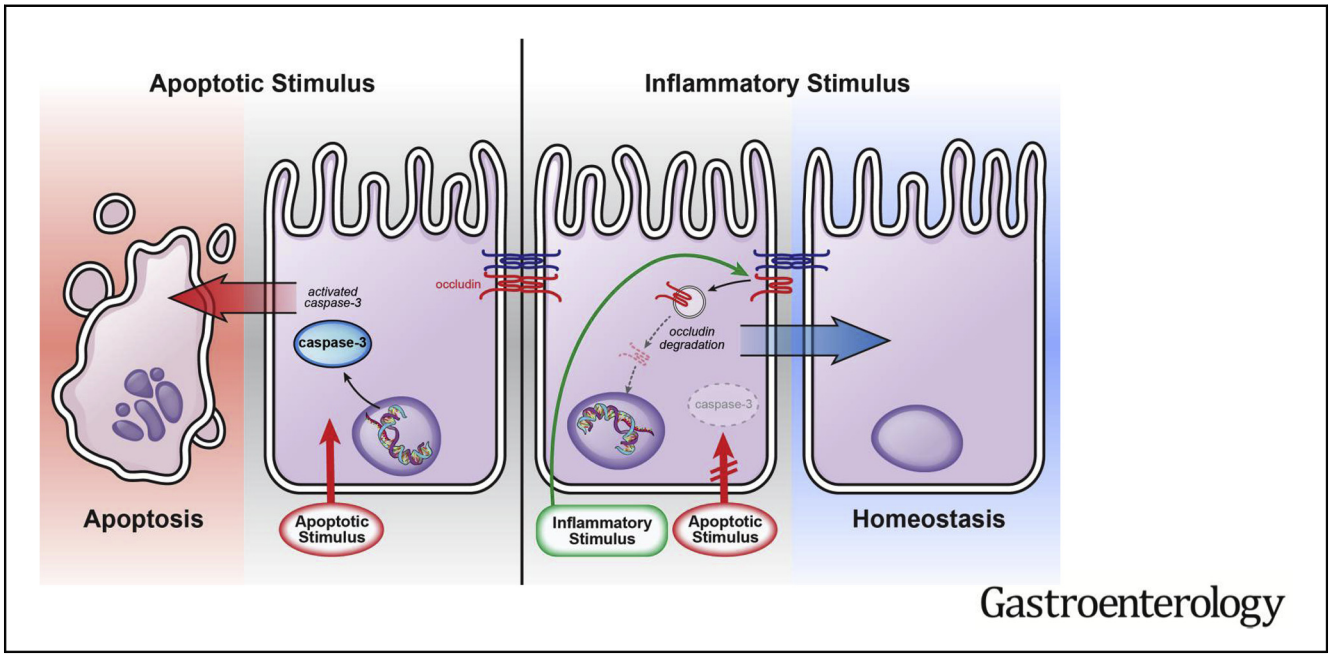
Gastroenterology 2019;157:1323–1337

Inflammation-induced Occludin Downregulation Limits Epithelial Apoptosis by Suppressing Caspase-3 Expression

Wei-Ting Kuo,[1,*] **Le Shen**,[2,*] Li Zuo,[1,3] Nitesh Shashikanth,[1] Ma. Lora Drizella M. Ong,[1] Licheng Wu,[2] Juanmin Zha,[2,4] Karen L. Edelblum,[2,5] Yitang Wang,[2] Yingmin Wang,[2] Steven P. Nilsen,[1] and Jerrold R. Turner[1,2]

[1]Laboratory of Mucosal Barrier Pathobiology, Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts; [2]Department of Pathology, The University of Chicago, Chicago, Illinois; [3]Anhui Medical University, Hefei, Anhui, China; [4]Soochow University and Department of Oncology, The First Affiliated Hospital of Soochow University, Suzhou, China; and [5]Department of Pathology and Laboratory Medicine, Center for Inflammation and Immunity, Rutgers New Jersey Medical School, Newark, New Jersey



BACKGROUND & AIMS: Epithelial tight junctions are compromised in gastrointestinal disease. Processes that contribute to the resulting barrier loss include endocytic occludin removal from the tight junction and reduced occludin expression. Nevertheless, the relatively-normal basal phenotype of occludin knockout (KO) mice has been taken as evidence that occludin does not contribute to gastrointestinal barrier function. We asked whether stress could unmask occludin functions within intestinal epithelia. **METHODS:** Wildtype (WT), universal and intestinal epithelial-specific occludin KO, and villin-EGFP-occludin transgenic mice as well as WT and occludin knockdown (KD) Caco-2_{BBe} cell monolayers were challenged with DSS, TNBS, staurosporine, 5-FU, or TNF. Occludin and caspase-3 expression were assessed in patient biopsies. **RESULTS:** Intestinal epithelial occludin loss limited severity of DSS- and TNBS-induced colitis due to epithelial resistance to apoptosis; activation of both intrinsic and extrinsic apoptotic pathways was blocked in occludin KO epithelia. Promoter analysis revealed that occludin enhances *CASP3* transcription and, conversely, that occludin downregulation reduces caspase-3 expression. Analysis of biopsies from Crohn's disease and ulcerative colitis patients and normal controls demonstrated that disease-associated occludin downregulation was accompanied by and correlated with reduced caspase-3 expression. In vitro, cytokine-induced occludin downregulation resulted in reduced caspase-3 expression and resistance to intrinsic and extrinsic pathway apoptosis, demonstrating an overall protective effect of inflammation-induced occludin loss. **CONCLUSIONS:** The tight junction protein occludin regulates apoptosis by enhancing caspase-3 transcription. These data suggest that reduced epithelial caspase-3 expression downstream of occludin downregulation is a previously-unappreciated anti-apoptotic process that contributes to mucosal homeostasis in inflammatory conditions.

Keywords: Permeability; IBD; Gene Regulation; Cell Death.

The first transmembrane tight junction protein discovered, occludin, is present within tight junction strands[1] and, when overexpressed, induces intracellular, multilamellar bodies with fused, tight junction–like membranes.[2] The occludin C-terminal coiled-coil domain binds directly to zonula occludens-1 (ZO-1)[3] via interactions regulated by phosphorylation within and adjacent to that region.[4–7] A range of in vitro studies, including analyses of peptides corresponding to occludin domains,[8] occludin knockdown,[9–11] or expression of occludin mutants (eg, those lacking the C-terminal tail),[12] suggest that occludin limits paracellular permeability to macromolecules. The observation that occludin removal from the tight junction, either in response to F-actin depolymerization or cytokine stimulation,[10,13,14] increases leak pathway permeability is consistent with this function. Conversely, cytokine stimulation of occludin-deficient monolayers does not reduce barrier function beyond the increased permeability already present as a consequence of occludin deletion.[10,11] These data indicate that this form of cytokine-induced barrier loss is largely, and perhaps entirely, caused by occludin removal from the tight junction.

Despite extensive in vitro data, the viability and absence of obvious structural or functional tight junction defects within the small intestine, colon, and bladder of occludin knockout (KO) mice[15,16] has led many to conclude that occludin does not contribute to mucosal barrier function or homeostasis. This conclusion fails to account for the multiple abnormalities of occludin KO mice, including hearing loss, growth retardation, chronic gastritis, cerebellar and basal ganglia calcification, male sterility, and inability of females to suckle their pups.[15,17] In addition, transgenic enhanced green fluorescent protein (EGFP)-occludin expression within the intestinal epithelium, that is, overexpression, limited tumor necrosis factor-α (TNF)-induced depletion of tight junction–associated occludin, leak pathway permeability increases, and diarrhea.[18] Nevertheless, the lack of baseline intestinal dysfunction in occludin KO mice suggests that compensatory changes might be masking relevant deficits. Experience with other KO mice has shown that underlying phenotypes can often be exposed by stress.[19–21]

We hypothesized that occludin KO mice might be hypersensitive to colitogenic stimuli. In contrast, mice lacking intestinal epithelial occludin were markedly protected from disease. This unexpected result reflected insensitivity of occludin-deficient intestinal epithelial cells to a variety of intrinsic and extrinsic apoptotic pathway agonists. Protection was due to loss of occludin-dependent *CASP3* transcription that led to reduced caspase-3 expression. Moreover, TNF-induced occludin downregulation protected epithelia from apoptosis by reducing caspase-3 expression. Finally, we confirmed previous reports of intestinal epithelial occludin downregulation in Crohn's disease and ulcerative colitis and found that this was strongly correlated with reduced caspase-3 expression. These data indicate that the adaptive process initiated by occludin downregulation is active and may enhance epithelial survival in human disease. We conclude that occludin serves as a critical regulator of epithelial apoptosis and survival by modulating *CASP3* transcription and caspase-3 expression.

WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

Intestinal barrier loss in patients with inflammatory bowel disease (IBD) is associated with reduced expression of the tight junction protein occludin. We investigated the function of occludin in cell lines, mice, and patient biopsies.

NEW FINDINGS

Occludin increases expression of caspase-3, which induces apoptosis. Reduced occludin expression leads to caspase-3 downregulation that limits epithelial apoptosis and attenuates colitis. Occludin downregulation correlates with lower levels of caspase-3 expression in biopsies from patients with IBD.

LIMITATIONS

These studies were performed in mice, cell lines, and human tissue samples. Studies are needed to define the mechanisms by which occludin regulates transcription.

IMPACT

Occludin downregulation by inflammatory stimuli may be a mechanism of epithelial preservation that promotes mucosal homeostasis.

Materials and Methods

Mice

Occludin KO,[15] intestinal epithelial specific EGFP-occludin transgenic,[18] caspase-3 KO,[22] and ZO-1 KO^{IEC28} mice have been described. Occludin KO^{IEC} mice were generated using *Ocln*$^{tm1a(EUCOMM)Wtsi}$ embryonic stem cells to create C57BL/6J-*Ocln*$^{f/f}$ mice, which were crossed with villin-Cre[23] mice. Littermate *Ocln*$^{f/f}$ mice lacking *Cre* were used as wild-type (WT) controls in experiments with occludin KO^{IEC} mice.

Further experimental details are available in the Supplemental Methods.

* Authors share co-first authorship.

Abbreviations used in this paper: **DSS, dextran sulfate sodium; EGFP, enhanced green fluorescent protein; ERK, extracellular signal-related kinase; 5-FU, 5-fluorouracil; ISOL, in situ oligonucleotide ligation; KD, knockdown; KO, knockout; MAP, mitogen-activated protein; mRNA, messenger RNA; STS, staurosporine; TNBS, 2,4,6-trinitrobenzene sulfonic acid; TNF, tumor necrosis factor-α; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; WT, wild-type; ZO-1, zonula occludens-1.**

Most current article

0016-5085/$36.00
https://doi.org/10.1053/j.gastro.2019.07.058

Results

Epithelial Occludin Expression Exacerbates Dextran Sulfate Sodium (DSS) Colitis

Extensive in vitro studies indicate that the tight junction protein occludin is involved in epithelial barrier regulation.[8–12] We initially asked if occludin expression contributes to epithelial homeostasis by comparing mucosal architecture, epithelial proliferation, and epithelial migration in WT and occludin KO mice; no differences were detected (Supplementary Figure 1). Nevertheless, reduced occludin expression in a range of intestinal disorders[24] suggests that occludin may be involved in disease. To test this hypothesis, we assessed the sensitivity of occludin KO mice to epithelial damage. Exposure to DSS induced expected weight loss (Figure 1*A*, Supplementary Figure 2*A*), clinical disease activity (Figure 1*B*, Supplementary Figure 2*B*), colon shortening (Figure 1*C*), histologic damage (Figure 1*D*), and neutrophil infiltration (Figure 1*E*) in WT mice. However, universal occludin KO mice, in which all tissues lacked occludin, were remarkably resistant to DSS-induced colitis by all of these measures (Figure 1*A*–*E*). Complementation by transgenic intestinal epithelia-specific EGFP-occludin expression[25] restored sensitivity to DSS colitis (Figure 1*A*–*E*). These data thereby link the observed phenotype specifically to intestinal epithelia and demonstrate that intestinal epithelial occludin expression promotes pathogenesis of DSS colitis.

Although complementation with transgenic occludin demonstrates the essential role of intestinal epithelial occludin in DSS sensitivity, tissue-specific occludin KO is more definitive. We therefore generated intestinal epithelial-specific occludin KO (occludin KO^{IEC}) mice. As with universal occludin KO mice, intestinal mucosal architecture of occludin KO^{IEC} (*Ocln$^{f/f}$ x Vil-Cre*) mice was indistinguishable from WT (*Ocln$^{f/f}$*) mice (Supplementary Figure 3*A*). Occludin KO^{IEC} did not, however, demonstrate male infertility or any of the other phenotypic abnormalities reported in universal occludin KO mice.[15,17] Similar to universal KO mice, DSS colitis severity was reduced in occludin KO^{IEC} mice. The protection observed included attenuation of DSS-induced increases in intestinal permeability (Figure 1*F*) and inflammatory cytokine production (Figure 1*G*) relative to occludin-expressing controls.

Intestinal Epithelial Occludin Promotes DSS-induced Apoptosis

The damage that characterizes DSS colitis represents an imbalance between chemical injury and mucosal repair. Occludin could potentially affect either or both of these processes. Epithelial proliferative responses to DSS-induced injury were, however, indistinguishable in occludin KO^{IEC} and WT mice (Figure 1*H*). We therefore turned our attention to damage. Epithelial cell death, assessed as DNA fragmentation using terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL), was similar in unstressed WT and occludin KO^{IEC} mice. DSS markedly increased the number of TUNEL-positive epithelial cells in WT mice, but this increase was largely suppressed in occludin KO^{IEC} mice (Figure 1*I*). To better assess the mechanism of cell death, sections were labeled by in situ oligonucleotide ligation (ISOL), which specifically detects double-strand DNA breaks that are blunt-ended or have a 1-base 3′-overhang. This is more specific for apoptosis than TUNEL, which detects DNA fragmentation induced by multiple processes. As with TUNEL, DSS-induced increases in ISOL labeling were significantly attenuated in treated occludin KO^{IEC} mice (Figure 1*J*). Finally, cleaved caspase-3 staining, a hallmark of apoptosis, was increased by DSS treatment of WT, but not occludin KO^{IEC}, mice (Figure 1*K*). Collectively, these data indicate that intestinal epithelial occludin expression sensitizes cells to DSS-induced apoptosis.

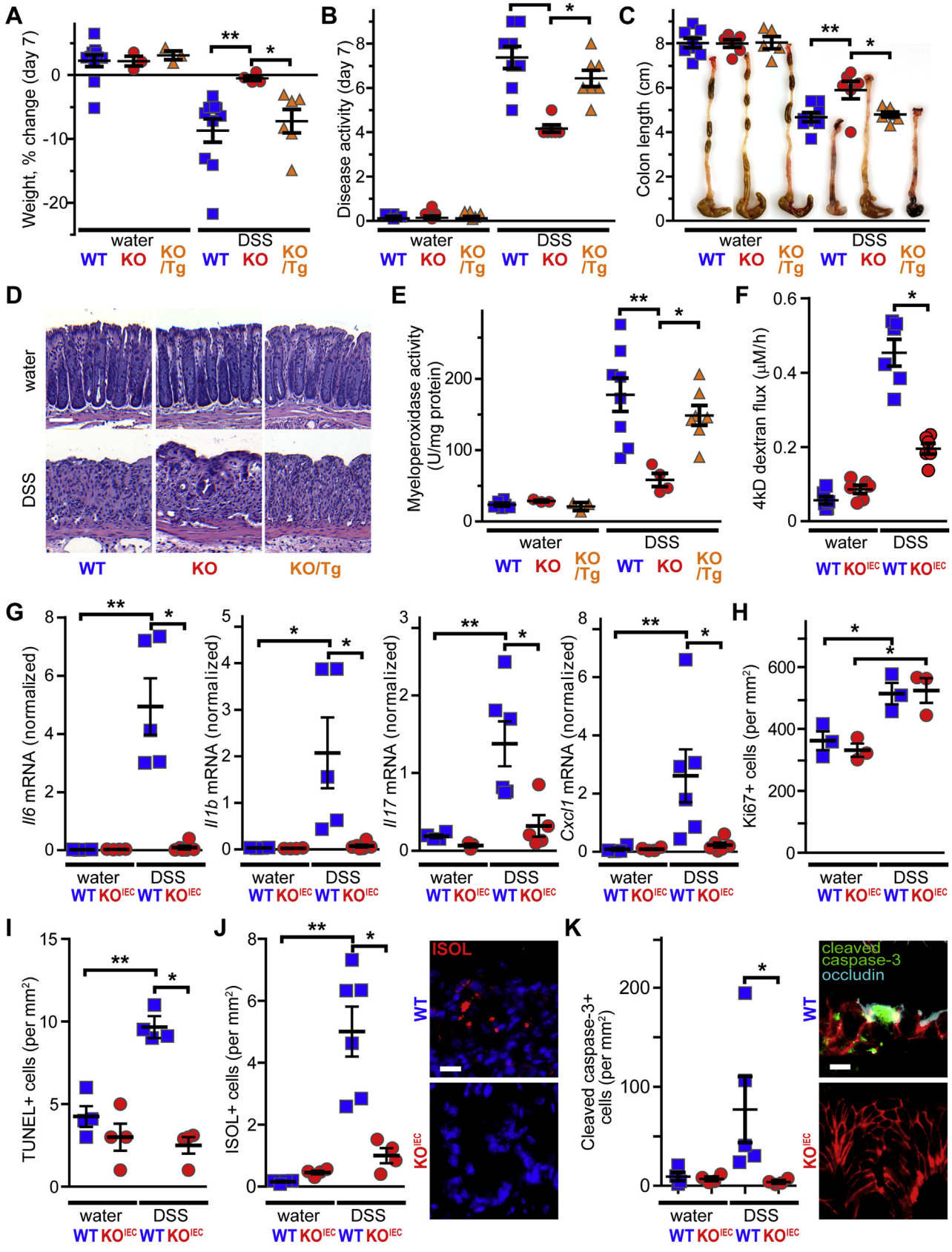
Intestinal Epithelial Occludin Deletion Limits 2,4,6-Trinitrobenzene Sulfonic Acid (TNBS)-induced Colitis and Tissue Damage

DSS induces colitis via direct chemical injury to intestinal epithelial cells.[26] We therefore considered the possibility that the protection afforded by occludin deletion was specific to direct chemical injury. To determine whether the effects of intestinal epithelial occludin deletion extended to other forms of injury, we assessed the sensitivity of occludin KO^{IEC} and control mice to TNBS colitis. TNBS haptenates autologous and luminal proteins to trigger immune-mediated damage. Despite this distinct pathogenic mechanism, resistance of occludin KO^{IEC} mice to TNBS was similar to that after DSS challenge. This protection was obvious on physical examination (Figure 2*A*) and could be validated quantitatively by reduced disease activity scores (Figure 2*B*). Histologic damage (Figure 2*C*), intestinal barrier loss (Figure 2*D*), and cytokine production (Figure 2*E*) were also limited in TNBS-treated occludin KO^{IEC} mice relative to occludin-sufficient WT controls.

TNBS-induced injury caused similar epithelial proliferative responses in occludin KO^{IEC} and WT mice (Figure 2*F*, Supplementary Figure 3*B*). As with DSS, epithelial occludin deletion attenuated TNBS-induced apoptosis. Moreover, TUNEL (Figure 2*G*, Supplementary Figure 3*B*), ISOL (Figure 2*H*, Supplementary Figure 3*B*), and cleaved caspase-3 (Figure 2*I*, Supplementary Figure 3*B*) staining were markedly reduced in TNBS-treated occludin KO^{IEC} mice relative to WT mice. These data indicate that the protection from apoptotic cell death afforded by intestinal epithelial occludin deletion extends to multiple forms of epithelial injury.

Occludin KO Limits Activation of Both Intrinsic and Extrinsic Apoptotic Pathways

The results demonstrate that occludin-deficient epithelia are resistant to colitis-associated apoptosis. Given the immune-mediated mechanism of TNBS colitis, it is likely that this resistance to apoptosis includes insensitivity to extrinsic pathway activation by pro-apoptotic


A
Weight, % change (day 7)
water
DSS
WT KO KO/Tg WT KO KO/Tg
B
Disease activity (day 7)
C
Colon length (cm)
D
water
DSS
WT KO KO/Tg
E
Myeloperoxidase activity (U/mg protein)
F
4kD dextran flux (μM/h)
WT KOIEC WT KOIEC
G
Il6 mRNA (normalized)
Il1b mRNA (normalized)
Il17 mRNA (normalized)
Cxcl1 mRNA (normalized)
H
Ki67+ cells (per mm2)
I
TUNEL+ cells (per mm2)
J
ISOL+ cells (per mm2)
ISOL
WT
KOIEC
K
Cleaved caspase-3+ cells (per mm2)
cleaved caspase-3
occludin

mediators, such as TNF. In contrast, the relative contributions of extrinsic, versus intrinsic (ie, cellular stress) pathways to DSS-induced epithelial apoptosis have not been defined. To determine whether occludin expression facilitates apoptosis via the intrinsic pathway (Figure 3*A*), mice were treated with the chemotherapeutic nucleoside analogue 5-fluorouracil (5-FU), which increased epithelial apoptosis, as demonstrated morphologically (Figure 3*B*), by TUNEL staining (Figure 3*C*), and by cleaved caspase-3 staining (Figure 3*D*). These increases were attenuated in occludin KO mice. Occludin therefore facilitates, and occludin deletion prevents, apoptosis via the intrinsic pathway.

To assess the effect of occludin loss on extrinsic pathway apoptosis (Figure 3*A*), mice were treated with recombinant TNF, which increased small intestinal epithelial apoptosis 30-fold (Figure 3*E*). As with DSS- and 5-FU–induced epithelial apoptosis, TNF-induced apoptosis was almost entirely blocked by occludin deletion. We also assessed small intestinal epithelial apoptosis in response to a more complex stimulus, cytokine storm induced by systemic T-cell activation.[27,28] Small intestinal epithelial apoptosis was readily detected 2 days after anti-CD3 administration, as indicated by TUNEL (Figure 3*F*) and ISOL (Figure 3*G*) labeling, in WT mice. Occludin KO^{IEC} mice were, however, protected. Occludin deletion therefore prevents apoptosis of intestinal epithelia in response to intrinsic and extrinsic pathway stimuli.

Caspase-3 Activation Is Defective in Occludin-deficient Epithelia In Vivo and In Vitro

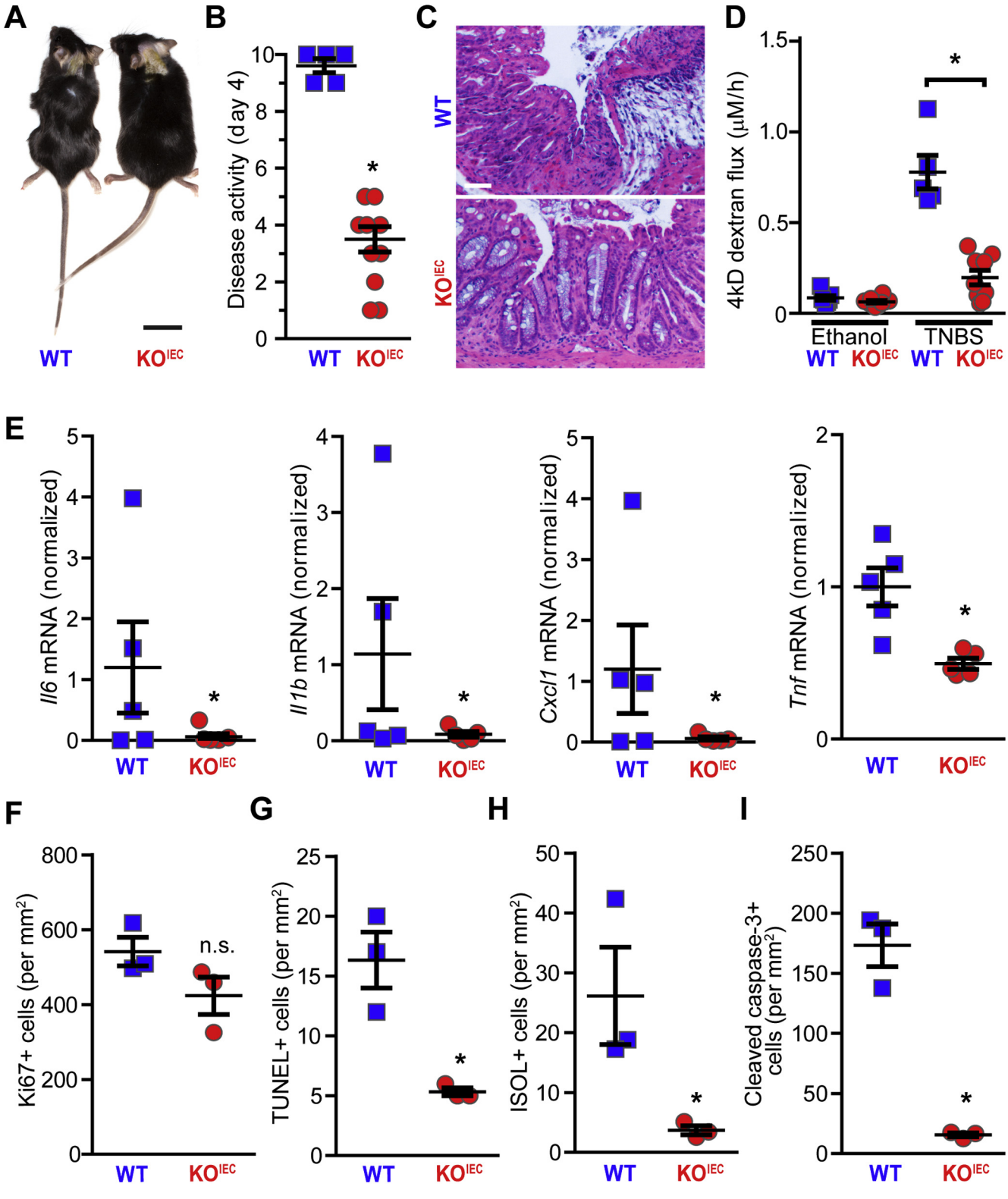
To identify the events downstream of TNF receptor activation affected by occludin, small intestinal epithelial cells from WT and occludin KO mice that received vehicle or recombinant TNF were isolated and probed for extracellular signal-related kinase (ERK) and p38 mitogen-activated protein (MAP) kinase activation, IκB degradation, and caspase cleavage (Figure 3*H*). ERK and p38 MAP kinase activation as well as IκB and caspase-8 degradation were similar in epithelia from TNF-treated WT and occludin KO mice (Figure 3*H*, Supplementary Figure 4*A–D*). Caspase-9 was not degraded, consistent with direct TNF-induced caspase-8 activation (Figure 3*E*, Supplementary Figure 4*E*). In contrast to WT mice, in which substantial caspase-3 cleavage was present, almost no cleaved caspase-3 was detected in intestinal epithelial cells from TNF-treated occludin KO mice (Figure 3*H*; Supplementary Figure 4*F*). These data suggest that insufficient caspase-3 activation may be responsible for the apoptotic resistance of occludin KO epithelia. Because caspase-3 is a terminal executioner caspase, this single defect induced by occludin deletion can explain resistance to both intrinsic and extrinsic pathway activation.

Although the in vivo data are striking, they do not address whether the effects of occludin KO on intestinal epithelial apoptosis reflect interactions with other cell types, such as immune cells, or are cell autonomous. To assess this, occludin knockdown (KD) intestinal epithelial monolayers were tested for sensitivity to intrinsic and extrinsic pathway-induced apoptosis induced by staurosporine (STS) or TNF and cycloheximide, respectively (Supplementary Figure 5). Both epithelial loss, as demonstrated by reduced nuclear density, and caspase-3 activation were limited in occludin KD monolayers (Supplementary Figure 5). ERK and p38 MAP kinase activation were similar in occludin-sufficient and KD monolayers following STS or TNF treatment. Caspase-3 activity, indicated by cleavage of poly (ADP-ribose) polymerase, a caspase-3 substrate and key intermediate in apoptotic progression, was also significantly reduced in both STS- and TNF-treated occludin KD, relative to WT, monolayers. These data demonstrate that in vitro occludin KD accurately recapitulates the effects of occludin KO in vivo and suggest that this is due to reduced capase-3 activity. The results further indicate that occludin deficiency limits epithelial apoptosis in a cell autonomous manner.

To measure caspase activation directly, cytosolic extracts from occludin-sufficient and occludin-deficient epithelia were studied. In the absence of activating stimuli,

Figure 1. Intestinal epithelial occludin expression promotes DSS-induced intestinal disease. (*A*) DSS-induced weight loss was attenuated in occludin KO mice. Transgenic intestinal epithelial EGFP-occludin expression (Tg/KO) restored DSS sensitivity to that of WT mice. Analysis at day 7 is shown (n $\geq$ 3 for water groups and n $\geq$ 4 for DSS-treated groups). The complete time course is shown in Supplementary Figure 2*A*. (*B*) Disease activity clinical scores were attenuated by occludin KO and restored by in Tg/KO mice (day 7 data, n $\geq$ 6 per group). The complete time course is shown in Supplementary Figure 2*B*. (*C*) Colonic shortening occurred in DSS-treated WT and Tg/KO mice but was markedly reduced in occludin KO mice (n $\geq$ 6 per group). (*D*) Histopathology shows preservation of surface epithelium and some crypts in DSS-treated occludin KO, relative to WT or KO mice with transgenic occludin expression. Bar = 50 μm. (*E*) DSS-induced increases in myeloperoxidase activity were attenuated in occludin KO mice but restored to WT levels in Tg/KO mice (n $\geq$ 3 for water groups and n $\geq$ 4 for DSS-treated groups). (*F*) DSS-induced increases in intestinal permeability to 4 kD dextran were markedly attenuated in occludin KO^{IEC} mice (n $\geq$ 5 per group). (*G*) DSS enhanced *Il6*, *Il1b*, *Il17*, and *Cxcl1* (an IL-8 ortholog) expression in WT, but not occludin KO^{IEC}, mice (n $\geq$ 5 per group). (*H*) DSS increased colonic epithelial proliferation, as assessed by Ki-67 staining, similarly in WT and occludin KO^{IEC} mice (n $\geq$ 5 per group). (*I*) DSS increased numbers of TUNEL-positive cells in WT, but not occludin KO^{IEC}, mice (n = 4 per group). (*J*) DSS increased numbers of ISOL-positive cells (red) in WT, but not occludin KO^{IEC}, mice. Nuclei shown in blue. Bar = 20 μm (n = 4–6 per group). (*K*) DSS increased numbers of cleaved caspase-3-positive cells (green) in WT, but not occludin KO^{IEC}, mice. Occludin (cyan) and E-cadherin (red) are shown for reference. Bar = 10 μm (n $\geq$ 4 per group). *Ocln*$^{+/+}$ littermates were used as WT controls for *Ocln*$^{-/-}$ and *Ocln*$^{-/-}$ $\times$ villin-EGFP-occludin (Tg/KO) mice. *Ocln*$^{f/f}$ littermates were used as WT controls for *Ocln*$^{f/f}$ $\times$ *Vil-Cre* (occludin KO^{IEC}) mice in this and all other figures. Statistical analyses by one-way analysis of variance with Bonferroni posttest. *P < .05; **P < .01.

caspases-8, -9, and -3 all displayed low protease activity that was quantitatively similar in WT and occludin KD extracts (Supplementary Figure 6). Cytochrome C and dATP activated caspases-8 and -9 in all extracts. In contrast, caspase-3 activity increased 12.0 ± 1.6-fold in occludin-sufficient extracts but only 4.6 ± 0.4-fold in cytosolic extracts from occludin-deficient epithelia. These data indicate that occludin depletion reduces caspase-3 activity and further supports the hypothesis that occludin deficiency prevents apoptosis by suppressing caspase-3 activation.



Occludin Enhances Transcription From the CASP3 Promoter

Because the results point to a defect at the level of caspase-3 activation, we tested expression of genes that could affect caspase-3 activity downstream of caspase-8 and -9 activation. Intestinal epithelial caspase-3 protein and *Casp3* messenger RNA (mRNA) expression were both markedly reduced in intestinal epithelia from occludin KO mice (Figure 4*A* and *B*). This was specific for caspase-3, as caspase-8 and -9 expression were not affected by occludin KO (Figure 3*H*, Supplementary Figure 4*D*–*F*). Moreover, Western blot analyses comparing expression of a panel of apoptosis-related proteins, including the inhibitor of apoptosis family, pro- and anti-apoptotic Bcl-2 proteins, and apoptosis-related mitochondrial proteins, failed to identify differences between WT and occludin KO epithelia (Figure 4*C*). We also considered the possibility that occludin promotes caspase-3 expression by interacting with ZO-1, as ZO-1 is known to regulate gene expression via the ZO-1–associated nucleic acid binding protein/DbpA.[29] However, *Casp3* transcription was indistinguishable in WT and intestinal epithelial specific ZO-1 KO (ZO-1 KO^{IEC}) mice[30] (Supplementary Figure 4*G*). These data indicate that occludin specifically promotes caspase-3 expression via a mechanism that is unrelated to ZO-1 and further suggests that reduced caspase-3 expression is the primary means by which occludin deficiency limits apoptosis.

To determine whether occludin regulates *CASP3* mRNA content via transcriptional activation, we took advantage of apoptosis resistance in occludin KD Caco-2_{BBe} intestinal epithelial cells (Supplementary Figure 5). Western blot analyses confirmed that, like native epithelia, occludin loss in vitro reduced caspase-3, but not caspase-8 or -9, protein expression (Figure 4*D*), indicating that, in this context, the in vitro model accurately recapitulates in vivo biology. *CASP3* mRNA was markedly reduced in occludin KD relative to WT Caco-2_{BBe} (Figure 4*E*). To further analyze *CASP3* transcriptional regulation, the human *CASP3* promoter was used to drive luciferase expression in transfected Caco-2_{BBe} cells. Luciferase activity in occludin KD cells was only 31% ± 3% of that in occludin-sufficient cells (Figure 4*F*). The specificity of this effect was confirmed by analyzing the activity of the same reporter after induction of EGFP or EGFP-occludin expression in occludin KD cells. Luciferase activity was significantly increased by induction of EGFP-occludin, but not by EGFP, expression, confirming occludin-dependent *CASP3* promoter regulation (Figure 4*G*). In contrast, a reporter construct containing the *CASP3* 3′ untranslated region generated similar luciferase activity in occludin-deficient and occludin-sufficient epithelial monolayers (Figure 4*H*). These data show that occludin expression results in increased *CASP3* promoter activation and mRNA transcription.

Reduced Caspase-3 Expression Is Sufficient to Confer Apoptosis Resistance

To determine if reduced caspase-3 expression was sufficient to explain the apoptotic resistance of occludin KO mice, we analyzed mice in which one *Casp3* allele was deleted ($Casp3^{+/-}$). Intestinal epithelial caspase-3 expression in $Casp3^{+/-}$ mice was reduced by 50% (Figure 5*A*), similar to the reduction in occludin KO epithelia. $Casp3^{+/-}$ mice were protected from DSS-induced apoptosis of colonic epithelia (Figure 5*B*) and TNF-induced apoptosis of small intestinal epithelia (Figure 5*C*). The effects of deleting one *Casp3* allele are therefore similar to the effect of occludin KO in terms of intestinal caspase-3 protein expression and apoptotic sensitivity. Although these data cannot be interpreted as indicating that reduced caspase-3 expression is the sole means by which occludin modulates apoptosis, they do show that the partial caspase-3 downregulation induced by occludin loss is sufficient to explain apoptotic resistance.

Caspase-3 Expression Is Reduced in Parallel With Occludin Loss in Crohn's Disease and Ulcerative Colitis

Intestinal epithelial occludin expression is reduced in Crohn's disease and ulcerative colitis.[22,31,32] If, as suggested by in vivo studies of mice and in vitro studies of human cell lines, occludin downregulation leads to reduced *CASP3* transcription, occludin downregulation in disease should be accompanied by reduced caspase-3 expression. Quantitative morphometry confirmed reduced epithelial expression of occludin and caspase-3 in ileal biopsies from patients with Crohn's disease, relative to healthy control subjects (Figure 6*A*–*C*). Similarly, epithelial occludin and caspase-3 expression were both reduced in colonic biopsies from patients with ulcerative colitis (Figure 6*D*, Supplementary Figure 7*A* and *B*). Caspase-3

Figure 2. Intestinal epithelial-specific occludin KO limits epithelial apoptosis and overall severity of TNBS colitis. (*A*) TNBS induced wasting and loss of normal fur texture in WT ($Ocln^{f/f}$) mice. At day 4 after rectal TNBS instillation these features were almost undetectable in occludin KO^{IEC} ($Ocln^{f/f}$ × *Vil-Cre*) mice. Bar = 1 cm. (*B*) TNBS-induced increases in disease activity scores were attenuated in occludin KO^{IEC} mice (day 4 data, n = 5 WT, 10 KO^{IEC}). (*C*) Histopathology shows severe damage in TNBS-treated WT, but not occludin KO^{IEC}, mice. Bar = 50 μm. (*D*) TNBS-induced increases in intestinal permeability to 4 kD dextran were markedly attenuated in occludin KO^{IEC}, relative to WT, mice (n ≥ 5 per group). (*E*) TNBS-induced *Il6*, *Il1b*, *Cxcl1*, and *Tnf* upregulation in WT, but not occludin KO^{IEC}, mice (n = 5 per group). (*F*) TNBS increased colonic epithelial proliferation similarly in WT and occludin KO^{IEC} mice (n = 3 per group). Representative photomicrographs are shown in Supplementary Figure 3*B*. (*G*) TNBS increased numbers of TUNEL-positive cells in WT, but not occludin KO^{IEC}, mice (n = 3 per group). Representative photomicrographs are shown in Supplementary Figure 3*B*. (*H*) TNBS increased numbers of ISOL-positive cells in WT, but not occludin KO^{IEC}, mice (n = 3 per group). (*I*) TNBS increased numbers of cleaved caspase-3–positive cells in WT, but not occludin KO^{IEC}, mice (n = 3 per group). Representative photomicrographs are shown in Supplementary Figure 3*B*. Statistical analyses by Student *t* test or one-way analysis of variance with Bonferroni posttest. $^{*}P < .05$; $^{**}P < .01$.

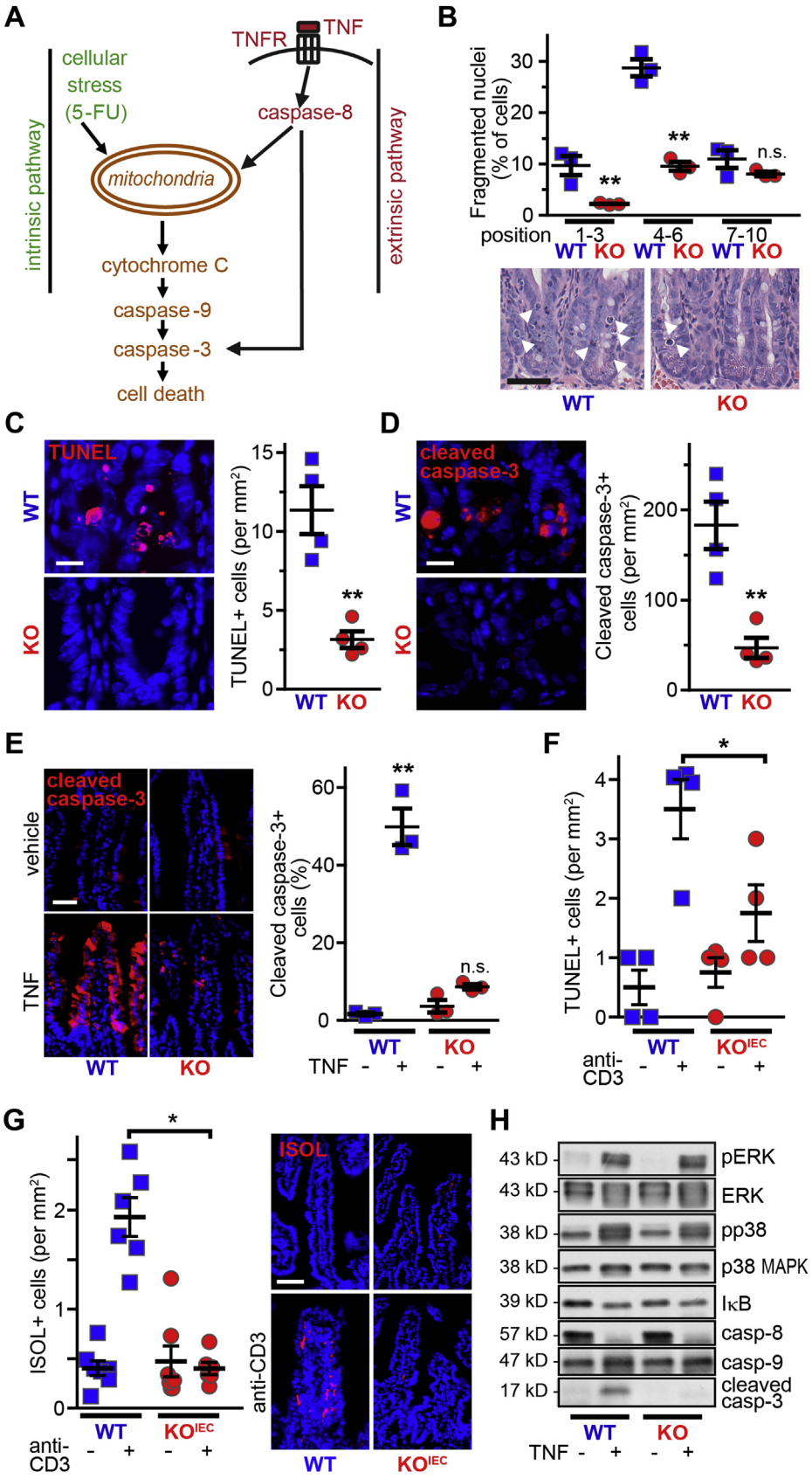


Figure 3. Occludin increases intrinsic and extrinsic pathway apoptosis in vivo. (*A*) Schematic highlighting the intrinsic and extrinsic apoptotic signaling pathways, which are activated by 5-FU and TNF, respectively. (*B*) 5-FU-induced crypt epithelial apoptosis (arrowheads) was reduced in occludin KO mice, particularly within the lower and middle crypt (positions 1–6). Bar = 50 μm (n = 4 per group). (*C*) TUNEL staining (red) shows fewer 5-FU-induced DNA breaks in occludin KO crypts. Nuclei are shown in blue. Bar = 20 μm (n = 4 per group). (*D*) Fewer cleaved caspase-3 positive cells (red) were present in WT, relative to occludin KO, mice after 5-FU treatment. Nuclei are shown in blue. Bar = 20 μm (n = 4 per group). (*E*) Acute TNF treatment-induced caspase-3 cleavage (red) within villus epithelia was markedly reduced in occludin KO mice. Nuclei are shown in blue. Bar = 50 μm (n = 3 per group). (*F*) Systemic T-cell activation (anti-CD3 immunoglobulin G) resulted in increased numbers of TUNEL-positive small intestinal epithelia in WT mice. This was markedly attenuated in occludin KO^{IEC} mice (n = 4 per group). (*G*) Systemic T-cell activation increased ISOL-positive (red) cell numbers in WT, but not occludin KO^{IEC}, mice. Nuclei are shown in blue. Bar = 50 μm (n = 4–6 per group). (*H*) Western blot of intestinal epithelia isolated from vehicle- and TNF-treated WT and occludin KO mice (as in [*E*]). With the exception of cleaved caspase-3, markers of TNF signaling and caspase activation were not affected by occludin KO. Quantitative analyses are shown in Supplementary Figure 4. Statistical analyses by Student *t* test or one-way analysis of variance with Bonferroni posttest. $^{*}P < .05$; $^{**}P < .01$.

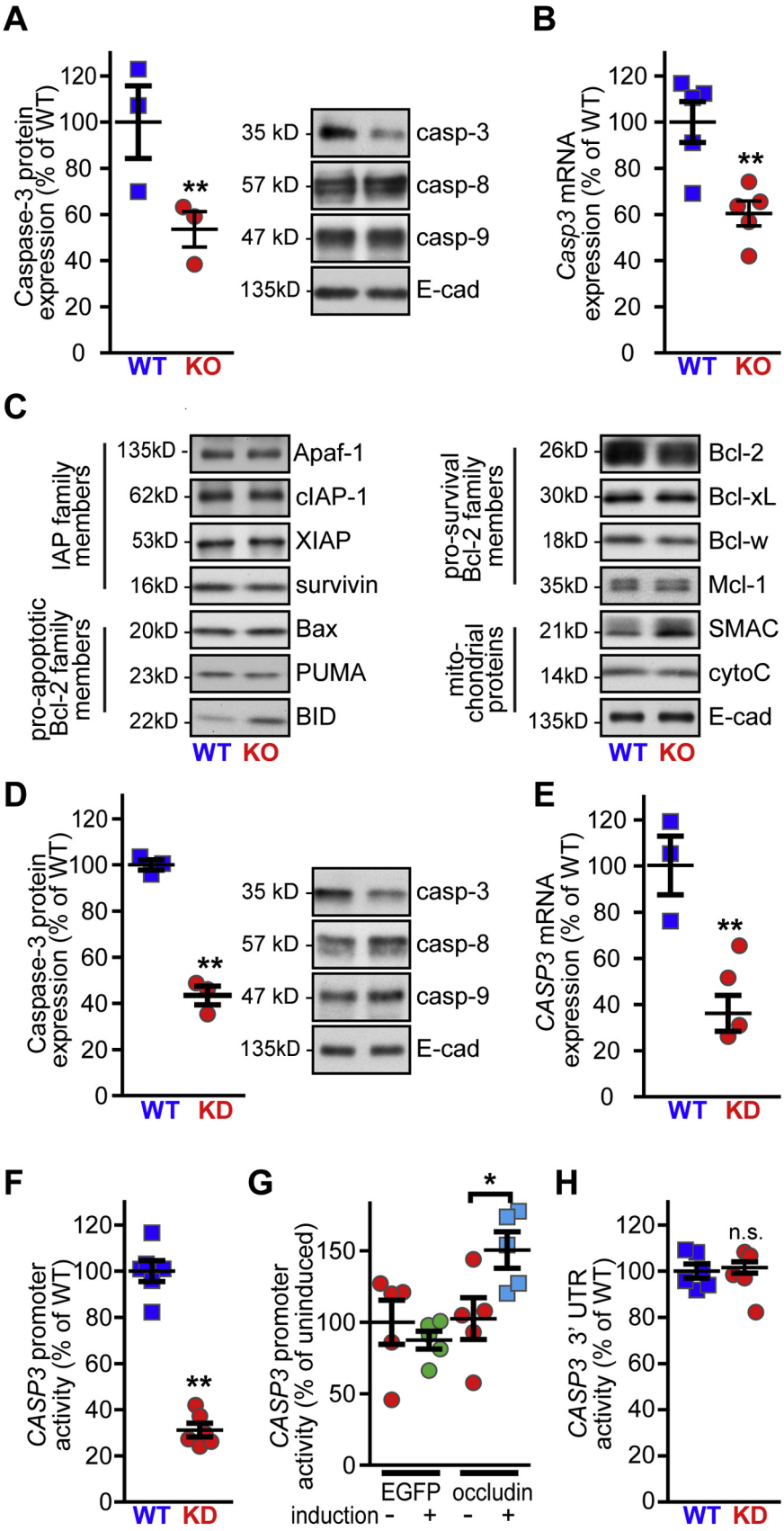
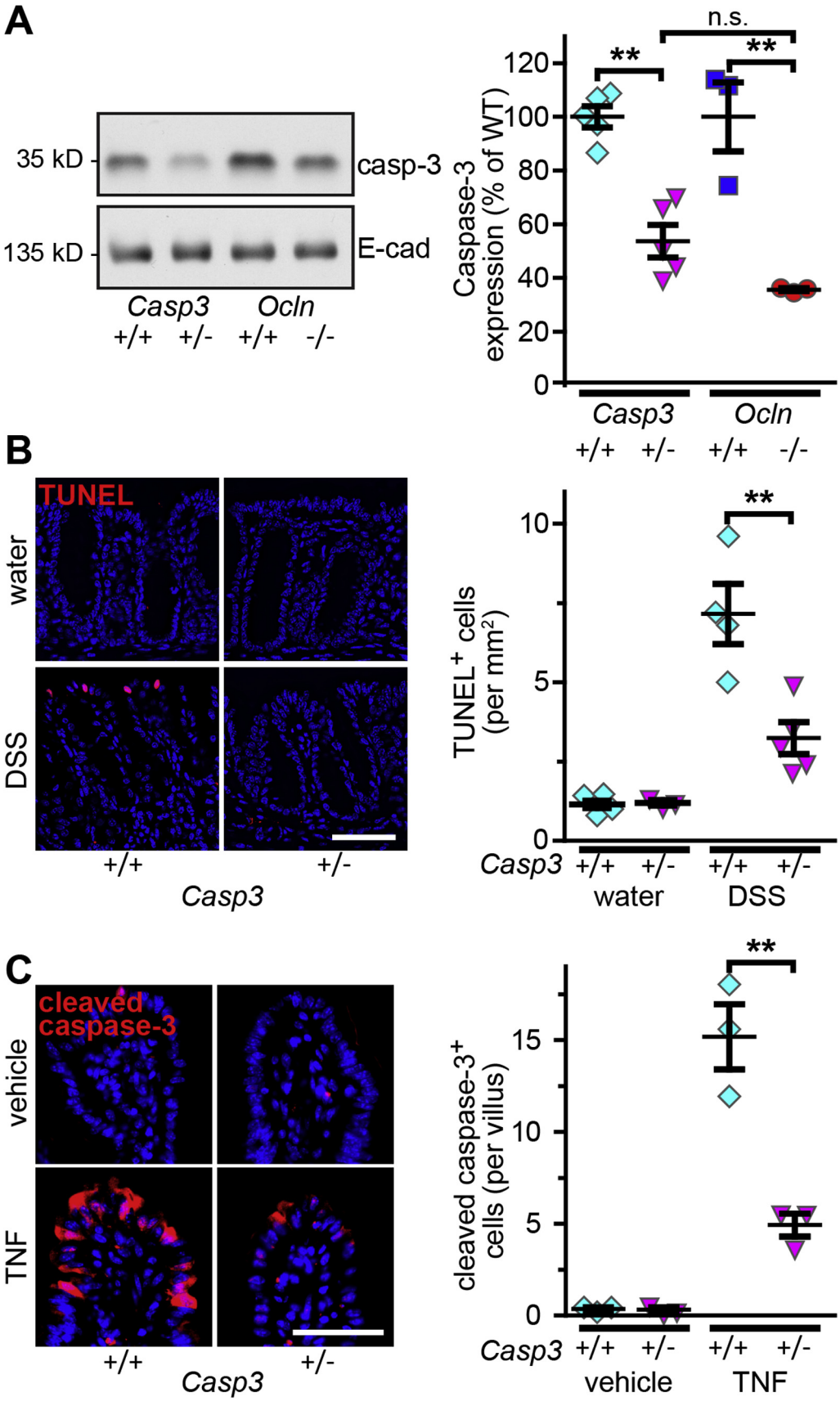


Figure 4. Occludin promotes *CASP3* transcription. (*A*) Caspase-3 expression was reduced in jejunal epithelial cells isolated from occludin KO mice. Caspase-8, caspase-9, and E-cadherin expression were not affected (n = 3 per group). (*B*) Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) of jejunal epithelial cells from WT or occludin KO mice shows reduced *Casp3* mRNA abundance in occludin KO mice (n = 5 per group). (*C*) Expression of apoptosis-related proteins was similar in jejunal epithelial cells from WT and occludin KO mice. Data shown are representative of n ≥ 3 per group. (*D*) Caspase-3 protein expression was reduced in occludin KD Caco-2_{BBe} monolayers. Caspase-8, caspase-9, and E-cadherin expression were not affected (n = 3 per group). (*E*) qRT-PCR shows reduced *CASP3* mRNA abundance in occludin KD, relative to WT, Caco-2_{BBe} (n = 3 per group). (*F*) Luciferase reporter assay shows reduced activity of the *CASP3* promoter in occludin KD compared to WT monolayers (n = 6 per group). (*G*) Induction of EGFP-occludin, but not EGFP, expression, increased *CASP3* promoter activity in occludin KD Caco-2_{BBe} (n = 5 per group). (*H*) Caco-2_{BBe} were transfected with PGK promoter-driven luciferase expression plasmid containing the *CASP3* 3′ untranslated region. Luciferase activity was similar in WT and occludin KD monolayers (n ≥ 5 per group). Statistical analyses by Student *t* test or one-way analysis of variance with Bonferroni posttest. **P* < .05; ***P* < .01.


A
35 kD
casp-3
135 kD
E-cad
Casp3
Ocln
+/+ +/- +/+ -/-
n.s.
**
**
Caspase-3 expression (% of WT)
120
100
80
60
40
20
0
Casp3
Ocln
+/+ +/- +/+ -/-
B
TUNEL
water
DSS
+/+
+/-
Casp3
**
TUNEL+ cells (per mm2)
10
5
0
Casp3 +/+ +/- +/+ +/-
water
DSS
C
cleaved caspase-3
vehicle
TNF
+/+
+/-
Casp3
**
cleaved caspase-3+ cells (per villus)
15
10
5
0
Casp3 +/+ +/- +/+ +/-
vehicle
TNF

downregulation correlated directly with reduced occludin expression in both forms of inflammatory bowel disease. These data suggest that occludin downregulation is the mechanism of reduced caspase-3 expression in human disease.

Proinflammatory Cytokines Decrease Occludin and Caspase-3 Expression to Cause Apoptotic Resistance

Previous studies have shown that prolonged (ie, >20 hours) TNF treatment reduces occludin transcription in HT29 intestinal epithelial cells.[33] Consistent with this, treatment of WT Caco-2_{BBe} monolayers with low-dose TNF (0.5 ng/mL) for 24 hours reduced occludin expression by 32% (Figure 7*A* and *B*) and was accompanied by a 41% reduction in caspase-3 expression. As a result, caspase-3 expression in TNF-treated WT monolayers was similar to that of untreated occludin KD epithelia (Figure 7*A* and *B*). In contrast, low-dose TNF treatment did not affect caspase-3 expression in occludin-deficient monolayers (Figure 7*A* and *B*). Cytokine-induced occludin downregulation is, therefore, sufficient to reduce caspase-3 expression in vitro in a manner similar to that observed in inflammatory bowel disease.

We next assessed the effect of cytokine-induced caspase-3 downregulation on activation of intrinsic and extrinsic apoptotic pathways. After STS or high-dose TNF and cycloheximide challenge, 23% and 25% of occludin-sufficient cells stained positively for cleaved caspase-3, respectively, whereas only 9% and 6% were positive after low-dose TNF pretreatment (Figure 7*C* and *D*). This low level of apoptosis was similar to both untreated and high-dose TNF-treated occludin KD epithelia, which were not affected by low-dose TNF pretreatment (Figure 7*C* and *D*). The data demonstrate that mild inflammatory stimuli that trigger occludin downregulation also downregulate caspase-3 and, in turn, confer resistance to intrinsic and extrinsic pathway apoptosis. The failure of low-dose TNF to affect caspase-3 expression or apoptotic sensitivity of occludin-deficient epithelia confirms that occludin is required for this mechanism of caspase-3 downregulation and apoptotic resistance. Cytokine-induced occludin downregulation may therefore be an adaptive process that enhances epithelial survival in inflammatory environments.

Discussion

In vivo and in vitro studies have demonstrated that occludin loss is critical to epithelial tight junction leak pathway permeability increases induced by inflammatory stimuli.[10,11,14,18,25,34] Nevertheless, the observation that occludin KO mice have essentially normal tight junction structure and barrier function[15,16] has caused many to question the importance of this protein. A possible explanation for the lack of intestinal defects in occludin KO mice could be partial compensation by tricellulin or MARVELD3, which together with occludin comprise the tight junction–associated MARVEL protein (TAMP) family.[35] In order to unmask such compensation, we asked if occludin KO mice would display an intestinal epithelial phenotype in response to stress.

We hypothesized that mild barrier defects might render occludin KO mice hypersensitive to injury, as has been shown in other mice lacking tight junction proteins.[36,37] Instead, occludin KO mice were markedly protected from DSS-induced chemical injury. We initially considered that this unexpected result might be explained by occludin loss in nonepithelial cells, such as intraepithelial lymphocytes.[38] Transgenic occludin expression within intestinal epithelia was, however, able to complement universal occludin KO. Moreover, we developed intestinal epithelial-specific occludin KO (KO^{IEC}) mice and found that they too were protected from both DSS-induced chemical damage and immune-mediated damage triggered by TNBS. Finally, in vitro studies using occludin KD intestinal epithelial cell lines confirmed that apoptotic resistance was a cell autonomous consequence of occludin loss.

To better characterize the mechanism by which occludin deletion confers apoptotic resistance, we challenged mice with prototypic activators of intrinsic and extrinsic apoptotic pathways. Occludin-deficient epithelia were protected from both, in vivo and in vitro. Events upstream of caspase-3 cleavage were excluded as potential causes of this resistance to apoptosis. Occludin depletion did, however, reduce *CASP3* transcription, protein expression, and enzymatic activity. Consistent with this, cleavage of poly (ADP-ribose) polymerase, a proteolytic target of activated caspase-3, was reduced in occludin-deficient epithelia. We asked whether a ~50% reduction in caspase-3 expression was sufficient to limit apoptosis using $Casp3^{+/-}$ mice. Remarkably, these mice, in which caspase-3 expression was also reduced by ~50%, were protected from apoptotic stimuli to the same extent as in occludin KO mice. These data demonstrate that the downregulation of capase-3 expression induced by occludin loss is sufficient to limit epithelial apoptosis, thereby indicating that occludin downregulation prevents apoptosis by reducing *CASP3* transcription.

Our studies are not the first to link occludin to transcriptional regulation. Previous work demonstrated that transformation of a salivary gland epithelial cell line, by transfection of oncogenic Raf-1, downregulated occludin and claudin-1 expression while inducing epithelial-mesenchymal transformation and disrupting monolayer growth in vitro.[39,40] Remarkably, transfection-induced occludin overexpression

Figure 5. Partial loss of caspase-3 expression is sufficient to limit DSS-induced intestinal epithelial apoptosis. (*A*) Caspase-3 expression was reduced in colonic epithelia from $Casp3^{+/-}$ (+/−), relative to WT (+/+), mice (n = 5 per group). Intestinal epithelial caspase-3 expression was similar in in $Casp3^{+/-}$ and occludin KO mice (n = 3 per group). (*B*) TUNEL staining (red) shows DSS-induced epithelial apoptosis in colonic mucosa from $Casp3^{+/-}$, relative to WT, mice. Bar = 100 μm (n = 4 per group). (*C*) Cleaved caspase-3 staining shows attenuated TNF-induced apoptosis of small intestinal epithelium of $Casp3^{+/-}$, relative to WT, mice. Bar = 100 μm (n = 3 per group). Littermates from $Casp3^{+/-} \times Casp3^{+/-}$ matings were used in these experiments Statistical analysis by one-way analysis of variance with Bonferroni posttest; $^{**}P < .01$.

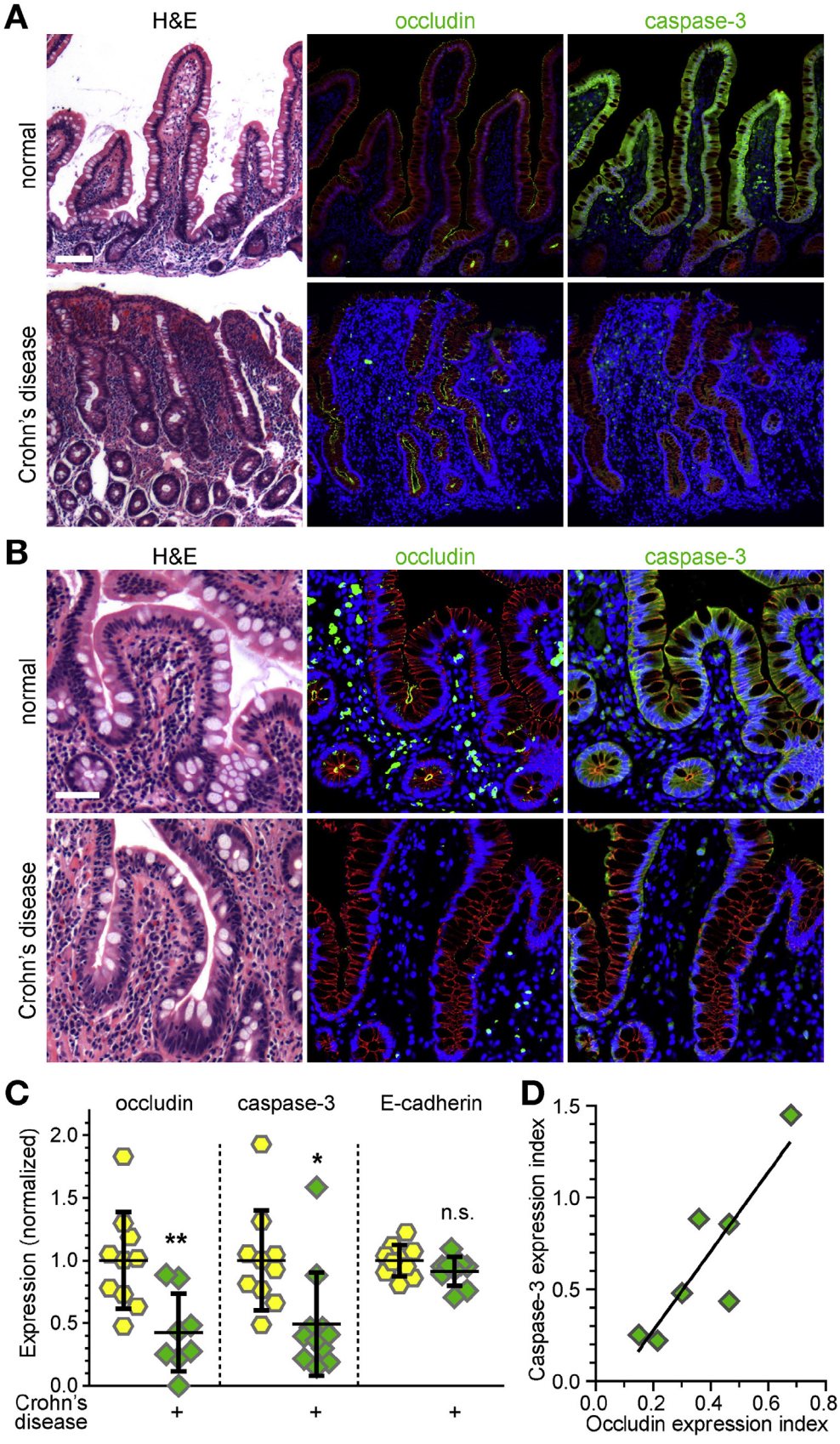


Figure 6. Ileal epithelia from patients with Crohn's disease demonstrate reduced expression of occludin and caspase-3. (*A, B*) Hematoxylin-eosin (H&E) and multiplex immunohistochemical staining of ileal biopsies from healthy subjects and patients with Crohn's disease. Expression of occludin and caspase-3 (green, as indicated) were reduced in Crohn's disease. E-cadherin (red) and nuclei (blue) are shown for reference. Bars = 100 μm (*A*), 50 μm (*B*). (*C*) Quantitative morphometry of occludin, caspase-3, and E-cadherin staining intensity within the intestinal epithelium (n = 8 to 11). (*D*) Ileal epithelial caspase-3 expression correlated with occludin expression in biopsies from patients with Crohn's disease ($r^2 = 0.76$). Statistical analysis by Student *t* test. *$P < .05$; **$P < .01$.

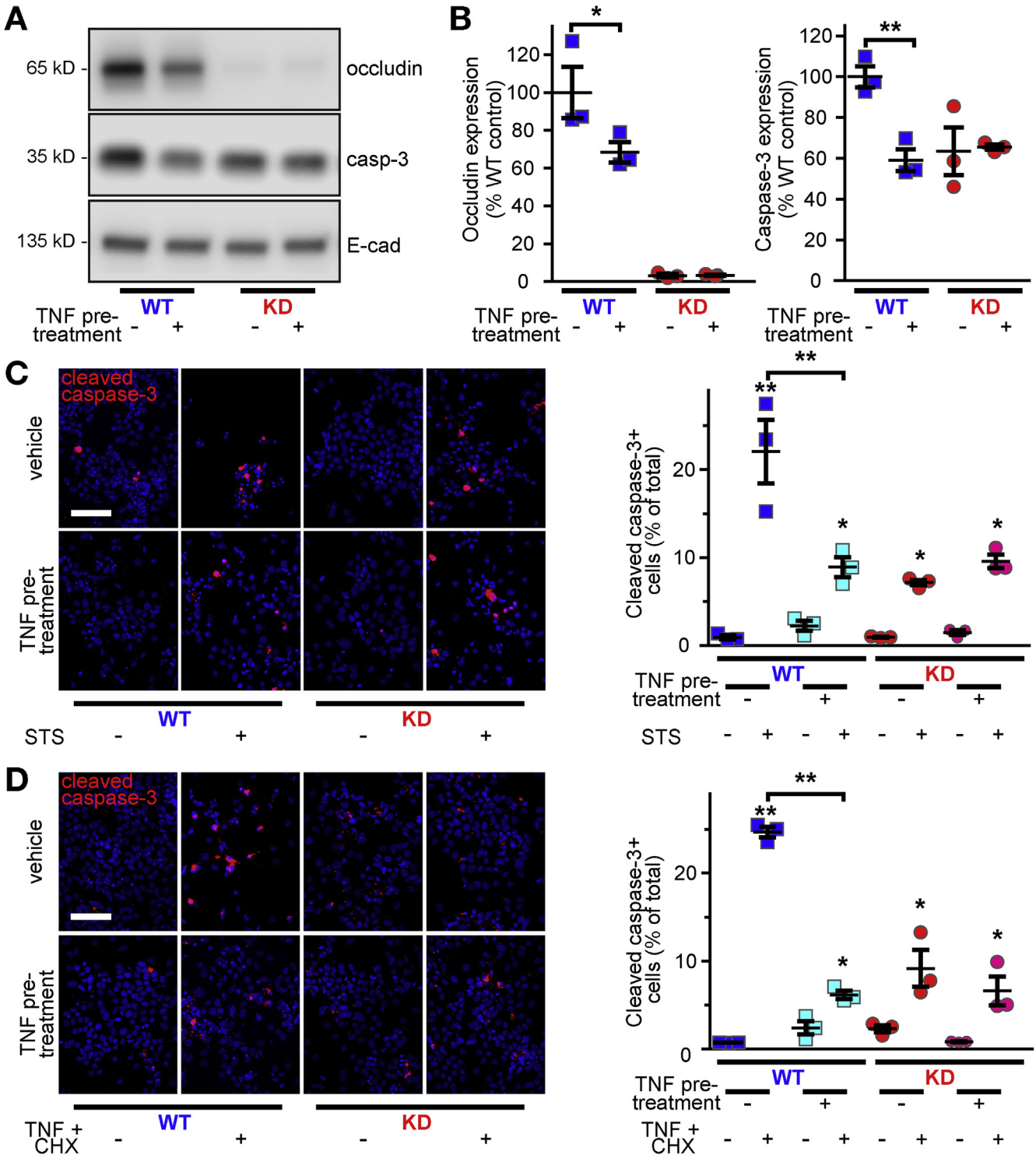


Figure 7. TNF-induced occludin downregulation prevents apoptosis of occludin-sufficient cells. (*A, B*) Low-dose (0.5 ng/mL) TNF treatment decreased occludin and caspase-3 expression in occludin-sufficient, but not occludin-deficient, cells. (*C, D*) TNF pretreatment diminished apoptosis and cell loss induced by STS (C) or TNF and cycloheximide (CHX). Cleaved caspase-3 (red) and nuclei (blue) are shown. Low-dose TNF pretreatment did not affect occludin KD monolayers. Bar = 100 μm (n = 3 per group). Statistical analysis by one-way analysis of variance with Bonferroni posttest; *$P < .05$; *$P < .01$.

restored claudin-1 expression, reversed epithelial-mesenchymal transformation, and prevented in vivo growth of the transformed line.[39,40] The mechanisms by which occludin reversed neoplastic transformation were not defined, but the extent to which occludin protein expression was restored did correlate directly with claudin-1 mRNA content.[39] Thus, in addition to our discovery of occludin-dependent caspase-3 transcription, occludin may also be a

transcriptional activator of claudin-1 and, possibly, other genes that regulate epithelial-mesenchymal transformation.

In hepatocellular carcinoma cell lines, occludin overexpression has been reported to enhance caspase-3 expression and apoptotic sensitivity[41] (ie, the inverse of occludin deletion). Those studies linked occludin effects to the C-terminal coiled-coil domain, as an occludin splice variant lacking exon 9, which includes that domain, did not affect apoptosis.[41] Interestingly, this region also includes the occludin-ELL (OCEL) domain.[42,43] ELL encodes an elongation factor that increases the catalytic rate of RNA polymerase II transcription; chromosomal translocations that create a bifunctional protein by fusing *ELL* to an RNA polymerase II, *MLL*, are common in acute myeloid leukemia.[44] As a whole, these studies, our results, and data indicating that a C-terminal occludin fragment enhances epithelial apoptosis[45] suggest that the occludin OCEL domain may regulate caspase-3 transcription.

We have previously reported that the occludin OCEL domain is essential for TNF-induced reductions in occludin anchoring at the tight junction and subsequent endocytosis.[10] Although not fully characterized, it is thought that this endocytosis results in lysosomal degradation and reduced occludin expression in colitis.[24] Our data indicate this occludin downregulation promotes mucosal homeostasis. How this effect interacts with the occludin loss induced increases in leak pathway permeability, which has been hypothesized to exacerbate disease, remains to be defined.

Overall, our data indicate that inflammation-associated occludin downregulation leads to reduced caspase-3 expression and generalized apoptotic resistance. This may, therefore, represent an adaptive mechanism that limits epithelial damage in the context of disease. We further speculate that, in chronic inflammation (eg, inflammatory bowel disease), this apoptotic insensitivity might also allow accumulation of cells with genetic defects and, in turn, facilitate neoplastic transformation. This hypothesis will need to be tested, but the results presented here demonstrate that occludin downregulation has beneficial effects. It also will be important for future studies to better define this new relationship between tight junction molecular structure and epithelial survival.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2019.07.058.

References

1. Furuse M, Hirase T, Itoh M, et al. Occludin: a novel integral membrane protein localizing at tight junctions. J Cell Biol 1993;123:1777–1788.
2. Furuse M, Fujimoto K, Sato N, et al. Overexpression of occludin, a tight junction-associated integral membrane protein, induces the formation of intracellular multilamellar bodies bearing tight junction-like structures. J Cell Sci 1996;109:429–435.
3. Fanning AS, Jameson BJ, Jesaitis LA, et al. The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton. J Biol Chem 1998;273:29745–29753.
4. Bolinger MT, Ramshekar A, Waldschmidt HV, et al. Occludin S471 Phosphorylation Contributes to Epithelial Monolayer Maturation. Mol Cell Biol 2016;36:2051–2066.
5. Elias BC, Suzuki T, Seth A, et al. Phosphorylation of Tyr-398 and Tyr-402 in occludin prevents its interaction with ZO-1 and destabilizes its assembly at the tight junctions. J Biol Chem 2009;284:1559–1569.
6. Raleigh DR, Boe DM, Yu D, et al. Occludin S408 phosphorylation regulates tight junction protein interactions and barrier function. J Cell Biol 2011;193:565–582.
7. Wong V. Phosphorylation of occludin correlates with occludin localization and function at the tight junction. Am J Physiol 1997;273:C1859–C1867.
8. Wong V, Gumbiner BM. A synthetic peptide corresponding to the extracellular domain of occludin perturbs the tight junction permeability barrier. J Cell Biol 1997;136:399–409.
9. Yu AS, McCarthy KM, Francis SA, et al. Knockdown of occludin expression leads to diverse phenotypic alterations in epithelial cells. Am J Physiol Cell Physiol 2005;288:C1231–C1241.
10. **Buschmann MM**, **Shen L**, Rajapakse H, et al. Occludin OCEL-domain interactions are required for maintenance and regulation of the tight junction barrier to macromolecular flux. Mol Biol Cell 2013;24:3056–3068.
11. Van Itallie CM, Fanning AS, Holmes J, et al. Occludin is required for cytokine-induced regulation of tight junction barriers. J Cell Sci 2010;123:2844–2852.
12. Balda MS, Whitney JA, Flores C, et al. Functional dissociation of paracellular permeability and transepithelial electrical resistance and disruption of the apical-basolateral intramembrane diffusion barrier by expression of a mutant tight junction membrane protein. J Cell Biol 1996;134:1031–1049.
13. Shen L, Turner JR. Actin depolymerization disrupts tight junctions via caveolae-mediated endocytosis. Mol Biol Cell 2005;16:3919–3936.
14. **Schwarz BT**, **Wang F**, Shen L, et al. LIGHT signals directly to intestinal epithelia to cause barrier dysfunction via cytoskeletal and endocytic mechanisms. Gastroenterology 2007;132:2383–2394.
15. Saitou M, Furuse M, Sasaki H, et al. Complex phenotype of mice lacking occludin, a component of tight junction strands. Mol Biol Cell 2000;11:4131–4142.
16. Schulzke JD, Gitter AH, Mankertz J, et al. Epithelial transport and barrier function in occludin-deficient mice. Biochim Biophys Acta 2005;1669:34–42.
17. Kitajiri SI, Katsuno T, Sasaki H, et al. Deafness in occludin-deficient mice with dislocation of tricellulin and progressive apoptosis of the hair cells. Biol Open 2014;3:759–766.
18. Marchiando AM, Shen L, Graham WV, et al. Caveolin-1-dependent occludin endocytosis is required for TNF-induced tight junction regulation in vivo. J Cell Biol 2010;189:111–126.
19. Ferrary E, Cohen-Tannoudji M, Pehau-Arnaudet G, et al. In vivo, villin is required for Ca(2+)-dependent F-actin

disruption in intestinal brush borders. J Cell Biol 1999; 146:819–830.
20. Shinkai Y, Rathbun G, Lam KP, et al. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. Cell 1992;68:855–867.
21. Ma A, Datta M, Margosian E, et al. T cells, but not B cells, are required for bowel inflammation in interleukin 2-deficient mice. J Exp Med 1995;182:1567–1572.
22. Kuida K, Zheng TS, Na S, et al. Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. Nature 1996;384:368.
23. el Marjou F, Janssen KP, Chang BH, et al. Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. Genesis 2004;39:186–193.
24. Heller F, Florian P, Bojarski C, et al. Interleukin-13 is the key effector Th2 cytokine in ulcerative colitis that affects epithelial tight junctions, apoptosis, and cell restitution. Gastroenterology 2005;129:550–564.
25. Pinto D, Robine S, Jaisser F, et al. Regulatory sequences of the mouse villin gene that efficiently drive transgenic expression in immature and differentiated epithelial cells of small and large intestines. J Biol Chem 1999; 274:6476–6482.
26. Wirtz S, Neufert C, Weigmann B, et al. Chemically induced mouse models of intestinal inflammation. Nat Protoc 2007;2:541–546.
27. Clayburgh DR, Barrett TA, Tang Y, et al. Epithelial myosin light chain kinase-dependent barrier dysfunction mediates T cell activation-induced diarrhea in vivo. J Clin Invest 2005;115:2702–2715.
28. Vyas D, Robertson CM, Stromberg PE, et al. Epithelial apoptosis in mechanistically distinct methods of injury in the murine small intestine. Histol Histopathol 2007;22:623–630.
29. Nie M, Balda MS, Matter K. Stress- and Rho-activated ZO-1-associated nucleic acid binding protein binding to p21 mRNA mediates stabilization, translation, and cell survival. Proc Natl Acad Sci U S A 2012;109:10897–10902.
30. Odenwald MA, Choi W, Kuo WT, et al. The scaffolding protein ZO-1 coordinates actomyosin and epithelial apical specializations in vitro and in vivo. J Biol Chem 2018;293:17317–17335.
31. Zeissig S, Burgel N, Gunzel D, et al. Changes in expression and distribution of claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active Crohn's disease. Gut 2007;56:61–72.
32. Kucharzik T, Walsh SV, Chen J, et al. Neutrophil transmigration in inflammatory bowel disease is associated with differential expression of epithelial intercellular junction proteins. Am J Pathol 2001;159:2001–2009.
33. Mankertz J, Tavalali S, Schmitz H, et al. Expression from the human occludin promoter is affected by tumor necrosis factor alpha and interferon gamma. J Cell Sci 2000;113:2085–2090.
34. Mir H, Meena AS, Chaudhry KK, et al. Occludin deficiency promotes ethanol-induced disruption of colonic epithelial junctions, gut barrier dysfunction and liver damage in mice. Biochim Biophys Acta 2016;1860:765–774.
35. Raleigh DR, Marchiando AM, Zhang Y, et al. Tight junction-associated MARVEL proteins marveld3, tricellulin, and occludin have distinct but overlapping functions. Mol Biol Cell 2010;21:1200–1213.
36. Laukoetter MG, Nava P, Lee WY, et al. JAM-A regulates permeability and inflammation in the intestine in vivo. J Exp Med 2007;204:3067–3076.
37. Vetrano S, Rescigno M, Cera MR, et al. Unique role of junctional adhesion molecule-a in maintaining mucosal homeostasis in inflammatory bowel disease. Gastroenterology 2008;135:173–184.
38. Edelblum KL, Shen L, Weber CR, et al. Dynamic migration of gammadelta intraepithelial lymphocytes requires occludin. Proc Natl Acad Sci U S A 2012;109:7097–7102.
39. Li D, Mrsny RJ. Oncogenic Raf-1 disrupts epithelial tight junctions via downregulation of occludin. J Cell Biol 2000;148:791–800.
40. Wang Z, Mandell KJ, Parkos CA, et al. The second loop of occludin is required for suppression of Raf1-induced tumor growth. Oncogene 2005;24:4412–4420.
41. Gu JM, Lim SO, Park YM, et al. A novel splice variant of occludin deleted in exon 9 and its role in cell apoptosis and invasion. FEBS J 2008;275:3145–3156.
42. Sakurai K, Michiue T, Kikuchi A, et al. Inhibition of the canonical Wnt signaling pathway in cytoplasm: a novel property of the carboxyl terminal domains of two Xenopus ELL genes. Zoolog Sci 2004;21:407–416.
43. **Li Y**, **Fanning AS**, Anderson JM, et al. Structure of the conserved cytoplasmic C-terminal domain of occludin: identification of the ZO-1 binding surface. J Mol Biol 2005;352:151–164.
44. Shilatifard A, Lane WS, Jackson KW, et al. An RNA polymerase II elongation factor encoded by the human ELL gene. Science 1996;271:1873–1876.
45. Beeman NE, Baumgartner HK, Webb PG, et al. Disruption of occludin function in polarized epithelial cells activates the extrinsic pathway of apoptosis leading to cell extrusion without loss of transepithelial resistance. BMC Cell Biol 2009;10:85.

Author names in bold designate shared co-first authorship

Received January 3, 2019. Accepted July 22, 2019.

Reprint requests
Address requests for reprints to: Jerrold R. Turner, MD, PhD, Brigham and Women's Hospital and Harvard Medical School, 77 Avenue Louis Pasteur, HNRB 730B, Boston, Massachusetts 02115. e-mail: jrturner@bwh.harvard.edu.

Acknowledgments
We thank the Brigham and Women's Hospital Pathology Core and University of Chicago Human Tissue Resource Center for their expert assistance, Linda Degenstein and the University of Chicago Transgenic Core for assistance with development of occludin KO^{IEC} mice, Margaret Neville (University of Colorado) and Sachiko Tsukita (Osaka University, Japan) for providing occludin KO mice, and Mingtao Li (Sun Yat-sen University, China) for the human *CASP3* promoter-containing vector.

Author contributions: Conceptualization: WK, LS, JRT; Experimentation: WK, LS, LZ, NS, MLDMO, LW, KLE, JZ, YtW, YmW, JRT; Data analysis: WK, LS, LZ, NS, SPN, JRT; Manuscript preparation and revision: WK, LS, LZ, NS, MLDMO, LW, KLE, JZ, YtW, YmW, SPN, JRT.

Conflicts of interest
The authors disclose no conflicts.

Funding
This work was supported by National Institutes of Health grants R01DK61931 (JRT), R01DK68271 (JRT), R24DK099803 (JRT), and K01DK092381 (LS), and the Harvard Digestive Disease Center (P30DK034854) and by National Science Foundation of China grant 81800464 (LZ).