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Review article

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Paracellular permeability and tight junction regulation in gut health and disease

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Abstract

Epithelial tight junctions define the paracellular permeability of the intestinal barrier. Molecules can cross the tight junctions via two distinct size-selective and charge-selective paracellular pathways: the pore pathway and the leak pathway. These can be distinguished by their selectivities and differential regulation by immune cells. However, permeability increases measured in most studies are secondary to epithelial damage, which allows non-selective flux via the unrestricted pathway. Restoration of increased unrestricted pathway permeability requires mucosal healing. By contrast, tight junction barrier loss can be reversed by targeted interventions. Specific approaches are needed to restore pore pathway or leak pathway permeability increases. Recent studies have used preclinical disease models to demonstrate the potential of pore pathway or leak pathway barrier restoration in disease. In this Review, we focus on the two paracellular flux pathways that are dependent on the tight junction. We discuss the latest evidence that highlights tight junction components, structures and regulatory mechanisms, their impact on gut health and disease, and opportunities for therapeutic intervention.

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Key points

- Increased intestinal permeability occurs in a wide range of disorders, including inflammatory bowel disease, coeliac disease and graft-versus-host disease, but the relative contributions of barrier dysfunction and immune responses are unclear.
- Intestinal barrier loss can be a consequence of tight junction dysfunction or of epithelial damage; in most studies, these mechanisms are not distinguished.
- Paracellular transport across the tight junction can occur via the pore pathway or the leak pathway, which have distinct size-selectivity and charge-selectivity and are differentially regulated by immune signalling.
- Claudin-2 increases Na^+ and water flux across the pore pathway but larger molecules are unable to traverse claudin-2 channels.
- The leak pathway allows macromolecules to cross the epithelial barrier and is regulated by the cytoskeleton and epithelial long myosin light chain kinase splice variant 1 (MLCK1).
- Blocking MLCK1 recruitment to the tight junction limits tight junction barrier loss without interfering with essential MLCK functions in epithelial cells and cells of other types.

Introduction

Epithelia separate the organism from the external environment and define individual compartments within tissues. At some sites, the epithelia form a nearly complete barrier, disruption of which is catastrophic. For example, massive disruption of the epidermal (skin) barrier by burn injury or mutagenesis in animal models can be fatal. However, at other sites, such as renal tubules and intestines, the balance between permeability and barrier function is nuanced, as selective permeability is essential for physiological processes but must also be precisely regulated.

Flux across the epithelial barrier occurs via transepithelial transport, which involves transcellular and paracellular pathways. Transcellular transport involves movement of molecules through cells, and is mediated by apical and basolateral transmembrane transporters with exquisite substrate specificity. Paracellular transport is less selective and can involve movement of molecules across the epithelial barrier via the pore pathway or the leak pathway. A third permeability route, the unrestricted pathway, is created by epithelial damage (Box 1). Flux across the pore and leak pathways reflects permeability of tight junctions, which seal the space between adjacent epithelial cells and are the rate-limiting components of paracellular transport. Both pore and leak pathways are size-selective, and the pore pathway is charge-selective. However, both pathways lack the structural specificity of transcellular transport. For example, L-glucose can be absorbed paracellularly but is not recognized by transmembrane transport proteins and, therefore, cannot be absorbed via the transcellular route.

Although barrier loss is considered most often in the context of disease, insufficient selective permeability – that is, barrier enhancement – can also contribute to disease. For example, the first monogenic tight junction disease to be discovered – familial hypomagnesaemia with hypercalciuria and nephrocalcinosis – is caused by mutation of claudin-16, which forms paracellular cation channels within the renal tubule. In the absence of claudin-16, paracellular Mg^{2+} and Ca^{2+} absorption in the thick ascending limb of the nephron fails[1,2]. Conversely, loss of claudin-14, which enhances paracellular barriers, in the organ of Corti causes deafness in mice and autosomal recessive nonsyndromic hearing loss 29 (DFNB29) in humans[3–6].

In the intestines, the epithelial monolayer separates subepithelial immune cells from the luminal microbiome[7]. The balance between paracellular permeability and barrier function is, therefore, especially delicate because the epithelial monolayer must prevent unregulated paracellular flux of potentially pathogenic luminal materials[8] while also allowing the selective paracellular permeability that is required for nutrient and water absorption[9]. This situation contrasts with that in the nephron, where both the lumen of the renal tubule and the interstitial space are sterile under normal conditions. In the gut lumen, which contains a diverse microbiome, disruption of the balance between selective permeability and barrier function is associated with a wide range of intestinal and systemic disorders[10,11].

In this Review, we consider several disorders that involve intestinal barrier dysfunction, focusing on changes to paracellular permeability and the function of tight junctions. We delineate molecular mechanisms that alter paracellular permeability and cause intestinal barrier loss. We also endeavour to differentiate between barrier loss as a contributor to disease pathogenesis and barrier loss as a consequence of disease processes.

Intestinal permeability in disease

Intestinal permeability is increased – that is, barrier function is reduced – in many intestinal and systemic diseases (Table 1). Of these diseases, the most well studied intestinal disorders are inflammatory bowel disease (IBD) and coeliac disease. Although tight junction permeability is increased in IBD, the extensive barrier loss seen in advanced, active disease is more likely to reflect epithelial damage[12–16]. Similarly, increases in intestinal permeability in advanced graft-versus host disease (GVHD) can reflect tight junction regulation or immune-mediated epithelial damage[17]. Although discrimination between these disparate mechanisms of barrier loss is possible, most studies have relied on the use of only a single probe, such as fluorescein isothiocyanate–4-kDa dextran in mouse models, to measure permeability. As a result, the data are insuffcient to differentiate between leak pathway and unrestricted pathway flux. Thus, correlations observed between the extent of barrier loss and the severity of disease in IBD, coeliac disease and GVHD are most likely to be secondary to epithelial damage. By contrast, increased intestinal permeability in Crohn's disease during remission is more likely to reflect increased tight junction permeability[18,19], particularly as increased permeability can occur up to 1 year before disease reactivation. Notably, psychological stress, which can increase intestinal permeability in rodents, is a risk factor for reactivation of Crohn's disease in patients[20,21].

Reports that some healthy first-degree relatives of patients with Crohn's disease have modest intestinal barrier dysfunction led to the hypothesis that loss of intestinal barrier function is a primary event in Crohn's disease pathogenesis. The idea that loss of barrier function is an early pathogenic event is supported by evidence that increased intestinal permeability in healthy first-degree relatives of patients with Crohn's disease is associated with the risk-associated *NOD2* 3020insC polymorphism[22]. Perhaps the most convincing evidence comes from another study of healthy relatives of patients with Crohn's disease,

Box 1

The pore, leak and unrestricted permeability pathways

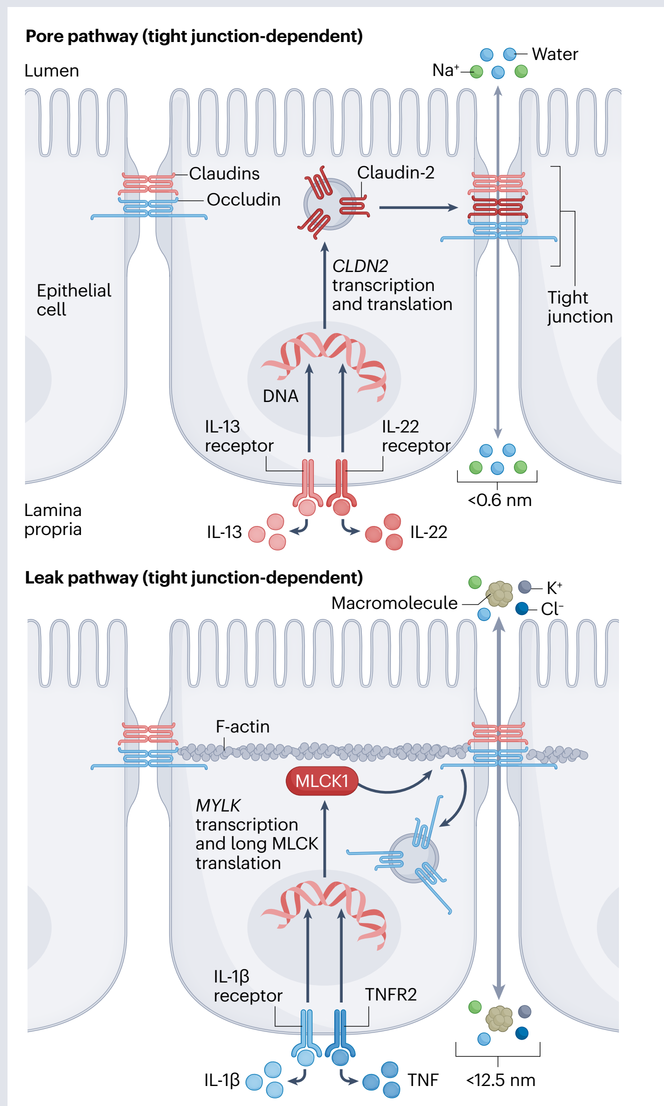
Intestinal permeability can reflect contributions of three distinct pathways: the pore pathway, the leak pathway and the unrestricted pathway (see the figure). The pore and leak pathways reflect flux across tight junctions, whereas the unrestricted pathway is independent of tight junctions.

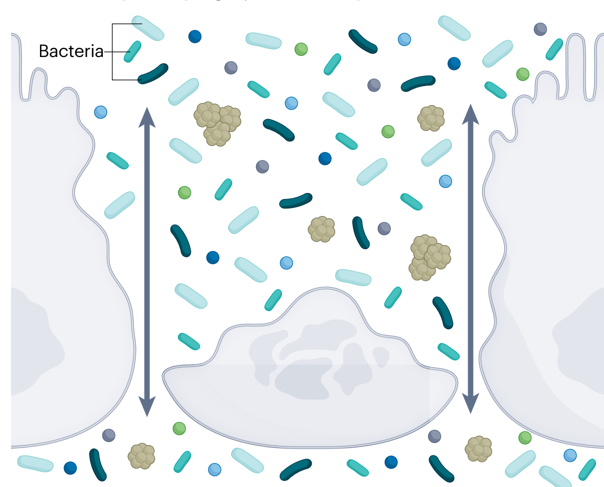
Pore pathway permeability is defined by claudin proteins, which form either channels or barriers at the tight junction[291–294]. Channels generated by pore-forming claudins are charge-selective and size-selective; the maximum diameter of solutes that can pass through them is 0.6 nm. Claudins form only cation-selective channels in the gastrointestinal tract, but anion-selective claudin channels are present at other sites, such as the nephron. Immune signals, including IL-13 and IL-22, lead to increased transcription and expression of intestinal epithelial claudin-2, which increases pore pathway permeability (see the figure, top)[45,50,86].

The tight junction leak pathway allows molecules with diameters up to ~12.5 nm to traverse the epithelial barrier. The molecular structure of the leak pathway is poorly understood, but its permeability can be regulated by long myosin light chain kinase splice variant 1 (MLCK1)[125,140,141,157]. MLCK1 phosphorylates myosin regulatory light chain to trigger endocytosis of the tight junction protein occludin, leading to an increase in tight junction permeability (see the figure, bottom left). MLCK1 expression and enzymatic activity can be activated by cytokines that include IL-1β and tumour necrosis factor (TNF)[124,125,135,140,158,295]. Altered expression of other tight junction proteins, including tricellulin or angulin 1, might also modify leak pathway permeability[55,146,147,296].

The unrestricted pathway refers to the diffusion of material across regions that lack a continuous epithelial barrier owing to epithelial cell damage or death (see the figure, bottom right). This route is independent of tight junctions, as they are either absent or severely damaged at these sites. The unrestricted pathway allows flux of very large molecules and even intact bacteria.

In summary, the pore pathway is a high-capacity pathway that is exquisitely charge-selective and size-selective, whereas the leak pathway is a low-capacity pathway, is not charge-selective, and, although size-selective, allows flux of molecules 20-fold larger than those accommodated by the pore pathway. Thus, the two tight-junction-dependent pathways are complementary. The unrestricted pathway is tight-junction-independent, high-capacity and non-selective.





Adapted from ref. 297, Springer Nature Limited.

Table 1 | Diseases associated with intestinal barrier defects

Disease	Findings in patients	Findings in animal models
IBD (Crohn's disease and ulcerative colitis)	Increased permeability is a risk factor for IBD in healthy relatives of patients with Crohn's disease and for relapse in patients with Crohn's disease[18,19,22,24,174,182,183]	*Il10*-knockout mice develop a permeability defect before disease onset; experimental IBD is more severe in genetically modified mice with increased intestinal permeability; genetic or pharmacological reduction of pore pathway or leak pathway function limits the severity of experimental IBD[59,98,117,134,141,163,184,185]
Graft-versus-host disease	Positive correlation of pre-conditioning gastrointestinal toxicity (presumed to indicate degree of transient barrier loss) with disease activity[17,186]	Gut damage is an essential driver of experimental disease; intestinal permeability is increased and tight junction protein organization is altered in experimental disease; disease progression and severity are attentuated in long MLCK-knockout mice[137,187–191]
Type 1 diabetes mellitus	Permeability is increased in patients with pre-diabetes and diabetes mellitus[192–194]	Permeability increases precede disease; barrier restoration can delay disease onset[195,196]
Metabolic syndrome (including type 2 diabetes mellitus, obesity and nonalcoholic fatty liver disease)	Increased intestinal permeability is a risk factor for type 2 diabetes mellitus and is associated with obesity and nonalcoholic fatty liver disease[194,197–201]	Hyperglycaemia, high-fat diet, nonalcoholic fatty liver disease and obesity are associated with increased intestinal permeability[202–208]
HIV/AIDS	Increased permeability in HIV enteropathy; positive correlation with disease stage; increased in patients with untreated HIV infection[90,209,210]	Increased intestinal permeability in simian immunodeficiency virus infection is associated with microbial translocation and systemic immune activation[211,212]
Multiple organ dysfunction syndrome	Correlates with increased disease severity[213]	Associated with shock; disease progression is limited in knockout mice that are protected from leak pathway permeability increases[214–217]
IBS	Increased in diarrhoea-predominant, post-infectious and non-post-infectious IBS; unaltered in constipation-predominant IBS[28,218–229]	Increased intestinal permeability is associated with and can cause changes in visceral sensitivity[31,230,231]
Coeliac disease	Positive correlation between increased permeability and disease activity; increased permeability in patients and healthy relatives; gluten-free diet can lead to barrier restoration[232–235]	Barrier loss is associated with disease in models of coeliac disease[236–238]
Environmental enteric dysfunction	Increased permeability; altered expression of absorptive and barrier-enhancing proteins[239–247]	Barrier loss is associated with malnutrition[240,248,249]
Food allergy	Increased in people with food allergy[250–252]	Permeability increased in mice after food antigen challenge[253–257]
Sepsis	Increased permeability in sepsis; increased plasma zonulin[258–261]	Permeability increased in experimental sepsis; relationship to disease progression is not defined[262–266]
SARS-CoV-2 infection	Permeability is increased in severe systemic disease; some data suggest that barrier restoration using a zonulin antagonist is beneficial[267–276]	No direct measures of intestinal permeability
Parkinson disease	Permeability increased in a subset of patients[277,278]	No direct measures of intestinal permeability
Asthma	Permeability increased in people with asthma; IBD associated with increased risk of asthma[279,280]	Correlation between disease and intestinal permeability in some models[281,282]
Multiple sclerosis and amyotrophic lateral sclerosis	Increased intestinal permeability in a subset of patients[283–285]	Increased intestinal permeability in experimental allergic encephalitis model[286]
Rheumatic diseases (arthritis and ankylosing spondylitis)	Increased intestinal permeability in some patients[287,288]	Increased permeability in mouse models; barrier restoration can limit disease[287,289,290]

IBD, inflammatory bowel disease; IBS, irritable bowel syndrome; MLCK, myosin light chain kinase.

in which the risk of developing disease was twofold to threefold higher among those with increased intestinal permeability than among those with normal permeability[23].

In healthy relatives of patients with Crohn's disease, increased intestinal permeability was also associated with reduced microbial diversity and alterations in specific genera and microbial metabolic pathways[24]. By contrast, faecal calprotectin – a marker of mucosal inflammation but not of barrier loss – was increased in some healthy relatives but was not an independent risk factor for development of Crohn's disease[25]. Reports of increased intestinal permeability up to 3 years before onset of Crohn's disease suggest that barrier loss is the initial trigger that activates intermediate events, such as mucosal immune activation, that culminate in disease. Together, these observations suggest that barrier loss is a primary event in Crohn's disease pathogenesis.

Some data suggest that increased intestinal permeability is associated with poorly understood conditions, including irritable bowel syndrome and autism spectrum disorders[26–30]. The mechanistic underpinnings of these associations have not been defined and, in both patients and animal models, the question of whether barrier loss is a cause or consequence of disease remains. The observation that mice with genetically induced increases in intestinal permeability develop

anxiety-like behaviours, hyporesponsiveness to rectal distension and activation of neurons within the thalamus, hypothalamus and hippocampus[31] demonstrates that increased intestinal permeability can effect changes in behaviour, visceral sensation and neurological activity. These mice also have an altered microbiome composition.

Tight junctions and intestinal barrier function

In the absence of epithelial damage, the tight junction is the rate-limiting determinant of passive paracellular transport. At the tight junction, the intercellular spaces between adjoining cells are eliminated and the outer leaflets of the plasma membrane lipid bilayer of adjacent epithelial cells are closely apposed and appear to fuse[32] (Fig. 1). Subapical to the tight junction are the adherens junctions and desmosomes, which are linked to actin-based microfilaments and cytokeratin-based intermediate filaments, respectively. These cytoskeletal structures provide the tensile strength that supports tight junctions and maintains cell shape.

Tight junction pathways and proteins

The first tight junction protein to be discovered was zonula occludens 1 (ZO-1)[33] (Fig. 1), followed by the two related proteins ZO-2 and ZO-3, and the unrelated protein cingulin[34–37], though all of these proteins are intracellular peripheral membrane proteins. These discoveries were followed by the discovery of the tetraspan transmembrane tight junction proteins occludin[38] and the claudins, which are encoded by 27 genes in mammals[39,40].

When expressed in non-epithelial cells, claudins can self-assemble into large polymers to form structures that are reminiscent of tight junction strands seen by freeze–fracture electron microscopy[41] (Fig. 1). Many claudins are critical for barrier function, but others form charge-selective and size-selective paracellular channels. The ensemble of expressed claudins dictates the size-selectivity and charge-selectivity of specific sites within tissues. Detailed characterization has demonstrated that the charge-selectivity of claudin channels is determined by specific residues within the first extracellular loop[42,43]. Regardless of whether they are cation-selective or anion-selective, all claudin channels studied to date are size-selective and allow paracellular flux of only molecules with a diameter of <0.6 nm (refs. 43–46). These channels define the pore pathway[47] (Box 1) and are exemplified by claudin-2, which mediates paracellular flux of small cations, such as Na^+, and water[42,43,48,49]. Claudin-2 cannot, however, accommodate the commonly used macromolecular probes lactulose, mannitol or 4 kDa dextran[48,50,51], emphasizing the need to consider the physical characteristics of the solute being measured when assigning mechanisms of changes in paracellular permeability.

Although rejected by the pore pathway, molecules larger than 0.6 nm, including lactulose, mannitol and 4 kDa dextran, can cross tight junctions via a second paracellular flux route known as the leak pathway (Box 1), which can accommodate molecules with diameters of up to ~12.5 nm and is not charge-selective[47,52]. The specific molecular structure of the leak pathway has not been identified, but its permeability is regulated by occludin[53,54], tricellulin (also known as MARVEL domain-containing protein 2, a member of the tight junction-associated MARVEL protein (TAMP) family)[55], ZO-1 (refs. 52,56) and perijunctional actomyosin[53,57–59].

Pore and leak pathway functions in health

The major pore-forming claudins expressed within the intestinal epithelium are claudin-2 and claudin-15 (refs. 9,60), both of which form cation-selective channels[49,61,62]. Mice that lack either claudin-2 or claudin-15 are viable, but mice that lack both claudins die before weaning[9], consistent with the idea that these proteins are, at least partially,

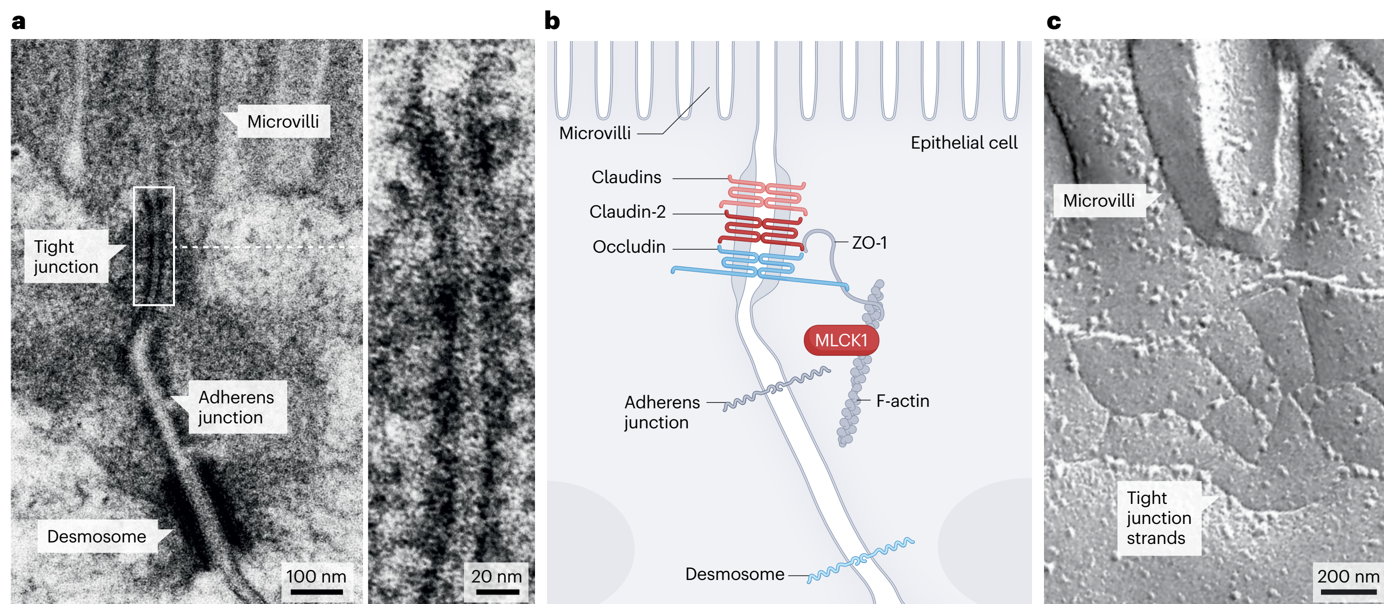


Fig. 1 | Tight junction structure and morphology. a, Transmission electron micrograph showing the tight junction, adherens junction and desmosome, which, together, comprise the apical junctional complex. The tight junction is located just below the base of the microvilli. The magnification shows the transition from the luminal space, between the microvilli, into the tight junction, where morphologically detectable paracellular space is obliterated. **b**, Schematic of the apical junctional complex shown in part **a**, showing the location of the tight junction proteins zonula occludens 1 (ZO-1), occludin, claudin-2 and other claudin family members. Long myosin light chain kinase 1 (MLCK1) is associated with perijunctional F-actin and is a key regulator of tight junction permeability. **c**, Freeze–fracture electron micrograph showing tight junction strands at the base of apical microvilli.

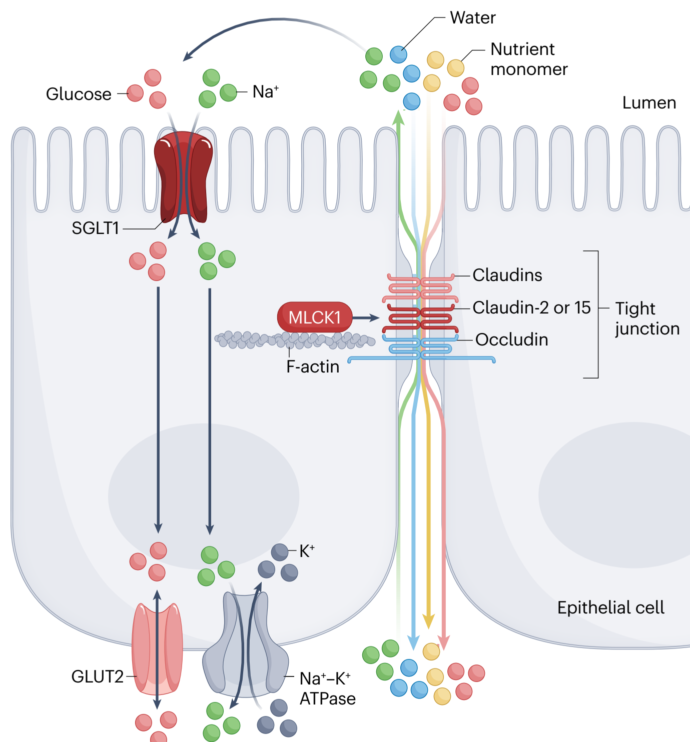


Fig. 2 | Coordination of transcellular and paracellular transport. The gradient of Na^+ between the gut lumen and the cytoplasm of epithelial cells provides the driving force for nutrient absorption across the apical brush border membrane, such as glucose absorption via the intestinal epithelial Na^+–glucose cotransporter SGLT1. Nutrients then exit the cell via facilitated exchange proteins, such as the glucose transporter GLUT2, and Na^+ exits via the Na^+–K^+ ATPase. Na^+–glucose cotransport also triggers signal transduction pathways that activate long myosin light chain kinase 1 (MLCK1) and increase tight junction permeability. The osmotic gradient generated by transcellular nutrient and Na^+ transport draws water across the tight junction and, owing to the high concentration of nutrient monomers in the unstirred layer, nutrients are carried along with this fluid in a mechanism known as solvent drag[71]. This process would quickly exhaust luminal Na^+ if not for claudin-2 and claudin-15, which form paracellular Na^+ channels that enable efflux of absorbed Na^+ in order to provide the driving force for continued transcellular nutrient absorption[9,63].

functionally redundant. Nevertheless, intestinal hypertrophy occurs in claudin-15-knockout, but not claudin-2-knockout, mice[63,64].

Paracellular Na^+ efflux via claudin-2 and claudin-15 channels is critical to transcellular nutrient transport. Brush border absorption is largely driven by Na^+–nutrient cotransporters that rely on the Na^+ gradient between the intestinal lumen and the cytoplasm of epithelial cells (Fig. 2). During this process, Na^+ enters the cytoplasm and is exported across the basolateral membrane into the lamina propria by the Na^+–K^+ ATPase[65]. In the absence of paracellular Na^+ transport, transcellular transport rapidly depletes luminal Na^+ and nutrient cotransport across the apical brush border membrane stops. Flux across claudin-2 and claudin-15 channels allows Na^+ efflux from the lamina propria to the lumen, where it can drive additional cycles of Na^+–nutrient cotransport.

The requirement for Na^+ efflux in the intestine explains the critical roles of claudin-2 and claudin-15 in nutrient absorption but does not explain why expression of these claudins is so precisely regulated. At birth, intestinal epithelial claudin-2 expression is high throughout the crypt–villus axis of the intestinal epithelium[60,63,66], but claudin-2 expression is markedly diminished and limited to crypt epithelium after weaning. Concurrently, claudin-15 expression increases throughout the crypt–villus axis. Although only subtle functional differences between these claudins have been detected in vitro[48,62], this precise developmental regulation suggests that the in vivo properties of claudin-2 and claudin-15 channels must differ substantially. Hypothetically, claudin-2 might allow greater paracellular Na^+ flux than claudin-15, which would be advantageous for the rapid growth that occurs during early postnatal development and depends on Na^+–nutrient cotransport. Alternatively, channel open state probabilities or other subtle functional characteristics of claudin-2 and claudin-15 might differ. This question could be resolved by single-channel analysis but, unfortunately, such data have only been reported for claudin-2 (ref. 46).

Na^+–nutrient cotransport triggers downstream signalling in the epithelial cell. These signalling events activate MLCK, which phosphorylates perijunctional myosin regulatory light chain (MLC) and increases leak pathway permeability[67–69]. This process amplifies paracellular nutrient transport via solvent drag (Fig. 2), whereby the osmotic gradient created by transcellular transport drives paracellular water absorption[70]. The absorbed water comes from the unstirred layer immediately adjacent to the epithelium, which is rich in small nutrient monomers owing to the activity of brush border digestive enzymes. This paracellular fluid absorption allows paracellular nutrient absorption to amplify transcellular transport[71–74]. Importantly, solvent drag only contributes substantially to nutrient absorption when luminal concentrations of nutrient monomers are high[72]. This subtlety probably explains why passive paracellular nutrient absorption via solvent drag has been detected in some studies and not others[75–84]. In contrast to transcellular transport, paracellular absorption via solvent drag is not stereospecific and can accommodate molecules for which there are no apical transporters, such as mannitol[72,85].

Paracellular amplification of transcellular absorption, or solvent drag, probably explains why the rate of nutrient transport across the intestinal epithelium cannot be saturated[72]. Clinically, solvent drag contributes to the efficacy of simple Na^+ and carbohydrate-containing oral rehydration solutions that have been used to treat countless individuals with potentially fatal, high-volume diarrhoeal diseases, such as cholera. By contrast, the glycosuria that occurs in diabetes mellitus suggests that no corresponding paracellular pathway for glucose resorption exists within the renal tubule.

The pore pathway in disease

Intestinal epithelial claudin-2 has been the subject of intense scrutiny owing to its markedly increased expression in a broad range of inflammatory disorders. Claudin-2 upregulation was first described in the contexts of ulcerative colitis and Crohn's disease[86,87]. Subsequent work demonstrated that intestinal epithelial claudin-2 is also upregulated in coeliac disease[88], irritable bowel syndrome[89], HIV enteropathy[90], enteric infection[50], necrotizing enterocolitis[91] and Whipple disease[92]. The factors that mediate claudin-2 upregulation are incompletely characterized, but in vitro and in vivo studies have implicated IL-1, IL-6, IL-13, IL-22 and tumour necrosis factor (TNF) as potential enhancers of claudin-2 expression[50,86,93–96]. By contrast, some in vitro studies suggest that butyrate can suppress claudin-2 expression and increase barrier function via an IL-10 receptor-dependent mechanism[97]. Together, these data

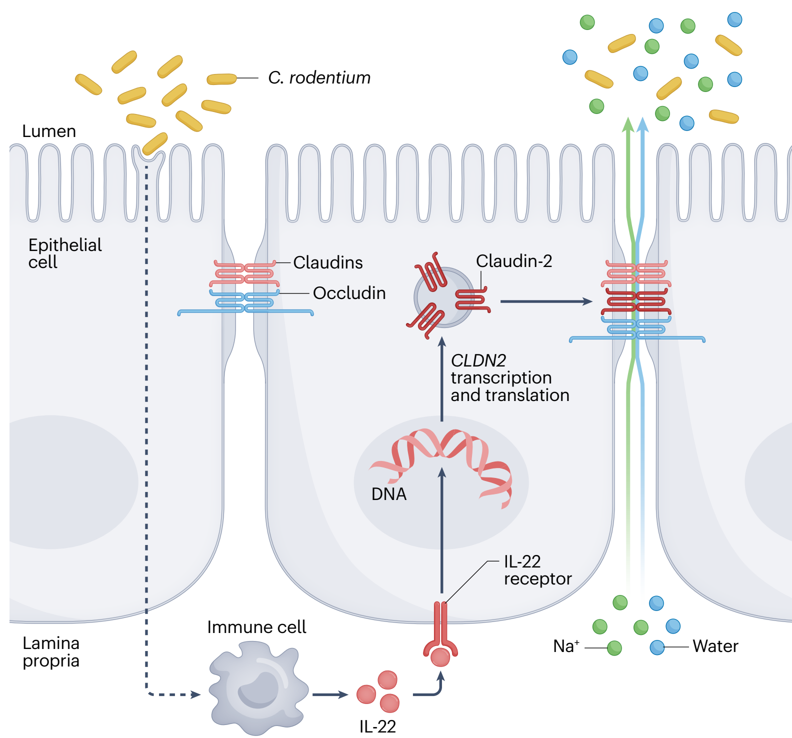


Fig. 3 | Promotion of pathogen clearance by paracellular fluid efflux. *Citrobacter rodentium* infection triggers an immune response that leads to IL-22 release within the lamina propria within 2 days of infection. IL-22 signalling activates claudin-2 transcription and increases claudin-2 channel-mediated Na^+ and water efflux via the tight junction pore pathway, resulting in diarrhoea that promotes clearance of the infection. Adapted with permission from ref. 50, Elsevier.

indicate that the mucosal immune system can fine-tune claudin-2 expression.

Administration of recombinant IL-13 to mice increases claudin-2 expression and augments intestinal paracellular cation permeability[98]. Similarly, transgenic claudin-2 overexpression within the intestinal epithelium increases paracellular permeability to cations to levels similar to those in IL-13-treated wild-type mice[98]. By contrast, IL-13 has no effect on intestinal permeability in mice in which the *Cldn2* gene, which encodes claudin-2, is knocked out. Thus, claudin-2 upregulation is both necessary and sufficient to increase paracellular cation permeability in vivo. The impact of this effect on disease is discussed in the following sections.

Claudin-2 attenuates diseases induced by luminal insults

A pair of studies of *Cldn2*-transgenic and *Cldn2*-knockout mice provided initial evidence that increased claudin-2 expression is beneficial in dextran sulfate sodium (DSS)-induced colitis[98,99]. However, claudin-2 overexpression also increases faecal water content[50], suggesting that increased claudin-2 expression might reduce the severity of colitis simply by diluting DSS within the distal colon. An alternative possibility is that claudin-2 expression promotes epithelial growth and mucosal repair[100–102], as discussed below (see 'Claudin-2 and epithelial proliferation').

Claudin-2 expression is increased during enteric infection in humans[103]. Similarly, the model pathogen *Citrobacter rodentium* triggers IL-22-dependent claudin-2 upregulation within 2 days of infection in mice[50] (Fig. 3). Although IL-22 is pleiotropic, an increased number of mucosa-associated *C. rodentium*, delayed pathogen clearance and a greater severity of mucosal damage in *Cldn2*-knockout mice relative to wild-type mice demonstrate that IL-22-dependent claudin-2 upregulation contributes to host defence[50]. The observation that transgenic claudin-2 overexpression limits *C. rodentium*-induced colitis provides further support for this conclusion.

The passage of either Na^+ or water through claudin-2 channels[104,105] could mediate the increased pathogen clearance and reduced disease severity associated with claudin-2 expression. In studies to dissect these mechanisms, wild-type, *Cldn2*-transgenic and *Cldn2*-knockout mice were infected with *C. rodentium*. Polyethylene glycol (PEG) was added to their drinking water 4 days later to create an osmotic gradient that increased fluid flow into the intestinal lumen. PEG treatment normalized the number of mucosa-associated *C. rodentium*, pathogen clearance and mucosal damage across genotypes, demonstrating that paracellular water efflux is the primary means by which claudin-2 promotes enteric pathogen clearance.

The effect of PEG was unlikely to have been a result of fluid efflux simply washing bacteria off the epithelial surface, as *C. rodentium* is an attaching and effacing pathogen that forms pedestals and is not easily displaced from intestinal epithelial cells. Moreover, intestinal epithelial cells turn over every few days during infection, so the cells present at the peak of disease (11 days after infection) are not the same cells that were initially colonized. Therefore, one possible explanation for the findings is that claudin-2-mediated paracellular water efflux reduces the efficiency with which new cells are infected. This hypothesis remains to be tested. Nevertheless, evidence indicates that the diarrhoea induced by claudin-2 upregulation is beneficial in the context of enteric infection. These results provide the first experimental data to show that diarrhoea promotes enteric pathogen clearance, an idea that has persisted for centuries despite a lack of supporting evidence[106].

Claudin-2 exacerbates immune-mediated colitis

Claudin-2 transcription is exquisitely responsive to cytokine stimulation and is increased in a wide range of human and experimental

disorders associated with mucosal inflammation. Given its protective role during enteric infection and DSS-induced injury, claudin-2 upregulation might also be expected to be beneficial in inflammatory disease. This hypothesis was tested by inducing immune-mediated colitis by T cell transfer in immunodeficient, claudin-2 wild-type, transgenic and knockout mice[98]. In contrast to the effects of claudin-2 in infectious colitis, its overexpression exacerbated immune-mediated colitis and was associated with severe weight loss, increased cytokine production, mucosal T cell infiltration and histopathological damage. Conversely, *Cldn2* knockout attenuated all measures of colitis severity. Together with the effects of claudin-2 expression on pathogen clearance, these data suggest that claudin-2 upregulation triggers defence mechanisms that include immune activation and, therefore, exacerbates immune-mediated disease. Although the mechanisms by which claudin-2-mediated paracellular Na^+ and water flux enhances immune activation is unknown, this phenomenon could explain the observed exacerbation of immune-mediated disease by high-Na^+ diets[107–114].

The findings in *Cldn2*-knockout mice also provide further support for the idea that substantial functional differences exist between claudin-2 and claudin-15 in vivo. Although claudin-15 expression is not altered in human IBD or experimental immune-mediated colitis, it was upregulated in colitic *Cldn2*-knockout mice[98]. However, *Cldn2*-knockout mice remained protected from immune-mediated colitis, indicating that claudin-15 cannot compensate for claudin-2 loss in this context.

Although disease severity was lower in *Cldn2*-knockout mice than in wild-type mice, survival was inferior[98]. The cause of death among *Cldn2*-knockout mice was intestinal obstruction. This observation could reflect an inability to increase luminal hydration, owing to lack of claudin-2-mediated water transport, that synergized with colitis-associated epithelial proliferation, mucosal expansion and luminal narrowing to allow formation of luminal faecaliths and intestinal obstruction. Consistent with this interpretation, induction of mild osmotic diarrhoea increased faecal water content, prevented intestinal obstruction and increased survival of the *Cldn2*-knockout mice[50]. Osmotic diarrhoea did not, however, affect overall disease severity in claudin-2 wild-type, transgenic or knockout mice[98]. Therefore, the increased survival due to osmotic diarrhoea does not reflect direct mitigation of immune activation or tissue damage.

The protection afforded by *Cldn2* knockout suggests that pharmacological inhibition of claudin-2 function might be effective in immune-mediated disease. Of several reported approaches to claudin-2 channel inhibition[115–118], only one has been assessed in vivo[98]. This approach relies on casein kinase 2 (CK2) inhibition, occludin dephosphorylation and assembly of a claudin-2–ZO-1–occludin complex that inactivates claudin-2 channels[98,117]. CK2 inhibition did not interfere with IL-13-induced increases in claudin-2 expression but did prevent IL-13-induced changes in paracellular Na^+ permeability. CK2 inhibition markedly attenuated the severity of immune-mediated colitis in claudin-2 wild-type mice[98]. Although CK2 is a ubiquitously expressed, promiscuous kinase, CK2 inhibition did not affect disease severity in *Cldn2*-knockout mice, indicating that its therapeutic benefit is largely due to claudin-2 channel inactivation. Notably, CK2 inhibition did not cause intestinal obstruction in *Cldn2* wild-type mice, probably owing to incomplete claudin-2 channel inactivation. Together with the fact that the intestinal lumen diameter is much greater in humans than in mice, this observation suggests that pharmacological claudin-2 channel inhibition is unlikely to cause intestinal obstruction in humans.

Claudin-2 and epithelial proliferation

Some evidence suggests that claudin-2 promotes epithelial proliferation[100,101,119,120]. For example, the rate of intestinal epithelial proliferation was nearly doubled in one strain of mice with transgenic claudin-2 overexpression[100]. As mentioned above, this proliferation might contribute to the protection that claudin-2-transgenic mice have from DSS-induced colitis[100]. However, epithelial proliferation was not increased in a different *Cldn2*-transgenic mouse model[50]. The reasons for this discrepancy are unclear, as *Cldn2* expression was under the control of the 9 kB villin promoter[121] and pore pathway permeability was increased in both models. However, the transgenic mice differed in that one expressed human claudin-2 at high levels[100], whereas the other expressed EGFP-tagged mouse claudin-2 at lower levels[50]. Although further study is needed, this difference could underlie the discrepancy between the two models and could also explain increases in leak pathway permeability that occurred in the first, but not the second, model.

Other data that suggest a role for claudin-2 in regulating epithelial proliferation include studies of SW480 and HCT116 human colon cancer cells, which demonstrated that claudin-2 overexpression increases proliferation in vitro, accelerates tumour growth in vivo and reduces apoptosis triggered by the chemotherapeutic agent 5-fluorouracil[122]. In a study in patients with colon cancer, high claudin-2 expression correlated with lower overall and disease-free survival, further supporting the notion that claudin-2 can promote epithelial proliferation[123]. Thus, although the mechanisms are not defined, claudin-2 overexpression might promote intestinal epithelial cell proliferation in some contexts.

The leak pathway in disease

Although the leak pathway is activated physiologically during Na^+–nutrient cotransport, far greater increases in leak pathway permeability are induced by TNF[124–126] (Box 1). The reasons for this difference between physiological and pathophysiological tight junction regulation are unclear because both depend on MLCK activation, but they might relate to the fact that occludin endocytosis occurs during TNF-induced barrier loss but occludin distribution is unaffected during Na^+–nutrient cotransport-induced permeability increases[53,68,125,127]. Occludin is also internalized during MLCK-mediated barrier loss induced by the TNF-related cytokine LIGHT, IL-1β or lipopolysaccharide[128–133].

In experimental, immune-mediated IBD, activation of intestinal epithelial MLCK accelerates disease progression, whereas genetic deletion of intestinal epithelial MLCK attenuates disease[59,134]. Interestingly, the claudin-2 upregulation that normally occurs in experimental, immune-mediated IBD is reduced in mice that lack intestinal epithelial MLCK[134] and is restored by transgenic expression of constitutively active MLCK within intestinal epithelia[134]. Thus, the leak pathway and pore pathway are linked in disease. Notably, transgenic expression of constitutively active MLCK within intestinal epithelial cells, which modestly increases leak pathway permeability but does not induce disease, increases both mucosal IL-13 production and epithelial claudin-2 expression. Thus, increased leak pathway permeability can, via mucosal immune activation, trigger claudin-2 upregulation[45]. Conversely, as noted above, leak pathway permeability is increased in one of two claudin-2 overexpressing transgenic mice despite the absence of overt disease. Although the precise relationship between pore pathway and leak pathway regulation in the context of disease remains to be determined, the ability of distinct cytokines to specifically and independently regulate pore pathway or leak pathway permeability is striking.

TNF, LIGHT and IL-1β all trigger transcriptional and enzymatic activation of MLCK[135–137], although reports differ as to whether the transcriptional activation is mediated by nuclear factor-κB, p38 mitogen-activated protein kinase (MAPK) or the transcription factor activator protein 1 (AP-1)[135,138–140]. Regardless of these discrepancies, MLCK activation clearly leads to perijunctional MLC phosphorylation and occludin endocytosis[125,140,141]. Despite ongoing debate regarding the functional significance of occludin, the consensus is that occludin, along with other proteins, is a critical regulator of leak pathway permeability. This role of occludin has been demonstrated in several in vitro studies[52,142–144], but the strongest evidence comes from in vivo studies that have shown that blockade of occludin endocytosis or transgenic occludin overexpression limits TNF-induced leak pathway barrier loss[54]. This observation suggests that reduced occludin expression could explain the increased permeability of the leak pathway observed in human disease, including IBD[86,145].

The tricellular tight junction proteins tricellulin and angulin 1 might also be important regulators of leak pathway permeability. In vitro studies have shown that deletion of either tricellulin or angulin-1 increases leak pathway permeability[146–148]. Similar to tricellulin, siRNA-mediated knockdown of the other TAMPs, MARVELD3 (refs. 146,149) or occludin[142,143], also increased leak pathway permeability. Tricellulin redistribution from tricellular to bicellular tight junctions[150] following occludin loss[54,125] could, therefore, be an intermediate event that allows occludin to regulate leak pathway permeability. Thus, although a great deal has been learned about proteins that contribute to leak pathway barrier function and mechanisms of experimental and pathophysiological leak pathway regulation, the molecular structure of the leak pathway remains enigmatic.

Diverse occludin functions

Unexpectedly, intestinal epithelial specific occludin knockout protects mice from experimental colitis and epithelial injury driven by intrinsic and extrinsic TNF signalling pathways. This finding was ultimately explained by the observation that occludin enhances activity of the promoter for *CASP3*, which encodes caspase 3, through an undefined mechanism[145]. In cultured cell lines and mice, occludin downregulation led to a reduction of ~50% in caspase 3 expression that conferred protection from a diverse range of pro-apoptotic stimuli[145,151]. Analyses of biopsy samples suggests that this process also occurs in human disease, as occludin downregulation correlates with reduced epithelial caspase 3 expression in patients with ulcerative colitis or Crohn's disease.

Thus, in addition to increasing leak pathway permeability, occludin downregulation can promote epithelial survival. However, this effect might not be entirely beneficial, as it could allow evolution of deleterious mutations that would have otherwise been eliminated by apoptosis. Consistent with this hypothesis, in vitro studies suggest that occludin functions as a tumour suppressor in some contexts[151–155]. Further exploration will, therefore, be required to fully understand extra-junctional functions of occludin.

Distinct functions of long MLCK splice variants

Epithelial MLCK is expressed from the same gene (*MYLK*) that encodes smooth muscle MLCK[156]. However, epithelial (long) MLCK is ~225 kDa (refs. 156,157), whereas smooth muscle (short) MLCK is only ~130 kDa (Fig. 4). Long MLCK transcription, which is activated by TNF, IL-1β and other stimuli[135,158], generates mRNA transcripts that include additional 5′ exons that are not present in short MLCK transcripts[156]. This difference reflects the location of the short MLCK promoter within an intron of long MLCK. Nevertheless, the carboxy-terminal catalytic and calmodulin-binding regulatory domains are identical in long and short MLCK. The 5′ region that distinguishes long MLCK from short MLCK undergoes extensive alternative splicing[156]. Of the splice variants generated, only two – long MLCK1 and MLCK2 – are expressed in intestinal epithelial cells[157] (Fig. 4). Although the underlying mechanisms have not been defined, splicing seems to be precisely regulated during differentiation, as MLCK2 is expressed throughout the crypt–villus axis but MLCK1 expression is limited to the upper villus[157]. Moreover, the increased MLC phosphorylation in active Crohn's disease is specifically associated with perijunctional MLCK1 recruitment[159,160] (Fig. 4).

The two intestinal epithelial long MLCK splice variants differ by a single exon that is present in MLCK1 but not in MLCK2 (ref. 157). The 69 amino acids encoded by this exon complete the third immunoglobulin–cell adhesion molecule domain (IgCAM3)[157]. This domain must, therefore, contribute to preferential perijunctional localization of MLCK1 relative to MLCK2, which is distributed more diffusely through the cytoplasm[141,157]. TNF triggers even greater recruitment of MLCK1 to the perijunctional actomyosin ring[141]. Similarly, MLCK1 is concentrated within the perijunctional actomyosin ring in intestinal biopsy samples from patients with active IBD[141,160,161]. This inflammation-inducible perijunctional MLCK1 recruitment, together with increased barrier function after MLCK1-specific knockdown[157], suggests that this splice variant is central to tight junction regulation.

These findings prompted solution of the IgCAM3 crystal structure, which was then used for in silico screening of small drug-like molecules that were predicted to bind to IgCAM3 (ref. 141). In vitro testing identified one compound that diverts MLCK1 from the perijunctional actomyosin ring and reverses cytokine-induced MLCK1 recruitment in vivo and in excised human intestine[141]. This molecule, known as divertin, blocks MLCK1-mediated phosphorylation of perijunctional MLC, thereby preventing subsequent occludin endocytosis and increases in leak pathway permeability[141]. Divertin does not, however, interfere with other functions of long or short MLCK, including its involvement in epithelial cell migration and smooth muscle contraction, because IgCAM3 is not present in short MLCK and is distant from the MLCK1 catalytic and regulatory domains[141]. This functional selectivity is critical, as in vivo inhibition of MLCK activity leads to hypotension and visceral paralysis, including aperistalsis, thereby precluding therapeutic use of enzymatic inhibitors[162].

Therapeutic targeting of MLCK1 recruitment

Divertin was remarkably effective in a mouse model of immune-mediated IBD – its effects were equal or superior to those of anti-TNF therapy by all measures, including survival[141]. This result supports the hypothesis that divertin-mediated interference with IgCAM3-mediated protein–protein interactions prevents perijunctional MLCK1 recruitment and, ultimately, disease progression.

A screen for potential MLCK1 binding partners was used to identify protein–protein interactions targeted by divertin. This process led to the discovery of tacrolimus binding protein FKBP8 as an MLCK1-interacting protein[160] (Fig. 4). MLCK1–FKBP8 interactions were specifically increased in TNF-treated intestinal epithelial monolayer cultures[160]. These interactions tended to occur near the perijunctional actomyosin ring[160]. Similarly, increases in perijunctional MLCK1–FKBP8 interactions were detected in biopsy samples from patients with Crohn's disease[160]. Tacrolimus also prevented TNF-induced perijunctional MLCK1 recruitment, MLCK1–FKBP8 interactions and

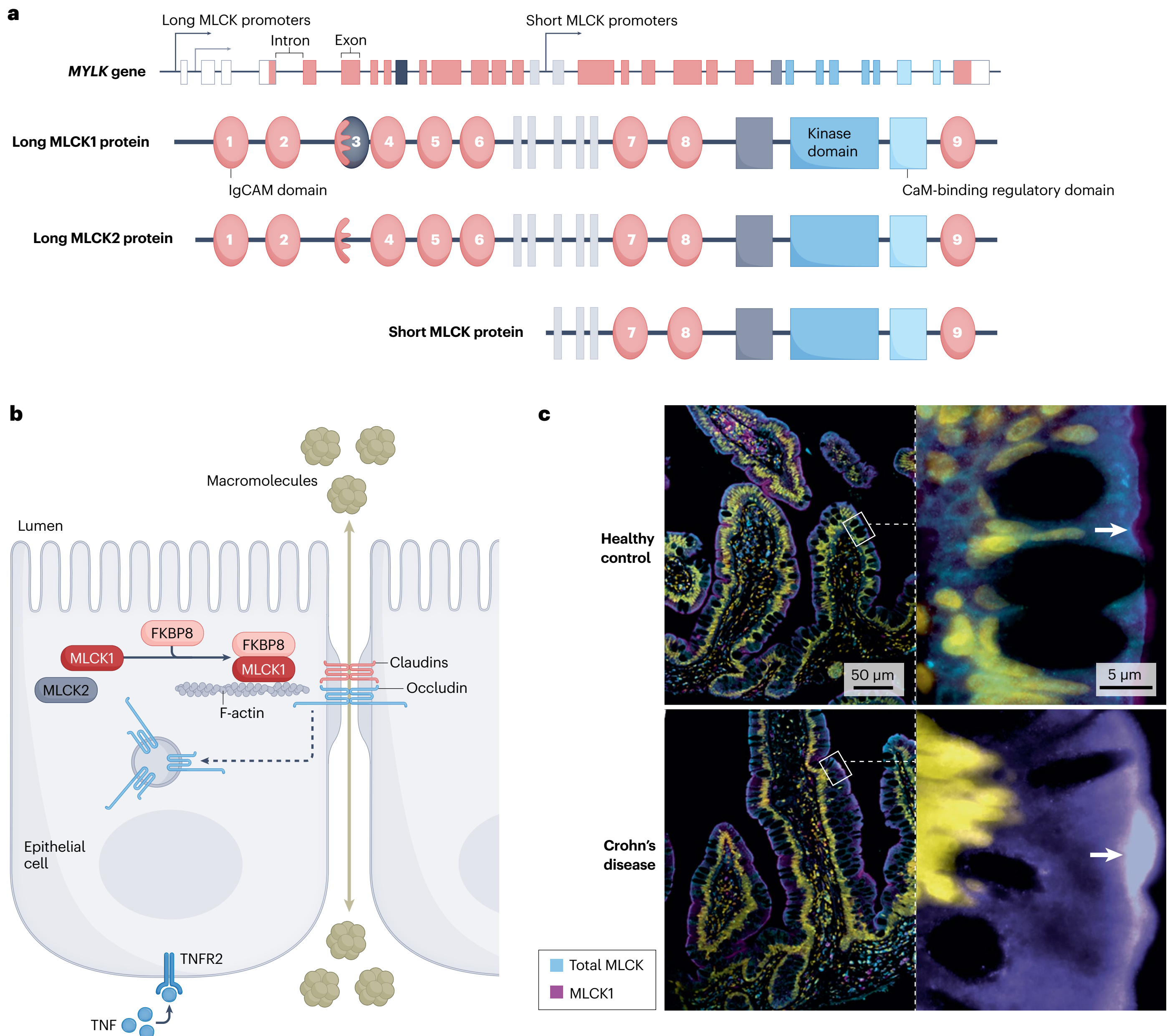


Fig. 4 | Epithelial and smooth muscle myosin light chain kinase. a, The human *MYLK* gene encodes long (non-muscle) and short (smooth muscle) isoforms of myosin light chain kinase (MLCK) protein. Two long MLCK transcriptional start sites that result in expression of the same protein have been identified. However, extensive alternative splicing within the 5′ half of the transcript occurs, which, in intestinal epithelial cells, results in expression of two long MLCK splice variants, MLCK1 and MLCK2. These variants differ by a single exon (black), removal of which causes the third of the nine immunoglobulin-cell adhesion molecule (IgCAM) domains to be incomplete in long MLCK2. The short MLCK promoter is located within a long MLCK intron and drives transcription of smooth muscle MLCK, which lacks the six amino-terminal IgCAM domains that are present in long MLCK1. The kinase and calmodulin (CaM)-binding domains are encoded by sequences within the 3′ half of *MYLK* and are identical in long and short MLCK proteins. **b**, Inflammatory signals, such as tumour necrosis factor (TNF), trigger MLCK1 binding to FKBP8. This binding facilitates MLCK1 recruitment to the perijunctional actomyosin ring, where it phosphorylates MLC. This phosphorylation causes occludin internalization to increase leak pathway permeability. In contrast to MLCK1, MLCK2 distribution is not affected by TNF. **c**, MLCK1 expression and recruitment to the perijunctional actomyosin ring (arrows) are increased in Crohn's disease. The insets show the boxed areas. MLCK1 and total MLCK are shown, as the absence of unique MLCK2 sequences prevents generation of MLCK2-specific antibodies. Nuclei appear yellow. Part **a** adapted from ref. 141, Springer Nature Limited.

perijunctional MLC phosphorylation in human intestinal organoids[160]. Finally, tacrolimus prevented MLCK1 recruitment, occludin internalization and barrier loss after acute T cell activation in mice[160]. Surprisingly, divertin did not interfere with FKBP8 binding to recombinant MLCK1 IgCAM domains one to four in vitro. Thus, despite the efficacy of divertin in experimental colitis, agents that prevent MLCK1 interactions

with FKBP8 or other MLCK1 binding partners should also be sought as potential therapeutics.

Conclusions

The first tight junction protein, ZO-1 (ref. 33), was discovered nearly 40 years ago, and numerous other tight junction proteins have been identified since[37–39,149,163–167], leading to substantial data describing the molecular interactions responsible for selective permeability and barrier regulation[144,146,147,168–172]. This work has led to conceptual advances, including the pore and leak pathway model of paracellular permeability[47,173], and foundational understanding of tight junction cell biology, physiology and pathobiology.

In the same year that ZO-1 was discovered[33], increased intestinal permeability was identified in a subset of first-degree relatives of people with Crohn's disease[174]. More recently, these modest leak pathway permeability increases were validated as an independent risk factor for IBD[24]. However, all human studies to date have relied on probes, such as lactulose and mannitol, that are too large to cross the pore pathway. Thus, despite increased claudin-2 expression in human disease[64,86,87,92,175,176] and experimental data showing that claudin-2-dependent pore pathway permeability increases exacerbate disease in mice[50,98,100], the relevance of claudin-2 to human intestinal disease remains to be determined. Our understanding of how barrier function and disease are affected by polymorphisms in barrier-related genes associated with IBD, including *INAVA*[177–179] and *CDH1* (refs. 180,181), which encode innate immune activator and E-cadherin, respectively, is even more limited.

In conclusion, our understanding of how permeability of the pore and leak pathways contributes to health and disease remains relatively rudimentary. Thus, although much has been accomplished, much more remains to be discovered. Remaining challenges include identification of the sites and molecular structure of the leak pathway, elucidation of the differences that must exist between seemingly redundant claudins, and the definition of non-canonical tight junction protein functions. Nevertheless, the promise of tight junction-targeted therapeutics remains compelling, and implementation of such therapeutic approaches is growing progressively closer.

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Author contributions

A.H. and J.R.T. wrote the manuscript. All authors researched data for the article, made substantial contributions to discussion of content and reviewed and edited the manuscript before submission.

Competing interests

J.R.T. is a consultant for Entrinsic and Kallyope. The other authors declare no competing interests.

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