Claudin-18 Loss Alters Transcellular Chloride Flux but not Tight Junction Ion Selectivity in Gastric Epithelial Cells

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SUMMARY
Claudin-18 loss during Helicobacter pylori infection promotes neoplastic progression. This phenotype was not due to barrier dysfunction, because paracellular permeability was not affected by claudin-18 deletion but may be secondary to transcellular anion transporter expression/function in the absence of claudin-18.

BACKGROUND & AIMS: Tight junctions form a barrier to the paracellular passage of luminal antigens. Although most tight junction proteins reside within the apical tight junction complex, claudin-18 localizes mainly to the basolateral membrane where its contribution to paracellular ion transport is undefined. Claudin-18 loss in mice results in gastric neoplasia development and tumorigenesis that may or may not be due to tight junction dysfunction. The aim here was to investigate paracellular permeability defects in stomach mucosa from claudin-18 knockout (Cldn18-KO) mice.

METHODS: Stomach tissue from wild-type, heterozygous, or Cldn18-KO mice were stripped of the external muscle layer and mounted in Ussing chambers. Transepithelial resistance, dextran 4 kDa flux, and potential difference (PD) were calculated from the chambered tissues after identifying differences in tissue histopathology that were used to normalize these measurements. Marker expression for claudins and ion transporters were investigated by transcriptomic and immunostaining analysis.

RESULTS: No paracellular permeability defects were evident in stomach mucosa from Cldn18-KO mice. RNAseq identified changes in 4 claudins from Cldn18–KO mice, particularly the up-regulation of claudin-2. Although claudin-2 localized to tight junctions in cells at the base of gastric glands, its presence did not contribute overall to mucosal permeability. Stomach tissue from Cldn18–KO mice also had no PD versus a lumen-negative PD in tissues from wild-type mice. This difference resulted from changes in transcellular Cl− permeability with the down-regulation of Cl− loading and Cl− secreting anion transporters.

CONCLUSIONS: Our findings suggest that Cldn18-KO has no effect on tight junction permeability in the stomach from adult mice but rather affects anion permeability. The phenotype in these mice may thus be secondary to transcellular anion transporter expression/function in the absence of claudin-18.

Keywords: Claudin-2; Paracellular Permeability; Genomic Profile; Ussing Chambers; RNAseq; Gastric Cancer.
One essential function of epithelial cells is to regulate ion transport between apical (mucosal) and basolateral (serosal) surfaces via transcellular and paracellular pathways. Whereas the transcellular pathway requires specific transporters that move ions into (ion loading) and out (ion secretion) of cells, the specificity and selectivity of paracellular transport occur at the apical tight junction complex. The main contributors to selective permeability at tight junctions are claudin molecules, a family of membrane-bound, tetraspanning, and pore-forming proteins. There are currently 27 annotated members of the claudin family in rodents; each is thought to confer a unique selectivity to the passage of charged and uncharged solutes. Claudins are expressed in a tissue-dependent manner; in a given tissue the dynamics of paracellular permeability are largely defined by the distinct combination of expressed claudins.

In the human and mouse stomach, the major claudin molecule expressed in epithelial cells is claudin-18. Claudin-18 has 2 isoforms; Cldn18A2.1 is primarily expressed in the stomach, and Cldn18A1.1 is expressed in the lung. A previous study of mice lacking the stomach isoform (stCldn18-KO) reported increased mucosal to serosal H⁺ flux and concluded that claudin-18 forms a paracellular barrier to cation permeation at tight junctions. Consistent with this, transmucosal Na⁺ (cation) and Cl⁻ (anion) permeabilities were also increased in stCldn18-KO mice. This increased cation and anion permeability was proposed to be caused by the up-regulation of claudin-2 and claudin-7, respectively. Cation permeability defects at tight junctions, promoting inflammation and mucosal injury that do not resolve, were thought to cause spasmodic polypeptide-expressing metaplasia and were proposed to account for the development of gastric tumors in stCldn18-KO mice. Permeability defects also occurred at tight junctions in cultured cells incubated with Helicobacter pylori, and tight junction dysfunction is thought to be a risk factor for H. pylori-induced gastric cancer development.

Although tight junction proteins typically reside within the apical tight junction complex, we showed using super-resolution microscopy techniques in archived formalin-fixed paraffin-embedded (FFPE) tissues that stomach-specific claudin-18A2.1 localized mainly to the basolateral membrane of gastric epithelial cells. It was previously demonstrated that Cldn-18A1.1 (lung isoform), which is not normally expressed in the adult stomach, was highly expressed in adult stCldn18-KO mice and localized to tight junctions. Because the lung isoform was highly expressed at tight junctions, it was unclear to us why a cation leak occurred in stCldn18-KO mice unless the lung and stomach isoforms function differently. It was also demonstrated that gastric tissues from stCldn18-KO mice were actively proliferating along the gland base, which caused a significant increase in the number of cells lining the gastric glands. We observed a similar pattern of proliferation and cellular hyperplasia in mice lacking both lung and stomach isoforms of claudin-18 (Cldn18-KO mice). Because an increase in the number of cells, which increases mucosal surface area, is known to affect tissue conductance, it was unclear whether the transepithelial resistance (TER) and

Permeability data reported for stCldn18-KO mice were due to cellular hyperplasia or reflected differences in tight junction function, per se.

Therefore, the present study was designed to explore the regulation of permeability changes in Cldn18-KO mice, which show progressive neoplasia development with significant premalignant lesions in the gastric mucosa by 7 weeks after birth. This model is clinically relevant, because the stomach mucosa from gastric cancer patients without claudin-18 have neither stomach nor lung isoforms. We demonstrate here that Cldn18-KO has no effect on gastric paracellular permeability when the measured TER, dilution potential, and paracellular flux data are corrected for surface area and mucosal cell number. Cldn18-KO mice had increased expression of claudin-2, which was mainly localized to cells at the base of gastric glands, but despite this result had no alterations in ion conductance or paracellular permeability. Furthermore, tissues from Cldn18-KO mice showed considerable differences in Cl⁻ but not Na⁺ permeability because of gastric atrophy and impairment of transepithelial Cl⁻ transport. Our data suggest that adult Cldn18-KO mice lack significant permeability defects to cause progressive neoplasia development and gastric tumorigenesis, but instead the phenotype in these mice may thus be secondary to transcellular anion transporter expression/function in the absence of claudin-18.

**Results**

**Cldn18 Deficiency Results in the Reorganization of Tight Junction Claudins**

To determine whether the deletion of Cldn18 affects the expression of other claudin or tight junction components, genomic profiling of the neck region from the gastric mucosa of claudin-18 wild-type (Cldn18⁺/+ ) and Cldn18-KO (Cldn18⁻/-) mice was done. Our mouse database annotated 26 of 27 rodent claudin genes (Figure 1). For claudin-18 wild-type mice, claudin gene expression could be stratified into 3 groups: (1) low (<100 normalized counts, 18/26 claudins); (2) medium (101–2000 normalized counts including Cldn 3, 4, 7, 8, 12, and 25); and (3) high (>25,000 normalized counts, limited to Cldn18) (Figure 1A and B). Significant changes in claudin gene expression for Cldn18-KO mice included the down-regulation of Cldns 8 and 15 and the up-regulation of Cldns 2 and 23 (Figure 1C and D, supplementary Table S1).

Abbreviations used in this paper: ANOVA, analysis of variance; Cldn, claudin; Cldn18-KO mice, claudin-18 knockout mice that are mice deficient in both stomach and lung isoforms of claudin-18; FD-4, fluorescein isothiocyanate-dextran, average molecular weight 4000; FFPE, formalin-fixed paraffin-embedded; MIT, Massachusetts Institute of Technology; NaK-ATPase, sodium (Na⁺)-potassium (K⁺)-ATPase; NKCC1, sodium (Na⁺)-potassium (K⁺)-2 chloride (2 Cl⁻) cotransporter-1; PD, potential difference; SE, standard error; stCldn18-KO mice, claudin-18 knockout mice with the knockout directed specifically to the stomach isoform of claudin-18; TER, transepithelial resistance.

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Claudin-8 is barrier-enhancing, and claudins-8 and -15 form a barrier to cation permeation like claudin-18. In addition, claudin-2 forms cation channels. Overall, these data suggest that remodeling of tight junction claudins occurs after the deletion of Cldn18 to facilitate a mucosa leaky to cations, particularly with the highly significant up-regulation of claudin-2. There was also significant up-regulation of tight junction associated MARVEL proteins, adherens junction, desmosome, and gap junction gene expression in Cldn18-KO mice (Table 1). These results likely reflect a significant increase in cell number in Cldn18-KO mice.

Transepithelial Resistance and Paracellular Permeability Are Unaltered in Cldn18-KO Mice at 7 Weeks After Birth

Because the genomic profile from Cldn18-KO mice suggests a leaky mucosa, particularly to Na⁺ and other cations, we examined barrier properties of the stomach mucosa from wild-type, heterozygous (Cldn18⁺⁻/⁻), and Cldn18-KO mice by measuring TER and the rate of mucosal to serosal dextran 4 kDa (FD-4) flux. Calculating both TER and FD-4 flux requires a measurement of the surface area of epithelial cells, which is typically assumed to be the same in each treatment.

However, because of the physical shape of the mammalian stomach, tissues from the greater curvature were unable to be stretched flat, thus causing folds in the mucosa that changed the overall mucosal length of the chambered tissue from 9 mm (calculated diameter or reservoir opening length of CHM2 Ussing chambers; World Precision Instruments, Sarasota County, FL) to about 10 mm, which was calculated from the actual length of tissues taken from the chamber after the experiment (Figure 2A–C). Although no significant difference was found in the length of tissues from wild-type, heterozygous, or Cldn18-KO mice (Figure 2C), we calculated TER and FD-4 flux using the correct surface areas derived from the length measurement for each tissue. In addition, the mucosa from Cldn18-KO mice was significantly thicker (Figure 2D–F),
containing longer glands with a greater number of cells per area and thus a greater surface area. To account for this difference, we measured the mucosal height from each tissue and used this figure to normalize TER and FD-4 flux measurements. It should also be noted that each tissue was also evaluated in H&E sections after the chamber experiment (Figure 2D and E) to ensure that proper nutrient and gas perfusion occurred; in a few of the tissues, poor perfusion led to a band of necrotic tissue in the center. This feature was used to exclude tissues from the study because cells in the tissue center were presumed to be nonviable. Tattering of the apical surface (Figure 2E) is a feature of the Cldn18-KO mouse stomach mucosa and not related to running tissues in the Ussing chamber.

When the data were normalized to mucosal height, there was no significant difference in TER, conductance, or luminal to basolateral FD-4 flux in Cldn18-KO mice (Figure 3A, C, and E). In addition, there was no effect of gender on TER (Figure 3A; $F_{1,29} = 1.492; P = .223$) or on the mucosal to serosal FD-4 flux (Figure 3E; $F_{1,31} = 0.529; P = .752$). In contrast, when differences in mucosal height were not considered in the calculations, the results showed a significant decrease in TER, an increase in conductance (Figure 3B and D), and a significant increase in FD-4 flux (Figure 3F) in Cldn18-KO compared with wild-type or heterozygous mice. Hence, the results obtained by calculating TER and permeability measurements using the surface area from each individual tissue and normalizing the data to mucosal height suggest that the loss of Cldn18 does not affect tight junction function in the ex vivo stomach at 7 weeks after birth.

### Claudin-2 Protein Is Expressed in the Stomach Mucosa of Cldn18-KO Mice

The results indicating no increase in TER or paracellular permeability in Cldn18-KO mice were inconsistent with

| Gene designation | Log2 | Significance (adjusted P value) | Gene name |
|------------------|------|-------------------------------|-----------|
| Tight junctions  |      |                               |           |
| (a) Scaffolding proteins |      |                               |           |
| Tjp1  | +0.84 | 8.66E-04 | Zonula occludins-1 (ZO-1) |
| Tjp2  | +1.35 | 8.36E-09 | Zonula occludins-2 (ZO-2) |
| Tjp3  | +1.07 | 1.20E-05 | Zonula occludins-3 (ZO-3) |
| Cgn   | +1.49 | 1.05E-07 | Cingulin |
| Mtl4  | +0.78 | .003  | Afadin |
| Mag1  | -0.78 | .003  | Membrane associated guanylate kinase-3 |
| Mag3  | +0.29 | NS (0.53) | Multiple PDZ domain protein (MUPP-1) |
| Mpdz  | +0.06 | NS (1.0) |           |
| (b) Junctional adhesion molecules |      |                               |           |
| F11r  | +0.18 | NS (0.82) | Junctional adhesion molecule-A (JAM-A) |
| Jam2  | -0.49 | NS (0.45) | Junctional adhesion molecule-B |
| Jam3  | -1.18 | .016  | Junctional adhesion molecule-C |
| (c) Tight junction associated MARVEL proteins (TAMPS) |      |                               |           |
| Ocln  | +0.91 | .0002 | Occludin |
| Marvd2| +0.60 | .05  | Tricellulin |
| Marvd3| -0.85 | NS (0.17) | MarvelD3 |
| (d) Claudins |      |                               |           |
| Cldn2 | +7.97 | 4.84E-61 | Claudin-2 |
| Cldn8 | -4.71 | 1.12E-06 | Claudin-8 |
| Cldn15| -1.89 | .012  | Claudin-15 |
| Cldn23| +5.02 | 8.68E-09 | Claudin-23 |
| Adherens junctions |      |                               |           |
| Cdh1  | +1.03 | 8.23E-05 | E-cadherin |
| Ctnna1| +0.92 | 6.80E-04 | $\alpha$-catenin |
| Ctnna2| -0.81 | NS (0.88) | $\beta$-catenin |
| Pvr1  | +0.67 | .0067 | Nectin-1 |
| Pvr2  | +1.69 | 6.92E-15 | Nectin-2 |
| Desmosomes |      |                               |           |
| Dsg2  | +1.07 | 8.58E-06 | Desmoglein 2 |
| Gap junctions |      |                               |           |
| Gjb1  | +1.81 | 5.77E-06 | Connexin-43 |
| Gja1  | +1.07 | .013  | Connexin-32 |
| Gja2  | +2.53 | .001  | Connexin-26 |
| Gjc1  | -1.56 | 1.4E-04 | Connexin-45 |

NS, no significant difference.

*Claudins that changed significantly are included.
tissues expressing high levels of claudin-2, so we next determined whether claudin-2 protein expression increased concomitant with its mRNA in Cldn18-KO mice, and whether the localization of claudin-2 was tight junction associated.

In tissues from Cldn18-KO compared with wild-type mice, there was a significant increase in claudin-2 protein expression (Figure 4A and B), with no difference between male or female mice (P = .394). Immunostaining for claudin-2 localization was difficult to do because of considerable variability in the antibodies commercially available for this protein. After testing numerous antibodies, we used the mouse monoclonal 12H12 antibody because it was considered to be most reliable of the claudin-2 antibodies commercially available.19 In FFPE mouse colon, this antibody stained tight junction-associated claudin-2 as expected at the base of crypts (Figure 5), but there was also staining at the basolateral membrane of epithelial cells and strong staining in the subapical cytoplasm of epithelial cells and in the lamina propria that was described as nonspecific in FFPE human colon tissues.19 Because tight junctions stained well in FFPE mouse colon, we used tissues prepared similarly from stomach to determine the extent of tight junction localization in Cldn18-KO compared with wild-type mice.

Claudin-2 immunostaining of stomach mucosa using FFPE tissues from wild-type mice, which should have little to no claudin-2 expression (Figure 4A), showed robust labeling of claudin-2 with the monoclonal anti-claudin-2 antibody including strong punctate staining between epithelial cells, very bright spots in the lamina propria thought to be nonspecific staining in macrophages,19 and strong co-localization of claudin-2 with Griffonia simplicifolia II lectin-expressing mucous in neck mucous cells that were confirmed to be nonspecific by using Cldn2-KO mice (Figure 4C, Figure 6).

Using FFPE tissues from Cldn18-KO mice, which had bright spots in the lamina propria but no Griffonia simplicifolia II lectin off-target staining, claudin-2 localized to the basolateral membrane and apical supranuclear
Figure 3. Significant differences in TER, conductance, and mucosal to serosal ($J_{ms}$) FD-4 flux rates between genotypes are eliminated when mucosal thickness is taken into account. (A–C) When normalized to account for individual differences in tissue height, no significant differences in (A) TER, (B) conductance, or (C) mucosal to serosal FD-4 flux were observed. (D–F) In contrast, when differences in individual tissue height are not taken into consideration when calculating the results, (D) TER in tissues from wild-type and heterozygous mice was significantly higher than in tissues from Cldn18-KO mice; (E) tissue conductance in wild-type and heterozygous mice was significantly lower than in Cldn18-KO mice; and (F) luminal to basolateral FD-4 flux was significantly higher in Cldn18-KO mice when compared with tissues from wild-type or heterozygous mice. Statistical results for all TER and conductance data (normalized and non-normalized) were obtained by using two-way ANOVA with Tukey test for post hoc comparisons and are plotted as means ± SE from n = 13 (7 M/6 F) wild-type, n = 12 (6 M/6 F) heterozygous, and n = 10 (4 M/6 F) Cldn18-KO Ussing preparations. Statistical results for mucosal to serosal FD-4 flux data were determined using Kruskal-Wallis test with Dunn’s test for post hoc comparisons and were plotted as means ± SE from n = 13 (7 M/6 F) wild-type, n = 12 (6 M/6 F) HET, and n = 12 (6 M/6 F) KO Ussing preparations. Statistical results for normalized mucosal to serosal FD-4 flux data were determined using two-way ANOVA with Tukey test for post hoc comparisons and are plotted as means ± SE from n = 13 (7 M/6 F) wild-type, n = 12 (6 M/6 F) HET, and n = 12 (6 M/6 F) KO Ussing preparations. Dotted line represents the mean for each group; female mice are denoted by red dots and male mice with blue dots. HET, heterozygous.
cytoplasm of epithelial cells. There were infrequent patches of positive cells in the neck region, but most of the positive cells were deep in the gland base (Figure 4E). To quantify this result in tissues from male and female mice, the total amount and the intensity of signal for claudin-2 were significantly greater in epithelial cells at the base of gastric glands (Figure 4F and G), which did not occur in FFPE tissues from Cldn2-KO mice (Figure 6A). This result suggested that claudin-2 expression was localized mainly to epithelial cells in the base of gastric glands and that claudin-2 was not
tight junction–associated in FFPE tissues from Clcn18-KO mice (Figure 4E).

To address a concern that stomach tight junction organization may not be amenable to FFPE methods, we immunostained unfixed frozen tissues from wild-type and Clcn18-KO mice and analyzed them for co-localization with tight junction zonula occludens 1 (Figure 7A). To provide the most robust analysis of tight junction localization, we used Figure 5. Tight junction expression occurs in colonic mucosa from control mice with claudin-2 antibody; FFPE tissues. (A) Representative images of colonic mucosa from wild-type mice (n = 3) stained with E-cadherin (E-cad) antibody to identify lateral cell membranes, claudin-2 (Clcn2) antibody to identify tight junctions, and Hoechst 33342 (HOE) to identify nuclei. As has been determined before in mice,32 in our hands the 12H2 antibody to claudin-2 localized to cells in the crypt base in colon in paraffin sections. (B and C) In the crypt base, claudin-2 co-localized with the tight junction protein ZO-1 (white signal in B, arrows in C) and was also found along the basolateral membrane (arrowheads). Claudin-2 was excluded from the base of cells in colon from FFPE tissues (asterisk). Scale bars: (A) 100 μm; (B) 20 μm; (C) 10 μm.

Figure 4. (See previous page). Increased claudin-2 protein expression in gastric epithelium accompanies up-regulation of Clcn2 in Clcn18-KO mice. (A) Representative claudin-2/actin immunoblot results performed on gastric mucosal tissues. Little claudin-2 is expressed in the normal stomach, whereas there is significant expression in Clcn18-KO mice. Single images represent observations from n = 10 (5 M/5 F) wild-type and n = 10 (5 M/5 F) Clcn18-KO mouse stomachs. (B) Quantification of claudin-2 protein expression normalized to actin levels from n = 10 (5 M/5 F) wild-type and n = 10 (5 M/5 F) Clcn18-KO mouse stomachs confirms significant increase in claudin-2 expression in Clcn18-KO mice compared with wild type mice with no effect of sex. ** denotes significance of P = .001. (C and D) Confocal microscopy analysis of claudin-2 protein expression in gastric mucosa from wild-type mice (n = 10; 5 M/5 F) shows robust claudin-2 expression in FFPE tissues. However, this is nonspecific binding of the antibody to different cells, particularly to mucus in neck mucus cells as confirmed by using similar sections from Clcn2 knockout mouse stained with claudin-2 antibody and Griffonia simplicifolia II lectin (Figure 6). Scale bars: 50 μm (low mags), 20 μm (pit, neck, and base insets). Clcn2, claudin-2; E-Cad, E-cadherin; HOE, Hoechst 33342 to stain nuclei; L, gastric lumen; SM, submucosa. (E–G) Confocal microscopy analysis of claudin-2 expression in FFPE tissues from Clcn18-KO mice (n = 10; 5 M/5 F) shows strong staining in the neck and base with claudin-2 localized to the basolateral membrane. Statistical analysis for pixel sum and fluorescence intensity was obtained using 2-way ANOVA with Tukey test for post hoc comparisons and were plotted as means ± SE. Scale bars: 50 μm (low mags); 5 μm (insets i and ii). Arrows denote the position of tight junctions. Arrowheads denote the position of basolateral membranes. (D, E, F) ** denotes significance from Surface/Pit at P = .01, *** denotes significance from Surface/Pit at P = .001, and † denotes significance from Neck at P = .05. In B - *** denotes significance of P = .001.
near super-resolution confocal methods (Figure 7B). In frozen sections from wild-type mice, the background staining was significantly reduced, with bright spots still present in the lamina propria and submucosa and some off-target staining in the lamina propria (data not shown). However, tissues from Cldn18-KO mice showed basolateral membrane staining and clear but weak claudin-2 associated tight junction staining in cells at the gland base in patches of cells that were positive for claudin-2 (Figure 7A and B). The frozen sections also showed infrequent patches of cells in the neck region that were positive for claudin-2 (Figure 7A). Overall, the data using frozen sections suggested that claudin-2 is expressed at tight junctions of some cells in the neck and at the gland base of Cldn18-KO mice but is most highly localized to the basolateral membrane.

**Cldn18 Loss Results in Decreased Transcellular Serosal to Mucosal Chloride Transport**

Although TER and FD-4 flux data showed no differences between genotypes (Figure 3A–C), the PD of tissues from wild-type and heterozygous mice differed significantly from
Cldn18-KO mice (Figure 8A). The PD was strongly lumen negative in wild-type and heterozygous mice, whereas the PD was nearly 0 in Cldn18-KO mice (Figure 8A). This difference in PD may reflect altered paracellular ion permeability at tight junctions or a significant reduction in tranacellular permeability. We measured dilution potentials to determine which pathway was affected in Cldn18-KO mice.

Dilution potential experiments showed significantly different Na⁺ and/or Cl⁻ movement in tissues for each genotype (Figure 8B, $P_{CI}/P_{Na}$). Substitution of NaCl from the mucosal solution resulted in changes in PD (Figure 8C), whereas substitution from the serosal solution did not (Figure 8D). This result alone confirms the lack of tight junction alterations in these mice because tight junctions are passive conduits in which permeability properties are symmetrical (see Discussion). Calculations of absolute permeabilities indicated no significant differences in Na⁺ movement between
genotypes (Figure 8A, PNa), whereas Cl\textsuperscript{–} movement was significantly slower in Cldn18-KO compared with wild-type and heterozygous mice (Figure 8B, PCl). These results suggest that an anion transport deficit exists in Cldn18-KO mice that is not tight junction associated but is rather a defect in transcellular chloride transport in a serosal to mucosal direction. With sodium movement unchanged, the slower rate of chloride movement results in neutralization of the charge and thus a near-zero PD in tissues from Cldn18-KO mice.

**Loss of Cldn18 Results in the Down-Regulation of Major Chloride Transporters in the Gastric Mucosa of Cldn18-KO Mice**

Our genomic database annotated numerous genes encoding proteins for neck cell region chloride transporters, many of which differ significantly in Cldn18-KO compared with wild-type mice (Figure 9). When further separated into ion transporters located at the basolateral membrane (used for Cl\textsuperscript{–} loading), we observed that Slc4A2 (AE2) was down-regulated, whereas Slc12A2 (NKCC1) was unchanged (Figure 9A and B). In addition, Slc12A6 (KCC3a), an ion transporter that binds to the sodium potassium adenine triphosphatase (NaK-ATPase), targets it to lipid raft domains on the basolateral membrane, and regulates its activity,\textsuperscript{10} was down-regulated (Figure 9A and B). Ion transporters used for Cl\textsuperscript{–} secretion at the apical membrane include Clcn2 (CIC-2) and Cftr, which were up-regulated, whereas Slc26A9 and Slc12A7 (KCC4) were down-regulated (Figure 9A and B).

We previously showed that Cftr localized to the apical membrane of metaplastic cells in the gland base from Cldn18-KO mice.\textsuperscript{15} For the mucosa to secrete Cl\textsuperscript{–} into the gastric lumen, NKCC1 must be targeted to the basolateral membrane for Cl\textsuperscript{–} loading. We thus confirmed that NKCC1

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**Figure 8.** Electrophysiological measurements indicate increased luminal positivity in Cldn18-KO mice as a result of decreased basolateral to apical chloride permeability. (A) Stomachs from Cldn18-KO mice were characterized by significantly higher (less negative) mean potential differences than stomach from wild-type or heterozygous (HET) mice. (B) PCl/PNa significantly differed between all groups, attributable to significant reduction in chloride permeability (PCl) in the stomach from Cldn18-KO mice compared with wild-type and heterozygous mice with no differences in sodium permeability (PNa). (C) Significant differences between all groups were observed in dilution potential experiments with apical NaCl substitution. (D) No differences between groups were observed in dilution potential experiments with basolateral NaCl substitution. PD data were determined by using Kruskal-Wallis test with post hoc comparisons performed using Dunn’s method and were plotted as means ± SE from n = 13 (7 M/6 F) wild-type, n = 12 (6 M/6 F) HET, and n = 12 (6 M/6 F) KO Ussing preparations. Dilution potential (basolateral NaCl substitution) data were determined by using Kruskal-Wallis test and are plotted as means ± SE from n = 6 (3 M/3 F) Ussing preparations per genotype. Dilution potential (apical NaCl substitution) data were determined by using one-way ANOVA with post hoc comparisons performed by using Tukey test and were plotted as means ± SE from n = 6 (3 M/3 F) Ussing preparations per genotype. PCl and PNa results were calculated by using the Goldman-Hodgkin-Katz and Kimizuka-Koketsu equations, with statistical analysis performed using one-way ANOVA with post hoc comparisons with Tukey test and were plotted as means ± SE from n = 6 (3 M/3 F) Ussing preparations per genotype. Dotted line in box plots represents the mean for each group; female mice are denoted by red dots and male mice with blue dots.
targeted to the basolateral membrane of cells at the base of metaplastic glands in Cldn18-KO mice (Figure 9C). This localization was not typical, because NKCC1 targeted to the basolateral membrane of parietal and neck cells in wild-type mice and not to gland base cells (Figure 9D). NKCC1 membrane targeting is regulated by phosphorylation via Wnk/Spak/Osr1, for which the mRNA expression (P values were calculated for Log2 data) was not different in Cldn18-KO vs wild-type mice (Figure 9E). For NKCC1 to function in Cl− loading, the NaK-ATPase must also localize to the basolateral membrane in Cldn18-KO mice to create an ion gradient. We thus confirmed that NaK-ATPase targeted to the basolateral membrane of cells at the base of metaplastic glands in Cldn18-KO mice (Figure 9F). However, parietal cells in the mucosa from Cldn18-KO mice had abnormal expression of NaK-ATPase along the basolateral membrane (Figure 9F, inset i), resembling internalization and/or glycosylation defects described previously. Furthermore, NaK-ATPase was homogeneously localized along the basolateral membrane in metaplastic cells (Figure 9F, inset ii), which was similar to chief cells (Figure 9G, inset ii). This localization pattern was in distinct contrast to the basolateral membrane localization in parietal and neck cells from wild-type mice, which was “beaded” along the entire membrane (Figure 9G, inset i). These data demonstrate that major ion transporters for Cl− loading and Cl− secretion localize to a compartment consistent with supporting Cl− secretion in Cldn18-KO mice but that the subcellular organization is not the same in metaplastic compared with wild-type cells.

Discussion

Although it is unclear how Cldn18 loss causes progressive neoplasia development, one hypothesis is that permeability defects result in inflammation and mucosal damage to start the cancer cascade. This concept was supported by data using stCldn18-KO mice, which showed cytokine upregulation, mucosal injury, and metaplasia development as a result of barrier defects caused by the loss of stomach-specific claudin-18. In contrast, using different methods of analysis and a different mouse model, namely a global Cldn18-deficient mouse, our work demonstrates that (1) global Cldn18 loss in mice at 7 weeks after birth does not cause tight junction dysfunction or paracellular permeability; (2) despite changes in claudin gene expression that should result in impaired cation specificity at tight junctions, mucosa from mice lacking claudin-18 show deficient transepithelial Cl− permeability that may be due to changes in the expression or activity of apical and basolateral transporters for Cl− loading and Cl− secretion, respectively; and (3) Cldn18 loss results in a number of significant structural alterations in the gastric mucosa, such as the loss of parietal cells, that impact ion conductance and thus require consideration when analyzing physiological data from Cldn18-KO mice.

TER and solute permeability, studied in Ussing chambers, are dependent on cell geometry, emphasizing the importance of identifying relevant histopathology when studying these parameters in genetically altered mice. Differences in surface area (cell number/mucosal height) and tight junction length, for instance, are not typically considered when using a monolayer of epithelial cells grown on Transwell filters that are structurally the same. However, in tissues from Cldn18-KO mice, a significant increase in cell number per area increases cell-cell contact length per area that permits the passage of solutes and electrical current. The increase in cell number/cell-cell contact length per area would theoretically increase paracellular permeability and decrease TER, which likely explains our results without normalization. Thus, significant changes in cell geometry occur in the stomach mucosa of Cldn18-KO mice that require consideration when calculating electrophysiological data.

An additional geometric consideration when using ex vivo mouse stomach tissues in Ussing chambers is that they are not flat, but rather they assume a half-ellipsoid shape if taken from across the greater curvature. It is necessary to use stomach mucosa from across the greater curvature to fill the entire Ussing chamber opening because the mouse stomach is small. Even when stretching the stomach, the mounted mucosa is a half-ellipsoid rather than flat. This means that in comparison to cells grown on Transwell filters, the surface area cannot be calculated from the radius of the chamber opening but instead using surface area measurements of a half-ellipsoid. We discovered that this calculation needed to be performed individually for each tissue because of variability in stretch, with the resultant surface area used in calculations of the electrophysiological measurements for that tissue.

Forte and Machen showed that when the mammalian stomach ex vivo is incubated with identical solutions facing mucosal and serosal surfaces, such as we had here, there was a spontaneous electrical PD, with the mucosal solution negative with respect to the serosal solution. In the case of stomach mucosa from wild-type and heterozygous mice in our study, this PD was −8.83 mV. PD is generated in the mammalian stomach by active sodium transport in the mucosal to serosal direction that is offset by chloride transport in the serosal to mucosal direction. In the case of gastric mucosa from wild-type and heterozygous mice in our study, mean Na+ permeability was less than Cl− permeability, thus generating a net negative PD. In contrast, mean Na+ permeability was not different from Cl− permeability in Cldn18-KO mice, resulting in little PD across the mucosa. Of interest is why Cl− permeability was slower in Cldn18-KO mice.

Recent computer modeling studies indicate that additional geometric characteristics impact predicted ion conductances in the stomach including the location of parietal cells (distance from the bathing solution), the presence of parietal cell canaliculi, the number of gastric glands per linear centimeter, length and diameter of the gland lumen, and the distance of the gland lumen from the bathing solution. If any of these parameters are different in genetically altered mice, differences in ion transport mechanisms and/or tissue conductance may occur and be due solely to changes in geometry. In addition to increased mucosal
height/cell number, Cldn18-KO mice show nearly complete mucosal atrophy (no parietal cells) by 7 weeks after birth.15 Because parietal cells are thought to dominate the total ion conductance of the gastric mucosa,26,27 ion secretion in mouse tissues from Cldn18-KO mice with atrophy should have altered electrical parameters, as well as Cl\textsuperscript{−}, Na\textsuperscript{+}, and K\textsuperscript{+} transcellular flux compared with wild-type mice.

Although Na\textsuperscript{+} and Cl\textsuperscript{−} transport can occur via paracellular or transcellular routes, dilution potential results with mucosal versus serosal NaCl substitution in our study were asymmetric, indicating changes in transcellular and not paracellular permeability.28 With the specific changes in claudin expression that occurred in Cldn18-KO mice, including the down-regulation of claudins-8 and -15 and the up-regulation of claudin-2, all changes that would generate a significant Na\textsuperscript{+} permeability across the mucosa, it would be easy to argue that the near-zero PD was due to high levels of Na\textsuperscript{+} permeation through tight junctions that offset Cl\textsuperscript{−}
movement across the mucosa. However, permeability calculations showed that Cl\(^{-}\), rather than Na\(^{+}\), permeability was affected in our model. This result is in contrast to an increase in both Na\(^{+}\) and Cl\(^{-}\) permeabilities previously reported in stCldn18-KO mice.\(^{15}\) Although Cldn18-KO mice do not express lung claudin-18 at tight junctions,\(^{15}\) making them different from stCldn18A2.1-KO mice,\(^{11}\) one potential difference is that we corrected all permeability measurements for individual tissue surface area and normalized to mucosal height.

In the mouse stomach, transcellular ion secretion is either acidic, which occurs during agonist-stimulated acid secretion, or nonacidic.\(^{26}\) Because we used famotidine to inhibit stimulated acid secretion in all tissues, TER and PD measurements in wild-type mice represent the situation for nonacidic electrogenic ion secretion. However, gene profiling of the corpus mucosa from Cldn18-KO mice demonstrated that at 7 weeks after birth, genes typically found in the antrum rather than corpus are expressed,\(^{15}\) suggesting that secretions in the same region from wild-type and Cldn18-KO mice would be different. In the corpus mucosa, 2 major Cl\(^{-}\) loading transporters are basolateral in parietal cells; one is Slc4A2 (AE2), which is a Cl\(^{-}\)/HCO\(_3^{-}\} \) exchanger, and the other is Slc12A2 (NKCC1), which is a Na\(^{+}\), K\(^{+}\), 2Cl\(^{-}\) co-transporter.\(^{26}\) AE2, a specific parietal cell marker that is mainly involved in Cl\(^{-}\) loading during acid secretion,\(^{26}\) is down-regulated at the mRNA and protein level in Cldn18-KO mice because of parietal cell loss.\(^{15}\) NKCC1 is the dominant basolateral pathway for Cl\(^{-}\) loading in parietal cells that are involved in nonacidic electrogenic ion secretion and in Cl\(^{-}\) secretion from mucous cells in the antral gland base.\(^{29}\) For NKCC1 to function as a co-transporter, low intracellular Na\(^{+}\) has to be generated by the proper membrane targeting and activation of the NaK-ATPase. Aided by KCC3a in parietal cells, an isofrom of the K\(^{+}\).Cl\(^{-}\} cotransporter, the NaK-ATPase is transported into distinct lipid-raft domains along the basolateral membrane, which significantly increases its ATPase activity.\(^{20}\) KCC3 mRNA and protein were down-regulated in metaplastic cells from Cldn18-KO mice, and concomitantly the NaK-ATPase was localized homogeneously along the basolateral membrane rather than to distinct membrane structures (beading) as we showed here in parietal cells. This result suggests that NaK-ATPase activity may be reduced, thus reducing serosal to mucosal Na\(^{+}\), K\(^{+}\), and Cl\(^{-}\) flux and providing a potential mechanism for the altered rate of Cl\(^{-}\) flux in Cldn18-KO mice. This reduction in Cl\(^{-}\) flux would occur despite the up-regulation of Cfr at the apical membrane of cells at the gland base in Cldn18-KO mice.\(^{15}\) Thus, Cldn18-KO mice express apical and basolateral transporters to facilitate Cl\(^{-}\) secretion, but because of parietal cell loss, changes in geometry, a reduced Na\(^{+}\) gradient, or other factors are unable to transport Cl\(^{-}\) in excess of Na\(^{+}\) to maintain a negative lumen in the resting state. Because deficits in ion transporter expression and function can be associated with gastric cancer development or progression,\(^{30}\) further experiments should be done to examine the relationship between ion transporters and the phenotype of Cldn18-KO mice.

In summary, this study demonstrates that in contrast to current thinking, barrier defects at tight junctions are not likely an adult phenotype after the loss of claudin-18. This result is important because it suggests that progressive neoplasia development in Cldn18-KO mice is initiated by factors other than permeability defects that drive inflammation and mucosal injury. Because claudin-18 is mainly a basolateral membrane protein, our results also suggest that it does not function as an accessory pore protein to augment tight junction function in adult tissues but instead may organize cellular signaling or have some other function in cells that is required for cellular homeostasis. Because the loss of CLDN18 expression is associated with the development of gastric cancer in human patients, determining how it regulates gastric homeostasis and pathways affected by its loss may inform

Figure 9. (See previous page). Loss of Cldn18 results in dysregulation of gastric mucosal chloride transporter expression. (A) mRNA expression for gastric mucosal chloride transporters that (A) normally display a specific basolateral or apical distribution pattern and (B) significantly differed (Log2, P < .0001) between tissues from wild-type and Cldn18-KO mice in the RNASeq analysis. Normalized counts data were determined by using Kruskal-Wallis test with post hoc comparisons performed using Dunn’s method and are plotted as means ± SE from n = 3 (3 M) wild-type and n = 3 (3 M) Cldn18-KO mice. (C) Confocal microscopy analysis of NKCC1 expression and localization in tissues from Cldn18-KO mice (n = 4; 2 M/2 F) shows strong membrane localization in intestinalized SPEM cells (SPEM) at base of gastric glands, a position that was shown to express Cfr.\(^{15}\) Inset (i) is higher magnification image of the gland base to better show NKCC1 localization. Hoechst 33342 to identify nuclei; PC, parietal cells. Scale bars: 50 μm (low mag); 20 μm (inset i). (D) Confocal microscopy analysis of NKCC1 expression and localization in tissues from wild-type mice (n = 4; 2 M/2 F) shows strong membrane localization along the basolateral membrane of PC with no membrane localization in chief cells (CC) at the base of gastric glands. Scale bar: 50 μm. (E) mRNA expression for effectors that regulate NKCC1 in tissues from wild-type and Cldn18-KO mice. P values represent the Log2-fold change in expression between wild-type and Cldn18-KO mice. (F) Confocal microscopy analysis of NaK-ATPase expression and localization in tissues from wild-type and Cldn18-KO mice (n = 4; 2 M/2 F) shows strong membrane localization in isPEM at the base of gastric glands. Inset (i) is higher magnification image of PC, demonstrating thickened signal along the basolateral membrane (arrows). At the gland base, NaK-ATPase localizes in a homogeneous manner to the basolateral membrane of isPEM cells (arrows). Scale bars: 50 μm (low mag); 10 μm (insets i and ii). (G) Confocal microscopy analysis of NaK-ATPase expression and localization in tissues from wild-type mice (n = 4; 2 M/2 F) shows strong membrane localization in all cells in the gastric corpus mucosa. Inset (i) is higher magnification image of PC and neck mucous cells (MNC), demonstrating a beaded localization of signal along the basolateral membrane (arrows). In contrast, NaK-ATPase localizes in a homogeneous manner to the basolateral membrane of CCs (arrows). Note that in CCs, NaK-ATPase does not localize to the basal membrane (asterisks). Scale bars: 50 μm (low mag); 10 μm (insets i and ii).
the development of new therapeutic treatments for gastric cancer.

Materials and Methods

Mice

B6.129S5-CLDN18^{tm1Lex}/Mmucd mice were purchased as frozen embryos from the Mutant Mouse Resource and Research Center (Davis, CA) and re-derived, bred, and housed at Massachusetts Institute of Technology (MIT) in an Association for Assessment and Accreditation of Laboratory Animal Care International approved facility. For each experiment, equal numbers of male and female wild-type, heterozygous, or Cldn18^KO mice were used at 7 weeks of age. Genotyping was performed by Transnetyx (Cordova, TN) via ear punches taken at weaning. Rapid cervical dislocation was used as a method of euthanasia to minimize tissue hypoxia. Alternative methods, such as the use of CO2 dislocation was used as a method of euthanasia to minimize tissue hypoxia, which are not suitable for maintaining maximum tissue viability during setup and throughout experiments. Animal experiments were performed according to institutional guidelines, and the animal protocol was approved by the Committee on Animal Care at MIT.

Stomachs from Cldn2-deficient mice were provided by Dr Sachiko Tsukita, Osaka University, Japan.

Reagents and Antibodies

FD-4, tetrodotoxin, indomethacin, and famotidine were purchased from Sigma-Aldrich (St Louis, MO). Mouse anti-claudin-2 antibody (12H12; Invitrogen, Carlsbad, CA; cat #32-5600), raised against 26 amino acids at the C-terminus, was purchased from Thermo Fisher Scientific (Rockford, IL), rat anti-ZO-1 was made by Dr Daniel Goodenough, and rabbit anti-E-cadherin antibody (AF648) was purchased from Thermo Fisher Scientific (Rockford, IL). Tetrodotoxin was purchased from R&D Systems (Minneapolis, MN). Tetrodotoxin was purchased and used according to institutional guidelines with a protocol approved by the MIT Biosafety Office.

Tissue Preparation and Ussing Chamber Setup

After euthanasia, the stomach was rapidly excised, opened along the lesser curvature, and gently washed in ice-cold Ringer’s solution (containing in mmol/L: 145.5 NaCl, 4 KCl, 1.2 CaCl2, and 0.05 indomethacin) to remove the contents. The stomach was pinned mucosal side down on a thick Sylgard 184 (Sigma-Aldrich) plate, covered with ice-cold Ringer’s solution that was continuously gassed with 100% O2, and the muscularis externa was removed from the underlying mucosa using a stereomicroscope as described previously.29,33 Tissues were then mounted between 2 Lucite halves of a Ussing-type chamber with an exposed mucosal area of 0.636 cm², with care taken to exclude mucosa from the squamous forestomach and antrum. In all Ussing chamber experiments, luminal and serosal sides of the tissue were bathed with a solution containing (in mmol/L): 108 NaCl, 22 NaHCO3, 3 KCl, 1.3 MgSO4, 2 CaCl2, 2.25 KH2PO4, 8.9 glucose, 10 Na-pyruvate, 0.03 indomethacin, 0.001 tetrodotoxin, and 0.3 famotidine (pH 7.4), and continuously gassed with 95% O2-5% CO2.33,34 Indomethacin and tetrodotoxin were used to inhibit prostaglandins and to abolish neuronal influences in mammalian stomach, respectively.33,34 Institutional approval was obtained for the use of tetrodotoxin, which is a federal select agent toxin that is regulated by the Centers for Disease Control and Prevention. Famotidine, a histamine H2 receptor antagonist, was used to block gastric acid secretion because acid secretion drives transepithelial electrical characteristics of the gastric epithelium.26,27 The sum of interventions using indomethacin, tetrodotoxin, and famotidine was important to minimize variation to ensure reproducible transepithelial electrical data that could be interpreted between genotypes. After the completion of experiments, stomach tissues were fixed in 10% neutral buffered formalin (Thermo Fisher Scientific, Hampton, NH) and processed for paraffin histology by the Beth Israel Deaconess Medical Center Histology core.

Electrophysiological Measurements

Measurements of PD and TER from male (n = 5–6) and female (n = 5–6) mice from each genotype were monitored by KCl-saturated agar bridges connected via 2 calomel electrodes to a voltmeter. TER was calculated from Ohm’s law using the change in voltage between mucosal and serosal sides during the application of a 50 µA current every 5 minutes. Five consecutive measurements of PD and TER were taken at 30-minute intervals, beginning 1.25 hours after mounting, after all electrical parameters were stabilized. TER and PD values are reported as the average of these 5 measurements.

Flux Measurements

Fluorescein isothiocyanate–labeled FD-4 (1 mg/mL) was added to the mucosal side, and 1 mL of sample was collected from the serosal side every 30 minutes with replacement using unlabeled buffer. Flux measurements were taken in conjunction with TER and PD from male and female mice from each genotype above. Samples were analyzed using a Spectromax M5 spectrophotometer (Molecular Devices, Sunnyvale, CA). FD-4 concentration was calculated from a standard curve. Flux rates were calculated from the slope of linear regression equation determined by plotting FD-4 concentration versus time.

Dilution Potentials, Calculation of $P_{Cl}$/$P_{Na}$ and Calculation of Absolute Permeabilities

In separate experiments, tissues from male (n = 5–6) and female (n = 5–6) mice from each genotype were mounted and allowed to equilibrate in the chamber for 1.25 hours, after which both sides were drained of buffer. The basolateral side was refilled with nutrient buffer, and the luminal side was filled with an osmotically balanced buffer solution that contained (in mmol/L): 54 NaCl, 3 KCl, 22 NaHCO3, 1.3 MgSO4, 108 mannitol, 2.25 KH2PO4, 10.0 Na-pyruvate, 0.001 tetrodotoxin, 0.03 indomethacin, 2 CaCl2, 8.9 dextrose, and 0.3 famotidine, pH 7.4 (from here forward referred to as dilution buffer). After substitution, PD and TER values were recorded immediately and then twice at 15-minute intervals.
After this, both chamber sides were drained and filled with nutrient buffer. After a 30-minute equilibration period, the substitution process was repeated, with nutrient buffer in the luminal side and dilution buffer in the basolateral side. Respective luminal and basolateral dilution potential values were calculated as the difference between PD recorded immediately before and 30 minutes after buffer substitution. The PD was calculated as the difference between the luminal side and dilution buffer in the basolateral side. Respective luminal and basolateral dilution potential values were calculated as the difference between PD recorded immediately before and 30 minutes after buffer substitution.

**Surface Area Determination**

Mouse stomach in our study was not flat when mounted on a Ussing chamber. Instead, it was naturally curved so to individual mucosal thickness, as described above. For Eqn 1 (Figure 10C) defines the surface area of a half-ellipsoid, \( S_{A_{issue}} \). The surface area represented that of an oblate spheroid on a Ussing chamber. Instead, it was naturally curved so to individual mucosal thickness, as described above.

\[ x = e^{-VF/RT} \]

\[ P_{Cl}/P_{Na} = \beta = \frac{\alpha - x}{\alpha x - 1} \]

\[ P_{Na} = G \cdot \left( \frac{RT}{F^2} \right) \frac{1}{(a(1 + \beta))} \]

\[ P_{Cl} = P_{Na} \cdot \beta \]

\( e \) = Mathematical constant, 2.71828
\( V \) = Dilution potential, calculated as described above.
\( G \) = Tissue conductance (1/TER) per unit surface area
\( \alpha \) = Activity ratio. The calculated activity of NaCl in the dilution buffer divided by the calculated activity of NaCl in the nutrient buffer
\( a \) = Na⁺ activity in nutrient buffer. This number is the same as the activity of NaCl in the nutrient buffer.\(^{37} \)
\( F \) = Faraday constant (96,485.3329 C/mol)
\( R \) = Ideal Gas constant (8.314 J/mol K)
\( T \) = Temperature (310.15 K).

Activity coefficients first require conversion of molarity to molality and then extrapolating the activity coefficient at 37°C from Trusdell.\(^{25} \) At 37°C, 108 mmol/L NaCl is 0.109 molal, and the activity coefficient is 0.774175. Thus, the calculated activity of NaCl at 108 mmol/L is 0.0844. Likewise at 37°C, 54 mmol/L NaCl is 0.05417 molal, and the activity coefficient is 0.8155. Thus, the calculated activity of NaCl at 54 mmol/L is 0.0442. All TER/conductance measurements used in these calculations were first normalized to individual mucosal thickness, as described above.

**Measurements of “d”**

After fixation, tissues were halved along the 2-dimensional plane defined by the axis \( a \) (Figure 10A), embedded in paraffin, and cut along the long axis. After sectioning and staining with H&E, digital images were taken along the entire length using an Axiomager wide-field microscope system (Carl Zeiss AG, Oberkochen, Germany) and then stitched into one image by using FUJI software (Tokyo, Japan). The final image was measured from end to end, and the total length was calculated by using ImageJ software.

**Measurements of Mucosal Thickness**

Using the stitched images, mucosal height (measured from the base of gastric glands to the mucosal surface) was measured at 5 equidistant positions along the length. The average height per tissue was calculated and used for normalization. In preliminary data, the number of cells per height was quantified by staining tissues with DAPI (to identify nuclei), and Volocity Image Processing Software (Quorum Technologies, Puslinch, Ontario, Canada) was used to calculate the number of cells per area as described by Hagen et al.\(^{15} \) Because height measurements were correlated to cell number (data not shown), all measurement corrections were done using mucosal height.

**Confocal Microscopy and Image Analysis**

Antigen retrieval using citrate buffer, pH 6.0, was used on paraffin sections from male (n = 5) or female (n = 5) Cldn18-KO or wild-type mice or from Cldn2-KO mice (n = 2, male) before staining with mouse anti-claudin-2 (Invitrogen 32-5600) and rat anti-ZO-1 (obtained from Dr Goodenough) or rabbit anti-E cadherin (R&D Systems, AF648), followed by secondary antibodies with complementary probes. Frozen sections were stained with the same antibodies after blocking for nonspecific staining in male (n = 3) or female (n = 3) Cldn18-KO mice or (n = 2) wild-type mice. Claudin-2 was always imaged in far red to reduce background staining. The same strategy was used for paraffin sections from male (n = 2) or female (n = 2) wild-type or Cldn18-KO mice stained with anti-NKCC1 (LSBio, Seattle, WA; LS-C313276-100) or anti-NaK-ATPase (Abcam, Cambridge, UK; ab76020 [EP1845Y]). Sections were mounted in Prolong Gold anti-fade mounting medium containing Hoechst 33342 (Invitrogen) to stain nuclei and evaluated by using an LSM880 confocal system with or without Fast Airyscan (Carl Zeiss).
The sum of pixels that were positive for claudin-2 was quantified using Volocity image analysis software. For this, two ×20 image tiles (3 × 2) from each tissue section were used. Within each image, 3 separate regions of interest were defined for analysis: (1) the surface and gastric pit, (2) the neck region, and (3) the gland base region. The sum of pixels within a specified intensity range with exclusion of background was collected and normalized to area. Likewise, the average pixel intensity per region of interest was quantified.

**Western Blotting and Protein Quantification**

Gastric mucosal samples were collected from male (n = 5) and female (n = 5) wild-type or Cldn18-KO mice. After excision, stomachs were opened along the greater curvature and washed in ice-cold phosphate-buffered saline. The mucosa was scraped into pre-weighed cryotubes on ice, taking care to exclude the esophagus, antrum, and duodenum. Directly after scraping, tubes containing tissue were weighed and immediately frozen in liquid nitrogen, followed by storage at −80°C.

To prepare gel samples, each mucosal sample was transferred into a Dounce homogenizer containing 9 volumes of ice-cold sample buffer containing (in mmol/L) 150 Tris-Cl, 2 EDTA, 0.1% sodium dodecyl sulfate, 1% sucrose containing Complete Mini protease inhibitor (Roche Biosciences, Indianapolis, IN) as described previously. Determination of protein levels was carried out using the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA). Proteins were resolved by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The blots were incubated with mouse anti-claudin-2 (1/1000), followed by washing and incubation with secondary horseradish peroxidase conjugated anti-mouse (1/3000), and the signal was imaged by chemiluminescence using a Bio-Rad Chemidoc Imaging System (Bio-Rad). Membranes were stripped, washed, and reincubated with primary antibody (mouse anti-actin, 1/10,000) as a loading standard. Normalization and quantification of claudin-2 protein levels were carried out using Bio-Rad Image Lab Software v. 5.2.1 (Bio-Rad).

**Preparation of RNA and RNASeq**

Tissues from wild-type (male, n = 3) or Cldn18-KO (male, n = 3) mice at 7 weeks after birth were frozen (unfixed) in optimal cutting temperature medium and prepared for RNASeq analysis as described in detail. Normalization was done during the bioinformatics pipeline analysis to convert raw read counts into measures of gene expression.

**Statistical Analyses**

Data were analyzed by one-way or two-way analysis of variance (ANOVA), followed by post hoc comparisons using SigmaPlot 14 software (Systat Software, San Jose, CA). Analysis of variance on ranks was performed if variances were not equal. Outliers were identified using the ROUT method at Q = 1% (GraphPad Software, San Diego, CA). Outlying data were omitted from analysis and graphs. Graphs were produced by using SigmaPlot 14 software. The data were considered significant at P < .05.

All authors had access to the data and reviewed and approved the final manuscript.

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Received February 12, 2020. Accepted October 13, 2020.

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Acknowledgments
The authors thank Professor Ursula Seidler for advice on running mouse tissues in Ussing chambers and Professor Sachiko Tsukita for allowing us to use the Cldn2 knockout mice that were developed in her laboratory.

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Conflicts of interest
This author discloses the following: JRT is a cofounder of and shareholder in Thelium Therapeutics. The remaining authors disclose no conflicts.

Funding
Supported by the Harvard Catalyst | The Harvard Clinical and Translational Science Center, National Center for Research Resources, and the National Center for Advancing Translational Sciences, National Institutes of Health (NIH) award 1UL1 TR001102-01, and financial contributions from Harvard University and its affiliated academic health care centers, Department of Surgery Bridge funds, and NIH grants R01 DK103046 and P30 DK04854 (S.J.H.); NIH grants R01 CA093405, P30 ES002109, R35CA21088, and P01CA028842 (J.G.F.); NIH and DOD grants R01DK088271, R01DK061931, and DOD CDMRP PR181271 (J.R.T.); and a Research Science Institute/Center for Excellence in Education Summer Research Fellowship (N.S.). T.J.C. and K.E.S. were supported by NIH T32 OD0109978 (J.G.F.).