, caveolae flattening in response to mechanical tension was demonstrated in the smooth muscle [115].

More recent studies have demonstrated the mechanoprotective role of caveolae in vivo. Cheng et al. [116] used dobutamine, a β-1 adrenoreceptor agonist, to increase heart contractility and cardiac output in mice. They observed caveolar disassembly in both heart and lung tissues. Importantly, endothelial cells showed a significant damage in Cav-1 KO mice, whereas no damage was observed in WT control mice [116]. Others have shown that the notochord, which comprises cells particularly abundant in caveolae, becomes unstable when caveolae are depleted by cavin-1b KO [117, 118]. Here, the notochord of cavin-1b KO zebrafish shows a significant cellular damage, cell necrosis and the appearance of lesions. Interestingly, damage to the notochord was increased when mechanical stress was applied to the notochord during swimming, further demonstrating the importance of caveolae as mechanoprotective.

In some tissues the ability of caveolae to attenuate membrane tension is facilitated by the formation of multi-lobed, rosette-like caveolar superstructures [105]. These superstructures are composed of multiple caveolae which have fused due to membrane curvature and tension [119, 120], are more sensitive to tension, disassemble more rapidly and release a far greater pool of PM upon flattening than individual caveolae [105, 121]. This hypothesis is supported by computational modelling and experimental data which demonstrate that caveolar rosette formation is promoted by low tension [119]. Further, cells can actively respond to force by altering the abundance or properties of caveolae. For example, caveola numbers are increased by almost 50% in bovine aortic endothelial cells (BAECs) experiencing chronic shear stress [122], highlighting the importance of caveolae in protecting tissues from physical damage. Mechanical stress applied to vascular endothelial tissue, such as is experienced during hypertension, dampens the eNOS-binding capacity of Cav-1, promoting vasodilation to attenuate elevated shear stress [123]. It has also been suggested that the mechanosensitive properties of caveolae regulate cell volume, as ectopic Cav-1 expression in cells lacking caveolae can promote caveolar biogenesis, allowing cells to swell to a greater extent following hypo-osmotic exposure [124].

Interestingly, caveolae flattening also alters the microenvironment of these structures beyond gross morphology. Under acute membrane tension, caveolae flattening results in the dissociation of the cavin protein complex [112] and the EHD2 ATPase [125] but not the caveolin proteins [112], from caveolae. This suggests that caveolae may actively respond to mechanical stress using dissociated proteins as signalling intermediates. Indeed, recent studies have demonstrated that release of EHD2 upon caveolae flattening suppressed transcription of caveolins 1 and 2 and cavins 1 and 2, which are required for caveolar biogenesis [126]. Furthermore, the

mechanosensitivity of caveolae, specifically the Cav-1 and cavin-4 proteins, influences the activity of RhoA [127]. Caveolae flattening promotes the phosphorylation of Cav-1 at tyrosine-14 (Y14) [128, 129]. pY14 phosphorylation of Cav-1 can enhance Cav-1-RhoA interaction to directly activate RhoA [127, 130] and negatively regulate the Src-p190RhoGAP pathway [131], suggesting that mechanotransduction via caveolae could influence RhoA for remodelling of the cytoskeleton. Furthermore, low tension at the rear of migrating cells promotes caveolar formation, activating RhoA-ROCK1-PKN2 signalling via Ect2 [132]. This drives local F-actin organisation and contractility, allowing the cell's posterior region to complete the migration cycle.

Caveolae flattening can also influence other aspects of membrane organisation with consequences for mechanoregulatory mechanisms. Ariotti et al. [133] demonstrate that perturbation of caveolar biogenesis by either Cav-1 or cavin-1 KD alters the cellular lipid composition, particularly the distribution of phosphatidylserine at the PM. This resulted in enhanced K-Ras expression and organisation and increased MAPK activity. Interestingly, these phenomena were found to be due to the loss of intact caveolae, as the acute dissociation of cavin-1 by hypo-osmotic shock mimicked these findings. Thus, this study implies that the mechanosensitive nature of caveolae may allow cells to rapidly alter the lipid composition of the PM in response to mechanical stress.

More recently, caveolae were found to modulate mechanical tension at the tissue level. Teo et al. [110] demonstrated that caveolae modulate tissue tension within epithelial monolayers. Here, caveolae were found to be at the apex of a novel signalling pathway, where intact caveolae suppress the availability of phosphatidylinositol-4,5-bisphosphate ($PtdIns(4,5)P_2$) at the PM. By inhibiting caveolar biogenesis, $PtdIns(4,5)P_2$ levels are enhanced, directly increasing cortical recruitment of the formin-like protein, FMNL2. In turn, enhanced FMNL2 increased the pool and organisation of cortical F-actin, resulting in elevated tension within the tissue. A significant ramification of this dysregulation was perturbation of oncogenic cell extrusion, resulting in the formation of large cell masses when a minority of oncogenic cells were incorporated into an otherwise healthy epithelial monolayer.

Intriguingly, recent studies show that the mechanosensitivity of caveolae and the Hippo-YAP/TAZ signalling pathway influence each other. The Hippo pathway, a major regulator or cell proliferation, migration and survival [134], is sensitive to mechanical stimuli such as tension [135], cell density [136] and stiffness of the extracellular matrix (ECM) [17], all of which affects the localisation and activity of YAP/TAZ. In 2018, Moreno-Vincente et al. [137] revealed that the mechanoresponsiveness of YAP to ECM stiffness was positively modulated by Cav-1, whose transcription, as well as that of cavin-1, is also dependent on the presence of YAP/TAZ [138]. Furthermore, caveolae are protective against mechanical stresses which arise from haemodynamic force [116, 139], a process which activates YAP/TAZ signalling [140, 141]. It has been suggested that interplay between caveolae and the Hippo pathway has significance in the pathogenesis of diseases such as atherosclerosis and vascular malformations, both of which result from dysfunctional endothelial shear stress sensing [142].

Conclusion

The past decade has witnessed exciting, rapid progress in understanding how cell-cell junctions contribute to the mechanobiology of epithelia. We now appreciate that epithelia are mechanically active tissues, which themselves generate forces and respond to forces generated extrinsically, and such changes in force can indicate events that challenge tissue integrity or homeostasis. In closing, we would identify a number of directions that we think warrant attention in the future. First is integrating molecular mechanisms into systems. We have begun to identify the molecular mechanisms that allow epithelia to detect changes in force and elicit compensatory homeostatic responses. Many more molecular details will inevitably be revealed in the next few years. As this molecular picture grows in richness and specificity, it will be important to consider how they may be integrated by feedback into functional networks rather than individual, linear pathways. For example, the contractile protein non-muscle Myosin II, which is activated by RhoA signalling, can also participate in mechanochemical feedback to support RhoA [143, 144]. Insights from statistical physics and mathematical modelling provide valuable resources to tackle such systems analysis. Second, can the homeostatic mechanisms of epithelial mechanotransduction be disrupted in disease? Clues include the observation that inflammatory cytokines such as TNF-α can increase mechanical tension at cell-cell junctions, including TJ [145]. Also, depletion of caveolae, which has been implicated in cancer, increases epithelial tension to compromise the elimination of transformed cells [110]. Therefore, elucidating the mechanobiology of junctions may provide many new insights into the biology and pathobiology of epithelia.

Acknowledgements The authors were supported by grants and fellowships from the National Health and Medical Research Council of Australia (GNT1164462, 1136592 to ASY; APP1156489 to RGP; GNT1140090 to ASY and RGP) and the Australian Research Council (DP19010287 to ASY).

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Intracellular Traffic and Non-canonical Roles of ZO-2 Protein

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Abstract *Zonula occludens 2* (ZO-2) is a peripheral tight junction (TJ) protein of the MAGUK family. This chapter describes how ZO-2 together with ZO-1 forms a platform for the polymerization of claudins into TJ strands and explains how ZO-2 participates in TJ formation during embryogenesis and the signaling pathway involved in ZO-2 assembly at TJs. The particular role of ZO-2 in the testis, liver, and inner ear and its impact on human disease are discussed. ZO-2 nuclear location, the mechanism and signals that regulate the intracellular traffic of the molecule, and ZO-2 role at gene transcription and translocation of proteins to the nucleus are analyzed. In addition, the chapter describes the role of ZO-2 in cell size regulation and cancer, describing the altered expression and location of the protein in several carcinomas and in conditions that favor a malignant phenotype.

Keywords ZO-2 · Tight junctions · Cell polarity · Cancer · Gene transcription · Cell size

Introduction

Zonula occludens 2 (ZO-2) is a 160-kDa peripheral membrane protein of tight junctions (TJs), first identified by Gumbiner for its co-immunoprecipitation with ZO-1 in MDCK cells [1]. ZO-2 belongs to the MAGUK (membrane-associated guanylate kinase homolog) protein family. ZO-2 contains three PDZ domains, a SH3 module, and a GUK domain. In addition, its carboxyl segment that ends with the PDZ-binding motif TEL exhibits an actin-binding and proline-rich region (Fig. 1). The presence of these protein-protein binding domains and motifs allows ZO-2 to

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L. González-Mariscal (ed.), *Tight Junctions*,
https://doi.org/10.1007/978-3-030-97204-2_4

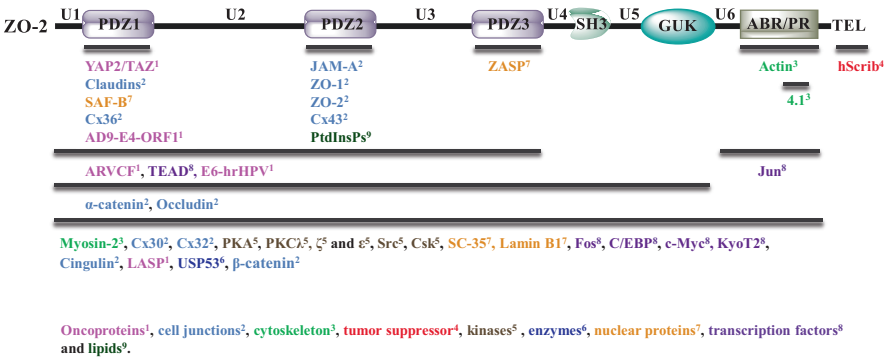

Fig. 1 Schematic organization of ZO-2. ZO-2 has three PDZ domains, an SH3 module, a GUK domain, and an actin-binding and proline-rich region (ABR/PR), and its carboxyl segment ends with the PDZ-binding motif TEL. The unique (U) regions located between the different domains and segments of ZO-2 are numerated. The lines indicate the regions of ZO-2 that associate with different molecules, including oncoproteins, cell junction proteins, tumor suppressors, kinases, nuclear and transcription factors, and lipids

function as a scaffold that brings together multiple proteins at the TJ and the nucleus and impacts diverse physiological processes.

This chapter describes how ZO-2 participates in establishing TJs in embryos and cell cultures and how this relates to adherens junction (AJ) formation. We discuss the signaling pathways involved and how phase separation of ZO proteins recruits junctional proteins. We pay special attention to tissues in which the presence of ZO-2 appears to be crucial for TJ sealing and where no other junctional protein can fulfill its role. We also analyze the participation of ZO-2 on cell polarity and the evidence of its interaction with lipids. ZO-2 is a protein that moves from the cytoplasm to the TJ and the nucleus. Hence, we will describe the numerous signals and posttranslational modifications that regulate this intracellular traffic and the non-canonical functions of the protein discovered as a consequence of ZO-2 presence at the nucleus. In addition, we report unexpected alterations observed in epithelial cells that do not express ZO-2 and the evidence for the proposal of ZO-2 as a potential tumor suppressor.

ZO-2 and Adherens Junction Formation

TJs do not form if AJ formation is blocked [2, 3]. However, new studies have enriched this view suggesting that AJs also regulate TJ composition by modulating the membrane lipid composition. This proposal is based on the observation that loss of α-catenin alters the plasma membrane's lipid composition, leading to claudin endocytosis [4].

ZO-2 also localizes in AJs in non-epithelial cells like fibroblast and the intercalated discs of cardiac muscle cells [5], and in cultured MDCK ZO-1/ZO-2 double knock-out (dKO) cells, the localization of AJ proteins E-cadherin and afadin becomes discontinuous and fragmented [6]. Likewise, in MDCK cells, ZO-2 silencing retards the arrival of E-cadherin to the plasma membrane triggered by a Ca^{2+}-switch [7], thus indicating that the assembly of the AJ also requires ZO proteins.

ZO-2 also associates with several AJ proteins, including α- [5] and β-catenin, and proximity ligation assays (PLAs) show the interaction with β-catenin at sites of cell-cell contact in MDCK cells [8].

ZO-2 and Tight Junction Formation

ZO-2 in Tight Junction Formation in Cultured Cells

In renal epithelial MDCK cells, ZO-2 silencing delays the arrival of occludin to the plasma membrane triggered by the transfer of monolayers from a medium with low (LC, 1–5 μM) to normal calcium (NC, 1.8 mM), also known as Ca^{2+}-switch [7]. In addition, the development of transepithelial electrical resistance (TER) is retarded in ZO-2 KD cells, and these monolayers reach a much lower peak TER than parental cells. This effect is due to the lack of ZO-2, as ZO-2 re-expression reversed the effect [9].

In epithelial cells in culture where the expression of both ZO-1 and ZO-2 was knocked down (KD)/KO, like Eph4 ZO-1KO/ZO-2KD [10] or MDCK ZO-1/ZO-2 dKO cells [6], no TJ strands were detected by freeze-fracture replica electron microscopy, unless the expression of one of these ZO proteins was restored. Hence, it was concluded that ZO-1 and ZO-2 function as a platform for the polymerization of claudins. Moreover, in ZO-1/ZO-2 dKO cells, ZO-3 and occludin delocalize from the apical junctional region, while JAM-A and claudins distribute throughout the apical and lateral membranes [6], highlighting the importance of ZO-1/ZO-2 for tethering TJ proteins into a complex at the apicolateral region.

Close membrane apposition and kissing points at the TJ appear to determine the paracellular permeability of two different pathways. Thus, the membrane apposition is proposed to act like a 60 A°-diameter sieve in the size-selective pathway that blocks the transit of molecules larger than 4 kDa. In contrast, the TJ kissing points constituted by claudins form the charge-selective small-pore pathway with an estimated diameter of 4 A° that regulates ion conductance (for review, see [11]). In ZO-1/ZO-2 dKO cells, the membrane appositions and kissing points at the uppermost portion of the lateral membrane are lost, and the adjacent intercellular space is widened [6] (Fig. 2a and b). The latter observation was also observed in MDCK ZO-2 KD cells [7, 9] (Fig. 2c). Interestingly, in claudin quintuple (quin) KO cells (claudin-1, claudin-2, claudin-3, claudin-4, and claudin-7 KO) that lack TJ strands and membrane kissing points, membrane appositions are still formed, but if JAM-A

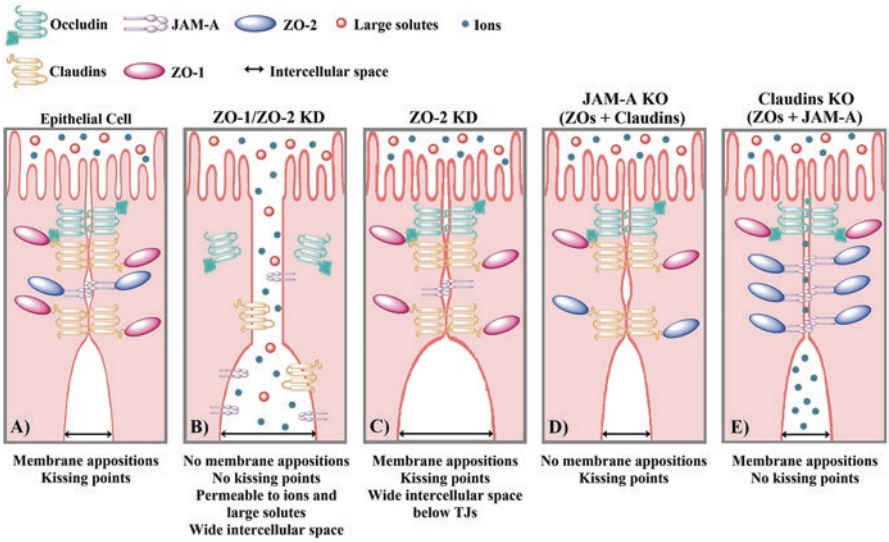

Fig. 2 Changes in membrane apposition and kissing points at the TJ and in the width of the intercellular space below the TJ generated by the absence of different TJ proteins. (**a**) In epithelial cells, close membrane apposition and kissing points that appear at the TJ restrict the passage of large solutes and ions through the paracellular pathway. (**b**) The lack of ZO-1/ZO-2 blocks TJ strand formation. No membrane appositions and kissing points are detected at the uppermost portion of the lateral membrane, and the intercellular space is widened. Large solutes and ions flow through the paracellular pathway. (**c**) In ZO-2 KD cells, TJs are formed, but the intercellular space below this region is wider than in parental cells. (**d**) TJ kissing points depend on the expression of both ZO-1/ZO-2 and claudins. (**e**) Membrane apposition at the upper lateral membrane requires the presence of both ZO-1/ZO-2 and JAM-A. Without claudins, ions can move through the paracellular route

is simultaneously eliminated, the appositions are lost [6]. Altogether these observations suggest that membrane apposition at the upper lateral membrane requires the presence of both ZO-1/ZO-2 and JAM-A, the TJ kissing points depend on the expression of both ZO-1/ZO-2 and claudins (Fig. 2d and e).

ZO proteins interact with both JAM-A and claudins. Immunoprecipitation experiments revealed that JAM-A associates directly with ZO-2 and the latter to afadin, [12], questioning previous results that support a direct interaction between afadin and JAM-A [13]. In the case of claudins, the three ZO proteins bind via their first PDZ domain to the C-terminal YV sequence of claudins 1–8 [14].

Recently, ZO proteins were reported to form condensed liquid-like compartments due to phase separation [15]. These condensed membrane-attached compartments that ZO proteins form become strongly enriched with TJ transmembrane proteins occludin and claudin, the adaptor proteins afadin and cingulin (not in the case of ZO-3), and signaling molecules including the transcription factor ZONAB in the case of ZO-1. Hence, the early recruitment of ZO-1/ZO-2 at primordial adhesion sites via AJ is proposed to allow these ZO proteins to reach a concentration

above the threshold for phase separation. This phase change could allow the recruitment of claudins and other TJ proteins into these compartments and subsequently trigger the polymerization of claudins and TJ strand formation. It is, however, striking that these condensations of ZO proteins did not sequester JAM-A [15] since, as stated above, the latter is the first TJ integral protein to be recruited to nascent TJ sites (for review, see [16]).

Phase separation of ZO proteins is negatively regulated by phosphorylation [15], which is noteworthy since we had previously found by two-dimensional phosphoamino acid analysis that ZO-2 derived from cells with disassembled TJs, due to treatment with the calcium chelator EGTA, is strongly phosphorylated in serine residues in comparison to ZO-2 from cells with TJs in a steady state [17].

The middle segment of ZO proteins, containing the third PDZ, SH3, and GuK regions (3PSG), constitutes a supra-domain needed to condensate ZO proteins into liquid compartments. In contrast, the U6 segment located downstream of the GuK domain inhibits this process [15]. ZO proteins switch between opened and closed states, where the closed state is attained when the U6 region acts as a hinge that folds back the C-terminus of ZO proteins on their N-terminal part and interacts with the GuK domain. The open state allows ZO protein dimerization and subsequent multimerization, leading to the phase transition [15]. In the case of ZO-1, the release of the closed state was achieved through stretching by the contractile actomyosin network [18]. In addition, actomyosin tension can trigger the movement of non-junctional clusters of ZO-1 formed by phase separation toward the TJ in a process mediated by direct actin binding of ZO-1 [19].

ZO-2 in Tight Junction Formation During Embryogenesis

The pivotal work by Fleming on early mammalian development revealed that once the eight-cell embryo undergoes compaction due to the expression of E-cadherin, TJ constituents assemble at the apicolateral portion of the membrane of adjacent blastomeres (for review, see [16]). This process occurs in a 24-h period that goes from embryo compaction to blastocoel cavitation, in a step-by-step manner divided into 8-, 16-, and 32-cell stages. In compact eight-cell embryos, the process begins with the arrival of the apical polarity complex Par-3/Par-6/atypical protein kinase C (aPKC) and cdc42 to the apical membrane of blastomeres. Then, the small GTPase Rab13 and ZO-1 isoform α^- assemble at the uppermost portion of the lateral membrane. Next, at the 16-cell stage, cingulin and ZO-2 [20] assemble at this region, until at the 32-cell stage, ZO-1α^+, occludin, and claudins integrate, generating a permeability seal between trophectoderm cells that allows the formation of the blastocoel cavity. Until this late stage, proteins of the AJ and TJ no longer co-localize and instead segregate into two distinct domains with their classical ultrastructural morphology.

In mouse zygotes, maternal ZO-2 protein is present at the nuclei, but at the 16-cell stage, it is embryonic ZO-2, which assembles at blastomeres' apicolateral

junctions [20]. In the trophectoderm layer, ZO-2 silencing delays the formation of the blastocoel cavity [20], but ZO-2 KO in mice embryos is lethal shortly after implantation, as the embryos show a decreased proliferation at embryonic day 6.5 (E6.5) and increased apoptosis at E7 [21]. ZO-1 KO mice are not viable beyond E11.5, exhibiting at E9.5 massive apoptosis in the notochord, neural tube area, and allantois and an impaired formation of the vascular tree in the yolk sac [22]. While ZO-3 KO mice display no obvious phenotype [21], ZO-3 silencing with morpholinos in zebrafish embryos leads to edema, loss of blood circulation, tail fin malformations, increased permeability of the enveloping cell layer, and high sensitivity to osmotic stress [23]. These observations suggest nonredundant roles of ZO proteins in the developing embryos that can vary according to the species.

In the case of ZO-2 KO mice, embryonic lethality was later circumvented by injecting ZO-$2^{(-/-)}$ embryonic stem cells into wild-type blastocysts to generate viable ZO-2 chimeras. Thus, indicating that ZO-2 is crucial for the proper function of the extra-embryonic tissue, but not for embryo development *per se* [24].

ZO-1 strongly stains the vessels in the parenchyma of the chorionic villi in human term placentae, whereas ZO-2 is instead present at the syncytiotrophoblast cell layer [25], suggesting that during embryogenesis, ZO-1 is crucial for placental angiogenesis and ZO-2 participates in the sealing of the trophectoderm.

ZO-2 Subcellular Distribution

The subcellular distribution of ZO-2 is sensitive to extracellular calcium and cell density. Thus, in cells cultured in LC, where TJs cannot assemble [26], ZO-2 is present in low amounts at the cytoplasm and concentrated at the nucleus [27, 28]. Instead, in NC, confluent monolayers concentrate ZO-2 at the cell borders, whereas sparse cultures also display a strong presence of nuclear ZO-2 [27].

At the TJ ZO-2 is associated with a complex array of proteins that includes the integral TJ proteins occludin [5], JAM-A [12], and claudins [14], as well as the peripheral TJ proteins ZO-1 [5, 29, 30] and cingulin [31]. At the cytoplasm, ZO-2 associates with 14-3-3 proteins [28]. These are small adaptor proteins that bind to target proteins preventing their proteasomal degradation. Of the seven different 14-3-3 proteins found in mammals and commonly called isoforms, the α, γ, ζ, θ, and σ isoforms bind to ZO-2 [28, 32–34]. Canine and human ZO-2, respectively, have 25 and 22 putative 14-3-3-binding sites with the consensus R[S/ϕ][+]pSXP or RX [S/ϕ][+]pSXP (where pS is the phosphoserine that 14-3-3 proteins bind, ϕ is an aromatic residue, + is a basic residue, and X is any type of residue, typically Leu, Glu, Ala, and Met) [28]. The interaction between ZO-2 and 14-3-3 in the cytoplasm is proposed to protect ZO-2 from degradation.

When the TJ is not formed due to the absence of extracellular calcium, ZO-2 is unstable, with a half-life of 7 h compared to 19.7 h found in monolayers cultured in NC media, and is constantly degraded in the proteasome [28]. However, in LC condition, a portion of ZO-2 is protected from proteasomal degradation and preserved

in the cytoplasm sequestered by 14-3-3. The U2 segment of ZO-2 localized between PDZ-1 and PDZ-2 contains almost half of 14-3-3 binding motifs of ZO-2 and is critical for the establishment of the ZO-2/14-3-3 complex. 14-3-3 inhibition with BV02 in LC cultured cells induces the appearance of ZO-2 at the cell borders, suggesting that the detachment of ZO-2 from 14-3-3 promotes its recruitment to the TJ [28]. Since the amount of ZO-2 at the membrane does not increase after BV02 treatment, the dissociation of the 14-3-3/ZO-2 complex may allow ZO-2 to polymerize at the cell border, making feasible its detection by immunofluorescence [28].

The use of quantitative microscopy in MDCK cells with endogenous ZO proteins containing amino-terminal insertions of a fluorescent tag revealed that ZO-2 has an average cytoplasmic concentration of 0.27 μM and an estimated junctional concentration of 5.8 μM, whereas ZO-1 is 2.5-fold and sevenfold more abundant than ZO-2 in the cytoplasm and TJs, respectively [15]. In addition, fluorescence correlation spectroscopy revealed that in the cytoplasm, 20% of the total ZO-2 fraction corresponds to monomers with a fast diffusion, while the rest conform to a slower component of oligomers with an average stoichiometry 1.5 [15].

ZO-2 is 22-fold more concentrated in the TJ than in the cytoplasm of confluent MDCK cells. However, junctional ZO-2 is rapidly exchanged with the cytoplasmic pool, as fluorescence recovery after photobleaching shows a $t_{½}$ of 126 s, slightly faster than the 161 s observed for ZO-1 [15]. Moreover, it was estimated that 69 ± 5% of TJ-associated ZO-1 is available for exchange with intracellular pools, explaining why fluorescence recovery occurs at similar rates at the center and edges of a bleached cell border [35]. Instead, occludin fluorescent recovery begins at the edges, indicating that this protein moves by diffusion within the membrane [35]. Accordingly, fluorescence recovery of occludin is sensitive to changes in membrane fluidity induced by low temperature and cholesterol depletion, whereas ZO-1 is not and only responds to ATP depletion [35].

Signaling Pathway Leading to ZO-2 Assembly at Tight Junctions

Since four decades ago, the pioneering work of Cereijido demonstrated the crucial requirement of extracellular Ca^{2+} for TJ formation, showing that Ca^{2+} removal with chelators and its restoration, respectively, opened and resealed the TJ in confluent monolayers of epithelial cells [36–38]. When cells are switched from the LC to NC condition, intracellular calcium increases [39]; however, TJ sealing does not depend on this increase, as incubation with La^{3+} [39], or the calcium channel blocker verapamil [40], which inhibit Ca^{2+} influx during the Ca^{2+}-switch, does not impair TJ formation and sealing. Hence, these observations suggested that Ca^{2+} triggers TJ formation by binding to a receptor in the membrane that subsequently initiates a signaling cascade in the cytoplasm.

E-cadherin was first considered as the putative Ca^{2+} membrane receptor [40] due to two main observations: first, that TJ assembly requires the initial formation of AJs [2, 3] and, second, that the five extracellular repeats of E-cadherin, the integral

protein of the AJs, need to bind Ca^{2+} ions (for review, see [41]) to form a rigid structure that permits the establishment of a stable cell-cell adhesion through the formation of *cis* and *trans* dimers [42]. However, the discovery that the activation of the G-protein-coupled Ca^{2+}-sensing receptor (CaSR) with agonists (e.g., neomycin, Gd^{3+}, and R568) induces the appearance of ZO-1 [43], ZO-2 [28], and occludin [43] at the cell borders of MDCK cells cultured in LC uncovered the existence of a more complex signaling pathway for TJ assembly. This observation also validated three decade-old studies showing the involvement of G-protein signaling in TJ assembly [44, 45].

Activation of the CaSR appears to be crucial for developing the permeability barriers as the over-expression of this receptor in the undifferentiated cells of the epidermis accelerates differentiation and the expression of claudin-6, claudin-11, and claudin-18 [46]. CaSR silencing downregulates claudin-14 expression in the thick ascending limb in the kidney, triggering hypercalcemia [47], as claudin-14 physically blocks the establishment of the paracellular cation channel made of claudin-16 and claudin-19 [48]. In this model, CaSR regulates the expression of claudin-14 by modulating the expression of two microRNA molecules that induce mRNA decay and translational repression of claudin-14 [48].

Instead, in MDCK cells, CaSR activation triggers the arrival of ZO-2 to the cell border by a downstream signaling pathway involving the G-protein $\alpha_{q/11}$ subunit that induces the activation of novel (n) PKCε, which phosphorylates ZO-2 and activates with no lysine kinase-4 (WNK4). This kinase binds and phosphorylates ZO-2, leading to its movement to the cell borders and dissociation from 14-3-3 [28]. At the membrane, ZO-2 polymerizes in newly formed TJs. At the same time, molecules of ZO-2 that cannot integrate into the junction are retrieved by endocytosis and degraded in the lysosome [28] (Fig. 3).

In isolated inner cell masses from murine embryos, PKC activation also promoted ZO-2 delivery to the cell borders [49]. However, in this model, the effect appeared to be mediated by nPKCδ.

Another kinase involved in TJ assembly triggered by extracellular calcium is AMP-activated protein kinase (AMPK). This kinase that senses energy status in eukaryotic cells is activated by elevated AMP/ATP ratios [50]. During a Ca^{2+}-switch, AMPK is activated, and in cells cultured in LC, AMPK activation with AICAR (5-aminoimidazole-4-carboxamide riboside), a nucleoside that converts to a nucleotide that mimics AMP [51] [52], triggers the appearance of ZO-1 [50] and ZO-2 [28] at the cell borders. The defects in TJ assembly observed in cells expressing a dominant-negative AMPK are partially rescued by mTOR inhibition with rapamycin, suggesting that mTOR is an upstream regulator or this pathway [50]. This observation is noteworthy because in MDCK ZO-2 KD cells signaling through the AKT/mTOR/S6K pathway is increased, thus suggesting that ZO-2 negatively regulates the repressive effect of mTOR on TJ assembly mediated by AMPK.

In human placental BeWo cells, long-term treatment with forskolin triggers TJ formation through AMPK activation mediated by LKB1-dependent phosphorylation [53]. In intestinal epithelial cells, polarization is achieved even in the absence of cell-cell contacts, through the activation of LKB1 mediated by the specific

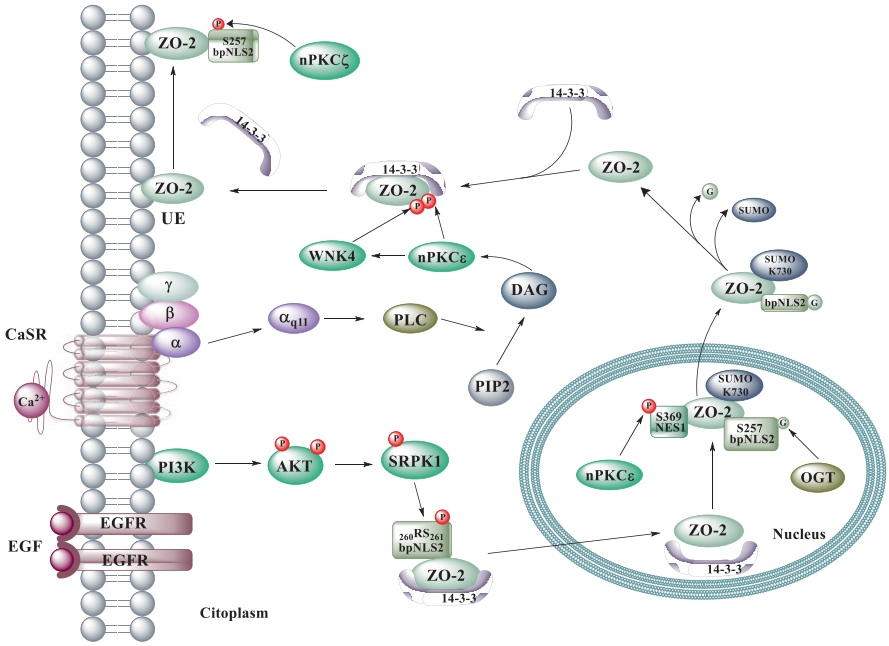

Fig. 3 NLS, NES, SR repeats, and posttranslational modifications, including phosphorylation, SUMOylation, and O-GlcNAcylation, regulate the intracellular traffic of ZO-2. In proliferating cells, activation of EGFR triggers a signaling cascade that leads to the phosphorylation by SRPK1 of SR repeats in ZO-2. In particular, the presence of S261 in bpNLS2 is required for the interaction between ZO-2 and 14-3-3 that facilitates the nuclear importation of ZO-2. ZO-2 nuclear exportation requires four intact NES, the phosphorylation of S369 in NES1 by nPKCε, the O-GlcNAcylation of S257 located in bpNLS2, and the SUMOylation of K730. The presence of extracellular Ca^{2+} activates the CaSR, which signals through $\alpha_{q/11}$ and PLC to activate nPKCε that phosphorylates cytoplasmic ZO-2 and WNK4. The latter also phosphorylates ZO-2 allowing it to separate from 14-3-3 and integrate at the plasma membrane to TJs. There, aPKCζ phosphorylates S257 as the TJ matures

adaptor protein STRAD [54]. Hence, it appears that the AMPK signaling is involved in both TJ assembly and cell polarization.

Although PKC stimulates adenylyl cyclase activity [55, 56], and activation of the latter with forskolin induces AMPK activation [53], the appearance of ZO-2 at the border of cells cultured in LC triggered by CaSR/PKC signaling does not require the indirect activation of AMPK [28]. However, cells cultured in NC might assemble ZO-2 at the TJ by activation of both the CaSR/G$\alpha_{q/11}$/nPKCε/WNK4 pathway and AMPK signaling triggered by LKB1 and adenylyl cyclase activation.

ZO-2 Impact on Epithelial Polarity and Three-Dimensional Organization

One of the canonical functions of TJs is their role as a fence that blocks that free diffusion of proteins within the membrane, from the apical to the basolateral surface and vice versa [57]. This way, the TJ maintains a polarized distribution of receptors, channels, pumps, and transporters, between the apical surface in contact with the content of cavities and ducts in the body and the basolateral membrane that faces the internal environment of the organism, allowing, in consequence, a vectorial transport across epithelial cell layers.

The view of TJs as critical players on membrane polarization is reinforced by observing that claudin-4 reconstitution into giant unilamellar vesicles by microfluidic jetting generates adhesive membrane interfaces sufficient to drive the partitioning of extracellular membrane proteins [58]. However, some years ago, it was surprisingly found that single intestinal epithelial cells display a polarized distribution of apical and basolateral markers upon activation of the serine/threonine kinase LKB1, which induced the precise localization of ZO-1 in a dotted circle peripheral to the apical brush border [54]. This observation raised the question of the role of ZO proteins in cell polarization.

In MDCK cells, ZO-1 is necessary for the formation of polarized cysts with single lumens in three-dimensional (3-D) cultures [59, 60], and in particular, the interaction of occludin with the U5-GUK domain of ZO-1 is crucial to avoid the formation of multiple lumens [60]. Likewise, MDCK ZO-2 KD and ZO-1/ZO-2 dKO cell cysts developed in 3-D cultures display an altered membrane polarity [6, 7] and multiple lumens [6, 9] (Fig. 4). Since ZO-2 heterodimerization with ZO-1 promotes the stretched conformation of the latter that exposes the U5-GUK domain to its ligands [18], it is possible that the absence of a single lumen in cysts of cells lacking ZO-2 appears as a result of a folded conformation of ZO-1 that cannot allow the interaction with occludin. These changes in polarity and lumen formation are not present in claudin quintuple KO cells, indicating that ZO-1/ZO-2 regulates epithelial polarity and 3-D cytoarchitecture in a manner independent of TJ strand formation [6].

In MDCK cells, the formation of cysts with multiple lumens also happens upon transfection with a ZO-2 S257E phosphomimetic mutation or the substitution of this residue by alanine (S257A) [61] (Fig. 4). The phosphorylation of this residue by aPKCζ occurs at the plasma membrane and develops as TJs mature, suggesting that this residue might be critical for the interaction of ZO-2 with other TJ proteins also involved in 3-D organization of epithelial cells [61].

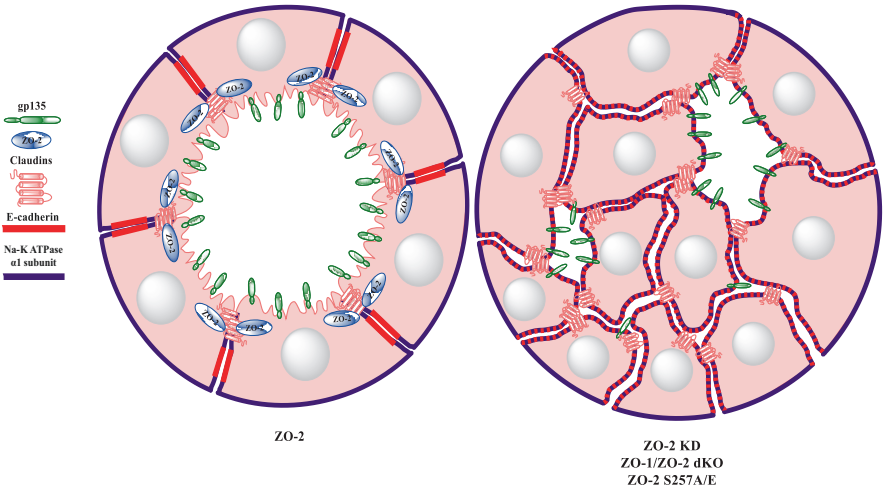

Fig. 4 ZO-1 KO, ZO-2 silencing, ZO-1/ZO-2 dKO, and ZO-2 mutation S257A/E alter the three-dimensional organization of epithelial cells. Epithelial cells, with a regular expression of ZO-1 and ZO-2, form cysts with a single lumen and a polarized epithelial membrane when grown on collagen or Matrigel. Instead, ZO-1 KO cells, ZO-2 KD cells, ZO-1/ZO-2 dKO cells, and cells expressing ZO-2 mutation S257A/E display a deficient polarization of membrane markers like E-cadherin and Na-K-ATPas e α1 subunit and exhibit multiple or non-expanding lumens

ZO-2 and Lipids

The observation that membrane lipids present in the exoplasmic leaflet of the membrane cannot move from the apical to the basolateral membrane when TJ's seal gave rise to the fence concept for proteins and lipids [62]. To explain this fence, a TJ lipid microdomain model proposed the formation of a lipid microdomain with an asymmetric organization between the exoplasmic and cytoplasmic membrane leaflets, induced by clustering of TJ membrane proteins [11].

Observations that highlight the importance of lipids for TJ assembly and stability include the following:

1. Sphingomyelin with long-chain fatty acids and cholesterol is enriched in the membrane fraction containing TJs [4].
2. Occludin and ZO-1 are present in lipid microdomains rich in cholesterol, from which they dissociate upon TJ disassembly by calcium chelation [63].
3. TJ sealing is altered by changes in the lipid composition of the cell. Thus, removal of cholesterol from the membrane triggers occludin phosphorylation [64], reduces TER [65], augments paracellular permeability [65], and diminishes occludin and ZO-1 staining at TJs [65]. In addition, cholesterol presence is essential for retaining claudins in the plasma membrane and the formation of TJs [4]. Also, a substantial reduction in very-long-chain ceramides that co-localize

with TJ complexes [66] increases intestinal permeability and triggers loss of ZO-1 in colon epithelium [67].
4. The palmitoylation of claudins [68] and JAM-C [69] promotes their location at TJs.

Moreover, multiple adaptor proteins of the TJ proteins like ZOs, PALS1, MAGIs, MUPP1 PATJ, Par-3, and Par-6 contain PDZ domains [70] that through their interaction with phosphoinositides (PIs), can function as lipid-binding modules (for review, see [71]). In ZO-1 and ZO-2, the second PDZ domain that binds lipids and forms a domain-swapping dimer displays a minor degree of specificity for PI: PI (3,4)P_2 over PI (3,4,5)P_3, PI (4,5)P_2, and PI (3,5)P_2 [72]. In ZO-1-PDZ-2 residues, K253, R201, R251, and K246 are involved in binding to the PI head group, whereas R193 and R194 also contribute but with non-specific electrostatic interactions [72]. Although ZO-1 and ZO-2 co-localize with membrane-bound PI (4,5)P_2, mutations of their abovementioned lipid-binding sites do not block their concentration at the plasma membrane, indicating that targeting of ZO-1 and ZO-2 to the cell border is not driven by lipid-mediated interactions [72].

At the nucleus, ZO-2 concentrates in nuclear speckles [27], where it co-localizes with PI (4,5)P_2 [72]. There, ZO-2 works as a scaffold for PIs since they disperse upon ZO-2 depletion [72].

Nuclear ZO-2

In 1996, ZO-1 was found at the nuclei of sparse epithelial cells in culture [73]. Although this observation has lately been questioned (for review, see [74]), it raised the interest to explore if other adaptor proteins of the TJ displayed the same behavior. In silico analysis revealed that the DNA sequences of ZO-1, ZO-2, and ZO-3; PALS1; MAGI-1, MAGI-2, and MAGI-3; Par-6; cingulin; symplekin; and ubinuclein contain both nuclear localization and exportation signals (NLS, NES) [75], thus suggesting that TJ adaptor proteins move between the nucleus and the plasma membrane.

ZO-2 presence at the nuclei is inversely related to the degree of cell-cell contact, as ZO-2 accumulated in the nuclei of cells in sparse cultures or present at the border of an inflicted wound [27] (Fig. 5). In addition, ZO-2 relocates to the nucleus following chemical stress with $CdCl_2$ and heat shock (42°C) [76] (Fig. 5). Likewise, E6 oncoprotein from high-risk human papillomavirus-16 (hrHPV-16) delocalizes ZO-2 from the TJ and triggers its accumulation at the nuclei and cytoplasm [77] (Fig. 5). This effect might contribute to cancer development since the forced concentration of ZO-2 at the nuclei due to the addition of SV40 NLS increases cell proliferation and the expression of M2 type of pyruvate kinase (M2-PK), a key enzyme of the glycolytic pathway associated with various cancers and malignancy degree [78].

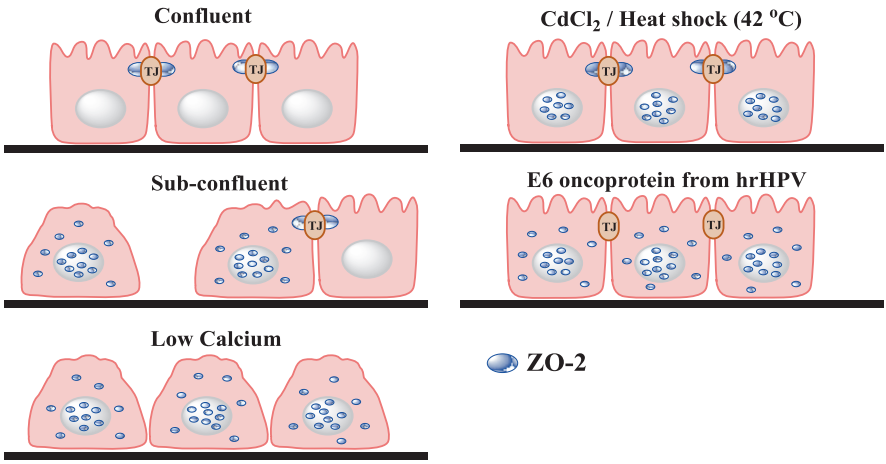

Fig. 5 Different conditions induce the nuclear accumulation of ZO-2. ZO-2 is abundant at the nucleus in sparse cultures, in monolayers incubated in LC (low calcium) condition, in cultures subjected to stress by treatment with $CdCl_2$ or heat shock, and in the presence of the oncogenic protein E6 from hrHPV

Treatment of epithelial cells with mimosine, which blocks cell cycle progression at the late G1 phase, or with nocodazole that arrests the cell cycle at the beginning of mitosis revealed that ZO-2 enters the nucleus at the late stages of G1 and departs at mitosis, thus explaining why in quiescent cells the nuclei are devoid of ZO-2, whereas cells in proliferation display a strong ZO-2 signal at the nucleus [79].

Upon ZO-2 transfection into MDCK cells cultured in NC, around 80% of the cells express the protein at the nucleus at the earliest times after transfection, but this percentage diminishes with time as the monolayer becomes confluent [80]. This initial arrival of ZO-2 to the nucleus only takes place in sparse cultures. In confluent monolayers, instead, blocking the nuclear exportation of ZO-2 does not induce the nuclear accumulation of the protein, indicating that only in sparse cultures ZO-2 travels to the nucleus [61].

A nuclear microinjection assay, in which the nucleus served as a container of anti-ZO-2 antibody, further showed that endogenous newly synthesized ZO-2 goes first to the nucleus and then travels to the plasma membrane [80]. Interestingly, of all the newly synthesized ZO-2, a much higher amount reaches the nucleus than eventually the plasma membrane, making the nucleus a cellular reservoir of ZO-2 [80].

ZO-2 is present in the nuclear matrix in association with lamin B1 [81]. In the nuclear speckles, ZO-2 co-localizes with SAF-B [76] that organizes chromatin [82] and acts as a transcriptional repressor (for review, see [83]) and SC-35 involved in alternative processing of pre-mRNA [27]. These interactions suggest that ZO-2 is a protein of the transcriptosome involved in a process that couples the organization of chromatin, transcription, and RNA processing (Fig. 6).

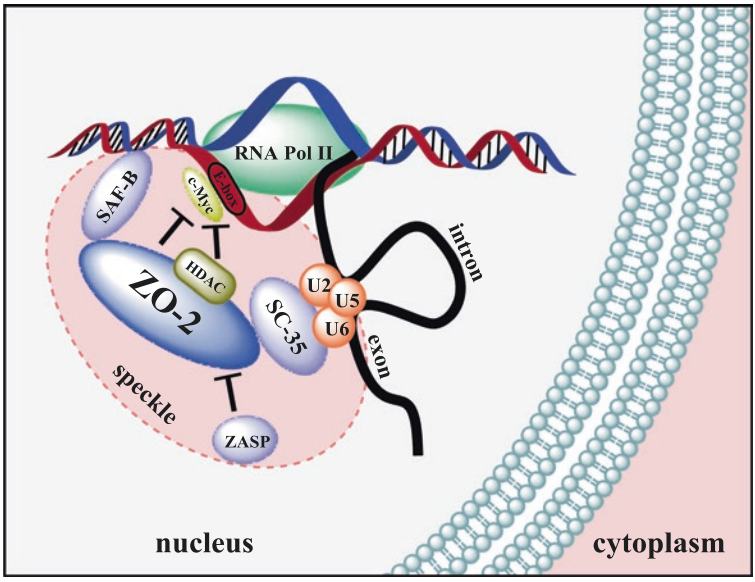

Fig. 6 ZO-2 is a protein of the transcriptosome. At the nucleus, ZO-2 is present in speckles where it associates with SAF-B, an organizer of chromatin and transcriptional repressor; histone deacetylase (HDAC); SC-35, a critical factor in the alternative processing of pre-mRNA; and transcription factors like c-Myc, suggesting its participation in a process that involves chromatin organization, gene transcription, and RNA processing

NLS, NES, and SR Repeats in ZO-2 and Their Post-Transcriptional Regulation

NLSs are present in ZO-2 DNA sequences derived from humans, dogs, mice, and chickens, whereas NESs are present in all these sequences except for humans [75]. Canine ZO-2 sequence exhibits four NESs, 2 at the PDZ-2 domain and the other 2 at the GuK domain [84], whereas the U2 segment displays 2 bipartite (bp) and a monopartite NLS and 16 serine-arginine (SR) repeats that localize the protein to the nuclear speckles [61] (Fig. 7). These signals were tested following the intracellular movement of reporter protein ovalbumin chemically coupled to peptides homologous to the NES [81, 84]/NLS [61] of ZO-2 upon microinjection into the nucleus/cytoplasm of epithelial cells. The nuclear export assay revealed the functionality of each NES of ZO-2. However, transfection of cells with ZO-2 carrying a single mutated NES blocked an efficient ZO-2 nuclear exportation, indicating that the presence of the four intact NESs in ZO-2 is required to move ZO-2 out of the nucleus [84]. In addition, the nuclear export assay showed that ZO-2 NES-1 required a phosphomimetic mutation in S369 to be functional [84]. Accordingly, further studies revealed that the departure of ZO-2 from the nucleus needed the phosphorylation of S369 by nPKCε [80] (Fig. 3).

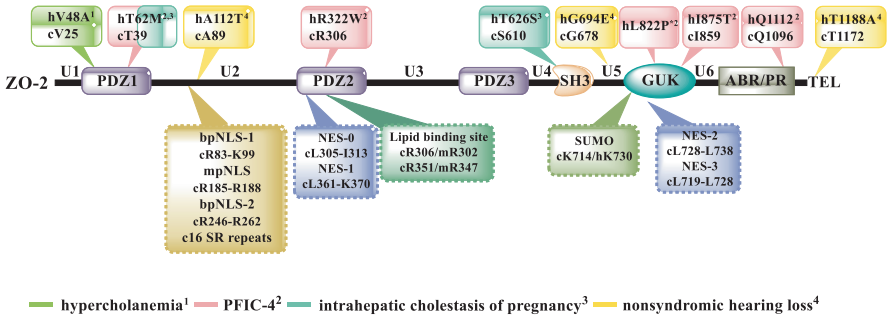

Fig. 7 Schematic representation of ZO-2 indicating the location of NLS, NES, and SR repeats and mutations that lead to liver damage and hearing loss. h, human; c, canine; bp, bipartite; mp, monopartite; PFIC-4, progressive familial intrahepatic cholestasis type 4; *, mutation present only in hZO-2 isoform 3

In ZO-2, the SUMOylation of K730 located a few residues upstream of NES-2 in the GuK domain regulates the nuclear exportation of the protein [8]. Mutation of this site to arginine results in a prolonged nuclear localization of ZO-2 as the cell culture becomes confluent, while transfected ZO-2 constitutively SUMOylated localized in the cytoplasm, suggesting that ZO-2 SUMOylation happens in the nucleus and promotes the exportation of the protein [8] (Fig. 3).

A nuclear importation assay with reporter protein ovalbumin revealed that while the bpNLS-1 of ZO-2 was functional, bpNLS-2 was not [61]. Further analysis showed that phosphomimetic mutations in ZO-2 serine residues 257, 259, and 261, present within a cluster of three arginine residues in the carboxyl part of bpNLS-2, delayed the nuclear importation of the protein. This observation suggested that bpNLS-2 is functional, but its positive charges were neutralized in the nuclear importation assay by the phosphorylation of serine residues 257, 259, and 261, which are putative PKC phosphorylation sites [61]. It is noteworthy that ZO-2 S261 present in bpNLS-2 is also a crucial binding site for 14–3-3, required for the efficient importation of the ZO-2/14-3-3 complex into the nucleus [28] (Fig. 3). S257 is also an interesting residue, as a Yin-Yang site that can be competitive and alternatively occupied by an O-phosphate and an O-GlcNAc. ZO-2 S257 is phosphorylated by aPKCζ, whereas the O-GlcNAcylation of ZO-2 at the nucleus triggers the nuclear exportation of the protein (Fig. 3). However, a treatment that inhibits an enzyme that removes O-GlcNAc from proteins induces ZO-2 proteasomal degradation in the cytoplasm [61].

The cluster of SR repeats observed in ZO-2 is also present in SR proteins that function as splicing factors [85], where the kinases SRPK phosphorylate the SR motifs (for review, see [85]). ZO-2 has in its U2 segment an SRPK1 docking motif and in the cytoplasm associates with SRPK1 [61]. Moreover, over-expression of SRPK1 induces hyperphosphorylation of ZO-2 and triggers the latter's movement into the nucleus in sparse cultures but not in confluent monolayers. In the former, epidermal growth factor (EGF) induces ZO-2 nuclear importation by inducing a

signaling cascade that, through AKT, triggers the activation of SRPK1, which promotes the serine phosphorylation of ZO-2 and its concentration in nuclear speckles [61]. In ZO-2 bpNLS1, S261, present in an SR repeat, is critical for 14-3-3 interaction and the nuclear importation of ZO-2 [28]. Therefore, the signaling cascade triggered by EGF that leads to ZO-2 nuclear importation might include the phosphorylation of S261 by SRPK1 (Fig. 3).

ZO-2 as a Regulator of Gene Transcription

The presence of ZO-2 in nuclear speckles, in association with proteins of the transcriptosome, posed the query of the possible participation of ZO-2 in gene transcription through its interaction with transcription factors [27]. Employing various assays, including pull-downs, gel-shifts, co-localizations, and immunoprecipitations, the nuclear association of ZO-2 with Jun, Fos, and C/EBP (CCAAT/enhancer-binding protein) transcription factors was discovered [86]. Unexpectedly, these studies also revealed that at the plasma membrane, these transcription factors also interacted with ZO-2 [86].

Previously, ZO-1 sequestration of the Y box transcription factor ZONAB/DbpA at the TJ was proposed as a mechanism to regulate its nuclear content [87]. However, this was questioned when the SH3 domain of ZO-1, but not full-length ZO-1, interacted with ZONAB/DbpA [88]. This apparent inability of in vitro-translated ZO-1 to bind ZONAB/DbpA might nevertheless be explained by a lack of intermolecular interactions that lead ZO-1 to acquire a closed conformation that limits the access of ZONAB/DbpA to the central GUK-SH3 module of ZO-1 (for review, see [89]). Accordingly, it has been shown that ZO-1 heterodimerization with ZO-2 promotes the stretched conformation of ZO-1 that allows its interaction with ZONAB/DbpA, whereas loss of either actin filament organization or myosin activity in cells that lack ZO-2 results in a folded conformation of ZO-1 where the GUK-SH3 module cannot associate with ZONAB/DbpA [18]. Since junctional ZONAB/DbpA diminishes by the depletion of both ZO-1 and ZO-2, and proteasomal inhibition reverts this effect [88], it appears that ZO proteins can regulate gene transcription by providing junctional retention and stability of transcription factors.

ZO-2 Regulation of AP-1 Sites

ZO-2 inhibits gene transcription of artificial promoters regulated by AP-1 sites [86]. However, when the human cyclin D1 (CD1) promoter was tested in a reporter gene assay, ZO-2 blocked gene transcription but not by interaction with the AP-1 site but by association with transcription factor c-Myc that interacted with an E-box present in CD1 promoter and through the recruitment of histone deacetylase 1 [90]. The inhibitory activity of ZO-2 on CD1 transcription and protein expression is blocked

by the over-expression ZASP, a speckle protein that, through its PDZ-binding motif, associates with the PDZ-3 domain of ZO-2 [91] (Fig. 6).

ZO-2 Regulation of β-Catenin/TCF Signaling

ZO-2 over-expression diminishes the endogenous mRNA for *Axin-2*, a target of the WNT pathway [79]. Accordingly, ZO-2 represses gene transcription regulated by β-catenin/TCF-4 signaling in reporter gene assays using both the artificial TOPflash/FOPflash promoter system and the promoter for *Siamois*, another WNT target [79]. Consequently, in ZO-2 KD cells, the transcriptional activity of TOPflash/FOPflash system is much higher than in parental cells [92]. The nuclear presence of ZO-2 is critical for gene repression, as a construct mimicking the constitutive SUMOylation of ZO-2 that concentrates in the cytoplasm exerts no effect. In contrast, a ZO-2 construct that concentrates in the nucleus due to the addition of a viral NLS completely inhibits β-catenin/TCF-4-mediated transcription [8]. Surprisingly, these effects can also be observed using a TCF/LEF construct with a deleted β-catenin-binding site, thus suggesting that ZO-2 can also mediate transcriptional repression regulated by TCF in a manner independent of β-catenin [8]. However, since ZO-2 and β-catenin form a complex [8], it is tempting to speculate that their interaction might somehow serve to induce transcriptional repression when the TCF/LEF construct with a deleted β-catenin-binding site was employed.

The ZO-2 effect was also tested in vivo in a murine model of podocyte dysfunction induced by the antibiotic Adriamycin (ADR) that activates the WNT signaling pathway. ZO-2 over-expression, by hydrodynamic transfection, reversed in the glomerulus the effects of ADR, including the decrease in phosphorylated β-catenin and nephrin content and the increase in snail transcription factor and restrained podocyte effacement and proteinuria. It is, however, not known how much of these effects were due to ZO-2 inhibition of gene transcription regulated by β-catenin/TCF signaling or by ZO-2 blockade of the inhibitory phosphorylation of GSK-3β [93].

ZO-2 and Transcription Regulated by YAP Coactivator and Transcription Factors TEAD and RUNX2

Transcriptional enhanced associate domain (TEAD) transcription factors that play an important role in tumor progression, metastasis, cancer metabolism, and drug resistance (for review, see [94]) employ Yes-associated protein (YAP), the final effector of the Hippo pathway, as a coactivator. Since YAP concentrates at the nuclei of ZO-2 KD cells, it became of interest to study the impact of the absence of ZO-2 on transcription by a reporter regulated by artificial TEAD-binding sites and on the promoter of the connective tissue growth factor (CTGF), a direct target gene of

YAP/TEAD. Results revealed a higher transcriptional activity of these promoters in parental than ZO-2 KD cells and showed that ZO-2 transfection blocked the activity of these promoters in both parental and ZO-2 KD cells [92].

ZO-2 also has an inhibitory effect on the reporter gene assay of the osteocalcin gene, regulated by YAP2 and runt-related transcription factor 2 (RUNX2) [95]. The latter binds DNA at a consensus RUNX motif and acts as a transcriptional activator or repressor depending on additional binding factors and posttranslational modifications (for review, see [96]). Altered expression of RUNX2 associates with osteosarcoma development and breast and prostate bone metastasis (for review, see [96]).

ZO-2 as a Translocator of Proteins to the Nucleus

ZO-2 is an adaptor protein with multiple protein-protein interaction domains. This characteristic has allowed ZO-2 to act as a platform capable of translocating to the nucleus, the AJ protein ARVCF (armadillo repeat gene deleted in velo-cardio-facial syndrome), the transcriptional coactivator YAP2, one of the three isoforms of YAP, and the transcription factor TEAD (Fig. 8).

ARVCF, which binds to ZO-1 and E-cadherin at the plasma membrane, localizes to the nucleus upon disruption of cell-cell adhesion. Nuclear localization of ARVCF requires the interaction with ZO-2 between ARVCF PDZ-binding motif and the amino segment of ZO-2 and relies on the NLS of ZO-2, thus indicating that ARVCF

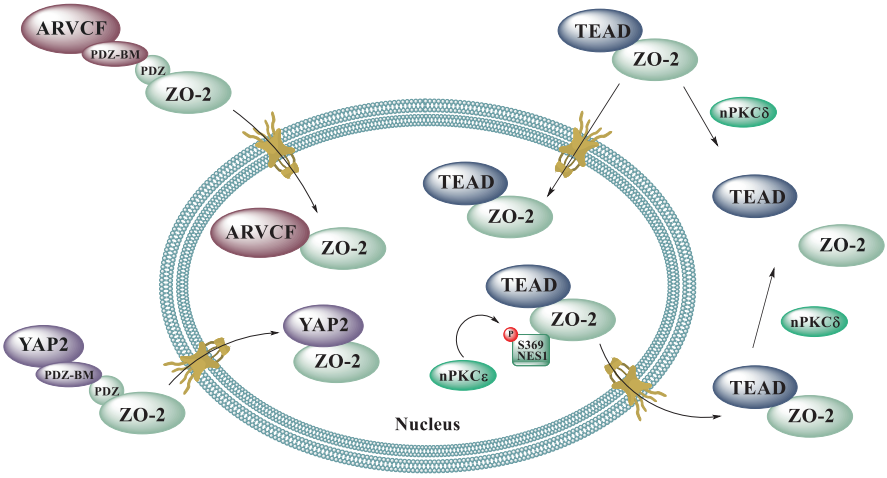

Fig. 8 ZO-2 acts as a translocator of proteins to the nucleus. The interaction between the PDZ-binding motifs (PDZ-BM) of ARVCF and YAP2 to ZO-2 PDZ domains allows ARVCF and YAP2 to enter the nucleus. ZO-2 and the transcription factor TEAD associate with the cytoplasm. This interaction, negatively regulated by nPKCδ, favors their nuclear importation as a TEAD/ZO-2 complex. Activation of ZO-2 NES-1 by nPKCε phosphorylation of S369 favors the nuclear exportation of TEAD

enters the nucleus through a piggyback mechanism with ZO-2 [97]. A similar situation is found with YAP2, which through its C-terminus PDZ-binding motif, associates with the PDZ-1 domain of ZO-2 [95]. ZO-2 over-expression induces YAP2 nuclear localization and requires the presence of the U2 segment containing the NLS of ZO-2 [95].

The subcellular localization of TEAD regulates its transcriptional activity. Thus, environmental factors, including osmotic stress, high cell density, and cell suspension, induce the cytoplasmic translocation of TEAD [98]. Likewise, ZO-2 modulates the localization of TEAD in epithelial cells [99]. Inhibition of nPKCδ induces ZO-2 and TEAD interaction in the cytoplasm, which allows their importation into the nucleus as a complex. Instead, the activation of ZO-2 NES-1 by nPKCε phosphorylation promotes TEAD nuclear exportation (Fig. 8). These observations provide a novel mechanism through which ZO-2 regulates YAP/TEAD-mediated transcription.

ZO-2 as a Regulator of Cell Size

An unexpected outcome of ZO-2 silencing in epithelial MDCK cells was finding that most cells displayed a diameter of 55–60 μm instead of the 35–40 μm regularly present in parental cells [92]. This change of cell size was not the result of an off-target effect of shRNAs, as ZO-2 transfection to ZO-2 KD cells restored regular cell size. The 52% higher protein/DNA ratio observed in ZO-2 KD cells, in comparison to parental cells, suggested that cells were experimenting an increase in physical growth in the early G1 phase of the cell cycle, without a subsequent entry into the S phase, and in effect, ZO-2 KD cells moved into the S phase at a slower pace. This slow transition into the S phase might reflect an imbalance between CD1 and cyclin E, as the lack of ZO-2 increases the cellular content of CD1 [92].

A second mechanism involved in developing hypertrophy in cells lacking ZO-2 involves the decrease in YAP phosphorylation and its nuclear concentration and activity, promoting the transcription of the catalytic subunit of PI3K and the expression of miR-29 that inhibits the translation of PTEN phosphatase [92]. Together, these changes generate an increase in PIP_3 and the subsequent activation of the AKT/mTORC1/S6K1 signaling pathway, leading to increased protein synthesis and cell hypertrophy.

Interestingly, in compensatory renal hypertrophy, where a uninephrectomy triggers an increase in size in the remaining kidney, cells in the proximal tubule augment their size, decrease ZO-2 expression, and increase the nuclear content of YAP [92]. These observations confirm ZO-2 role as a cell size modulator by inhibiting YAP translocation to the nucleus, which leads to the activation of the AKT/mTORC1/S6K1 signaling pathway.

Tissues Where ZO-2 Plays Crucial Roles at Tight Junctions

ZO-2 at the Testis

ZO-2$^{(-/-)}$ embryonic stem cell injection into wild-type blastocysts generated viable chimeric mice [24]. However, adult chimeric males displayed reduced fertility with seminiferous tubules of smaller diameter that sometimes lacked spermatogenesis. The analysis of the blood-testis barrier (BTB) proteins in the chimeras revealed that ZO-1, ZO-3, claudin-11, and occludin were not affected. Likewise, ZO-1 and occludin maintained the expected localization at the basal section of the tubules, while claudin-11 staining spread into the adluminal region. In the ZO-2 chimeras, the paracellular marker lanthanum diffused into the adluminal space between Sertoli cells, indicating a compromised BTB [24]. Hence, these observations indicated that ZO-2 plays a unique role in the adult testis that other TJ proteins cannot replace.

Methamidophos is an organophosphate pesticide extensively used in agriculture in numerous countries, diminishing fertility in male human field workers [100]. In mice, treatment with methamidophos opens the BTB and perturbs spermatogenesis [101]. Mass spectrometry revealed that methamidophos forms covalent bonds with ZO-2 present in seminiferous tubules. These modifications formed in lysine residues might affect the acetylation and ubiquitination of ZO-2, whereas adducts formed in serine, threonine, and tyrosine residues could impact ZO-2 signaling normally triggered by phosphorylation. Accordingly, methamidophos treatment reduced the interaction of ZO-2 and occludin in the testis. Likewise, the TER diminished in MDCK cells transfected with ZO-2 lacking a methamidophos target residue, which is also a ubiquitination site [101].

ZO-2 at the Liver

The crucial role of ZO-2 in the liver was first hinted by the presence of a familial hypercholanemia, characterized by a high concentration of bile acids in serum, itching, and fat malabsorption, in a group of Amish people with mutation V48A in the *TJP2* gene that encodes ZO-2 [102] (Fig. 7). This mutation reduced the stability of the ZO-2 PDZ-1 domain and, when tested in vitro, diminished binding of ZO-2 PDZ-1 module to C-terminal PDZ-binding motifs of claudins [102]. These observations suggest that V48A substitution blocked the proper function of ZO-2, allowing liver TJs to leak bile acids from the canalicular lumens into plasma.

Progressive familial intrahepatic cholestasis (PFIC) was known to be the result of mutations in genes encoding proteins involved in bile formation [103]. Thus, type 1 PFIC is due to mutations in *ATP8B1* that encodes the aminophospholipid flippase FIC1; type 2 to mutations in *ABCB11*, which encodes the bile salt export pump (BSEP); and type 3 to mutations in *ABCB4* coding for the multidrug resistance

P-glycoprotein MDR3. Now, mutations in *TJP2* are recognized as the cause of PFIC classified as type 4.

A study of adults with cryptogenic cholestasis found three mutations in *TJP2* characterized as highly pathogenic, according to an in silico analysis that evaluates their influence on the structure and function of the protein [85]. These mutations identified as T62M, R322W, and I875T localized in PDZ-1, PDZ-2, and GuK domains of ZO-2 (Fig. 7). In a teenager with type 4 PFIC, the additional compound heterozygous L822P mutation in ZO-2 was reported [104] (Fig. 7). Likewise, in a consanguineous family where both parents carry the heterozygous *TJP2* variant 3334C > T that likely induces nonsense-mediated decay and degradation of ZO-2, the state of their adult children was analyzed [105]. Of five siblings with the variant in a homozygous state, two had cirrhosis and hepatocellular carcinoma (HCC), and three others only displayed elevated liver enzymes without cirrhosis. In addition, five sisters had intrahepatic cholestasis of pregnancy that was severe or mild according to their homozygous and heterozygous states, respectively [105]. This pregnancy-specific liver disease, characterized by pruritus, hepatic impairment, and elevated serum bile acids, can give rise to adverse pregnancy outcomes, including preterm labor, prolonged neonatal intensive care, and third-trimester intrauterine death [106]. Other heterozygous mutations in *TJP2* (T62M and T626S) have also been observed in other patients with intrahepatic cholestasis of pregnancy [106] (Fig. 7).

Homozygous [103, 107, 108] and compound heterozygous [109, 110] mutations in *TJP2* that alter the reading frame and generate premature terminator codons are present in children with severe PFIC that in some cases has led to HCC requiring early liver transplantation [108, 109]. A substantial reduction in the *TJP2* mRNA level by nonsense-mediated mRNA decay (NMD) and no ZO-2 expression was found in these livers. Instead, claudin-1 was expressed but failed to localize at the TJ region, whereas claudin-2 displayed a standard pericanalicular staining [103, 107]. The lack of ZO-2 in these children only causes significant disruption in cholangiocyte-cholangiocyte borders and biliary canaliculus margins, suggesting that ZO-2 is essential for liver TJs in humans, whereas in other tissues, ZO-2 function can be substituted by other TJ proteins.

In mice, liver-specific deletion of *TJP2* does not generate an overt disruption of the blood-bile barrier (BBiB). However, it results in lower claudin-1 protein levels and mild progressive cholestasis accompanied by a lower expression level of bile acid transporter Abc11/Bsep and detoxification enzyme Cyp2b10 [111]. Mice lacking *TJP2* are, however, more susceptible to liver injury, as a cholic acid diet tolerated by control mice-induced cholestasis and liver necrosis in them [111]. Instead, in ZO-1/ZO-2 double-mutant mice, the BBiB is disrupted, and the animals die by 6 weeks of age [112]. The hepatocytes in these animals fail to form TJs and to establish cellular polarity. In addition, the expression and localization of transporters were disorganized. Altogether, these findings suggest that ZO-2 is important for the BBiB in mice but is not as critical and irreplaceable as for humans. Instead, ZO-1 and ZO-2 are redundant in mice liver, requiring inactivation of both proteins to disrupt the hepatic barrier.

ZO-2 at the Inner Ear

At the inner ear, the TJs present between the sensory epithelial hair cells and in the *stria vascularis* are crucial to keep a barrier between K^+-rich endolymph and low K^+ perilymph, which allows the formation of the endocochlear potential. Accordingly, mutations in several TJ proteins lead to hearing loss [113–115]. In *TJP2*, two pathogenic mutations, A112T and T1188A, and ten single nucleotide polymorphisms (SNPs) were uncovered in a genetic screen of the Korean population, with nonsyndromic hearing loss [116] (Fig. 7), and in a Chinese family with unconditional autosomal dominant nonsyndromic hereditary hearing impairment, the pathogenic missense mutation G694E was identified [117] (Fig. 7).

In mice cochlea, ZO-2 localizes at the membranes connecting the hair cells and supporting Deiters cells, both at the apical edge and along the basolateral side [118], since in the inner ear TJs and AJs form a combined structure [119]. ZO-2 expression diminishes with age in mice inner ear, being the level in the adult mice around 50% to that at birth. In humans, the dominant, adult-onset, progressive nonsyndromic hearing loss DFNA51 is due to a tandem inverted genomic duplication of the wild-type gene *TJP2* [118]. In the lymphoblast of individuals carrying the duplication compared to non-carrying relatives, the endogenous expression of *TJP2* mRNA and ZO-2 protein augments 1.7-fold and twofold, respectively. This change is concurrent with a decrease in the inhibitory phosphorylation of GSK-3β. Since the activity of GSK-3β promotes apoptosis, the expression of genes related to apoptosis was tested, finding a pro-apoptotic expression profile [118]. Therefore, since hair cells of the inner ear are non-regenerative, it was hypothesized that ZO-2 over-expression induced hearing loss through an increase in the susceptibility of the inner ear to apoptosis mediated by GSK-3β activation.

In a search for genes linked to hearing loss, random mutations were introduced with ethylnitrosourea in mice. A mutation in *Usp53* was identified as the cause of progressive hearing loss [120]. *Usp53* codes for a catalytically inactive deubiquitinating enzyme present in cochlear hair cells and in a group of supporting cells, where it co-localizes with ZO-1 and ZO-2. In these animals, hair cells degenerate after the first post-natal week, but this process can be rescued in organotypic cultures in low K^+ medium. These observations suggest that the TJ barrier that separates endolymph from perilymph is compromised and that a disorder in the ubiquitination of ZO proteins in the cochlea could be implicated.

ZO-2 and Cancer

During epithelial transformation, TJs are reorganized or lost. This process involves a change in the expression pattern of some proteins like claudins and the silencing of others, including the ZO proteins (for reviews, see [121, 122].

Epithelial cells have two isoforms of ZO-2 named A and C, transcribed at comparable levels [123]. Transcription of these isoforms is driven by upstream promoter P_C and downstream promoter P_A. The longer cDNA of ZO-2C starts with a unique 377-bp non-translated region, and the first ATG of this cDNA corresponds to the second ATG in ZO-2A cDNA. The shorter cDNA of ZO-2A has a unique 189-bp sequence at the 5`-end with the first ATG in ZO-2A. The two proteins encoded by these cDNAs are different in that ZO-2A contains 23 amino acid residues at the amino terminus, not present in ZO-2C [124]. In the ductal type, pancreatic adenocarcinoma ZO-2A is absent through a process that does not involve mutations, lack of transcription factors, or methylation of the immediate promoter region [123, 125]. In breast cancer, where the level of ZO-2 is downregulated [126], both isoforms are regularly silenced [123]. In contrast, isoform ZO-2A is rarely absent in prostate and colon cancer [123]. However, other studies reported that the *TJP2* gene that codes for ZO-2 is hypermethylated in prostate cancer cell lines [127], while treatment of prostate cancer cells with hepatocyte growth factor (HGF), a promoter of tumor progression and metastasis, decreases the expression of ZO-2 and induces its redistribution away from the TJs [128] (Fig. 9).

ZO-2 expression also diminishes in a hypoxia-resistant cancer cell line derived from scirrhous gastric carcinoma [129] and HCC cell lines and specimens [130]. In testicular carcinoma in situ [131] and bronchopulmonary cancer [132], ZO-2 expression decreases (Fig. 9) and displays a preferential localization in the cytoplasm. In the latter, this localization correlates with invasion and is associated with an increase of membrane-type 1 matrix metalloproteinase (MT1-MMP) [132]. Likewise, in lung adenocarcinoma A549 cells, ZO-2 silencing increases cell migration and the activity of MMP-2 [133] (Fig. 9). In these lung cells, where claudin-18 expression is downregulated, the ectopic expression of claudin-18 increased the level of ZO-2 [133].

In some carcinomas, the over-expression or redistribution of claudins alters the interaction between ZO-2 and YAP1, promoting the nuclear translocation and transcriptional activity of YAP1. In HCC, the expression of claudin-6, a protein found in embryonic stem cells but not in normal tissues, is associated with multidrug resistance [130]. The mechanism is competition between claudin-6 and YAP1 for the association through their respective PDZ-binding motifs with the PDZ-1 domain of ZO-2. Thus, upon claudin-6 over-expression, the amount of YAP1 that co-immunoprecipitates with ZO-2 diminishes, allowing cytoplasmic YAP1 to translocate to the nucleus and induce a transcriptional program that triggers a phenotypic shift of HCC cells from a hepatic lineage to a biliary lineage that is more refractory to sorafenib [130], an inhibitor of multiple kinases involved in tumor cell proliferation and angiogenesis [134] (Fig. 9). These observations are in line with our previous results showing that ZO-2 silencing in epithelial MDCK cells decreased YAP phosphorylation and augmented its nuclear concentration and transcriptional activity, whereas ZO-2 transfection blocked in parental and ZO-2 KD cells, the transcription of genes regulated by TEAD-binding sites [92] (Fig. 9).

The presence of claudin-4 at the nucleus in oral squamous cell carcinoma (OSCC) correlates with cancer progression and is found frequently in the tissue

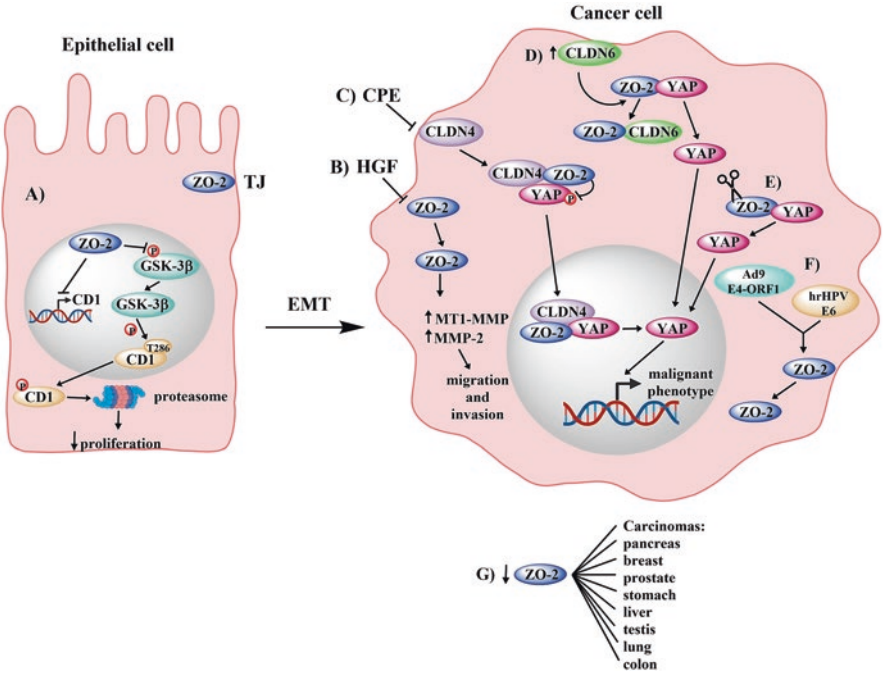

Fig. 9 ZO-2 inhibits cell proliferation, is downregulated in multiple carcinomas, and is a target of oncogenic viral proteins. (*A*) In epithelial cells, ZO-2 inhibits cell proliferation by blocking CD1 transcription and GSK-3β inhibitory phosphorylation leading to CD1 proteasomal degradation. (*B*) HGF induces ZO-2 delocalization from the membrane, which augments MT1-MMP and MMP-2 concentration leading to migration and invasion. (*C*) CPE triggers the nuclear translocation of claudin-4 in a complex with ZO-2 and YAP that favors YAP transcriptional activity. (*D*) Claudin-6 liberates YAP1 from ZO-2, allowing its nuclear translocation and gene transcriptional activity that leads to a malignant phenotype. (*E*) ZO-2 silencing increases YAP transcriptional activity. (*F*) Oncogenic proteins E4-ORF1 from adenovirus 9 (Ad9) and E6 from hrHPV target ZO-2 and delocalize it from the plasma membrane. (*G*) ZO-2 expression diminishes in numerous carcinomas

positive for *Clostridium perfringens* [135]. Accordingly, treatment of OSCC cell lines with *Clostridium perfringens* enterotoxin (CPE) induces claudin-4 nuclear translocation and the formation of a complex of YAP1, claudin-4, and ZO-2 that suppresses YAP1 phosphorylation and promotes the expression of YAP1 target genes that favor the development of a malignant phenotype [135] (Fig. 9). ZO-2 sequestration from the TJ in a complex with YAP1 and claudin-4 might favor cancer progression and relate to effects triggered by some cancer-inducing virus. Thus, the oncogenic E4 region-encoded ORF1 (E4-ORF1) of adenovirus type 9, which elicits mammary tumors in animals, associates through its C-terminal PDZ-binding motif with the first PDZ domain of ZO-2, resulting in aberrant sequestration of ZO-2 within the cytoplasm that is key for the tumorigenic properties of this virus [136] (Fig. 9). Moreover, an analysis of the targets of the C-terminal PDZ-binding motif of E6 protein from HPV revealed that while hDlg is a common target of all HPV E6

proteins, regardless of their oncogenic potential, only the PDZ-binding motif of E6 from hrHPV-16, hrHPV-18, hrHPV-31, hrHPV-51, hrHPV-56 and the possibly carcinogenic HPV-70 associate with ZO-2 [137]. In MDCK cells, transfection with E6 from HPV-16 blocks ZO-2 degradation and triggers its translocation away from the cell borders into the cytoplasm and nucleus [77] (Fig. 9). Accordingly, in transgenic mice expressing HPV-16 E6, ZO-2 is over-expressed [77], whereas, in HeLa cells, the ablation of HPV-18 expression diminishes ZO-2 expression [137]. These results highlight that sequestration of ZO-2 away from the TJ, more than the amount of ZO-2 present in the cell, is critical for cell transformation to develop.

ZO-2 can be considered a potential tumor suppressor due to its silencing in cancerous tissues, because it inhibits cell proliferation, and is a target of oncogenic viral proteins. Thus, in epithelial MDCK cells, ZO-2 over-expression inhibits CD1 protein expression and cell proliferation and blocks cell cycle progression from G0/G1 into S phase but exerts no effect in apoptosis. Remarkably, in synchronized cultures, ZO-2 transfection does not diminish the level of CD1 mRNA. Instead, ZO-2 overexpression decreases the amount of CD1 protein by proteasomal degradation [79]. ZO-2 interacts with GSK-3β, and the cytoplasmic but not the nuclear fraction of ZO-2 decreases the inhibitory phosphorylation of GSK-3β at S9 [8]. Consequently, GSK-3β can phosphorylate CD1 at T286, inducing CD1 nuclear exportation, ubiquitination, and proteasomal degradation [79] (Fig. 9).

ZO-2 is also proposed as a potential diagnostic marker of certain cancers, including the bladder, where ZO-2 in urinary extracellular vesicles is upregulated in bladder cancer patients [138]. The same suggestion relies on a transposable element in the *TJP2* gene (*TJP2*-Alu transcript), which is more abundant in colorectal cancer than in the normal tissue [139].

Future Directions

The view of ZO-2 has greatly expanded since three decades ago the protein was discovered in a ZO-1 immunoprecipitate. Uncovering the signaling cascade that regulates how ZO-2 moves and integrates into TJs revealed the existence of a highly tuned intracellular traffic of the protein modulated by multiple posttranslational modifications, of which we have only started to scratch the surface. The studies reporting phase separation of ZO proteins open a new window of research where the interaction of ZO-2 with several ligands including lipids should be tested. The presence of ZO-2 at the nucleus and its role as transcriptional repressor and translocator of proteins incites the search for novel mechanisms modulating signaling pathways like Wnt and Hippo. Finally, the altered expression of ZO-2 observed in cancerous tissues highlights the need of testing the role of ZO-2 as a tumor suppressor protein.

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Modulation of Intestinal Disorders by Claudin-2 and Occludin Via Canonical and Noncanonical Mechanisms

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Abstract The past 35 years, beginning with the identification of ZO-1 in 1986, have been an exciting time during which critical tight junction proteins were discovered. We have, however, only begun to define the mechanism by which tight junctions are regulated, their impact on health and disease, and noncanonical functions of individual tight junction-associated proteins. Here, we provide an overview of advances in understanding mechanisms of tight junction barrier regulation within the intestinal epithelium and discuss recent discoveries related to claudin-2 and occludin in greater detail. We anticipate that the next 35 years will yield major advances in fundamental understanding of tight junction protein interactions, regulation, and canonical and noncanonical functions that result in translational applications in which tight junction modulation is established as a therapeutic approach.

Keywords Intestinal permeability · Intestinal barrier · Tumor necrosis factor · IL-13 · IL-22 · Inflammatory bowel disease · Tight junction

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L. González-Mariscal (ed.), *Tight Junctions*,
https://doi.org/10.1007/978-3-030-97204-2_5

Abbreviations

CK2	Casein kinase 2
DSS	Dextran sulfate sodium
FRAP	Fluorescence recovery after photobleaching
MLC	Myosin II regulatory light chain
MLCK	Myosin light -chain kinase
TER	Transepithelial electrical resistance
TNBS	Trinitrobenzenesulfonic acid
TNF	Tumor necrosis factor

Introduction

The viability of multicellular life forms requires the presence of barrier-forming tissues that partition distinct compartments within the organism and separate the external environment from the internal milieu. In mammals, examples of surfaces that interact with the external environment include the skin as well as oral, gastrointestinal, and genitourinary mucosae. At each of these sites, the tight junction creates a selectively permeable seal that restricts flux across the paracellular space between cells. Tight junctions in the skin are nearly impermeant, and, in burn patients, the extent of epidermal barrier loss is a strong prognostic indicator of outcome. The gastrointestinal epithelium, particularly within the distal gut, interfaces with a dense and complex luminal microbiome that can include invasive pathogens as well as potentially harmful bacterial components and metabolites. Dysregulated systemic exposure to these luminal materials, which could lead to immune-mediated, metabolic, and infectious diseases, emphasizes the essential nature of the intestinal epithelial barrier. However, in contrast to the skin, the intestinal barrier must allow paracellular flux of water, ions, and nutrients. The charge and size selectivity of this barrier are determined by the tight junction, which is the rate-limiting step in paracellular flux.

Tight Junctions Are Regulated by Pharmacological and Physiological Stimuli

Farquhar's descriptions of the tight junction and apical junctional complex were remarkably consistent across samples and tissues [1]. Together with other data, this led many to conclude that tight junctions are static structures with unchanging permeabilities. Ussing's report [2], only 1 year after Farquhar's landmark publication, that application of an osmotic gradient could markedly increase paracellular flux across was interpreted as tight junction disassembly. Subsequent studies showing

that osmotic gradients reduced [3], while phalloidin and proliferative stimuli increased [4, 5], the complexity of strand networks were generally dismissed as non-physiological. This changed in 1987 when Pappenheimer et al. published a series of papers demonstrating that increased intestinal epithelial tight junction permeability was a physiological response to Na^+-nutrient cotransport [6–8]. These first studies showing physiological tight junction regulation also led to the discovery of "solvent drag" [8–10], a mechanism by which paracellular flux allows continued nutrient absorption despite saturation of transcellular transport pathways. Although initially controversial [11–15], subsequent in vitro and in vivo studies demonstrated that solvent drag occurs in many species, including humans [16, 17], as a result of increased paracellular permeability [18–20]. Subsequent work has documented similar paracellular amplification of transcellular transport in the renal tubule [21, 22].

Ex vivo studies of rodent mucosae showed that Na^+-nutrient cotransport-induced tight junction permeability increases were associated with morphological changes including expansion of spaces between tight junction strands and condensation of perijunctional microfilaments [6]. This led to the hypothesis that actomyosin contraction might contribute to Na^+-nutrient cotransport-induced tight junction regulation. However, methods available at that time precluded mechanistic analysis in intact mucosae. This obstacle was overcome by the development of a cell culture model of intestinal epithelial tight junction regulation in response to Na^+-glucose cotransport [23]. Using a model facilitated the discovery that actomyosin contraction driven by myosin light chain kinase (MLCK)-mediated phosphorylation of myosin II regulatory light chain (MLC) was essential for physiological tight junction regulation. Moreover, enzymatic MLCK inhibition blocked Na^+-glucose cotransport-induced tight junction regulation in cultured monolayers and isolated rodent mucosae [23]. Subsequent studies showed that Na^+-glucose cotransport led to increased tight junction permeability and MLC phosphorylation in human intestinal mucosae [19]. Importantly, both in vitro and in vivo studies showed that these alterations were size selective in that mannitol (~7 Å diameter) permeability was increased, but there were no detectable changes in paracellular inulin (30–40 Å diameter) flux [20, 23]. Thus, MLCK activation by physiological stimuli is a canonical mechanism of epithelial tight junction regulation.

Tight Junctions Are Differentially Regulated by Inflammatory Stimuli

The discovery of MLCK as the mediator of Na^+-glucose cotransport-induced permeability increases provided the first mechanistic insight into physiological tight junction regulation. This also created opportunities to discover mechanisms of pathophysiological barrier regulation. Of these, the most well-established is the discovery that MLCK also mediates tight junction regulation induced by

proinflammatory cytokines, including TNF, LIGHT, and IL-1β [24–26]. The efficacy of anti-TNF therapies and their ability to restore barrier function in patients highlights the clinical relevance of this discovery, although efficacy is more likely due to immunomodulation [27, 28]. Experimentally, barrier loss induced by TNF was completely reversed by a specific membrane-permeant inhibitor of MLCK (PIK). MLC phosphorylation, which was increased following TNF treatment, was also corrected by PIK [29]. PIK was, however, only able to restore barrier function in response to modest degrees of barrier loss [29], indicating that the effect was due to reversal of TNF-induced tight junction regulation rather than inhibition of TNF-induced apoptosis [30–32]. Moreover, PIK or genetic epithelial MLCK inhibition each prevented acute, TNF-induced barrier loss and diarrhea in vivo [33]. Further, epithelial MLC phosphorylation and MLCK expression were found to be increased in human inflammatory bowel disease and to correlate with disease activity [34]. Thus, TNF and more complex inflammatory stimuli hijack physiological mechanisms of tight junction regulation to increase intestinal permeability. There are, however, differences between tight junction regulation by physiological Na^+-glucose cotransport and that induced by TNF in that only TNF increases permeability of molecules larger than small sugars [23, 30]. Thus, although both are driven by MLCK, the barrier loss induced by TNF is qualitatively different from that induced by Na^+-glucose cotransport. This may reflect a second signal induced by TNF, as TNF, but not Na^+-glucose cotransport, triggers occludin endocytosis in vivo [33, 34].

Once claudins were discovered and antibodies became available, several groups began to assess patterns of expression in health and disease [35–38]. These demonstrated markedly increased claudin-2 expression during active inflammatory bowel disease and showed that IL-13, which is elevated in both ulcerative colitis and Crohn's disease, was able to induce claudin-2 expression in cultured intestinal epithelial monolayers [36, 37]. Further study showed that, although IL-13, like TNF, reduced transepithelial electrical resistance (TER), only TNF increased paracellular permeability to 4 kDa dextran (28 Å diameter) and reduced charge selectivity [39]. In contrast to TNF, IL-13-induced barrier loss, was due to a claudin-2-mediated increase in paracellular Na^+ permeability and was unaffected by PIK [39]. In vivo studies confirmed that IL-13 was sufficient to increase cation permeability, and subsequent studies demonstrated that IL-13 was unable to affect paracellular permeability of claudin-2 knockout mice, while transgenic claudin-2 overexpression was sufficient to replicate the effects of IL-13 in the absence of cytokine treatment [40]. These data demonstrated that the IL-13 and TNF modify paracellular permeability by different mechanisms, claudin-2 upregulation and MLCK activation, respectively, that have distinct functional effects.

Distinct Tight Junction Pore and Leak Pathways

In parallel with the studies above, analyses of genetically modified MDCK monolayers showed that claudin-2 expression increased paracellular permeability of small molecules across pores with diameter of ~8 Å but had no effect on

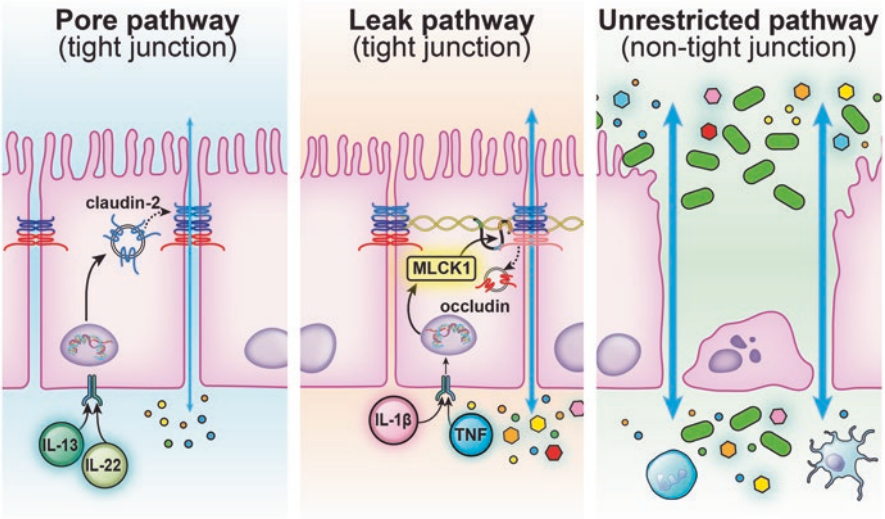

Fig. 1 The intestinal barrier. The space between epithelial cells is sealed by selectively permeable tight junctions that allow paracellular flux by two distinct pathways. The pore pathway (left) is a size- and charge-selective, high-conductance pathway that accommodates molecules up to 6–8 Å in diameter. Upregulation of claudin-2, which forms paracellular channels, increases pore pathway permeability. The mucosal immune system can induce claudin-2 transcription via IL-13 or IL-22 secretion. The leak pathway (center) is a charge-nonselective, low-capacity macromolecular flux route that accommodates molecules up to 100–125 Å in diameter. TNF and IL-1β are each able to increase leak pathway permeability by upregulating myosin light chain kinase (MLCK) expression and activity and recruiting the MLCK1 splice variant to the perijunctional actomyosin ring. This triggers removal of occludin by caveolar endocytosis and leads to increased leak pathway flux. A potential third route, the unrestricted pathway (right panel), is tight junction-independent, size-nonselective, and charge-nonselective. It is created by epithelial damage and can become the dominant route of intestinal barrier loss

paracellular flux of larger probes [41]. In contrast, knockdown of either ZO-1 or occludin increased paracellular permeability to larger macromolecules [42, 43]. Specifically, occludin knockdown increased flux across a pathway that accommodates macromolecules up to ~125 Å in diameter [43]. Thus, TNF treatment or occludin knockdown increased macromolecular permeability and reduced charge selectivity, while IL-13 treatment or claudin-2 overexpression increased cation selectivity without affecting macromolecular permeability. This confluence of observations led to the hypothesis that two distinct pathways, termed pore and leak, mediate paracellular flux (Fig. 1) [44, 45]. The pore pathway, exemplified by claudin-2 channels, is a high-conductance route that is both charge- and size-selective, with a maximum diameter of 6 to 8 Å. The low-capacity leak pathway is size-selective, with an estimated maximum diameter of 100 to 125 Å and is not charge-selective. In contrast to the pore pathway, which is created by claudin-based, gated channels [46], the anatomy of the leak pathway has not been defined; some have suggested that leak pathway flux may occur primarily at tricellular tight junctions [47].

The increases in intestinal epithelial tight junction permeability induced by Na^+-nutrient cotransport to amplify transcellular nutrient absorption are charge-nonselective, permit flux of molecules too large to traverse claudin channels, and are regulated by the cytoskeleton [8, 19, 23, 48]. These changes, therefore, reflect leak pathway flux. In an analogous fashion, claudin-2-mediated pore pathway flux allows paracellular Na^+ absorption to amplify transcellular Na^+ absorption in the renal proximal tubule [21]. Thus, defined flux across both pore and leak pathways is of physiological benefit.

Claudin-2: A Paracellular Cation and Water Channel

In normal human and rodent development, intestinal epithelial claudin-2 expression is highest in the immediate perinatal period, where it is expressed in villous and crypt epithelial cells [35, 49, 50]. Claudin-2 expression is then progressively down-regulated until, by weaning, expression is low and restricted to crypt epithelial cells [35, 49, 50]. Claudin-15, which also forms a paracellular cation and water channel [51–55], has a reciprocal temporal expression pattern and is present at low levels prior to weaning but is expressed at higher levels in both villous and crypt epithelia by adulthood [35, 49, 50].

Although the reasons for developmental regulation of claudin-2 expression have not been defined, it may reflect differences in nutrient demand, which is greatest during the rapid growth that characterizes the neonatal and pre-weaning periods. This is best understood by recognizing that expression of claudin-2 or claudin-15 is required for ongoing Na^+-nutrient cotransport; mice lacking either claudin survive while those lacking both die of malnutrition by 3 weeks of age [56]. It is also notable that paracellular Na^+ conductance is significantly greater in neonatal mice, relative to adults [49]. Thus, it may be that claudin-2 is able to more effectively conduct Na^+ than claudin-15. This has not, however, been demonstrated experimentally. In the context of inflammatory stimuli, including disease, intestinal epithelial claudin-2 expression is upregulated, while claudin-15 expression is largely unchanged (Fig. 2) [36, 39, 40, 50, 57–60]. This could therefore represent a compensatory mechanism that restores nutrient absorption in the face of mucosal damage. However, the observation that claudin-2 is not upregulated in claudin-15-deficient mouse intestine [61] suggests that alternative explanations should also be considered.

Claudin-2-Dependent Fluid Efflux Promotes Pathogen Clearance

One major limitation of most in vivo studies of permeability is that the probe or probes used do not allow discrimination between changes in pore, leak, and damage-induced (unrestricted pathway) permeability. Lactulose and mannitol, the most

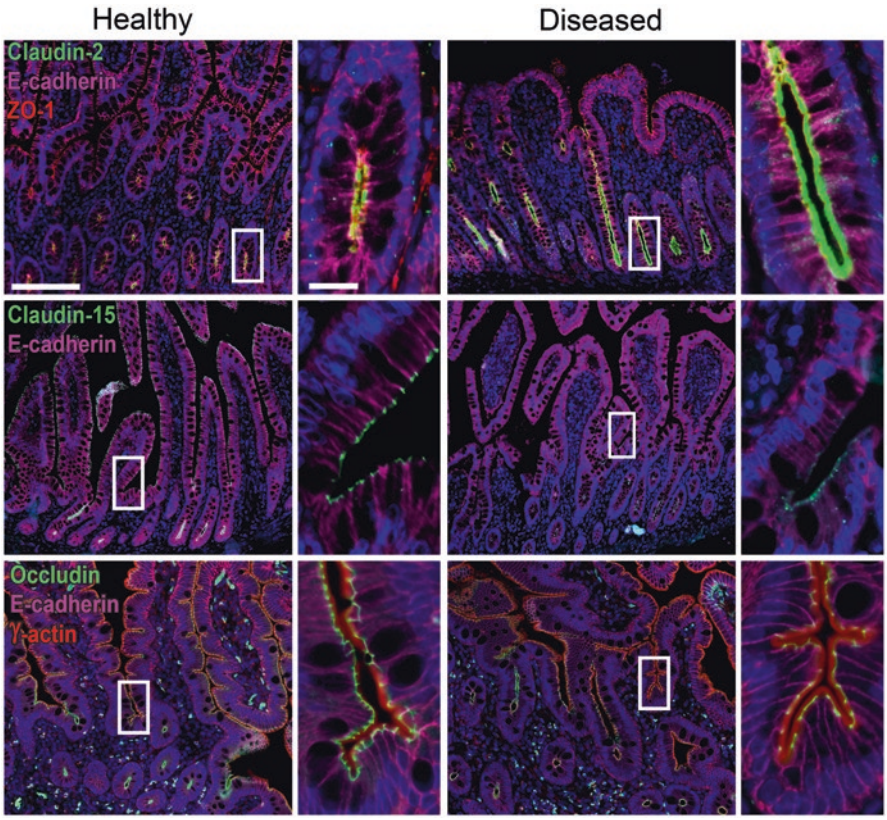

Fig. 2 Regulation of claudin-2, claudin-15, and occludin expression in healthy and diseased adult human intestine. Claudin-2 (green) is primarily expressed in the crypts of the small intestine and colon, and this is increased in inflammatory disease. In contrast, expression of claudin-15 (green), which also forms paracellular cation-selective pores, is present throughout the crypt-villus axis and is unchanged in disease. Occludin (green) expression is reduced in inflammatory disease. E-cadherin (magenta), ZO-1 or γ-actin (red), and nuclei (blue) are shown for reference. Bar, 50 μm; 20 μm (insets)

extensively used probes in human studies, are both able to traverse leak and unrestricted pathways. Mannitol is too large to be accommodated by claudin-2 channels in vitro, but it remains to be determined if it can cross the pore pathway in vivo [41]. In mouse models, most investigators simply use 4 kDa FITC-conjugated dextran. Serum recovery of this probe can reflect increased permeability across both leak and unrestricted, but not pore, pathways [62]. Experimental data suggest that greater than two-fold increases in 4 kDa dextran flux are most likely to reflect unrestricted pathway activation as consequence of epithelial damage [62]. To overcome this limitation, we established a method using creatinine (6 Å diameter), 4 kDa dextran (28 Å diameter), and 70 kDa dextran (120 Å diameter), to measure flux across pore, leak, and unrestricted pathways, respectively [60, 62]. This approach, which has

been validated by other groups [63], readily distinguishes between barrier defects induced by *C. rodentium* infection, TNF, and DSS [62].

Using the three-probe in vivo permeability assay, we discovered that creatinine flux is uniquely increased 2 days after *C. rodentium* infection in mice [60]. This correlated with increased epithelial expression of claudin-2, but not other tight junction proteins, and elevated mucosal IL-22 levels [60]. Analyses using recombinant IL-22 demonstrated that this member of the IL-10 superfamily is responsible for the increased intestinal epithelial claudin-2 transcription and translation seen after *C. rodentium* infection.

In order to understand the impact of claudin-2 upregulation in *C. rodentium* infectious colitis, we took advantage of claudin-2 knockout and claudin-2 transgenic mice [60]. The very low claudin-2 expression observed in healthy adults was similar to the absence of claudin-2 in knockout mice, while the increased expression in claudin-2 transgenic mice was comparable to levels detected at the peak of *C. rodentium* infection [60]. The genetically modified mice therefore model the extremes of claudin-2 expression in wild-type mice.

The severity of *C. rodentium* colitis was exacerbated by claudin-2 knockout, as indicated by increased histopathology scores, increased mucosa-associated *C. rodentium*, prolonged *C. rodentium* shedding, and markedly elevated mucosal TNF, IL-1β, IL-6, and IL-22 (Fig. 3) [60]. The observation that fecal water and Na^+ were increased in healthy claudin-2 transgenic mice suggested that the greater severity of disease in claudin-2 knockout mice might be secondary to reduced water and Na^+ efflux. Consistent with this hypothesis, induction of mild osmotic diarrhea by adding polyethylene glycol to the drinking water reduced histopathology scores, mucosa-associated *C. rodentium*, fecal *C. rodentium* shedding, and mucosal TNF, IL-1β, IL-6, and IL-22 levels such that all measures of disease were similar in polyethylene glycol-treated claudin-2 knockout, transgenic, and wild-type mice [60]. These data suggest that the primary means by which claudin-2 upregulation accelerates pathogen clearance and reduces infectious enterocolitis severity is by promoting paracellular water and Na^+ efflux [60]. Because epithelial cell turnover is accelerated in *C. rodentium*-induced colitis, this cannot be simply due to bacterial washing from the epithelial surface but is more likely to reflect disruption of the cycle by which newly formed epithelial cells are infected (Fig. 3a). The increased fecal water resulting from claudin-2 overexpression likely also dilutes and reduces local DSS concentrations to attenuate mucosal damage, thereby explaining the protection fromm DSS-induced injury observed in claudin-2 transgenic mice [64]. Thus, claudin-2 increases luminal water efflux to promote pathogen clearance and dilute offending chemical agents in infectious and chemical colitis, respectively. Intestinal claudin-2 upregulation and the water efflux that follows can therefore be considered a primitive form of innate immunity.

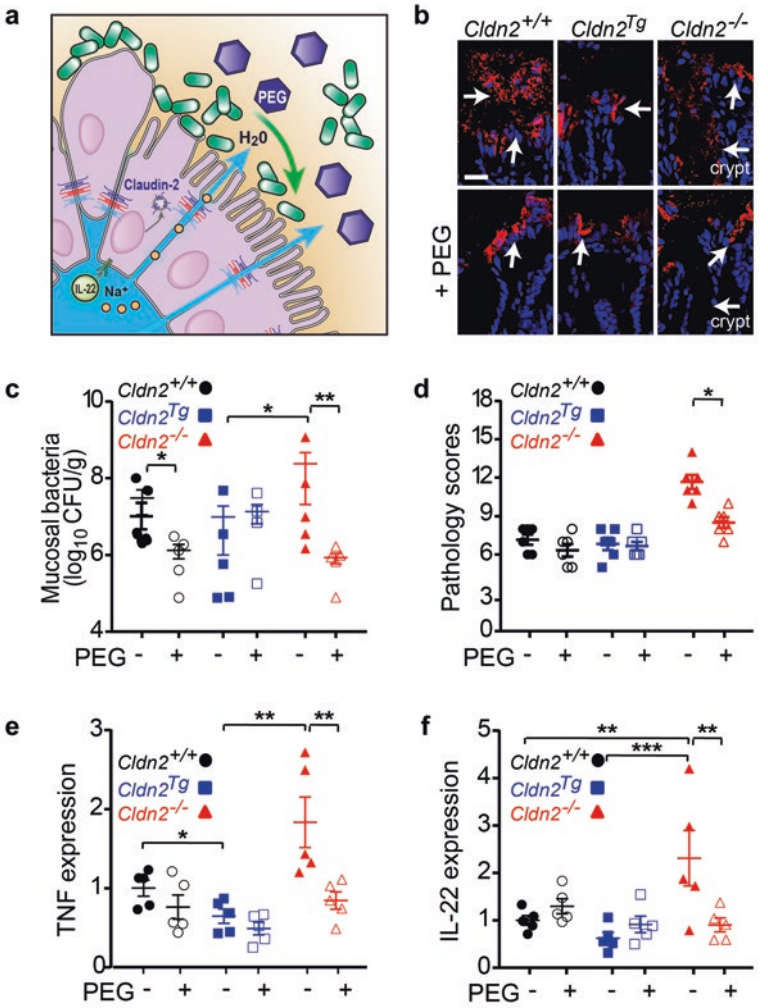

Fig. 3 IL-22 upregulates epithelial claudin-2 to drive diarrhea and enteric pathogen clearance. (**a**) IL-22 released in response to enteric infection upregulates claudin-2 expression to enhance paracellular Na^+ and water efflux and promote pathogen clearance. Here, polyethylene glycol (PEG) is shown as an inducer of osmotic diarrhea. (**b**) *C. rodentium* (red) and nuclei (blue) 11 days after infection. Colonization of crypt spaces in claudin-2 knockout mice is prevented by PEG treatment. Bar, 50 μm. (**c**) Mucosa-associated *C. rodentium* numbers are reduced in *Cldn2*^{Tg} and increased in *Cldn2*$^{-/-}$, relative to wild-type (*Cldn2*$^{+/+}$), mice. PEG treatment eliminated differences between genotypes. (**d**) Histopathology was increased in *Cldn2*$^{-/-}$ mice and reduced by PEG treatment. (**e** and **f**) Mucosal TNF and IL-22 were markedly greater in *Cldn2*$^{-/-}$ mice relative to wild-type or *Cldn2*^{Tg} mice, at day 11 after infection in the absence, but not presence, of PEG treatment. (Data from Tsai et al. Cell Host Microbe, 2017, with permission)

Claudin-2 Exacerbates Immune-Mediated Colitis

Although claudin-2 upregulation reduces severity of infectious and chemical colitis, it is not clear if it represents a beneficial adaptation or contributes to progression of immune-mediated colitis. To address this, claudin-2 knockout and transgenic mice were crossed onto an immunodeficient *Rag1* knockout background and studied using the adoptive T-cell transfer colitis model [40, 65]. In contrast to infectious and chemical colitis models, immune-mediated disease severity was increased by transgenic claudin-2 expression and reduced by claudin-2 knockout, as measured by weight loss, disease activity index, cytokine production, T-cell infiltration, and histopathology scores (Fig. 4) [40]. Conversely, the same measures showed that claudin-2 knockout ameliorated immune-mediated colitis [40]. It therefore appears that claudin-2 upregulation is a maladaptive response to mucosal immune activation in the absence of offending exogenous agents, such as pathogenic bacteria or toxic chemicals. Further study is needed to understand how claudin-2 modifies mucosal immune activation and whether this contributes to the relative protection afforded by claudin-2 overexpression in *C. rodentium* colitis.

Despite reduced disease severity, survival after T-cell transfer was reduced in claudin-2 knockout mice. This was unexpected and, in most cases, followed an unusual clinical pattern. Wild-type and claudin-2 transgenic mice displayed progressive increases in disease activity and weight loss over weeks. In contrast, claudin-2 knockout mice appeared well until they became acutely ill in the few days before death. Necropsy showed that these mice suffered from intestinal obstruction [40]. This was not due to fibrosis or dysmotility. We therefore considered the possibility that insufficient luminal hydration could result in fecalith formation and obstruction. In contrast to humans, this led to obstruciton in mice, where the luminal space is dramatically narrowed by mucosal inflammatory expansion. To prevent obstruction, polyethylene glycol was added to the drinking water of claudin-2 knockout and wild-type mice. This did not affect disease severity but improved survival of claudin-2 knockout mice [40]. Thus, increasing luminal water was sufficient to prevent obstruction and death in claudin-2 knockout mice. Notably, claudin-2 knockout, but not wild-type, mice upregulated intestinal epithelial claudin-15 expression during colitis. This can be taken as an additional piece of evidence that, despite our inability to detect functional differences at present [52], claudin-2 and claudin-15 are not functionally interchangeable, as such claudin-15 upregulation did not disrupt the effects of claudin-2 knockout.

Occludin S408 Phosphorylation Regulates Claudin-2 Channel Function

The occludin C-terminal tail can be separated into membrane-proximal and distal portions. The distal half (residues 373 to 522) is most highly conserved [66] and can be broken into an unstructured region (373–412) and a coil-coil domain (413–522) composed of three alpha helices [67]. Biochemical studies found that the

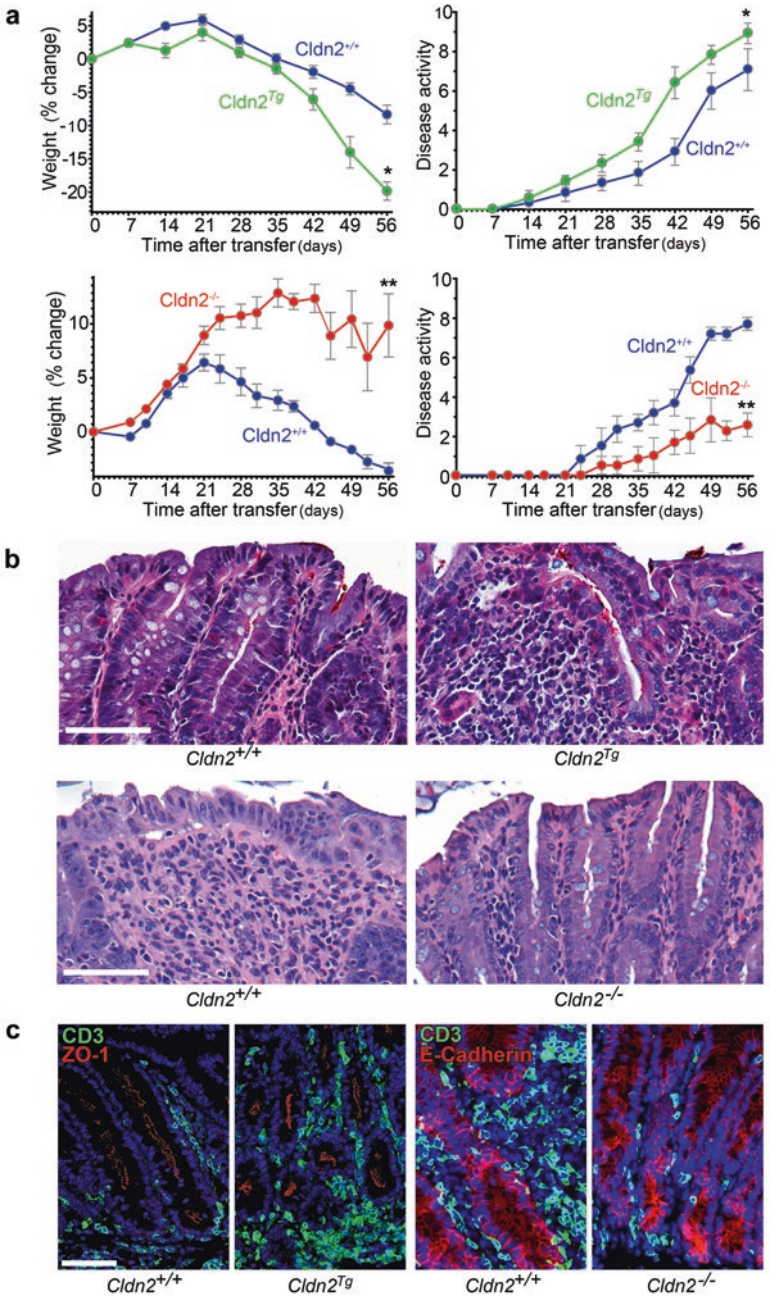

Fig. 4 Intestinal epithelial claudin-2 overexpression exacerbates, and claudin-2 knockout limits immune-mediated colitis severity. (**a**) Weight loss and disease activity induced by T-cell transfer in *Cldn2*$^{+/+}$*Rag1*$^{-/-}$ mice (blue circles), *Cldn2*$^{-/-}$*Rag1*$^{-/-}$ mice (red circles), and *Cldn2*^{Tg}*Rag1*$^{-/-}$ mice (green circles). (**b**) Histopathology of mice sacrificed at day 56 after T-cell transfer shows more severe disease in *Cldn2*^{Tg}*Rag1*$^{-/-}$ mice and milder disease in *Cldn2*$^{-/-}$*Rag1*$^{-/-}$ mice, relative to *Cldn2*$^{+/+}$*Rag1*$^{-/-}$ mice. (**c**) T-cell recruitment (CD3, green) parallels disease severity. ZO-1 or E-cadherin (red) and nuclei (blue) are shown for reference. Bars, 50 μm (Data from Raju et al. J Clin Invest, 2020, with permission)

unstructured region within the distal occludin tail contains multiple sites that can be phosphorylated by casein kinase 2 (CK2) [68–70]. The development of highly specific CK2 inhibitors as pharmacological therapies for neoplastic disease created an opportunity to assess the impact of CK2-mediated occludin phosphorylation on a barrier function. In Caco-2 intestinal epithelial cell monolayers, enzymatic CK2 inhibition reduced paracellular permeability of small cations, consistent with inhibition of the claudin-2 channel [71]. CK2 inhibitors had no effect in the absence of CK2, occludin, or claudin-2 expression [71]. Mutagenesis studies showed that serine-408 dephosphorylation was required for the effect of CK2 inhibition on barrier function. Fluorescence recovery after photobleaching (FRAP) studies showed that CK2 inhibition reduced the occludin mobile fraction and that this behavior also mapped to serine-408 [71]. Further analyses using pull-down assays showed that the non-phosphorylatable tail of occludin^{S408A} captured greater amounts of endogenous occludin, claudin-1, and claudin-2 from Caco-2 cell lysates than the phosphomimetic occludin^{S408D} [71]. Although no direct binding sites between claudins and occludin are known, ZO-1 includes PDZ1 and U5-GuK domains that bind to each of these, respectively [72, 73]. Consistent with an essential role for ZO-1-mediated interactions between occludin and claudins, neither S408A nor S408D occludin tails effectively recovered claudin-2 from ZO-1-deficient Caco-2 cell lysates [71]. Moreover, CK2 inhibitors had no effect on TER of ZO-1-deficient Caco-2 monolayers. The TER response to CK2 inhibition could be restored by expression of full-length ZO-1, but not ZO-1$^{\Delta PDZ1}$ or ZO-1$^{\Delta U5GuK}$ [71]. Together, these data demonstrate that dephosphorylation of occludin^{S408} triggers assembly of an occludin-ZO-1-claudin-2 complex that interferes with claudin-2 channel function (Fig. 5a) [73]. As a final test of this hypothesis, T84 cell monolayers, which do not ordinarily express significant levels of claudin-2, were treated with IL-13 to induce claudin-2 expression. This reduced TER and increased cation selectivity, and both of these changes could be reversed by CK2 inhibition. Thus, occludin phosphorylation at S408 is a molecular switch that, indirectly, regulates claudin-2 channels [71].

Fig. 5 (continued) channels. (**b**) IL-13 administration increased claudin-2 (green) expression in proximal colonic crypt epithelium. This was not affected by CK2 inhibition. Nuclei (blue) are shown for reference. (**c**) Ussing chamber analyses of proximal colonic mucosae from IL-13-treated mice (red squares) shows specific increases in permeability of small cations relative to vehicle-treated mice (blue circles). Treatment with CK2 inhibitor (green-outlined symbols) blocked IL-13-induced permeability increases but had no effect in the absence of IL-13. (**d**) Weight loss following T-cell transfer was attenuated in *Rag1*$^{-/-}$ mice treated with a CK2 inhibitor (green diamonds) relative to controls receiving vehicle (blue circles). (**e**) Histopathology was reduced by CK2 inhibitor treatment. (**f**) T-cell (CD3, green) infiltration was reduced by CK2 inhibition. E-cadherin (red) and nuclei (blue) are shown for reference. Bars, 50 μm (Data from Raju et al. J Clin Invest, 2020, with permission)

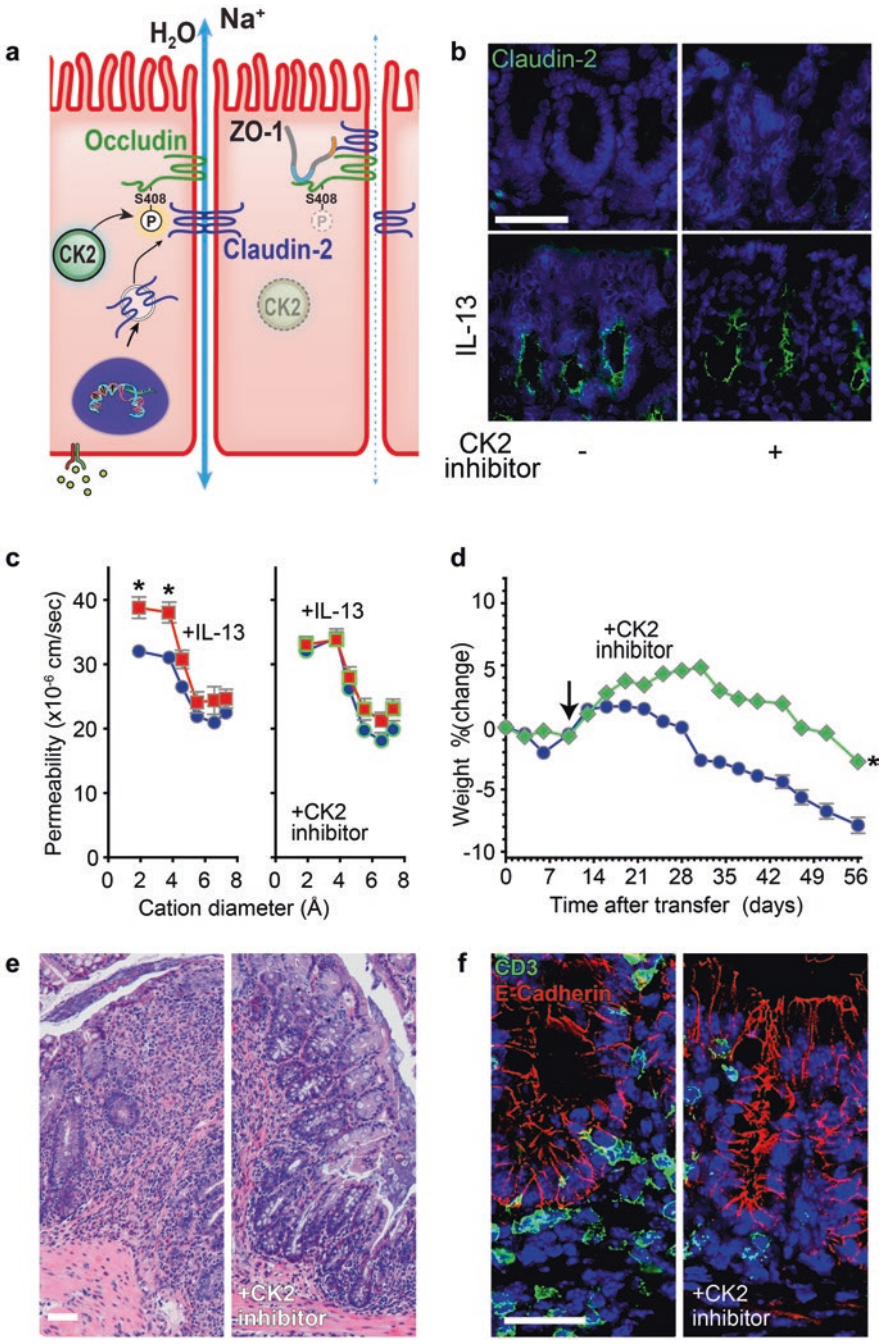

Fig. 5 CK2 inhibition limits immune-mediated colitis severity by inactivating claudin-2 channels. (**a**) Casein kinase-2 (CK2) inhibition results in occludin S408 dephosphorylation and triggers assembly of a trimolecular complex composed of occludin, ZO-1, and claudin-2 to inactivate claudin-2

Inhibition of Occludin Phosphorylation Is Therapeutic in Experimental, Immune-Mediated Colitis

In order to determine whether CK2 inhibition could also block claudin-2 channel function in vivo, mice were treated with saline or IL-13, to induce claudin-2 expression, in combination with a CK2 inhibitor or vehicle [40]. Bi-ionic potential measurements were then used to characterize excised proximal colonic mucosae [40, 53, 74, 75]. As expected, IL-13 specifically increased claudin-2 expression (Fig. 5b) as well as paracellular permeability to Na^+, methylamine, and, to a lesser degree, ethylamine (Fig. 5c) [40]. CK2 inhibition failed to prevent increased claudin-2 expression but reversed changes in paracellular permeability (Fig. 5b, c). In contrast, CK2 inhibition had no effect on permeability in the absence of IL-13 treatment. CK2 inhibition is, therefore, able to block claudin-2 channel function in vivo.

Daily treatment with an orally bioavailable CK2 inhibitor, beginning 10 days after T-cell transfer, dramatically reduced immune-related colitis severity by all measures in claudin-2 wild-type, *Rag1*$^{-/-}$ mice [40]. In contrast, CK2 inhibition afforded no benefit to claudin-2 knockout *Rag1*$^{-/-}$ mice [40]. Thus, although the nearly ubiquitous expression and substrate promiscuity of CK2 make it impossible to fully exclude other targets, the requirement for claudin-2 expression suggests that the effect of CK2 inhibition reflects claudin-2 channel inactivation [40]. Thus, in vivo inhibition of CK2-mediated occludin phosphorylation prevents progression and attenuates severity of immune-mediated colitis [40]. CK2 inhibition can affect many processes and, therefore, is not an ideal therapeutic approach. However, future definition of how S408 phosphorylation regulates occludin tail interactions may lead to more specific means of inhibiting claudin-2 function.

Occludin Endocytosis Is Required for TNF-Induced, MLCK-Dependent Leak Pathway Permeability Increases

Remarkably, beyond the perijunctional actomyosin condensation seen by transmission electron microscopy, occludin internalization was the primary morphologic change associated with T-cell activation-induced, TNF-dependent barrier loss [33]. Further study showed that occludin was internalized into caveolin-1-positive endosomes. Although TNF-induced MLCK activation and MLC phosphorylation were increased, caveolin-1 knockout mice failed to internalize occludin and were protected from TNF-induced barrier loss [76]. Caveolar endocytosis is, therefore, required for TNF-induced, MLCK-mediated leak pathway permeability increases. Occludin was the only tight junction protein consistently identified within these endosomes, but the data do not exclude the possibility that occludin is merely a marker of some other critical change in tight junction structure. To assess this, transgenic mice that overexpress occludin within intestinal epithelial cells were treated with TNF. Although endocytosis did occur, tight junction-associated occludin pools

were preserved in transgenic, relative to wild-type, mice [76]. Moreover, the magnitude of barrier loss was attenuated, and TNF-induced diarrhea was prevented by transgenic occludin expression [76]. Occludin is, therefore, an essential regulator of leak pathway permeability. Finally, this result is consistent with leak pathway barrier loss and reduced occludin expression in human inflammatory bowel disease [36, 77, 78].

Occludin Regulates Epithelial Survival

The initial reports of occludin knockout mice failed to identify intestinal or renal barrier defects or spontaneous disease involving these organs [79]. Nevertheless, occludin knockout was associated with male infertility, inability of females to effectively suckle pups, gastric epithelial hyperplasia, brain calcifications, testicular atrophy, and osteoporosis, and subsequent studies showed that occludin knockout mice became deaf over time [80]. Finally, our unpublished experience that the frequency of knockout pups when occludin heterozygotes were bred was far less than the expected 25% predicted by Mendelian genetics suggests embryonic loss; crosses of occludin floxed and occludin floxed;villin-cre transgenic mice resulted in equal numbers of cre-positive and cre-negative pups. Embryonic loss of occludin-deficient embryos is, therefore, not due to *Ocln* deletion within the intestinal epithelium.

The absence of intestinal disease in occludin-deficient mice could be due to compensatory mechanisms that overcome occludin loss. Universal and intestinal epithelial-specific occludin knockout mice were, therefore, stressed in order to unmask functional deficits. Remarkably, dextran sulfate sodium (DSS) failed to induce weight loss in occludin knockout mice (Fig. 6a) [81]. Thus, intestinal epithelial occludin expression sensitizes mice to chemically induced colitis.

Further analyses showed that histopathology, inflammatory cytokine production, and epithelial apoptosis induced by DSS or trinitrobenzenesulfonic acid (TNBS) were suppressed in intestinal epithelial occludin knockout mice (Fig. 6b). To better define the mechanism by which occludin loss reduced epithelial apoptosis, mice were treated with 5-fluorouracil, which triggers intrinsic pathway apoptosis, systemic T-cell activation using anti-CD3, or TNF treatment to activate the extrinsic apoptotic pathway [81]. Occludin knockout protected intestinal epithelial cells from all three stimuli (Fig. 6c). Biochemical analysis of apoptotic signaling induced by TNF showed that ERK, p38 MAPK, IκB, and caspase-9 were all activated similarly in occludin-sufficient and knockout intestinal epithelia (Fig. 6d). However, TNF-induced caspase-3 cleavage was only detected in occludin-expressing epithelia (Fig. 6d). Further characterization showed that this was due to a ~ 50% reduction in overall caspase-3 mRNA and protein expression in the absence of occludin. Although it was surprising that an only 50% decrease in expression would be sufficient to prevent apoptosis, mice heterozygous for *Casp3* knockout (*Casp3*$^{+/-}$), which express caspase-3 at ~50% of normal levels, were also protected from intestinal epithelial apoptosis induced by DSS or TNF [81]. Thus, occludin promotes

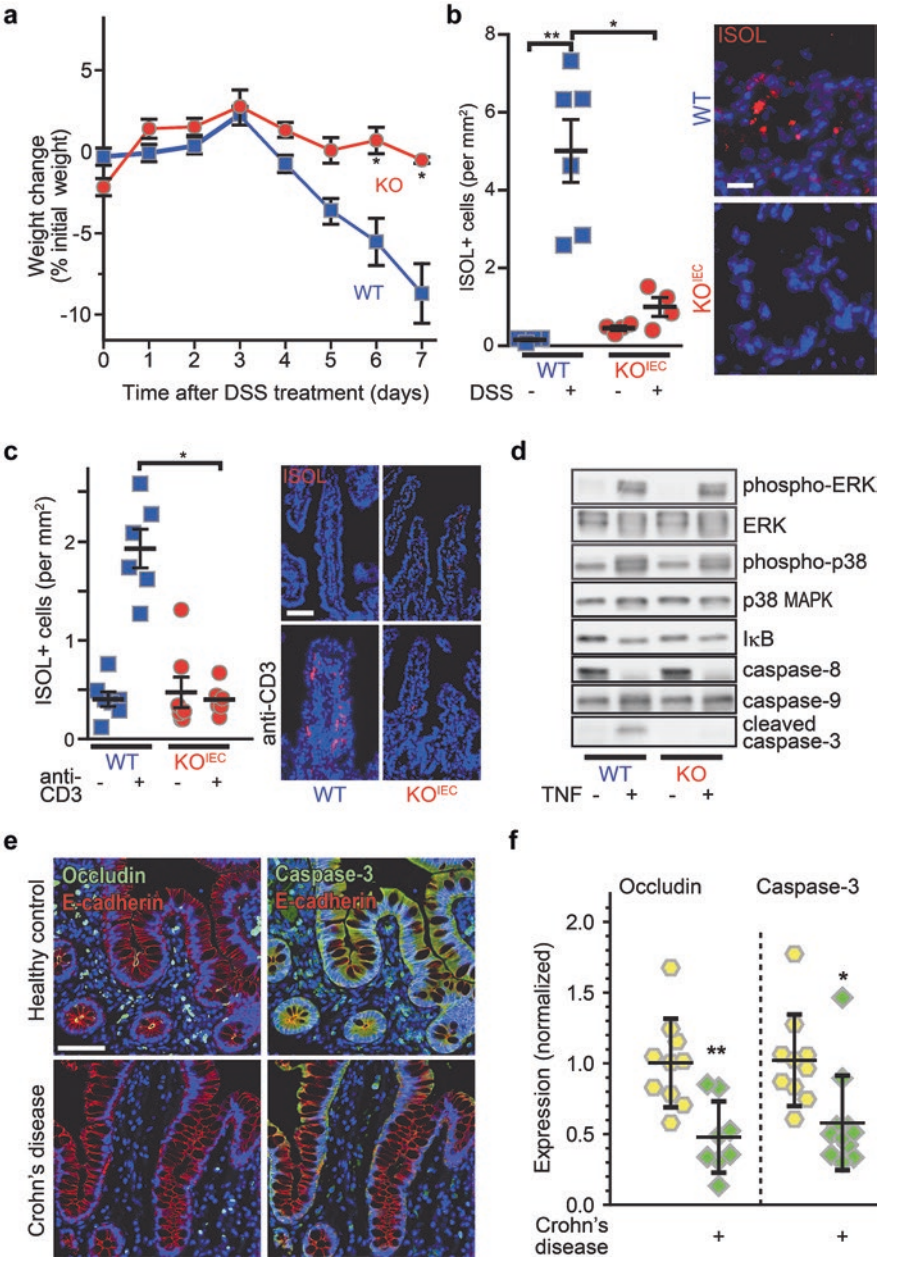

Fig. 6 Occludin deletion reduces caspase-3 expression, limits epithelial apoptosis, and attenuates experimental disease in mice; both occludin and caspase-3 expression are reduced in Crohn's disease. (**a**) DSS-induced colitis severity and weight loss were reduced in *Ocln*$^{-/-}$ (red circles) relative to wild-type (WT, blue squares) mice. (**b**) Numbers of apoptotic (ISOL-positive, red) epithelial cells were increased in DSS-treated wild-type, but not intestinal epithelial-specific occludin knock

caspase-3 transcription, and the reduced caspase-3 expression that follows occludin loss is sufficient to prevent intrinsic and extrinsic pathway apoptosis.

Similar to mice, Caco-2 cells lacking occludin displayed reduced *CASP3* transcription and protein expression. Inducible occludin expression in these cells activated the *CASP3* promoter and restored transcription to normal levels [81]. Thus, although occludin has not been detected within nuclei, it promotes *CASP3* transcription, either directly or indirectly [81]. To assess the effect of inflammation-induced loss of occludin expression on epithelial survival, Caco-2 monolayers were treated with TNF at a low concentration that was insufficient to cause apoptosis. This was, however, sufficient to reduce both occludin and caspase-3 expression and protect cells from staurosporine-induced intrinsic pathway or high-dose TNF-induced extrinsic pathway apoptosis. In contrast, pretreatment of occludin-deficient Caco-2 monolayers with low-dose TNF had no effect on subsequent responses to staurosporine or high-dose TNF. Thus, low-grade inflammation, modeled in vitro by low-dose TNF, may lead to reduced occludin and caspase-3 expression that results in cytoprotection [81].

In order to determine whether occludin downregulation in human disease is associated with reduced caspase-3 expression, small intestinal and colonic biopsies from Crohn's disease and ulcerative colitis patients and age- and sex-matched healthy control subjects were analyzed by quantitative immunohistochemistry (Fig. 6e). Occludin expression was significantly reduced in both Crohn's disease and ulcerative colitis and was accompanied by caspase-3 downregulation (Fig. 6f). Moreover, reductions in caspase-3 expression correlated directly with decreased occludin expression. Thus, occludin downregulation in inflammatory disease may be an adaptive response that promotes epithelial survival. Further studies will be needed to understand how this cytoprotective function interfaces with the role of occludin in tight junction barrier regulation.

Fig. 6 (continued) out (KO^{IEC}), mice. Nuclei (blue) are shown for reference. Bar, 20 μm. (**c**) Systemic T-cell activation induced by anti-CD3 treatment increased ISOL-positive (red) cell numbers in wild-type, but not KO^{IEC}, mice. Nuclei (blue) are shown for reference. Bar, 50 μm. (**d**) Western blot of intestinal epithelia isolated from vehicle- and TNF-treated WT and *Ocln*$^{-/-}$ mice. With the exception of cleaved caspase-3, signaling events downstream of TNF signaling were not affected by occludin deletion. (**e**) Ileal biopsies from healthy subjects and Crohn's disease patients show reduced occludin (green) and caspase-3 (green) expression in disease. E-cadherin (red) and nuclei (blue) are shown for reference. Bar, 50 μm. (**f**) Quantitative morphometry confirms similar reductions in occludin and caspase-3 expression intensity in ileal epithelium from Crohn's disease patients (green diamonds) relative to healthy controls (yellow circles) (Data from Kuo et al. Gastroenterology, 2019, with permission)

Summary and Future Directions

The studies discussed here demonstrate the important contributions of pore and leak pathway permeability to intestinal disease. They also highlight the importance of considering these mechanisms as separate processes that are regulated by different signaling events and have distinct effects on barrier function and pathophysiology. The data also show that the impact of increased tight junction permeability may not be the same in infectious and immune-mediated disorders. Although available data cannot explain the reasons for these differences, one might hypothesize that increased claudin-2 expression in infectious disease both enhanced water efflux and augmented mucosal immune activation. Evolutionarily, where infectious diseases had much greater impact on survival than immune-mediated disorders, increased pathogen clearance and immune activation might be considered synergistic. The contradiction only occurs in the context of immune-mediated disease. Although the effects of occludin downregulation in infectious disease have not been defined, one could hypothesize that increased leak pathway permeability might promote pathogen clearance while simultaneously downregulating caspase-3 expression to limit tissue damage. Thus, regulatory mechanisms that evolved over billions of years to promote survival may now have unintended consequences in the context of modern maladies.

The growing synergy between in vitro and in vivo models makes this an exciting time for tight junction biology in which future studies will address the questions raised in this text and many others. Ultimately, we hope that both fundamental, foundational advances and translational studies will lead to clinical trials in which barrier and tight junction modulation are validated as therapeutic interventions for a broad range of intestinal and systemic diseases.

Acknowledgments We thank previous and current members of the Laboratory of Mucosal Barrier Pathobiology for their intellectual contributions to the development of studies described herein and for allowing the reuse of their published data. We are also indebted to Tiffany S. Davanzo (Slaybaugh Studios) and Heather Marlatt (Nationwide Histology) for their contributions to the figures. Finally, although we have attempted to cite previous work correctly and completely, we are certain to have omitted some studies, either unintentionally or due to space limitations, and offer our apologies to those we have failed to acknowledge.

Funding: This work was supported by the National Institutes of Health grants R01DK061931 (JRT), R01DK068271 (JRT), and P30DK034854 (The Harvard Digestive Disease Center).

Conflict of Interest Statement JRT is a founder and shareholder of Thelium Therapeutics and has served as a consultant for Entrinsic, Immunic, and Kallyope.

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Tight Junctions in the Inflamed Gut

Maria del Rocio Encarnacion-Garcia and Porfirio Nava

Abstract The gastrointestinal system digests and absorbs nutrients while integrating exogenous and endogenous signals that contribute to create immune tolerance. Epithelial cells forming the physical barrier regulate ion, solutes, and water absorption in the gut. Tight junctions (TJs) are specialized cell-cell junctions that bring adjacent gastrointestinal epithelial cells together and seal the paracellular space. Additionally, TJs are important for maintaining cellular polarity in epithelial cells and in the gastrointestinal epithelium itself. Dysfunctional gastrointestinal TJ enhances leakage of luminal contents into the interstitium, a process that stimulates the development of inflammatory disorders. Therefore, the pathophysiology of the TJs has gained attention in the medical field. Several studies aimed to investigate the viability of those structures are now used in the diagnosis for patients with chronic gastrointestinal inflammation. This chapter focuses on analyzing the role of the TJs in the gut, the techniques employed to study these structures, as well as various mechanisms responsible for compromising the TJs and the intestinal epithelial barrier.

Keywords Gastrointestinal tract · Inflammation · Claudins · Occludin · Epithelial cells

Abbreviations

AJ	Adherens junctions
AJC	Apical junctional complex
CK1	Casein kinase 1
cPKC	$Ca2^{+}$-dependent protein kinase C

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L. González-Mariscal (ed.), *Tight Junctions*,
https://doi.org/10.1007/978-3-030-97204-2_6

ECL1	First extracellular loop
ECL2	Second extracellular loop
GI	Gastrointestinal
GuK	Guanylate kinase homology
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cells
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
IP	Intestinal permeability
JAMs	Junctional adhesion molecules
KO	Knockout
MUPP1	Multi-PDZ domain protein 1
PDZ	*P*SD95, *D*lgA, *Z*O1 homology
PKA	Protein kinase A
QIIME	Quantitative Insights Into Microbial Ecology
SH3	Non-catalytic SRC homology 3
TJs	Tight junctions
ZO	Zonula occludens

Introduction

The gastrointestinal (GI) tract encompasses all organs of the digestive system from the mouth to the anus (Fig. 1). The principal function of the GI system is to digest the food to extract nutriments and then to compact the waste and expelled it as feces [1]. Anatomically, the GI tract contains a series of hollow muscular tubes fully interconnected that can be divided into upper (mouth, pharynx, esophagus, stomach, and duodenum) and lower (colon, rectum, and anus) tracts. Accessory structures of the GI tract include the liver, pancreas, and gallbladder [2]. Histologically, the GI tract is formed by four concentric layers: the mucosa, the submucosa, the muscularis, and the adventitia also referred to as serosa (Fig. 1). In the mucosal layer, millions of intestinal epithelial cells (IEC) line the luminal space and create an absorptive surface of approximately 100 m^2 [3]. IEC are polarized specialized cells with two biochemically and functionally distinct domains, the "apical" and the "basolateral" plasma membranes (Figs. 1 and 2). The apical membrane directly faces the luminal fillings, while the basolateral domain is anchored to the lamina propria. To create a functional physical barrier, epithelial cells connect to each other through a series of intercellular junctions that include tight junctions (TJ), adherens junctions, desmosomes (DMs), and gap junctions [4–7] (Fig. 2). At the uppermost part of the basolateral side, TJ proteins create an adhesive structure that closes the intercellular space and strongly influences epithelial barrier properties [7–10].

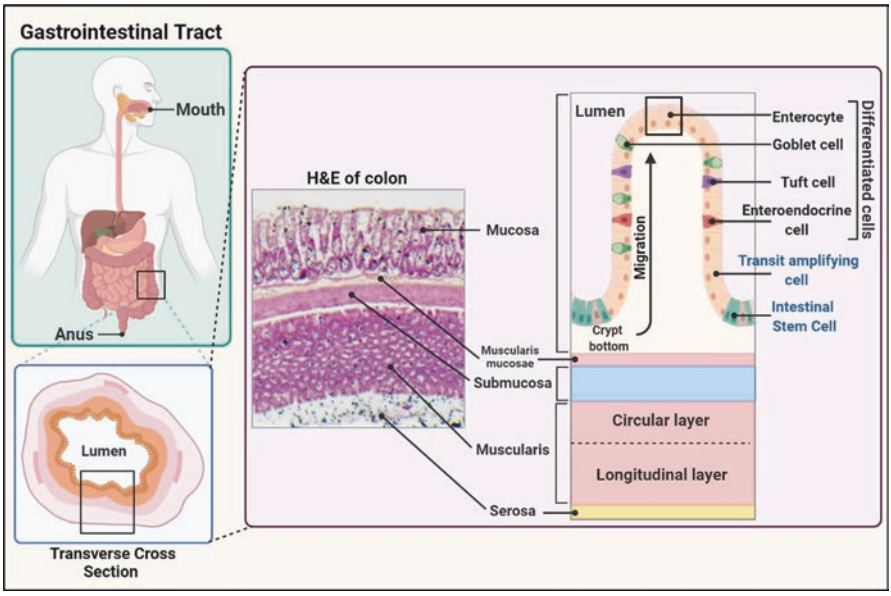

Fig. 1 The gastrointestinal system. A diagram of the gut in the average human, a transverse section of the hollow intestines, a hematoxylin-eosin staining of the colon, and a schematic illustration for the general structure of the gut wall are presented. The migration axis and the cellular populations present at the colonic crypt are also presented

In the gut, toxic luminal compounds damage superficial epithelial cells and/or the TJs in between compromising epithelial barrier functions. Leakage of intestinal fillings following epithelial barrier disruption builds up an acute inflammatory response mediated by innate and adaptive immune cells residing in the lamina propria. Uncontrolled acute inflammatory events in the intestine often progress to chronic diseases [11–13]. To overcome these challenges, the body created a protective mechanism where old or damaged epithelial cells are continuously replaced by newly born cells arising from stem cell progenitors residing at the base of the crypt. New cells migrate upward to the crypt surface where they replace exfoliated cells and, in the process, undergo differentiation and specialization [14–16] (Fig. 1). However, the homeostatic process that renews the epithelium in the GI tract requires cellular adaptations at the microscopical level that preserve the macroscopic properties of the epithelial barrier. Then, to maintain epithelial barrier functions in the gut, the TJs must evolve and change at the same rate or faster than the IEC. Consistent with this, gastrointestinal TJs are envisioned as highly dynamic entities that rapidly adapt to exogenous and endogenous signals. Therefore, pathological episodes compromising the gastrointestinal epithelial barrier often induce disorganization, destruction, or dysfunction of the TJs [7, 11, 17]. In this overview, we will analyze some of these events when occurring in the inflamed gut.

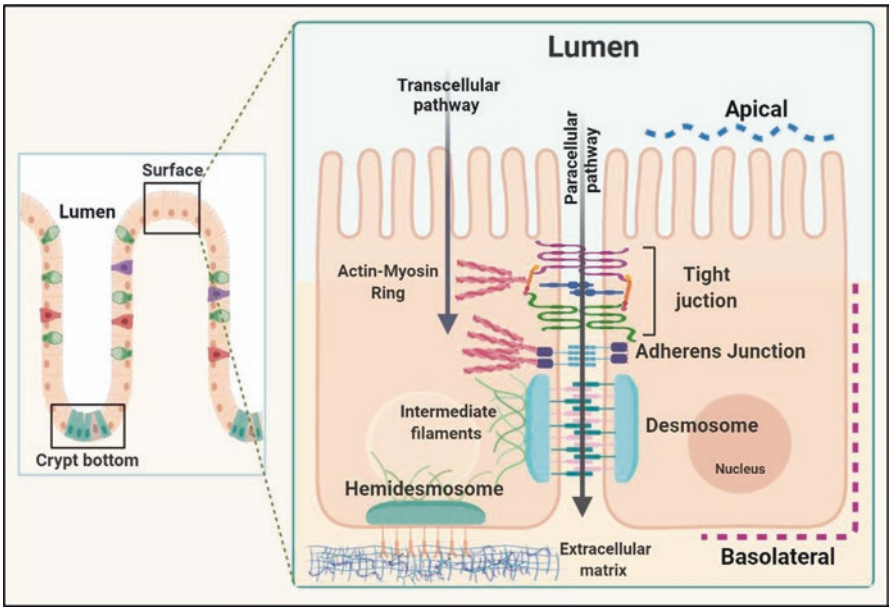

Fig. 2 Intestinal epithelial cells. Drawing depicting the bottom-surface axis in the colonic crypts where millions of polarized intestinal epithelial cells regulate the unidirectional transcellular and paracellular flow of the luminal contents. Intercellular junctions encompassing tight junctions (TJs), adherens junctions (AJ), and desmosomes (DMs) bring cells together, and hemidesmosomes connect epithelial cells to the extracellular matrix. Actin and intermediate filaments constitute the cytoskeleton network in the intestinal epithelial cells

Epithelial Barrier in the Gut

The gastrointestinal epithelium is the most extensive mucosal barrier in the body. Therefore, the single layer of polarized columnar cells lining the organs of the digestive tract and the TJ holding the cells together represent the most important physical barrier in the organism. Two main functions have been attributed to this barrier: (a) halting the entry of toxic materials and (b) facilitating the uptake of nutrients. Thus, an intrinsic property of the epithelial barrier is the ability to consent the unidirectional pass of molecules in a highly selective manner, a process known as intestinal permeability (IP) [1, 18, 19]. The movement of luminal contents through the epithelium follows two major routes: the transcellular and the paracellular pathways [20] (Fig. 2). Transcellular permeability is mediated by specialized transporters or by endocytic/exocytic mechanisms for the translocation into and out of the cell of sugars, peptides, lipids, and several other molecules. Then, transcellular transport involves an active process that uses energy to move substances across the hydrophobic barriers created by the apical and basolateral membranes [21–26]. In contrast, paracellular permeability refers to the free flow of hydrophilic substances across the intercellular space generated between the lateral membranes of

two neighbored cells [9, 10, 27, 28]. The passive absorption at the paracellular route follows a concentration gradient and is limited by the channels created by the extracellular domains of the integral proteins forming the TJ. Contrary to the transcellular transport, the absorption rate at the paracellular pathway cannot be saturated because it is transporter independent.

Tight Junctions

More than 50 different types of proteins have been detected at the TJ. Despite the high number and diversity of the molecules forming these structure, the basic core encompasses tetraspan and PDZ (PSD95, postsynaptic density protein 95; DlgA, *Drosophila* disc-large tumor suppressor; and ZO1, a mammalian tight junction protein) containing proteins [29] (Fig. 3). *Cis* and *trans* polymerization mechanisms

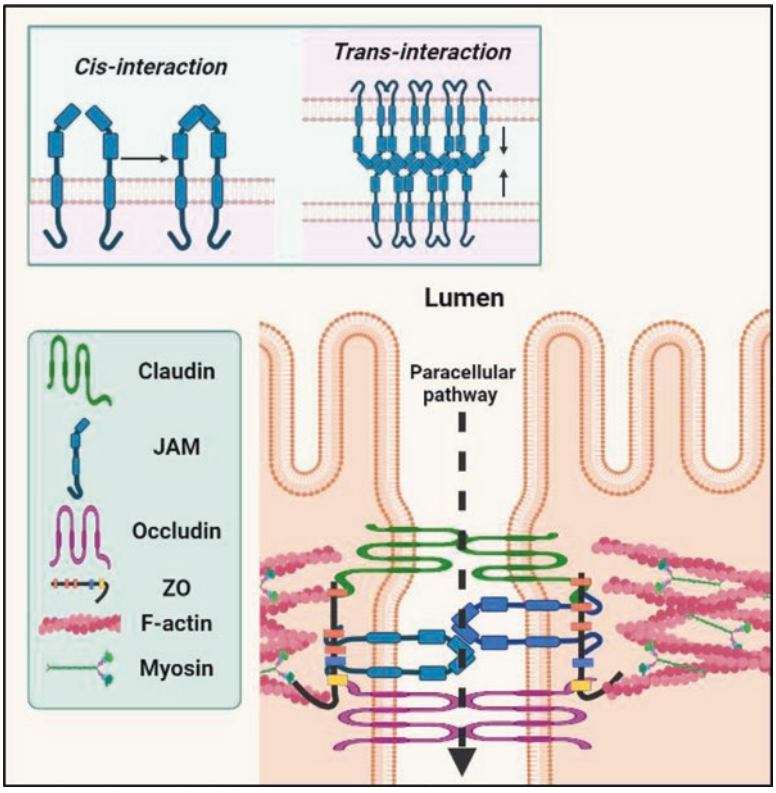

Fig. 3 Tight junctions. Illustration showing the proposal mechanism for *cis* and *trans* interactions that control the assembling of the TJ junctions in the intestinal epithelial cells. Cytosolic scaffolding proteins constitute the cytosolic platforms linking the TJ strands with the actin cytoskeleton

allow transmembrane TJ proteins to produce the paracellular channels that regulate the flow of hydrophilic molecules moving across the intercellular cleft [9, 30, 31] (Fig. 2). C*is* interactions occur within the same membrane and produce non-linear fibrils that traverse the uppermost region of the lateral plasma membrane. In contrast, the *trans* interface is created when fibrils of polymeric TJ proteins bind to the analogous structures present in adjoining cells. *Cis* polymerization of transmembrane TJ proteins entails intracellular adapters to connect the cytosolic C-terminal domain of the integral proteins with the actomyosin ring surrounding the cell. Thus, the vast majority of cytosolic TJ proteins are essentially submembrane protein adaptors linking transmembrane molecules with the actin fibers [32, 33] (Fig. 3). A direct association of the TJ proteins with the perijunctional actomyosin belt confers rigidity and shape to the cells but also plays an important role in epithelial mechanotransduction. In that context, circumferential contractions generated by the actin belt can change the composition of the TJ, the paracellular permeability, and even the turnover rate in the epithelial sheet. Then, the actomyosin belt is the perfect companion for the TJ in controlling epithelial barrier function [32, 34–36]. Scaffolding TJ molecules also create intracellular membrane-bound platforms where signaling molecules are "stationed." In consequence, another function of the tight junctions is to create an extracellular sensing system that transduces information about the external environment. However, by targeting some core components at the TJ, the signaling molecules associated with the scaffolding molecules may also deliver plasticity to the epithelial barrier [32, 33, 37].

Claudins

Claudins are a family of integral membrane proteins (20–34 kDa) comprising approximately 27 members and whose molecular structure includes a short cytoplasmic N-terminal region, four transmembrane domains, two extracellular loops, and a long cytoplasmic C-terminal tail [29, 31, 35, 38] (Fig. 4). The intramembrane and extracellular domains allow *cis* dimerization between claudins within the same plasma membrane and *trans* interactions among claudins expressed in neighboring cells, respectively. Claudins are promiscuous, and therefore *cis* and *trans* interactions can be established by a single type of claudin (homophilic) or different types of claudins (heterophilic interaction) [29, 33, 39, 40].

The first extracellular loop (ECL1) in the claudins forms the central core of the paracellular channels, and its highly charged amino acids confer the anionic or cationic selectivity [41]. In contrast, conserved aromatic residues (phenylalanine 147, tyrosine 148, and tyrosine 158) in the second extracellular loop (ECL2) form the stable *trans* hydrophobic interactions. ECL2 inter-claudin interactions narrow the paracellular cleft and play an important role in the pore size formation [40, 42]. Intracellularly, a C-terminal PDZ-binding motif allows claudins to partner with PDZ-containing scaffold proteins such as zonula occludens (ZO)-1 to ZO-3, multi-PDZ domain protein 1 (MUPP1), and MAGUK with inverted domain structure-1 to

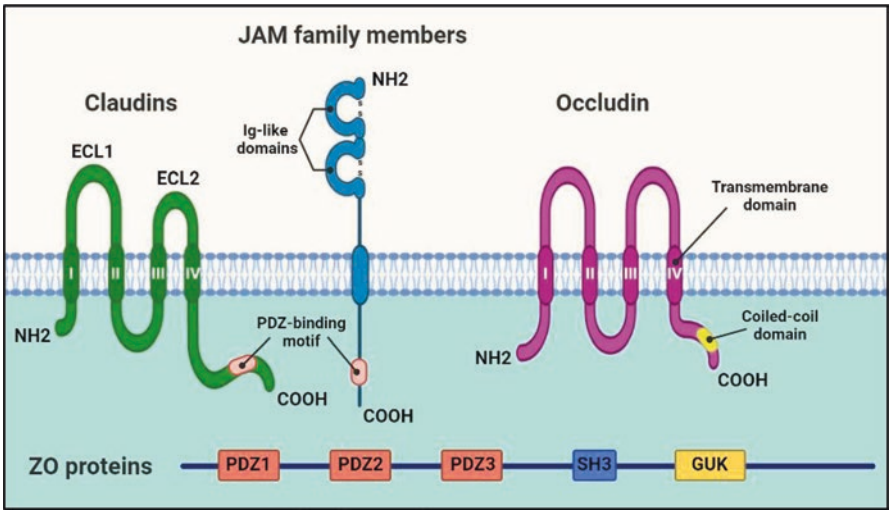

Fig. 4 Tight junction proteins. General depiction of the molecules that make up the central core of the tight junctions: claudins, JAMs, occludin, and ZO proteins

−3 [29, 35] (Fig. 4). By linking the transmembrane TJ proteins with the actin cytoskeleton, the scaffolding proteins perform as a stabilizing spring for the claudin-based TJ strands [32, 37]. Regulatory mechanisms adjusting those interactions regulate TJ efficiency. In this regard, upstream of the PDZ-binding motif, claudin family members contain amino acid residues susceptible of posttranslational modifications such as phosphorylation, sumoylation, and palmitoylation [43–45]. Therefore kinases, phosphatases, small ubiquitin-like modifier (or SUMO) proteins, and DHHC domain-containing proteins recruited to the TJ are decisive factors in the maintenance/establishment of the epithelial barrier. Distribution and expression of claudin family members along the GI tract are notoriously complex with some proteins exhibiting a regional occurrence along the intestine, while other members are ubiquitously expressed (Fig. 5). In connection with this of the 27 claudin family members identified, just claudin-6, claudin-9, claudin-10, claudin-11, claudin-14, claudin-16, claudin-18, claudin-19, and claudin-24 are not to present in the adult murine intestine. Furthermore, each claudin expressed in the murine gut displays a distinctive distribution along the crypt-villus axis with its own characteristic subcellular localization [46–50] (Table 1). For instance, claudin-2 is uniformly expressed in epithelial cells at the bottom of the gastrointestinal crypts; meanwhile, only surface colonocytes and surface duodenal cells are positive for claudin-3 [48, 50] (Fig. 5). Like in the mouse intestine, claudin family members are also partitioned in the human gut (Fig. 5).

In the GI tract, the expression patterns of claudins dictate the biophysical properties of the epithelial barrier. For example, claudin-2 expression induces the formation of cationic channels [54, 58], and claudin-7-based TJs form anionic pores [56, 60]. However, the myriad of hetero-interactions stablished among the claudin

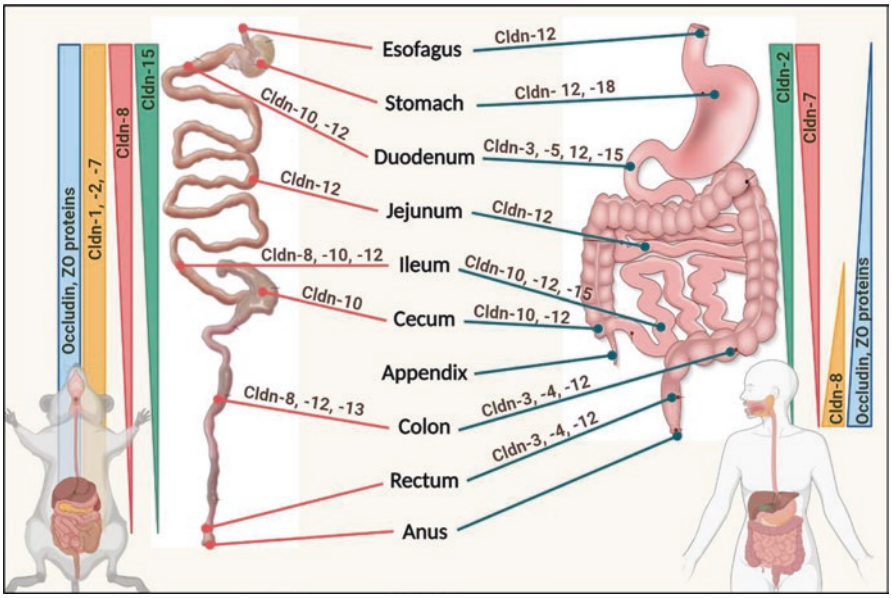

Fig. 5 Tight junction proteins in the gut. Schematic representation for the distribution of TJ proteins in the mouse and human gut

extracellular domains may affect the selectivity properties of the intestinal barrier as shown for claudin 12 [46, 47, 61]. Notable, the intermolecular attractions generated between the extracellular domains of the claudins are also involved in epithelial cohesiveness. Therefore, changes in the expression of claudin family members differentially affect epithelial barrier capabilities [41, 54, 60, 62] (Table 1).

Occludin

Occludin is a 65-kDa tetraspan molecule and was the first integral membrane protein identified in the TJ. Structurally, occludin contains a long cytoplasmic N-terminal region, two extracellular loops, four transmembrane domains, and a long cytoplasmic C-terminal tail [8] (Figs. 4). Homophilic *trans* interactions mediated by highly conserved tyrosines and glycines in the first extracellular loop of occludin create a flexible intermolecular bridge that confers the size selectivity to the TJ [63, 64]. In contrast, the strong and robust interactions mediated by the second extracellular domain stabilize occludin TJ strands [65]. A direct interaction between ZO proteins and the occludin COOH-terminal region allows occludin-based fibrils to be anchored to the actin cytoskeleton [66, 67] (Fig. 3).

N- and C-terminal domains of occludin are prone to posttranslational modifications that affect the localization and function of the molecule. For example, the

Table 1 Gastrointestinal claudins

Claudin	Intestine section	Function	Animal models	Phenotype	Disease	Refs.
1	Ubiquitously expressed throughout the GI tract	Barrier	Global Cldn1$^{-/-}$ mice	Dehydration. Died on postnatal day 1	Reduced expression in colorectal cancer (CRC)	Garcia-Hernandez et al. [48], Nakagawa et al. [51], Yoshida et al. [52]
			Villin-claudin-1 transgenic mice (intestinal claudin-1 overexpression)	Increased IEC proliferation Reduced differentiation of goblet cell		
			APC-Cldn1 mice (apc^{min} mice overexpressing claudin-1)	Enlarged colonic tumor growth and size Poor survival		
2	High levels at birth. The expression decreases in the first 90 days after birth and is restricted to the crypt base	Cationic channel regulates Na + and water. Regulatory functions in Ca^{2+} excretion	Cldn2$^{-/-}$ (global claudin-2 deletion)	Reduced intestinal permeability for Na^{+} and K^{+}. Reduced intestinal calcium secretion Highly susceptible to experimental colitis	Highly expressed in IEC of patients with celiac disease, infectious enterocolitis, and IBD Increased in CRC	Curry et al. [53], Raju et al. [54]
			Cl^{-}2TG (villin-claudin-2 transgenic mice (intestinal claudin-2 overexpression)	Protected against experimental colitis and increased colonocyte proliferation		
3	Distal colon	Barrier			Altered in IBD Increased in CRC	Lameris et al. [50]
4	Distal colon	Barrier				Lameris et al. [50]
5	Duodenum	Barrier			Decreased in CD Augmented in CRC	Xing et al. [55]

(continued)

Table 1 (continued)

Claudin	Intestine section	Function	Animal models	Phenotype	Disease	Refs.
7	Highly expressed in the distal colon	Anionic channel	Global claudin $7^{-/-}$ mice	Intestinal inflammation and mucosal ulcerations Salt wasting, dehydration, and growth retardation, dies within 12 days after birth	Decreased in CRC Reduced expression in invasive and metastatic cells	Ding et al. [56], Lameris et al. [50], Tabariès and Siegel [57]
			Villin-claudin-7 KO mice (intestinal claudin-7 ablation)	Colon inflammation Dies within 12 days after birth		
8	Expression progressively increases from the small intestine to the colon	Barrier			Decreased in CD	Garcia-Hernandez et al. [48]
10	Base of the crypt in the ileocecal junction	Pore				Garcia-Hernandez et al. [48]
12	Ubiquitously expressed throughout the GI tract	Functions depend on interactions with other claudins			Decreased in CD	Lameris et al. [50]
13	Colon, highly expressed in luminal epithelial cells					Fujita et al. [47]
14	Expression progressively increases from the duodenum to the distal colon	Barrier				Holmes et al. [49]

(continued)

Table 1 (continued)

Claudin	Intestine section	Function	Animal models	Phenotype	Disease	Refs.
15	Strong expression in the duodenum and jejunum. Weak expression in the ileum and colon	Cationic pore (Na^+ transport and water)	$Cldn15^{-/-}$ (global claudin-15 deletion)	Mega-intestine Reduced intestinal permeability for K^+ Reduced glucose absorption via the apical sodium glucose co-transporter SGLT		Tamura et al. [58]
18	Duodenum and jejunum				Increased in ulcerative colitis and CRC	Xing et al. [55]
19	Transient expression in mouse jejunum (first 2 weeks after birth)	Barrier				Holmes et al. [49]
23	Superficial epithelial cells of the large intestine	Barrier function remains unexplored			Reduced in CRC	Garcia-Hernandez et al. [48], Maryan et al. [59]

phosphorylation of occludin C-terminal tail impairs the association of the molecule with ZO proteins and disrupts occludin-based TJ [63]. Consequently, protein kinases targeting occludin C-terminal region compromise epithelial barrier functions. Conventional $Ca2^+$-dependent protein kinase C (cPKC), novel PKCs (nPKCs), casein kinase 1 (CK1), CK2, p34cdc2/cyclin B-complex, extracellular signal-regulated kinase 1, and nonreceptor tyrosine kinase c-Yes are among the kinases targeting occludin C-terminal domain [43, 66]. At the opposite end, ubiquitination of occludin enhances the turnover and degradation of the protein. Then an intact N-terminal domain must be necessary to preserve and maintain TJ properties [68]. The expression of occludin 1B, an occludin splice variant missing part of the N-terminal region, may therefore signal the presence of additional regulatory mechanisms in the intestinal epithelial barrier [69].

Occludin is homogeneously expressed in gastrointestinal epithelial cells (Fig. 5), and its loss compromises epithelial barrier function [70, 71]. The mechanisms underlying the process still debatable but, losing occludin from the TJ outcomes in

the removal of the "pacemaker" at the paracellular channels. In supporting the theory, phosphorylation of occludin C-terminal end by CK2 induces its subsequent removal from the paracellular channels formed by the complex occludin/ZO-1/claudin-2 and elevated the permeation of cations, resulting in the so-called leaky gut [72]. Therefore, during inflammation occludin removal from the TJ plays a critical role in the disturbance of the epithelial barrier [73] (Table 2). Within this context, IFNγ increases the paracellular permeability in IEC by promoting the fast removal of occludin from the TJ in a PI3K and Rho-associated kinase (ROCK)-dependent manner [73, 82, 83], and the process occurs without affecting occludin protein levels [83, 84]. Other cytokines enriched in the inflamed gastrointestinal mucosa including TNFα, IL-6, IL-1β, and IL-22 not only stimulate the removal of occludin from the TJ strands but also promote its turnover or lessen its expression [71, 73, 85–88]. However, the multifaceted role of the inflammatory mediators has prevented the full understanding of the mechanisms controlling the formation/maintenance of the occludin-based TJ. For instance, disintegration of occludin-based TJ downstream of IFNγ is lessened by the ubiquitously expressed proinflammatory cytokine IFNβ [89], and IL-17 stimulates the synthesis of occludin and its subsequent recruitment to the TJ strands (J. S. [90], p. 23). Understanding the unique

Table 2 Occludin defects during gastrointestinal diseases

Modification	Disease	Section of the gastrointestinal tract affected	Refs.
↓mRNA, ↓protein Relocated to the basolateral membrane ↑staining in cytoplasm	Irritable bowel syndrome with diarrhea (IBS-D)	Duodenum Jejunum Descending colon Rectosigmoid colon	Hanning et al. [74], Martínez et al. [75]
↓protein	Irritable bowel syndrome with constipation (IBS-C)	Colon	Annaházi et al. [76]
Relocated to the basolateral membrane	Crohn's disease	Colon	Oshitani et al. [77]
↓mRNA	Ulcerative colitis remission	Colon	Yamamoto-Furusho et al. [78]
↑mRNA	Active ulcerative colitis	Colon	Yamamoto-Furusho et al. [78]
↓protein Displaced from the tight junction	Crohn's disease	Colon	Zeissig et al. [79]
↓protein	Collagenous colitis	Sigmoid colon	Bürgel et al. [80]
↓protein	Infection with enteroaggregative or enterohemorrhagic *Escherichia coli* and *rotavirus*	Intestine	Krug et al. [81]

functions of occludin at the inflamed gut is also masked by the redundant roles displayed of the other MARVEL (MAL and related proteins for vesicle trafficking and membrane link) proteins, marvelD3 and tricellulin [91, 92]. In fact, it is well known that marvelD3 can partially compensate for occludin loss [93]. Thus, in the inflamed mucosa, the functions of occludin might be partially substituted by structurally similar proteins and consequently lessen the effects engendered by occludin alterations.

Formation and maintenance of the TJ are not the only role of occludin in the organism. For instance, occludin is strongly expressed in cells lacking TJ, such as immune cells, neurons, and myocytes [94–97]. Additionally, the global occludin knockout (KO) mice reveal no significant abnormalities in either TJ morphology or function. Nevertheless, occludin KO mice exhibit growth retardation, chronic gastric inflammation, and intestinal epithelial hyperplasia [98]. The evidence, therefore, indicates that occludin plays unique roles in the body, such as NADH oxidation [99] or regulation of immune cell transmigration [97]. Hence, although the idea of occludin directly contributing to the formation and maintenance of the TJ is highly accepted, it may be too simplistic and should be reevaluated. In fact, given the additional biological mechanisms where the molecule is involved, a broad study may be necessary to fully solve this puzzle.

Junctional Adhesion Molecules (JAMs)

Junctional adhesion molecules were described in 1998 as immunoglobulin (Ig) superfamily members expressed along the lateral membrane of polarized cells [100]. Some JAM proteins are enriched at the TJ of endothelial and epithelial cells of different origins, including the endothelium and the epithelium of the small intestine, liver, and colon. Classically JAM family members include JAM-A (JAM-1) mainly expressed in endothelial and epithelial cells, JAM-B (JAM-2, VE-JAM) expressed only in endothelial cells, and the ubiquitously expressed JAM-C (JAM-3). Other JAM family members currently identified are JAM-4 and JAM-L (AMICA1). JAM proteins are important regulators of cell-cell contact maturation, epithelial homeostasis, epithelial morphogenesis, and assembly/disassembly of the TJ [101–104].

JAM family members have certain common structural features: a short N-terminal signal peptide, two extracellular Ig-like domains, and a single membrane-spanning element followed by a short cytoplasmic tail (~40 aa) that contains consensus phosphorylation sites and ends with a classic PDZ-binding motif [29, 104] (Fig. 4). *Cis*-dimerization and *trans*-homophilic interactions in JAM proteins are mediated by charged and polar amino acids present in the first Ig domain [105]. Then, the adhesive interface formed by the *cis* dimers establishes hetero- and homophilic *trans* interactions with proteins expressed in neighbored cells (Fig. 3). Physiologically, *cis* and *trans* interactions launched by JAM extracellular domains are necessary for creating and maintaining intercellular contacts. Of note, at the inflammation site,

those interactions also control leukocyte transmigration, angiogenesis, and platelet activation [103]. Direct association of the JAM extracellular domain with occludin is important for TJ assembly, epithelial barrier maintenance, pore formation, and in the regulation of ion conductance [103, 106]. The C-terminal PDZ-binding motif in JAM enables their association with PDZ-containing scaffold proteins such as ZO-1, AF-6, MUPP1, and PAR-3 (also known as ASIP) and with signaling molecules like PDZ-GEF1 [102, 106, 107]. Posttranslational modifications in the carboxyl-terminal domain of JAM-A, JAM-B, and JAM-C not only regulate the interaction of JAMs with the scaffolding proteins but also affect several physiological processes governed by the molecules [43, 108]. For instance, the phosphorylation of JAM-A at S285 by the atypical protein kinase C (aPKC) is necessary for stabilization of nascent TJ strands and epithelial barrier formation [109], and the phosphorylation of threonine 273 and tyrosine 280 is required for hepatocyte polarization and endothelial morphogenesis/angiogenesis, respectively [110–112]. Also, tyrosine phosphorylation of the JAM-A C-terminal region modulates integrin signaling transduction [111]. Meanwhile, the phosphorylation of serine 281 in JAM-C promotes TJ assembly, and the phospho residues in the coxsackie and adenovirus receptor (CAR) C-terminal region control E-cadherin recycling [113, 114]. In the contrary, dephosphorylation of the carboxyl-terminal of JAM family members often impairs TJ assembly and compromises epithelial barrier functions [109, 115]. Thus, the interplay stablished between the kinases and phosphatases targeting the C-terminal domain of JAM proteins controls the assembly and maintenance of the TJ.

In the inflamed GI tract, removal of JAM proteins from the TJ is a very common event induced by protein kinases activated downstream of proinflammatory mediators (e.g., TNFα, IFNγ, IL-22, or IL-17A), the microbiota, and pathogens [103]. PKC, Yes-1, protein tyrosine phosphatase non-receptor type 13 (PTPN13), protein kinase A (PKA), and CK2 are among the kinases that stimulate the turnover of JAM proteins in the inflamed mucosa [109, 116]. Additionally, during inflammation loss of JAM proteins is also reported [103]. Indeed, some pathogens such as adherent-invasive *Escherichia coli* can chronically reduce JAM protein levels in epithelial sheets by changing their gene expression programs [117]. Thus, multiple events compromising the epithelial barrier function in the inflamed gut arise from alterations in JAM proteins.

Zonula Occludens (ZO)

ZO proteins are ubiquitous membrane-associated guanylate kinase (MAGUK) family members comprising ZO-1 (~220 kDa) [118], ZO-2 (~160 kDa) [119], and ZO-3 (~130 kDa) [120]. Originally described as peripheral TJ proteins, the ZO proteins link integral proteins (TJ proteins, adherens junction proteins, and channels) with the filamentous actin cytoskeleton and cytoskeleton-associated proteins [29, 32] (Fig. 3). ZO proteins encompass an amino-terminal half with three PDZ (*P*SD95,

*D*lgA, *Z*O1 homology) domains. Following the PDZ domains, ZO proteins contain a non-catalytic SRC homology 3 (SH3) module and a guanylate kinase homology (GuK) domain [29, 121] (Fig. 4). The large plasma membrane-associated platforms encompassing ZO proteins enable integral TJ molecules to interact and polymerize into proteinaceous fibrils referred to as the TJ strand. Then, in the absence of the ZO proteins, epithelial barrier function is compromised since transmembrane TJ proteins fail to polymerize [122–124]. However, other mechanisms governed by the ZO proteins might also be involved in the epithelial barrier breakdown. For example, the multi-domain arrangement in the ZO proteins consents the formation of signalosomes as well as facilitates the intracellular shuttling of diverse molecules, including transcription factors [125, 126]. Then, modifications in the localization or expression of the ZO proteins might affect a diverse set of mechanisms that influence epithelial barrier functions.

Changes in the expression of ZO proteins during inflammatory disorders are not as remarkable as reported for the integral molecules. However, direct posttranslational modifications or rearrangements of the integral TJ proteins affect the solubility and distribution of these proteins (Table 3) [127, 128, 130]. In the inflamed GI tract, the subcellular distribution of the ZO proteins is often altered [77], and the process directly impacts intestinal epithelial homeostasis, protein expression, and epithelial barrier function. Therefore, minor changes in the distribution of the ZO proteins stemming from inflammatory events result in key biological alterations that compromise organ functionality.

Fence and Gate Functions of the TJ

Two intrinsic properties of the TJ, the gate and fence functions, are essential for the optimal performance of the gastrointestinal tract. Fence and gate functions are controlled by the large array of proteins forming the central core of the junction [29]. For instance, the gate function is generated by a web of paracellular channels established by the extracellular domains of the transmembrane TJ proteins [9, 27, 131]. Paracellular channels discriminate hydrophilic molecules moving across the paracellular pathway based on charge and size, and their formation is primarily

Table 3 Alterations of ZO proteins during intestinal inflammation

Protein	Disease/model	Alteration	Refs.
ZO-1	Crohn's disease	Relocated to the basolateral membrane ↓ protein levels	Das et al. [127], Oshitani et al. [77]
	Ulcerative colitis	↓ protein levels	
	DSS-induced colitis	↓ protein levels	Poritz et al. [128]
	TNBS-induced colitis	↑ protein levels	Han et al. [129]
	Lactobacilli administration	↑ protein levels	Landy et al. [130]

determined by claudin interactions [28, 46, 62]. The highly charged amino acids in the first extracellular loop of the claudins confer charge selectivity and electrical resistance to the channels [29, 41]. By contrast, size discrimination is dependent on the pore density rather than pore composition or pore size. Direct interactions among the extracellular domains of the integral TJ proteins (claudins, occludin, and JAM) limit the rate of pore formation and its size [72, 106]. However, the pore density reflects the pattern of claudin expression. Therefore, it is basically the type of claudins expressed by each epithelium that dictates the gate properties of the TJ.

Transmembrane proteins at the TJ organize themselves in membrane microdomains enriched in cholesterol, also known as lipid rafts [132–134]. The TJ-lipidic microdomains are not randomly stationed in the plasma cell membrane and rather limit the boundary between the apical (luminal side) and the basolateral membranes (interstitial side), two functionally and compositionally distinct domains [4, 135, 136] (Fig. 2). The TJs are not directly responsible for the establishment of the apical-basolateral polarity, but instead create a natural hurdle that restricts the mixing of lipids and proteins freely diffusing in the exoplasmic plasma cell membrane of both domains. Also, TJs specify the location of docking sites for the exocyst and represent regions of active basolateral membrane addition. Thus, in polarized epithelial cells, the fence function of the TJ restricts the intermixing of plasma membrane molecules in the apico-basolateral domains while creating a "touchdown spot" for the intracellular machinery of molecular recognition and sorting [4, 137].

Inflammatory Pathologies in the Gut

The GI tract is normally exposed to multiple insults that need to be properly controlled, and therefore mild inflammatory events are constantly observed in the intestinal mucosa of individuals of all ages [138]. Acute inflammation is also frequent and mostly linked to food allergy, food poisoning, intestinal infection (viral, bacterial, and/or parasitic) or to organ and tissue damage (appendicitis, gallstones, or anal fissures). Episodes of acute inflammation in the GI tract produce a variety of symptoms ranging from abdominal pain, stomach cramps, and diarrhea (sometimes blood can be detected in the stools) to the presence of ulcers/erosions and anorexia [139–141]. In that context, an early and probably transitory epithelial barrier disturbance allows intestinal contents to breach into the submucosa and activate intestinal immune and support cells. Cellular and molecular responses in the inflamed mucosa are aimed to minimize injury and restore tissue homeostasis. However, some inflammatory mediators further weaken the intestinal barrier, enhance intestinal permeability, and cause a "leaky gut" syndrome that exacerbates the influx of luminal antigens [139, 142, 143]. The inability of resolving the acute inflammatory process and reconstituting the GI barrier progresses into a chronic stage [141, 144]. Chronic inflammation is a hallmark of several recognized intestinal pathologies, such as inflammatory bowel disease (IBD) [144], irritable bowel syndrome [141], pancreatitis [145], diverticulitis [146], hemorrhoids, and gastroesophageal reflux disease

[140, 147]. Of note, inflamm-aging [148, 149], obesity [150], metabolic syndrome, and food allergies promote a chronic low-grade inflammation in the gut and should be considered as chronic inflammatory pathologies of the GI tract. The etiology of all those diseases is complex, but, as mentioned before, a compromised intestinal epithelial barrier is the unifying factor.

A compromised intestinal epithelial barrier is also involved in the occurrence of inflammatory pathologies affecting other organs, such as multiple sclerosis, systemic lupus erythematosus, Parkinson's disease, and Alzheimer's disease [143, 151]. The mechanism behind the process is not fully defined although it is possible that local intestinal immune responses triggered after epithelial barrier disruption [152] can spread to other organs. In that premise, acute production/secretion of inflammatory mediators by local-resident immune cells can disseminate and elicit a long sustained immune response in other tissues [143]. Thus, secretory epithelial cell lineages, tissue-resident innate immune cells, and support cells in the GI tract are active mediators in inflammatory processes in the entire body. Then, a healthy and functional epithelial barrier is important for preventing chronic inflammatory diseases in the organism.

Mechanisms Compromising Epithelial Barrier in the GI Tract

The gastrointestinal epithelial barrier separates, physically and functionally, intestinal contents from resident immune cells and consequently favors the induction of oral tolerance toward environmental antigens [152]. A single layer of polarized cells and the TJs sealing the paracellular pathway are the main constituents of the intestinal epithelial barrier. Failing on preserving the integrity of any of those components increases intestinal permeability and stimulates inappropriate inflammatory responses. A hallmark in chronic gastrointestinal inflammation is the presence of a leaky barrier arising from nonoperational TJ [139]. In the inflamed gut, the mechanisms compromising the TJ comprise:

Microbiome

Age-related changes in the intestinal microbial ecology encompassing alterations in microbial diversity and bacterial load create asymptomatic chronic low-grade inflammation in the gut that progresses over time [153]. Inflamm-ageing occurs because of the weakening in the response of the adaptive immune system toward the microbiota which in turns make the innate immune mechanisms to be more responsive [148, 149]. Consequently, chronic activation of the innate immune system and changes in the composition/response of tissue-resident immune cells promotes hypersecretion of proinflammatory mediators and compromises epithelial barrier function [144, 154]. Additionally, the intrinsic properties of the newly generated

microbiome could also affect epithelial barrier capabilities. For instance, bacterial proteases can damage or alter the extracellular domains of the TJ proteins. Also, intracellular signaling originating from the association of pattern-recognition receptors with bacterial bioproducts can adversely affect TJ composition [155]. In the contrary, an abrupt decompensation in the microbiota caused by pathogenic bacteria often implies sudden alterations in the TJ and is characterized by the appearance of diarrhea, abdominal pain, and loss of the epithelial barrier function [156]. Various mechanisms triggered by pathogenic bacteria induce tight junction alterations and include rearrangement of the actin cytoskeleton [157–159]; proteolytic degradation of TJ proteins [160–162]; redistribution, internalization, and dephosphorylation occludin and other integral TJ proteins [163–165]; changes in the association of the TJ proteins that causes aberrant TJ strand formation [166]; binding to the extracellular domains of TJ proteins [163, 167–169]; and activation of signaling transduction pathways that induce internalization and disorganization of the TJ [155, 170, 171].

The pathogenesis in both scenarios presents its own specificities but also shares a common important characteristic, and intestinal mucosal inflammation often progresses to a chronic stage and is accompanied by a severe dysbiosis that mainly reduces the load of butyrate-producing bacteria [172–178]. In that context, we can assume that low-fiber diets, high processed food, or the drastic inflammatory responses during infection stimulate the transition/adaptation of intestinal bacterial species and reduces the bacterial community composition. The imbalance in the composition of pro- and anti-inflammatory bacterial species creates a pathological environment that disrupts homeostasis and differentiation in the epithelium. Such changes modify the composition of the TJ, compromise the epithelial barrier function, and further extend the scope and duration of the immune response. Then, in a scenario of gastrointestinal dysbiosis, the process will eventually accentuates local inflammatory responses that degenerate in a chronic inflammatory disease [153, 179–182].

Changes in the microbiome, including the negligible modifications in the epithelial barrier function experienced during inflamm-ageing, increase the susceptibility or worsen the course of other inflammatory pathologies such as Alzheimer's disease, atherosclerosis, obesity, type II diabetes, and chronic heart diseases [148, 180, 183–186]. Then, dysbiosis is linked to the development of many diseases ailing the organism and is therefore clinically relevant to characterize the imbalance existing in the intestinal microbiota of human patients. Advanced technologies for microbiota/microbiome analysis including Quantitative Insights Into Microbial Ecology (QIIME) and multi-omics analyses will be helpful in accurately measuring and identifying changes in individual microbial populations in the gut. Meaningful information in the changes in microbial ecology will clarify the contribution of different pathogens into the development of various inflammatory disorders [187–190].

Cytokines

Inflammatory mediators enriched in the GI tract encompass a normal response to a wide variety of tissular insults [154]. The regulation of enteric TJ by cytokines, chemokines, and chemical intermediaries has been widely studied, and their contribution in the epithelial barrier disruption well stablished. Mechanisms leading to epithelial barrier deterioration in response to those mediators are vast and include internalization, relocation, and degradation of TJ proteins, stimulation of the actomyosin ring activity, disruption of epithelial homeostasis, destabilization of TJ strands, induction of posttranslational modifications on TJ proteins, disruption of lipid rafts and changes in the organization, and expression and degradation of other cell-cell junction proteins [144, 154, 191–203]. However, new evidence suggests that in IEC the mechanism of action of the inflammatory mediators is more convoluted than previously thought. In connection with this, it has been proven that epithelial polarization plays an important role in the inflammatory response in the epithelial sheets. For instance, cytokine receptors are dissimilarly expressed in the apical and basolateral domains [204–206], and the numerous cell types expressing the inflammatory mediators at the GI mucosa and submucosa differentially target these receptors [192, 207]. Then, it is tempting to speculate that any inflammatory response in the epithelium is highly influenced by the targeted domain and the source of the stimulus. This hypothesis fits in with the regulation of the colitic epithelial barrier by interleukine-6 (IL-6). In the colitic mucosa, IL-6 is produced by surface epithelial cells and by macrophages bordering the basolateral side of the epithelial cells at the base of the crypt. Upon stimulation with IFNγ, IL-6 is apically released by surface IEC and in a non-polarized manner by the pyroptotic macrophages [206, 207]. As a result, IL-6 stimulates apical or basolateral receptors expressed in the surface of epithelial cells. The IL6-IL6 receptor interaction in the apical domain stimulates synthesis of claudin-1, a protein that tightens up the paracellular pathway [206]. However, IL-6-IL-6 receptor association at the basolateral side robustly influences the expression of a leaky claudin, claudin-2 [208]. So, in the inflamed epithelium, IL-6 can parallelly stimulate the expression of leaky and tight claudins.

Adding more complexity to the system, in the inflamed mucosa, the mechanisms for passive cellular release of intracellular contents that usually forge epithelial homeostasis boost the release of commonly secreted proteins [209–213] and create a more sophisticated and intertwined landscape in the system [144, 214]. In such situation, the final outcome will arise from the integration of multiple inputs. Therefore, we must conceive that proinflammatory environments create specific scenarios that differentially affect the role/function of the TJ and ultimately shape epithelial barrier properties.

Intracellular Signaling Pathways

Intestinal epithelial cells express a wide variety of receptors coupled to different transduction signaling pathways. Inflammatory signals activate more than one signaling pathway in the same cell, and targeted cells integrate the inputs through a series of positive or negative feedback loops. Then, the cellular machinery controlling signaling thresholds determines cause-effect sequences that modify or adapt the cell behavior by encouraging posttranslational modifications in different proteins. Posttranslational changes in TJ proteins (e.g., phosphorylation, ubiquitination, sumoylation, and proteolytical processing) instantly impair epithelial barrier functions [43]. Then, the cytosolic platforms bringing signaling molecules and intracellular proteases directly in contact with the integral TJ proteins are responsible for the plasticity observed in the paracellular channels [215]. Additionally, intracellular signaling programs modify the expression of TJ components [215]. Adjustments in the expression of integral TJ proteins create new *trans* interactions among newly synthesized proteins and enables the exclusion or relegation of incompatible molecules. For that reason, the changes in the transcriptional programs gradually modify and adapt the intestinal epithelial barrier [216]. Therefore, the heterogeneous epithelial barrier properties observed along the gut arise from the integration of multiple local inputs created by the specific microenvironments in each portion of the GI tract.

Epithelial Homeostasis

The number of epithelial cells in the gastrointestinal tract must remain constant during the lifetime of an individual to maintain the epithelial integrity that prevents unregulated leakage of intestinal contents into the organism. Surface epithelial cells are continuously damaged by ravaging luminal chemicals and must be replaced by new cells derived from highly proliferative progenitors. The highly proliferative progenitor compartment arises from the asymmetric cell division of local populations of stem cells residing at the base of the crypt. Expansion of the proliferative cell progenitors transiently creates a multiple cell layer until out-of-plane daughter cells migrate and return into the original stratum. The robust signaling program that triggers migration from the crypt base to the lower part of the villus in the progenitor cells also stimulates the commitment of the cells into the appropriate cell lineage. Thus, microenvironmental signaling gradients controlling proliferation, migration, and cell death act in concert to stablish the homeostatic processes that sustain the architecture and lineage compartmentalization in the epithelial sheets in vivo [14]. Changes in the cell physiology or in the environmental cues disrupt epithelial homeostasis and trigger adaptive responses. For example, cell damage accelerates cell turnover at its fastest rate to maintain epithelial homeostasis [15].

Inherently, during cell proliferation, migration, and differentiation and even during the shedding of the damaged cells, the macroscopic properties of the epithelia barrier must remain intact. Consequently, the intracellular signaling programs sustaining epithelial homeostasis must influence the formation and maintenance of the TJ. In connection with this, it has been observed that pro-proliferative signaling pathways control the expression of claudin-1, claudin-2, and claudin-4 ([217, 218, 224]). In contrast, signaling proteins governing epithelial cell differentiation stimulate the expression of claudin-1 and claudin-5 ([219–221]). Based on that, it could be easy to understand the presence of specific absorption profiles along the crypt-villus axis [222] and the changes in the TJ composition exhibited by the different intestinal cell types [223]. However, in an organism the mechanisms controlling cellular homeostasis are never that simple. For example, a particular set of cell-cell adhesion proteins activate signaling molecules that can modify IEC behavior. For instance, claudin family members strongly influence cell cycle progression ([224, 225]). Therefore, we must assume that macro- and micro-intestinal epithelial barrier properties along the gut encompass a heterogeneous response that is influenced by the machinery controlling epithelial homeostasis.

Actomyosin Cytoskeleton

The apical junctional complex (AJC) consisting of the TJ and the AJ multiprotein complexes associates with the perijunctional F-actin myosin ring to confer rigidity and cell shape but also modulates the assembly and disassembly of the AJC. The association between AJC proteins and the apical actomyosin cytoskeleton is tightly regulated by signaling molecules (e.g., mTORC2, PKC, Rho GTPases) and a broad diversity of modulatory actin-binding proteins that include actin-related protein 2/3 complex (Arp2/3 complex), coronin, moesin, cortactin, cofilin-1, and the motor proteins, the myosins (myosin light-chain kinase and non-muscle myosin IIA). The spatiotemporal formation of the actomyosin cytoskeleton provides the mechanical forces necessary for stabilizing, repairing, and disassembling the AJC. Then proper functioning of the actomyosin ring is essential for limiting the paracellular permeability and maintaining intestinal epithelial homeostasis [226–230]. Within this context, it is understandable that any decompensation in the proteins controlling the actomyosin cytoskeleton can compromise epithelial barrier function and induce organ/tissue failure. For example, a dysfunctional intestinal epithelial barrier has been observed in several animal models lacking actin-binding proteins implicated in the actin polymerization (Table 4) [231–235, 237]. Incidentally, the activation of compensatory mechanisms in these animal models allowed the formation and maintenance of semifunctional TJ and prevented the induction of dramatic alterations in the crypt cytoarchitecture. Contrary to the observation for the actin-binding proteins, the removal of the molecules conferring the tensile and contractile forces to the actomyosin ring induces profound intestinal changes that cannot be compensated. For instance, ablation of the non-muscle myosin II, unconventional myosin

Table 4 Defects in regulators of actin filament turnover and effect over TJ proteins

Protein	Animal model and genotype	Phenotype	Tight junction proteins altered	Disease related	Refs.
Neural Wiskott-Aldrich syndrome protein (N-WASP)	Villin-*Wasl* KO mice (intestinal WASP ablation)	Spontaneous increase in intestinal permeability, abnormal microvilli, and decreased perijunctional actin	Occludin mislocalization Impaired assembly and disassembly of AJC proteins		Garber et al. [231]
Cortactin	Cortactin$^{-/-}$ (Global cortactin deletion)	Increased intestinal permeability and augmented epithelial proliferation Deformation of the colonic crypts Increased susceptibility to extran odium ulfate (DSS)-induced colitis	Reduced levels of ZO-1, claudin-1, and E-cadherin Upregulation of claudin-2	Decreased cortactin in IEC of IBD patients	Citalán-Madrid et al. [232]
Actin-depolymerizing factor (ADF) also known as destrin	ADF $^{-/-}$ (Global ADF deletion)	Increased intestinal permeability Increased susceptibility to extran odium ulfate (DSS)-induced colitis			Wang et al. [233]

(continued)

Table 4 (continued)

Protein	Animal model and genotype	Phenotype	Tight junction proteins altered	Disease related	Refs.
Non-muscle IIA (NM IIA) heavy chain	Intestinal epithelial-specific knockout mice	Increased intestinal permeability. Spontaneous rectal prolapses, intestinal lymphoid aggregates, enhanced mucosal cytokine expression, and neutrophil infiltration Increased susceptibility to extran odium ulfate (DSS)-induced colitis	Internalization of claudin-7	Mislocalization of NM IIA in colonic mucosa of Crohn's disease patients	Naydenov et al. [234]
Myosin IXb	Villin-*Myo9b*$^{-/-}$ KO mice (intestinal *Myo9b*$^{-/-}$ ablation)	Impaired weight gain and intestinal bleeding Increased IEC apoptosis Intestinal mucosal damage (ulceration and neutrophil infiltration in the ileum)	Reduction of ZO-1, claudin-1, and occludin in IEC	Myosin IXb is an IBD susceptibility gene	Hegan et al. [235]
Myosin 1d	Myo1d$^{-/-}$ (Global cortactin deletion)	Disruption in intestinal homeostasis and epithelial barrier function Impairment of actin remodeling			McAlpine et al. [236]
Myosin 1a	Myo1a$^{-/-}$ (Global cortactin deletion)	Disruption in intestinal brush border			Tyska et al. [237]

IXb, or unconventional myosin 1D results in spontaneous intestinal inflammation, spontaneous colitis, and high susceptibility to DSS-induced colitis, respectively (Table 4) [234–236].

In the case of inflammatory events, most of our understanding concerning the regulation of the TJ proteins by the actomyosin ring comes from in vitro studies. Defects in the TJ that are associated with the impairment of the actomyosin ring are diverse and include increased endocytosis of integral TJ proteins, changes in the tensile forces at the AJC, mistargeting of TJ proteins, changes in epithelial homeostasis, and modifications in the cellular transcriptional program [36, 82, 216, 227]. However, with the advent of transgenic animal models, other incidents driven by the actomyosin ring decompensation that went longer undetected have been revealed. For example, in vivo models showed that the acute responses of the actomyosin ring prevented tissue damage and/or organ failure (Table 4).

Functional Analysis of the TJ in the Gut

Acute recurrent inflammatory episodes evolve into a chronic pathology. Therefore, in patients with intestinal inflammation and its asymptomatic relatives, it is highly recommendable to continuously monitor and evaluate epithelial barrier function. Several techniques are commonly used to analyze the contribution of TJ proteins to the maintenance of the gastrointestinal barrier (Fig. 6). However, other techniques mostly used by experimental pathologists can provide useful information to complement the diagnosis. Many of these assays are based on the analysis of cultured epithelial cells since they provide a clearer system for quantification and visualization of the TJ. Therefore, for their use as a diagnostic tool, we must adapt the assays to the clinic.

Intestinal Permeability in Patients

Sugars and hydrophilic radioactively labeled molecules orally administered are rapidly absorbed in the small intestine through the paracellular pathway [238, 239]. Once consumed the levels of the molecules rapidly increase in the bloodstream, and non-metabolized compounds are subsequently cleansed by the urinary system (Fig. 6). The amount of the tracer retrieved from blood or urine samples over a certain period will be a direct reflection of the IP and the integrity of the TJ [21]. Clinically, IP is mostly assessed by monitoring renal excretion as a noninvasive test. Although practical and safe, the use of this approach is rather limited due to inherent and methodological problems [139]. For example, the sensitivity for some tracers is low, and the villous atrophy induced by inflammatory events reduces the absorptive area and artificially decreases intestinal absorption of the probes. Additionally, some of the sugars used as tracers are widely present in nature, especially in edible

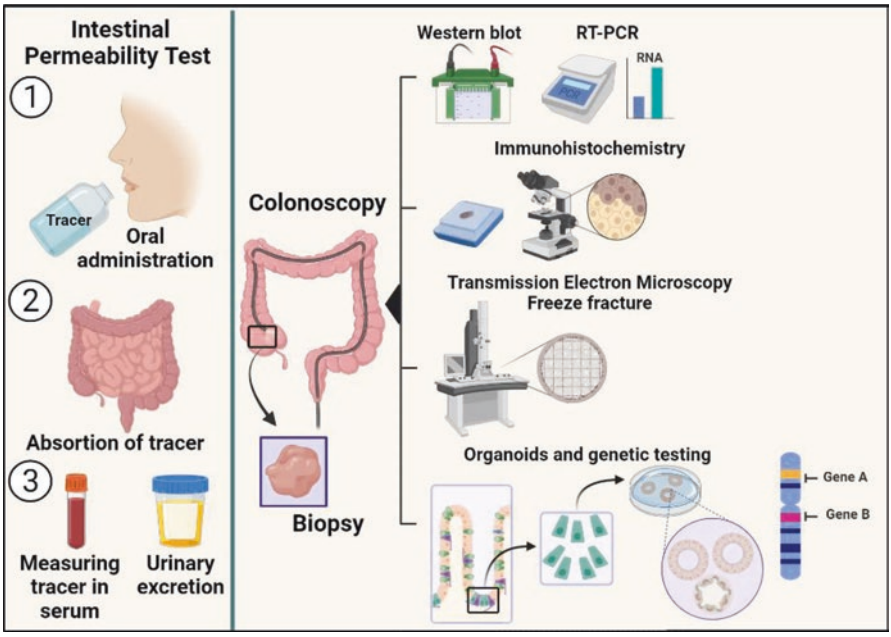

Fig. 6 Functional analysis of the TJ. Illustrations corresponding to current and suggested methods useful to analyze the function of the TJ in the human gut

plants, and repeatedly affect the measurements. Intestinal intolerance to sugars and the danger posted by radioactive probes are also inconveniences limiting the number of functional tracers available.

TJ Proteins in Biopsies

A broad number of TJ proteins are fully characterized, and therefore evaluating posttranslational modifications or expression changes in these molecules represents a viable method to explore the integrity of the TJ [43]. Also, the full arsenal of commercial tools raised against TJ proteins will enable experimental pathologists to assess subtle changes in the molecules when analyzing fresh biopsies of patients with increased IP. Western blot and RT-PCR analysis can be used to efficiently identify alterations in TJ proteins. In the inflamed gut, claudin-1, claudin-2, claudin-3, and claudin-4 are the most studied TJ proteins, but alterations in occludin and ZO molecules are also fully documented [74, 76, 79]. The wide range of mechanisms reshapes the TJs in the inflamed mucosa, and it is well known that TJ proteins display continuous or discontinuous patterns of modifications along the gastrointestinal tract. Specimens harvested from different areas from the same individual may even show different posttranslational modifications for the same protein. Different modifications in the same molecule may impair its function at different levels. Then,

tracking the site of the biopsy and analyzing biopsies from different regions are important to accurately evaluate the degree of commitment of the different TJ proteins within the GI tract.

Immunohistochemistry

During endoscopy it is common to observe normal-looking areas with abnormal histology [240]. In these areas, epithelial barrier function impairment arises from changes that minimally affect TJ proteins and therefore undetectable by quantitative methods. Therefore, under these circumstances, alternative methods such as immunohistochemistry (IHC) analyses of paraffin embedded sections represent an excellent alternative to analyze changes in the TJ proteins. In that context, IHC analysis allowed experimental pathologists to identify changes in the distribution of claudin-1, claudin-2, claudin-3, claudin-4, and claudin-7 in the colitic mucosa of IBD patients [127, 241]. Alterations in the distribution of occludin and ZO proteins were also appreciated during IHC analysis [127]. However, it is important to point out that identifying an anomalous distribution in the TJ proteins cannot deliver an etiological diagnosis. Therefore, IHC analyses may only be used as a complementary tool to acquire additional information pertaining to the changes experienced by the TJ.

Other Methods

Using unconventional or more sophisticated methodology for analyzing biopsied tissues from patients emerges as a good approach to fully identify the mechanisms compromising intestinal TJ. Then, transmission electron microscopy, freeze-fracture, and organoid cell culture assays may have to be adapted and included as routinely evaluation tools.

Light microscopy is a fast and useful tool to grossly analyze TJ proteins. However, electron microscopy offers information on the TJ structure at the nanometer level and is effective to characterize TJ components and their precise distribution [242]. Furthermore, by incorporating electron-dense tracers of different sizes into the analysis, we may be able to determine some of the permeability capabilities exhibited by the specimens and evenly delineate the extracellular spaces. Electrodense probes may be also used to define the specific site of the TJ concerning to the other intercellular junctions.

In the freeze-fracturing process [243], a biopsy will be quickly frozen in liquid nitrogen and then tensile or knife fractured. The fracture of the specimen will occur along the weak portions of the tissue, usually the center of the lipid bilayer, making the transmembrane proteins of the TJ partition in one of the sides of the lipid bilayer.

Then, the labile-frozen surface will be shadowed with a thin layer of platinum carbon before the organic material is removed. The composition and properties (leaky or tight) of the TJ can be predicted by analyzing the two replicas of the membranes that were obtained during the process. The type of strands present within the replicas, the face where those fibrils are present, and the shape exhibited for the strands indicate specific characteristics in the TJ [244]. For example, at the inflamed gut, strand breaks indicate a disruption in the continuity of the TJ, and alterations in the typical pattern of the TJ strands show abnormal assembly of the junction [245].

The etiology of the inflammatory diseases targeting the GI tract considers genetic and epigenetic components that predispose specific individuals to develop these pathologies. Whole-genome association studies identified genetic variants and alterations in epithelial signaling pathways that could be accounted for the genetic susceptibility. Novel models addressing epithelial biology will be useful in assessing these specific dysfunctions. The growth of organoids, a technological breakthrough, allows to re-create three-dimensional intestinal epithelial tissue in vitro, starting from intestinal epithelial stem cells recovered from biopsied specimens [16, 246–248]. Advantageously, the organoid retains the properties from the parental tissue allowing to study its natural properties in a strictly controlled environment [249]. Therefore, culturing organoids constitutes a viable method to investigate and validate mechanisms affecting the composition and function of the TJ including genetical/epigenetical alterations.

The current methodology for analyzing ion conductance in the paracellular channels and the fence function of the TJ involves monitoring and measuring the diffusion of ions and probes in real time. Additionally, such analysis heavily depends on the use of single-cell monolayers with a homogenous population. Then, functional assays for investigating the fence and gate function are circumscribed to in vitro studies. Nevertheless, with the advent of the organoid growth, the pathological mechanism(s) affecting the function of the TJ in the inflamed mucosa of each individual might be certainly replicated in a controlled environment. Then, tissue-like structures cultured from biopsied human pluripotent epithelial stem cells and single-cell monolayers using 2D cultures derived from organoids represent a good approach to overcome the problem (Y. [250, 251]).

Acknowledgments We want to extend our gratitude to Dr. Zayda Piedra Quintero for sharing her expertise in the inflammation field and for her critical discussion of the manuscript. The project was supported by the sectoral funding for research and education via the grant for Basic Science from Conacyt (No. A1-S-20887 to PND) and the SEP-Conacyt grant (No.179 to NV/PND). MREG is the recipient of a doctoral fellowship from Conacyt (860508).

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The Myriad Ways Enteropathogenic *Escherichia coli* (EPEC) Alters Tight Junctions

Rocio Tapia and Gail Hecht

Abstract Enteropathogenic *Escherichia coli* (EPEC) is considered one of the most important enteric pathogens infecting children and one of the main causes of diarrhea worldwide. EPEC uses a type 3 secretion system (T3SS) to inject effector proteins into host intestinal epithelial cells, causing diarrhea. Through a coordinated action of virulence factors, EPEC translocates effectors into host cells, resulting in the perturbation of cellular structures and functions by altering cell signaling pathways. Epithelial cells are held together by apical junctional complexes, including tight junctions (TJs), adherens junctions (AJs), and desmosomes. TJs contribute to the establishment of barrier function and maintenance of apico-basal cell polarity. TJ integrity relies on several cell structures and functions including the actin cytoskeleton, microtubule networks, membrane integrity, inflammation, and cell survival. EPEC perturbs TJ structure and function, leading to impairment of the intestinal barrier. This chapter summarizes the various mechanisms employed by EPEC that contribute to TJ disruption.

Keywords Enteropathogenic *E. coli* · Tight junctions · Microtubules · Intestinal permeability · Apico-basal polarity · Transepithelial electrical resistance

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L. González-Mariscal (ed.), *Tight Junctions*,
https://doi.org/10.1007/978-3-030-97204-2_7

Introduction

Historically, enteropathogenic *Escherichia coli* (EPEC) has been considered an important cause of infantile diarrhea in developing countries. EPEC, however, are divided into typical and atypical strains. Typical EPEC expresses bundle-forming pili (BFP) encoded on the *E. coli* adherence factor plasmid (EAF). These structures allow for microcolony formation and enhanced efficiency of T3SS effector delivery into host cells via BFP retraction [1]. Atypical EPEC now accounts for ~95% of isolated clinical EPEC strains [2]. Atypical EPEC causes diarrhea not only in children but also in adults. Interestingly, up to 22% of healthy asymptomatic individuals test positive for EPEC by multiplex PCR assays [2–4]. Most of the studies regarding the effect of EPEC on tight junctions have used typical EPEC strains, most notably E2348/69. Therefore, the information presented here concerns typical EPEC.

EPEC adheres to the apical surface of host intestinal epithelial cells and produce attaching and effacing (A/E) lesions, characterized by intimate attachment and the loss of microvilli [5, 6]. The factors responsible for A/E lesion formation are encoded by a 35-kilobase pathogenicity island called the locus of enterocyte effacement (LEE) [7, 8]. The LEE encodes both the T3SS and bacterial effector proteins it delivers into host cells. The molecular structure of the T3SS shows that EspB and EspD form a pore in the host membrane and EspA forms a hollow filamentous structure that assembles as a physical bridge between bacteria and the host cell surface, allowing the translocation of EPEC effectors into host cells [9, 10]. LEE EPEC-secreted effector proteins, including translocated intimin receptor (Tir), EspF, EspG, EspH, mitochondrial-associated protein (Map), among others, interfere with a variety of host cells functions. EPEC also uses effectors encoded outside of LEE, known as non-LEE-encoded effectors such as NleA, NleB, NleC, NleH, EspG2, EspM, and EspT, among others. The synergistic effects of effectors, either encoded inside or outside of LEE, contribute to EPEC pathogenesis [11–15].

Molecular Structure and Function of Epithelial Tight Junctions

Tight junctions (TJs) are part of the cell-cell adhesion complex localized to the most apical portion of the lateral membrane of epithelial cells. Ultrathin-section electron microscopy reveals that TJs are discrete membrane fusions, involving the outer leaflet of the plasma membrane of adjacent cells sealing the intercellular space in epithelial and endothelial cellular sheets [16]. Freeze-fracture electron microscopy shows that TJs are a set of continuous intramembranous particle strands composed of integral membrane proteins [17, 18]. TJ strands contribute to the establishment of a permeable-selective barrier for charged ions and uncharged solutes through the paracellular space [19, 20]. TJs are also involved in creating and establishing apical and basolateral membrane domains, inhibiting the free movement of proteins and lipids through the different membrane surfaces [21].

At the molecular level, TJs consist of transmembrane cell-cell adhesion molecules located between cells in epithelial sheets (occludin, claudins, tricelulin, JAMs, CAR, etc.), and scaffold submembrane proteins (ZO-1, −2, −3, cingulin, paracingulin, MAGI-1-3, MUPPI-1, etc.), associated with integral TJ membrane proteins through multiple interactions. In this chapter, we briefly describe the adhesion proteins involved in regulating barrier function that are targeted by EPEC.

Occludin was the first transmembrane TJ protein identified [22–24]. Although occludin appears to not be involved in TJ strand formation or in intestinal barrier function, occludin-deficient mice show histological abnormalities in several tissues [24]. The claudin family of proteins is composed of 27 four-transmembrane domain proteins and constitutes the backbone of TJ strands [25–27]. Intracellular interactions between claudins create the paracellular barrier and/or channels with specific characteristics that define TJ function [28–33]. Tricellulin is a transmembrane protein preferentially localized at the vertically oriented TJs of tricellular contacts. Tricellulin has been reported to be critical in the formation of the epithelial barrier and the organization of bi- and tricellular TJ contacts [34]. Junctional adhesion molecules (JAMs) are single-span TJ membrane proteins. JAM proteins contain immunoglobulin (Ig)-like ectodomains and are found in epithelial cells, endothelial cells, leukocytes, and myocytes. JAM-A has been reported to coordinate TJ development and epithelial polarity [35, 36].

The TJ scaffold proteins ZO-1, ZO-2, and ZO-3 are multidomain proteins that belong to the membrane-associated guanylate kinase homologs (MAGUKs) consisting of PDZ, SH3, and enzymatically inactive guanylate kinase (GUK) domains [37–39]. ZO-1 and ZO-2 are required for TJ strand assembly and epithelial polarity [36, 40]. Cingulin is a coil-coiled domain peripheral membrane protein that binds directly to ZO-1 [41, 42]. Cingulin is involved in the recruitment of GTPase regulatory proteins to TJs; its phosphorylation promotes the junctional association of microtubules [43–45]. Cingulin has also been reported to regulate gene expression and cell proliferation [46, 47]. Afadin is localized at both TJ and AJs and has a critical role in the early polarization of the apical junctional complex [48, 49]. MAGI (membrane-associated guanylate kinase inverted-1 and 3) and MPDZ (multiple PDZ domain protein, MUPPI-1) are PDZ domain scaffolding proteins that interact with a large number of TJ proteins [50–52]. MAGI-3 has been implicated in the regulation and phosphorylation of the JNK signaling pathway [53]. Although there is vast evidence regarding its numerous protein interactions, the role of these multiple PDZ molecules in TJ physiology is yet not clear.

Impact of EPEC Effectors on TJs

TJs are dynamic structures under normal physiological conditions and are altered in a variety of disease states. Infection with microorganisms, such as bacteria, viruses, parasites, and bacterial toxins, can disrupt TJ barrier. Increased intestinal TJ permeability may contribute to diarrhea and to inflammatory responses. EPEC perturbs intestinal epithelial cell (IEC) function as demonstrated by increased permeability

to ions and solutes and loss of transepithelial electrical resistance (TEER), a conventional barrier assessment used to detect changes in paracellular barrier properties. These changes are associated with the redistribution of TJ proteins. EPEC effectors cause such changes by activating a variety signaling pathways that induce cytoskeletal rearrangements that impact the localization and functionality of membrane-associated proteins (e.g., adhesion components, cotransporters, channels, microtubules, polarity complexes, etc.). In this chapter, we focus only on the EPEC effectors that play an important role in the disruption of TJs.

In vivo and *in vitro* studies show that EPEC changes the localization of TJ proteins and perturbs intestinal barrier function. EPEC infection diminishes ileal and colonic mucosal barrier function in murine models and augments paracellular permeability demonstrated by the ability of the molecular tracer biotin to traverse the intestinal epithelium into the lamina propria. These changes correlate with the redistribution of occludin, claudin-1, and ZO-1 from cell-cell contacts into cytoplasm [54–56]. *Citrobacter rodentium*, an A/E-inducing pathogen of mice, has similar pathogenic mechanisms as EPEC and shows the redistribution of claudin-1, claudin-3, and claudin-5 requires EspF [57]. *In vitro*, EPEC effectors exert synergistic effects to increase intestinal permeability, redistribute TJ proteins, and alter transport functions [58–61].

Tir is delivered into the plasma membrane of host cells by the T3SS and serves as a receptor for the EPEC outer membrane adhesion protein, intimin, a bacterial surface adhesion encoded by the *eae* gene housed in the LEE pathogenicity island [11]. Initial Tir/intimin binding promotes intimate EPEC adhesion to host cells and A/E lesion formation. A/E lesions are characterized by effacement of microvilli in the area of attached EPEC and a dense concentration of actin microfilaments beneath intimately attached bacteria [6]. EPEC infection stimulates the production of inositol phosphates such as PI(4,5)P_2 and PI (3,4,5)P_3, which accumulate beneath EPEC microcolonies and are required for EPEC adherence to the host cell surface and actin pedestal development in a Tir-dependent manner [62–69].

Tir is phosphorylated at its COOH-terminus by host tyrosine kinases [70, 71]. Tir phosphorylation (Y474) facilitates binding to the SH2 domain of Nck, a host adaptor molecule, and the activation of Neural Wiskott-Aldrich syndrome protein (N-WASP) and Arp2/3 actin-nucleation complex, promoting reorganization of the host cytoskeleton leading to the formation EPEC pedestals [72–77]. The NH-2 terminus of Tir binds to cytoskeletal components found within pedestals, such as α-actinin, talin, vinculin, and ezrin [73, 78–82]. Additionally, Tir interacts with and recruits components of intermediate filaments (IF), CK8 and CK18, to pedestals [83]. EPEC effectors also redistribute TJ proteins to these structures. N-WASP activation triggered by Tir/intimin binding contributes to the recruitment of ZO-1 to EPEC pedestals via its proline-rich region (PRR) [84]. These findings suggest that Tir promotes the local accumulation of inositol phosphates beneath EPEC pedestals and, through its protein-protein interactions, stabilizes pedestals by anchoring EPEC to the cytoskeleton of host cells. The recruitment of TJ proteins mediated by Tir to pedestals may be an important step to indirectly destabilize the structure and function of TJs.

EPEC effectors cooperate in a coordinated manner to cause TJ disruption. For example, Tir and intimin interactions play a crucial role in mediating EPEC intestinal epithelial TJ disruption [85, 86]. Infection of intestinal cell monolayers with a *tir* deletion strain does not alter TEER, suggesting that the Tir/intimin interaction is needed for attachment and delivery of EPEC effectors into host cells that ultimately impact permeability. In addition, EPEC alters the distribution of β1-integrin, which is typically restricted to the basolateral membrane. Interestingly, the apical positioning of β1-integrin from the basal domain upon EPEC infection allows it to interact with Tir, substituting for the natural EPEC ligand intimin. The interaction of β1-integrin with Tir results in impaired barrier function [85]. However, EPEC-mediated loss of TEER does not require Tir as expression of intimin alone can induce intestinal barrier dysfunction and remove occludin from TJs. The absence of Tir, however, does not prevent the delivery of Map or EspF effectors into host cells, two well-known effectors associated with TJ disruption [59, 60, 87, 88]. These data suggest that both Tir and intimin are required to initiate the downstream signals that perturb TJs function, and that intimin, probably by interacting with host cell proteins, contributes to the ability of EPEC effectors to disrupt intestinal barrier function in a Tir-independent manner.

EspF has multiple functions in host cells including nucleoli disruption, multinucleation, and cell hypertrophy [89, 90]. The NH-2 region of EspF functions as a mitochondrial targeting signal (MTS) that targets host cell mitochondria and induces cell death [91–94]. The COOH-terminus of EspF contains binding sites for the SH3 domain of the endocytic modulator sorting nexin 9 (SNX9) through its RxAPxxP motif [95, 96]. EspF also interacts with SNX18 and SNX33, and WIPF1 (WAS/WASL interacting protein family member 1) proteins [97]. EspF possesses N-WASP-binding sites and directly stimulates the actin-polymerizing activity of N-WASP [96, 98]. EspF from rabbit EPEC (REPEC) associates with N-WASP and Arp2/3 and recruits ZO-1, ZO-2, occludin, and claudins to actin-pedestals, promoting pedestal biogenesis [99]. In addition, EspF induces the sequential removal of ZO-1 and afadin from cell-cell contacts with relocalization to actin-pedestals, driving pedestal maturation [100]. Recent evidence demonstrates that EspF, through its binding partners SNX9 and N-WASP, promotes the redistribution of ZO-1 and ZO-2 from TJs to actin-pedestals [101]. Interestingly, although mutations in neither SNX9- or N-WASP-binding domains of EspF affect EPEC actin-pedestal formation, the binding of EspF to SNX9 alone is sufficient to contribute to the actin-pedestal organization and increased colocalization of aPKCζ and F-actin in those structures [101, 102].

The essential role of EspF in EPEC pathogenesis has been demonstrated. Infection of mice with an EPEC strain deficient of *espF* has no effect on barrier function or on ileal TJ morphology. Only minor alterations of colonic TJ structure are seen at 1-day postinfection, but these defects disappear at later times postinfection [54]. In polarized intestinal epithelial cell culture monolayers, delivery of EspF correlates with a decrease in TEER, increased intestinal permeability, and redistribution of occludin [103]. Although the mechanisms by which EspF perturbs TJs are

still unknown, data suggest that the EspF chaperone CesF plays a crucial role in the disruption of intestinal epithelial barrier function [104, 105].

Although EPEC is characterized as a noninvasive pathogen, EPEC utilizes membrane microdomains (lipid rafts) of the host cell to invade IECs, leading to the disruption of TJ structure, altered composition of TJ proteins associated with lipid raft-membrane fractions, and a drastic drop in TEER [96, 106, 107]. EPEC invasion has been reported to be dependent on EspF, which accumulates in patches at the cell surface and colocalizes with clathrin. EspF interacts with SNX9, causing the formation of elongated plasma membrane tubules, as well as the internalization of EPEC into IECs [96, 107]. Recent evidence shows that EPEC prompts the recruitment of clathrin and AP2, early (Rab5a, Rab7, and EEA1) and recycling (Rab4a, Rab11a, Rab11b, FIP2, Myo5b) endocytic proteins, to the sites of bacterial attachment [97, 108]. Additionally, transferrin receptor (TfR), β1-integrin, Exo70, a major component of the exocyst complex, and the basolateral protein VAMP3, involved in docking and fusion vesicles containing basolateral cargo, and aquaporins (AQPs), are also recruited to pedestals. The recruitment of these proteins to sites of infection correlates with increased endocytosis, recycling, and transcytosis to the infected plasma membrane [97, 108]. The movement of endosomes and associated endocytic proteins in polarized epithelial cells depend on EspF. SNX9 is recruited to clathrin-coated pits and, in conjunction with N-WASP and associated proteins (dynamin, Arp2/3 and AP2), promotes the endocytosis and recycling of plasma membrane proteins [96, 97, 109–113]. For example, Crumbs3 (Crb3), a polarity protein, is internalized via Rab5 vesicles and it is driven to the lysosome compartment in an EspF- and dynamin-dependent endocytic process. The association of EspF with SNX9 causes displacement of Crb3 from the membrane surface to the cytoplasm [114]. EspF/SNX9 interaction also alters the localization of occludin, ZO-1, and JAM-A/Ser285, a crucial phosphorylation step for TJ assembly. Ablation of the EspF/SNX9 interaction or mutations in the SNX9- and N-WASP-binding sites restore the junctional localization of these TJ proteins [35, 101, 102, 114].

Several studies have independently demonstrated EspF binding to SNX9 and N-WASP and their impact on TJs. An *espF*-SNX9-binding-deficient mutant strain disrupts barrier function in polarized T84 cells but does not alter the membrane localization of occludin, suggesting that this interaction may be dispensable for EPEC-induced junction disruption [96]. However, recent studies have shown that although mutations in either the SNX9- or N-WASP-binding domain of EspF, or an *espF* mutant, failed to bind SNX9, both attenuated EPEC-induced TJ disruption and do not alter the junctional localization of occludin in infected cells [101, 102]. The discrepancy in these data suggests that redundancy in sorting nexin proteins in colon cancer cell lines may influence the impact of EPEC on barrier function. The ability of EspF to bind both SNX9 and N-WASP is important, however, for the full effects of EPEC on intestinal permeability.

EspF also regulates the expression of TJ proteins through transcriptional and posttranslational mechanisms. Ectopic expression of EspF disrupts TJ integrity and prevents the recruitment of occludin, claudin-1, and − 4 and ZO-1 into TJs during junction assembly, leading to their cytoplasmic accumulation and eventual removal

by lysosomes [115]. These data suggest that EspF promotes EPEC invasion of IECs, regulates the transcription of TJ proteins, and induces endocytosis, recycling, and trafficking of vesicles destined for the basolateral membrane to the apical sites of bacterial attachment. This may contribute to EPEC attachment and subsequent microcolony growth.

Besides its role in TJ perturbation, EspF dismantles IF architecture although other secreted proteins may be involved. EPEC infection increases the solubility of CK18, a component of IF cytoskeleton, and promotes its binding with the adaptor protein 14–3-3ζ in infected cells. EspF interacts with host cell protein CK18 and it has been suggested that EspF forms a complex with CK18 and 14–3-3ζ during EPEC infection [116].

Map is a 203 amino acid protein that contains a mitochondrial-targeting sequence that directs it to mitochondria, causing dysfunction [117, 118]. Map also possesses a WxxxE motif that induces filopodia formation through the activation of Cdc42, functioning as a GEF [14, 119, 120]. A functional PDZ ligand, the TRL motif, at the COOH-terminus, is also involved in filopodia formation and remodeling and elongation of the brush border in infected cells [119, 121]. This motif allows Map to interact with the host cell protein NHERF. During EPEC infection, NHERF1 is recruited to sites of bacterial attachment colocalizing with ezrin, which is activated by EPEC, resulting in altered barrier function [121, 122]. The binding of NHERF1 to ezrin functions as a molecular scaffold that links Map to the actin cytoskeleton. This interaction assembles a positive feedback loop that amplifies Cdc42 signaling within membrane microdomains [123].

Map has been shown to play a major role in disruption of intestinal barrier function as *map* deletion strains have less impact on both TEER and loss of occludin from cell junctions. Although the mechanisms by which Map disrupts TJs are not known, it likely occurs through modulation of the actin cytoskeleton. Map cooperates with EspF to mediate TJ disruption [88]. Epithelial cells constitutively expressing EspF and Map have been used to elucidate the role of these effectors on TJs. Ectopic expression of Map increases epithelial permeability and prevents junctional recruitment of TJ proteins, ZO-1, occludin, claudin-1, and claudin-4 into TJs during *de novo* TJ assembly. In addition, expression of EspF and Map downregulates the expression of claudin-1, claudin-4, and occludin and inhibits their junctional recruitment, leading to depletion at the plasma membrane. Interestingly, Map significantly downregulates the transcription of claudin-1. Map interacts with all isoforms of nonmuscle myosin II and actin, and EspF binds to ZO-1 and actin [115]. These data suggest that these EPEC effectors regulate TJs by interacting with cytoskeletal proteins and regulating contractility of the actomyosin cytoskeleton.

EspG is an EPEC effector with multiple functions. EspG binds to host ADP-ribosylation factor (ARF), p21-activated kinases (PAKs), and Rac/Cdc42-binding domain of PAK1, all important regulators of the actin cytoskeleton and cell motility [124–126]. Recent findings indicate that EspG, via binding to ARF and PAK, plays an essential role in EPEC attachment and pedestal formation in host cells [127, 128]. EPEC activates Rac and Cdc42 RhoA GTPases in an EspG-dependent manner. EPEC promotes the activation of Rho GTPases by recruiting Fabrin, a host

Cdc42-specific GEF and important regulator of the actin cytoskeleton, to actin-pedestals [128, 129]. PAK activation, pedestal formation, and bacterial attachment are reduced in Fabrin-deficient cells. EspG localizes to actin-pedestals via Arf6 binding. The recruitment of Fabrin to sites of EPEC attachment depends on EspG and requires the enrichment of PIP2 and host ARF6 [128]. These data suggest that EspG contributes to pedestal biogenesis by binding and activating cellular GTPases required for actin pedestal formation.

It has been demonstrated that microtubule (MT) networks contribute to TJ maintenance. Disruption of MT prevents the movement of occludin-containing vesicles to the plasma membrane and leads to a disruption of cell barrier function [130, 131]. In addition, disruption of MT or loss of myosin IIA and B results in impaired TJ function [132]. EspG and its homolog Orf3 (EspG2) cause progressive fragmentation and loss of the MT network and induction of stress fibers formation, which impact intestinal barrier function [133–138]. EspG and Orf3 induce disruption of the MT network beneath adherent bacteria by direct association with tubulins, stimulating MT destabilization. The regulation of MT disassembly is mediated by activation of the RhoA/ROCK signaling pathway, which enhances myosin contractility and actin stress fiber formation [139–141]. EPEC activates RhoA in an EspG- or EspG2-dependent manner [133, 134]. GEF-H1, a RhoA-specific guanine nucleotide exchange factor, binds to MT in its inactive form and switches to an active form when released from MT. [142] Interestingly, both EspG and Orf3 release GEF-H1 from the cytoskeleton into the cytosol, possibly increasing its activity. EPEC-induced actin stress fiber formation is prevented by expression of dominant-negative form of GEF-H1 and RhoA and by ROCK inhibition, suggesting that EspG and Orf3 disrupt MT integrity, triggering activation of the RhoA-ROCK signaling pathway via GEF-H1 activity [133]. EspG1/G2 causes the progressive movement of occludin, claudin-1, and ZO-1 away from the membrane into the cytosol and reduces the expression of tricellulin, leading to epithelial barrier disruption [134, 135, 139, 143]. Interestingly, EspG1/G2 regulates size-selective paracellular permeability without altering the TJ architecture during EPEC infection in MDCK monolayers [134]. In contrast, in polarized intestinal epithelial cells, depletion of *espG1/G2* induces a gradual loss of TEER and eradication of EPEC with gentamicin promotes barrier function recovery [138]. These data suggest that the effects of EspG1/G2 on MT delay the recovery of TJs damaged by EPEC infection, thus perpetuating the loss of barrier function. In addition to its effect on MT and TJ disruption, EspG also contributes to the arrest of vesicle trafficking and blocks the recycling of vesicle cargo to the cell surface [144, 145].

EspH is translocated into host cells by the T3SS and localizes beneath EPEC microcolonies. EspH decreases filopodia formation, disrupts stress fibers, and modulates pedestal formation and pedestal elongation during EPEC infection, suggesting its potential function in modulating actin dynamics [15, 146]. EspH associates with the plasma membrane of host cells and causes disruption of filamentous actin structures. EspH binds directly to the guanine nucleotide exchange factor for Rho, p115RhoGEF. This binding prevents Rho activation, thereby inhibiting downstream Rho signaling and actin cytoskeleton dynamics [147]. Recently, it was found that

EspH cooperates with Tir to promote actin polymerization at the bacterial attachment sites. EspH then promotes the recruitment of N-WASP and Arp2/3 to bacterial attachment sites via a mechanism involving the COOH-terminus of Tir and the WH1 domain of N-WASP [146]. Additionally, WASP-interacting protein (WIP), which binds the N-WASP WH1 domain, is crucial in EspH-mediated actin polymerization. EspH induces the colocalization of Tir, WIP, and N-WASP at actin-rich structures. These data suggest that Tir- and EspH-mediated actin signaling pathways contribute to pedestal biogenesis.

Interestingly, EPEC controls Rho GTPase activity by translocating one effector to inactivate mammalian RhoGEFs and replacing them with bacterial RhoGEFs that promote cell survival. EspH induces focal adhesion disassembly, cell detachment, and induces cytotoxicity. EPEC translocates EspH, which inactivates mammalian RhoGEFs, and, at the same time, translocates the bacterial RhoGEFs, EspM2, and EspT, which inhibit the EspH-induced focal adhesion disassembly, cell adhesion, and survival [148]. Furthermore, EspH perturbs desmosomes structures, which are intercellular junctions that are connected to IF components of the cytoskeleton. It has been reported that desmosomal proteins, desmoglein, desmocollin, and desmoplakin, as well as desmosome morphology, are unaltered during EPEC infection [149]. However, recent evidence demonstrates that EPEC induces a dramatic separation of apposing lateral membranes of adjacent cells by perturbing desmosomes, compromising cell-cell adhesion and barrier function of IECs. The EPEC effector EspH is responsible for these changes [150]. EspH inhibits RhoA GTPase, resulting in actin depolymerization, in turn perturbing IF stability and ultimately leading to destabilization of desmosomes and desmoglein-2 (DSG2) downregulation. EspH induces DSG2 redistribution and subsequent degradation within lysosomes. These changes precede the loss of occludin from cell-cell contacts [150]. EspH-induced DSG2 loss and desmosomal perturbation compromise epithelial monolayer integrity, leading to loss of epithelial barrier function.

NleA effector (non-LEE-encoded effector A) binds and inhibits mammalian COPII through direct interaction with the COPII component Sec24 [151]. COPII is a protein complex that mediates the packaging and trafficking of proteins in the endoplasmic reticulum (ER). NleA inhibits protein secretion from the ER to the Golgi by direct interaction with Sec24. NleA increases intestinal permeability by disrupting occludin and ZO-1 from cell-cell contacts [152]. Knockdown of COPII components in epithelial cells is sufficient to disrupt TJ-associated occludin and ZO-1, suggesting that NleA inhibits the transport of newly synthesized TJ proteins, thus disrupting the barrier. Murine models infected with *C. rodentium* show that NleA mutants unable to bind Sec24 have normal TJs and their fecal water content is similar to that of uninfected mice [153]. Therefore, NleA and its interaction with components of the COPII complex promotes TJ disassembly and diarrhea in murine models.

EspM is an effector harboring WxxxE motifs, thereby possessing the ability to modulate actin cytoskeleton dynamics [154, 155]. EspM induces the formation of actin pedestals and stress fibers that are linked to the plasma membrane through focal adhesions. Actin stress fiber formation is regulated by the GTP-binding

protein RhoA. Infection of cells expressing $RhoA^{N19}$, a mutant that inhibits activation of the small GTPases, leads to the formation of typical actin-rich pedestals, but there is a mark reduction in formation of stress fibers, suggesting that formation of stress fibers by EspM is RhoA-dependent [154, 155]. Additionally, EspM increases the phosphorylation of cofilin, a protein that binds actin filaments, and is a downstream ROCK target, suggesting that EspM regulates stress fiber formation through RhoA-ROCK-cofilin signaling. EspM has also been demonstrated to be an important modulator of pedestal formation. Ablation of both *espM1* and *espM2* induces highly developed actin pedestals, whereas those induced by the wild-type strain were poorly developed [156]. Importantly, EspM causes dramatic changes in TJ architecture in infected polarized monolayers. In addition, cells infected with EPEC expressing EspM2 show disruption of ZO-1 from TJs to the apical and basolateral membrane surface but without a reduction in barrier function.

EPEC Perturbs Cytoskeletal Networks and Adhesion Complexes Affecting Barrier Function

EPEC Infection Stimulates Contraction of the Actomyosin Perijunctional Ring

TJ permeability is regulated in part by contraction of the perijunctional actomyosin ring, leading to decreased TEER [157]. Phosphorylation of myosin light chain (MLC) stimulates contraction of the actomyosin ring, thereby increasing TJ permeability. EPEC infection enhances the phosphorylation of MLC and its association with the cytoskeleton [158–161]. MLC is distributed between cytosolic and cytoskeletal cell fractions; its association with the cytoskeleton increases with the duration of EPEC infection. Analysis of phosphopeptide mapping indicates that MLC is phosphorylated at different sites, strongly suggesting that PKC and MLC kinase (MLCK) are involved in MLC phosphorylation in response to EPEC infection [160]. PKC activators or ectopic expression of the catalytic domain of MLCK increase MLC phosphorylation in a manner similar to levels observed during EPEC infection [162, 163]. Phosphorylation of MLC by MLCK is associated with increased intestinal TJ permeability during EPEC infection [161]. Inhibition of MLCK with ML-7/9 pretreatment, or a membrane-permeant inhibitor of MLCK (PIK), or the neuropeptide vasoactive intestinal peptide (VIP), which regulates epithelial paracellular permeability, decreases intracellular MLC phosphorylation, prevents the redistribution of occludin, claudin-3, and ZO-1, and ameliorates EPEC-induced disruption of the colonic epithelial barrier [161, 164–166]. These data suggest that MLC phosphorylation by MLCK is involved in the perturbation of TJs by EPEC.

EPEC Disrupts AJs by Activation of PKCα

AJs are linked to the actin cytoskeleton through β and α-catenins. EPEC dissociates β-catenin from the membrane and moves it to the cytosol, thus increasing intestinal epithelial paracellular permeability [167]. EPEC phosphorylates PKCα, which induces its association with E-cadherin, thus dissociating the E-cadherin/β-catenin complex [167, 168]. Expression of dominant-negative PKC or treatment with a PKCα-inhibitory peptide blocks these effects. Therefore, targeting of AJs by EPEC may undermine the integrity of intestinal epithelial barrier function [167].

EPEC Alters the Phosphorylation State of Adhesion and Cytoskeleton Molecules

Phosphorylation of occludin is required for its association with TJ complex. EPEC induces a progressive decrease in occludin phosphorylation, correlating with an increase in the nonphosphorylated form and its subsequent dissociation from TJs. These changes correlate with a leaky intestinal barrier [87]. Inhibition of serine/threonine phosphatases prevents EPEC-induced changes in both occludin and TEER, implicating their involvement in the regulation of TJs. EPEC promotes the accumulation of ezrin in A/E lesions, increasing its activity and association with the actin cytoskeleton by promoting ezrin phosphorylation [122]. Ezrin belongs to the Ezrin-Radixin-Moesin (ERM) protein family, which mediates the dynamic linkage between the plasma membrane and cortical actin. Ezrin is reorganized by EPEC infection, leading to impaired TJs [78]. EPEC-induced association of ezrin with the cytoskeleton as well as the drop in TEER depend on the presence of EspB and EspF. Expression of dominant-negative ezrin attenuates the effect of EPEC on ZO-1 and the drop in TEER [122]. These findings suggest that EPEC regulates the phosphorylation states of several proteins important in mediating EPEC-induced signals that result in perturbation of the TJ barrier.

EPEC Perturbs Apico-Basal Polarity

TJs are crucial for the establishment and maintenance of epithelial apico-basal polarity, which is controlled by Crb (Crumbs3/Pals1/Patj), Par (Par3/Par6/aPKC/Cdc42), and Scribble (Scrib/Lgl/Dlg) polarity complexes. Apico-basal polarity contributes to cell morphology, directional vesicle transportation, ion and solute transport, and specific localization of proteins and lipids to different membrane domains [169]. The impact of EPEC on intestinal epithelial polarity has been studied. EPEC alters the distribution of β1-integrin and Na^+/K^+ ATPase, which are typically restricted to the basolateral membrane. EPEC infection caused the movement of

these proteins to the apical surface, resulting in impaired barrier function [85, 114]. Furthermore, cells infected with EPEC expressing EspM exhibit apical β1-integrin staining at bacteria attachment sites and at the cell-cell contacts, presumably just above TJs [156]. These data suggest that changes in apical domain morphology induced by EspM are associated with redistribution of some basolateral membrane proteins. EPEC also recruits basolateral endocytic and recycling membrane proteins (Rabs), AQPs, Tnf, Exo70, and VAMP3 to the apical surface beneath sites of EPEC microcolony attachment [97, 108]. In addition, EPEC alters plasma membrane lipids by inducing the formation of PI(3,4,5)P3 at actin pedestals [69]. In polarized MDCK monolayers, PI(3,4,5)P3 is restricted to the basolateral membrane domain where it functions as a regulator of the basolateral membrane formation [170]. Therefore, it appears that basolateral proteins are specifically trafficked to EPEC microcolonies at the apical membrane by intracellular vesicle transport, indicating failed fence function in host cells.

In addition to the redistribution of basolateral proteins, EPEC infection induces the relocalization of occludin, claudin-1, and ZO-1 from the TJ region to the lateral membrane and cytoplasmic compartment, impacting the architecture and function of TJs [171]. Freeze-fracture replicas of EPEC-infected monolayers reveal aberrant strands containing claudin-1 and occludin extending down the lateral membrane surface well below the TJ area. These structural changes correlate with both increased paracellular permeability and decreased TEER [171]. Interestingly, EspM causes host cells to take on an abnormal round shape, with the main mass of the cell body bulging out and the TJs located at the lower part of the cell, close to the basal membrane. In addition, EspM changes the localization of ZO-1 from the TJs to the apical and basolateral membranes but without a reduction in barrier function [156]. These findings suggest that EPEC-induced perturbation of apico-basal polarity and TJ structure allow the free diffusion of cytoplasmic and membrane proteins to inappropriate cellular domains, further contributing to EPEC pathogenesis.

The impact of EPEC on apico-basal polarity complexes was reported recently. Par6 and aPKCζ, both Par polarity members, are crucial for the establishment of cell polarity and TJ formation. Par6 is a scaffolding protein that interacts with all polarity complexes, thus allowing aPKCζ to phosphorylate its kinase substrates. EPEC displaces Par6 and aPKCζ, but not Par3, from the cell-cell contacts to the cytoplasm and basolateral membrane of IECs [102]. The interaction of Par6 with Cdc42-GTP activates aPKCζ to phosphorylate Par3, which binds 14–3-3 protein; this interaction regulates cell polarity and TJ formation [172–177]. Par6 interacts with Pals1 and Crb3, the latter being target of aPKCζ, and this complex is crucial for TJ formation [178–180]. Detailed *in vitro* and *in vivo* analyses show that EPEC redistributes Crb3 and Pals1, but not Patj, from cell-cell contacts to the cytoplasm of IECs [114]. Both *espF* and *map* are involved in this phenotype. Interestingly, in a cyst morphogenesis assay, ectopic expression of EspF leads to the formation of 3D cysts with multiple lumens, indicating disruption of the formation of cell polarity [114]. These findings indicate that EspF perturbs cell polarity in intestinal epithelial cells.

Several studies have demonstrated the direct effect of EPEC on the activation and translocation of aPKCζ in infected epithelia. EPEC significantly increases aPKCζ activity in T84, but not SKCO-15, polarized monolayers. Inhibition of aPKCζ with a pseudosubstrate protects against disruption of the TJ barrier by EPEC, suggesting that aPKCζ is involved in EPEC-induced barrier disruption. EPEC also induces the translocation of aPKCζ from the cytoplasm to membrane and from cell-cell contacts to actin-rich pedestals of infected monolayers; both situations correlate with loss of barrier function [102, 165, 181, 182]. Interestingly, aPKCζ recruitment to EPEC pedestals is not diminished by deletion of *map* or *espF* or by mutation of the SNX9-binding domain of EspF. However, the interaction of EspF with SNX9 plays an important role in actin-pedestal organization and contributes to the colocalization of active aPKCζ and F-actin to the plasma membrane and within pedestals in a cell-specific manner [102].

EPEC impairs both cell polarity and intestinal epithelial TJ barrier function. Recent evidence indicates that the temporal sequence of TJ disruption following EPEC infection correlates with the redistribution of polarity proteins. EPEC induces the recruitment of aPKCζ to pedestals colocalizing with actin almost immediately upon bacterial attachment (5–15 min). TJ proteins are then removed from cell-cell contacts to cytoplasm. JAM-A S285 is internalized by 30 min and TEER significantly decreases 45 min postinfection. Phosphorylation of JAM-A at tyrosine 280, which is related to loss of barrier function, is detectable after 1–2 h post EPEC infection, corresponding with leaky TJs [102, 183]. Occludin and ZO-1 disruption occurs at 1–2 h postinfection, respectively, corresponding with a more significant drop in TEER. aPKCζ has direct and indirect roles in the formation and maintenance of polarity and TJ structure and function. aPKCζ phosphorylates Crb3, occludin, claudins, JAM-A, ZO-1, and ZO-2 to establish and maintain TJ structure and barrier function [35, 184–187]. EPEC alters the phosphorylation state and localization of these proteins, leading to a loss of barrier function [87, 102, 114]. The displacement of aPKCζ away from TJs results in perturbation of barrier function, suggesting that the progressive dismantling of TJ by EPEC and the corresponding impact on barrier function occur after the early recruitment of aPKCζ to actin pedestals [102].

EPEC Activates Various Signaling Pathways

Inflammation in response to infection by enteric pathogens contributes to TJ disruption. For example, IL-13, a pro-inflammatory cytokine, which is increased in the mucosa of patients with ulcerative colitis and Crohn's disease, is able to induce expression of claudin-2, which increases TJ permeability to both ions and small nonionic solutes [188, 189]. Interferon (IFN)-γ and tumor necrosis factor (TNF)-α, both pro-inflammatory cytokines, are critical regulators of barrier function via MLC phosphorylation and TJ remodeling [164, 190–192]. TNF-α induces occludin internalization, paracellular barrier loss, and diarrhea [190, 193]. Infection of T84 cells

by enteric bacterial pathogens has been shown to increase the expression of both TNF-α and IL-1β pro-inflammatory molecules [194]. EPEC and REPEC infection increases TNF-α, IL-1β, IL-8, and IL-6 cytokines [195, 196]. EPEC infection induces only a modest inflammatory response due in part to the presence of anti-inflammatory EPEC effectors including non-LEE-encoded NleB, NleC, NleD, NleE and the homologs NleH1 and NleH2 [197–200]. EPEC infection also activates the mitogen-activated protein (MAP) kinases ERK1/2, p38 and JNK, and nuclear factor-κB (NF-κB) signaling pathways that upregulate expression of the pro-inflammatory cytokine IL-8 [201–204]. aPKCζ has also been reported to be involved in ERK1/2 and NF-κB activation; both pathways are activated by EPEC [202–209]. aPKCζ regulates NF-κB activation by binding and activating IκB kinase (IKK), which phosphorylates the NF-κB inhibitor IκB, triggering its degradation. Inhibition of aPKCζ or expression of a dominant-negative mutant significantly suppresses EPEC-induced IκBα phosphorylation but does not impact the EPEC-induced stimulation of ERK1/2 [181]. Interestingly, inhibition of ERK1/2 and p38 attenuates the phosphorylation and degradation of IκBα and expression of IL-8 but does not affect A/E lesion formation or protect against the decrease in barrier function associated with EPEC infection [202, 204]. These data suggest that EPEC-activated ERK1/2 and aPKCζ signaling pathways contribute to the inflammatory response during EPEC pathogenesis.

EPEC Alters Intestinal Transport

EPEC-induced loss of intestinal barrier function is believed to contribute to diarrhea by disrupting intestinal epithelial ion and fluid transport. The loss of TJ barrier function would prevent the creation of ion gradients needed for effective ion and solute transport. In addition, infectious diarrhea can be caused by increased chloride secretion, decreased NaCl absorption, or both. EPEC infection attenuates secretagogue-induced net ion transport and impacts chloride secretion [210–212]. Both electroneutral Na^+/H^+ and Cl^-/OH^- exchange activities are altered by EPEC [213]. EPEC modulates intestinal epithelial cell electrolyte transport, reducing the expression of the Na^+/H^+ exchanger-3 (NHE3), the major intestinal transporter of Na^+ absorption in an EspF-dependent manner, and stimulating the apical NHE2 and basolateral NHE1 activity [213, 214]. Analysis of the signal transduction cascades responsible for the increased NHE2 activity during EPEC infection shows that PLC, PKCα, and PKCε signaling pathways are implicated [215]. Map interacts with NHERF1 (Na^+/H^+ exchanger regulatory factor I), Map and NleH1 bind to NHERF2 altering its function [121, 216]. EPEC infection decreases Cl^- absorption in intestinal cells by reducing the activity of the $Cl^-/HCO3^-$ exchanger, SLC26A3 (downregulated in Adenoma, DRA), resulting in a reduced Cl^- uptake and its accumulation in the lumen, driving water loss. Mortality in *C. rodentium*-infected mice is associated with downregulation of DRA and other genes involved in intestinal transport, decreased uptake of chloride, and fatal diarrhea [217–219]. EPEC reduces DRA

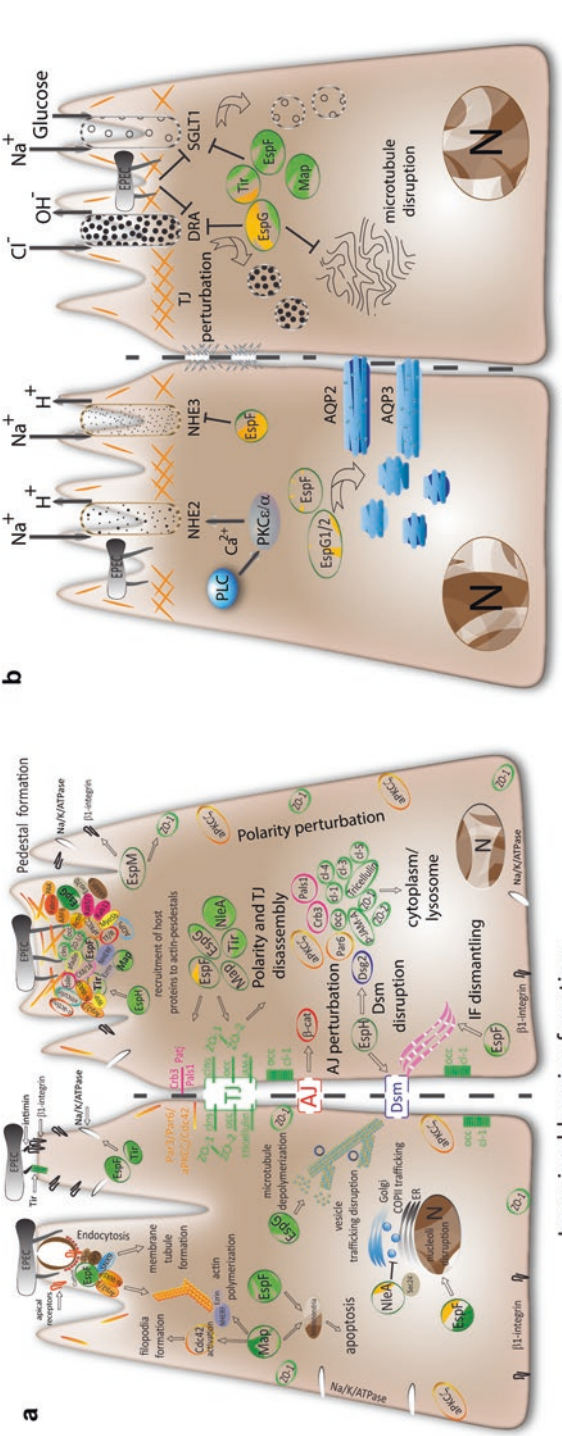

Fig. 1 Model displaying the mechanisms by which EPEC perturbs TJs and induces diarrhea. EPEC injects a large repertoire of effector proteins into the host cell. EPEC effectors mediate actin rearrangement and contribute to pedestal formation. (**a**) EPEC induces the recruitment of several host proteins to actin pedestals immediately upon bacterial attachment. Effectors dismantle cytoskeletal cell structures (MTs and IF) and induce disruption of vesicle trafficking, impacting TJs function. Proteins from TJs, AJs, desmosomes, and polarity complexes are displaced from cell-cell contacts and internalized into the cytoplasm, and some are degraded by lysosomes. EPEC alters the apico-basal polarity, evidenced by redistribution of basolateral proteins (β-1 integrin and Na^+/K^+ ATPase) to the apical membrane, and TJ proteins to the lateral and apical domains (occludin, claudin-1, and ZO-1). All these events contribute to TJs disassembly. (**b**) EPEC alters intestinal transport. EPEC infection regulates apical Na^+, Cl^-, and glucose absorption, impacting the activity of the cotransporters NHE2 and NHE3, DRA, and SGLT1. EPEC also induces the endocytosis of DRA in an EspG-dependent manner. The basolateral water channel proteins AQP2 and AQP3 are redistributed to the cytoplasm after EPEC infection. EPEC alters the activity and localization of cotransporters, contributing to increased diarrhea during EPEC pathogenesis

protein expression and redistributes its localization from the apical membrane surface to intracellular compartments. This process is mediated by EspG1/G2 [220, 221]. EPEC alters the activity of the sodium-D-glucose cotransporter (SGLT-1), a major water pump in the small intestine [222]. EPEC causes a rapid movement of SGLT-1 from the apical membrane into intracellular vesicles. EspF, Map, Tir, and intimin cooperate to decrease activity of SGLT-1. AQPs function as a water channels to maintain the dehydration of fecal contents. *C. rodentium* infection alters the localization of AQP2 and AQP3, from the lateral membrane to the cytoplasm. The change in localization correlates with the diarrhea in infected mice [223]. The altered distribution of AQPs is partially dependent on EspF and EspG effectors, suggesting that AQPs may also contribute to diarrhea during bacterial infection. These data suggest that during EPEC infection, the ability of the intestinal epithelium to regulate absorption and secretion of ions and water is compromised, contributing to diarrhea.

Conclusions

The findings presented in this chapter support the contention that EPEC-induced perturbation of TJs is a complex process driven by the downstream effect of multiple signaling pathways activated by EPEC effectors, disruption of cytoskeletal networks, increased endocytosis, changes in the localization of proteins to different cell compartments and membrane domains, and altered apico-basal polarity (Fig. 1a). EPEC-induced loss of intestinal epithelial barrier function is believed to contribute to diarrhea by disrupting intestinal epithelial ion and fluid transport (Fig. 1b). All of these changes converge disrupting intestinal epithelial host cell homeostasis, leading to TJ disruption that contributes to EPEC-associated diarrhea.

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Microbial Metabolite Regulation of Epithelial Tight Junctions and Barrier

J. Scott Lee, Ruth X. Wang, and Sean P. Colgan

Abstract The intestine harbors trillions of metabolically active microbes, collectively termed the microbiome, that is generally symbiotic with the needs of the host. A single layer of intestinal epithelial cells provides a physiologic barrier that facilitates the selective permeability to microbial components. Paracellular transport is regulated by specialized intercellular contact points known as the apical junctional complex, and recent studies have identified microbial metabolites that regulate paracellular permeability in the intestine. Analyses of these pathways have provided a multitude of opportunities for understanding epithelial tight junction biology and tissue barrier function. In this chapter, we summarize our current understanding of how microbial-derived molecules directly and indirectly influence the regulation of epithelial tight junctions.

Keywords Inflammation · Microbiota · Short chain fatty acid · Purine · Indole · Mucosa · Colitis

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The authors declare no financial interests in any of the work submitted here.

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https://doi.org/10.1007/978-3-030-97204-2_8

Abbreviations

AJC	Adherens junction complex
AMP	Antimicrobial peptide
Cldn	Claudin
HBD-1	Human beta defensin-1
HDAC	Histone deacetylase
HIF	Hypoxia-inducible factor
IEC	Intestinal epithelial cell
PMN	Polymorphonuclear leukocyte
SCFA	Short chain fatty acid
SYNPO	Synaptopodin
TJ	Tight junction
ZO-1	Zonula occludens-1

Introduction

Intestinal epithelial cells (IECs) regulate intestinal homeostasis through isolating the host immune system from the external environment of pathogenic and commensal microorganisms. IECs form the largest single-cell layered physical and biochemical barrier in the human body [1]. The intestinal epithelium is continually regenerated every 3–5 days from the base crypt stem cells. These stem cells give rise to multiple differentiated epithelial cell subsets that participate in specific functions of the mucosal barrier. Aside from the absorptive enterocytes and colonocytes, secretory IECs include the enteroendocrine cells, Paneth cells, goblet cells, and tuft cells that each provide specific functions to the host [2]. For simplicity, IECs will refer to enterocytes/colonocytes unless otherwise noted.

The mammalian gastrointestinal tract is host to trillions of microbes, including bacteria, viruses, and fungi. This finely tuned host-microbe relationship coexists with epithelial cells atop the mucus layer of the intestinal mucosa, where microbes provide essential components for host health [3]. Conversely, microbes can initiate and perpetuate both acute and chronic diseases [4]. In this chapter, we summarize some of the important microbial-derived components that regulate mucosal barrier function, with a specific focus on IEC tight junction structure and function.

Unique Environment of the Mammalian Intestine

The mammalian intestine has evolved to function as a uniquely suited environment for the growth and survival of anaerobic and facultative anaerobic microbes [5]. Given the central role of oxygen to mammalian metabolism, such an austere

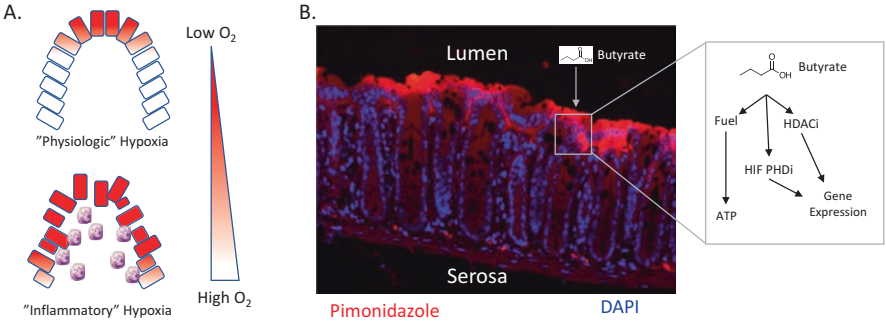

Fig. 1 Butyrate impacts a multitude of functions in intestinal epithelial cells. Panel **A** depicts the differences between "physiologic" and "inflammatory" hypoxia evident at the surface of the mucosa. Panel **B** shows a histologic section of healthy mouse colon documenting low oxygen regions (red) visualized by pimonidazole staining. Nuclear counterstain with DAPI is shown in blue. Low oxygen regions within the tissue are enriched in butyrate utilization, where butyrate is used as a fuel for the generation of ATP. Butyrate also functions to inhibit HIF-prolyl hidroxylase domain proteins (PHDs) as well as HDACs

environment demands metabolic adaptation like no other barrier surface in the body. The gastrointestinal (GI) tract, for example, harbors a quite unique oxygenation profile [6]. Even at baseline, barrier epithelial cells that line the mucosa exist at a low oxygen tension environment, defined as "physiologic hypoxia" [7] (see Fig. 1). Original studies revealed that the countercurrent oxygen exchange mechanisms of the GI tract provide for arterial blood supply diffusion to adjacent venules, along the crypt villus axis, resulting in graded hypoxia [8]. This steep oxygen gradient has been well-documented in the distal colon of the GI tract, spanning from the anaerobic lumen, across the epithelium, to the richly vascularized subepithelial mucosa [9]. Given the high-energy requirement of the gut and the integral role of the epithelium in maintaining intestinal homeostasis, it is not surprising that these cells have evolved a number of mechanisms to cope with this austere metabolic environment [10]. During active inflammation, the combination of recruited leukocytes, edema, and vasculitis enhances the hypoxic gradient to become "inflammatory hypoxia" [7] (see Fig. 1).

Tissue oxygenation has been tracked using 2-nitroimidazole dyes (e.g., pimonidazole, see Fig. 1), a class of compounds known to undergo intracellular metabolism dependent on the level of tissue oxygenation [11]. These dyes were developed to image the low O_2 environment of growing tumors [12] and have subsequently been used as tools to monitor levels of tissue oxygenation ex vivo. Nitroimidazoles form adducts with thiol groups with various tissue macromolecules where all atoms of the ring and side-chain of the 2-nitroimidazole are retained at pO_2 <10 mmHg. Antibodies specific for these conjugated adducts provide a histochemical approach to estimation of tissue pO_2. It is notable that this approach has not been established to titer tissue pO_2, rather it is a quantitative estimate of tissue hypoxia (e.g., above or below pO_2 of ~10 mmHg).

Given the rather unique environment of the intestine, particularly the colon, a number of studies have shown that stabilization of the transcription factor hypoxia-inducible factor (HIF) in low oxygen environments triggers the expression of genes that are essential to epithelial barrier function [13–16]. Additionally, HIF is one of the central regulators of overall tissue metabolism [17] and has profound influences on the inflammatory response [10]. HIF function is dependent on stabilization of an O_2-dependent degradation (ODD) domain expressed on the α-subunit and subsequent nuclear translocation to form a functional complex with HIF-1β [18]. In normally oxygenated tissues, iron-, alpha-ketoglutarate-, and O_2-dependent hydroxylation of two prolines (Pro564 and Pro402 of HIF-1α in humans) within the ODD of the alpha subunit initiates the association with the von Hippel-Lindau tumor suppressor protein (pVHL) and the recruitment of a ubiquitin-E3 ligase for degradation via proteasomal targeting [19, 20]. An in-depth review of the adaptive role of HIF in the context of barrier function has been reviewed in detail elsewhere [5].

Microbial Short-Chain Fatty Acids and Barrier Regulation

The intestinal microbiota has the capacity to harvest large amounts of energy from fibers unable to be digested by the human gut through anaerobic fermentation that produces SCFAs as end products that exert beneficial influence not only on host energy metabolism but also on systemic health. Humans lack enzymes to degrade the majority of dietary fibers, and thus these nondigestible carbohydrates pass through the small intestine undisturbed to reach the large intestine for fermentation by anaerobic bacteria. Nondigestible dietary fibers include inulin, oat bran, wheat bran, cellulose, Guar gum, and pectin. These substrates provide fuel for bacterial fermentation. Fermentation by-products include multiple groups of metabolites, of which SCFAs dominate. For the microbial community, SCFAs are a requisite waste product to balance redox equivalent production in the anaerobic gut lumen. SCFAs are classified as saturated aliphatic carboxylic aids between one and six carbons in length that include acetate (C2), propionate (C3), butyrate (C4), valerate (C5), and hexanoate (C6). Acetate, propionate, and butyrate are the most abundant and comprise >95% of SCFAs and exist in a molar colonic ratio of approximately 60:20:20, with total SCFAs reaching 140 millimolar (mM) in the proximal colon and 70 mM in the distal colon [21]. The majority of SCFAs are rapidly absorbed by colonocytes, with only 5–10% secreted in feces. These SCFAs have significant impact on host physiology as energy substrates, gene expression regulators, and signaling molecules for specific receptors [22–25] (also see Fig. 1).

Sequestration of butyrate to the colon is due in large part to the different affinities of the apical (K_m = 1.5 mM) and basolateral (K_m = 17.5 mM) SCFA-HCO_3^- exchange transporters, which confine butyrate to colonocytes [26, 27]. Similarly, the affinity of the apical monocarboxylic acid transporter-1 (MCT1) for butyrate is also higher than the basolateral transporter MCT4, and the higher intracellular pH renders all

SCFAs in the dissociated form, which eliminates any passive diffusion across the basolateral membrane. Peripheral systemic availability of colon-derived butyrate has been shown to be less than 2%, where the vast majority of butyrate is utilized by colonocytes [28].

Another aspect of butyrate sequestration lies in that it is the preferred energy source of the colonic epithelium, with oxidation of this SCFA accounting for over 70% of the cellular oxygen consumption in the distal colon [29]. Colonocytes utilize butyrate over acetate and propionate, where it is oxidized to ketone bodies and CO_2. More than 95% of produced butyrate is utilized by colonocytes for energy. Butyrate metabolism stimulates mitochondrial respiration and has been shown to be important in preventing IEC autophagy [30]. As an energy substrate, butyrate undergoes β-oxidation to form acetyl-CoA, which enters into the tricarboxylic acid (TCA) cycle to produce the reducing factors that drive the electron transport chain (ETC) and oxygen consumption to ultimately regenerate ATP. Maintenance of the mucosal barrier requires cytoskeleton stability, which needs substantial energy reserves, and the rapid utilization of butyrate not only prevents butyrate from escaping into systemic circulation but also provides the requisite energy for IECs to rapidly polarize and form strong AJCs [31]. In fact, the colon of germ-free (GF) animals has been shown to exist in a state of energy deficiency [30].

Due to the specificity of butyrate to the colon, multiple studies have shown butyrate to influence the intestinal barrier. An mRNA-based screen of intestinal epithelial cells exposed to physiologic concentrations of butyrate revealed the repression of CLDN2, a "leaky" claudin that increases permeability. The mechanisms of butyrate activity were subsequently traced to induction of the IL-10 receptor on IEC through mechanisms involving butyrate regulation of histone deacetylase (HDAC) inhibition [32] (see Fig. 2). Other studies have also shown butyrate to induce the expression of other "sealing" TJ proteins such as CLDN1 also through HDAC inhibition [33]. More recently, a newly characterized intestinal epithelial tight junction protein synaptopodin (SYNPO) was identified via a single cell RNA sequencing approach. Like claudins, the mechanisms of butyrate regulation of SYNPO were via HDAC inhibition (see Fig. 2). SYNPO was additionally shown to regulate wound healing and to be a critical component to butyrate promoting wound healing in vivo [34]. Butyrate can coordinate the repression of "leaky" TJ proteins and the induction of "tight" TJ proteins, all through HDAC inhibition. While HDAC inhibition impacts the expression of ~2% of mammalian genes [35], butyrate is seemingly able to regulate barrier function through influencing multiple genes through HDAC inhibition. In the aforementioned studies, SYNPO was induced at the protein level after butyrate treatment after 6 h, while the other studies showed that CLDN2 levels were reduced by butyrate after 24 h, which corresponded with peak IL-10RA induction at 24 h, and CLDN1 was increased after 36 h [32, 33]. While these time signatures are dependent on the experimental limitations set during the separate analyses, it could be that butyrate may temporally organize the induction and repression of specific TJ proteins to ultimately promote barrier function. Wang et al. showed in their scRNAseq that multiple TJ and actin-associated genes were upregulated by butyrate not limited to cingulin (CGN) and claudin 3 (CLDN3), as well as genes

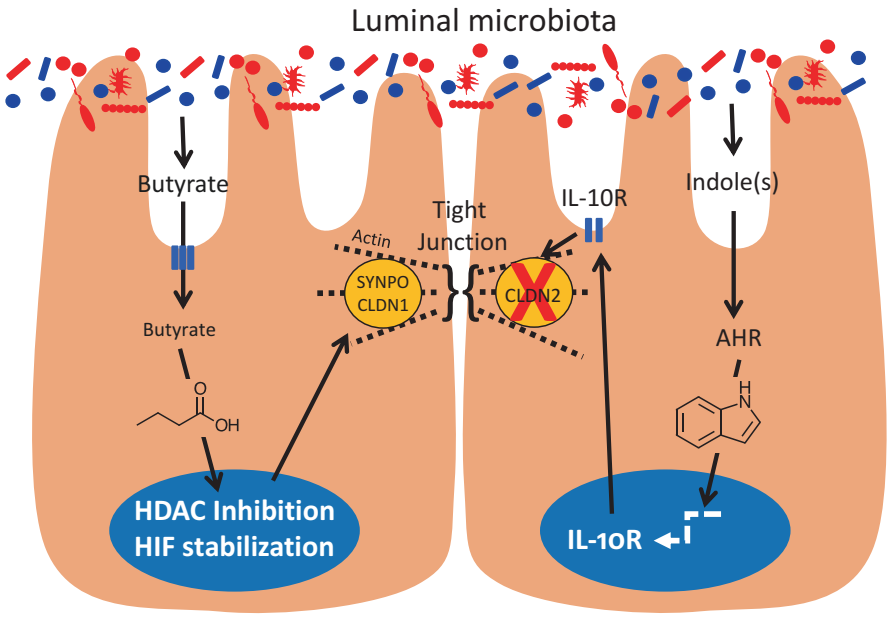

Fig. 2 Microbial-derived butyrate and indole regulate epithelial tight junction expression and function. Shown here is the influence of butyrate (left cell) and indole (right cell) on tight junction protein expression. Through actions on HDAC and HIF, butyrate influences the expression of TJ proteins (e.g., claudin-1, CLDN1) as well as TJ adapter proteins (e.g., synaptopodin, SYNPO). Indoles act through the aryl hydrocarbon receptor (AHR) to induce the apical IL-10 receptor, which, when activated, represses "leaky" claudin-2 (CLDN2) to promote barrier function during inflammation

related to cellular motility including MYLIP and KIF11 [34]. This suggests that butyrate could simultaneously and purposefully influence multiple genes related to these processes, as naturally, the AJC is already comprised of many components and active epithelial restitution to begin wound healing requires many proteins working in concert.

As HIF is a well-known transcription factor stabilized in hypoxia, the influence of butyrate metabolism on oxygen availability in the colon regulates HIF stabilization in the intestinal mucosa. For example, mice lacking microbiota-derived butyrate (e.g., germ-free mice) have diminished HIF stabilization at baseline [36]. β-oxidation of butyrate increases oxygen consumption of IECs to the extent that HIF is stabilized [36]. In recent extensions of this work, it was revealed that butyrate stabilizes HIF independent of β-oxidation. Further analysis using a combination of recombinant HIF prolyl hydroxylase enzyme and 1D-NMR found that butyrate regulates HIF by functioning as a direct, noncompetitive inhibitor of the HIF PHD in vitro and in vivo. This implicates butyrate as a significant endogenous regulator of HIF in IECs [37]. It is notable that intestinal epithelia are responsible for iron (Fe) absorption and thus support red blood cell production. The HIF-2α isoform

regulates key IEC proteins involved in iron absorption including duodenal cytochrome B (DCYTB), which apically reduces luminal iron to the transportable form Fe^{2+}, divalent metal transporter 1 (DMT-1), which mediates uptake of Fe^{2+} from the lumen, and ferroportin (FPN), which mediates basolateral iron efflux into systemic circulation from IECs [38]. Additionally, HIF-1α regulates the expression of ectonucleoside triphosphate diophosphohydrolase 1 (CD39) and 5′-nucleotidase (CD73), which enzymatically convert adenosine triphosphate (ATP)/adenosine diphosphate (ADP) to adenosine monophosphate (AMP) and AMP to adenosine, respectively. Adenosine signaling plays a key role in the perfusion of the intestinal mucosa and promotes intestinal barrier function through activating the adenosine 2B receptor (A2BR), which is highly expressed in the intestinal mucosa and is transcriptionally regulated by HIF-1α [39].

HIF contributes to epithelial barrier function in a number of ways. Selective knockdown of HIF-1α in murine IECs demonstrated major defects in the mucosal barrier integrity. This could partly be due to HIF-1α directly regulating the expression of CLDN1, a crucial TJ protein [40]. HIF-2α regulates creatine kinase B (CKB), which colocalizes with AJs and supplies energy at junctional sites for tasks such as tight junction assembly, maintenance, and restitution. HIF-1α also upregulates MUC2, the major component of the mucus layer, as well as human β-defensin 1 (HBD-1), which is the only constitutively secreted antimicrobial peptide in the intestine [41, 42]. Likewise, HIF is a transcriptional regulator of intestinal trefoil factor (i.e., TTF3), a 40 amino acid lectin protein that binds and crosslinks mucins [13].

Aside from the physiologic hypoxia of the intestinal mucosa, HIF activation is also significant in the phenomenon of "inflammatory hypoxia," in which infiltration of inflammatory polymorphonuclear cells (PMNs) can deplete the local oxygen stores due to the respiratory burst and production of reactive oxygen species (ROS) to clear out pathogens and cellular debris. During inflammatory insult, it is estimated that ROS generation by PMNs can consume up to 10 times more O_2 than any other cell in the body. This oxidative burst is robust and not hindered even in low O_2 tensions, as ROS can be generated in the relatively low O_2 environments of inflamed intestinal mucosa [43]. Specifically in the colon, this inflammatory depletion of oxygen compounds with the physiologic hypoxia already present, and further stabilizes HIF, which is a crucial component in appropriately stopping the inflammatory response and beginning the processes of barrier reformation and epithelial repair [44]. Activation of HIF serves as an alarm signal for the resolution of inflammation in various murine disease models. Indeed, inflammatory hypoxia and lack of return to baseline oxygenation levels has been shown to be a major component of intestinal disease [45]. Thus, this interface between intestinal oxygenation and microbial SCFA production appears to be an essential component of a healthy mucosal barrier.

Microbial Indoles and Tight Junction Regulation

Recent metabolomic analysis has revealed that gut microbiota impacts host mammalian metabolism through a variety of metabolites, including amino acid metabolites [46]. Lack of dietary tryptophan, for instance, impairs intestinal immunity in mice and alters the gut microbial community [47], suggesting that tryptophan metabolism is an important component of mucosal homeostasis.

Dietary tryptophan is utilized directly by the microbiota to synthesize the aromatic heterocyclic compound indole and is made by a variety of both Gram-positive and Gram-negative bacteria [48]. To date, 85 indole-producing bacterial species have been identified [49]. Indole is synthesized from tryptophan by the bacterial enzyme tryptophanase (tnaA, EC 4.1.99.1) in the following reaction:

$$\text{L-tryptophan} + H_2O \rightarrow \text{indole} + \text{pyruvate} + NH_3$$

Several derivatives of indole (e.g., indole propionate, indole aldehyde, indole acetate) are also made by select members of indole-producing bacteria. Indoles can be produced at concentrations of up to 1 mM in the human, rat, and mouse intestines [50, 51]. Indole is a well-known signaling molecule that modulates bacterial activity including plasmid stability [52], cell division [53], antibiotic tolerance [54, 55], and even spore formation [56, 57]. Many non-indole-producing bacteria as well as eukaryotes can modify or degrade indole using oxygenases and P450 family members [46, 58]. Consequently, indole derivatives are widely present in prokaryotic and eukaryotic communities [48], though less is known about their biological role, metabolism, or mechanisms of biological action. It has been indicated that high concentrations of particular metabolites, specifically indoxyl sulfate, contribute to kidney damage [59]. However, others have clearly shown the benefit of indole metabolites, such as by suppressing central nervous system inflammation [60], and most notably in the intestinal milieu [61, 62].

Two indole metabolites, namely, indole-3-aldehyde (IAld) and indole-3-propionic acid (IPA), are known for their intercellular signaling activity. IAld was recently identified as a ligand for the aryl hydrocarbon receptor (AHR) [63], a ligand-dependent transcription factor activated by a variety of biological and synthetic molecules that serve important roles in immunological and inflammatory responses [64]. AHR contributes to immune homeostasis through various methods, including T cell differentiation and Th17 development [65, 66], as well as the upregulation of IL-22 production [67]. In mucosal epithelial cells, indoles have been shown to specifically induce the interleukin-10 receptor (IL-10R) [68], which was previously shown to be regulated by cytokines such as interferon-gamma and expressed predominantly on the apical surface of IEC in vitro and in vivo [69]. These same studies in mice lacking IEC IL-10R revealed significant increases in susceptibility to colitis and a prominent increase in intestinal permeability. Studies in cultured Caco2 and T84 cells have revealed that ligation of the IL-10R variably regulates expression of several TJ proteins, most prominently the repression of Cldn2 mRNA and protein [32]. Knockdown of the IL-10R using short hairpin RNA

resulted in increased in Cldn2, while cDNA-mediated overexpression of IL-10R fulfilled this hypothesis to repress Cldn2. Therefore, indoles provide an example of a microbial amino acid metabolic pathway that impacts, among other targets, IEC tight junction expression and function.

Microbial Purine Salvage and Tight Junction Interactions with the Cytoskeleton

Tight junctions reside at the most apical region of the complex and regulate the paracellular flux of solutes and macromolecules (gate function), while also polarizing the cells through isolating apical and basolateral plasma membrane domains (fence function) [70, 71]. Membrane claudins, zonula occludens (ZO) adapter proteins, and filamentous actin (F-actin) interact and have indispensable roles in tight junction assembly and maintenance [72, 73]. Claudins regulate paracellular permeability by forming charge-selective, small pores with a diameter of ~4 Å, while ZO proteins are a family of multidomain scaffolding proteins that form oligomers and link tight-junction-associated membrane spanning proteins to the actin cytoskeleton [71, 74, 75]. Accommodating the various cell morphologies and movements that occur in a monolayer requires junctions to be both strong and plastic, a functionality that requires an exceptionally active cytoskeleton rich in actin filaments working to stabilize and cycle junction proteins [70, 76, 77]. To this end, the apical junctional complex is supported by a network of tight-junction-associating F-actin bundles and a dense circumferential actomyosin ring contiguous with adherens junctions to form one of the most organized and active actin networks found in nature [78]. Over the last two decades, an understanding of the mechanistic role of actin in the adherens junction has emerged, with the ATP-dependent actomyosin ring providing the stability needed for tight junction formation and cellular polarization, in addition to strong lateral adhesions and intercellular tension that forces paracellular flux through the tight junction [78–81]. Despite extensive identification of tight-junction-associated proteins, a similar understanding of the interactions between these proteins and F-actin is largely unresolved [82]. The formation of tight junctions is dependent upon and regulated by the actomyosin cytoskeleton [71], which constitutes a network that transduces adhesive and mechanical signals from the membrane, into the cell, and back to mediate this regulation [77, 83]. It was recently reported that ZO-1 function is controlled by mechanical cytoskeletal force [84], suggesting an important role for such force in tight junction assembly, but the degree of tension requires fine-tuning as excess strain can disrupt barrier [85–87]. Furthermore, in contrast to the high-affinity cytoskeletal associations with adherens junctions, recent findings show loose and dynamic couplings between ZO-1 and F-actin, with inhibition of actin polymerization by cytochalasin D and B detrimental to barrier resistance [82, 88, 89]. In this, it appears that F-actin does not work to apply strong forces at the tight junction, but instead to stabilize and organize tight

junction components in the regulation of paracellular permeability, and readily facilitate claudin cycling through F-actin extension and contraction to rearrange pore size and charge selectivity in response to various physiological or pathophysiological stimuli [90, 91]. Such dynamic cytoskeletal functionality demands substantial energy input, as actin polymerization is driven by ATP and stimulates ATP hydrolysis 42,000-fold [92–94]. The dependence of the tight junction on energy is highlighted by the loss of gate and fence functionality under conditions of ATP depletion, concomitant with dysregulated paracellular flux [89, 95].

The energetic requisite of the gut commands ~20% of total cardiac output while consuming 10–20% of the available oxygen [96–98], with microbiota-derived metabolites also significantly contributing to energy procurement. This is exemplified in that germ-free (GF) mice lacking a microbiota are lean in comparison to conventionally raised (CR) mice, with the colonization of GF mice inducing rapid weight gain and increased adiposity [99]. The contribution of the microbiota to the human energy requirement is estimated to be 5–10% [100], with a considerable local impact on the large intestine. For example, analyses of the total available energy (TAE), a metric that accounts for the total available chemical energy in a system as ADP, ATP, and phosphocreatine [95], from colon tissue extracts, reveal that GF mice have ~55% ($p < 0.001$) of the TAE shown in CR counterparts [101]. As a preferential fuel source of the colonic epithelium accounting for over 70% of the cellular oxygen consumption in the distal colon [29], microbiota-derived butyrate strongly contributes to intestinal energy balance and barrier function. As mentioned above, humans consume a wide range of complex carbohydrates; many of these dietary polysaccharides endure digestion and pass through the stomach and small intestine. These resistant starches (RSs) and nonstarch polysaccharides (NSPs, the major component of dietary fiber) reach the colon, where they are fermented by the microbiota to end products such as short chain fatty acids (SCFAs), including butyrate [102]. Clostridia are the major butyrate-producing class, and particularly, *Eubacterium rectale, Eubacterium hallii,* and *Faecalibacterium prausnitzii* are among some of the most abundant and dominant butyrate-producing species [22, 103, 104]. This energetic supply provides critical support to the cytoskeleton and thus tight junction, facilitating actin polymerization, apical junction complex formation, and regulation of paracellular permeability [105].

Microbiota-sourced purine metabolites, notably the nucleobase hypoxanthine, and their impact on tight junction formation and regulation has only recently been appreciated (see Fig. 3). Hypoxanthine provides a readily available substrate for efficient nucleotide biogenesis via purine salvage [101, 106], with previous studies demonstrating that the gut mucosa preferentially salvages purines in lieu of the energy and nutrient-consuming de novo pathway in the presence of available purine substrate [101, 107–110]. Hypoxanthine is readily salvaged by intestinal epithelial cells to support energy balance and nucleotide biosynthesis [95, 101]. For example, colonic enterocyte model T84 cells show a substantial decrease in tight junction barrier resistance when subjected to hypoxia, an energetically depleting state of oxygen deprivation representative of their natural environment. Hypoxanthine supplementation significantly increased the TAE in hypoxic cells, coinciding with

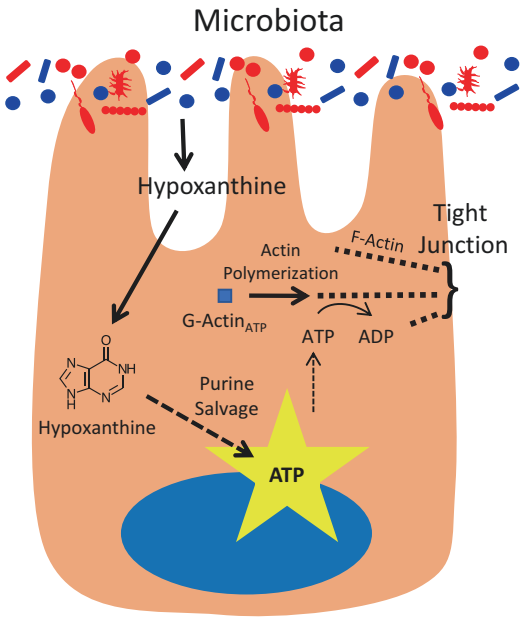

Fig. 3 Purines derived from microbial sources directly influence the epithelial apical junction complex. Shown here is a depiction of IEC procurement of microbial purines, especially hypoxanthine. These purines are salvaged to generate ATP, which supports actin polymerization of G-actin to F-actin in the epithelial TJ

complete recovery of tight junction barrier. Further analyses revealed that the energetic benefit afforded by hypoxanthine supplementation promoted actin polymerization and apical junctional complex stability, and increased the rate of tight junction formation from a depolarized cell state [95]. Taken together, regulation and maintenance of the tight junction requires significant cytoskeletal capacity and ATP availability, with the colonic epithelium critically dependent upon microbiota-derived metabolites as fuel to meet this energy requisite.

Conclusions

In the past decade, significant attention has been paid to understand the contribution of the microbiome to health and disease. Of particular interest is the unique locale in which microbial metabolites influence tissue function. Juxtaposed between a microbial-rich anaerobic lumen and the highly vascularized submucosa, the epithelium has evolved and adapted to providing a selective barrier to the microbiota. Numerous studies have revealed that microbial-derived molecules, including SCFA and indoles, promote intestinal barrier function through a multitude of mechanisms. Ongoing studies that identify new metabolites and refine our understanding of these important host-microbial crosstalk pathways will undoubtedly provide new avenues for the treatment of intestinal diseases.

Acknowledgments This work was supported by NIH grants DK104713, DK050189, DK DK122741, DK1200720, DK09549, and VA Merit Award 1I01BX002182.

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Non-tight Junction Functions of Claudin Proteins: Roles in Cell-Matrix Interactions and Stem Cell Regulations

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Abstract Tight junctions (TJs) are the most apical components of the junctional complex in epithelial cells, and they play an essential role in maintaining epithelial tissue integrity. Claudins are the major structural and functional components of TJs that regulate paracellular permeability by forming barriers or size- and charge-selective channels. However, claudins have been increasingly highlighted in processes occurring outside of the TJ structure. These include interactions with cell adhesion receptor protein integrins and epithelial cell adhesion molecule EpCAM to modulate various cellular activities, such as supporting cell-matrix adhesion, regulating the blood-brain barrier, and modulation of cancer metastasis. In addition, recent studies illuminated the novel function of TJ proteins in stem cell regulations. The identification of TJ protein's activities beyond traditional TJs underscores the new insights of the importance of these junctional proteins. This chapter focuses on the interactions of TJ proteins, especially the claudins, with cell-matrix molecules and stem cell niche in health and diseases.

Keywords Tight junctions · Claudins · Integrins · Stem cells · EpCAM · Cell-matrix interactions

Abbreviations

AT2	Type 2 alveolar epithelial cells
BBB	Blood-brain barrier
cCldn7$^{fl/fl\text{-}T}$	Inducible, intestinal epithelial-specific conditional claudin-7 knockout mice
Cl-1 Tg	Overexpression of claudin-1

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L. González-Mariscal (ed.), *Tight Junctions*,
https://doi.org/10.1007/978-3-030-97204-2_9

CRC	Colorectal cancer
EMT	Epithelial-to-mesenchymal transition
gKO	Global claudin-7 knockout mice
IESCs	Intestinal epithelial stem cells
JAMs	Junctional adhesion molecules
KD	Knockdown
MDCK	Madin-Darby canine kidney
MET	Mesenchymal-to-epithelial transition
OSP	Oligodendrocyte-specific protein
TEMs	Tetraspanin-enriched membrane microdomains
TER	Transepithelial electrical resistance
TJs	Tight junctions
ZO	Zonula occludens

Introduction

Cell-cell junctions form connections between adjacent cells and include apical tight junctions (TJs), adherens junctions, desmosomes, and gap junctions, while cell-matrix junctions include focal adhesions and hemidesmosomes [1–4]. TJs form a seal between adjacent cells to regulate the diffusion of solutes and ions through both barrier and fence functions [5, 6]. The "fence" refers to separation of apical and basolateral compartments; the "barrier" functions to regulate the paracellular pathway [7, 8]. TJs are made up of three major integral membrane proteins: claudins, occludins, and junctional adhesion molecules (JAMs) and are vital for regulating permeability of epithelial barriers [4, 9, 10]. Claudins form the backbone of the TJ strands and largely determine the epithelial permeability as they function to regulate paracellular penetrability to small molecules and ions [11, 12]. Aside from maintaining TJ integrity, claudin proteins have been implicated in many processes such as epithelial-to-mesenchymal transition (EMT), cell proliferation, signal transduction, cell-matrix interactions, stem cell functions, and cancer progression [11, 13–15]. Besides traditional localization at the apical TJ region, many claudins also reside in regions outside of the TJs in which they exhibit unique functions regulating various cellular activities [16–18]. Furthermore, the differential expression of claudins between healthy and cancerous tissues has highlighted claudins as potential prognostic indicators, therapeutic targets, and/or cellular markers for cancer therapies [19, 20]. Due to the increased evidence demonstrating that TJ proteins have important non-TJ functions, this chapter focuses on the roles of TJ proteins, especially claudin proteins, outside of the TJs, including interactions with integrins and EpCAM to regulate various cellular activities, altered expression in EMT and cancers, as well as regulation of stem cell functions.

Claudin and Integrin Interactions in Normal Cells/Tissues

While claudins are largely localized to the apical TJ structure, claudin proteins often exhibit altered localization in various tissues and interact with cell-adhesion proteins such as integrin molecules and EpCAM, among many others [10, 17, 18, 21]. Multiple claudins, including claudin-1, claudin-2, and claudin-7, have been associated with extracellular matrix regulation through integrins in the focal adhesion structure [22]. Singh et al. reviewed this partnering of claudin proteins with integrins in the regulation of "outside-in" and "inside-out" signaling, highlighting the roles of claudin proteins in cell signaling through integrins [9].

Although several claudins interact with other cell-cell adhesion proteins, claudin-7, in particular, localizes to the basolateral membrane in intestinal tissues where it colocalizes with integrin molecules and is involved in cell-matrix adhesion [16]. Our previous study highlighted claudin-7 interaction and colocalization with integrin α2 at the epithelial basolateral compartment of the small intestine [16]. Though the apical TJs remained largely intact, loss of claudin-7 led to intercellular gaps and cell-matrix loosening in global claudin-7 knockout mice (gKO) as seen by electron microscopy (Fig. 1), as well as altered integrin α2 distribution and localization where integrin α2 formed clusters and/or moved toward the apical lateral surface [16]. In addition to changing cell-matrix protein localization, claudin-7 knockout also disrupted the formation of the claudin-7/integrin α2/claudin-1 complex, which normally forms in the intestines [16].

In the intestines, integrins play important roles in regulating cell proliferation and intestinal inflammation [23, 24]. For example, conditional knockout of integrin β1 in the intestines led to the increased epithelial cell proliferation with dysplasia, reduced Hedgehog expression, and mislocalization of Tcf-4, a claudin-7 expression regulator and a transcription factor required for intestinal epithelial stem cell proliferation [24, 25]. In addition, integrin β7 knockout mice exhibited the reduced leukocyte homing and weakened protective immunity against helminth infection, highlighting the importance of the integrin in host protection during intestinal inflammation [23]. Integrins can also modulate the epithelial TJ permeability via rotavirus. Treatment of Madin-Darby canine kidney (MDCK) cells with VP8, a rotavirus surface protein, induced the redistribution of integrin $\alpha_v\beta_3$ and integrin β1 from the basolateral membrane to the apical surface [26]. This redistribution of integrins caused by rotavirus protein resulted in the epithelial permeability change by opening the paracellular space normally sealed by TJs, therefore diminishing transepithelial electrical resistance (TER) and inhibiting the formation of new TJs [26]. The importance of integrins extends into pathologic conditions as some integrins, such as integrin α4 and integrin β7, have been utilized as therapeutic targets in the treatment of intestinal bowel disease (IBD) [27, 28]. Particularly, specific inhibition of integrin α4β7 activation may provide a more efficient IBD treatment than completely blocking integrin α4β7 function, as integrin β7 knock-in mice were found to be resistant to T-cell-transfer-induced chronic colitis [28]. These studies demonstrate the many vital functions, including TJ permeability regulation, of integrins in the intestinal epithelium.

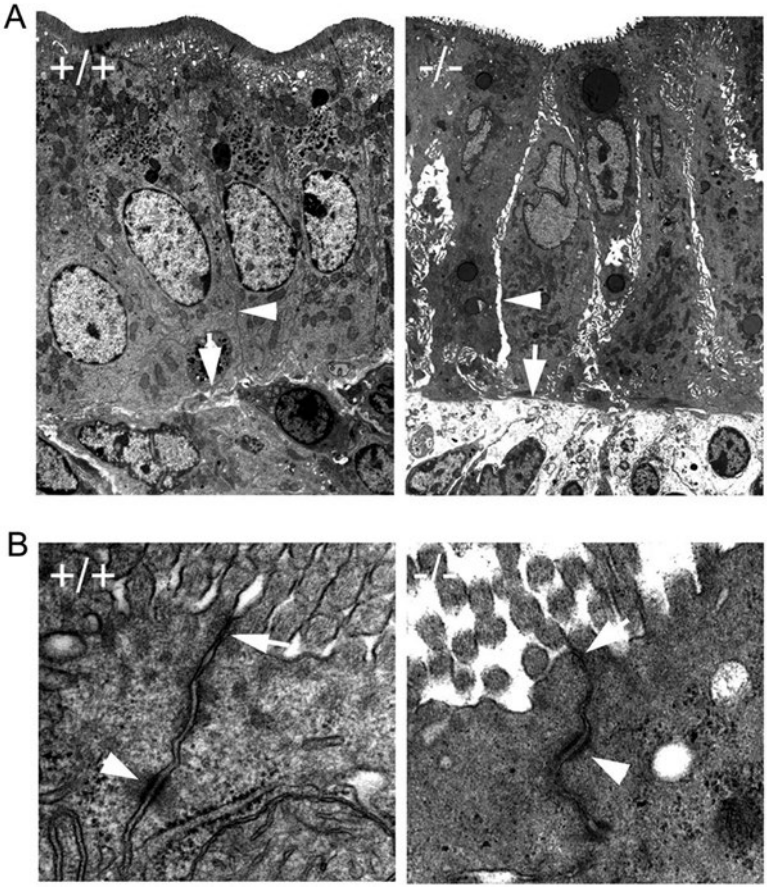

Fig. 1 Disruption of cell adhesion in claudin-7 gKO intestines. Electron micrographs show postnatal day 5 wild-type (WT, +/+) and gKO (−/−) small intestines. (**a**) The arrowhead in (−/−) pointed to the intercellular gap along the gKO lateral membrane compared to that of WT (+/+, arrowhead). The arrow in (−/−) revealed the loosening of cell-matrix connection in gKO versus the close contact between cell and matrix in WT intestines (+/+, arrow). (**b**) The arrows in (+/+) and (−/−) pointed to the apical TJ. The arrowheads indicated the desmosome. Magnifications: **a**, ×5000; **b**, ×50,000. Reprinted from *Gastroenterology*, 2012, 142(2):305–315**,** with permission from Elseiver

Claudin and integrin interactions regulate many processes during development including oligodendrocyte myelination, placentation, and kidney tubular permeability [29–31]. Oligodendrocyte-specific protein (OSP)/claudin-11, OAP-1, and integrin β1 form a complex, which functions in modulating the proliferation and migration of oligodendrocytes during myelination, as well as in maintenance and repair of the myelin sheath [31]. Oligodendrocytes deficient in OSP/claudin-11 exhibited reduced migration ability [31]. Claudin and integrin expression patterns are also clinically relevant as distinguishing markers of trophoblast lineages during

the process of placentation, which is a major factor in the success of pregnancy [29]. Claudin-1, claudin-3, claudin-4, claudin-5 and integrin α1β1 have been found to be upregulated in the placenta of pre-eclamptic women, while integrin α6β4 is down-regulated [29]. This knowledge highlights the potential use of claudins and integrins as markers to manage malplacentation-related diseases, therefore reducing obstetric complications [29].

Furthermore, it has been reported that integrin β1 regulates the paracellular permeability of kidney proximal tubules by altering the composition and function of their TJs [30]. Claudin-2 is a paracellular monovalent cation-selective channel, which plays an important role in the kidney proximal tubular function. Deletion of integrin β1 results in transition from a "loose" epithelium exhibiting high claudin-2 expression and low claudin-7 and E-cadherin expression to a "tight" epithelium with reduced claudin-2 expression and increased E-cadherin and claudin-7 expression [30]. This loss of renal integrin β1 negatively influences paracellular transport in the proximal tubule, leading to a major defect in the process of urine concentration [30]. Additionally, claudin-4, claudin-7, and integrin β4 were identified to be involved in endogenous protection mechanisms against acute pancreatitis in mouse models [32].

One factor regulating these interactions between claudin proteins and integrins is zonula occludens 2 (ZO-2) [33]. ZO-2 regulates Rho, Rac, and Cdc42 proteins, all of which are vital for TJ sealing and development of epithelial cytoarchitecture [33]. Knockdown of ZO-2 reduced the expression of claudin-7 and integrin β1, increased the intercellular gaps and stress fiber formation, as well as decreased the cell attachment to the substratum, indicating the role of ZO-2 in modulating the development of cellular architecture and barrier function through claudins and integrins [33].

Claudin and EpCAM Interactions Regulating Multiple Cellular Activities

Claudin proteins regulate many cellular functions including cell migration, invasion, and proliferation via interactions with EpCAM in the basolateral compartment [9, 34]. Formation of an EpCAM-tetraspanin-claudin-7 complex alters homotypic cell-cell adhesion and supports resistance to apoptosis [35, 36]. Screening of primary colorectal cancer tissue and liver metastasis confirmed coexpression and coimmunoprecipitation of EpCAM and claudin-7, as well as colocalization in the basolateral compartment [35, 37]. Additionally, EpCAM and claudin-7 coexpression with the tetraspanin CO-029 and CD44 variant isoform v6 (CD44v6) inversely correlated with disease-free survival and induced complex formation and recruitment into tetraspanin-enriched membrane microdomains (TEM) [35]. Results of this study suggest that claudin-7 is required for EpCAM recruitment into TEMs as the absence of claudin-7 resulted in decreased association of EpCAM with CO-029 and CD44v6 and lack of EpCAM recruitment into TEMs. [35, 38]. Formation of the

EpCAM/claudin-7 complex was found to support proliferation and promote tumor progression and metastasis by blocking EpCAM oligomerization, which is vital for EpCAM-mediated cell-cell adhesion [38]. This TEM-associated EpCAM/claudin-7 complex also upregulates antiapoptotic proteins and is correlated with significantly reduced disease-free survival [35, 38, 39]. Furthermore, EpCAM-claudin-7-expressing cells exhibit enhanced motility due to claudin-7 association with actin bundles, which further promotes tumorigenicity and accelerates tumor growth [38].

While claudin-7 and EpCAM association affects an array of cell functions, EpCAM itself also regulates adhesion and TJ composition and function through modulating the degradation and localization of claudin proteins [40]. EpCAM coimmunoprecipitated with both claudin-7 and claudin-1, and physical interaction of claudins with EpCAM was required for claudin stabilization [40]. The shRNA knockdown of EpCAM in human colorectal adenocarcinoma Caco-2 cells resulted in altered morphology, increased cell proliferation, increased resistance to disruption by calcium chelation, and enhanced TER, highlighting the role of EpCAM in regulating claudin dynamics and TJ function and composition [40].

In addition, palmitoylation of claudins is essential for effective TJ localization and barrier function. For example, expression of palmitoylation-deficient claudin-14 significantly reduced TER increase and was less localized to TJs and more found in lysosomes, suggesting the altered trafficking or stability [41]. Following palmitoylation, claudin-7 is recruited into glycolipid-enriched membranes in which it associates with EpCAM, integrins, proteases, and cytoskeletal linker proteins to support cell-matrix degradation, induce cleavage of claudin-7-associated EpCAM, and enhance motility and invasiveness [34]. Palmitoylated claudin-7 also plays a role in tumorigenicity and increased motility, as palmitoylation of claudin-7 impedes its integration into the TJ and inhibits cell-cell adhesion as demonstrated in human embryonic kidney HEK293 cells [34].

Claudin and Integrin Interactions in the Blood-Brain Barrier

While TJs and claudins greatly contribute to the structure of cerebral microvessel endothelial cells, adequate cell-matrix adhesion is also crucial in development and maintenance of the blood-brain barrier (BBB) [42]. Integrin β1 directly regulates interendothelial claudin-5 expression and brain microvascular permeability, as blocking integrin β1 function results in decreased claudin-5 expression, reduced TER of endothelial cell monolayers, and significantly increased permeability [42]. Disruption of endothelial integrin β1-mediated matrix adhesion led to reorganization of claudin-5, occludin, and zonula occludens 1 (ZO-1), leading to increased vascular endothelial permeability [43]. Interestingly, the expression of these proteins changes throughout development according to the needs of the developing brain [44]. Claudin-5 and integrin β1 both exhibited an “uphill-type” expression pattern in that they continuously increased in expression from postnatal day 1 to

postnatal day 56 in developing rat brain capillaries, demonstrating the increasing importance of these proteins in development of the BBB [44].

Claudin and integrin expression and localization patterns are also used to examine alterations in the BBB-associated ECM in the case of injury [45]. There was a significant increase in the expression of integrin α5 in the striatum of mice who have undergone bilateral carotid artery stenosis to model vascular dementia [45]. These mice also exhibit altered expression patterns of claudin-5 within the striatum and significantly reduced expression of occludin in the cortex and striatum [45].

Claudin and Integrin Interactions in EMT and Cancer Cells

Claudins have been strongly implicated in epithelial-to-mesenchymal transition (EMT), the process by which cells lose their epithelial properties and gain mesenchymal characteristics, therefore enhancing their motility and invasive potential [12]. A myriad of studies have highlighted altered claudin expression in various epithelial-derived cancers in a stage-, tumor-, and tissue-specific manner [20, 46–48]. The loss of several claudin proteins has been linked to decreased intercellular adhesion and enhanced tumor cell metastasis [13, 14, 48, 49]. Wang et al. reported that the expression of claudin-7 in colorectal cancer (CRC) is downregulated as differentiation grade decreases. The low expression level of Claudin-7 corresponds to the downregulation of E-cadherin and upregulation of vimentin and snail-1, which promotes the invasion and metastasis of CRC through the regulation of EMT [50]. Claudin-7 knockdown (KD) in colon cancer cells, HT-29 and DLD-1, induced EMT and colony formation in vitro, as well as increased xenograft-tumor growth in vivo [51]. Moreover, forced expression of claudin-7 in highly metastatic SW620 colon cancer cells induced mesenchymal-to-epithelial transition (MET), in which cell growth in soft agar and tumor growth in vivo were both inhibited [51].

Our previous studies demonstrate a tumor-suppressive function for claudin-7 due to its role in cell-matrix adhesion and proliferation [52, 53]. Claudin-7 colocalizes with and forms a stable complex with integrin β1. The siRNA silencing of claudin-7 in human lung adenocarcinoma HCC827 cells reduced integrin β1 expression and diminished cell-matrix adhesion [7, 53, 54]. Claudin-7 KD in these lung cancer cells affected both cell-matrix interactions and cell growth as these cells exhibited impaired cell-matrix adhesion and grew on top of each other forming spheroids (Fig. 2), as well as resulted in significantly larger tumor formation when inoculated into nude mice, compared to control cells [53]. Injection of claudin-7-expressing human lung carcinoma NCI-H1299 cells into nude mice resulted in significantly reduced tumor size compared to cells without claudin-7, further suggesting a tumor-suppressive role for claudin-7 through the ERK/MAPK signaling pathway [52]. Claudin-7 KD in human colorectal carcinoma HCT116 cells increased the migration ability and enhanced the tumor growth [7]. To elucidate if these proteins regulate cell proliferation and invasion independently or synergistically, further investigation into claudin-7 and integrin β1 interactions suggests that claudin-7

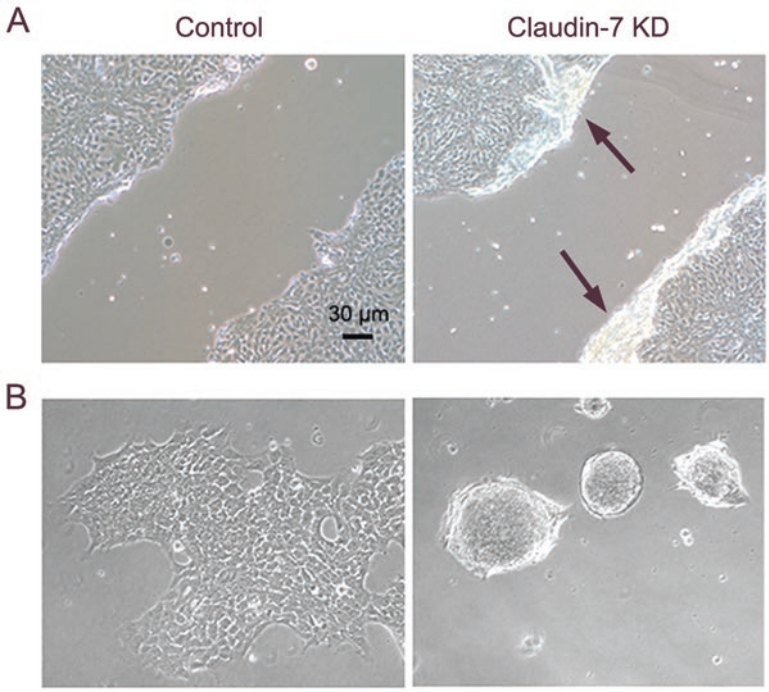

Fig. 2 Reduced cell-matrix adhesion in claudin-7 KD cells. (**a**) Scratches were made on the confluent HCC827 control and claudin-7 KD cell monolayer. Claudin-7 KD cells were easily peeled off along the scratch as shown in arrows, while the control cells were well attached to the plate. (**b**) When cultured on uncoated glass coverslips, HCC827 claudin-7 KD cells formed spheroids, while the control cells were able to spread out and formed a monolayer. Reprinted from *Molecular Cancer*, 2015, 14:120, 1–15**,** published as open access by BioMed Central

regulates cell motility through integrin β1 [54]. Overexpression of claudin-7 in HCC827 claudin-7 KD cells resulted in reduced cell proliferation, increased expression of focal adhesion proteins, and improved ability to attach to cell culture plates, while ectopic expression of integrin β1 in HCC827 claudin-7 KD cells exhibited increased migration and adhesion but not the cell proliferation [54]. Taken together, these data identified a new function of TJ protein claudin-7 in maintaining epithelial cell-matrix attachment and cell motility via integrin β1 [7, 16, 53, 54].

Other claudins have also been shown to interact with integrin proteins to influence cancer metastasis [55–57]. Claudin-2 promotes the formation of breast cancer metastases by increasing the cell surface expression of α5β1 and α2β1 integrin complexes, which enhances the ability of breast cancer cells to adhere to type IV collagen and fibronectin in the ECM, therefore facilitating adhesion and survival in secondary tissues [57]. Claudin-18 exhibits basolateral localization and regulates cell lineage differentiation and signaling within the mouse stomach [56]. Loss of claudin-18 resulted in increased cell proliferation, altered cell signaling, and intraepithelial neoplasia to induce development of gastric cancer [56]. Additionally,

decreased claudin-1 and claudin-11 expression and increased integrin β3 expression are associated with primary oral mucosal melanoma. These proteins may play a role in the pathogenesis and invasive potential of oral melanomas [55]. Interestingly, the expression of claudins is predominantly located in the cytoplasm, rather than on the cell membrane in oral mucosa melanoma tissues [55].

Specific targeting of TJ and focal adhesion proteins suggests a new and promising mechanism for more effective cancer treatments [58, 59]. In poorly differentiated gastric cancer TMK-1 cells, the non-TJ claudin-4 interaction with integrin β1 leads to increased stem cell markers including nucleostemin or WNT [59]. Targeting of claudin-4 along with cis-diamminedichloroplatinum treatment increases tumor apoptosis and inhibits cell proliferation in a synergistic manner [59]. This increased chemotherapeutic sensitivity occurs via the structural disintegration of claudin-4, which is overexpressed in gastric cancer [59]. DZ-50, a quinazoline-based Doxazosin derivative, impairs prostate cancer tumor growth, survival, migration, and invasion through downregulation of fibronectin, integrin α6, and claudin-11 [58]. Targeting of these TJ and focal adhesion intercellular interactions and loss of signaling effectors including integrin linked kinase sensitize human prostate cancer cells to the process of anoikis [58]. These studies shed light on the potential for utilizing TJ and focal adhesion proteins in targeted cancer therapies.

Regulation of Intestinal Stem Cell Functions by TJ Protein Claudin-7

Besides the barrier function, TJs have been found to interact with many associated proteins to modulate cell differentiation and proliferation by regulating signaling pathways, transcription factor expression, and gene expression [60]. When intestinal epithelial differentiation is induced in intestinal enteroids, claudin proteins distribute heterogeneously among the various cell types [61]. Claudin-1 is the most highly expressed in goblet cells and Paneth cells in organoids, while claudin-7 is enriched in the differentiated cell types such as enterocytes, goblet cells, and Paneth cells [61]. Claudin-2 is detected in both crypt and villus cells of the small intestine but is restricted to the undifferentiated crypt cells in the colon [21]. These studies suggest that, in addition to their tradition roles in regulating epithelial barrier function and polarity, claudins also regulate specific cell functions that contribute to cell proliferation and differentiation.

Our previous study demonstrated that claudin-7 is essential in maintaining intestinal epithelial stem cell (IESC) functions and intestinal epithelial self-renewal [15]. The essential role of claudin-7 was revealed in both global *Cldn7* knockout mice (gKO) and inducible, intestinal epithelial-specific conditional *Cldn7* knockout mice (cCldn7$^{fl/fl\text{-}T}$) as both mice were unable to survive for more than 2 weeks [15, 16, 62]. Importantly, deletion of claudin-7 reduced the number of IESCs and disrupted epithelial differentiation and proliferation in both gKO and cCldn7$^{fl/fl\text{-}T}$ mice. Loss of

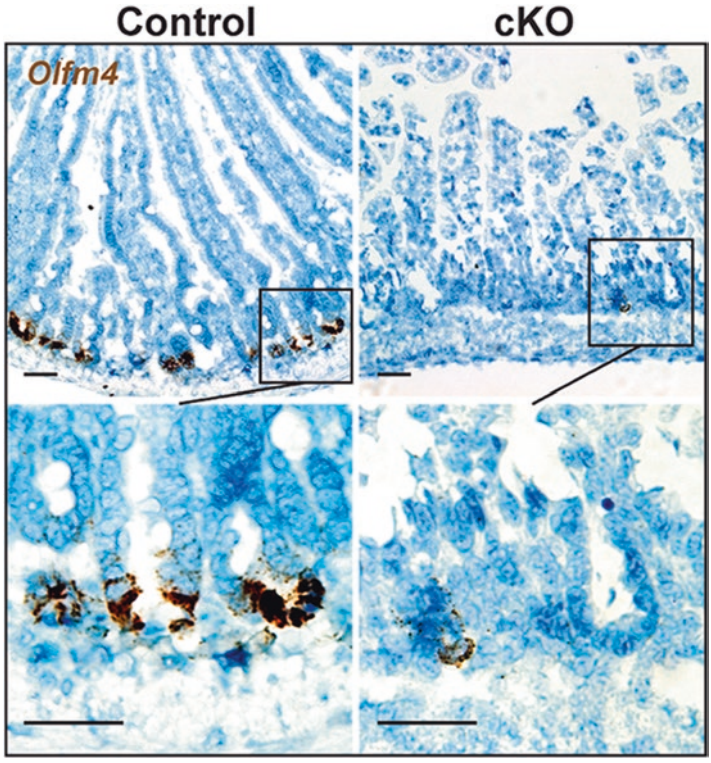

Fig. 3 Loss of active crypt stem cells in cCldn7$^{fl/fl\text{-}T}$ small intestines. Active intestinal epithelial stem cells (IESCs) were labeled with crypt stem cell marker *Olfm4* mRNA using fluorescence in situ hybridization (FISH) in 3-month-old WT (+/+) and cCldn7$^{fl/fl\text{-}T}$ (−/−) small intestines. Scale bar: 100 μm. Reprinted from *Cellular and Molecular Gastroenterology and Hepatology*, 2020, 9(4):641–659, published as open access by Elsevier on behalf of the AGA Institute

claudin-7 led to significantly decreased OLFM4-positive IESCs, as seen in both gKO and cCldn7$^{fl/fl\text{-}T}$ mouse intestinal crypt region and claudin-7-deficient enteroids (Fig. 3) [15]. Isolated crypts from gKO mice were unable to form budding enteroids and enteroid survival was greatly reduced with claudin-7 deletion, suggesting a critical role for claudin-7 in supporting IESC survival [15]. More importantly, the Wnt/β-catenin signaling pathway – an essential signaling pathway for IESC survival and self-renewal – was suppressed in claudin-7-deficient intestines [15]. These exciting findings revealed that claudin-7 is vital in the process of replenishing daily epithelial cell loss at the tips of villi by sustaining the stable pools of IESCs for epithelial self-renewal, and by promoting differentiation into mature epithelial cells [15].

Given the critical role of claudin-7 in maintaining IESC functions, it is essential to understand the regulation of claudin-7 in the epithelial self-renewal. HNF-4α directly interacts with the *Cldn7* promoter, which in turn upregulates the claudin-7 protein during IESC differentiation [63]. Additionally, the transcriptional factor

Hopx was found to stimulate claudin-7 expression in the mouse colonic epithelium [64]. Tcf-4 also maintained the low expression level of claudin-7 at the bottom of colonic crypts via Sox-9 to suppress the tumor cell polarization [25]. The receptor and downstream targets of Wnt/β-catenin signaling pathway, *Olfm4 and Lgr5,* were suppressed in claudin-7-deficient small intestines [15]. Interestingly, they are both active stem cell markers in the mouse small intestine. The Wnt/β-catenin signaling pathway is essential for IESC survival, and its defect will lead to the depletion of IESCs [65]. It has been reported that claudin-7 overexpression enhances β-catenin/Tcf activity and promotes tumor formation in xenograft mice [51]. These studies demonstrate that claudin-7 interacts with the Wnt/β-catenin signaling pathway in controlling intestinal epithelial cell differentiation and proliferation [15]. Moreover, EpCAM, the epithelial cell adhesion protein that directly binds to and stabilizes claudin-7, is required for stem cell survival and proliferation [37, 66]. Deletion of EpCAM resulted in significantly decreased protein expression of claudin-7, and epithelial integrity was severely compromised [37, 66]. Additionally, enteroid survival and growth were attenuated, suggesting that IESC function was compromised [67].

Regulation of Epithelial Self-Renewal Function by Other TJ Proteins

Besides claudin-7, an increasing number of publications have highlighted the importance of claudins in stem cell functions [15, 16, 68, 69]. Overexpression of claudin-1 in a transgenic mouse (Cl-1 Tg) colon led to the suppression of goblet cell number and increase in colonocyte number [68]. Due to the compromised protection of mucin, Cl-1 Tg mice were more susceptible to dextran sodium sulfate (DSS)-induced colitis; of note, these mice exhibited significantly decreased claudin-7 expression [68]. In our study, claudin-7 deletion caused severe intestinal damage, which could partially explain the sustained intestinal inflammation in the Cl-1 Tg mice [15, 16, 68]. In addition, Notch signaling was disrupted in the Cl-1 Tg mice [68]. Hes1, the transcriptional factor of Notch signaling, directly binds to the *Cldn1* promoter and inhibits claudin-1 expression in human and rat colon crypts [69].

An emerging body of literature suggests that claudin-18 controls cell proliferation [70–72]. Claudin-18 is highly expressed in the lung epithelium [71]. Mice lacking claudin-18 exhibited increased alveolar epithelial permeability to ions and solutes, altered cytoskeleton organization, and large lung size [71, 72]. This finding revealed an important mechanism that claudin-18 regulates proliferation of type 2 alveolar epithelial (AT2) cells. Claudin-18 interacts with both ZO-1 and YAP and deletion of claudin-18 disrupted the ZO-1/YAP membrane-protein complex, leading to nuclear translocation of dephosphorylated YAP and induction of the AT2 cell proliferation [72]. Interestingly, this cell cycle stimulation effect is organ-specific. Deletion of the stomach-specific *Cldn18.2* isoform in mice leads to the loss of TJ

strands and increased paracellular H^+ leakage into the stomach, resulting in atrophic gastritis and metaplasia; however, there was no evidence of tumor formation. [70].

ZO-1, which is essential for TJ assembly and cytoskeletal structures, is homogenously expressed among different cell types under normal conditions but is redistributed away from the cell membrane during injury [61, 73]. ZO-1 has also been shown to suppress Stat3 and Smad1/5/8 activities and ERK activity to promote mouse embryonic stem cell differentiation [74]. Additionally, decreased ZO-1 expression reduced trophoblast cell-cell fusion and differentiation [75].

In the embryonic cerebral cortex, occludin is localized to the apical surface of neuroepithelial cells at the chick neural plate [76]. An *occludin*-null mouse model displayed a complex phenotype including abnormalities of gastric epithelium, bone, testes, brain calcification, and postnatal growth retardation [77]. Occludin mutation was identified in a rare human neurologic disorder with band-like calcification in the gray matter [78]. Later, it was discovered that loss of full-length occludin in the mouse embryonic cortex led to development of microcephaly due to prolonged M-phase, a transient burst of apoptosis, and early neuronal differentiation at the expense of the progenitor pool [79]. Furthermore, the hESC-derived cortical organoids from occludin mutant mice were significantly smaller with reduced cell proliferation, premature differentiation, increased apoptosis, and increased aneuploidy [79]. These studies not only highlight a novel role for occludin in regulating neural progenitor proliferation and neuron survival, but also illuminate that full-length occludin is required for the promotion of early progenitor self-renewal through proper neural stem cell mitotic spindle function [78, 79]. Additionally, occludin deletion promotes the differentiation of mucus cells, but reduces the production of parietal and chief cells. These studies suggest that occludin also regulates gastric epithelial differentiation in the homeostasis of the gastric environment [77].

A number of studies have highlighted the possible correlation between TJ proteins and stem cell functions. Claudin-2 localizes to both crypt cells and villus cells in the small intestine, but localizes only to undifferentiated crypt cells in the colon [80]. Interestingly, claudin-2 gene expression is regulated by GATA-4 – a transcriptional factor essential for the survival of embryos and regulation of intestinal development [81]. The *Cldn2* promoter shares similarity with *sucrase-isomaltase*, a marker gene of enterocytes [82]. Moreover, claudin-4-positive intestinal epithelial cells exclusively express the enteroendocrine cell marker gene *Chga* and other enteroendocrine-cell-related hormone genes [83]. These studies suggest that different claudins may have fundamental roles in epithelial cell differentiation. Lastly, claudin-6 triggers epithelial morphogenesis in mouse embryonic stem cells and induces expression of claudin-7, occludin, and ZO-1α + [84]. While the significance of this research is highly recognized in the field, further studies are needed to provide insight into the unique role of TJ proteins in stem cell functions. TJ proteins contribute not only to the epithelial barrier and cell-matrix adhesion, but also function as a niche-like environment for stem and progenitor cells.

Conclusion

This chapter highlights the roles of claudin and other TJ proteins outside of the TJ structure, including their interactions with integrin signaling molecules, functions in development, involvement in cancer and metastasis through EMT processes, impact on cellular activities through interaction with EpCAM, and regulation of stem cell functions in many different tissues. While there is a wealth of information illuminating these unique non-TJ functions, additional studies are necessary to further understand the complex roles of TJ-related proteins outside of the traditional TJ structure and the mechanisms by which they perform these functions.

Acknowledgments This study is supported by NIH DK103166.

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Roles for Claudins in Regulating Lung Barriers and Function

Michael Koval

Abstract The lung is a unique barrier forming tissue that has a large surface area exposed to the outside environment and that requires exquisite fluid balance in order to be properly hydrated to enable efficient gas exchange between the atmosphere and the bloodstream. Central to the maintenance of a pulmonary air-liquid barrier are tight junctions. Different anatomic regions of the lung differ in tight junction protein composition and morphology. The epithelia can be broadly divided into the conducting airways and alveolar epithelium, both of which are composed of heterogeneous epithelial monolayers. The alveolar epithelium also coordinates with the pulmonary vasculature to maintain the air-liquid barrier. This chapter focuses specifically on claudin-family tight junction proteins, which have been shown to form paracellular ion channels with different characteristics, including the formation of charge-specific ion channels. How the context of claudin expression influences their role in control of paracellular permeability is discussed. In addition to their ability to control tight junction paracellular permeability, claudins serve several functions to regulate lung tissue repair and epithelial cell phenotype. The involvement of claudins in lung epithelial homeostasis, as well as their potential as therapeutic targets to prevent lung disease, is discussed.

Keywords Alveolar epithelium · Airway epithelium · Vascular endothelium · Tight junction · Acute respiratory distress syndrome

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L. González-Mariscal (ed.), *Tight Junctions*,
https://doi.org/10.1007/978-3-030-97204-2_10

Introduction

The respiratory system critically regulates fluid balance in an air-filled lumen in order to support gas exchange needed to sustain life. This requires the coordinated function of multiple epithelial and endothelial cell types, interconnected by tight junctions. The primary function of tight junctions is to regulate paracellular permeability to water, ions, and other solutes. It is becoming increasingly apparent that tight junctions can also serve to regulate other aspects of epithelial function, including the control of gene expression, cell proliferation, and tissue morphology.

Several insults have been associated with general impairment of lung tight junctions, including hyperoxia [1, 2], exposure to toxicants [3, 4], cigarette smoke [5–9], viral infections [10, 11], inflammation/asthma [12–14], and mechanical injury [15, 16]. Barrier dysfunction has also been associated with cystic fibrosis [17–22].

This chapter focusses on a key structural component of tight junctions: claudin family transmembrane proteins. Claudins serve as the main structural element that regulates paracellular permeability by forming paracellular ion channels. In addition to roles for specific claudins in regulating barrier function, this chapter also describes the ability of claudins to regulate signal transduction pathways that are required to maintain healthy lung epithelia.

Multiplicity of Epithelial Cells Lining the Respiratory Tract

As a conduit for gas exchange, the respiratory tract consists of functionally distinct segments ranging from the conducting airways to the terminal airspaces where gas exchange occurs (Fig. 1). The conducting airways are further divided into functionally distinct anatomical segments: the upper, lower, and distal airways. The upper airway consists of the nasal and oral cavities, pharynx, and larynx and is predominantly involved in sensory function, air humidification, and gross filtration of airborne particulates. The lower airway begins with the trachea, which then branches into two primary bronchi. Primary bronchi extend and branch through secondary and tertiary bronchi followed by 5–20 generations of bronchioles. The bronchial tree provides a semirigid cartilaginous framework to support the terminal airspaces that have the flexibility and compliance required for air influx and efflux.

The main airspace surface of the trachea, as well as conducting airways distal to the trachea, is lined by a pseudostratified epithelium, which includes ciliated cells, mucus-secreting goblet cells, columnar cells, serous cells, and basal cells, which altogether form a permeability barrier. Conducting airways are also peppered with submucosal glands containing goblet, duct, and serous cells, which contribute to mucus secretion. A major function of the lower airway is clearance of particulates from the lower airspaces. This is facilitated by the concerted efforts of goblet cells and ciliated cells that serve as a “mucociliary escalator” moving foreign material upward and out of the lung [23]. Regulation of the airway surface liquid underneath

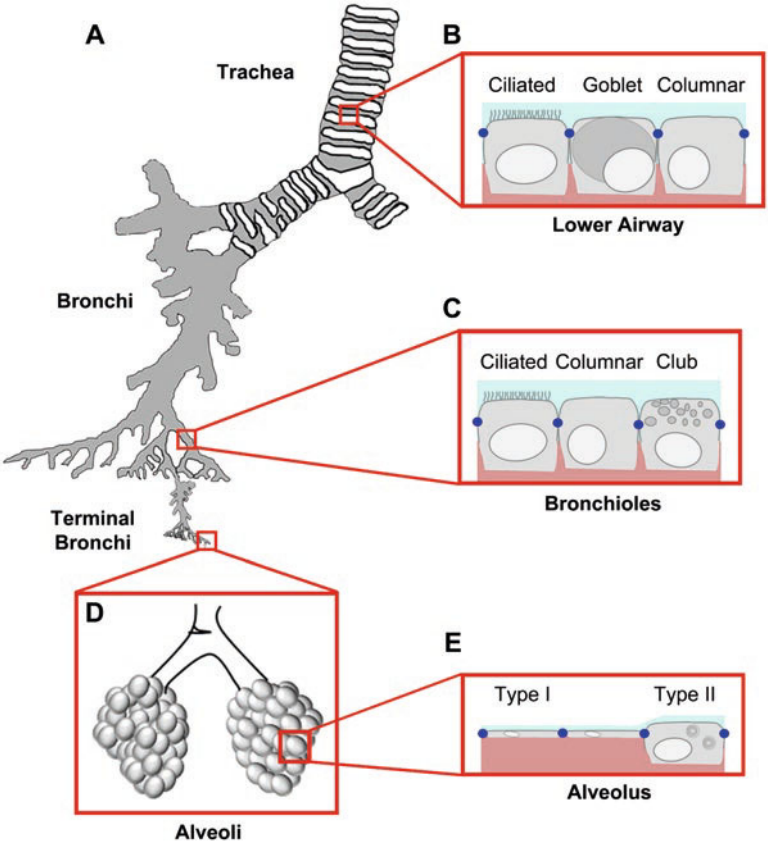

Fig. 1 Epithelial diversity along the respiratory tree. (**a**) Airways are divided into four main segments: the trachea, the branching bronchi, the terminal bronchi, and the alveolar space. Each segment contains a unique mix of cell types that have specialized functions. (**b**) The lower airway, proximal to the bifurcation of the left and right bronchus, consists of mostly ciliated cells whose main function is to sweep mucus (light blue) secreted by goblet cells out of the airways. Columnar and other cells (e.g., serous cells) also contribute to the airway barrier. Basal cells (not shown) are localized to the basement membrane but do not contribute to the tight junction barrier. (**c**) Distal to the tracheal bifurcation are bronchiolar cells that consist mainly of ciliated, columnar, and club cells. Club cells secrete a specialized form of pulmonary surfactant as opposed to mucus and provide a transition zone between the airway and alveolar space. (**d**) The alveolar space is the location of gas exchange and consists mainly of squamous type I and cuboidal type II cells. Tight junctions between these cells form at apical cell-cell interaction sites. The alveolar sac maintains surface tension through surfactant secreted by type II cells preventing alveolar collapse. (**e**) Gas exchange occurs efficiently through type I cells, which make up the vast majority of alveolar surface area. In **b**, **c**, and **e**, dark blue disks represent tight junctions; in each case, the cells form a high resistance barrier. Reproduced from [131] with permission

the mucus layer is critical to proper mucociliary clearance [24]. Airway epithelial cells maintain airway surface liquid balance by the concerted action of plasma membrane channels (which regulate transcellular fluid and ion transport) and tight

junctions (which regulate the paracellular route). Roles for claudins in regulating paracellular ion and water diffusion are described below.

Conducting lower airways are kept open to the atmosphere by supportive cartilaginous rings, which gradually thin as the airway diameter decreases. At the most distal portion of the conducting airways are terminal bronchioles, the smallest branches of the conducting airway with a diameter of 5 mm or less. Terminal bronchioles interconnect the conducting airways with the terminal airspaces, known as alveoli, where gas exchange occurs. In contrast to the conducting airways, the terminal airspaces of the alveolar epithelium are coated with pulmonary surfactant, which serves to reduce the surface tension of the air-filled alveoli under the pressure of the fluid-filled tissue. Thus, bronchioles serve as transition zones from the mucus-dominated airspace as reflected by their distinct cellular composition [25, 26]. In contrast to larger bronchioles, which are dominated by ciliated and goblet cells, terminal bronchioles lack submucosal glands and are enriched for club cells. Instead of mucus, club cells are secretory cells that produce a form of pulmonary surfactant containing surfactant protein A (SP-A), SP-B, and Club-Cell-specific protein 10 (CC-10) [27]. The surfactant produced by club cells differs from surfactant produced by alveolar epithelial cells, which lacks CC-10 but instead contains SP-C and therefore has different surface active properties [28].

The terminal airspaces are covered by the alveolar epithelium, which consists of type I and type II alveolar epithelial cells (Fig. 1). Although type II alveolar epithelial cells constitute 60% of the alveolar cells, they only cover <5% of the alveolar surface because of their size and cuboidal shape [29]. Conversely, type I alveolar epithelial cells are large, squamous, and extremely thin to allow the diffusion of gases between the terminal airspaces and capillaries. Given their large surface area, type I cell junctions are mainly responsible for alveolar epithelial barrier function [30]. However, pulmonary microvascular endothelial cell tight junctions also contribute to alveolar barrier function by maintaining a tight seal to prevent leakage from the bloodstream. Failure of the microvascular barrier while the alveolar epithelial barrier is maintained leads to tissue edema [31, 32], whereas failure of the alveolar epithelial barrier causes airspace flooding and increases susceptibility to acute respiratory distress syndrome (ARDS) [33]. Besides the prevention of fluid leak, alveolar epithelial cells maintain lung fluid balance by regulating ion transport to promote fluid resorption, comparable to conducting airway epithelium [34–36].

A key point in considering intercellular junctions throughout lung epithelia is that their nature as intermixed cell populations (such as pseudostratified monolayers) means that they are enriched for heterocellular interfaces between cells of different phenotype. Thus, in order to form functional tight junctions, cells of different phenotype that are in direct contact need to express compatible claudins. Consistent with this, Flynn et al. [37] showed by confocal immunofluorescence microscopy that claudin-1, claudin-3, and claudin-7 localize to tight junctions formed between goblet and ciliated airway epithelial cells. Moreover, type I and type II alveolar epithelial cells were shown to have multiple claudins localized to tight junctions despite having differential claudin expression [38, 39].

Claudin-1

Claudin-1 is predominantly associated with epithelial cells in the conducting airway [40, 41]. Claudin-1 is particularly enriched in airway basal stem cells. A clue to the regulation of claudin-1 by airway epithelial cells comes from studies of cultured cell models, where it has been appreciated that in order for airway cells to fully differentiate, they must be cultured on Transwell permeable supports at an air-liquid interface [42]. One effect of culturing basal airway cells at air-liquid interface as opposed to a submerged liquid-liquid interface is a specific increase in claudin-1 expression that was found to cause a twofold increase in barrier function as measured by transepithelial resistance [43]. Paracellular permeability to solutes was also reduced by increased claudin-1 expression by cells cultured at air-liquid interface. On the other hand, cells cultured at a liquid-liquid interface showed less claudin-1 expression. Mechanistically, submerged cells exhibited increased retinoic acid synthesis and signaling, which, in turn, inhibited the ability of the transcription factor AP-1 to stimulate claudin-1 expression [43].

Although claudin-1 was associated with improved barrier function, there is also evidence that stimulation of claudin-1 expression by acute TGF-β treatment has the ability to induce epithelial mesenchymal transition and increased migration of airway cells [44]. This is a somewhat paradoxical result, since claudins are more typically associated with epithelial differentiation. One possibility is that exceptionally high levels of claudin-1 could impact airway epithelial cell proliferation and migration in a manner comparable to the effect of claudin-1 on cancer cells, where it increases metastasis and cell growth [45]. High levels of claudin-1 expression have also been associated with asthma [46]. Consistent with this possibility, claudin-1 expression by human airway smooth muscle cells, which lack tight junctions, is enhanced by proinflammatory cytokines and thus could be a causative factor in the progression of asthma [47], raising the possibility that a nonjunctional role for claudin-1 may be involved.

Claudin-3

Claudin-3 is expressed by most lung epithelial cells, most prominently in the conducting airway [40, 41]. A role for claudin-3 in regulating paracellular permeability of primary bronchiolar epithelial cells was identified when it was determined that chronic TGF-β treatment caused a decrease in barrier function that correlated with a specific decrease in junction-associated claudin-3 [48]. Claudin-3 mRNA and total protein levels were unaffected by TGF-β. Instead, claudin-3 translocated from tight junctions to the nucleus. Although the nucleus is an atypical site for claudin localization, several claudins have nuclear localization signals [49]. Also, there is precedent for nuclear localized claudin-1 [45], claudin-2 [50], and claudin-3 [51] in

regulating cancer cell proliferation and metastasis, consistent with a potential direct role for claudins in regulating gene expression and/or cell growth.

Claudin-3 expression in the alveolus is most prominent in type II cells and significantly lower in type I cells [39–41]. This means that the claudin composition of type I-type I cell interfaces is going to differ from that of type I-type II cell interfaces, which is expected to affect their function. In fact, transducing type I alveolar epithelial cells to express increased claudin-3 results in increased barrier permeability [38]. This is in contrast with other evidence demonstrating that claudin-3 has a sealing function that increases in barrier function [48, 52, 53], reflecting a specific role for claudin-3 in regulating tight junction permeability in the context of other alveolar epithelial claudins. The ability of claudin-3 to increase alveolar epithelial tight junction permeability is likely due to the unique ability of claudin-3 to heterotypically interact with several other claudins, including claudin-1, claudin-2, and claudin-5 [54, 55]. The physiological implications of differential claudin-3 expression in the alveolar epithelium remain undetermined at present.

Claudin-4

Claudin-4 is associated with a protective effect by improving lung epithelial barrier function [38, 56, 57]. This is supported by data from patient samples where increased claudin-4 was associated with increased lung fluid clearance and decreased injury [57]. A protective effect for claudin-4 is underscored by the observation that claudin-4 is upregulated 12–16-fold in response to acute injury [56, 58]. Claudin-4 was also required for the ability of the chemical chaperone 4-phenylbutyrate to prevent lung damage due to hyperoxia [59], suggesting that claudin-4 expression is sensitive to the unfolded protein responses associated with cell stress [60].

Claudin-4-deficient mice have a mild lung phenotype, showing only decreased Na^+/K^+-ATPase activity, which impairs steady-state fluid balance [58]. Loss of claudin-4 is further exacerbated by ventilator-induced lung injury and hyperoxia, which causes significant pulmonary edema. Increased susceptibility to injury in wild-type mice can be revealed using a *Clostridium perfringens* enterotoxin (CPE)–derived peptide, which binds to claudin-4 and exacerbates the injurious effects of high tidal volume mechanical ventilation on lung injury [56].

Injured claudin-4-deficient mice have increased activation of TNFα and IL-1β proinflammatory cytokine signaling when stratified into a severe injury group using bronchoalveolar lavage (BAL) fluid protein content to stratify samples [58]. Severely injured claudin-4-deficient mice increase expression of the Early Growth Response 1 (Egr1) transcription factor [58]. The activation of these signal transduction pathways in the absence of claudin-4 is consistent with increased IL-1β and low Egr1 observed in claudin-18-deficient mice [61, 62] and underscores the ability of claudins to regulate cell behavior beyond serving as structural components of the tight junction barrier.

Claudin-7

Claudin-7 is also prominently expressed by airway and alveolar epithelia [40, 41]. A role played by claudin-7 in preserving lung barrier function was demonstrated by treating alveolar epithelial cells with EGF, which caused an increase in claudin-7 expression that correlated with decreased paracellular permeability [63]. Like many claudins, the impact of claudin-7 on paracellular permeability depends on the context of expression [64]. LLC-PK1 cells overexpressing claudin-7 simultaneously show an increase in sodium permeability and a decrease in chloride permeability [65]. By contrast, experiments using mIMCD-3 cells suggest a more general role for claudin-7 in decreasing paracellular permeability, since RNAi depletion of claudin-7 resulted in a 40–45% increase in both sodium and chloride permeabilities [66].

In addition, claudin-7 permeability is sensitive to posttranslational modifications. For instance, WNK4 kinase activation increased chloride permeability, an effect that correlated with claudin-7 phosphorylation [67]. This could be due to altered regulation of tight-junction-associated claudin-7. However, it could also reflect a change in the assembly of claudin-7 into tight junctions, comparable to the role of palmitoylation in shifting claudin-7 from a tight-junction-associated protein to a nonjunctional, glycolipid-enriched membrane-microdomain-localized protein where it serves to organize a signaling hub critical for regulation of cell proliferation by forming a complex associated with integrin β1, integrin α2, and other focal adhesion proteins [68–70].

Claudin-7-deficient mice are severely dehydrated due to a defect in fluid homeostasis, and so, they die soon after birth [71]. This is due to differential expression of renal ion and water channels, including increased Na^+/K^+-ATPase-α1, aquaporin-2, and epithelial sodium channel (ENaC), resulting in an increase in sodium absorption. The combination of an increase in sodium absorption and a decrease in chloride secretion is a hallmark of the impaired regulation of airway surface liquid associated with cystic fibrosis [72, 73]. Since claudin-7-deficient mice have a fluid imbalance phenotype comparable to that found when the cystic fibrosis transmembrane regulator chloride channel (CFTR) is impaired, this suggests that claudin-7 may preserve airway surface liquid chloride by limiting paracellular chloride flux. In fact, claudin-7 and CFTR are coordinately regulated in the uterus, as demonstrated in Kiss1r deficient mice [74]. Whether they are also coregulated in the lung is an open question at present, but these findings suggest that targeting the paracellular route in general, and claudin-7 in particular, may prove to be an important adjunct to therapeutic approaches to treat fluid imbalance in cystic fibrosis.

Claudin-18

Claudin-18 is differentially spliced, where the claudin-18.1 variant is primarily expressed by lung epithelia and another (claudin-18.2) is mainly expressed by the stomach [75]. MDCK cells overexpressing claudin-18.2 demonstrate a selective

proton and sodium sealing function but allow chloride permeability [76]. The first extracellular domain of claudin-18.1 is 87% identical to that of claudin-18.2, suggesting that both splicing isoforms have comparable paracellular permeability. Of note, paracellular chloride permeability has been proposed to counterbalance sodium flux across lung epithelial monolayers [34]. Claudin-18.1 is predominantly expressed by the alveolar epithelium [77, 78] and, to a lesser extent, by conducting airway cells [79, 80].

Two different claudin-18.1 knockout mice have been produced [61, 81]. In both cases, there was little effect on net lung fluid balance, which was surprising, although it could be explained by an upregulation of sodium transport via ENaC and Na^+/K^+-ATPase [61]. Because fluid clearance was enhanced, claudin-18 knockout mice were resistant to ventilator-induced lung injury, which is counterintuitive considering the prominent expression of claudin-18 in the alveolus [61].

Although claudin-18-deficient mice maintain lung fluid balance, barrier function to macromolecules, such as albumin, is impaired *in vivo* [61, 81]. This is not the case for claudin-18.2 knockout mice [62], suggesting that claudin-18 more critically regulates paracellular barrier function in lung than stomach. Claudin-18-deficient mice also show increased expression of claudin-3 and claudin-4; however, these two claudins are unable to compensate for the loss of claudin-18 in regulating lung epithelial permeability [61].

The permeability deficit in claudin-18-deficient mice is likely linked to a role for this claudin in regulating tight junction architecture, since immunofluorescence analysis of claudin-18-deficient alveolar type I cells shows gaps in ZO-1 localization that represent areas with significantly increased permeability [61]. By transmission electron microscopy, tight junctions between type I cells in claudin-18 knockout mice appeared ruffled and disrupted as compared with normal overlapping junctional morphology, which also suggests that they are sites with increased permeability [81].

In addition to changing lung epithelial permeability, claudin-18 deficiency leads to changes in cell architecture and gene expression [61, 81]. Central to this is the discovery that the transcription factor YAP directly binds to the C terminus of claudin-18 [82], which parallels other junction-associated transcription factors, including ZO-2 [83] and β-catenin [84] that have the capacity to shuttle between intercellular junctions and the nucleus to act as sensors of intercellular contact. High levels of claudin-18 expression dampen the ability of YAP to localize to the nucleus, whereas in the absence of claudin-18, YAP readily translocates to the nucleus where it can promote cell growth.

In the terminal airspace, normal regulation of claudin-18 enables YAP-mediated alveolar epithelial repair. However, in the absence of claudin-18, unchecked YAP activation leads to adenocarcinomas due to unregulated alveolar cell proliferation [82, 85]. Consistent with this model, tumor formation in claudin-18-deficient mice is preceded by alveolar type II cell hyperplasia as well as significant type I cell damage [81].

YAP also has been found to play a key role in lung epithelial differentiation and branching morphogenesis [86, 87] acting as a switch between basal cell

proliferation and epithelial cell differentiation [88]. In this context, abnormally low claudin-18 expression in the airway is associated with asthma in patients and in claudin-18-deficient mice [79, 80]. In addition to its direct effects on airway cell growth and differentiation, low claudin-18 is associated with a proinflammatory environment. In this context, claudin-18-deficient mice have increased IL-1β [62], which has the capacity to cause emphysema and aberrant airway remodeling [56, 89], in addition to compromising airway barrier function [90].

These data underscore the concept that alveolarization is heavily regulated by claudin-18, which, in turn, is likely to act as a sensor for cell-cell contact in addition to its role as a barrier-forming protein. Whether it is feasible to target claudin-18 to treat lung diseases where alveolar morphology is impacted, including pulmonary fibrosis and chronic obstructive pulmonary disorder, remains an open question.

Claudin-5

Claudin-5 is classically associated with vascular endothelial tight junctions [40, 91]. A role for claudin-5 in regulating the blood-brain barrier was first discovered by an analysis of claudin-5 knockout mice, which show a neonatal lethal permeability defect leading to the leakage of molecules less than 0.8 kDa into the cerebral spinal fluid [92]. Since then, there have been multiple studies correlating claudin-5 expression with vascular barrier function [93–99]. Claudin expression by endothelial cells is not restricted to claudin-5, since claudin-1, claudin-3, and claudin-12 are also present in low levels. Of note, claudin-11 is expressed by venous endothelial cells, but not arterioles [100]. Claudin-11 is also found in the blood-brain barrier [101] and is likely to be a component of the pulmonary microvasculature as well.

The human CLDN5 gene has an interesting high-frequency single-nucleotide polymorphism downstream from a cryptic start codon, where the G allele contains codon for glutamine (Q) and the A allele has a stop codon [102]. Theoretically, the G allele represents an open reading frame that encodes for a version of claudin-5 that is 303 amino acids in length, whereas the A allele encodes for the more typical 218 amino acid form of claudin-5. Based on immunoblot analysis of genotyped human lung tissue, only the 218 amino acid form of claudin-5 was detected, even in samples homozygous for the G allele of *CLDN5* [102]. A cDNA construct of the 303 amino acid form of claudin-5 can be forcibly expressed by transfected cells, however, it does not properly traffic to the plasma membrane and instead is retained in the endoplasmic reticulum. This suggests that in a disease state where the long form of claudin-5 is aberrantly expressed, it would have a pathological effect on the host cells by being trapped in the secretory pathway, perhaps by causing an unfolded protein response. It also suggests that caution should be used when using claudin-5 cDNAs for experimental purposes, since several vendors supply the 303 amino acid form in expression vectors.

Lung barrier function is due to the concerted effects of pulmonary epithelial and microvascular tight junctions [36, 103–105]. Claudin-5 expression by the

pulmonary microvasculature is high, making it difficult to detect in other cells in histologic sections, for example, alveolar epithelial cells [78, 91]. Nonetheless, claudin-5 is clearly detected in isolated fetal human alveolar epithelial cells [77] and rodent alveolar epithelial cells [38, 106–108]. Claudin-5 is also expressed by human airway epithelial cells [18, 40, 41, 109].

In contrast with a role for claudin-5 in promoting endothelial barriers, there is a body of evidence that increased claudin-5 impairs lung epithelial barrier function [107–112]. This is a key point underscoring the importance of the context of expression, where claudin-5 in endothelial tight junctions interacts with a different cohort of claudins than claudin-5 expressed by epithelial cells.

Increased lung epithelial claudin-5 is associated with a condition known as alcohol use disorder (AUD) associated lung syndrome, where a baseline deficit in alveolar epithelial barrier function leads to increased morbidity and mortality in response to acute respiratory distress syndrome (ARDS) [113–116]. At baseline, patients with AUD-associated lung syndrome have increased paracellular permeability, however, this increased leak is compensated by increased sodium-driven fluid efflux, due to the increased function of ENaC and Na/K-ATPase [34]. However, in response to a second insult, such as sepsis, pneumonia, toxicant exposure, or ventilator-induced lung injury, there is a catastrophic failure of the alveolar barrier in the AUD lung which is then unable to further upregulate sodium transport [117, 118], leading to alveolar flooding in the most severe cases.

Using cultured primary alveolar epithelial cells, it was shown that increased claudin-5 expression was necessary and sufficient to increase alveolar paracellular permeability [112]. Specifically, using normal alveolar epithelial cells transduced to express increased claudin-5 caused increased paracellular leak. In addition, alveolar epithelial cells isolated from alcohol fed rodents transduced with claudin-5-targeted shRNA had decreased paracellular leak [112]. This was revealed to be due to an interaction between claudin-5 and claudin-18. Normally, claudin-18 interacts with ZO-1, as well as other scaffold proteins, to assemble into tight junctions that form a high resistance barrier. However, in the presence of high levels of claudin-5, claudin-18 dissociates from ZO-1 and becomes reorganized into structures called tight junction spikes [119] (Fig. 2). Critically, claudin-5/claudin-18 interactions can be

Fig. 2 (continued) supports that were either untreated controls (**a**, **c**) or transduced with adenovirus encoding untagged claudin-5 (**b**, **d**) were processed for immunofluorescence analysis of claudin-5 (**a**, **b**) or claudin-18 (**c**, **d**). Bar – 20 μm. Cells transduced with claudin-5 showed a significant increase in tight junction spikes containing claudin-18 (* $p < 0.001$ vs. control; # $p < 0.001$ vs. EGFP transduced AECs, $n = 9$ fields from two independent experiments, one way ANOVA with Tukey multiple comparisons test) (**e**). (**f**) AEC barrier function was decreased by increased expression of untagged claudin-5, as assessed by transepithelial resistance (TER) (* $p = 0.0011$ vs. control AECs; # $p = 0.0002$ vs. EGFP transduced AECs, $n = 6$, one way ANOVA with Tukey multiple comparisons test). All quantitative data represents average ± SEM. Reproduced from [112] with permission under Creative Commons License CC BY 4.0. (**g**) Model for the impact of claudin-5 (blue) on claudin-18 (gold). In a healthy alveolar epithelium, tight junctions are composed largely of claudin-18 that engages ZO-1 (red ovals) and other scaffold proteins. Claudin-5 interacts with claudin-18, which changes its ability to interact with scaffold proteins, modeled here by a change in the conformation of the C terminal domain

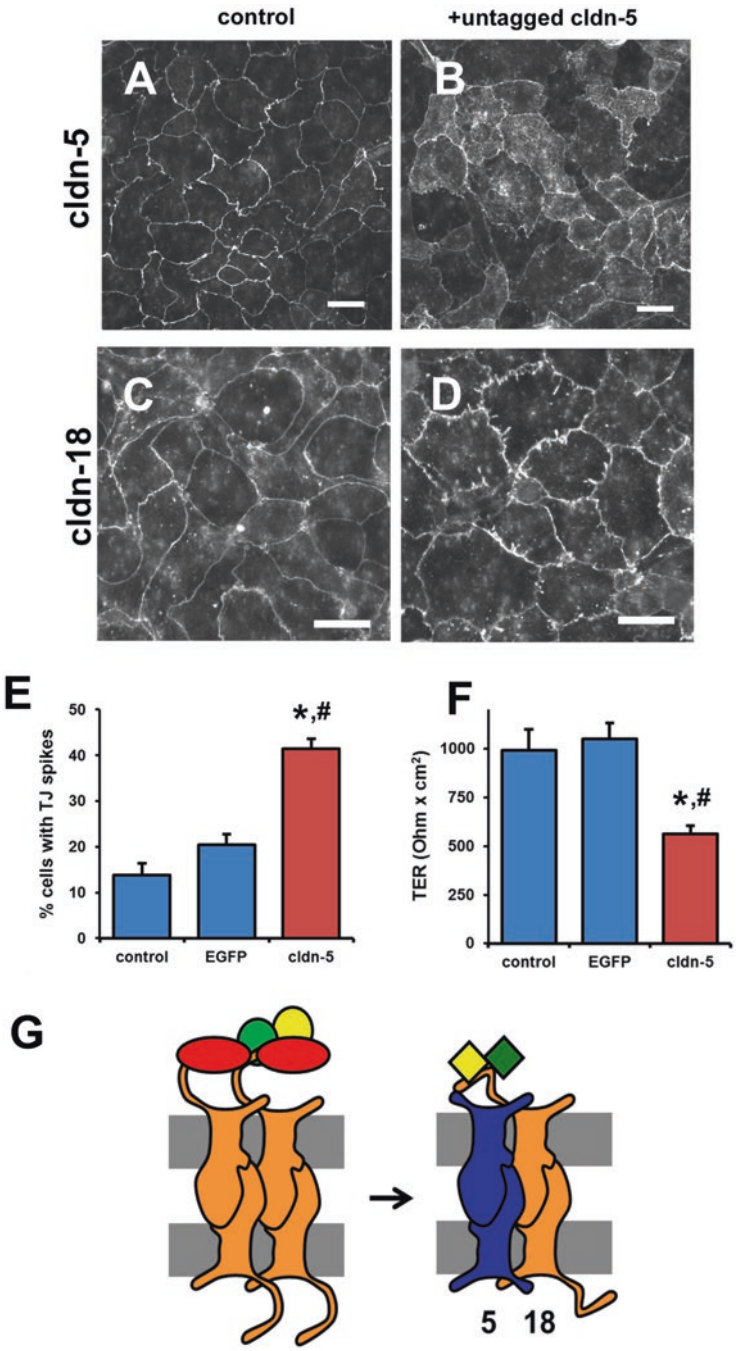

Fig. 2 Increased expression of untagged claudin-5 induces formation of tight junction spikes and impairs alveolar barrier function. (**a–d**) Primary rat alveolar epithelial cells on Transwell permeable

inhibited by a claudin-5 peptide mimetic, reducing spike formation and increasing alveolar barrier function, suggesting a potential therapeutic approach to treat AUD-associated lung syndrome and, perhaps, ARDS in general [112].

Tight junction spikes are not sites of increased permeability, but instead are composed of an asymmetric configuration of adherens junction proteins, dynamin-2, and actin that induce tension to cause stress on opposing tight junctions, increasing barrier leak [111]. Tight junction spikes may also serve as a signaling hub, where specific claudins nucleate the creation of signaling hubs, for example, claudin-18 recruitment of the transcription factor YAP [120]. In this model, tight junction spikes would be the alveolar counterpart to the tubulobulbar complex found in seminiferous tubules that is also enriched for dynamin, actin-binding proteins, and that helps regulate calcium-mediated signaling [121]. Further work is needed to confirm whether this is the case.

Other Claudins

In lung biopsies from patients with idiopathic pulmonary fibrosis (IPF), it was found that there was a specific increase in ***claudin-2*** that was accompanied by a decrease in claudin-4 [122]. This parallels changes in claudin expression that occur in inflammatory bowel disease [123], suggesting that inflammation is a driver of lung fibrosis. Since claudin-2 is pore forming, another implication of increased expression in IPF is that it would cause a deficit in lung barrier function through increased paracellular permeability. Claudin-18 was also found to be decreased in IPF, which would be expected to contribute to aberrant lung repair as well as impaired barrier function.

By expression profiling, Kielgast et al. found that ***claudin-8*** was expressed throughout the conducting airways, but was absent from the alveolar epithelium [124]. They found that stimulation of primary airway cells *in vitro* with the glucocorticoid dexamethasone resulted in an increase in barrier function that correlated with an increase in claudin-8 expression at the mRNA and protein levels [124]. Since claudin-8 has been shown to promote assembly of claudin-4 into tight junctions [66], this effect is likely to require both claudins, given the prominent role for claudin-4 in lung physiology described above. Interestingly, they found that claudin-8 depletion also impaired the ability to recruit occludin to tight junctions, another protein associated with the regulation of barrier function [125]. These findings that dexamethasone can enhance barrier function through regulation of claudin-8 have particular significance related to its mechanism of action, which complements its function as an anti-inflammatory agent used to treat chronic lung diseases associated with pulmonary edema, such as ARDS.

Claudin-10b is specifically expressed by club cells [126], but functional roles for this claudin remain elusive, as most studies have used it as a club cell marker [127,

128] rather than focus on roles for claudin-10 in regulating club cell barrier function. Interestingly, claudin-10b is one of several splicing isoforms that can serve as a pore-forming claudin [129]. Given the importance of club cells in regulating immunity at the interface between the conducting and respiratory airways [130], it is tempting to speculate that claudin-10b helps transmit cytokine signals across the epithelial barrier. Whether this is the case remains to be determined.

Summary

Epithelial and endothelial diversity requires a wide range of claudins that are differentially expressed to fine-tune and regulate the tight junction paracellular barrier. Different parts of the respiratory tree have different requirements to maintain fluid balance and an air-liquid interface, which is reflected by differences in claudin expression. Several disease states impair lung barrier function and frequently, this is the result of specific changes in claudin expression although there are also multiple examples where tight junctions are completely disassembled.

Transgenic mice lacking either claudin-18 or claudin-4 remain viable and effectively compensate for deficits in barrier function in unstressed animals. In many respects this kind of redundancy is not surprising, given the crucial need for the lung to maintain an air-liquid barrier. Given the impact of increased claudin-5 expression on claudin-18 and alveolar tight junction permeability in alcoholic lung syndrome, it may well be that dominant negative effects may be especially critical in the pathogenesis of pulmonary disease. Thus, claudin inhibitors and activators both need to be considered when considering approaches to maintain or improve lung barrier function.

In addition to their role as barrier-forming proteins, claudins are also being appreciated as playing roles in gene expression, an area that has been especially informed by the cancer literature, where aberrant claudin expression is associated with loss of control of cell proliferation and migration, processes that are also necessary for wound repair. This highlights the potential for claudins as key factors that organize signaling hubs, much in the same way that adherens junctions sequester transcription factors at sites of cell-cell contact. The regulation of lung repair by claudin-18/YAP interactions is a particularly clear example of how a claudin can serve in this capacity. Future work defining the claudin protein interacting proteomes and how they are influenced by different disease states is needed to better determine how claudins influence cell behavior beyond forming selective barriers.

Acknowledgments The section “Multiplicity of epithelial cells lining the respiratory tract” is excerpted from [131] with permission.

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Blood-Retinal Barrier Development and Potential for Regeneration in Blinding Eye Disease

Mónica Díaz-Coránguez and David A. Antonetti

Abstract The blood-retinal barrier (BRB) creates the defined retinal environment necessary for neuronal signaling and vision. The well-developed tight junctions in the vascular endothelium and pigmented epithelium control transport to the neural parenchyma protecting the neural tissue from potential blood-borne toxicity. Retinal endothelial cells are intimately linked to astrocytes, mural cells including pericytes, neurons, and microglia that collectively promote differentiation of endothelium to the BRB. Loss of the BRB contributes to the pathophysiology of several retinal diseases including diabetic retinopathy, central retinal vein occlusion, retinopathy of prematurity, and age-related macular degeneration. This chapter will detail the current understanding of the development of the BRB focusing on the vascular component, describe the alterations to this barrier in blinding eye diseases, and explore how loss of the barrier contributes to loss of retinal function. Finally, this chapter will address the potential for regenerative therapies targeting restoration of the BRB and vascular function.

Keywords Blood-retinal barrier · Retinal capillaries · Endothelial junctions · Barriergenesis · Retinopathy

Blood-Retinal Barrier

The retina represents a specialized component of the central nervous system and, like the brain, it has a highly differentiated barrier of the blood vessels to allow proper neuronal function. In humans, the constant metabolic demand of the retina

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L. González-Mariscal (ed.), *Tight Junctions*,
https://doi.org/10.1007/978-3-030-97204-2_11

requires an oxygen consumption of up to 8% of the total basal metabolic rate [1, 2]. Indeed, mammals are the only animals with vascularized retina. This high metabolic demand is addressed by three capillary layers in the retina including one in the ganglion layer called the inner capillary plexus and two deeper layers, one on the anterior side of the inner nuclear layer called the intermediate capillary plexus, and one on the posterior side of the inner nuclear layer called the outer capillary plexus (Fig. 1). The retinal capillaries that supply the inner neural retina make up the inner blood-retinal barrier (iBRB), while the retinal pigment epithelium makes up the outer blood-retinal barrier (oBRB), which controls exchange of nutrients and gases between the outer retina and choroidal vasculature.

The iBRB constitutes a highly specialized structure with similar properties to the vascular component of the blood-brain barrier (BBB). Retinal endothelial cells are connected by tight junction proteins, and vesicular transport is limited. The retinal vessels have limited fenestrae and are intimately linked to astrocytes, mural cells including pericytes, neurons, and microglia. Collectively, these components establish an anatomical and functional structure referred to as the neurovascular unit (NVU) (Fig. 2.). Reciprocal interactions between vascular and neuroglial components allow proper neural function. During development, these NVU interactions regulate the angiogenesis and confer unique vascular barrier properties. However, loss of the BRB contributes to the pathophysiology of a number of retinal diseases including diabetic retinopathy [3], central retinal vein occlusions [4, 5], retinopathy of prematurity [6], and age-related macular degeneration [7]. Understanding the

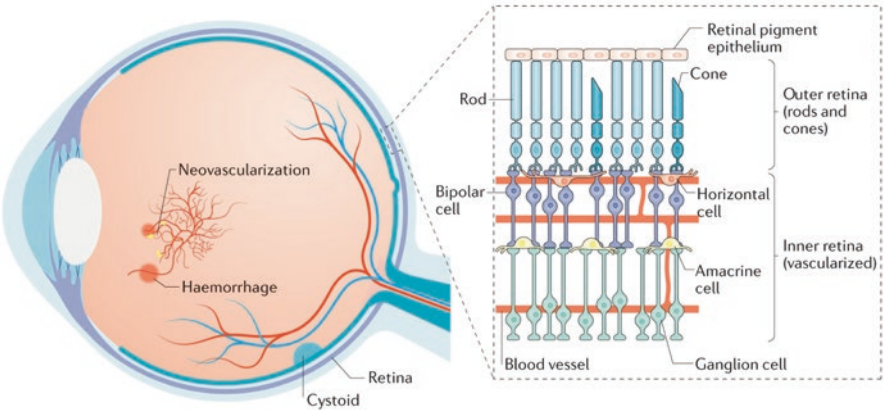

Fig. 1 Schematic diagram of the human eye. The retinal capillaries that supply the inner neural retina and make up the blood-retinal barrier emerge from the optic disk and are distributed into three capillary layers in the retina including the ganglion layer (inner capillary plexus) and on either side of the inner nuclear layer (intermediate and deep capillary plexus). Loss of the BRB contributes to the pathophysiology of several retinal diseases including diabetic retinopathy, which may include vessel leakage and cystoid formation leading to DME, as well as neovascularization as indicated. Material from: David A. Antonetti et al. Current understanding of the molecular and cellular pathology of diabetic retinopathy. Nature Reviews Endocrinology. Springer Nature. Jan 19, 2021

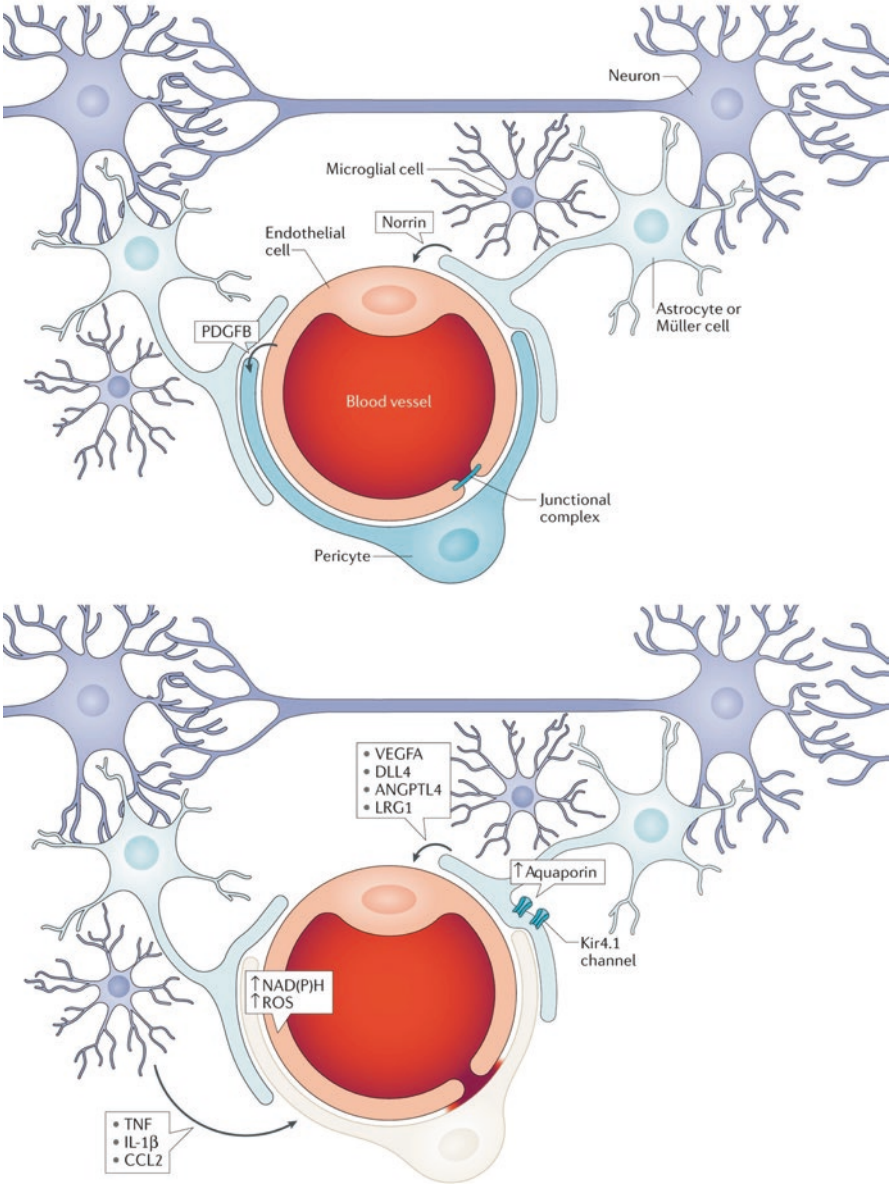

Fig. 2 The neurovascular unit and disruption of the blood-retinal barrier. The neurovascular unit is formed by retinal vessels, linked to astrocytes, mural cells (pericytes), basal lamina, and extracellular matrix, in association with neurons and microglia. Norrin is secreted by glia, while PDGFB is released from endothelial cells to promote recruitment of pericytes necessary for proper barrier formation. Collectively, these components promote tight junction complex formation and confer unique barrier properties to the retinal endothelium. Loss of the blood-retinal barrier contributes to the pathophysiology of several retinal diseases including diabetic retinopathy, in which aquaporin and Kir4.1 channel have been found elevated. In addition, vascular endothelial growth factor A

(continued)

specific cell signaling interactions of the BRB is therefore crucial to the development of restorative therapies for retinal vascular diseases.

This chapter will detail the current understanding of the development of the BRB, describe the alterations to this barrier in various blinding eye diseases, and explore how loss of the barrier contributes to loss of retinal function. Finally, this chapter will address the potential for regenerative therapies targeting restoration of the iBRB and vascular function.

Flux Across the BRB and Vascular Permeability

The iBRB regulates the transport of ions, water, solutes, and cells across the vascular bed. Flux of a solute or water describes the net movement over time across a defined area of the vascular endothelium, while permeability to a specified molecule describes the properties of the vessel wall allowing that flux, and may be given in cm/sec. Changes in permeability may occur through changes in transport through the cells, broadly termed transcytosis, or through changes in the junctional complexes connecting cells, leading to flux around the cells or paracellular permeability. Further, diffusion may drive paracellular flux of solutes or the solutes may be carried by fluid, called convection. Fluid flux is driven by both hydrostatic pressure and osmosis. Both transcellular and paracellular routes are composed of multiple specific pathways that may act simultaneously and are not mutually exclusive. These pathways collectively contribute to permeability for a given molecule across the vascular wall and are tightly controlled in the retina.

Transcytosis

Transcellular transport across retinal endothelial cells is necessary for regulation of the retinal environment's homeostasis [8]. There are a variety of pathways for this route. Small lipophilic molecules are able to passively diffuse along the retinal endothelial membrane, while larger lipophilic molecules and hydrophilic molecules require ATP-dependent processes, including receptor-mediated vesicular transport, non–receptor-mediated pinocytosis, transporters, and pumps that mediate transcytosis [9].

Fig.2 (continued) (VEGFA), delta-like ligand 4 (Dll4), angiopoietin-like 4 (ANGPTL4) and leucine-rich α2-glycoprotein 1 (LRG1) are secreted and promote permeability, angiogenesis, or both. Loss of pericytes increases endothelial cell sensitivity to VEGF signaling. Furthermore, microglia and inflammatory cells release cytokines including tumor necrosis factor (TNF), interleukin (IL) 1β, and CC-chemokine ligand 2 (CCL2), among many others. The hyperglycemic environment causes direct endothelial dysfunction through a change in redox state (NAD(P)H and reactive oxygen species (ROS)). Material from: David A. Antonetti et al. Current understanding of the molecular and cellular pathology of diabetic retinopathy. Nature Reviews Endocrinology. Springer Nature. Jan 19, 2021

Retinal endothelial cells selectively regulate the transcellular movement of molecules from the blood to the neural tissue by controlling the expression of molecules at both luminal and abluminal sides. Retinal endothelial cells express a low number of receptors, transporters, and vesicles, in combination with a high expression of efflux pumps [10], which collectively contribute to the BRB properties.

Evidence has been provided for caveolae in regulating transcytosis [11–13]. These vesicles appear as an electron-dense structure by electron microscopy due to the lipid rafts enriched in glycosphingolipids, sphingomyelin, cholesterol, and lipoproteins. Although caveolin-1 is the main component of caveolae, these vesicles are also covered by cavin [14–16]. Moreover, they contain receptors for the following: transferrin, insulin, albumin, advanced glycation end products, low- and high-density lipoprotein cholesterol (LDL and HDL, respectively), interleukin 1, endothelin, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and transforming growth factor-beta (TGF-β), and signal transduction molecules including small G-proteins, mitogen-activated protein kinases (MAPK), Src kinases, Raf, and protein kinase C (PKC) [17, 18].

More recently, clear evidence for a role for plasmalemma-vesicle-associated protein (PLVAP), also known as PAL-E antigen or PV-1, in transcytosis, has emerged. PLVAP was identified as the ligand of the human endothelium-specific monoclonal antibody APL-E [19, 20] and the mouse-specific monoclonal MECA-32 [21]. Its expression in retinal endothelial cells is absent or low, correlating with the low number of vesicles [22]. However, in pathologies where the barrier is lost, PLVAP expression increases specifically in capillaries with vascular leakage [23–25], supporting PLVAP expression as an indicator of transcytosis-mediated vascular leakiness.

Conversely, the major facilitator superfamily domain containing 2a (Mfsd2a) is a negative regulator of transcytosis. It is a sodium-dependent transporter to lysophosphatidylcholine (LPC) containing docosahexaenoic acid (DHA), one of the omega-3 fatty acids essential for brain growth and neuronal function [26]. Gene deletion of *Mfsd2a* in mice results in leakiness at the brain capillaries, accompanied with low levels of LPC-DHA and neuronal cell loss, cognitive deficits, and microcephaly. The specific mechanisms activated by Mfsd2a controlling transcytosis are still unknown; nevertheless, electron microscopy studies of Mfsd2a knockout mice show an increased vesicle transport without affecting tight junctions. Moreover, in animal models of pericyte loss, Mfsd2a is downregulated specifically at the endothelial cells uncovered by pericytes [27]. Together, these studies suggest that pericytes are required for Mfsd2a expression in endothelial cells and that Mfsd2a reduces transcytosis and increases LPC-DHA uptake.

Paracellular Transport

In addition to transcellular transport, molecules can move via the paracellular transport route, which occurs through the intercellular space between adjacent cells. The formation of junctional complexes opposes paracellular transport and may regulate

this process and consist of tight junctions, adherens junctions, gap junctions, and desmosomes, with tight and adherens junctions contributing a central role in barrier regulation.

Tight Junction

The remarkably tight control of solute and fluid flux across the BRB is conferred by well-developed tight junctions. Tissues with high barrier properties demonstrate complex and well-developed tight junctions as evidenced by high density of branch points on tight junction strands in scanning electron microscopic images [28]. In ultrathin sections, tight junctions appear as contact points of close apposition, or "kissing points," where the two lipid bilayers become indistinguishable. In endothelial cells, these are located at several points along the paracellular space between cells as well as contacts within a cell forming the capillary lumen [29].

Tight junctions serve two main functions: gate function, which restricts the passage of molecules through the paracellular space, and fence function, which confers cell polarity by restricting the movement of lipids and proteins between the apical and basolateral plasma membrane [30, 31]. However, more recent research reveals roles of tight junction proteins in several cell-signaling processes such as cell proliferation, and differentiation [32–34] potentially linking barrier formation with these fundamental cell processes. Both these traditional roles and new studies will be highlighted throughout this chapter.

At a molecular level, the tight junctions consist of over 40 proteins, which can be categorized into transmembrane proteins, including the tetraspan families of claudin and MARVEL (MAL and related proteins for vesicle trafficking and membrane link) proteins, and the single span proteins from the junctional adhesion molecules (JAM) family; and cytoplasmic scaffold proteins, including members of the membrane-associated guanylate kinase homologue (MAGUK) family like zonula occludens (ZO), and other membrane-associated proteins such as afadin (AF6) and cingulin [35, 36]. While a great number of studies have focused on the role of these tight junction proteins in epithelial cells, there are more limited studies demonstrating direct function in the BRB. The following section highlights the role of specific tight junction proteins that have been examined in relation to the BRB.

Claudins

The claudin family are tetraspan transmembrane proteins that play a crucial role in paracellular transport by forming ion-selective barriers and pores. In the retina, claudins are regulated during development. From the 27 known mammalian claudin members [37], mouse retinas from the postnatal day 15 (P15) show a strong mRNA expression of claudin-1, claudin-2, claudin-3, claudin-4, claudin-5, claudin-12, claudin-22, and claudin-23, coincident with the formation of the BRB in retinal capillaries [38]. After this, their expression decreases significantly except for

claudin-22. However, from these isoforms, only claudin-1, claudin-2, and claudin-5 are detectable by immunostaining at retinal vessels by P18 when the barrier is completely mature. The expression of these claudins in adult mice and the specific role of claudins in retinal development have not been fully determined, and the role of only a limited number of claudins has been defined.

Like in the brain, claudin-5 is highly abundant and localizes at the tight junctions of retinal vessels. Claudin-5-deficient mice die within 10 h after birth due to BBB disruption. These mice show no abnormal development or morphology of blood vessels and no abnormal bleeding or edema but do present a leaky BBB toward small molecules <800 Da [39]. Ongoing studies focusing on the BRB in pathologies that model diabetes, ischemia, and strokes have identified a loss of claudin-5 associated with an increase in permeability [40, 41], thus supporting the important role of claudin-5 in BRB.

Additional claudin isoforms have been reported to be expressed in endothelial barriers. Although claudins-1 [42, 43], claudin-2 [44], claudin-3 [45], and claudin-12 [46] have been deleted in mice, no brain or retinal vascular phenotype has been described other than for claudin-5, potentially implying redundancy in function. Some studies suggest that claudin-1 is expressed in the retinal vasculature of adult mice, and its content decreases in streptozotocin-induced diabetic rats, a model of diabetes with destruction of insulin-producing beta cells of the pancreas [47], as well as in a model of experimental autoimmune uveoretinitis [48]. However, the specific expression of claudin isoforms in endothelial barriers, in addition to claudin-5, remains to be determined. Immunodetection of claudins in knockout mice models suggest cross-reactivity of claudin-1 antibodies with claudin-3 [49], while antibodies with less cross-reactivity fail to detect colocalization of endothelial markers with claudin-1, claudin-3, or claudin-12 isoforms in brain [45, 46] or with claudin-3 and claudin-12 in the retina [38].Thus, the role of specific claudin isoforms in BRB requires further investigation.

MARVEL Proteins

MARVEL (MAL and related proteins for vesicle trafficking and membrane link) proteins contain conserved sequence adjacent to the four-transmembrane regions that define the MARVEL domain and are characterized by their involvement in vesicle trafficking [50]. The tight-junction-associated MARVEL protein (TAMP) family consists of occludin (MARVELD1), tricellulin (MARVELD2), and MARVELD3 [51].

Occludin

Occludin (MARVELD1), a ~60kD transmembrane protein, was first identified in tight junctions from chick liver junctional fractions. Hydrophilicity plot and cDNA sequence analyses indicated that occludin is a tetraspan transmembrane protein that has two extracellular loops and cytoplasmic NH_2 and COOH termini [52, 53]. The

N-terminus of occludin interacts with Itch, E3-ubiquitin-protein ligase that regulates occludin degradation [54] and endocytosis [55]. The distal C-terminus of occludin forms a coiled-coil region that binds to the scaffold proteins ZO-1 [56], ZO-2 [57], and ZO-3 [58], specifically to the guanylate kinase-like (GUK) domain of ZO-1 [59].

Occludin knockout mice revealed that occludin is not required for tight junction formation or basal intestinal epithelia barrier properties [60, 61]. These animals have phenotypic alterations including growth retardation, thinning of compact bone, testicular atrophy, male infertility, loss of cytoplasmic granules in salivary epithelial cells, females are not able to lactate, brain calcification [61], and hyperproliferation of mucous epithelial cells in the intestinal lining [62]. In vitro, the silencing of occludin using siRNAs had a limited effect on barrier properties, with increases in permeability to divalent organic cations and to small molecules under hydrostatic pressure [63, 64]. In ARPE-19 cells, a human retinal pigmented epithelial cell line, loss of occludin increases the DNA synthesis rate and cell proliferation [64]. Moreover, during neurogenesis, occludin loss has been found in neural tubes of chicken and mouse embryos at E9 [65].

More recent studies show that occludin has a more complex function that includes the regulation of cell signaling, tight junction protein trafficking, and cell growth. In these studies, specific occludin phosphosite changes and their role in barrier regulation and cell signaling have been identified [66, 67]. For example, PKCη-induced phosphorylation of occludin at T403 and T404 residues enhances tight junction assembly and maintenance in epithelial cells [68]. In contrast, phosphorylation of occludin at Y398 and Y402 by c-Src [69], or at S408 by casein kinase 2 (CK2), causes its dissociation from ZO-1, leading to tight junction destabilization. In the case of S408, this is due to an increased occludin-occludin interaction that results in small cation paracellular flux via claudin-1- and claudin-2-based pores [70]. Moreover, occludin phosphorylation on S490 in response to VEGF regulates occludin ubiquitination, endocytosis of occludin and other tight junction proteins, and endothelial permeability in cell culture [41, 55, 71] and in vivo [72]. Collectively, research from a variety of laboratories reveals that posttranslational modifications of occludin, including multiple phosphorylation sites and ubiquitination, regulate barrier properties associated with tight junction protein endocytosis. However, future research is needed to identify the molecular mechanisms by which occludin regulates barrier properties and junctional endocytosis.

Recent work further reveals an additional role of occludin in cell growth regulation. During mitosis, occludin phosphorylated at S490 localizes in centrosomes and the mutation of S490A mutant retarded MDCK cell growth and prevented proliferation and tube formation in endothelial cells in culture [73, 74]. A second phosphorylation site, also located in the coiled-coil domain of occludin, also contributes to proliferation and tight junction formation. It has been found that MDCK epithelial cells undergo postcontact proliferation necessary for proper epithelial maturation and tight junction formation. Inhibition of occludin Ser471 phosphorylation by either an alanine mutation or inhibition of the G-protein-regulated kinase that targets this site prevents this postcontact proliferation and subsequent epithelial

maturation and blocks tight junction formation with no effect on adherens junction formation [75].

Other studies support a role of occludin in proliferation and interaction with centrosomal proteins. *OCLN* mutations in human patients can lead to microcephaly and band-like calcifications with polymicrogyria characterized by loss of cortical convolutions, shallow or absent sulci, and multiple small gyri, giving the cortex surface a roughened irregular appearance [76–81]**.** Primary microcephaly (MCPH, for microcephaly primary hereditary) is a brain development disorder that results in abnormal head circumference more than 3 standard deviations below the mean for age and gender. Notably, many of the causative genes for MCPH encode centrosomal proteins involved in centriole biogenesis [82]. To date, 13 pathogenic mutations in occludin have been identified in 13 families and 7 mutations are situated in exon 3 [78–81].

Recently, a new isoform of occludin was discovered by Bendriem et al. with a specific function in neural progenitors that alter cortex size in the developing mouse brain [83]. The original occludin knockout mouse line was generated by excising exon 3 (Fig. 3) and was believed to be a null model. While mouse full-length occludin (mOCLN-FL) is no longer expressed, a truncated form that lacks its N-terminus and the first three of occludin's four transmembrane domains (mOCLN-ΔN) is still expressed. This 32–34 kDa protein results from a shorter ΔN transcript lacking exons 2 and 3. Both mOCLN-FL and mOCLN-ΔN isoforms localize to the centrosomes; however, in the homozygous mutant mouse line $Ocln^{\Delta N/\Delta N}$, mOCLN-ΔN only localizes to interphase and mitotic centrosomes in the embryonic mouse cortex but not at the plasma membrane, suggesting the C-terminal domain of occludin is important for this centrosomal localization. Consistent with occludin mutation in patients with microcephaly, depletion of full length occludin in mice led to a reduced brain size. An increased mitotic index, potentially indicating prolonged mitosis, was found in the $Ocln^{\Delta N/\Delta N}$ mutant mice along with a higher percentage of cells in $Ocln^{\Delta N/\Delta N}$ E12.5 cortices in prometaphase and metaphase compared to wild type. Moreover, a higher percentage of activated (cleaved) caspase 3 (CC3)-positive apoptotic cells in mutant embryos was observed compared to controls, prior to E14.5, potentially due to alterations in mitosis.

Occludin centrosomal localization was also confirmed in vitro in two hESCs lines that closely resemble the $Ocln^{\Delta N/\Delta N}$ mouse mutant. Mutant hESC-derived organoids displayed pronounced proliferation defects, premature differentiation, and apoptosis. Cells with a reduced ratio of basal neural progenitor marker HOPX (homeodomain-only protein homeobox), compared to early neuronal marker NeuroD1 (HOPX+/NeuroD1+), may be responsible for the reduced size of human organoids. It is important to note that centrosomal occludin colocalized and immunoprecipitated with mitotic spindle proteins NuMA and small GTPase RAN, two important proteins in mitotic spindle assembly and stabilization and mutant occludin hESCs exhibited impaired mitotic spindles and abnormal morphology at the spindle poles. These studies demonstrated an important role for occludin in neurogenesis through its centrosomal interaction and promotion of proper functioning neural progenitor mitotic spindles.

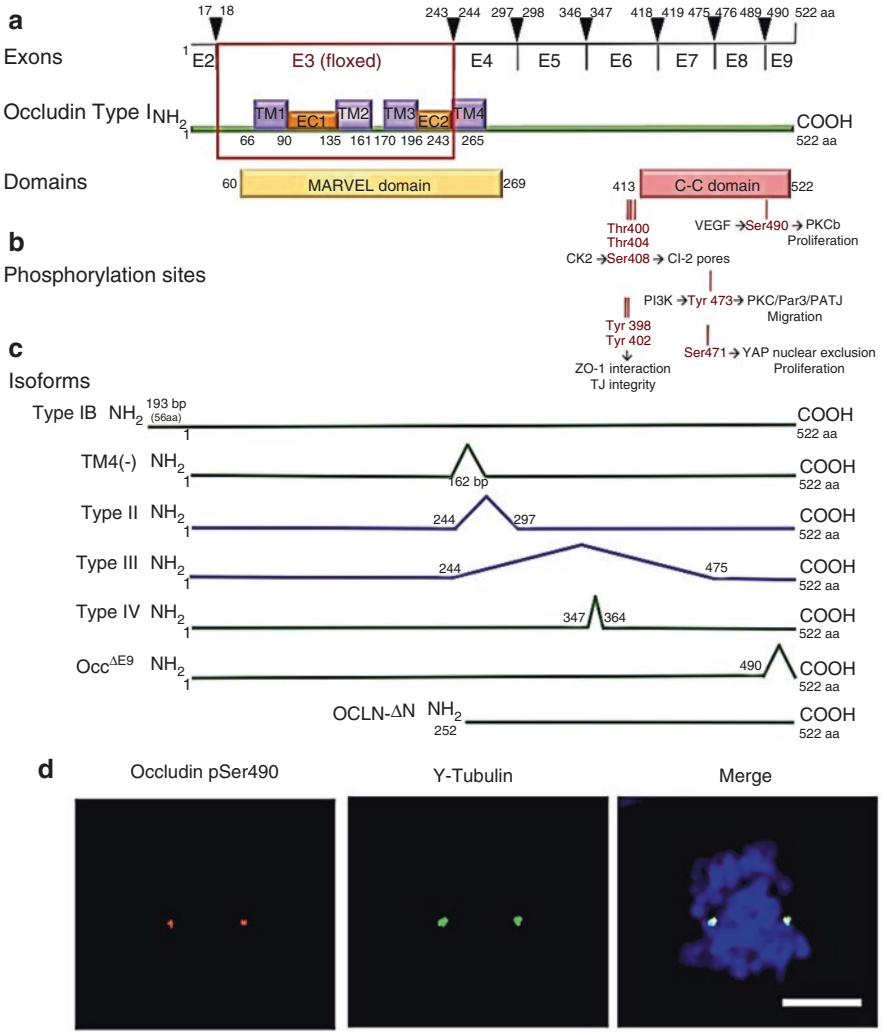

Fig. 3 Occludin structure and isoforms. Occludin is a 522 amino acids protein encoded by 9 exons (**a**). Occludin full length (type I) possesses four transmembrane (TM) domains and two extracellular (EC) loops with the MARVEL domain as homology on the cytoplasmic side after each TM region. At the C-terminus (COOH) of occludin, a coiled-coiled (C-C) domain can be phosphorylated at multiple sites (**b**). Phosphorylation of occludin identified and known or implied functions. Occludin can mediate proliferation through the phosphorylation of two sites: Ser471, which regulates postcontact proliferation in epithelial cells, and Ser490, which is promoted by VEGF-induced PKCβ activation and regulates both endothelial permeability and neovascularization. To date, several occludin isoforms have been described (**c**). The function of each isoform has not been fully elucidated, but most isoforms localize to the junctions except type II and III (blue lines). Interestingly, occludin deleted in exon 9 (OccΔE9) restricts cell migration. (**d**) In bovine retinal endothelial cells, occludin stained with a pS490-specific antibody (red) shows colocalization of phospho-occludin with the centrosome marker γ-tubulin (green) in pro-metaphase. Hoechst dye (blue). Scale bar = 5 μm. Material from: Díaz-Coránguez M, Liu X, Antonetti DA. Tight Junctions in Cell Proliferation. *International Journal of Molecular Sciences*. 2019; 20(23):5972. https://doi.org/10.3390/ijms20235972

Along with occludin localization at the centrosomes, studies have also shown that intracellular occludin-containing vesicles move along microtubules (MTs) and contribute to the regulation of cell proliferation [84]. MTs interacting with plasma membranes participate in the preservation of epithelial tight junction structure and function. This is regulated by the binding of MT plus-end-tracking proteins at the scaffold in the adherens junctions. They may also be achieved through microtubule minus end binding of nezha/calmodulin-regulated spectrin-associated protein (CAMSAP) and ninein to the adherens junctions [85–89]. The junctional localization of several centrosome-associated proteins suggests a crucial role of junctions as sites that orchestrate MT organization in polarized cells [90]. Glotfelty et al. reported that intracellular occludin-containing vesicles move along MTs and that the rate of movement depends on intact microtubule networks. This suggests a requirement for the dynein minus-end motor dynein. Consistent with this hypothesis, siRNA knockdown of dynein/dynactin induced occludin accumulation in the cytosol, whereas plus-end motor kinesin knockdown did not [84]. This model of microtubule-dependent tight junction trafficking was further supported by the results from the studies on dynein and Rab11 [91, 92]. Rab11 utilizes MTs for trafficking and has been shown to participate in occludin trafficking through the regulation of the Rab11 FIPs (Rab11 family interacting protein) [92].

Occludin-containing vesicles may traffic bidirectionally at MTs to regulate junction organization and cell proliferation. Previous studies suggest that trafficking along microtubules likely contribute to tight junction assembly by delivery of tight junction proteins through MT-associated vesicles. Particularly occludin-containing granules have been found near the tip of oolemma ingression in dividing *Xenopus* oocytes [93]. In epithelial cells, Rab13 or junction rab (JRab) is a key mediator of the endocytic recycling of occludin [94] through its binding partners, Rab13-binding protein and CasL-like 2 (MICAL-L2) [95, 96]. VAP-33 (VAMP-associated protein of 33 kDa), which is involved in vesicle docking/fusion, binds to occludin and overexpression promotes occludin movement along the lateral edge of the plasma membrane [97]. Identification and characterization of a homolog of VAP-33 in *Drosophila* (DVAP-33A) revealed this protein regulates the division of boutons at the synaptic terminals by stabilizing and directing the microtubule cytoskeleton during budding [98]. Thus, occludin trafficking along microtubules contributes an important role in both cell proliferation and junction organization.

Together these data reveal the complex role of occludin in barrier formation and permeability regulation, cell growth, and angiogenesis. Further, the studies point to an intimate relationship in growth and differentiation of cells that form tight junctions and a specific role for occludin in regulating these processes. The role for occludin trafficking in the blood-brain and blood-retinal barriers and vascular growth control will need to be further established as a number of these studies were conducted in other cell types. However, the transgenic point mutant of occludin regulating BRB properties in vivo establishes the essential role of this protein in vascular barrier control. Future studies will need to clarify the interaction of occludin with dynein and kinesin motors that regulate microtubule trafficking.

MARVELD2 and D3

Tricellulin (MARVELD2) is a ~ 64 kDa transmembrane protein localized primarily at regions where 3 cells converge [99, 100]. Like occludin, tricellulin has a long C-terminus tail, but they only share about a 32% homology. Interestingly, both tricellulin-deficient mice and occludin-deficient mice develop hearing loss [101–103]. A majority of the studies on tricellulin have been on epithelial cells, and research has suggested that tricellulin is only present in endothelial cells of the BRB but not in vascular beds of other tissues [99]. This observation suggests a unique role for tricellulin paracellular barrier properties of the BRB.

MARVELD3 is the third member of the MARVEL family and is also a tetraspan protein but lacks the carboxyl tail found in occludin and tricellulin [104]. The role of MARVELD3 in BRB remains unclear. However, MARVELD3 has been shown to contribute to regulation of the MEKK1-c-Jun NH_2-terminal kinase pathway and control of proliferation in epithelial cells [105]. Understanding whether MARVELD3 controls vascular cell signaling may likewise provide important insight into regulation of vascular growth and differentiation control.

Junctional Adhesion Molecules (JAM)

JAMs are single-span proteins that belong to the immunoglobulin superfamily, because they contain at least one IgG domain at its extracellular N-terminus [106, 107]. In the retinal endothelium, JAM-A is the predominant JAM isoform [108, 109]. The exact role of JAMs in the regulation of endothelial barriers is still unclear; however, studies demonstrate JAM plays a key role in diapedesis. JAM-A expression on monocytes facilitates its movement across the BBB [110], while JAM-A knockout and endothelial-specific JAM-deficient mice prevent neutrophil transmigration [111]. JAM-C global knockout mice showed increased JAM-A expression in the retina [112]. Mice pups with JAM-C endothelial-specific depletion, when exposed to hyperoxia during retinal vascular development (oxygen-induced retinopathy model), showed 50% more retinal vascularization coverage by P17, as compared with their littermates [113]. This was due to an increase in the number of endothelial sprouts, which also increased when JAM-C was knocked down using specific siRNAs in human retinal microvascular endothelial cells (HRMEC), suggesting a yet uncharacterized role for JAM in retinal angiogenesis.

These data indicate that JAMs contribute to several mechanisms including cell migration, immune cell infiltration, and angiogenesis in the BRB. However, further studies are clearly needed to determine the exact role of the different JAMs in the BRB formation and maintenance.

Zonula Occludens (ZO)

ZO are large >200 kDa scaffold proteins that connect transmembrane proteins with the cytoskeleton and contribute an important role in tight junction organization [58, 114, 115]. ZO-1-deficient mice are embryonic lethal and express defects in the

vasculature and neural tube development [116]. Several studies focusing on pathologies disrupting the BRB have revealed that loss/reduction of ZO-1 has a correlation with an increase in paracellular permeability [41, 117], thus supporting ZO-1 as a marker of leakiness in endothelial barriers. ZO-2 and ZO-3 are also expressed in BRB; however, their specific role in endothelial barriers has not been addressed yet.

Adherens Junctions

Adherens junctions play a critical role in cell-cell adhesion, cell polarity, contact inhibition, and paracellular transport regulation. The adherens junctions of the BRB include vascular endothelial (VE)-cadherin of the cadherin superfamily. VE-cadherin is a transmembrane Ca^{2+}-dependent cell adhesion protein with a conserved cytoplasmic tail that binds to β-catenin. In vivo studies of transgenic mice deficient of VE-cadherin, which is embryonic lethal by E9.5, have demonstrated that VE-cadherin is required for proper lumen formation and maintenance of newly formed vessels [118]. VE-Cadherin has an intimate relation to the vascular endothelial growth factor receptor (VEGFR) and is a major control point for regulation of barrier development, paracellular permeability, and growth control (for review, refer to: [36, 119, 120]).

Development of the BRB

The formation of the BRB requires the vascularization of the retina and differentiation of the retinal endothelium to the specialized BRB. Retinal vascularization occurs by two different mechanisms: (1) vasculogenesis, which refers to the maturation of progenitor cells to the primary vascular bed and (2) angiogenesis, which refers to the sprouting of vessels from the already existing vasculature. This process is followed by endothelial differentiation forming the barrier or barriergenesis.

Retinal Vascularization

The retinal vascularization occurs in three distinct phases. First, the cells from the optic artery migrate into the retina surface toward the periphery. Secondly, superficial vessels sprout into the deep outer plexiform layer, followed by the third phase wherein vessels spread into the inner plexiform layer, resulting in three distinct retinal layers. In mice, this process starts at P1, the superficial layer is covered by P8, the deep capillary layer is formed by P12, and the process is completed by P21 [121, 122]. In humans, this process starts around the week of gestation (WG) 14 and continues until the WG 32 [123].

VEGF signaling is crucial for vasculogenesis and angiogenesis. Deletion of a single allele of VEGFA is lethal in mice due to severe vascular abnormalities [124]. During retinal development, filopodia from tip cells extend toward VEGFA secreted by glia and neurons [125, 126]. The subsequent activation of the tyrosine kinase receptor VEGFR2 regulates endothelial migration in tip cells that signal through Notch, promoting the formation of proliferative stalk cells.

VEGFA responses in endothelial cells including proliferation and migration are driven by the activation of VEGR2 [127], with highest level of mRNA and protein found in tip cells [128]. Genetic studies of loss and gain of function of VEGFR1 demonstrate this receptor acts as a decoy, but additional studies suggest that VEGFR1 is a negative regulator of tip cell formation and arterial branching morphogenesis through signaling, since loss of VEGFR1 tyrosine kinase activity increased the number of tip cells in a Notch-dependent manner [129]. Although VEGFR3 is mainly expressed in lymphatic vasculature, it has been also found to be expressed in blood vessels during angiogenesis, controlling the anterior-venous remodeling of the primary vascular plexus in mice [130, 131] and the transition between tip-stalk cells by the activation of Notch signaling [132]. In the retina, VEGFR3 modulates vascular permeability and endothelial junction integrity by increasing VEGFR2 protein levels [133]. In vitro studies demonstrate that VEGFR3 can form heterodimers with VEGFR2 and this interaction promotes signaling through VEGFA and regulates angiogenic sprouting positively [134, 135]. Therefore, it is possible that VEGFR3 plays a role in the regulation of blood vessel growth by modulating VEGFR2-mediated signals. Nevertheless, further studies are required to elucidate the precise mechanism underlying the role of VEGFR3 in vascular growth.

Cell fate in vascular endothelial cells is controlled by Notch signaling [136–138]. There are 5 isoforms of Notch that can be activated by delta-like (Dll)-1 and delta-like-4 ligands and activated or antagonized by Jagged-1 and Jagged-2. In response to VEGF signal, endothelial cells that receive the highest VEGF concentration become tip cells and signal to neighboring cells through Dll4 to activate Notch. Upon activation, Notch is cleaved by the tumor-necrosis-factor-beta-converting enzyme and the gamma secretase complex to release the Notch intracellular domain (NICD). NICD acts as a transcription factor for mastermind-like coactivator proteins. Dll-4 stimulation of Notch signaling reduces but maintains VEGFR2 and leads to a proliferative stalk phenotype. In retinal endothelial cells, the transcription factor NF-E2-related factor 2 (Nrf2) promotes angiogenic sprouting by suppressing Dll4/Notch signaling [139] and as a consequence, tip cell markers, including VEGFR2, are overexpressed [140–142]. Formation of the deep and mid capillaries may be described by this VEGF and Notch signaling, but mechanisms of tube formation, branching, and anastomosis remain to be fully elucidated.

After capillary growth, the vasculature is remodeled by leukocytes through the induction of endothelial apoptosis in a Fas ligand (or CD18)-dependent process [143]. Pericytes contribute to vascular remodeling as well via endosialin (or CD248) [144]. Endosialin is a type I transmembrane glycoprotein whose expression in the retina is restricted to pericytes associated with newly forming vessels. Deletion of endosialin in mice causes increased vessel density during development due to

defects in endothelial cell apoptosis and detachment, without affecting pericyte recruitment. Thus, endothelial/pericyte cooperation controls vascular regression during development.

To protect neuronal function, the BRB properties are developed in parallel to retinal vascularization. Although the BRB of the contemporary vasculature is already established by P10 [145], the retinal capillaries are not fully mature until P18 [146]. Endothelial tip cells express high levels of PDGFB (Fig. 2a), thus inducing pericyte recruitment at the new vessels [147]. The pericytes wrap around the vessels, by connecting with endothelial cells through N-Cadherin [148], Conexin-43 [149], and fibronectin adhesion plaques [150]. Pericyte coverage of retinal vessels is required for proper formation of endothelial barrier function [151]. Studies in mice null of *Pdgfrb* gene, which are deficient in pericyte generation, demonstrate the requirement of pericytes to control vascular permeability. Deletion of the PDGFB retention motif, which ablated localization, led to low pericyte density in retinal vessels and promoted abnormal vessels [128]. Blocking PDGF receptor-β signaling using an antagonistic antibody showed similar vascular defects [152]. Importantly, deletion of pericytes in adult mice with targeted diphtherial toxin expression did not cause BRB impairment. However, endothelial cells become hyperresponsive to VEGFA-induced permeability. Uncovered endothelial cells showed increased expression of angiopoietin 2 (Ang-2) and VEGFR2 due to a high transcriptional activation of FOXO1, thus promoting elevated VEGFA signaling. Together, these data support the requirement of pericytes in BRB establishment and maintenance.

Pericyte-secreted factors maintain the integrity of the BRB by promoting the expression of tight junction proteins and reducing permeability [153]. Angiopoietin-1 (Ang-1) is secreted by pericytes and binds to Tie-2 receptor on retinal endothelial cells, promoting maturation and barrier properties [154–156]. Expression of Ang-2 antagonizes Ang-1 signaling to the Tie2 receptor, as well as potentially activates autocrine signals in endothelial cells. Together with VEGF, Ang-2 promote angiogenesis. Interestingly, Ang-2 alone promotes vascular regression [157]. Ang-2 promotes loss of endothelial barrier function, by the induction of VE-Cadherin phosphorylation and degradation [158], while Ang-1 prevents the degradation of the junction complex [159–161]. In summary, Ang-1 and Ang-2 have opposing effects in BRB properties with Ang-1 promoting patent BRB.

Barriergenesis

Müller cells [162–164] and endothelial cells [165] of the developing retina express norrin (Fig. 2a) that contributes to proper angiogenesis and the formation of the BRB [166]. Norrin is a secreted 131 amino-acid protein from the cysteine knot growth factor superfamily that includes TGF-β [167] and utilizes the wingless/integrated (Wnt) signaling pathway. Norrin forms a dimer that binds to the Frizzled 4 (FZD4) receptor and the low-density lipoprotein receptor–related protein (Lrp5–6)

coreceptor [168], activating the β-catenin canonical signaling pathway. In addition, the coactivator tetraspanin12 (TSPAN12) binds and stabilizes the FZD4 receptor at the cell membrane and enhances norrin-induced, but not Wnt-induced, β-catenin signaling [169–171]. The canonical pathway of FZD4 signaling involves β-catenin-mediated transcriptional regulation. The APC destruction complex, formed by adenomatous polyposis coli (APC), axin, protein phosphatase 2a (PP2A), casein kinase 1α (CK1α), and *glycogen synthase kinase 3* (GSK3), phosphorylates and targets β-catenin for ubiquitination and proteasomal degradation. Norrin binding to the FZD4 receptor complex inhibits GSK3 kinase and inactivates the APC degradation complex, stabilizing β-catenin, which migrates to the nucleus and promotes gene transcription (Reviewed in [172]).

Mutations in norrin and its receptors may cause a spectrum of inherited exudative retinopathies. Mutations in the norrin gene (*NDP*) cause an X-linked retinal dysplasia on the severe end of the spectrum that presents with congenital or early childhood blindness, called Norrie disease [173–176]. The retinal hypovascularization disorders, referred to as familial exudative vitreopathy (FEVR), are caused by mutations in the genes encoding for norrin receptor FZD4 [177–179], and coreceptors LRP5 [180–183], TSPAN12 [177, 178, 184, 185], β-catenin [186, 187], and some norrin mutations [188]. The hypovascular phenotype observed in both Norrie and FEVR diseases has been recapitulated in knockout mice models of norrin or the FZD4 receptor complex [162, 166, 169, 189–195], in which retinal vascular growth, mural cell recruitment, endothelial differentiation, and barrier properties are dramatically altered with reduced Sox7, Sox17, and Sox18 gene expression implicated [196]. Knockout of the norrin or FZD4 receptor complex components demonstrates high retinal vascular permeability that correlates with reduced border immunostaining of the tight junction protein claudin-5 and increased expression of the transcytosis marker PLVAP, a phenotype that can be largely reversed by the expression of stabilized, constitutively active β-catenin [166]. Within the brain, Wnt3a [197], Wnt7a/Wnt7b [198–205], and norrin [206] ligands regulate BBB integrity, with redundancy. However, in the retina, these Wnt ligands only have a small contribution with norrin as the major regulator of retinal barriergenesis [171]. Moreover, Wnt/β-catenin signaling might be coordinated through the expression of the negative regulator APCDD1, because *Apcdd1*-deficient mice have precocious retinal endothelial barrier formation [146]. Together, these data indicate that distinct from the brain, norrin signaling through FZD4/TSPAN12/β-catenin is required in deep capillary angiogenesis, BRB formation, and maintenance.TGF-β signaling has a role in deep capillary angiogenesis. It has been shown that astrocyte-endothelial interactions in the retina activate TGF-β signaling and promote barrier properties, as cocultures of astrocytes with endothelial cells increase TGF-β signaling along with occludin and claudin tight junction protein expression and transendothelial electrical resistance [207, 208]. According to this study, endothelial-specific deletion of TGF-βRII in mice at E11.5 results in cerebral hemorrhage with embryonic lethality at E15.5 [209]. Moreover, if deletion of TGF-βRII is induced postnatally, mice retinas show defects in vessel growth, with severe impairment of deep capillary plexus, presence of glomerular tufts close to the superficial plexus, and hemorrhage [210],

thus suggesting a role of TGF-β signaling in organized migration of endothelial cells as they begin to enter the deeper layers of the retina. Recent research suggests there are two populations of tip cells during retinal development that can be distinguished by specific molecular signatures: superficial (S) and deep (D) tip cells [211]. Notably, the D-tip cell specification depends on TGF-β signaling mediated by its receptor I (ALK5), because its deletion reverts D-tip cells to an S-like phenotype and inhibits neuroretina vascularization. Together, these studies indicate a role of TGF-β signaling in the formation of retinal deep capillary plexus.

BRB Dysfunction During Pathological Conditions

Diabetic Retinopathy and Diabetic Macular Edema

Diabetic retinopathy (DR) is the most common complication of diabetes and a leading cause of blindness in working age adults [212]. This progressive multifactorial disease has a complex pathogenesis in which the interaction between the cells of the NVU is disrupted. Changes in the neural retina have been implicated in the progression of this debilitating disease [213]; reviewed in [214]. However, vascular changes are most closely linked to vision loss and targeting vascular disruption has proven to be a highly effective treatment, reviewed in [215]. Therefore, changes to the vascular component will be the focus of this chapter.

DR has been classified according to vascular changes as nonproliferative and proliferative DR (Fig. 4). Clinically, the nonproliferative DR lesions show microaneurysms, venous beading, and hemorrhages, which reflect altered retinal blood-flow, vascular permeability, basement membrane thickening, loss of pericytes, and acellular capillaries. Some patients develop proliferative DR with neovascularization or dysregulated growth of highly leaky vessels. The vessels may grow into the vitreous and cause severe hemorrhaging and retinal detachment.

Macular edema (DME) increases the risk of vision loss in patients with retinal pathologies including central retinal vein occlusion, retinopathy of prematurity, age-related macular degeneration, and in diabetes, in which this is referred to as diabetic macular edema or DME. A hypoxic environment promotes high release of VEGF in patients, leading to increased permeability in the retinal capillaries. The excessive vascular permeability contributes to DME presumably through both fluid release and the deposition of albumin in the retina that retains fluid by osmotic forces, leading to the accumulation of extracellular fluid and resulting in a thickening of macular tissue. DME and vascular permeability are the clinical features most closely associated with vision loss [216, 217]. Moreover, the risk of vision loss increases with the location and extent of the retinal thickening in OCT scans, such as when the DME involves the center of the macula [218].

Current therapies to treat DR as well as retinal vein occlusion, retinopathy of prematurity, and age-related macular degeneration target both vascular permeability

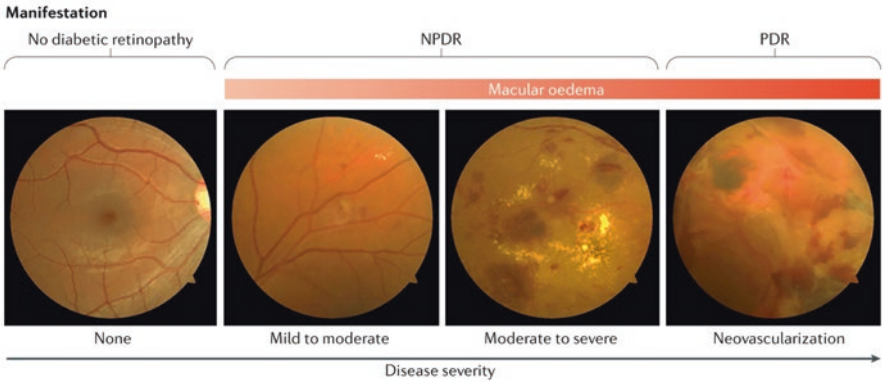

Fig. 4 Vascular abnormalities in diabetic retinopathy (DR). Fondus images of patients with DR lesions show microaneurysms, venous beading, and hemorrhages, which reflect altered retinal blood-flow, vascular permeability, basement membrane thickening, loss of pericytes, and acellular capillaries. This stage is known as nonproliferative diabetic retinopathy (NPDR). As the ischemic environment increases, patients might develop proliferative diabetic retinopathy (PDR), which is characterized by neovascularization that might lead to retinal detachment. Diabetic macular edema (DME) can occur in both NPDR and PDR. Material from: David A. Antonetti et al. Current understanding of the molecular and cellular pathology of diabetic retinopathy. Nature Reviews Endocrinology. Springer Nature. Jan 19, 2021

and neovascularization or VEGF directly. Laser photocoagulation controls vessel neovascularization and edema [219]. Targeting VEGF with antibodies or inhibitory receptor traps (a modified soluble-receptor-blocking VEGF action) reduces the occurrence of DME and improves vision or prevents further vision loss [220–226]. However, these trials reveal that only approximately half of treated patients respond to anti-VEGF therapy and this therapy requires multiple injections [227], which suggests additional factors that contribute to the pathophysiology of the disease. Consequently, additional therapeutic interventions are needed.

The multifactorial nature of retinopathies and DME makes treatment difficult. Using multiple strategies for the treatment of this disease might expand the options for patients, especially for those who do not respond to the current therapies. The potential new therapies that might help for the restoration of barrier properties will be summarized here.

Molecular Mechanisms of Barrier Loss

High permeability and active proliferation of new vessels occur in retinopathies, causing vitreous hemorrhage or fluid exudation from fragile new vessels. In DR, glia express high levels of aquaporin and Kir4.1 channels contribute to swelling [228] (Fig. 2b), in addition to vasoactive substances such as VEGFA, Dll4, ANGPTL4 and LRG1 that promote permeability, angiogenesis, or both. Loss of

pericytes leads to hyperresponsiveness of endothelial cells to VEGF signaling. Furthermore, inflammatory cytokines, such as TNF, IL-1β, and CCL2, among many others, are produced by microglia and other retinal and inflammatory cells.

Vascular Endothelial Growth Factor

Clinical data indicate that VEGF is a major driver in mediating active intraocular neovascularization in patients with ischemic retinal diseases [229], with high correlation of VEGF content with neovascularization stages in different retinopathies [6, 230, 231].

In response to a hypoxic environment and through the activation of the hypoxia inducible factor 1 (HIF-1), VEGFA is secreted to activate VEGFR2 signaling in retinal endothelial cells where it stimulates vascular permeability and angiogenesis [232, 233]. Among seven VEGF proteins, the pathogenicity of VEGFA has been shown in patients with proliferative DR and DME [232, 234, 235], while a natural antiangiogenic form of VEGF (VEGFxxxb) has been found significantly decreased compared with VEGFA [236].

Activation of VEGF/VEGFR2 signaling induces BRB leakiness mediated by occludin phosphorylation on S490 in a PKCβ-dependent mechanism. Studies in bovine retinal endothelial cells and in ischemia reperfusion models show that the occludin is phosphorylated specifically on Ser490 in the C-terminal coiled-coil domain allowing subsequent ubiquitination and endocytosed along with other junctional proteins like claudin 5, promoting gaps and retinal endothelial permeability [41, 55, 71].

The role of occludin phosphorylation on S490 in diabetes was recently demonstrated. This research reveals that conditional vascular-specific expression of the phosphorylation-resistant S490 to alanine (S490A) form of occludin prevents VEGF-induced permeability, edema, and tight junction disruption [72]. Moreover, expression of the S490A in vascular endothelial cells prevented diabetes-induced retinal vascular permeability and leukocyte accumulation in the retina. Importantly, diabetes-induced loss of visual acuity and contrast sensitivity were prevented by the vascular endothelial specific expression of the point mutant of occludin. Together, these studies support the role of occludin S490 phosphorylation in the regulation of barrier properties upon VEGF stimulation or in diabetes, and importantly demonstrate the role of compromised BRB in diabetes-induced vision loss.

VEGF can disrupt BRB properties by increasing transcytosis and by altering tight junction organization. In a model of VEGF-induced retinopathy, the transcytosis marker PLVAP expression increases and colocalizes with the capillary sites where the barrier is lost [23–25]. VEGF-induced PLVAP expression has been also demonstrated in vitro [237, 238] and in vivo [1, 25, 238]. Moreover, in vivo studies of barriergenesis of permeability using fluorescent molecules like sulfo-NHS-biotin have shown that PLVAP is highly expressed, specifically at the places of endothelial leakiness, and this expression inversely correlates with the expression of the tight junction protein claudin-5 in models of norrin/FZD4 deletion [166], suggesting a

coordination in regulation of both transcellular and paracellular permeability. Whether this coordination is maintained in disease process is unclear.

Platelet-Derived Growth Factor (PDGF)

PDGF has a role in PDR pathogenesis through the regulation of pericyte health [239, 240]. Chronic hyperglycemia reduces PDGF receptor signaling, which results in pericyte apoptosis and diabetic vasculopathy. This occurs through the activation of PKCδ and increased expression of the tyrosine phosphatase Src homology 2 domain–containing phosphatase 1 (SHP1) [241, 242]. Studies in mice show that PDGFB deficiency specifically causes similar vascular abnormalities as patients with DR, including changes in vessel diameter, microaneurysms, and vessel regression [243] [128]. A similar phenotype is caused by the administration of a PDGF receptor-β–blocking antibody, which induces hemorrhage and permeability [152]. Notably, this study demonstrates that pericytes control Ang-2 and VEGFR2 expression in endothelial cells, through the transcription factor FOXO1. Altered ratio of Ang-1 and Ang-2 has been observed in DR with increased Ang-1 and an even greater increase in Ang-2 observed in nonproliferative DR patients [244]. Mouse studies reveal that the overexpression of Ang-2 in the retina results in reduced pericytes and vascular sheaths, vascular denuded basement membrane without cells, referred to as acellular capillaries [245]. Together, these studies suggest that loss of pericytes in the diabetic retina is mediated by reduced PDGFB signaling leading to subsequent increase in Ang-2 making retinal vasculature highly susceptible to VEGFA, thus contributing to the progression of DR.

Inflammatory Mediators

Cell signaling through inflammatory factors might contribute to the pathogenesis of vascular retinopathies by influencing vascular changes. For example, IL-6, which promotes vascular permeability through the promotion of VEGF expression [246, 247]. IL-8 induces angiogenesis through the activation of the transcription factor nuclear factor kappa-B (NF-κB) [248–250]. IL-1β is cleaved by caspase-1 and induces retinal capillary degeneration in diabetic mice [251]. IL-8 and the monocyte chemoattractant protein (MCP-1/CCL2) together recruit inflammatory cells, which in turn secrete angiogenic and fibrotic factors [252]. And TNF-α can directly induce vascular permeability [253, 254]. Together, these data suggest that cytokines can contribute to vascular defects in retinopathies.

An increased pro-inflammatory environment accompanies the recruitment of peripheral immune cells. The intracellular adhesion molecule-1 (ICAM-1) regulates leukocyte infiltration in the retinal tissue. ICAM-1 expression in the vascular endothelium is promoted in diabetes by TNF-α [255]. In addition to ICAM-1, several

adhesion molecules have been found elevated in patients with DR, including soluble intercellular adhesion molecule-1 (sICAM 1), soluble platelet-endothelial cell adhesion molecule (sPECAM-1), selectin, and soluble vascular cell adhesion molecule (sVCAM-1) [256–258]. This suggests that in retinopathies, the expression of chemoattractants and enriched cytokines signal to recruit peripheral immune cells, which, in turn, might potentiate immune response and contribute to the progression of the disease.

Potential Mechanisms of BRB Restoration

Norrin

It is apparent from the discussion above that the factors involved in retinal vascular angiogenesis including VEGF, Ang-1 and Ang-2, and PDGF may contribute to the process of dysregulated vascular growth and permeability in retinopathies. Likewise, barrier-promoting factor Norrin may be able to restore barrier properties after DR. Loss of Norrin or its coreceptor TSPAN12 in mouse retinas reveal a phenotype that resembles some of the pathological features of DR. Retinas from mice with *Ndp* gene deletion show formation of cystoid edema, neovascularization, and inflammation [259]; whereas endothelium-specific loss of TSPAN12 induces cystoid edema formation and basement membrane collagen IV deposition [260]. Other studies suggest that Norrin [260] and β-catenin [261] expression increase with diabetes, as determined by retinal sections of postmortem human eyes. In Akita mice and streptozotocin-induced diabetic rats, both β-catenin and LRP5/6 were elevated [261]. Nevertheless, the role of norrin signaling during DR is not completely understood. The role of norrin signaling in BRB formation suggests that it may have the potential to restore BRB properties after induced retinal vascular permeability. Recent research supports this idea. In these studies, in vivo experiments demonstrate that norrin can completely prevent and restore BRB function in two models of vascular dysfunction. Norrin prevented VEGF-induced permeability when coinjected into the vitreous of rats and intravitreal injection of norrin reversed diabetes-induced retinal permeability in rats [262]. Moreover, in cell cultures of retinal endothelial cells, it was demonstrated that VEGF and norrin interact in regulating barrier properties. VEGF induces permeability but simultaneously primes norrin signaling by promoting TSPAN12 coreceptor localization at the cell membrane. Subsequent norrin signaling promotes claudin-5 organization at the cell border and BRB restoration. These studies suggest potential novel treatment paradigms for patients with DR or other retinal vascular diseases driven by VEGF-induced permeability, which focus on restoration of proper vascular function rather than prevention of further damage through binding VEGF.

Angiopoietin-like4 (ANGPTL4)

ANGPTL4 levels have been found in patients with DR and DME, correlating with high permeability [263–265]. The ability of ANGPTL4 to increase permeability has been demonstrated in a model of ischemic retinopathy, in which its expression increased in Müller cells under the control of HIF-1 [266]. Concomitant with this, ANGPTL4 expression localizes specifically in areas of retinal neovascularization of patients with PDR and in the ischemic areas of patients with DME [267]. In vitro studies show that ANGPTL4 promotes permeability when it binds to neuropilin receptor and activates the small G protein RhoA [268]. Interestingly, although neuropilin acts as a VEGFR2 coreceptor to increase vascular permeability as well, the effects of ANGPTL4 seem to be independent of VEGFR2. Indeed, inhibition of ANGPTL4 expression reduces vascular permeability and angiogenesis additively to the inhibition of VEGF [267].

Conflicting studies suggest a role of ANGPTL4 in the protection of vascular integrity. Mice genetically deleted in ANGPTL4 show defects in developmental angiogenesis [269]. At a molecular level, it has been proposed that ANGPTL4 interacts with αvβ3 integrin and sequestrates Src, thus resulting in decreased VEGFR2/Scr signaling and increased VEGFR2/VE-Cadherin interaction [270]. In a stroke model, ANGPTL4 can inhibit VEGF-induced permeability through the regulation of Src phosphorylation [271]. Further studies are needed to determine the specific role of ANGPTL4 in retinal vascular integrity.

Notch

Inhibition of Notch signaling has also been proposed as an alternative to control endothelial leakiness. Levels of both Dll4 and Jagged 1, a Notch antagonist, have been found elevated in diabetic mouse models and in human endothelial cells in response to glucose levels. While intraocular injection of the ligand molecule increased retinal permeability, a recently developed Notch-trap has been shown to reduce retinal permeability [272], thus suggesting a novel mechanism to decrease vascular leakiness that involves the regulation of Notch signaling.

Conclusions

Changes in retinal vascular permeability contribute to loss of vision and the pathophysiology of several blinding eye diseases including DR, with evidence for growth-factor- and cytokine-induced alterations to both the junctional complex and paracellular route and the vesicle trafficking and the transcellular route. Recent research has provided novel insight into the formation mechanisms of the unique

vascular barrier of the BRB. Understanding the factors that promote barriergenesis may lead to novel therapeutic options to restore the BRB and preservation of vision in vascular retinopathies.

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Role of Cerebral Endothelial Tight Junctions in the Formation of Brain Tumors

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Abstract Malignant brain tumors are devastating conditions, characterized by limited survival of the patients. In order to widen the therapeutic possibilities targeting cerebral malignancies, we have to understand the mechanisms of their development. In this chapter, we review the involvement of tight junctions (TJs) of cerebral endothelial cells in the formation of brain cancers. Two main aspects will be discussed. First, we cover the mechanisms of opening of the TJs of the blood-brain barrier (BBB) during paracellular extravasation of metastatic cells. In this process, proteolytic mechanisms, induction of endothelial-mesenchymal transition, release of extracellular vesicles, and modulation of cells of the neurovascular unit take the most important roles. Second, we introduce the blood-tumor barrier (BTB), that is, the altered vasculature of both primary and secondary brain tumors. Although leakier in general than the intact BBB, the BTB restricts the majority of drugs to reach cytotoxic concentrations in brain tumors.

Keywords Blood-brain barrier · Blood-tumor barrier · Brain metastasis · Extravasation · Paracellular transmigration · Primary brain tumor

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L. González-Mariscal (ed.), *Tight Junctions*,
https://doi.org/10.1007/978-3-030-97204-2_12

Introduction

As crucial structural elements of epithelial and endothelial cells, tight junctions (TJs) play an important role in many aspects of carcinogenesis and metastasis formation. These include epithelial-to-mesenchymal transition (EMT) of tumor cells involving loss of epithelial features, including TJ proteins, and consequent detachment from the initial site. In line with this, less differentiated and claudin-low (negative for claudin-3, claudin-4, and claudin-7) breast cancer cells are prone to initiate the metastatic cascade and form brain and lung metastases [1, 2]. Similarly, downregulation of claudin-1 in melanoma cells supports formation of brain metastases [3].

In addition, intravasation of metastatic cells into the vessels and extravasation from the circulation to the secondary tumor site may involve breakdown of vascular endothelial TJs. Implicitly, if the secondary site is the brain, TJs of the blood-brain barrier (BBB) will be compromised. Once in the metastatic site, cancer cells lose mesenchymal proteins and reacquire the epithelial phenotype through the process known as mesenchymal-to-epithelial transition (MET) [4]. This involves upregulation of TJ proteins. Moreover, in the brain, there is a specific role of vascular TJs in metastasis formation. The relative tightness of cerebral endothelial TJs forming the BBB or the blood-tumor barrier (BTB) inhibits penetration of several antitumor drugs to the central nervous system (CNS) and brain tumors, causing a yet unresolved therapeutic problem.

This chapter describes the response of the BBB to malignant cells with focus on cerebral endothelial TJs. Two main aspects will be presented. First, we discuss how cerebral TJs hinder diapedesis of metastatic cells into the brain. Second, we describe the differences between TJs of the healthy BBB and the BTB, which is formed by the vasculature of brain tumors. This second aspect refers to both metastases and also primary tumors of the brain, mainly different types of gliomas.

Brain Barriers in the Protection of the CNS

The CNS is protected by several active interfaces from the potential damaging effects coming from its environment. With a surface of approximately 12–18 m^2 [5], the BBB is the largest CNS/periphery interface protecting the brain parenchyma. The BBB is a complex function of the neurovascular unit (NVU) formed by cerebral endothelial cells, pericytes, endfeet of astrocytes, other glial cells, and neurons. By sealing the intercellular cleft and forcing most molecular and cellular traffic to take the transcellular, rather than the paracellular, route, continuous TJs interconnecting endothelial cells of cerebral microvessels and capillaries form the basis of the barrier.

TJs of cerebral endothelial cells share many similarities with epithelial TJs. Detailed description of epithelial TJs and TJs of the BBB has been excellently

discussed in other chapters; here, we only refer to a few aspects related to metastasis formation. Among the differences between epithelial and endothelial TJs, one of the most important is that TJs are located apically in comparison to adherens junctions (AJs) in polarized epithelial cells, while these two are often intermingled in endothelial cells [6]. In addition, claudin composition is cell type specific, the backbone of cerebral endothelial TJs being formed by claudin-5. Absence of claudin-5 practically opens the BBB to substances smaller than 800 Da [7]. Among endothelia of different organs, only brain and retina endothelial cells show a strong barrier function with high transendothelial electrical resistance (TEER) and these are the only endothelia, which develop tricellular contacts with high content of tricellulin and angulin-1/LSR [8]. Further important components of cerebral endothelial TJs include occludin and plaque proteins (ZO-1, ZO-2, MAGI-1, paracingulin/JACOP, etc.) [9]. JAM/ESAM family members should also be noted, because they play crucial functions in leukocyte trafficking to the brain; however, their role in brain metastasis formation is less well understood.

The paracellular barrier formed by interendothelial TJs is completed by three more defense lines of the BBB [10], including the transcellular barrier (given by the thick glycocalyx and the low level of transcytosis), the enzymatic barrier (provided by a complex set of enzymes), and the efflux transporters, which have a special importance in the impermeability of the BTB to anticancer agents. Importance of the BBB over other cerebral barriers in metastasis formation is indicated by the fact that the most common metastases are parenchymal lesions, where tumor cells invade the brain tissue across the BBB. Role of the blood-cerebrospinal fluid barrier—formed by epithelial cells of the choroid plexus—in brain metastasis formation is less relevant, since periventricular metastases are very rare [11, 12]. Further brain barriers include the arachnoid barrier between the subarachnoid space and the dura mater, through which cancer cells can spread to the membranes covering the brain to form leptomeningeal metastases.

Primary Tumors of the CNS

Primary brain tumors are rare malignancies, accounting for about 2% of all cancers. They represent a heterogeneous group, among which gliomas are the most frequent in adults [13]. Gliomas are malignant tumors of neuroectodermal origin and include astrocytomas, oligodendrogliomas, and ependymomas. The most common nonmalignant primary brain tumors are meningiomas. Among malignant brain tumors, glioblastoma multiforme (GBM), also known as grade IV astrocytoma, is the most aggressive and accounts for half of the cases. In this cancer type, despite new diagnostic and treatment opportunities, the prognosis is dismal and the median survival of the patients is approximately 1 year [14]. Although highly invasive and proangiogenic, GBM is not prone to metastases outside the CNS [15].

Formation of Brain Metastases

Metastases are the main forms of malignant processes in the brain, outnumbering primary tumors. They originate mainly from lung cancer (40–50%), breast cancer, especially the triple-negative subtype (i.e., negative for estrogen receptors, progesterone receptor, and Her2) (15–25%), and malignant melanoma (5–20%); these three types accounting for 70–80% of brain metastases [16]. Among these tumors, melanoma metastasizes to the brain with the highest frequency, being detected in approximately 75% of patients dying of melanoma [17]. Due to the lack of effective therapies, the median survival of patients who have developed brain metastasis is only a few months; thus, development of effective therapeutic or prevention strategies is one of the most critical unmet medical needs in oncology [18]. Among strategies of prevention or reduction of brain metastases, preserving BBB integrity may play an important role.

Since the brain parenchyma lacks a classical lymphatic vasculature, the only way for metastatic cells to reach the CNS is via the blood stream and the BBB. As discussed previously, the BBB constitutes a multiple defense line protecting the CNS from potentially harmful substances and penetration of cellular elements. In addition, the basal membrane and astrocytic endfeet also constitute a major obstacle for transmigrating cells.

Our knowledge of the cellular transmigration through the BBB originates mainly from studies using white blood cells. Classical steps of leukocyte extravasation are tethering, rolling, adhesion, and diapedesis. Although extravasation of metastatic cells shows considerable similarities to these mechanisms, differences also occur [19], as follows. As an initial step of the metastasis formation, tumor cells arrest at the level of capillaries and postcapillary venules, preferentially at vessel branches [20, 21]. Diameter of these vessels is comparable with the size of metastatic tumor cells. The time tumor cells spend in the vasculature differs from organ to organ. As a unique aspect of brain metastasis formation, tumor cells spend the longest time—3–5 days in average and up to 14 days, according to mouse studies—in the lumen of the vessels before extravasating into the parenchyma [21, 22]. During this time, tumor cells induce several morphological and functional changes in the vasculature, including vasoconstriction, development of intraluminal endothelial plugs, and reversible endothelial blebbing [22].

Upon tumor cell extravasation into the brain, cerebral endothelial TJs may also be compromised, but this seems to be tumor cell type specific. Diapedesis of tumor cells through the BBB is completed within a few hours either through the paracellular route between endothelial cells or transcellularly through the cytoplasm of a single endothelial cell [22, 23]. Transendothelial migration of melanoma cells was shown to be exclusively dependent on the paracellular pathway, while breast cancer cells could take the transcellular route as well. Since the paracellular pathway involves active participation of the TJs of the BBB, this will be discussed in more detail.

Changes in the TJs During Transmigration of Metastatic Cells Through the BBB

One of the main strategies used by metastatic cells to reach the brain parenchyma involves damage of the junctional integrity. In this respect, different cancer cells may differently affect the junctional complex, and the severity of the damage may be in correlation with the metastatic capacity of the tumor: more invasive phenotypes cause a more intense degradation. The protective role of an intact junctional complex against development of brain metastases is supported by the finding that physical exercise is able to reduce the number of tumor cells extravasating into the brain at both 48 h and 3 weeks postinjection by maintaining a higher occludin level [24]. Furthermore, substances that can damage the junctional complex, such as polychlorinated biphenyls, facilitate metastasis formation [25].

Direct damage of junctional proteins by metastatic cells was detected in a considerable number of studies. After 8 h of coculturing melanoma cells with cerebral endothelial cells, tumor cells were found attached to the endothelial cells in close proximity to TJs [23]. Furthermore, melanoma cells were able to degrade junctional proteins when meeting endothelial cells. At least under in vitro conditions, rapid degradation of claudin-5, occludin, and ZO-1 was demonstrated [26]. Interestingly, degradation of TJ proteins did not require direct contact of melanoma cells with the endothelium, which points to the importance of soluble factors released by metastatic cells. Changes in junctional proteins of the BBB were induced by lung cancer cells as well. A shift in occludin from insoluble to soluble phase was detected paralleled by the disruption of the characteristic continuous junctional staining for occludin, claudin-5, and ZO-1. This was accompanied by a decrease in TEER values. All these effects were reversed by ROCK inhibition, but not PKC or PI3K inhibition [27]. Thus, ROCK may affect the junctional complex of brain endothelial cells through regulation of the cytoskeleton to which TJ proteins are anchored. Importantly, ROCK can not only affect the junctional complex of brain endothelial cells, but can also regulate release of proteases from carcinoma cells, which in turn might degrade endothelial junctional proteins.

Evidently, there is a significant difference between different cancer cell types in their capacity to damage endothelial junctional complexes. Thus, melanoma cells with a high metastatic capacity are able to more effectively degrade claudin-5, decrease TEER of brain endothelial monolayers, and to transmigrate more rapidly compared to breast cancer cells. By targeting the PI3K/Akt pathway, adhesion and transmigration potential of melanoma cells and breast cancer cells was decreased without evident effects of the inhibitor on endothelial TJs [28].

Tumor-cell-induced junctional damage is induced by several mechanisms, among which proteolytic degradation and induction of endothelial-mesenchymal transition (EndMT) are the most important. The specific factors released by tumors and how they impact endothelial junctions are summarized in Table 1 and will be discussed further.

Table 1 Mechanisms of tumor-cell-induced opening of cerebral endothelial TJs during extravasation

Tumor type	Released factor	Effect	Reference
Melanoma		Claudin-5 ↓ Occludin ↓ ZO-1 ↓	[22]
	Seprase	Transmigration ↑	[22]
	uPA	Transmigration ↑	[27]
	TGF-β	EndMT in endothelial cells TEER ↓ Claudin-5 ↓ Transmigration ↑	[45]
Lung cancer		Claudin-5 ↓ Occludin ↓ ZO-1 ↓ TEER ↓ Occludin: From insoluble phase to soluble	[23]
	AKB1B10	Modulation of MMP-2 and MMP-9 Brain metastasis formation	[37]
	PLGF	TJ disassembly through occludin phosphorylation	[42]
	miR-143-3p	TJ protein expression ↓ EMT	[65]
Breast cancer	Cathepsin S	Proteolysis of JAM-B Higher probability of brain metastasis Occludin ↓ Claudin-3 ↓	[29]
	MMP-1	Claudin-5 ↓ Occludin ↓	[34, 35]
	ADAM8	Transcription of MMP-9 ↑ Transendothelial migration ↑	[36]
	CXCL13, CXCL1	BBB permeability ↑ Claudin-5 ↓	[38]
	VEGF	BBB permeability ↑ Disruption of endothelial monolayers ZO-1 ↓	[39, 40]
	TGF-β	N-cadherin (EndMT) ↑ Modulation of MMP-2 and MMP-9	[19, 43]
	SP	TEER ↓ Disintegration of TJs	[52]
	miR-105	Higher risk for brain and lung metastases ZO-1 ↓	[60]
	miR-181c	Brain metastasis formation BBB breakdown Redistribution of TJ proteins (claudin-5, occludin, ZO-1) to the cytoplasm	[61]
	lncRNA GS1-600G8.5	Silencing: BBB destructive effect of breast-cancer-cell-derived exosomes ↓ Silencing: Claudin-5, ZO-1 ↑	[66]

Proteolytic Mechanisms in the Interendothelial Junctional Damage Induced by Metastatic Cells

A plausible mechanism of junctional damage is proteolytic degradation of TJ proteins. There are two classical phenotypes of migrating tumor cells: the mesenchymal, characterized by an elongated cell morphology and increased Rac and proteolytic activity; and the amoeboid type, with rounded morphology and increased Rho/ROCK activity and contractility. Out of these, the mesenchymal phenotype seems to be more favorable for cancer cells to penetrate through cerebral endothelial cells [29], and proteolytic activity is probably a key element in this phenomenon.

It is well known that junctional proteins are substrates of different proteolytic enzymes. Despite a significant amount of investigations on the proteolytic profile of different cancer cells, only a few studies have addressed directly the involvement of proteases in the junctional damage during brain metastasis formation.

Role of Serine Proteases in the Migration of Tumor Cells Through Brain Endothelial Cells

One of the candidate proteases is the membrane-bound serine protease seprase. Melanoma cells were found to express high amounts of seprase, silencing of which decreased the number of transmigrated cells through cerebral endothelial monolayers [26]. Seprase was shown to colocalize with the urokinase plasminogen activator receptor (uPAR) in the membrane protrusions of melanoma cells and invadopodia of adhered cancer cells, leading to pericellular proteolytic activity. Formation of seprase-uPAR complex was found to be integrin dependent [30].

Other serine proteases shown to facilitate penetration of melanoma cells through the BBB are plasminogen activators, especially urokinase (uPA) [31]. On the other hand, plasminogen activator inhibitory serpins, including neuroserpin and serpin B2, were found to promote metastatic cell survival and vascular co-option in the brain [32]. This shows that tumor cells require activation of different mechanisms in order to overcome the BBB and to survive in the cerebral environment.

Taken together, serine proteases play an unambiguous role in the transensothelial migration process; however, the targets of these enzymes have not been clearly defined and we only presume that TJ proteins are among them.

Cathepsin S and Brain Tumors

Brain metastatic human breast cancer cells release the cysteine protease cathepsin S especially in early stages of metastasis formation, which induces proteolysis of JAM-B. Furthermore, elevated expression of cathepsin S at the primary tumor site

correlates with higher probability for brain metastasis. Elevated expression of this protease at the primary tumor site correlates with decreased brain metastasis-free survival in patients. Targets of this protease in the junctions are occludin and claudin-3, but not claudin-5, CD31, or VE-cadherin [33]. In addition, inhibition of cathepsin S in glioblastoma cells could reverse TGF-β-induced EMT and restore reduced occludin and ZO-1 levels in the tumor cells [34]. Thus, inhibition of cathepsin S might be used to control motility and spreading of both brain metastatic cells and primary brain tumor cells.

Involvement of Metalloproteinases (MMPs) in Opening of the TJs to Facilitate Brain Metastasis Formation

Metalloproteinases (MMPs) are key elements of cancer cell migration and metastasis. As an important aspect of brain metastasis formation, MMPs (especially MMP-2 and MMP-9) are able to degrade junctional proteins (claudin-5 and occludin) under pathological conditions [35]. In line with this, elevated MMP-9 activity was measured in the sera of patients with brain tumors [36]. MMP-2 and MMP-9 were described to be secreted by leukemic cells leading to impairment of the TJs, opening the BBB and contributing to the invasion of leukemic cells to the CNS [37]. In addition, MMP-1 and MMP-9 showed elevated mRNA expression in brain-seeking breast cancer cells compared to bone-seeking or parental cells [38]. MMP-1, highly expressed in brain metastatic breast cancer cells, is capable of degrading claudin-5 and occludin, increasing their ability to form metastases in vivo [39].

All these data underline the importance of MMP secretion from metastatic cells to open the TJs of the BBB, facilitating extravasation of tumor cells into the brain parenchyma. Moreover, other enzymes enhance transmigration of metastatic cells through the BBB by increasing expression of MMPs. For example, ADAM8 increases transcription of MMP-9 in brain metastatic breast cancer cells through a pERK1/2- and pCREB-dependent mechanism, promoting the metastatic processes, especially transendothelial migration [40]. In addition, the aldo-keto reductase family 1 B10 (AKR1B10), which is significantly elevated in non–small cell lung cancer, modulates MMP-2 and MMP-9 expression via MEK/ERK signaling and thus may influence the integrity of TJs and contribute to brain metastasis formation [41].

Nonprotease Mediators of Junctional Damage in Response to Tumor Cells

Several secreted factors including growth factors and cytokines mediate junctional damage and transmigration, partly through the activation of endothelial signaling pathways, which cause junctional damage. The role of factors released by

metastatic cells is indicated by the fact that sera of patients having breast cancer brain metastasis were found to increase permeability of the BBB with a parallel decrease in claudin-5 expression. CXCL13 and CX3CL1 were significantly elevated in the sera of these patients, which could mediate changes in TJs [42].

Tumor-Cell-Derived Vascular Endothelial Growth Factor (VEGF) and Impairment of Cerebral Endothelial TJs

Among the mechanisms, which lead to TJ breakdown, increased VEGF expression is one of the most important. VEGF is well known to increase vascular permeability and was shown to facilitate migration of MDA-MB-231 mammary tumor cells through the brain microvascular endothelium. Although endothelial permeability increased with the presence of tumor cells, and VE-cadherin continuity was found to be disrupted, TJ proteins were not directly investigated in this study [43]. In a further study, it was shown that integrin β4- and ErbB2- (also known as Her2/neu) signaling-mediated increase in VEGF expression in mammary tumor cells led to the disruption of endothelial monolayers involving a decrease of junctional ZO-1 and increased adhesion of tumor cells at or near endothelial junctions [44]. In later stages of metastasis development, VEGF can also play an important role; however, in these cases, its importance might rise from its angiogenic properties. In addition, in endothelial cells of GBM, a significantly higher expression of VEGF-A and TGF-β has been found, in comparison to microvascular endothelial cells of normal brain and low-grade glioma [45]. Besides solid tumors, leukemic cells also produce VEGF and this is a possible mechanism underlying leukemic infiltration of the brain [46].

Another member of the VEGF family, placental growth factor (PLGF) was shown to be elevated in small cell lung cancer, which has a high metastatic capacity to the brain. PLGF can bind to endothelial VEGFR1, leading to Ser/Thr phosphorylation of occludin, which results in disassembly of the TJs in these cells and promotes transendothelial migration of the tumor cells. The process is regulated via Rho/ROCK- and ERK1/2-mediated signaling, but not through PI3K or PKCα/β1 [47]. In line with this, elevated PLGF serum levels were detected in patients having small cell lung cancer brain metastasis [47].

Altogether, tumor-cell-induced activation of VEGFR signaling in cerebral endothelial cells induces disruption of the TJs and formation of new vessels in different stages of brain metastasis formation.

TGF-β Secretion and Induction of EndMT

Tumor cells can produce excessive levels of different cytokines, including TGF-β. TGF-β may disrupt the junctional complex by increasing MMP-2 and MMP-9 activity [48]. Moreover, TGF-β is a key player in the induction of EndMT. EndMT is a process similar to EMT, where endothelial cells lose their intercellular connections, increase motility, and acquire characteristics of mesenchymal cells [49]. It has been demonstrated that melanoma-cell-derived TGF-β induces EndMT in cerebral endothelial cells, which results in decreased TEER, downregulation of claudin-5, and increased transmigrating capacity of the tumor cells through the mesenchymal-transformed cerebral endothelial monolayers [50]. TGF-β is produced by breast cancer cells as well, leading to N-cadherin upregulation (i.e., EndMT) in cerebral endothelial cells [50], but this is dispensable for the extravasation of mammary carcinoma cells [23].

Involvement of Tumor-Cell-Derived Other Cytokines in the Regulation of Cerebral Endothelial TJs

By creating an inflammatory milieu, pro-inflammatory cytokines may promote formation of brain metastases through activating the endothelium before extravasation. On the one hand, by upregulation of cell adhesion molecules on the luminal surface of endothelial cells, such as vascular cell adhesion molecule-1 (VCAM-1) [51]. On the other hand, TJs are also targets of pro-inflammatory cytokines in several conditions [52]; however, little is known about the direct effect of inflammatory cytokines secreted by circulating or arrested tumor cells on cerebral endothelial TJs, opening of which facilitates the metastatic process. Nevertheless, injection of tumor necrosis factor (TNF) selectively induces BTB permeabilization at sites of brain metastases [53].

Melanoma cells are able to secrete a wide range of soluble factors, such as osteopontin, interleukin-8 (IL-8), growth differentiation factor-15 (GDF-15), and macrophage migration inhibitory factor (MIF) in high amounts, which favor tumor cell survival. Surprisingly, none of them had any effect on the barrier integrity of endothelial cells [54]. Hepatocyte growth factor (HGF), a regulator of epithelial cell transformation, tumor cell proliferation, survival, migration, and angiogenesis, was shown to decrease the expression of occludin, claudin-1, claudin-5, JAM-1, and JAM-2 in breast cancer cells [55]. Although HGF is a key regulator of both primary and metastatic brain tumor formation [56], its implication in breaking down cerebral endothelial TJs has not been shown so far.

It has to be mentioned here that cytokines secreted by tumor cells might also influence resident and infiltrating immune cells in the metastatic environment; however, a detailed description of this process is out of the scope of this chapter.

Substance P (SP)-Induced Opening of the TJs

Tumor cells can release high amounts of SP, while 10 ng/ml of SP was found to decrease TEER by 20–25% in an hour and rapidly disintegrate TJs within minutes. Inhibition of SP reduced the tumor-cell-induced BBB permeability increase and metastatic colonization of the brain [57]. Increased SP expression was observed 3 days after breast cancer cell inoculation, indicating the role of SP in early tumor cell extravasation into the brain [58].

The mechanism by which breast-cancer-cell-secreted SP opens endothelial TJs is activation of endothelial cells leading to secretion of TNF-α and angiopoietin-2 (Ang-2) from these cells [57].

Angiopoietin-2 (Ang-2): Opening of the TJs During Extravasation and Contradictory Role in Brain Tumor Angiogenesis

In line with the data shown in the previous paragraph, increased Ang-2 expression was observed in cerebral endothelial cells of mice injected with breast cancer cells into the carotid artery, leading to the disruption of the TJs. Interestingly, no secretion of Ang-2 was found in triple-negative breast cancer cells. Neutralization of Ang-2 in these mice prevented loss of BBB integrity and inhibited metastatic colonization of the brain [59].

In already formed brain metastases, Ang-2 together with VEGF drives vessel growth. Therefore, combined inhibition of them may reduce BTB permeability and brain metastatic burden [60]. However, another study showed that elevated Ang-2 expression leads to the formation of nonfunctional vessels with inadequate oxygenation of the tumor tissue, decreasing tumor growth together with increasing median survival of model animals [61]. Similarly, systemic administration of high levels of Ang-2 inhibited angiogenesis and tumor growth in mice with colon adenocarcinoma xenografts [62]. This contradictory role of Ang-2 might depend on the active remodeling of the tumor vessels during angiogenesis and vessel co-option [63].

Role of Extracellular Vesicles (EVs) and Noncoding RNAs Released by Cancer Cells in Disrupting the Endothelial Junctional Complex

Not only secreted soluble factors but also extracellular vesicles (EVs) and noncoding RNAs, especially some miRNAs, are involved in opening of the TJs and promoting metastasis formation in the brain [64]. Metastatic

breast-cancer-cell-derived exosomes contain miR-105, which binds to the 3′UTR of ZO-1 and downregulates its expression in cerebral endothelial cells recipient of the exosomes. As a result, cancer-secreted miR-105 induces vascular permeability and promotes lung and brain metastases formation [65]. In addition, tumor-cell-derived circulating EVs containing miR-181c were shown to disrupt intercellular junctions leading to BBB breakdown and promoting brain metastasis formation of breast cancer [66]. The mechanism through which miR-181c promotes the destruction of BBB is downregulation of 3-phosphoinositide-dependent protein kinase-1 (PDPK1), resulting in cofilin activation and consequently modulation of actin dynamics [66]. Although not directly connected to brain metastasis development, miR-939 is also a candidate molecule to modulate the integrity of endothelial junctional complexes during metastasis formation. Breast-cancer-secreted miR-939 has been shown to downregulate VE-cadherin in HUVECs, increasing the transmigration rate [67]. Moreover, some circulating miRNAs were suggested to be biomarkers of breast cancer brain metastatic disease [68, 69]. In lung cancer cells, upregulation of miR-143-3p reduced expression of junctional proteins, induced EMT in the tumor cells, and increased their invasiveness through an in vitro BBB model [70].

Other potential candidate molecules used by cancer cells to interfere with TJ integrity are long noncoding RNAs (lncRNAs). Exosomes derived from highly brain metastatic breast cancer cells contain lncRNA GS1-600G8.5, which decrease claudin-5 and ZO-1 protein expression in endothelial monolayers. Silencing of the lncRNA significantly reduced the BBB destructive effect with increased TJ protein expression [71]. In accordance with this, a recent in silico study also raised the possibility that lncRNAs can be involved in the regulation of BBB permeability and thus metastasis formation through claudin-5-dependent mechanisms [72].

Altogether, tumor-cell-derived EVs and noncoding RNAs are emerging as important modulators of the pre-metastatic niche in the brain to enhance transendothelial migration of cancer cells. EVs released by metastatic cells not only target cerebral endothelial cells, but can also cross them by transcytosis to be taken up by and modulate other cell types of the NVU, especially astrocytes [73].

Role of Cellular Components of the NVU in Modulating the TJs During Initial Steps of Brain Metastasis Development

Continuous TJs and explicit barrier properties are not intrinsic features of cerebral endothelial cells, but induced and maintained by the neural environment, especially pericytes and astrocytes [74]. Therefore, factors released by the cells of the NVU might also play a role in modulation of the TJs during metastasis formation especially as arrival of cancer cells to the brain induce rapid changes in the NVU.

Regulation of TJs by Astrocytes in Brain Metastases

Astrocytes are one of the cell types that are activated early in response to metastatic cells, even before their extravasation. In close relationship with extravasating cancer cells, upregulation of astrocytic MMP-9 was shown, which could contribute to the degradation of the junctional complex, thus supporting cancer cell progression [20]. Astrocytes also overexpress the extracellular matrix degradative enzyme heparanase in response to the presence of metastatic melanoma cells and this might contribute to brain colonization [75]. Astrocyte-secreted IL-23 was found to significantly enhance transendothelial migration of melanoma cells, while an IL-23-neutralizing antibody could block this increased migration Furthermore, knocking down the expression of MMP-2 in melanoma cells reduced their IL-23-mediated invasiveness [76].

In an in vitro three-dimensional BBB model, the astrocyte-secreted CCL2 (also known as monocytic chemotactic protein 1 or MCP-1) promoted transmigration of breast and lung cancer cells without inducing changes in vascular permeability [77]. Indeed, CCL2 is a strong chemoattractant and an important mediator of tumor cell and host cell interaction [78].

All these data indicate that astrocytes react to cancer cells very early in the brain metastatic process and influence the progression of the disease. In fact, astrocytes play a decisive role in the fate of the tumor cells in the CNS and have a Janus-faced attitude toward them. This means that several astrocyte-related mechanisms have been described, which are either harmful or supportive towards metastatic cells [29].

Involvement of Pericytes in Early Stages of Brain Tumor Formation

Contradictory roles have been linked to pericytes as well in brain metastasis development. An in vitro model using a rat primary BBB model and human lung cancer cells showed that tumor cells decreased TEER together with the disruption of ZO-1 in endothelial-astrocyte coculture models. In contrast, in the presence of pericytes, normal TEER values and integrity of the TJs could be preserved [79]. However, principally pericytes have very prominent prometastatic features. By secreting extracellular matrix proteins, pericytes were shown to enhance adhesion of extravasated melanoma and breast cancer cells on vessel walls, which is a prerequisite for their survival in the brain. Furthermore, pericytes released insulin-like growth factor 2, which has a prompt proproliferative effect on breast cancer cells [80]. In primary tumor sites, endosialin-expressing pericytes also promoted tumor cell dissemination, intravasation, and metastasis formation [81].

Microglia in the Regulation of TJs During Initial Steps of Brain Metastasis Formation

Microgliosis starts already during extravasation of metastatic cells. Microglia are able to secrete multiple cytokines, growth factors, and enzymes, which may influence the metastatic environment. Microglia/macrophages are the predominant stromal cells expressing cathepsin S; however, in contrast to the breast-cancer-cell-derived cathepsin S, which seems to be important for the step of BBB transmigration, stromal cathepsin S is rather involved in supporting survival of metastatic cells in the cerebral environment [33]. In melanoma brain metastases, the number and moving speed of microglia/macrophages were found to be increased. These cells expressed high amounts of MMP-3, which could degrade ZO-1. MMP-3 inhibition reduced TJ impairment and the number and size of melanoma brain metastases [82].

The Blood-Tumor Barrier (BTB)

Both primary and metastatic brain tumors alter the features of vessels, creating a unique microvasculature forming the BTB. It is generally accepted that the BTB is leakier than the healthy BBB, leading to vasogenic edema. While numerous findings are in line with this concept supported by advanced imaging technics, such as MRI and PET, the mechanisms are not completely known.

The morphology of the BTB differs significantly from the healthy BBB (Fig. 1). In glioma, tumor cells have been shown to utilize blood vessels for movement and

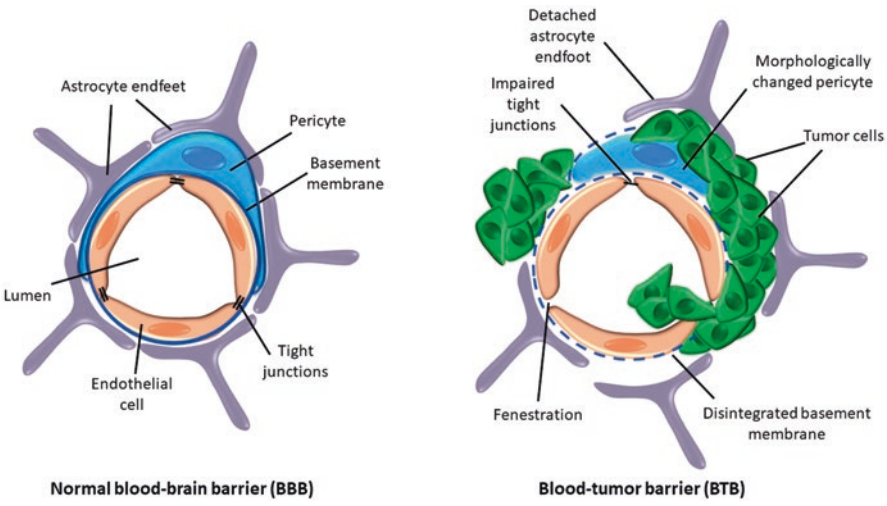

Fig. 1 Differences between the BBB and the BTB

invasion. During this process, the tumor co-opts existing blood microvessels and also remodels them. In this respect, intussusceptive angiogenesis, vessel dilation, formation of capillary loops, and glomeruloid bodies have been described in a mouse model [83]. Glioma cells are able to extend long membrane protrusions enhancing their invasion properties and also formation of a multicellular network interconnected by gap junctions, conferring resistance to damage and therapy [84]. Among metastatic tumors, melanoma and breast cancer cells have been shown to grow along the capillaries and acquire blood supply through vessel co-option, while lung cancers are characterized by early angiogenesis [21, 22]. Tumor-cell-induced barrier changes affect both astrocyte-endothelial and pericyte-endothelial interactions: astrocytic endfeet are displaced, while pericytes become hyperplastic [85, 86].

All these may affect TJs of infiltrated vessels. Early studies using brain implants of carcinosarcoma and electron microscopy revealed the presence of dissociated TJs [87]. Alterations of the vascular TJs in brain tumors seem to be dependent on the tumor type. In gliomas, the most frequent primary brain tumors, cell junctions of the capillaries are either short or elongated, while TJs have 2–7 strands, as shown with freeze-fracture technique. Changes are related to the malignancy grade, thus in GBM, endothelial TJs show a more altered morphology compared to those in benign astrocytomas. In nonglial tumors, TJs are composed of one or two strands and freeze-fracture investigations revealed that their structure is discontinuous [88]. In high-grade glioma, hypoxia-inducible factor 1 stimulates the production of VEGF. This leads to the breakdown of the pre-existing BBB structure, new vessel formation by several different mechanisms [89], and abnormal expression of membrane proteins, like RDC1/CXCR7 and plasmalemmal-vesicle-associated protein 1 (PV1) [90, 91]. In animal models of lung cancer and melanoma brain metastases, inhibition of sulfonylurea receptor 1 (SUR1), expression of which was significantly increased in the tumors, decreased endothelial ZO-1 gaps and halted increased BTB permeability [92]. An investigation focusing on peritumoral tissue in human glioma revealed that cells of the NVU displayed morphological alterations; however, they showed structurally normal TJs [93]. It is important to note that the reaction of the vasculature to brain tumors is heterogeneous. Not only different tumors affect the BTB differently, but also there are permeability differences even within the same tumor mass, both in primary and metastatic brain tumors [94, 95].

Permeability of the BBB and BTB is composed of multiple factors including paracellular permeability, transcellular permeability, and activity of efflux transporters. Paracellular permeability is largely determined by the TJs and affects mainly small hydrophilic molecules. Heterogeneity of permeability increase can be observed in brain tumors, as low-molecular-weight markers are distributed unevenly inside individual lesions, as well as among them [95]. Heterogeneous BTB permeability determines the growth rate of tumors. In a mouse melanoma model, increased vascular permeability to sodium fluorescein of individual brain metastatic lesions correlated with their growth [96]. In line with this, large size of brain metastatic lesions has been associated with increased BTB permeability [97], although recent findings failed to confirm the correlation between the size of the metastatic lesion

and the level of vessel leakiness [95, 98]. It has also been suggested that the BTB is more permeable in the tumor core in comparison to the periphery of the tumor [99].

Nevertheless, in Wnt-medulloblastoma, the fenestrated vasculature permits accumulation of administered therapeutic agents, rendering this type of tumor curable and benign. On the other hand, the intact barrier of Shh-medulloblastoma makes this tumor type resistant to chemotherapy [100]. Indeed, drug efficacy depends on BTB permeability. Distribution of ^{14}C-paclitaxel and ^{14}C-doxorubicin was followed in the brains from a mouse model of breast cancer brain metastasis. Uptake of these chemotherapeutic drugs was heterogeneous in the metastatic lesions, but higher than in the healthy brain tissue. However, average permeability of brain metastatic lesions to chemotherapeutic agents was almost an order of magnitude lower than that of peripheral tissues or peripheral metastases, and only reached the level of cytotoxicity in 10% of brain lesions [100]. Interesting results were provided by a study using quantitative fluorescence microscopy in preclinical models of primary and secondary brain tumors. Three molecules ranging from 100 Da to 70 kDa were found to permeate to the glioma tissue at rates proportional to their diffusion in water, suggesting a calculated vascular pore size of >140 nm in diameter. In contrast, in the breast cancer brain metastasis model, the calculated pore size of the vessels was approximately ten-fold smaller [101]. In line with animal model data, capecitabine and lapatinib penetrate to a significant though variable degree into human breast cancer brain metastatic tissues, as quantitatively determined from surgical specimen [102].

In order to identify mechanisms of junctional damage and permeability increase, changes in the expression of TJ proteins were investigated, showing that these are dependent on the tumor type [101]. In low-grade gliomas, there was no change in occludin expression compared to normal tissue, whereas in high-grade gliomas, 2/3 of the vessels were occludin negative. In metastatic adenocarcinoma vessels, occludin was absent [103]. A further study performed on cases of human glioblastoma multiforme indicated a significant downregulation of claudin-5 and occludin and an association of the TJ particles with the exocytoplasmic fracture face in freeze-fracture experiments [104]. Moreover, endothelial occludin expression was found to highly correlate to the development of peritumoral edema in human primary and metastatic brain tumors [105].

Beside clear indication of junctional damage, there is increasing evidence that the permeability increase is at least partially attributable to an increased transcellular permeability mediated by micropinocytosis, which can be independent of TJ opening [106]. On the other hand, TJ proteins may be regulated by elements of the transcellular pathway, for example, caveolin-1 [107].

Regulation of TJs in the BTB

Proteases, Cytokines, and Signaling Molecules Involved in the Regulation of BTB TJs

One of the primary mechanisms leading to an increased permeability of the vascular barrier is proteolytic degradation of junctional proteins. It has been shown that glioma-derived factors can increase endothelial MMP activity, which in turn downregulates occludin and claudins. One of the glioblastoma-derived factors involved in the upregulation of MMPs was suggested to be TGF-β [108].

Another important mediator of junctional damage in glioblastoma could be IL-17. In the CNS, IL-17 is synthesized by astrocytes and other cells as well (e.g., oligodendroglia and T helper 17 (Th17) cells). IL-17A was shown to correlate with BBB breakdown in nonmalignant brain pathologies [109]. GBM cells also expresses high amounts of IL-17A, which promotes their migration and local invasion via PI3K/Akt signaling and downregulation of ZO-1 in the tumor cells [110]. As MMP-2 and MMP-9 are widely regulated by IL-17, and MMPs can degrade junctional proteins, IL-17 could be involved in the BTB permeability increase observed in GBM [111].

Junctional proteins not only have structural roles in the TJs, but can also act as signaling molecules. In line with this, JAM-A has been shown to promote C/EBP-α expression through suppression of β-catenin transcriptional activity, and also through activation of EPAC (exchange protein directly activated by cAMP). On the other hand, C/EBP-α directly binds the promoter of claudin-5 to promote its transcription. However, in blood vessels of tissue biopsies obtained from patients with glioblastoma, JAM-A-C/EBP-α-mediated regulation of claudin-5 was shown to be lost [112].

Taken together, brain tumor cells secrete several factors, which can directly or indirectly regulate TJs of BTB endothelial cells.

Noncoding RNAs in the Regulation of the TJs of the BTB

Noncoding RNAs can also play an important role in the regulation of TJs, not only during extravasation of tumor cells, but also in already formed metastases or primary brain tumors. Especially miRNAs are important regulators of several steps of metastasis formation [64]. BTB endothelial cells express miR-181a, which leads to an impaired and permeability-enhanced BTB together with reduced ZO-1, occludin, and claudin-5 expression [113]. In addition, miR-18a was also shown to increase the permeability of BTB through the reduced expression of ZO-1, occludin, and claudin-5 [114]. While miR-181a caused these effects in a mechanism dependent on KLF6 downregulation, miR-18a controlled RUNX1 activity.

Recent studies indicate that circular RNAs are also able to regulate TJs under pathological conditions. From these studies, an interesting observation emerges: several circular RNAs upregulated in glioma BTB cells decrease junctional permeability. The circular RNA USP1 (circ-USP1) was found to reduce BTB permeability in an in vitro model, by regulating ZO-1, claudin-5, and occludin expression using a miR-194-5- and FLI1-dependent mechanism. Knockdown of circ-USP1 and upregulated miR-194-5 negatively regulated the transcription factor FLI1 and decreased transcription of TJ genes [115]. A similar regulatory mechanism was found in the case of the lncRNAs metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) and long intergenic non–protein-coding RNA 174 (linc00174), which were found to be highly expressed in microvessels of human glioma and glioma endothelial cells. Knockdown of either of the two led to an increased BTB permeability, as well as decreased ZO-1, occludin, and claudin-5 expression. In this process, miR-140 was involved with nuclear factor YA as a downstream target, for MALAT1, while for linc00174, the signaling partners were miR-138-5p, miR-150-5p, and FOSL2 [116, 117]. More specifically, knockdown of MALAT1 resulted in the upregulation of miR-140, consequent reduction in NFYA activity, and reduced TJ protein transcription [116, 117]. Similarly, knockdown of linc00174 resulted in the upregulation of miR-138-5p and miR-150-5p, consequent reduction in FOSL2 activity and reduced TJ protein transcription [116, 117]. LncRNA nuclear paraspeckle assembly transcript 1 (NEAT1) was also found to be increased in glioma endothelial cells. NEAT can bind miR-181d-5p. One of the downstream targets of miR-181d-5p is SOX-5 (sex-determining region Y-box protein 5), which can interact with the promoter region of ZO-1, occludin, and claudin-5. Thus, NEAT1 reduction can increase BTB permeability by targeting junctional proteins through SOX5 [111]. LncRNA TUG1 (taurine upregulated gene 1) also seems to play a role in the regulation of BTB. Its knockdown results in permeability increase caused by downregulation of ZO-1, occludin, and claudin-5 expression in cerebral endothelial cells via binding to miR-144 and heat shock transcription factor 2 (HSF2) [118].

Thus, noncoding RNAs including miRNAs and circular RNAs can be dysregulated in endothelial cells of the BTB regulating its permeability.

Cells of the NVU in the Regulation of the BTB

Astrocytes in Brain Tumors

Disruption of astrocyte-vascular coupling was observed in both primary and secondary brain tumors. Perivascular glioma cells adhere between astrocytic endfeet and vascular smooth muscle cells, displacing the astrocytic endfeet; thus, astrocyte-secreted factors no longer reach the vessels [85]. Similarly, in breast cancer metastases, astrocytes and astrocyte endfeet are gradually expelled from the vessels to the border of the growing tumor [22].

Astrocytes contribute through diverse mechanisms to permeability increase in tumors. Sphingosine-1 phosphate (S1P) receptor is upregulated in astrocytes in metastatic lesions of triple-negative breast cancer and mediates an increased permeability of the BTB by secretion of IL-6 and CCL2. Inhibition of S1P3-mediated IL-6 and CCL2 secretion strengthens the BBB and intensifies ZO-1 staining [119]. In glioblastoma, dislocation of aquaporin-4-containing orthogonal array of particles leads to compromised water transport and consequently to cytotoxic edema. Dislocation of aquaporin-4 might be probably due to the degradation of the proteoglycan agrin by MMP-3 and might lead to breakdown of the TJs and development of vasogenic edema [120]. Reduced expression of laminin α2 expression in the astrocytic basement membrane was also found to correlate with increased BTB permeability [121].

Regulation of the BTB by Pericytes

Brain metastatic cells tend to co-opt microvessels along their division in the metastatic environment. During this process, while endfeet of astrocytes are detached from vascular surfaces, pericytes are engulfed together with endothelial cells, as described in mouse and human breast cancer brain metastases [80]. Maintenance of pericytes in close association with the endothelium is probably crucial in the partial maintenance of barrier properties of endothelial cells of brain tumor vessels. Pericytes apparently regulate BTB permeability and this is largely dependent on their phenotype. When comparing low and highly permeable metastatic lesions of breast cancer, desmin-positive pericytes were mainly associated with a higher permeability. A higher endothelial permeability was also correlated with reduced CD13 positivity in pericytes [121]. This opens the possibility for the development of new therapies in which pericytes associated with tumors can be targeted specifically.

In nonbrain tumors, platelet-derived growth factor signaling (PDGF-BB-PDGFRβ) and pericyte-fibroblast transition have been shown to promote tumor growth and metastasis [122]. Pericytes express PDGFRβ, while endothelial PDGF-B is essential for pericyte recruitment [123]. Interestingly, PDGFB expression has been shown in metastatic breast cancer cells [4], suggesting a possible role of this signaling in brain-tumor-associated pericytes as well. In addition, hyperplasia of αSMA-expressing pericytes is characteristic to the tortuous vessels of malignant gliomas [86]. Abnormal vessels of GBM tumors are characterized by multilayered pericytes expressing specific NG2/CSPG4 isoforms [124]. GBM cells have been found to switch pericyte function from tumor-suppressor to tumor-promoter through diverse mechanisms. On the one hand, GBM cells employ Cdc42-dependent and actin-based cytoplasmic extensions, called flectopodia, to modify contractility of pericytes [125], induce expression of anti-inflammatory cytokines, and upregulate chaperone-mediated autophagy in pericytes to elicit their immunosuppressive function [126, 127].

As previously stated, the increased BTB permeability does not allow drugs to reach cytotoxic levels in the tumor mass. Thus, new antitumoral approaches are urgently needed. Targeting glioma stem cell (GSC)-derived pericytes in GBM resulted in disruption of BTB and increased drug effusion into brain tumors. Inhibiting the bone marrow and X-linked (BMX) nonreceptor tyrosine kinase in GSC-derived pericytes only targeted neoplastic pericytes, which disrupted the BTB but not normal pericytes of the BBB [128].

Microglia in Brain Tumors

Inflammatory processes and activation of microglia are also often associated with malignant processes of the brain. It has been demonstrated that microglial cells interacting with glioma cells secrete high levels of IL-6, which deteriorates endothelial barrier function by downregulating TJ proteins through the activation of the JAK2/STAT3 pathway in endothelial cells [129]. In metastases, inflammatory reaction is characterized by activation of both tumor-associated microglia and bone-marrow-derived macrophages [130], which are essential in the tumor development [82] and maintenance of an inflammatory milieu [131], which might influence the permeability of the BTB.

Traits of the BTB

In conclusion, the BTB is formed by vessels of primary or secondary brain tumors, and this barrier differs significantly from the intact BBB on both morphological and cellular levels. The BTB, although leakier in general than the BBB, shows a heterogeneous permeability with altered transporter and junctional protein expression. Reduced integrity of the barrier promotes cellular transmigration and also permeability to small and large molecules. Increased number of desmin-positive pericytes, decreased expression of laminin α2 in the basement membrane, and elevated expression of S1P3 in astrocytes are markers of increased BTB permeability. The BTB is diversely regulated by detachment of astrocytic endfeet and the phenotype of pericytes. Although most anticancer drugs are efflux transporter substrates, increased BTB permeability could be exploited in drug delivery strategies [99].

Conclusions

TJs of cerebral endothelial cells have two principal roles in brain tumor formation. By forming the first defense line of the BBB, TJs restrict the movement of metastatic cells from the circulation to the brain parenchyma. Therefore, by forming the

basis of the tightest endothelial barrier, cerebral endothelial TJs have a crucial role in determining the very low number of cancer cell types, which can form metastases in the CNS. Only tumor cells able to modulate and overcome continuous TJs or those, which are able to migrate transcellularly can successfully extravasate into the brain. Upon reaching the brain parenchyma, metastatic cells have to adapt to the very specific brain environment in order to survive and proliferate. Growing metastatic lesions co-opt existing vessels and use other forms of angiogenesis to form their own vasculature. These vessels, although still tighter than peripheral capillaries, are leakier than the intact BBB, and form the BTB. Similarly, primary brain tumors are also protected by the BTB. TJs of BTB-forming endothelial cells, together with other barrier mechanisms, restrict accumulation of chemotherapeutic agents in brain tumors. Understanding the specific mechanisms that drive changes in the BBB and BTB during brain tumor formation might pave the path toward the development of new therapies to either prevent or treat these cancers.

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Endocytosis of Tight Junction Proteins: A Pathway for Barrier Remodeling

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Abstract The tight junction complex is a central component of barrier tissues by occluding the paracellular space. Environmental and intrinsic signals constantly remodel this highly dynamic multiprotein structure. Endocytosis is a crucial regulator of tight junction dynamics, and it allows cells to adapt to stress/damaging stimuli. It has emerged as a central process for tight junction remodeling in physiological and pathological conditions. Tight junction protein endocytosis involves an interplay between signaling pathways and the vesicular machinery, and it is very cell type specific. This chapter summarizes current understanding of the molecular mechanisms involved in the endocytosis of tight junction proteins and the signaling pathways that regulate it. It discusses the role of tight junction protein endocytosis in barrier remodeling and maintenance in normal physiology and disease, and the potential to control tight junction protein endocytosis to enhance drug delivery. The chapter also addresses the relationship between tight junctions and cell polarity and how tight junction protein endocytosis may alter the latter with potential implications for cancer.

Keywords Endocytosis · Clathrin · Caveolae · Claudins · Occludin · Barrier remodeling · Barrier maintenance · Cell polarity complex

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L. González-Mariscal (ed.), *Tight Junctions*,
https://doi.org/10.1007/978-3-030-97204-2_13

Introduction

The tight junction (TJ) complex is a large multimeric and highly elaborated structure, arranged as multiple anastomosing strands on the cell lateral membrane [1, 2]. The fully developed and functional complex expressed in epithelial, endothelial, and Schwann cells provides two important features: a) it helps build a barrier that protects the organism from the environment, separates two different tissue compartments, and controls paracellular transport, and b) it determines cell behavior by controlling apicobasal cell polarization [1–3].

This multiprotein structure is composed from ~50 different proteins, organized in three structural groups. *Transmembrane proteins* include claudins (e.g., claudin-1, claudin-3, claudin-4, claudin-5), occludin, tricellulin, and MarvelD3 [tight-junction-associated Marvel proteins (TAMPs)], an IgG type of protein junctional adhesion molecule (JAM-A, JAM-B, and JAM-C), endothelial-cell-selective adhesion molecules (ESAMs), and coxsackievirus and adenovirus receptor (CAR). Homophylic and heterophilic cis- and trans-interactions of these membrane-spanning TJ proteins are the basis for adhesive properties and paracellular space occlusion. *Scaffolding proteins* comprise cytoplasmic plaque proteins, for example, *zonula occludens* ZO-1, ZO-2, ZO-3, afadin-6, cingulin, and cytoplasmic signaling molecules [e.g., protein kinase C λ (PKCλ)]. They are linked intracellularly to the transmembrane proteins and provide structural support and regulate the adhesive properties of the transmembrane proteins. *Actin cytoskeleton and actin-binding proteins* ensure attachment of TJ complex to the actin cytoskeleton and deliver essential physical support for the complex [1, 4, 5].

The morphological appearance and properties of the TJ complex in tissue barriers suggest a very stable seal of the paracellular space, providing a size- and charge-selective semipermeable barrier. The occlusive properties of the complex are determined mainly by claudin composition, which shows cell specificity and determines unique barrier characteristics [1, 6, 7]. In addition to regulating barrier formation, the TJ complex is an integral component of an evolutionarily conserved signaling mechanism that controls epithelial and endothelial-cell polarization, establishing two functionally distinct parts of the cell membrane, the apical and basal surfaces, and promoting intracellular polarity.

The TJ complex is not static but rather a very dynamic structure capable of remodeling in response to various external and intrinsic stimuli. Emerging evidence suggests that endocytosis plays a prominent role in such remodeling. Endocytosis is an active process of absorbing molecules by engulfing them with the cell membrane and moving them inside the cell. It requires extensive cellular machinery (membrane transport vesicles for internalization and the endo-lysosomal membrane system), signaling molecules (enzymes, adaptor, and motor proteins), and the actomyosin cytoskeleton [7]. Endocytosis is mostly implicated in responses to external stimuli, although it can occur under steady-state conditions enabling protein turnover. At the TJ protein/complex, endocytosis is considered a key regulator of cell-cell interactions, barrier properties, and remodeling.

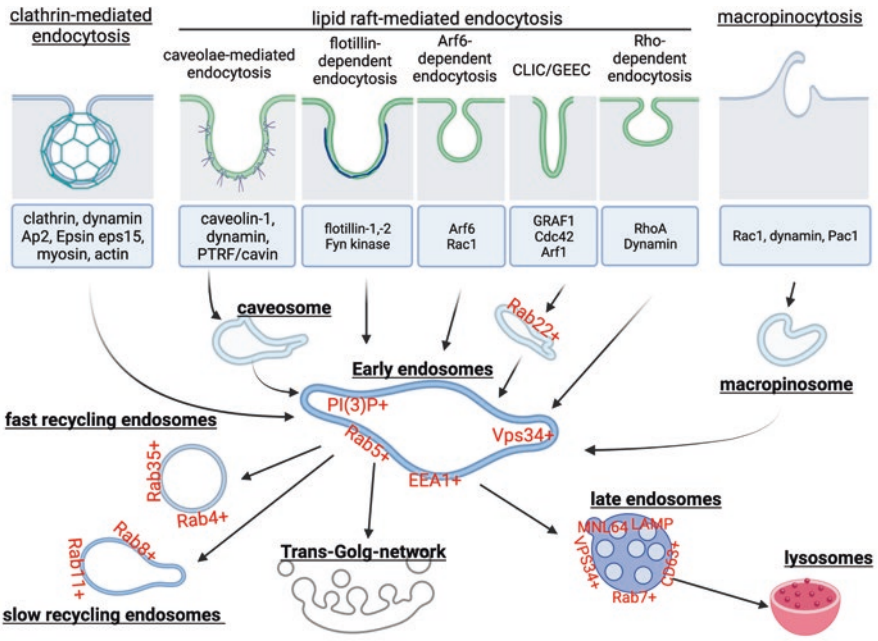

Fig. 1 Schematic summary of endocytosis pathways. This schematic was created using BioRender.com

Endocytotic Pathways and Sorting

Endocytosis is an adaptive cell mechanism that responds to extracellular and intracellular stimuli, controls cell function, shape, cell migration, and even cell defense. Generally, endocytosis initiates with the internalization of cargo from the extracellular space (fluid, nutrients) or membrane surface (transmembrane and membrane proteins, plasma membrane lipids). It continues with the incorporation of cargo into a tightly controlled network of endocytic vesicles with distinct phenotypes (distinct luminal and surface profiles) that allow differential modulation of intracellular pathways and delivery of cargo to distinct intracellular destinations [7, 8]. There are multiple pathways involved in endocytosis, as outlined below, and Fig. 1 summarizes their characteristics.

Internalization

The initial step in endocytosis is internalization. Cells use several internalization pathways generally classified into two categories: clathrin-dependent and clathrin-independent pathways [7, 8]. *Clathrin-dependent* internalization is mediated by

small clathrin-coated vesicles (CCVs) formed in plasma membrane domains termed clathrin-coated pits. CCVs are characterized by a triskelion coat containing three clathrin heavy and light chain proteins, specific profile of adaptor proteins [i.e., epsin, adaptor protein (AP) 1, -2)] and accessory proteins [N-BAR and BAR-domain containing protein amphiphysin, sorting nexin 9 (SNX9)] that coordinate CCV formation (clathrin nucleation, polymerization, stabilization) and budding off of vesicles from the plasma membrane (dynamin recruitment) [9, 10]. In clathrin-dependent internalization, CCV composition depends on many factors, including specificity of cargo (concentration, activation state, mobility in the plasma membrane) and adaptor/accessory protein (affinity to bind for cargo, concentration, and ability to interact with clathrin-polymerizing machinery) and trafficking destination [10].

Clathrin-independent internalization includes lipid-raft dependent [caveolae-, flotillin-, GTPase regulator associated with focal adhesion kinase-1 (GRAF1)-, adenosine diphosphate-ribosylation factor 6 (Arf6)- and RhoA-dependent endocytosis] and lipid-raft independent endocytosis (i.e., macropinocytosis) [7].

Caveolae-mediated internalization is a highly studied type of clathrin-independent internalization. This type of internalization occurs in specific membrane microdomains, called lipid rafts, rich in cholesterol and sphingolipids [11, 12]. In caveolae-mediated internalization, vesicles or tubulovesicles express a palmitoylated form of caveolin-1 protein that confers to the structure the ability to generate highly ordered oligomers and bind to cholesterol and fatty acid, supporting changes in membrane curvature. Other important components that build caveolae are the structural protein cavin-1/polymerase I and transcript release factor (PTRF), dynamin (involved in tethering and plasma membrane removal of vesicles) and the signaling kinases, Src kinase [that phosphorylates caveolin-1 tyrosine residue (Y14) facilitating caveolae biogenesis], RhoGTPAse (i.e., Cdc42), and protein kinase-C (PKC) (that regulate the process of internalization) [13–15].

Flotillin-mediated and *caveolin-mediated internalization* share similarities, such as the formation of raft microdomains and vesicles enriched in protein flotillin-1 (reggie-2) and -2 (reggie-1), proteins that make hairpins in the plasma membrane [16]. The Src family kinase Fyn and dynamin regulate flotillin-mediated internalization [17].

Other subgroups involved in clathrin-independent and caveolae-independent internalization are closely associated with specific membrane nanodomains enriched in certain lipids, such as cholesterol [18]. For example, *Rho-A dependent internalization* requires a particular membrane nanodomain-containing Rho GTPases, RhoA, and Rac1, which are involved in the initiation, internalization, and vesicle formation distinct from other types of endocytosis. The p21-activated kinases, Pak1 and Pak2, and dynamin are implicated in regulating Rho-A dependent internalization [18, 19]. Another GTPases, the ADP-ribosylation factor (Arf), regulates clathrin-dependent internalization (Arf1) and vesicle sorting and recycling (Arf6). It also participates in a different type of clathrin−/caveolae-independent internalization [20, 21]. In *Arf6-dependent internalization*, Arf6 (an Arf class III member) interacts with phosphatidylinositol-4,5-bisphosphate (PIP2) to from vesicles/tubules that are dynamin independent [7, 22].

A further non-clathrin-dependent and non-caveolin-dependent type of internalization is the *CLIC/GEEC (CG) pathway* [clathrin- and dynamin-independent carrier (CLIC)], which form glycosylphosphatidylinositol (GPI-AP) enriched early endosomal compartments (GEEC)]. This type of vesicle is indicated in the internalization of lipid-anchored proteins (GPI-Aps) and does not use any protein coat [23, 24]. The endocytosis is dynamin independent and results in tubulovesicular carriers called CLIC, derived from the cell surface and capable of internalizing fluid phase to the cell. The regulator/supporting proteins for CLIC/GEEC endocytosis include Arf1, ARHGAP10/21, Rho GTPase CDC42, as well focal adhesion kinase-1 (GRAF1) with RhoGAP-, BAR-, and SH3-domains for clustering regulatory molecules [24, 25].

Macropinocytosis is a lipid-raft-independent type of internalization characterized by the formation of a pocket-like structure mainly containing polymerized actin filaments. These vesicles mostly endocytose large nonselective cargo (extracellular fluid and molecules; >1 μm) [26, 27]. Like caveolae-dependent internalization, micropinocytosis depends on dynamin, but has unique machinery including Ras GTPase, phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) enzymes, sorting nexin (SNX) family adaptors (e.g., (SNX5)), and motor proteins [27–30].

Endosomal Vesicles

Internalized cargo moves through dynamic and interconnected membrane-bound endosome compartments that identify, collect, sort, and transfer a cargo to its final destination, where it can perform its function, be degraded, be recycled, or sent to the trans-Golgi network [31]. In general, different types of internalization share similar pathways in the endosomal network, although differences in endosome phenotype characterize particular types of endocytosis. Endosomes are classified as early, late, or recycling, based on luminal pH, alterations in phosphatidylinositol lipids, and differential recruitment and activation of members of the Ras superfamily of monomeric G proteins (Rab-GTPases) as well members of the ADP ribosylation factors (ARFs), family of small GTPases, like Arf1 or Arf6 [21, 32–35].

Early endosomes (enriched in Rab5+, EEA1+ phosphoinositide kinase Vps34+, phosphatidylinositol 3-phosphate PI(3)P+) represent the central sorting "station" for cargo and are a center of decision making [36–38]. Early endosomes arise by fusion and accumulation of internalized vesicles/cargo until endosomes reach a limit in size. The process that determines endosome size is ill-characterized. Cargo in the endosomes then undergoes further maturation and sorting, before delivery to its final destiny [36, 39]. The sorting process is active and complex, and depends directly upon endosomal pH and geometry, the type of cargo, existence of sorting signals [i.e., lipid-binding motifs including BAR domain and nexin family (SNXs) protein binding domain], supporting adaptor proteins like adaptor protein phosphotyrosine interacting with PH domain and leucine zipper 1 and 2 (APPL1, APPL2), membrane proteins, [i.e., Soluble N-ethylmaleimide attachment protein receptor (SNARE)] for fusion events in membrane trafficking and signaling molecules (i.e.,

PtdIns3P kinase, VASP34, Rab subfamily GTPases, Rab5, Rab5 effector Rabenosyn-5, Rab4) [35–37, 40, 41]. For clathrin-independent endocytosis, transforming steps between internalization and early endosomes include caveosomes and macropinocytotic vesicles, while CLIC/GEEC pathways also include Rab22 as an additional regulator of sorting [26, 41–43].

Recycling endosomes contain a network of three different types of recycling routes: a fast Rab4-dependent route, another slow Rab8/Rab11 route, while the last one is ARF6 dependent [8, 44] These differences in recycling route depend on the cargo as well recycling capacity, which is determined by the phenotype of the vesicles [recycling motif on the C terminal (i.e., PDZ barcode)], Rab proteins (Rab4, Rab8, Rab11, Rab35 in some cases), translocation carrier (i.e., nexin protein SNX4, SNX17, ACAP1 BA2R), retromer complex (VPS26, VPS29, and VPS35) for selection of cargo, epsin (clarthin-independent recycling) for the oligomerization and formation/stabilization membrane tubules [22, 35, 41, 45]. Estimations indicate that cells recycle back ~50% of newly endocytosed material to plasma membrane.

Early endosomes generate late endosomes [Rab7+, VPS34+, focally highly enriched in lysosome-associated membrane protein 1 (LAMP1) and metastatic lymph node gene 64 protein (MLN64), enriched in lysobisphosphatidic acid, mannose-6-phosphate receptor+, CD63+) [31, 35, 42, 46]. This transformation includes an increase in luminal acidification, endosome movement from the cell periphery toward a juxtanuclear localization, and the switching of two key endosome identity cues, Rab5 to Rab7 [31, 39].

One of the processes associated with clathrin-independent endocytosis is ubiquitination and proteasomal degradation. ESCRT ubiquitin recognition system and regulatory E3 ligases mediate ubiquitination of the cargo, which proceeds then from early endosomes toward proteasomes for degradation [47–50].

Other sorting pathways include bulk flow recycling and endosome-to-trans Golgi network (TGN) retrograde transport. The first occurs in narrow tubule-like formations with large surface areas that experience continuous fission and recycling. This type of sorting participates in recycling nutrient receptors to the cell surface in conditions of exceptional need [51]. A similar complex occurs in Endosome-to-TGN retrograde sorting. The vesicles share some phenotypes with clathrin-dependent endocytosis. Some of the clathrin pathway adaptor proteins, AP1, PACS1, epsinR, and SNARE, are associated with this pathway as well as Rab13 [52]. The sorting occurs between TGN-endosome and an endosome-TGN retrieval pathway, and it participates in biosynthetic processes [53–55].

TJ Protein Endocytosis

Endocytosis is a pivotal process that finely regulates the TJ complex in response to intrinsic and extracellular stimuli, maintains cell homeostasis, and imparts dynamic characteristics to the barrier. Internalization and intracellular trafficking of TJ proteins may in synchrony affect the bulk TJ complex or specific TJ proteins. This

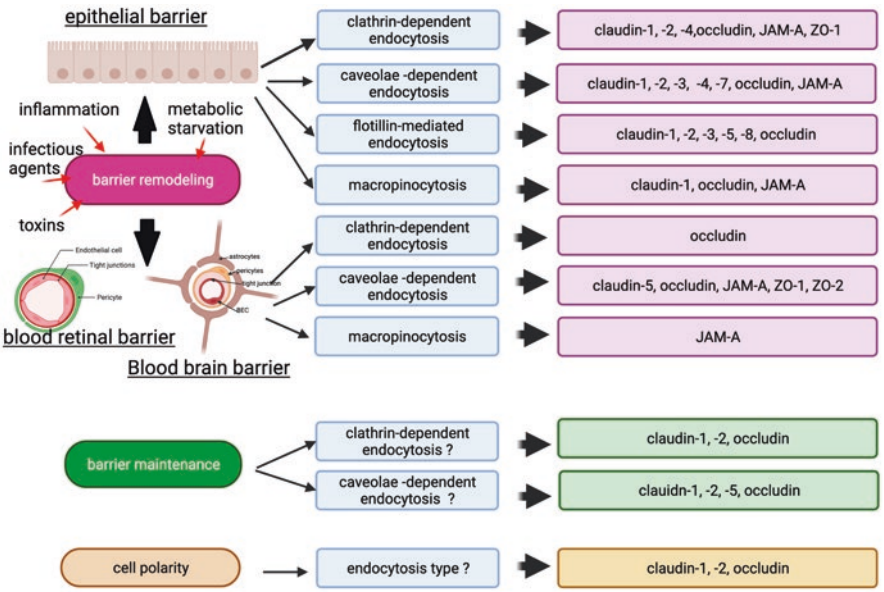

Fig. 2 Summary of the endocytosis pathways involved in uptake of TJ proteins during barrier remodeling, barrier maintenance, and regulation of cell polarization

enables barrier remodeling, contributes to barrier healing during stress and pathological conditions, regulates/maintains the junctional complex in physiological condition, and establishes cell polarity. In light of these essential processes, endocytosis of TJ proteins will be further addressed. The endocytosis pathways involved in TJ protein uptake and their consequences are summarized in Fig. 2.

Endocytosis of TJ Proteins in Barrier Remodeling

In response to a wide range of stimuli (e.g., inflammation, toxins, infectious agents, stress, and metabolic starvation), TJ complexes undergo rapid removal with redistribution of transmembrane TJ proteins from the lateral membrane to the cytoplasmic compartment, resulting in a loss of barrier properties and decreased expression of TJ proteins. This process depends on the type and duration of the signal(s) and can ultimately lead to barrier adaptation to the new condition.

Cytokines are potent stimulators of TJ protein endocytosis in endothelial and epithelial cells, contributing to the barrier remodeling and dysfunction in many inflammatory conditions. For example, tumor necrosis factor-α (TNFα) contributes significantly to Crohn's disease and several ischemia and immune-mediated intestinal diseases, promoting intestinal epithelial barrier dysfunction [56]. In this regard, TNF-α causes retraction of occludin and claudin-2, claudin-3, claudin-4, claudin-7

from the membrane in enterocytes [57–60]. Furthermore, interferon-γ (IFN-γ) and LIGHT (lymphotoxin-like inducible protein that competes with glycoprotein D for herpes virus entry on T cells) mediate endocytosis of occludin in intestinal epithelial cells (T84 cells); TGF-β3 induces internalization of JAM-A and occludin in Sertoli cells; chemokine CCL2 induces occludin, claudin-5, and JAM-A internalization in brain endothelial cells; vascular endothelial growth factor (VEGF) upregulates occludin endocytosis in retinal endothelial cells, while epidermal growth factor (EGF) induces endocytosis of claudin-2 in MDCK cells [32, 61–66]. The endocytic pathways involved in such barrier remodeling include predominately caveolin-dependent endocytosis (TNF-α, CCL2), macropinocytosis (INF-γ, CCL2 specifically for JAM-A), and clathrin-dependent endocytosis (VEGF, EGF, TGF-β) [32, 61, 63, 64, 66–69]. In most cases, the type of endocytosis depends on the type of cells and membrane microdomains and TJ protein properties rather than the type of cytokine.

Infectious agents are also potent regulators of barrier dysfunction via endocytic removal of TJ proteins. Binding of hepatitis C virus particles to glycosaminoglycans and/or lipoprotein receptors is associated with coordinated interactions with scavenger receptor class B type I, a major receptor of high-density lipoprotein, the CD81 tetraspanin, and the TJ proteins claudin-1 and occludin, which induce clathrin-dependent endocytosis of those two TJ proteins [70–73]. West Nile virus induces barrier dysfunction via clathrin- and dynamin-dependent endocytosis of claudin-1 and occludin, while cytotoxic necrotizing factor-1, a toxin from *Escherichia coli,* causes endocytosis of occludin via caveolin-dependent internalization [74, 75]. Similarly, enteropathogenic *Escherichia coli* (EPEC) induces barrier dysfunction and endocytosis of occludin, ZO-1, and JAM-A via caveolae- and flotillin-dependent internalization [16, 58, 74, 76]. Although flotillin and caveolin have distinct localizations and do not physically associate, they may cooperate in controlling TJ protein internalization [16]. An intriguing finding in EPEC-induced caveolae-dependent internalization is the involvement of polarity complex components Crb3, Pals1, aPKCζ, as well sorting nexin 9 (SNX9), in endocytosis of JAM-A and occludin [74, 76]. *Toxoplasma gondii* shows high dissemination and migration properties across biological barriers, infecting immunologically privileged organs by inducing TJ proteins internalization, triggered by the ESPs proteases [77]. Coxsackievirus modulates barrier function and occludin internalization by macropinocytosis in concert with caveolae-mediated endocytosis [78]. The trans-activator of transcription (Tat) protein from HIV induces occludin, ZO-1, and ZO-2 alterations in brain endothelial cells via a caveolae-dependent pathway [79]. *Clostridium difficile* toxins, TcdA and TcdB, increase paracellular permeability, mediating TJ disassembly via caveolae-mediated occluding internalization [80]. It is important to highlight that internalization of TJ proteins in the presence of infective agents may result from them acting as viral receptors as with occludin (Coxacievirus B, Hepatitis virus C, West Nile virus) and claudin (Hepatitis virus C, West Nile virus), or as consequence of intracellular signaling after cell exposure to the infective agent. The type of endocytosis/ internalization pathway engaged depends on the coreceptors involved,

ongoing cellular processes, and often cross-interaction between internalization pathways.

Toxins, like ethanol and methamphetamine, induce barrier remodeling via endocytosis of TJ proteins. Ethanol causes occludin endocytosis in intestinal epithelial cells via a macropinocytosis-like process. On the other hand, methamphetamine instigates occludin internalization in brain endothelial cells, an effect mediated by the actin-related protein 2/3 (Arp2/3) complex, which causes brain endothelial barrier dysfunction, neurotoxicity, and neuroinflammation [81, 82].

Cell metabolic status can induce TJ endocytosis and barrier remodeling, allowing cells to fine-tune paracellular permeability during metabolic starvation. Nutrient-starvation-induced claudin-3 and -4 endocytosis via dynamin-dependent, but clathrin- and caveolae-independent pathways in IPEC-J2 cells, a process reversed by amino acid supplementation [83]. Alterations in oxidative metabolism (ischemia or hypoxia) or nitric oxide can induce claudin-5 internalization in brain endothelial cells primarily via caveolin-dependent pathways [84].

Factors, such as hypotonic stress, high glucose, Ca^{2+} depletion, and dysregulation of the Na^{+}, K^{+}-ATPase ion pump, induce endocytosis of claudin-1 and claudin-2 in renal tubular epithelial cells (clathrin-dependent pathways), claudin-1 and occludin in retinal pigment epithelial monolayers (caveolae-mediated endocytosis), occludin, JAM-1, ZO-1, claudin-1, and claudin-4 in colonic T84 cells (clathrin-mediated endocytosis), and claudin-1, claudin-2, claudin-4 in MDCK cells [68, 85–88].

Endocytosis of transmembrane proteins presumably plays a role in long-term barrier dysfunction or adaptation to new conditions generated by pathological stimuli. Stimulus type (e.g., cytokines, viruses, or toxins) is the predominant trigger of endocytosis, while the endocytic mechanism is dependent on cell type and TJ cargo. Most epithelial and brain endothelial cells have lipid raft microdomains that support TJ protein localization and barrier formation. Thus, it is not surprising that TJ proteins utilize available caveolae- and lipid-raft-dependent endocytic machinery. An exception is retinal pigment epithelium that lacks lipid raft microdomains and utilizes clathrin-dependent internalization of TJ proteins [89]. Furthermore, TJ proteins could favor types of internalization. Occludin possesses a C-terminal coiled-coil /ELL (OCEL) domain, accounting for interaction with caveolin-1, prompting the caveolae-internalization on TNF-α exposure [90]. For other TJ proteins (e.g., claudins and ZOs), no specific binding motifs have been identified. However, there may be interactions of TJ proteins with vesicle adaptor proteins, as was indicated for ZO-2, and ZO-3, which possess a binding motif for SNX27 on their PDZ domains [86].

How do TJ proteins internalize during barrier remodeling? A hypothetical paradigm assumes that the first step is an alteration in the status of TJ transmembrane proteins as well alterations in the interaction with scaffolding proteins. Activation of different types of kinases (e.g., PKCs, MAPK, Rho kinase) is often an initial step in the dissociation of the TJ complex, mirrored in changes of the TJ protein phosphorylation to loss of interaction with ZO-1 and the actin cytoskeleton that provide key structural support for the adhesive properties of transmembrane proteins [15,

64, 85, 91–93]. Phosphorylation could potentially uncover binding motifs and interaction sites for endocytosis machinery, prompting TJ protein internalization. Although it is commonly believed that the TJ complex is removed intact from the cell membrane followed by later dissociation of TJ proteins, the diverse endocytosis pathways involved for different TJ proteins under the same stimulus favor a dissociation step prior to internalization.

Internalized TJ proteins are further directed toward the endosome system with a first stop in early endosomes, sorting and redirection to late endosomes, and, finally, delivery into lysosomes. Although the processes of sorting and endosomal trafficking of TJ proteins remain largely unknown, several studies pinpoint some routes. Internalized occludin and claudin-5 and claudin-1 are found in Rab5+ endosomes despite different internalization pathways [63, 78, 94]. The expression of dominant-negative Rab5-trapped occludin at the TJ, and constitutively active Rab5, led to the retention of occludin in cytoplasmic vesicles [78, 95, 96]. Similarly, JAM-A and occludin, after macropinocytotic internalization and accumulation in Rab34+ vesicles, are directed toward Rab5+ endosomes [64, 78]. As for a sorting process dependent on the interaction of cargo with specific microdomain in Rab5+ endosomes, there is a lack of evidence about specific binding motifs on any TJ proteins that could recognize microdomains on Rab5+ endosomes or specific regulatory and adaptor proteins [96]. The final destiny of endocytosed TJ proteins during barrier remodeling is either late endosomes and degradation or in some instances recycling endosomes. Claudin-2 is sorted via Rab14+ endosomes to late endosomes and lysosomes preventing claudin-2 inclusion in the TJ complex and barrier leakage, while claudin-1 localization in late endosomes associates with E3 ubiquitin ligase ligand of Numb-protein X1 (LNX1p80) [49, 97]. Claudin-3 and 4 in jejunal IPEC-J2 cells, as well claudin-1 in renal tubular epithelial cells, are sorted to lysosomes and degraded during metabolic induced TJ protein endocytosis [83, 85]. In addition, sorting toward lysosomes and degradation may intersect with other mechanisms of cargo removal like autophagy. Thus, autophagy-related ATG6/beclin-1 is involved in multiple vesicle trafficking/endocytic pathways and forms a complex with occludin, providing an additional mechanism of occludin endocytosis and increased targeting of occludin to lysosomes [98]. This may result in reduced total occludin levels during barrier remodeling.

The other destiny of endocytosed TJ proteins is the recycling route (slow or fast), which is dependent on the set of regulator proteins rather than on cargo and endosomal domain [44]. Dynamic and rapid barrier remodeling involving recycling of occludin, claudin-1, and claudin-2 occurs in a range of epithelial cells (kidney, colon, and lung) [57, 87, 99–101]. In brain endothelial cells, during rapid CCL2-induced endocytosis, claudin-5 and occludin were mostly associated with recycling endosomes (80%), while 20% was associated with Rab7 [63]. Due to the focal and brief effect of CCL2 on leukocyte chemoattraction, the high rate of occludin and claudin-5 recycling may represent transient barrier remodeling for leukocyte transmigration. An intriguing case is JAM-A recycling during barrier remodeling. JAM-A has a dual role in regulating barrier integrity and leukocyte adhesion. During inflammatory remodeling of the brain endothelial barrier (exposure to CCL2, LPS,

or ischemia/reperfusion condition), JAM-A rapidly recycles from the TJ complex toward to the luminal membrane, where it gains a role as a leukocyte adhesion molecule directing leukocyte transmigration [64, 102].

TJ proteins mostly recycle through the classical pathway involving Rab4+ and Rab11+ endosomes. However, there are some exceptions. Claudin-1 recycling depends on Endosomal sorting complex required for transport (ESCRT), which participates in the trafficking of transmembrane proteins to the lysosome, as well in autophagy and TGN trafficking [103]. Another example is claudin-16, where recycling depends on Rab11 and STX8 in kidney epithelial cells. Similarly, Rab13 plays a role in postendocytosis recycling of occluding to the membrane in cells treated with testosterone [45, 53].

TJ protein endocytosis is an efficient mechanism to adapt barriers to various physiological or pathological conditions. Mechanisms and extent of TJ protein endocytosis differ based on cell type and stimuli. Identifying and dissecting the endocytosis pathways involved in TJ protein uptake during barrier remodeling may elucidate new directions for efficiently maintaining TJ complex function in pathological conditions. There is currently no evidence of TJ proteins recycling toward the TGN network or recycling pathway via the bulk flow pathway.

Endocytosis in Barrier Maintenance

Besides remodeling barriers in response to pathological stimuli, endocytosis is important in maintaining the barrier and trafficking of TJ proteins in steady-state conditions by enabling the continuous renewal of junctional proteins. Occludin, claudin-1, and claudin-2 undergo constitutive exchange between membrane and cytoplasmic pools with a cyclic process of exocytotic delivery of newly synthesized or recycled junctional proteins to the cell surface and the removal of protein components of mature TJs via endocytosis [53, 57, 99, 100]. The proposed model for the renewal of TJs without affecting TJ stability is based on a process of accumulation of newly synthesized claudins at the strand breaks or free edges of strands on the basal side of TJs, followed by the endocytosis of the old claudins and fast incorporation of newly synthesized TJ proteins [104]. This can occur without losing cell-to-cell contact and causing paracellular barrier disruption.

There are questions awaiting elucidation. How does endocytosis of TJ proteins occur in steady-state conditions and are different endocytic pathways involved from those changing barrier function? Do individual TJ proteins use common pathways? Thus far, Rab3b + and Rab13+ endosomes, and ESCRT with Vps24/CHMP3, which bind the phosphoinositide PtdIns(3,5)P2, are part of the machinery that recycles TJs proteins [55, 99]. An important factor that may regulate this process is the half-life of TJ proteins, which varies from 1.5 to 13 hours for claudins (claudin-5, claudin-2, claudin-4) and occludin, dictating endocytosis and recycling dynamics [104, 105].

Endocytosis in the Regulation of Cell Polarity

The cell polarity complex plays a key role in establishing and maintaining functional barriers. Polarity complex proteins are categorized in three modules: Crumbs (Crumbs/Stardust/PATJ), Scribble (Scribble/Discs Large/Lethal Giant Larva), and PAR (Par6/Par3/aPKC) [106–108]. They regulate cell polarization in the apical-basal and anterior-posterior axes and in the plane of the tissue (planar cell polarity) [106–108]. There is a close relationship between TJ and polarity complex proteins, particularly among claudins, JAM-A, and ZO-1 in epithelial and endothelial cells and the apical-basal and cell planar complex [109–111]. Endocytosis also exists in a flexible, reciprocal relationship with polarity regulators [112]. It plays an important role in the proper localization of key polarity proteins, while some polarity proteins can also regulate endocytosis machinery [106, 112]. Recent evidence indicates intersections between the cell polarity complex and TJ endocytosis. Thus, in establishing and maintaining TJ complexes, TJ protein endocytosis and recycling can be modulated by polarity complex proteins, while in cell migration, changes in polarity induced by endocytosis of TJ proteins prompt cells into a migratory phenotype [99, 113]. Some recent evidence demonstrates the role played by TJ protein endocytosis in alterations in cell polarity. For example, Rab14 regulation of claudin-2 and occludin endocytosis is implicated in regulating MDCK cell polarization and cyst formation [57]. Opposite emerging evidence indicates that polarity complex proteins, such as ESCRT, Crumbs, and Myo5b, regulate claudin-1 and claudin-2 endocytosis [99, 113, 114]. Further studies should, hopefully, shed more light on the interactions between TJ protein endocytosis and the polarity complex as crucial cell processes.

Crossover Endocytosis

A separate type of endocytosis can occur where claudins and occludin in one cell are taken up into neighboring cells via large, double-membrane vesicles (crossover endocytosis). This uptake is via clathrin- and caveolin-dependent, dynamin-independent endocytosis, and it is closely associated with the autophagosomal pathway [115]. TJ remodeling via crossover endocytosis represents a general mechanism for degrading transmembrane proteins at cell-cell contacts and directly links junctional membrane turnover to autophagy.

Signaling Pathways Involved in Endocytosis of TJ Proteins

Multiple signaling pathways are involved in regulating endocytosis, controlling every step in internalization and postinternalization sorting. While general principles of endosome-based signaling are emerging, how signaling impacts the

endocytic sorting process is still poorly understood. The signaling pathways involved depend on the type of internalization. In clathrin-dependent internalization, MAP kinases (Mek/ERK1/2 in concert with Akt), protein kinase A (PKA), protein kinase C (PKC), and RhoGTPase (RhoA-ROCK, Cdc42) are involved [116–119]. In caveolae-mediated endocytosis, MAP kinase ERK1/2 and p38, in concert with c-Jun N-terminal kinase (JNK), PKC-α, small GTPase RhoA (RhoA, Rac1 Cdc42), and tyrosine kinases (c-Abl, Src, and Fyn), are important [120–125], whereas Rho GTPases regulate flotillin-mediated internalization and Rac-GTPase-, myosin light chain kinase (MLCK)-, and Ras-dependent actin signaling regulate micropinocytosis [28, 126, 127]. Endocytic sorting involves a variety of signaling pathways important for regulating cargo recognition and membrane fusion: SNAREs, recruitment of cargo in endosomes (nexin SNX), ESCRT, and Rab GTPases [40, 99, 128].

Regulation of TJ protein endocytosis occurs at several levels. To initiate endocytosis, both modification of TJ proteins (cargo) for retraction from the TJ complex and assembly of the vesicular network need to be synchronized. Thus, regulatory signaling pathways act on two targets: TJ proteins and vesicle organization. Initiation of TJ protein endocytosis/internalization requires posttranslational modification of TJ proteins, which could be essential for binding of those proteins to endocytic adaptors/ vesicular structural proteins and retrieval of TJ proteins from the TJ complex.

The major posttranslational TJ protein modification that prompts internalization is phosphorylation. Examples include cAMP-dependent protein-kinase-mediated phosphorylation at T192 of claudin-3; EPH receptor A2 (EphA2) tyrosine-kinase-induced phosphorylation of Tyr-208 in the cytoplasmic tail of claudin-4 causing internalization after calcium switch; hypotonic-stress-induced phosphorylation on T191 of claudin-1; and S208 phosphorylation of claudin-2 via MAPK kinase p38 and subsequent dephosphorylation in clathrin-dependent internalization [85, 129, 130]. Occludin phosphorylation at Ser^{490} by PKCβ prompts clathrin-dependent internalization in retinal endothelial cells, enabling ubiquitination of Ser^{490}-phosphorylated occludin and direction to clathrin-dependent endocytosis [81]. Myosin light-chain kinase (MLCK) is important in regulating occludin endocytosis in intestinal anoxia/reoxygenation injury and TNF-α- and LIGHT-induced caveolae-mediated internalization [62, 93]. RhoA and Rho-associated kinase (ROCK) regulate endocytosis of epithelial TJ proteins and JAM-A micropinocytosis [64, 93]. C-Jun N-terminal kinase modulates caveolae- and clathrin-dependent endocytosis and macropinocytosis of occludin, while MAP kinase ERK1/2 regulates internalization and degradation of claudin-2, claudin-4, occludin, and ZO-1 [88, 131, 132]. RabGTPases are also important regulators of the endocytosis. Rab activation (i.e., Rab5, Rab4, Rab11, Rab7, Rab13, Rab14) is closely associated with endosome organization guiding the endosomal sorting of TJ proteins to their final destination [63, 64, 87, 97]. Ubiquitin ligases have an important role in determining destiny after internalization. Occludin and claudin-1 undergo ubiquitination by Ube2j1 (ubiquitin-conjugating enzyme E2 J1) and ubiquitin ligases p80 isoform of the E3 ubiquitin ligase ligand of Numb-protein X1 (LNX1p80), respectively, that leads to TJ disassembly and internalization [49, 133].

It should be noted that all steps in endocytosis are tightly tuned. Even though the inducer of TJ protein endocytosis and the cell response to such endocytosis may differ between conditions and cells, there are commonalities in regulatory signaling pathways. Those include the MAP kinases ERK1/2 and JNK, which are often involved in cell stress responses. As signaling pathways are potential therapeutic targets for regulating TJ protein endocytosis and thus barrier function, an in-depth understanding of the signaling pathways involved is an important goal for treating disease conditions.

Diseases Associated with Endocytosis of TJ Proteins

Endocytosis of TJ proteins has a role in the pathogenesis of many diseases, particularly those associated with inflammation as well in cancer. Diseases such as inflammatory bowel disease (IBD), Crohn's disease, ulcerative colitis, and celiac disease are characterized by relocalization of occludin and JAM-A to subapical vesicle-like structures in mucosal biopsies, mostly due to their internalization [134, 135]. In experimental models of IBD, dextran sulfate sodium (DSS)-induced colitis, trinitrobenzenesulfonic-acid-induced colitis, intestinal epithelial barrier anoxia/reoxygenation injury, and in a mouse model of necrotizing enterocolitis (NEC), occludin, claudin-4, and claudin-3 are displaced from the TJ complex and internalized, causing barrier dysfunction and exaggerating ongoing inflammation [135–137]. In addition, mutations in the autophagy-related genes, *ATG16L1* and *IRGM,* are substantiated risk factors for Crohn's disease [138]. This may further implicate the importance of TJ protein endocytosis in disease progression as there is overlap in endocytic and autophagic machinery in regulating intracellular trafficking (early endosomes, clathrin pits, caveolin-1). Similarly, inflammation associated with traumatic brain injury and stroke trigger intense endocytosis of TJ proteins claudin-5 and JAM-A at the blood-brain barrier, accelerating neuroinflammation and edema formation [102, 139, 140].

In cancer, progression of epithelial-to-mesenchymal transformation (EMT) is associated with TJ protein removal from the plasma membrane, mostly through internalization. The retraction of ZO-1, occludin, and specific claudins from the TJ complex via endocytosis is accompanied by loss of cell polarization, transforming cells toward a more migratory and invasive phenotype in breast, gastric, colon, and other types of cancer [141–143]. This finding not only opens avenues for targeting TJ protein endocytosis as a potential cancer treatment but may also unveil molecules bearing prognostic value.

TJ Protein Endocytosis and Drug Delivery

While preventing TJ protein endocytosis and thereby barrier disruption may be important in different diseases, tissue barriers may also limit access of therapeutics to treat disease. This raises the question of whether manipulating TJ protein

endocytosis could be used to enhance drug delivery? This is particularly important for drug delivery to the brain due to the unmet need for a delivery system that will efficiently breach the blood-brain barrier. Inducing barrier opening by triggering endocytosis of TJ proteins might rapidly destabilize transmembrane protein interaction and allow passage of drugs via the paracellular route. This opening may be transient due to the recycling ability of TJ proteins. Recent publications on claudin-1 and claudin-5 using specific peptides to target TJ protein endocytosis show the feasibility of this approach [144]. Those peptides can bind to the extracellular loop of claudins, triggering claudin internalization and barrier disruption. Another potential approach might be linking those peptides to a therapeutic agent so that they will be internalized with claudin into the endothelium and may gain access to brain. Future studies should further explore the TJ as a potential carrier for drug delivery.

Conclusion

It seems highly likely that TJ protein endocytosis is a pivotal process in barrier remodeling in response to physiological and pathological conditions. Such remodeling in inflammatory and metabolic diseases states can induce barrier dysfunction, aggravating injury. Similarly, in cancer, alterations in cell polarity due to endocytosis of TJ proteins can change cell phenotype, impacting disease progression. Future characterization of the mechanisms of TJ protein endocytosis will provide insight into specific disease pathogenesis and potentially reveal new targets for recovery of barrier function. Equally important is understanding the role of TJ protein endocytosis in physiological conditions and barrier maintenance. Regulation of barrier integrity and permeability by the TJ complex makes transmembrane TJ proteins an attractive target for enhancing drug delivery, particularly across the blood-brain barrier. Targeting transmembrane proteins like claudins for rapid and transient barrier opening via endocytosis/internalization represents a promising tool for regulating paracellular permeability and efficient drug delivery.

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L. González-Mariscal (ed.), *Tight Junctions*,
https://doi.org/10.1007/978-3-030-97204-2