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CELL SCIENCE AT A GLANCE

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The mucosal barrier at a glance

Marion M. France[1] and Jerrold R. Turner[1,2,*]

ABSTRACT

Mucosal barriers separate self from non-self and are essential for life. These barriers, which are the first line of defense against external pathogens, are formed by epithelial cells and the substances they secrete. Rather than an absolute barrier, epithelia at mucosal surfaces must allow selective paracellular flux that discriminates between solutes and water while preventing the passage of bacteria and toxins. In vertebrates, tight junctions seal the paracellular space; flux across the tight junction can occur through two distinct routes that differ in selectivity, capacity, molecular composition and regulation. Dysregulation of either pathway can accompany disease. A third, tight-junction-independent route that reflects epithelial damage can also contribute to barrier loss during disease. In this Cell Science at a Glance article and accompanying poster, we present current knowledge on the molecular components and pathways that establish this selectively permeable barrier and the interactions that lead to barrier dysfunction during disease.

KEY WORDS: Barrier function, Mucosa, Epithelia, Tight junction, Intestinal disease, Permeability

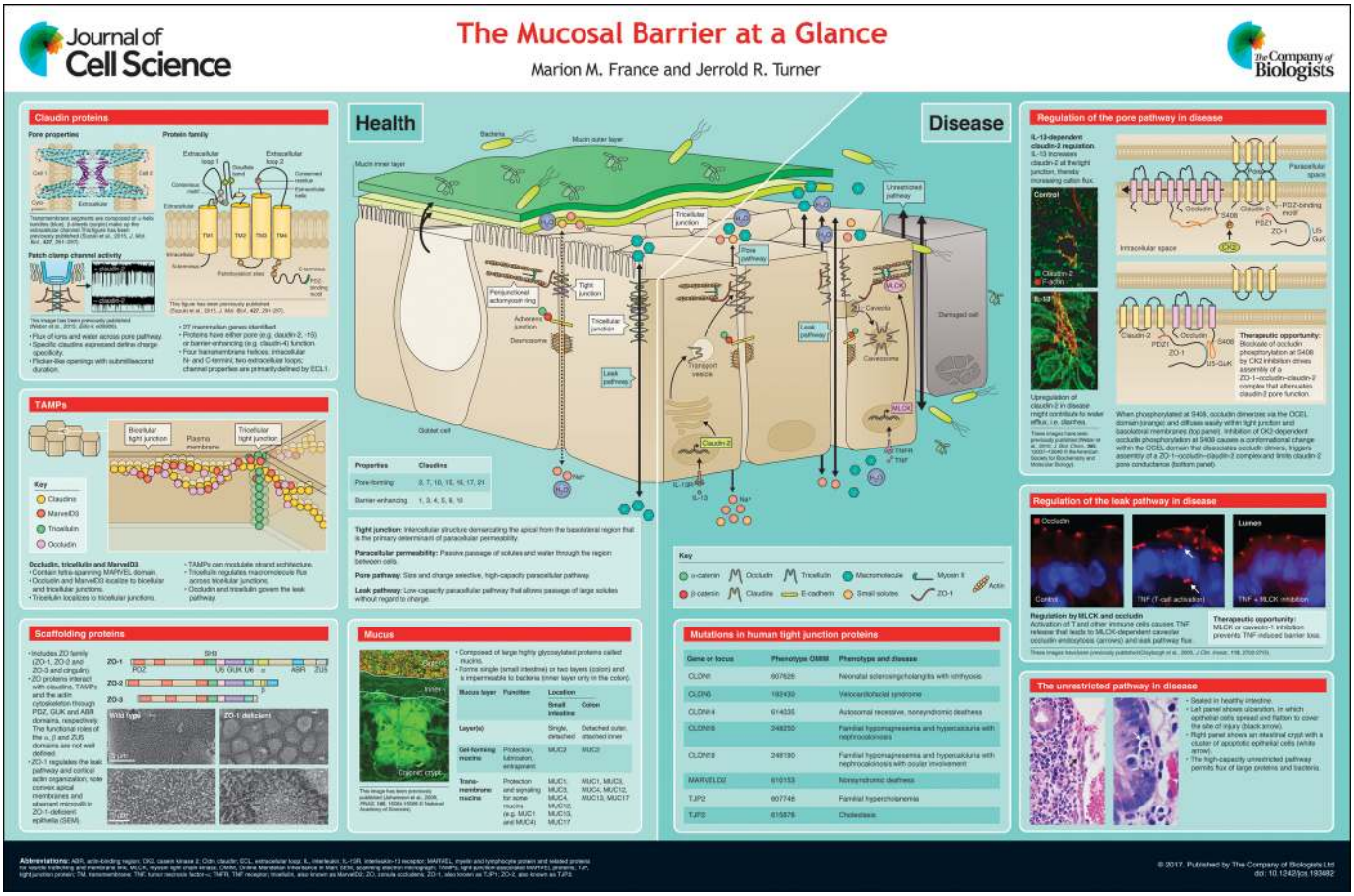
[1]Department of Medicine (Gastroenterology, Hepatology, and Endoscopy), Brigham and Women's Hospital and Harvard Medical School, 20 Shattuck St, TH1428, Boston, MA 02115, USA. [2]Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, 20 Shattuck St, TH1428, Boston, MA 02115, USA.

*Author for correspondence (jrturner@bwh.harvard.edu)

J.R.T., 0000-0003-0627-9455

Introduction

Mucosal barriers separate the external environment from the body's internal milieu. These selectively permeable barriers prohibit passage of bacteria and toxins while permitting flux of water, ions and solutes, including nutrients. Directional transport across epithelia is established through cell polarity, in which cells are asymmetrical in structure and function. The interface between the apical and basolateral membrane domains is demarcated by the apical junctional complex, which, from the apical to basolateral surfaces, is composed of tight junctions, adherens junctions and desmosomes (see poster) (Farquhar and Palade, 1963). In simple – i.e. non-stratified – epithelia, the tight junction serves as a molecular seal between adjacent cells. The core protein components of the



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tight junction support paracellular flux through two distinct routes, termed the pore and leak pathways (Anderson and Van Itallie, 2009; Turner, 2009). The pore pathway is a high-capacity route that is selective in terms of size and charge, with the maximal diameters of transported molecules ranging from approximately ∼5 Å to ∼10 Å (Krug et al., 2012; Tanaka et al., 2016; Van Itallie et al., 2008; Yu et al., 2009). Although less-well characterized, the best available evidence suggests that the complementary leak pathway supports the paracellular flux of molecules with diameters up to 125 Å and is not charge selective, but has a limited capacity (Buschmann et al., 2013; Turner, 2009). A third unrestricted pathway that opens only in particular circumstances as a consequence of epithelial damage is discussed below.

In molecular terms, tight junctions are composed of members of the claudin protein family (Furuse et al., 1998b, 1999; Morita et al., 1999), some of which function to create paracellular channels, whereas others are thought to seal the intercellular space. These channels are the anatomical site of flux through the pore pathway. Other integral membrane proteins – e.g. the tight-junction-associated MARVEL protein (TAMP) family (Raleigh et al., 2010), and periplasmic scaffolding proteins, such as the zonula occludens (ZO) protein family, are crucial to regulation and maintenance of the leak pathway. For example, ZO-1 (also known as TJP1) has an actin-binding domain which is, in part, responsible for regulation of the leak pathway through the perijunctional actomyosin ring (Van Itallie et al., 2009; Yu et al., 2010).

Epithelial cells also release protective substances. In the intestines, these include antimicrobial peptides and mucin, which are predominantly synthesized by Paneth and goblet cells, respectively. Mucins form a protective mucus layer that covers the apical surface and limits direct interactions of the epithelium with microbes and larger molecules, such as food particles (Johansson et al., 2014; Pelaseyed et al., 2014).

Precise orchestration of the components that make up the barrier is crucial for the development and maintenance of barrier function. The epithelial barrier is compromised under pathological conditions that are driven by infectious, ischemic or immune-derived stimuli. As a result, barrier loss can be detected as increased flux across the pore pathway, the leak pathway or the unrestricted pathway. The latter is a tight-junction-independent potential pathway that becomes functional in disease owing to direct epithelial damage (Nalle and Turner, 2015). The unrestricted pathway allows large quantities of large and small molecules, including microbes, to cross the damaged epithelial barrier (Aihara et al., 2016; Yu et al., 2016). When epithelial injury is limited and focal, for instance following a gastrointestinal mucosal biopsy, healing is rapid and long-term harm is avoided. This rapid reformation of the epithelial barrier is partly due to the brisk restitution response, whereby surviving epithelial cells spread to cover the basement membrane before new cells can be generated by proliferation (Aihara et al., 2016; Moore et al., 1989).

Recent years have seen substantial progress in understanding the means by which components of the mucosal barrier are coordinately regulated in health and disease. In this Cell Science at a Glance article and accompanying poster, we outline the essential factors that contribute to mucosal barrier function using the intestine as a model. We also explore how regulatory factors provide a foundation for normal function and how their associated pathways are perturbed in disease.

Tight junction composition

Intestinal epithelia establish a selective barrier that prevents passage of commensal bacteria and pathogens while permitting intercellular flux of molecules and ions (see poster) (In et al., 2016). As noted above, the tight junction forms the actual seal between adjacent cells (Farquhar and Palade, 1963). Adherens junctions and desmosomes provide the adhesive strength that holds cells together and prevent mechanical disruption of the epithelial sheet (Baum and Georgiou, 2011; Green and Simpson, 2007). Together, these structures form the apical junctional complex, which is also home to proteins that direct epithelial polarization (Anderson et al., 2004). Polarization is essential to epithelial function and involves proper sorting and delivery of both newly synthesized proteins and those trafficking through intracellular compartments to the apical or basolateral plasma membrane (Weis et al., 2016; Yeaman et al., 1999). This depends, in part, on binding of the exocyst complex to the apical junctional complex through cell-adhesion proteins (Yeaman et al., 2004) in order to direct basolateral exocytosis (Grindstaff et al., 1998).

When viewed by performing freeze-fracture electron microscopy, the tight junction is seen as a meshwork of anastomosing strands. It is generally accepted that these strands create the barrier. The biochemical composition of these strands is still a topic of investigation, but varied data support roles for both lipid and protein components (Furuse et al., 1998a, 1993; Kachar and Reese, 1982; Meyer, 1983; Pinto da Silva and Kachar, 1982; Stevenson et al., 1986). Consistent with an important role of lipids, tight junction proteins are concentrated within cholesterol-enriched raft-like microdomains whose density – i.e. composition – is altered during barrier regulation (Shen et al., 2006). Accordingly, depletion of plasma membrane cholesterol results in alterations in the structure of tight junction strands and barrier function (Francis et al., 1999). Cholesterol-dependent caveolar endocytosis is also essential for cytoskeletal-mediated internalization of occludin, which results in increased flux through the leak pathway, for instance following exposure to tumor necrosis factor-α (TNF) (Marchiando et al., 2010). Nevertheless, our understanding of the lipid structures within tight junctions is limited and in need of further study (Lingaraju et al., 2015).

Claudin proteins define the selectivity of paracellular permeability

The independent observations that expression of some claudin proteins in tight-junction-deficient fibroblasts results in assembly of tight-junction-like strands, whereas expression of other claudin proteins alters epithelial strand architecture, confirm that claudin proteins are a fundamental component of tight junction strands (Furuse et al., 1998b; Yamazaki et al., 2011). However, the exact biochemical composition of tight junction strands has not been defined. The consensus view is that the structure of the tight junction, which forms the barrier, comprises barrier-enhancing claudins that occlude the intercellular space and pore-forming claudins that form channels supporting solute flow. For example, pore-forming claudin-2 forms gated paracellular channels (Weber et al., 2015) that permit flux of Na^+ and small uncharged molecules but not of larger solutes (Van Itallie et al., 2008). Notably, although the gating behavior of these channels is similar to that of transmembrane ion channels, the relative selectivity of tight junctions for monovalent cations – including Na^+, K^+, methylamine and ethylamine, and even anions – is far more limited (Tamura et al., 2011; Weber et al., 2015; Yu et al., 2009). Claudin-2 pores also permit the flux of water through a common pore (Muto et al., 2010; Rosenthal et al., 2016, 2010). The specificity for Na^+ is governed by the first of two extracellular loops of claudin-2 that are adjoined to four transmembrane domains with

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intracellular N- and C-termini (Angelow and Yu, 2009; Li et al., 2014; Suzuki et al., 2014; Yu et al., 2009). Pore-forming claudins define the charge and size selectivity of the high-capacity pore pathway (Anderson and Van Itallie, 2009; Colegio et al., 2002; Shen et al., 2011; Turner, 2009; Van Itallie et al., 2008; Weber et al., 2010). Accordingly, the selective increase in Na^+ permeability resulting from expression of claudin-2 in cultured epithelial monolayers coincides with a reduction in transepithelial resistance (TER) (Amasheh et al., 2002; Furuse et al., 2001), a measure of barrier function. Consistent with this, the presence of claudin-2 has been identified as the primary factor responsible for the lower TERs observed in Madin-Darby canine kidney (MDCK) II compared to MDCK I monolayers, in that the latter lack claudin-2 (Furuse et al., 2001). Barrier function can also be modified by other claudins. For example, claudin-4 expression increases TER – i.e. reduces paracellular ion conductance – in cultured epithelial monolayers (Van Itallie et al., 2001). This has led to the categorization of claudin-4 as a barrier-enhancing claudin. Other data supporting the notion of barrier-enhancing claudins include the severe epidermal barrier loss in claudin-1-deficient mice (Furuse et al., 2002). However, the observation that claudin-4 expression reduces paracellular cation transport only in epithelial monolayers that express claudin-2, but not in monolayers that lack claudin-2 (Van Itallie et al., 2003), suggests that barrier-enhancing claudins reduce paracellular permeability by antagonizing the function of pore-forming claudins. Consistent with this, claudin-8 has been reported to displace claudin-2 from the tight junction, thus reducing claudin-2 channel function, and to modify tight junction structure (Angelow et al., 2007; Yu et al., 2003). These studies emphasize the importance of considering the function of claudin proteins in an individual epithelium as a reflection of the repertoire of claudin proteins expressed. The data also highlight the ability of claudin protein interactions to modulate barrier function and the importance of understanding the homotypic and heterotypic interactions between claudin proteins.

Non-claudin tight junction proteins regulate the paracellular barrier

Claudin-based tight junction strand structure and function can be modulated by other tight junction proteins, including the TAMPs – occludin, tricellulin (also known as MarvelD2) and MarvelD3 – which all contain the tetra-spanning 'myelin and lymphocyte protein and related proteins for vesicle trafficking and membrane link' (MARVEL) domain. TAMPs are recruited to distinct tight junction domains; occludin and MarvelD3 localize to bicellular and tricellular junctions, whereas tricellulin is primarily found at tricellular junctions (Ikenouchi et al., 2005; Raleigh et al., 2010; Riazuddin et al., 2006). These proteins form homotypic cis-interactions (Cording et al., 2013), and are also able to modulate strand architecture and claudin channel function (Cording et al., 2013; Raleigh et al., 2011). In this manner, occludin can regulate the paracellular pore pathway (Raleigh et al., 2011). For example, occludin can disrupt claudin-2 anchoring at the tight junction under certain conditions (Raleigh et al., 2011). Occludin also regulates the leak pathway; occludin knockdown in monolayers of MDCK (Yu et al., 2005) and human intestinal Caco-2 monolayers (Al-Sadi et al., 2011; Buschmann et al., 2013) enhances the permeability of large monovalent cations and uncharged solutes. Further, *in vivo* occludin overexpression limits cytokine-induced increases in leak pathway permeability (Marchiando et al., 2010). The role of occludin in barrier regulation is, nevertheless, controversial because two studies have reported normal intestinal barrier function in unstressed occludin-deficient mice (Saitou et al., 2000; Schulzke et al., 2005). Despite this, the most parsimonious conclusion suggests that occludin is a regulator of the tight junction, rather than an essential structural component. This interpretation could explain the absence of intestinal barrier defects in unstressed occludin-deficient mice.

Paracellular flux across tricellular junctions is regulated by tricellulin, a component of tricellular junctions (Ikenouchi et al., 2005; Krug et al., 2009). Tricellulin overexpression results in localization at tricellular as well as bicellular junctions and increases epithelial resistance by decreasing the paracellular flux of ions as well as of larger molecules (Krug et al., 2009). Tricellulin overexpression to a more limited degree only enhances localization of tricellulin exclusively at tricellular junctions and selectively decreases the permeability to macromolecules of 4–10 kDa in size (Krug et al., 2009). Further, macromolecule permeability increases when tricellulin is removed from the tricellular junction (Krug et al., 2013). These data indicate that tricellulin is a regulator of the leak pathway at tricellular junctions.

TAMPs interact directly with ZO-family proteins, including ZO-1, ZO-2 (TJP2) and ZO-3 (TJP3), which are often described as scaffolding proteins. The ZO-family proteins share similar structures, with protein-binding motifs within the N-terminus – including three PDZ domains that mediate binding to claudins, to other ZO-family members and to signaling proteins such as phospholipase C isoforms (see poster) (Fanning and Anderson, 2009; Fanning et al., 1998; Itoh et al., 1999; Meerschaert et al., 2009; Utepbergenov et al., 2006). The interactions of ZO-1 and ZO-2 with claudin proteins through the PDZ1 domain are thought to be required for claudin trafficking and tight junction biogenesis (Ikenouchi et al., 2007; Rodgers et al., 2013; Umeda et al., 2006; Yamazaki et al., 2008). The PDZ domains are followed by the U5 and GuK domains that mediate binding to occludin, a U6 domain that might regulate interactions with occludin, and finally, in the case of ZO-1 and ZO-2, actin-binding regions (Fanning et al., 2002; Muller et al., 2005). Despite these shared structures, ZO-family proteins must have unique functions given that deletion of either ZO-1 or ZO-2 results in embryonic lethality in mice (Katsuno et al., 2008; Xu et al., 2009, 2008). In terms of barrier function, ZO-1 contributes to regulation of the leak pathway, as has been demonstrated by the increased paracellular permeability of large solutes through the leak pathway when ZO-1 is knocked down in MDCK monolayers (Van Itallie et al., 2009). In addition, ZO proteins regulate cortical actin organization because cells that lack both ZO-1 and ZO-2 show cortical actin hypercontraction (Fanning et al., 2012; Ikenouchi et al., 2007). Other data indicate that ZO-1 interacts with additional cytoskeletal regulators. For example, knockdown of both ZO-1 and afadin results in reduced TER, consistent with the crucial role of the adherens junction in maintaining intercellular adhesion, which is a prerequisite for tight junction assembly (Choi et al., 2016). Conversely, some data suggest that ZO-1 also regulates adherens junctions (for review see Fanning and Anderson, 2009). Finally, recent studies indicate that ZO-1 interactions with F-actin and occludin orient epithelial polarization and direct morphogenesis in three-dimensional culture (Odenwald et al., 2017).

Adherens junctions contribute to epithelial barrier function by stabilizing the tight junction

Components of the adherens junction provide a foundation for cell–cell interactions and are essential for tight junction assembly. The major component of the epithelial adherens junction is E-cadherin (also known as CDH1), a single-spanning transmembrane protein

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capable of homotypic cell–cell interactions and intracellular interactions with other adherens junction components (Hartsock and Nelson, 2008). *In vitro* knockdown studies suggest that, although E-cadherin is essential for assembly, it might not be required for maintenance of tight junctions (Capaldo and Macara, 2007). In mice, functional disruption of E-cadherin through chimeric expression of a dominant-negative N-cadherin mutant that lacks an extracellular domain leads to aberrant epithelial differentiation, an active inflammatory response, crypt hyperproliferation and epithelial dysplasia (Hermiston and Gordon, 1995a,b). Tissue-specific E-cadherin knockout within the intestinal epithelium results in loss of both adherens junctions and desmosomes as well as deficiencies in the numbers of Paneth and goblet cells (Bondow et al., 2012; Schneider et al., 2010). Similar changes occur after deletion of other adherens junction components, such as p120 and afadin (Choi et al., 2016; Ikeda et al., 1999; Smalley-Freed et al., 2010; Tanaka-Okamoto et al., 2011). Although the functional impact is not well defined, it is also worth noting that E-cadherin (*CDH1*) polymorphisms are linked to inflammatory bowel disease (Barrett et al., 2009; Muise et al., 2009), and both gastric (Guilford et al., 1998) and colonic adenocarcinoma (Houlston et al., 2008).

Activation of the perijunctional actomyosin ring regulates tight junction barrier function

The perijunctional actomyosin ring is described as a dense ring of myosin and actin that runs around the circumference of the cell at the region of the adherens and tight junctions (see poster). Regulation of tight junction function is in part governed by myosin light chain kinase (MLCK), which phosphorylates myosin II regulatory light chain to drive actomyosin contraction and increase tight junction permeability. For example, the increase in paracellular permeability that follows activation of Na^+–glucose cotransport and supports paracellular nutrient absorption is blocked by inhibition of MLCK (Sadowski and Meddings, 1993; Turner et al., 1997), whereas expression of a constitutively active MLCK catalytic domain that lacks regulatory elements is sufficient to reorganize perijunctional actomyosin and increase paracellular permeability *in vitro* and *in vivo* (Hecht et al., 1996; Shen et al., 2006; Su et al., 2009). Further, genetic or pharmacological inhibition of MLCK can prevent acute cytokine-induced increases in flux through the leak pathway *in vitro* and *in vivo* (Clayburgh et al., 2005).

Mucin is an extracellular component essential in barrier function

Mucus provides a layer of protection between the luminal contents and the epithelium layer. In the colon, mucus is organized as a mucus bilayer composed of an adherent inner layer and a loose detached outer layer, whereas only a single detached layer is present in the small intestine (see poster) (Heazlewood et al., 2008; Johansson et al., 2011, 2008, 2013; McGuckin et al., 2011). Mucus is impermeable to commensal bacteria, with the exception of that forming the outer layer in the colon (Johansson et al., 2011, 2008, 2013). Mucins are highly glycosylated proteins produced and released by goblet cells that vary in structure, function and sites of expression (Fu et al., 2011; Johansson et al., 2013; Larsson et al., 2011; Sommer et al., 2014). For example, differences in their protein domains distinguish the transmembrane mucins anchored to the epithelial layer from the detached gel-forming mucins. Depletion of the dominant intestinal mucin gene, *Muc2*, leads to mucosal injury and diarrhea (Van der Sluis et al., 2006), emphasizing the essential protective role of the mucus layer.

Tight junction permeability pathways in disease

During disease, changes to the components of the barrier modify tight junction structure and function, ultimately leading to barrier dysfunction. In inflammatory bowel disease, the representative cytokines interleukin (IL)-13 and TNF are commonly upregulated (Kelsen et al., 2015; Kiesler et al., 2015), and induce barrier loss through distinct mechanisms (Shen et al., 2011). Studies of intestinal epithelia have shown that IL-13 and TNF reduce barrier function by targeting claudin-2 and MLCK, respectively (see poster) (Weber et al., 2010). Specifically, IL-13-dependent claudin-2 expression increases the flux of small cations across the pore pathway, both *in vitro* and *in vivo* (Weber et al., 2010). In contrast, TNF induces increases in the flux of larger solutes across the leak pathway that can be selectively blocked through genetic or pharmacological inhibition of MLCK (Clayburgh et al., 2005; Weber et al., 2010; Zolotarevsky et al., 2002) or by overexpressing occludin (Marchiando et al., 2010). Although they appear to be distinct, there is evidence that the pore and leak pathways converge at the level of intercellular signaling. For example, mucosal IL-13 and epithelial claudin-2 expression are elevated in mice with intestinal epithelial expression of a constitutively active MLCK catalytic domain (Su et al., 2009; Weber et al., 2010). Conversely, claudin-2 expression is not upregulated during experimental inflammatory bowel disease in mice that lack epithelial MLCK (Su et al., 2013). Finally, prolonged TNF treatment can enhance claudin-2 expression *in vitro* (Mankertz et al., 2009). In humans, claudin-2 can contribute to barrier dysfunction during disease, as colonic biopsies from individuals with inflammatory bowel disease exhibit increased claudin-2 expression (Heller et al., 2005; Prasad et al., 2005; Weber et al., 2008), and this could be a contributing factor to increased water-flux-associated diarrhea (Luettig et al., 2015). As such, targeting claudin-2 function could represent an untapped therapeutic opportunity (see poster). *In vitro* studies suggest that one such potential approach could be inhibition of casein-kinase-2-dependent occludin phosphorylation as this has been shown to limit claudin-2 pore function and reverse IL-13-induced barrier loss (Raleigh et al., 2011). This reversal is due to assembly of a complex containing dephosphorylated occludin, ZO-1 and claudin-2 that limits claudin-2 channel function (Raleigh et al., 2011). The potential of casein kinase 2 inhibitors as *in vivo* therapeutic interventions have not yet been reported.

In Crohn's disease, anti-TNF antibody (biologic) therapy has been shown to restore intestinal barrier function (Suenaert et al., 2002). Whether restoration is partly due to inhibition of TNF-induced MLCK activation in intestinal epithelial cells, or whether it only reflects the overall dampening of inflammatory activity, remains to be determined. It is, however, notable that intestinal epithelial expression of MLCK and its activity are increased during inflammatory bowel diseases – that is Crohn's disease and ulcerative colitis (Blair et al., 2006). Further, mice that lack intestinal epithelial MLCK are protected from increases in tight junction leak pathway permeability during experimental inflammatory bowel disease (Su et al., 2013).

The unrestricted pathway

In contrast to the pore and leak pathways, particles of almost any size can overcome the epithelial barrier through the unrestricted pathway; this potential route opens as a result of epithelial damage and allows the flux of large proteins, viruses and bacteria (see poster). A well-documented example of unrestricted pathway flux occurs during graft-versus-host disease (GVHD), in which the extent of bacterial flux across the unrestricted pathway correlates

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with the severity of the disease (Cooke et al., 1998). GVHD is a complication that can occur following a bone marrow or hematopoietic stem cell transplant, whereby the recipient's body cells are attacked by donor-derived immune cells. Using a clinically relevant mouse model of GVHD, epithelial damage has been determined to be essential for disease pathogenesis (Nalle et al., 2014). Consistent with the crucial role of the intestinal barrier in limiting GVHD, the requirement for epithelial injury could be circumvented through intraperitoneal administration of bacterial lipopolysaccharides – i.e. endotoxin (Nalle et al., 2014).

Isolated tight junction dysfunction is insufficient to cause disease

The causes of barrier dysfunction in disease are multifactorial; barrier loss can result from changes to the components that regulate and maintain distinct permeability pathways as well as the mucosal immune stimuli. Indeed, barrier loss is thought to be a driving force in the initiation and propagation of many intestinal disorders (Hollander et al., 1986). For example, the onset of experimental inflammatory bowel disease is accelerated and disease is more severe in transgenic mice that express constitutively active MLCK within the intestinal epithelium (Su et al., 2009). However, barrier loss itself alone does not cause intestinal disease, as demonstrated in multiple mouse models (Su et al., 2009; Vetrano et al., 2008). In humans, this is shown by the subset of first-degree relatives of individuals with Crohn's disease who have increased intestinal permeability but are healthy (Hollander et al., 1986; May et al., 1992). This increased permeability has been linked to specific NOD2 polymorphisms (Buhner et al., 2006), but it has not been determined whether these healthy relatives are at increased risk of developing disease.

Although this discussion has focused on inflammatory bowel disease and graft-versus-host disease, which has been studied extensively, other examples of disease-associated gut barrier loss abound, including those that occur within the contexts of intestinal infection (Halliez et al., 2016; In et al., 2016; Zolotarevsky et al., 2002), irritable bowel syndrome (Bertiaux-Vandaële et al., 2011; Martínez et al., 2013; Wu et al., 2016), celiac disease (Schumann et al., 2012; Setty et al., 2015; Szakál et al., 2010) and environmental enteric dysfunction (Kelly et al., 2016; Vinetz et al., 2016; Yu et al., 2016). Although studied to a lesser extent, barrier defects also occur in pulmonary, renal and dermatologic diseases.

Conclusions and perspectives

Mucosal barrier function is dependent upon a complex integrated network of numerous protein and lipid components that extend from the epithelium to the mucus layer. The tight junction, which defines much of the mucosal barrier function, comprises multiple proteins, including claudins, that have been characterized as either barrier-enhancing or pore-forming. This, however, remains incompletely understood, as does claudin regulation as a whole. Further research is needed to unveil interactions that are essential to mucosal barrier homeostasis in order to define how these components, as well as pore and leak pathways, are disrupted during disease. Important challenges include defining the means by which cytokines, including TNF and IL-13, signal to cause barrier loss and identifying potential therapeutic targets. Despite being insufficient to initiate disease, intestinal barrier loss is likely to play an important role in disease progression, and success in the challenges associated with understanding barrier loss is likely to be beneficial in treating many diseases.

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Competing interests

The authors declare no competing or financial interests.

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